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ESTABLISHMENT OF CELL POLARITY DURING MATING IN YEAST

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

DEDICATION

I dedicate this work
to the memory of my brother
John Edward Chenevert

ACKNOWLEDGEMENTS

I am grateful to Ira Herskowitz for providing just the right mixture of freedom and guidance during my development as a scientist. I thank Tim Mitchison and Bruce Alberts for their interest in my work and for helpful scientific advice, and I also thank Bruce for providing personal support. I am grateful to all the members of the Herskowitz lab, past and present, for useful discussions, technical help, and friendship.

Several of my colleague graduate students deserve special mention: John Chant encouraged me in the early years ("We'll have chemotaxis figured out in yeast before Dicty ever slimes out of the mud"); Aaron Neiman provided insight of many kinds and laughs in the bay and reminded me of "the awesome ease of yeast genetics"; Nicole Valtz, keeper of the "wild-type mutants", is a natural-born appreciator of shmoos; and Jody Rosenblatt restored an important but forgotten practice to our lab: giggling at "mating experiments".

I enjoyed many stimulating discussions and collaborations with John Pringle and David Drubin and members of their laboratories. Kathy Corrado especially deserves thanks for her generous gift of Bem1p-antibody and for general graduate student commiseration.

I thank my parents for their support and a great deal of timely babysitting. And finally my husband Gerard for appreciation and encouragement and also for making many of the figures in this thesis. In addition I am grateful to Gerard and to my son Valentin for providing incentive to finish up and for putting up with a certain amount of neglect while I was doing so.

ESTABLISHMENT OF CELL POLARITY DURING MATING IN YEAST

JANET CHENEVERT

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. Genes involved in pheromone-induced cell polarization were identified by isolating mutants defective in mating to an enfeebled partner. The mutants were divided into classes based on their morphological responses to mating pheromone. One classs is unable to polarize in reponse to pheromone and instead enlarges in a uniform manner. These mutants harbor special alleles of gnes required for cell polarization during vegetative growth, BEM1 and CDC24. Another class forms peanut-like shpaes when treated with pheromone and defines two genes, PEA1 and PEA2. 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 mutants in BEM1, denoted bem1-s, were analyzed in detail. Unlike other bem1 mutants, the bem1-s mutants are normal for vegetative growth. The bem1-s mutants encode truncated proteins which are present in reduced amount relative to wild-type. Mutants in BEM1 exhibit disorganized actin, and in some cases form aberrant actin structures. The predicted sequence of the BEM1

protein reveals two copies of a domain (denoted SH3) whose function is still mysterious but which is found in many proteins associated with the cortical cytoskeleton. A second genetic screen identified genes which interact with BEM1 during mating. Two genes were isolated which when overexpressed can partially restore mating and shmooing to the bem1-s alleles: the previously identified gene FUS2, which is required for cell fusion and a new gene, DRT1. DRT1, but not FUS2, is able to partially suppress the vegetative defect of a bem1 deletion strain. A model is suggested in which BEM1 is proposed to be an element that links the cytoskeleton to morphogenetic determinants on the cell surface. The DRT1 and FUS2 gene products may be involved in recruiting BEM1 protein towards the region of the cell surface which is most near the source of pheromone during mating and shmooing.

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CHAPTER 1

INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, two cells of opposite mating type orient towards one another during conjugation. The purpose of this spatial reorganization is to position certain molecules and organelles very near a mating partner so that cell fusion and nuclear fusion can ensue. The phenomenon of polarization towards an external stimulus is of widespread occurrence and importance in nature, and common principles are emerging among the polarization responses of many diverse systems. Rapid and powerful genetic techniques make yeast an attractive organism in which to investigate cell polarization. The goal of this work is to identify and analyze yeast genes involved in polarizing towards a mating partner and in so doing, to learn about how eucaryotic cells develop cellular asymmetry in response to extracellular signals.

Yeast cells polarize towards an extracellular signal for mating. When two yeast cells of opposite mating type come into contact, they grow towards each other in a polarized fashion (Tkacz and MacKay 1979; Byers and Goetsch 1975; Hasek *et al.* 1987; Field and Schekman 1980). Secretion and new cell-surface growth are concentrated in the direction of the partner (Tkacz and MacKay 1979). The actin and microtubule cytoskeletons are polarized towards the mating partner (Byers 1981; Ford and Pringle 1986; Hasek *et al.* 1987). This response facilitates efficient cell and nuclear fusion (reviewed in Cross *et al.* 1988) and also enables yeast cells to mate with high efficiency to cells of opposite mating type without making mistakes such as orienting or mating to more than one partner or to a cell of the same mating type in a crowded

mixture of cells (Bender and Pringle 1991; Jackson and Hartwell 1990a; Jackson and Hartwell 1990b; J. Chenevert, unpublished observations).

Mating yeast cells signal one another with cell-type specific mating pheromones. The pheromones are secreted peptides and are recognized by cell-surface receptors: **a** cells produce **a**-factor, which binds to a receptor on α cells (encoded by STE3), and α cells produce α-factor which binds to a receptor on **a** cells (encoded by STE2). Pheromone receptors belong to the large family of G protein-coupled receptors with seven transmembrane domains (reviewed in Marsh, Neiman and Herskowitz 1991; Kurjan 1992). 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 polarized cell shape ("shmoo").

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, Taylor and Ballou 1976; Tkacz and MacKay 1979; Field and Schekman 1980). This growth reflects the polarized orientation of the cytoskeleton (Read, Okamura and Drubin 1992; Barnes, Drubin and Stearns 1990; see Figure 1-1). 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 analogue of the centrosome or microtubule organizing center) orients towards the shmoo tip, and microtubules emanating from it extend into the tip (Byers 1981; Rose and Fink 1987; Gehrung and Snyder 1990; Meluh and Rose 1990). A ring of ten nanometer filaments

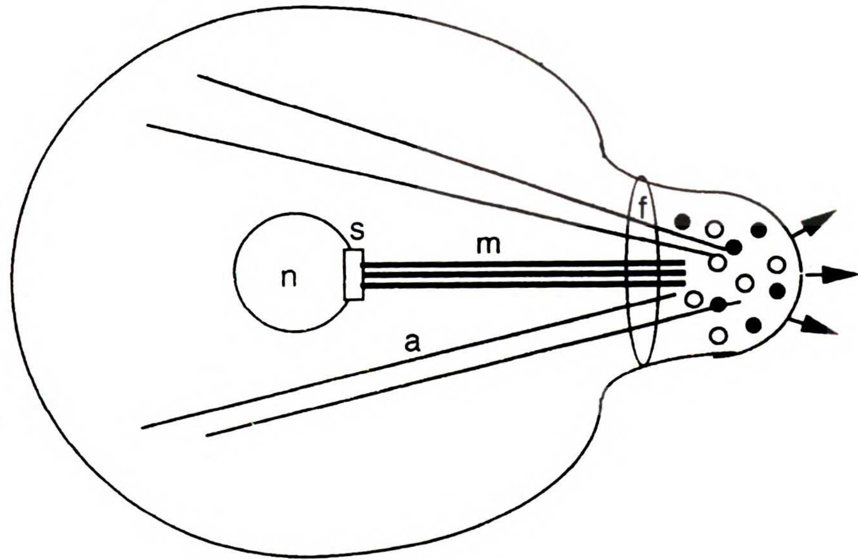
Figure 1-1: Polarized components in a yeast cell.

A: Pheromone-treated (shmooing) cell.

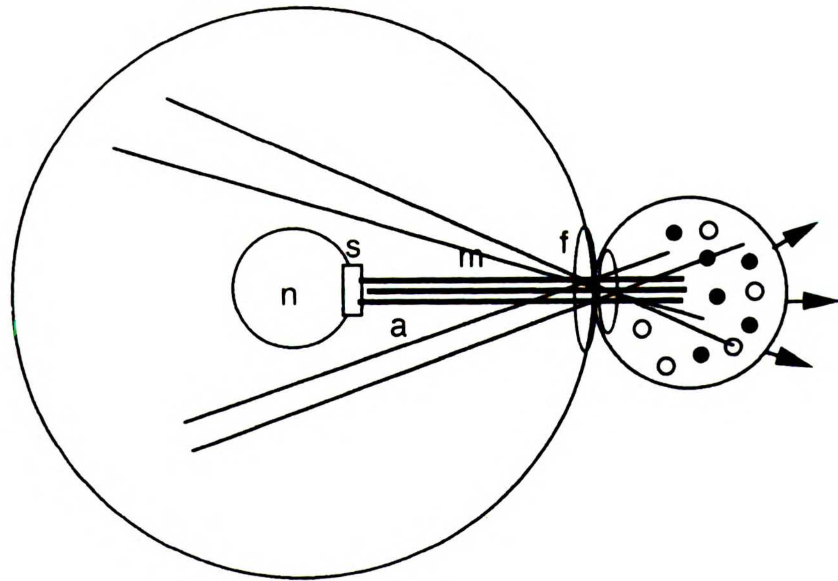
B: Vegetative (budding) cell.

All major cytoskeletal elements and organelles are organized around the site of cell-surface growth in the two types of polarized cells. The spindle pole body (s) on the nucleus (n) is positioned on the side of the nucleus near either the shmoo tip or the bud. Extranuclear microtubules (m) radiate from the spindle pole body into the shmoo tip as they do into the bud. Cortical actin patches (open circles) are concentrated in both the shmoo tip and the bud and actin cables (a) orient toward the shmoo tip or the bud. A ring of ten nanometer filaments (f) is found at the shmoo neck as well as at the mother-bud neck. Secretory vescicles (filled circles) are concentrated in the shmoo tip and the bud and the insertion of new cell-surface material (arrows) occurs at both the shmoo tip and the bud tip.

A



B



is observed at the base of the projection (Kim, Haarer and Pringle 1991; Ford and Pringle 1986). 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, Klis and Tanner 1988), Fus1p (Trueheart, Boeke and Fink 1987), Spa2p (Gehrung and Snyder 1990), Ste2p (Jackson, Konopka and Hartwell 1991), Fus2p (E. Elion, personal communication), and Ste6p (Kuchler, Dohlman and Thorner 1993). The shmoo is thus a manifestation of the pheromone-stimulated morphogenesis which normally occurs during mating but which becomes exaggerated in the absence of a mating partner.

The spatial signal emanating from the mating partner which directs localized growth during mating is apparently a high concentration of mating pheromone. Exogenously added pheromone cannot restore mating to mutant strains unable to produce pheromone (Kurjan 1985; Michaelis and Herskowitz 1988; Bender and Sprague 1989), indicating that the mating pheromone must be presented by a cell in a spatially meaningful manner. Given a choice between mating partners which do or do not produce pheromone, yeast cells mate almost exclusively with the pheromone-producing cells (Jackson and Hartwell 1990a; Jackson and Hartwell 1990b; Jackson, Konopka and Hartwell 1991). This phenomenon is termed "mating partner discrimination" and is thought to reflect cell morphogenesis towards a gradient of mating pheromone in the

environment. Mutants which are supersensitive to mating pheromones are defective in discrimination and mate equally well with the pheromone-producing or nonproducing cells, perhaps because they cannot determine the location of the source of the pheromone gradient and thus respond by choosing a cell at random.

A direct assay would be extremely useful in identifying and characterizing genes involved in polarizing toward a mating partner. Early reports that **a** or α cells orient bud growth toward cells of the opposite mating type (Herman 1971; Rine 1979) have proved difficult to reproduce (F. Chang, J. Chenevert, N. Valtz, unpublished observations). In addition, numerous attempts to observe shmooing of **a** cells towards a large variety of pheromone sources, including individual α cells, groups of α cells, spots and streaks of α-factor, micropipets filled with α-factor, and tiny beads coated with α-factor, have failed (J. Chenevert, unpublished). These assays used very high concentrations of α-factor (10^{-6} M), which far exceed the pheromone receptor dissociation constant of 5 X 10^{-9} M (Jenness, Burkholder and Hartwell 1986). Recently, oriented shmooing of **a** cells towards a micropipet filled with α-factor has been demonstrated (Segall 1993). The α-factor concentration required for optimal orientation (67 nM) was calculated to result in a gradient of 330 nM/mm, which would produce a difference in receptor occupancy between the front and rear halves of the cell of about 1% of the total number of receptors occupied. This sensitity approaches that seen for the orientation of movement of cells such as *Dictyostelium discoideum* or neutrophils in spatial gradients of chemoattractant (Zigmond 1977; Mato *et al.* 1975; Caterina and Devreotes 1991; see below).

Yeast cells polarize towards an intracellular signal for budding. 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, sites of growth during budding are determined by the genetic make-up of a cell and are not influenced by the presence of other cells. Haploid MAT**a** and MATα cells choose bud sites in a pattern called "axial" (Friefelder 1960; Hicks, Strathern and Herskowitz 1977). In the axial pattern, a mother cell produces a new bud immediately adjacent to its last daughter, and the daughter cell produces its bud towards its mother cell. Diploid MAT**a**/MATα cells choose bud sites in another pattern, called "bipolar". In this pattern, the mother cell is restricted to bud either adjacent to its last daughter or at the opposite end of the cell, and the daughter cell produces a bud away from its mother. Budding pattern is controlled by heterozygosity or homozygosity at the MAT locus and not by ploidy (Hicks, Strathern and Herskowitz 1977; Hartwell 1980; J. Chant, S. Sanders, unpublished observations).

The directed growth that occurs during budding has strong similarities to the growth that occurs during mating. Many of the same intracellular components are organized in a polarized manner in both cases (Figure 1-1). As the bud forms on the surface, cell-surface growth and secretion are concentrated to a small patch on the yeast cell surface (Tkacz and Lampen 1972; Tkacz and Lampen 1973; Field and Schekman 1980). Actin dots are clustered at the region of cell-surface growth, and actin cables run the length of the cell into the bud (Adams and Pringle 1984). The golgi apparatus (Segev, Mulholland and Botstein 1988) and the spindle pole body are

on the side of the nucleus facing the small bud, and extranuclear microtubules radiate from the spindle pole body into the bud (Byers 1981). A ring of 10-nm filaments forms at the site of bud emergence (Byers and Goetsch 1976; Byers 1981; Haarer *et al.* 1990; Kim, Haarer and Pringle 1991).

Role of the cytoskeleton in yeast cell polarization. Some of the components which become polarized during cell growth are also required for polarized growth. Actin filaments and microtubules have been shown to function in the morphogenesis of various cells that polarize. Studies on mating cells indicate that microtubules are not required for morphogenesis or cell fusion and that they are required for nuclear fusion (Delgado and Conde 1984; Hasek *et al.* 1987; Huffaker, Thomas and Botstein 1988). Recent studies using several mutant alleles of the actin and tubulin genes show that microtubules are essential for positioning the nucleus in the projection tip but not for polarized growth (Read, Okamura and Drubin 1992). During budding, a similar requirement for cytoskeletal components is seen. Mutants defective in actin cease to grow at any point during bud growth (Novick and Botstein 1985). Cell-surface growth during budding appears normal in tubulin mutants or in the presence of nocodazole sufficient to eliminate detectable microtubules (Jacobs *et al.* 1988; Huffaker, Thomas and Botstein 1988). Cells defective in the vesicle-mediated secretion pathway (sec^{-}) are blocked for bud growth at the restrictive temperature (Novick and Schekman 1979). To summarize, actin and the secretory apparatus are required for polarized growth in yeast whereas microtubules do not appear to be required.

Genes involved in polarity establishment. Organization of the cell and restriction of cell-surface growth to a specific site requires a group of genes known as polarity establishment genes: CDC24, CDC42, CDC43, and BEM1 (Sloat, Adams and Pringle 1981; Adams *et al.* 1990; Chant *et al.* 1991; Chenevert *et al.* 1992). Mutants in the polarity-establishment genes are unable to localize growth to form a bud but rather enlarge in a uniform manner and are slow growing or inviable (Sloat and Pringle 1978; Sloat, Adams and Pringle 1981; Adams *et al.* 1990; Johnson and Pringle 1990; Chenevert *et al.* 1992; Chant *et al.* 1991; Bender and Pringle 1991). These mutants have no apparent defects in cell-surface growth but rather in the ability to organize this growth, as analyzed by the depostion of chitin, the insertion of the cell-surface protein alkaline phosphatase, and the organization of actin (Sloat, Adams and Pringle 1981; Field and Schekman 1980). Thus the products of the polarity establishment genes appear to function as central organizers or regulators of cell polarity.

The sequences of the polarity establishment genes are suggestive of their possible functions. CDC42 encodes a RAS-like GTP-binding protein with a site for membrane attachment (Johnson and Pringle 1990). CDC24 protein contains a region of sequence similarity to the *dbl* oncogene product, which specifically catalyzes the dissociation of GDP from a human homologue of CDC42, CDC42Hs (Hart *et al.* 1991). The possibility that Cdc24p may be a guanine nucleotide exchange factor for Cdc42p has not been directly tested. Sequence analysis and the isolation of calcium-sensitive alleles suggest that CDC24 protein may bind calcium (Ohya *et al.* 1986;

Miyamoto *et al.* 1987). CDC43 encodes a subunit of the geranylgeranyl transferase essential for the correct posttranslational modification of CDC42 (Finegold *et al.* 1991). BEM1 protein contains two SH3 domains. Both Bem1p (Corrado 1992) and Cdc42p (D. Johnson, personal communication) localize to site of cell-surface growth in a cell cycle dependent manner. They are found in a small patch on the plasma membrane in the same region as actin dots just prior to bud emergence, at the tip of a bud in cells with a small bud, and then staining becomes diffuse throughout the rest of the cell cycle. Staining with anti-Cdc24p antibodies reveals punctate dots throughout the cell surface of the mother and the bud in all stages of the cell cycle (Ziman and Johnson 1993).

Polarity establishment in response to mating pheromones has been less well studied, but the fact that many of the same components are organized in a polarized manner in a budding cell and in a shmooing cell (see above and Figure 1-1) and the apparently common roles of the cytoskeleton and secretory apparatus suggest that the same organizational molecules may be involved in mating and budding. 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 alleles of BEM1 exist which are defective in mating and shmooing but not budding (Chenevert *et al.* 1992).

Genes involved in bud-site selection. Mutations which cause improper positioning of a bud but no growth defects have

identified five bud-site selection genes (BUD1-5) (Chant and Herskowitz 1991; Chant *et al.* 1991). Mutations in BUD1, 2, or 5 cause random budding in all cell types. Mutations in BUD3 or BUD4 cause bipolar budding in **a** and α cells and have no effect on **a**/α cells. The phenotypes conferred by mutations in the bud-site selection genes or the polarity-establishement genes and genetic interactions among the two groups of genes 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 a 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.

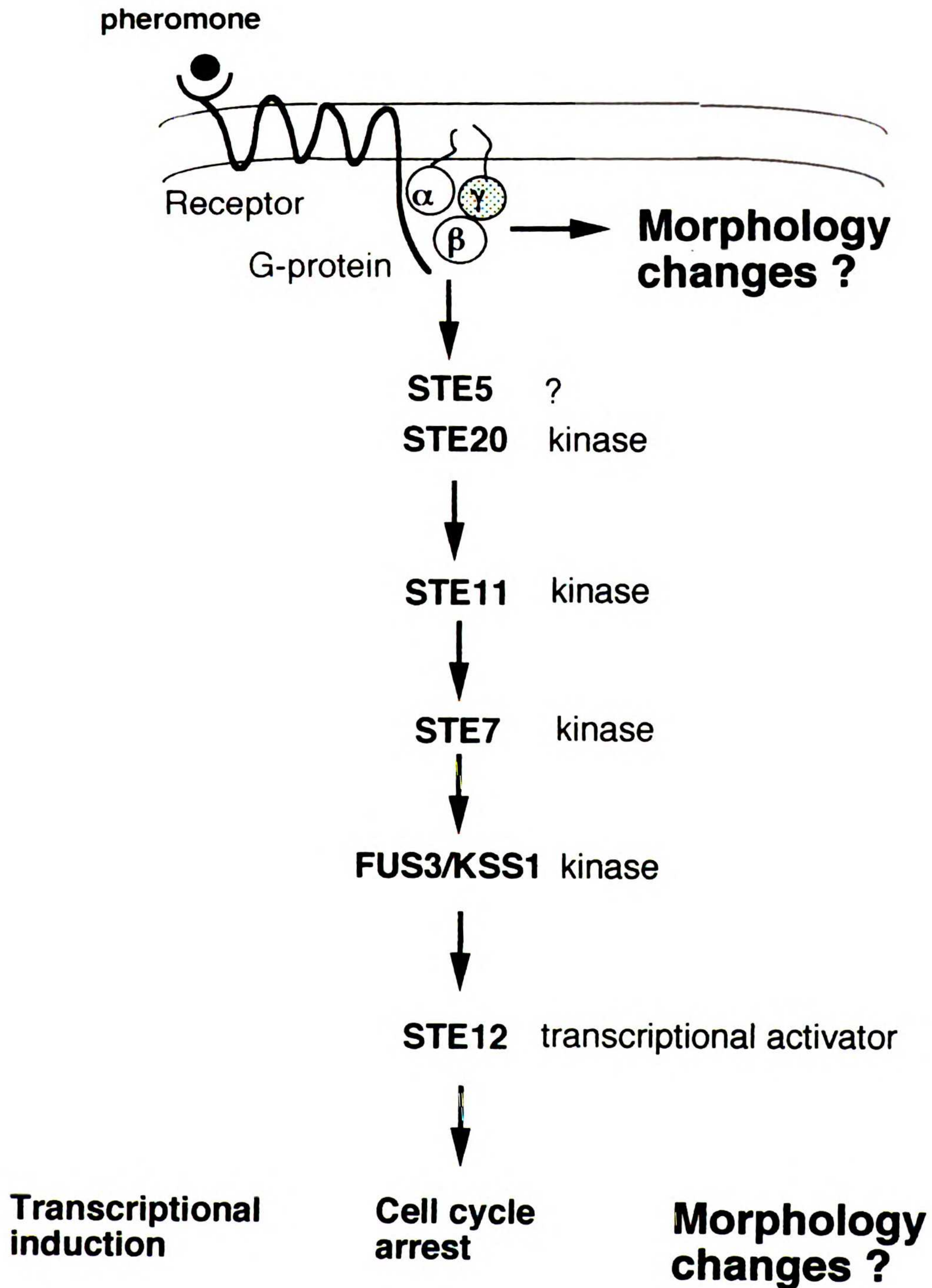
The sequences of three of the BUD genes suggest that they may function together in a nucleotide-exchange cycle. BUD1, also called RSR1, is a member of the ras family of small GTP-binding proteins (Johnson and Pringle 1990; Bender and Pringle 1989). Bud2p has homology to GAP proteins which promote the intrinsic GTPase acitivity of GTP-binding proteins, and has been shown to have GAP activity on Bud1p (Park, Chant and Herskowitz 1993). BUD5 is homologous to a GDP-GTP exchange protein (Chant *et al.* 1991; Powers *et al.* 1991). The mechanism by which the two small GTP-binding proteins (Bud1p and Cdc42p) function in cellular morphogenesis is not clear, but information about the roles of other small GTP-binding proteins suggests several possibilities. Bud1p and Cdc42p might direct or monitor a cycle of assembly and disassembly of a complex at the bud site. This cycle might be involved in proof-reading to improve fidelity of bud-site selection, or it might rapidly

amplify and communicate the position of the selected bud site to the rest of the cell.

The pheromone response pathway and cell polarization. A signal transduction pathway is activated when **a**-factor or α-factor bind to their respective receptors (Figure 1-2). The mating factors and receptors are the only cell-type-specific components of the pathway; signal transduction downstream of the receptors uses a common set of molecules in both **a** and α cells, including a heterotrimeric G protein, $G_{\alpha\beta\gamma}$ (Dietzel and Kurjan 1987; Miyajima *et al.* 1987). Stimulation of the receptor causes Gα to switch from the GDP-bound state to the GTP-bound state, which leads to release of Gβγ. Free Gβγ then initiaties the pheromone response (Blinder, Bouvier and Jenness 1989; Whiteway *et al.* 1989; Whiteway, Hougan and Thomas 1990). The direct target of Gβγ is not known, but it acts through STE5 and STE20 (Blinder, Bouvier and Jenness 1989) to activate a cascade of serine/threonine kinases, consisting of STE7, STE11, and FUS3 which is functionally redundant with another kinase KSS1 (reviewed in Neiman 1993).

The molecular details of how the pheromone-induced signal transduction pathway results in a specific response are partially understood for transcriptional induction and cell cycle arrest. A transcription factor encoded by STE12 is activated by phosphorylation (Song *et al.* 1991), probably by one or more of the pathway protein kinases. STE12 protein induces transcription of many genes involved in mating by its action at pheromone response elements present in the upstream regulatory regions of target genes. Cell cycle arrest results from the pheromone-induced activation of

Figure 1-2: Yeast pheromone signal transduction pathway. Stimulated receptors activate a G protein which triggers a cascade of protein kinases, resulting in several mating responses. The arrow from the G-protein to morphology changes is speculative and indicates the position at which the localized signal for cytoskeletal organization may branch off from the rest of the pathway. Some components required for morphological response may need to be induced by the pheromone response pathway, hence an arrow after STE12.


pheromone
Receptor
G-protein
α
γ
β
Morphology changes ?
STE5 ?
STE20 kinase
STE11 kinase
STE7 kinase
FUS3/KSS1 kinase
STE12 transcriptional activator
Transcriptional induction
Cell cycle arrest
Morphology changes ?

FAR1 and FUS3, which contribute to the inactivation of the three G1 cyclins (CLN1, CLN2, and CLN3) (Richardson *et al.* 1989; Wittenberg, Sugimoto and Reed 1990; Chang and Herskowitz 1990). FAR1 is activated by STE12-dependent transcriptional induction and FUS3-dependent phosphorylation and is responsible for inhibiting CLN2 (Chang and Herskowitz 1990; Peter *et al.* 1993) and presumably CLN1. FUS3 is transcriptionally induced by mating factors (Elion, Grisafi and Fink 1990) and is thought to inhibit CLN3 (Elion, Brill and Fink 1991).

The connection between the pheromone response pathway and molecules which act to polarize the cell is much less clear. Identification of signalling components required for cell polarization would be possible by creating yeast strains in which the pathway has been severed by mutation and activated downstream to allow mating. These strains could then be tested for ability to carry out pheromone-induced cell polarization. Such a scheme was used to examine the components required for mating partner discrimination (Schrick and Hartwell 1993). It was found that the pheromone receptor and the G protein are required for mating partner discrimination, but none of the other genes in the conventional signal transduction pathway (STE5, STE11, STE7, KSS1, and FUS3) are required. If the discrimination assay truly measures the ability of a cell to organize the cytoskeleton towards a mating partner, then these results suggest a functional branchpoint from the G protein to the cytoskeleton. The molecules which organize the cytoskeleton must remain localized in the cell to retain the directional information from the pheromone gradient. This requirement would be fulfilled

by a branchpoint at the level of the G protein, which is spatially coupled both to the receptor and to the membrane. An important challenge of this model is to test mutant strains defective in each gene in the pathway in the aforementioned shmoo orientation assay (Segall 1993).

Neutrophils and amoebae polarize toward extracellular signals during chemotaxis. The best-studied eucaryotic models of oriented movement toward a stimulus are chemotaxis in neutrophils and amoebae (reviewed in Devreotes and Zigmond 1988; Caterina and Devreotes 1991). Neutrophils, also called polymorphonuclear leukocytes, are mammalian phagocytic cells that carry out chemotaxis in response to bacterial products such as N-formylated peptides. *Dictyostelium discoideum* amoebae are unicellular slime molds that chemotax towards a source of cAMP during their developmental process of becoming a multicellular organism. The dissection of the molecular events involved in chemotaxis in these two cell types sheds light on how yeast and other eucaryotic cells are able to generate cellular asymmetry in response to chemical gradients in the environment.

In both neutrophils and amoebae, chemoattractants bind to surface receptors that contain seven transmembrane domains (Thomas, Pyun and Navarro 1990; Klein *et al.* 1988). There is good evidence that the chemotaxis signal is transmitted through a receptor-coupled G protein. In neutrophils, formyl peptides induce GTP hydrolyisis in membrane preparations (Hyslop *et al.* 1984), and preincubation with pertussis toxin inhibits chemotaxis (Becker *et al.* 1986). The formyl peptide receptor copurifies with two putative

GTP-binding proteins (Polakis, Uhing and Snyderman 1988; Polakis and Snyderman 1988), although these have not yet been cloned. *Dictyostelium* amoebae express numerous Gα subunits at different stages of development (Wu and Devreotes 1991) and mutants deleted for one of them (Gα2) are unable to carry out cAMP-mediated responses (Kumagai *et al.* 1991).

In both cell types, activation with chemoattractants triggers a cascade of molecular events and the production of several second messengers. Phospholipase C is activated to generate inositol triphosphate, causing release of intracellular calcium stores, and diaclyglycerol, which leads to activation of protein kinase C (reviewed in Newell *et al.* 1990; Sklar and Omann 1990). These responses are G protein-mediated: pertussis toxin-treated neutrophils and *Dictyostelium* Gα2 null mutants fail to elevate IP3 or cytoplasmic Ca^{2+} in response to chemoattractants (Bominaar *et al.* 1991). Within seconds after chemoattractant addition, rapid polymerization of actin occurs, resulting in doubling of the amount of filamentous actin in the lamellipodia of neutrophils (Fechheimer and Zigmond 1983; Wallace *et al.* 1984) and of amoebae (McRobbie and Newell 1984). Actin polymerization is an essential response, since cytochalasin treatment completely blocks chemotaxis in either cell type (Howard and Oresajo 1985; White, Naccache and Sha'afi 1983; Hall *et al.* 1989).

It is clear that actin polymerization is a result of a G protein-mediated signal: actin polymerization is blocked in neutrophils treated with pertussis toxin (Bengtsson, Stendahl and Andersson 1986), and GTP can stimulate actin polymerization in

electropermeabilized cells (Thierrien and Nacchache 1989). However, the relevant targets of the G protein and the second messengers which may promote actin polymerization are unclear. In neutrophils, protein kinase C and metabolism of phosphatidylinositol may be involved, as gradients of diaclyglycerol stimulate leukocyte chemotaxis (Wright *et al.* 1988). Elevation of cytoplasmic calcium is not required in neutrophils: depletion of extracellular and cytoplasmic calcium stores by EGTA does not inhibit the chemotactic response (Zigmond *et al.* 1988; Perez *et al.* 1989). In contrast, there is evidence that calcium could be involved in *Dictyostelium*. In saponin-permeabilized amoebae, the addition of IP_3 or Ca^{2+} causes rapid actin polymerization (Europe-Finner and Newell 1986).

In summary, chemotaxis in other eucaryotes exhibits great similarity to pheromone-induced cell polarization in yeast in that binding of chemoattractant to a cell-surface receptor activates a G protein and results in actin reorganization. The identity of the localized signal responsible for initiating cytoskeletal organization is not known in any system, but degradation or sequestration of messengers such as diacylglycerol, IP_3 or calcium may be involved.

Site selection and polarity establishment during mating: a model. All results reported so far are consistent with a view in which yeast cells achieve a high efficiency of mating with an appropriate mating partner by determining the location of a pheromone gradient and polarizing mating structures directly towards it. We hypothesize that activated pheromone receptors generate an intracellular spatial signal which is recognized by

molecules capable of locally organizing the cytoskeleton, the polarity establishment proteins. It is likely that information is passed from the pheromone receptor to the polarity establishment proteins via the G protein, considering the requirement for the G protein in mating partner discrimination (Schrick and Hartwell 1993) and the G protein dependence of chemotaxis in neutrophils and *Dictyostelium* (Devreotes and Zigmond 1988). It remains possible, however, that a conformational change or a clustering in the intracellular domains of pheromone receptors caused by pheromone binding could directly recruit one or more polarity establishment proteins.

The target of the G protein target and thus the nature of the spatial cue for cytoskeletal organization is completely unknown. A yeast phospholipase C has recently been identified (Payne and Fitzgerald-Hayes 1993), but mutants in the phospholipase C gene were not reported to display any mating or shmooing defects. A potential mediator of cytoskeletal organization in yeast is calcium. Addition of pheromone causes a rapid influx of Ca^{2+} from the media (Tachikawa *et al.* 1987; Ohsumi and Anraku 1985; Iida, Yagawa and Anraku 1990. A localized elevation in intracellular Ca^{2+} levels could cause organization of the cytoskeleton in the proper place by activating polarity-establishment gene products. CDC24 encodes a putative Ca^{2+} binding protein (Miyamoto *et al.* 1987), and a mutant in CDC24 is sensitive to high calcium (Ohya *et al.* 1986). Both calmodulin (Brockerhoff and Davis 1992) and BEM1 protein (Corrado 1992) are concentrated at sites of cell growth, raising the possibility that Ca^{2+}-bound calmodulin could influence the localization of Bem1p. An alternative possibility for the establishment of cell

polarity is that Gβγ interacts directly with cytoskeletal organizing molecules such as Cdc24p or Bem1p. Small molecule signals would not be required in this case.

In summary, the question of how external signals influence cell polarity is being approached from two directions: by searching for relevant targets of the receptor and the G protein and by investigating what molecules actin and polarity establishment proteins respond to in pheromone-stimulated cells. An eventual intersection of these two types of investigations will lead to an understanding of the connection between an extracellular cue and cell polarity.

The goal of this work was to learn about how mating factors influence cell polarity in yeast. A genetic screen for mating defects followed by a microscopic screen for morphology defects identified genes required for pheromone-induced cell polarization. One of these genes, BEM1, was studied in depth, and several others are being cloned and analyzed by Nicole Valtz. A second genetic screen identified other genes which interact with BEM1. Some of the genes and proteins identified in this study appear to comprise components of the link between mating factors and the cell polarization machinery.

CHAPTER 2

IDENTIFICATION OF GENES REQUIRED FOR PHEROMONE-INDUCED CELL POLARIZATION IN *SACCHAROMYCES CEREVISIAE*

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. In order 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*. 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.

INTRODUCTION

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 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 towards each other (Byers and Goetsch 1975; Byers 1981), 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; Jackson and Hartwell 1990b; 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, Neiman and Herskowitz 1991; Kurjan 1992).

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, Taylor and Ballou 1976; Tkacz and MacKay 1979; Field and Schekman 1980. This growth reflects the polarized orientation of the cytoskeleton (reviewed by Barnes, Drubin and Stearns 1990; Read, Okamura and Drubin 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 analogue 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, Klis and Tanner 1988), Fus1p (Trueheart, Boeke and Fink 1987), Spa2p (Gehrung and Snyder 1990), Ste2p (Jackson, Konopka and Hartwell 1991), Fus2p (E. Elion, personal communication), and Ste6p (Kuchler, Dohlman and Thorner 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 make-up 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 (*CDC24*, *CDC42*, *BEM1*) (reviewed in Drubin 1991). Mutations in the bud-site selection genes cause improper positioning of the bud but no growth defect (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 are slow growing or inviable (Sloat and Pringle 1978; Sloat, Adams and Pringle 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 signalling but produces uniformly enlarged cells rather than polarized shmoos (Konopka, Jenness and Hartwell 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. In order 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 2-1. Crosses, sporulation and tetrad dissection were performed as described previously (Sherman, Fink and Hicks 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).

Table 2-1

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	" *bem1-s1*	this study	mutant derived from JC2-1B
JC-F5	" *bem1-s2*	this study	mutant derived from JC2-1B
JC-J9	" *pea1-1*	this study	mutant derived from JC2-1B
JC-A10	" *pea2-1*	this study	mutant derived from JC2-1B
JC-G16	" *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	*MATa far1-c lys1*	this study	enfeebled mating tester
IH2351	*MATa fus1-D1 fus2-D3 ura3-52 trp1D1*	G. Fink	enfeebled mating tester
IH1793	*MATa lys1*	IH lab collection	mating tester
IH1792	*MAT***a** *lys1 cry1*	IH lab collection	mating tester
IH993	*MATa sst2-1 met1 his6 can1 cyh2*	IH lab collection	halo tester
IH414	*MAT***a** *bar1 arg9 ilv3 ura1* killer$^+$	IH lab collection	halo tester

IH2356	*MATa trp1 leu2 ura3 his4 FUS1::lacZ(URA3)*	IH lab collection	
IH2431 (E187Ja)	*MATa cdc24-3(ts)*	J. Pringle	Sloat, Adams and Pringle 1981
IH2433 (CJ198)	*MATa cdc43(ts) ura3 trp1*	J. Pringle	
KO1-1A	*MATa bem1::URA3 trp1 leu2 ura3 his4*	this study	
KO2-5B	*MATa bem1::LEU2 trp1 leu2 ura3 his4*	this study	Chenevert *et al.* 1992
JC108	*MATa trp1 ade2 ura3 bem1-s1* (G11 mutation)	this study	used for complementation tests
NVY14	*MATa leu1 trp5 ura3 bar1 pea1-1* (D6 mutation)	this study	used for complementation tests
NVY20	*MATa leu1 trp5 ura3 bar1 pea2-1* (I14 mutation)	this study	used for complementation tests
NV52a	*MATa leu1 trp5 ura3 bar1 tny1-1* (G16 mutation)	this study	used for complementation tests
JC117	*MATa bem1-s1 his4 leu2 ura3 FUS1::lacZ(URA3)*	this study	used for quantitative matings
NVY123	*MATa leu1 trp5 bar1*	this study	used for quantitative matings
NVY124	*MATa pea1-1 leu1 trp5 bar1*	this study	used for quantitative matings
NVY126	*MATa tny1-1 trp1 leu5 ade2 bar1*	this study	used for quantitative matings
NVY118	*MATa pea2-1 leu1 trp5 ade2 bar1*	this study	used for quantitative matings

Mutagenesis: Strain JC2-1B was mutagenized by exposure to ethylmethane sulfonate (EMS; Sigma). The genotype of this strain (see Table 2-1) 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 ul EMS was added to the remaining one ml culture. The treated tubes were incubated at 30°C 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 thiophosphate. The mutagenized cells were then washed twice with water, once with YEPD, and resuspended in one ml YEPD. These cultures were frozen for two 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°C 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°C 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 2-1) were patched in the middle of each streak with a toothpick. After overnight incubation at 30°C, 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 resistant): (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 2-1). The ability to produce **a**-factor was seen as a zone of growth inhibition surounding the patch.

In order to observe the morphological response of the mutant strains to pheromone, cultures of each were grown to mid-log phase in YEPD at 30°C (OD_{600} = 0.7), α-factor was added to a final concentration of 10^{-6} M, and the cultures were returned to 30°C 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°C instead of 37°C. Approximately 3 x 10^6 cells from exponentially growing cultures of each strain were mixed and then filtered onto 0.45 um nitrocellulose filters (Millipore). The filters were rinsed with 5 ml YEPD, placed on YEPD plates, and incubated for 4 hr at 30°C. 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 frequecy 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 HindIII 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 BglII fragment containing *FAR1* from pFC21 (F. Chang, unpublished) cloned into the BamHI 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 *Saccharomyces 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 towards a mating partner (F. Chang, thesis). *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, Boeke and Fink 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, Boeke and Fink 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. C., 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), 138 were defective in mating to the enfeebled tester strains (Figure 2-1; Table 2-2). 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 were not arrested by pheromone (Figure 2-1, Table 2-2); 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 52 pheromone-sensitive mutants, 9 were deficient in **a**-factor production and were not studied further.

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

Figure 2-1. Mutant phenotypes.

Three representative mating-defective mutants (A) 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.

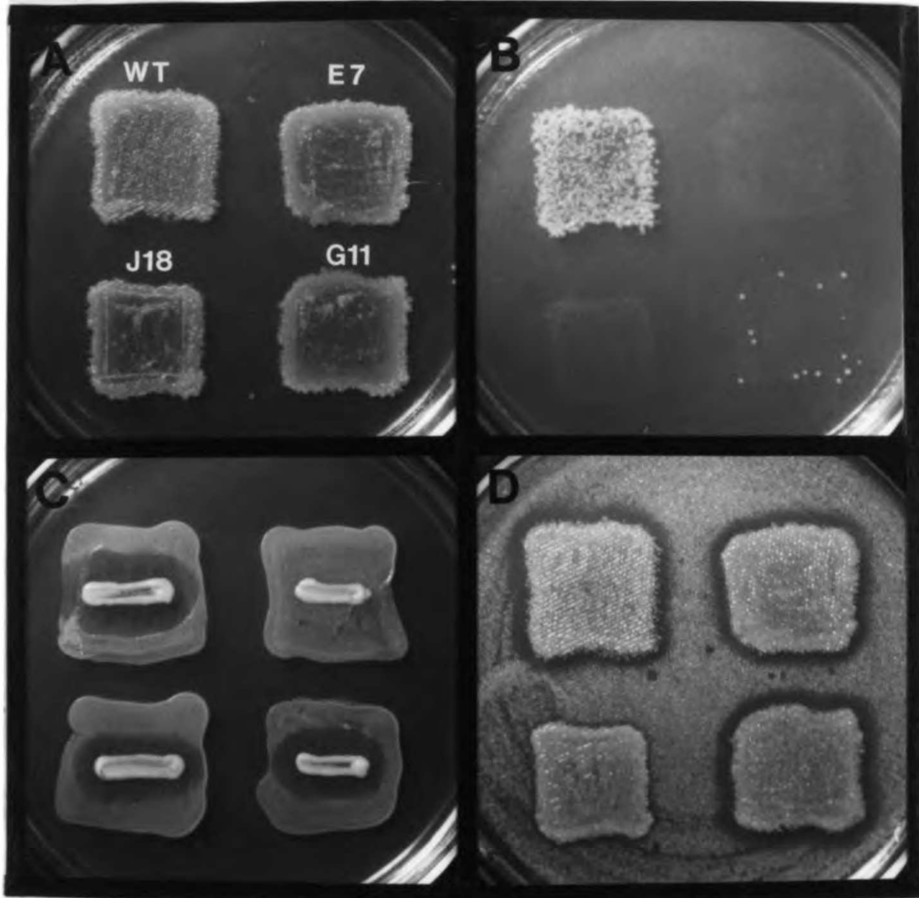

A
WT
E7
J18
G11
B
C
D

Table 2-2

Demographics of Mutant Hunt

Mutant Phenotype	Number 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

displayed a bipolar pattern (S. Sanders, personal communication). The final tally of candidates with potential defects in mating-related polarization was 34 (see Table 2-2). 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. These mutants were treated with pheromone and characterized microscopically. A variety of shapes was observed, falling into five broad categories (Table 2-3 and Figure 2-2): (1) One class of mutants (shmooless mutants, 3 isolates) appeared to be unable to form shmoos but instead enlarged in a uniform manner (Fig. 2-2B). (2) A second class (peanut mutants, 4 isolates) formed cells that were roughly symmetrical and had two lobes (Fig. 2-2D). (3) The third class (tiny mutants, 2 isolates) contained about 50% shmoos of unusually small size (Fig. 2-2C). (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.

Table 2-3

Morphologies of Mutants in the Presence of Pheromone

Mutants		Number	Designations	Genes
homogeneous terminal morphologies:				
Class 1	shmoo-less	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 2-2.

Figure 2-2: Morphologies of yeast strains in the presence of pheromone.

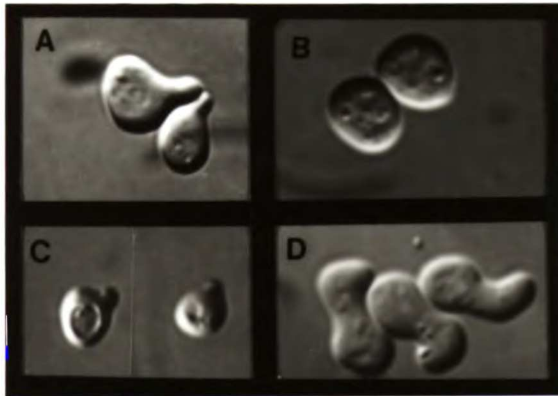
A: wild-type **a** strain JC2-1B

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.


A
B
C
D

We focussed on the shmooless, peanut shmoo, and tiny shmoo mutants because they displayed abnormal but homogeneous morphologies in the presence of pheromone.

Shmooless mutants are defective in polarity establishment genes *BEM1* and *CDC24*: Complementation tests were performed to determine whether the three 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 2-4).

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 Pringle 1991). 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-typerecombinants were not recovered (cross 1, Table 2-5).

Two observations on mutant I13 gave clues to its identity. First, it was unable to form colonies at high temperature (>33°C) and

Table 2-4

Complementation Test of Shmooless Mutants

A. *MAT*a/*MAT*a diploids derived from mating between	mating[a]
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	+

B. haploids:	growth at 37°C
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.

grew slower than a wild-type strain at 30°C. Second, it did not yield prototrophic diploids when mated with a *cdc24-ts* mutant at 37°C (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°C) to yield a diploid. This strain was unable to grow at nonpermissive temperature (37°C), indicating that I13 contains a mutation in *CDC24*. This mutation is recessive and did complement *cdc43* and *bem1* mutations (Table 2-4). The diploid formed by mating I13 to the *cdc24-ts* strain IH2431 yielded meiotic segregants all of which were temperature sensitive (Table 2-5), 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 2-5). A *MAT*α segregant containing the mutation present in D6 was obtained (strain NVY14, Table 2-1) 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

TABLE 2-5

Allelism Analysis

Cross	PD	NPD	T	% wild-type segregants	phenotype scored
(1) G11 X KO1-1A (*bem1*)	10	0	0	0[a]	mating
conclusion: mutant G11 contains a mutation in *BEM1*					
(2) I13 x IH 2431 (*cdc24*)	12	0	0	0[a]	growth at 37°C
conclusion: mutant I13 contains a mutation in *CDC24*					
(3) NVY14 (D6) x I14	3	0	3	17	mating[b] and peanut morphology
(4) NVY14 (D6) x A10	4	0	4	14	"
(5) NVY14 (D6) x J9	13	0	0	0	"
(6) NVY20 (I14) x J9	2	0	6	20	"
(7) NVY20 (I14) x A10	8	0	0	0	"
conclusion: D6 and J9 contain mutations that are allelic; A10 and I14 contain mutations that are allelic					
(8) NV52a (G16) x 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* strain JC31-7D or *fus1 fus2* strain IH2351

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 2-5 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*: In order 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 of the resultant diploid (Table 2-5) indicates that they are defective in the same gene, which we call *TNY1*.

Mutants in *BEM1* 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 2-6. 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*

Table 2-6

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.

mutant and a *MATα beml* mutant (.00001), however, is lower than the product of the mating frequencies observed for a *MAT***a** *beml* mutant to a *MATα BEM1* strain (.018) and a *MAT***a** *BEM1* strain to a *MATα beml* mutant (.002). Thus a mutation in *BEM1* confers an apparent bilateral mating defect. We have observed in this and other experiments that *MAT***a***/MATα beml/beml* diploids grow substantially slower than *MAT***a** *beml* or *MATα beml* 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** *peal* mutant and a *MATα peal* mutant, for example, is not significantly lower than the product of the mating frequencies observed for a *MAT***a** *peal* mutant to a *MATα PEA1* strain and a *MAT***a** *PEA1* strain to a *MATα peal* mutant.

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 apparently 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 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, Goldman and Hartwell 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.

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, Drubin and Stearns 1990; Byers 1981; Read, Okamura and Drubin 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 *cdc24-5*) 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, Adams and Pringle 1981). *cdc24-5* strains grow normally at 25°C, but slower than wild-type or *cdc24-4* at 30°C. Thus the function of the *CDC24* protein in the *cdc24-5* strain is compromised at 30°C, which could explain why it exhibits a defect

in shmooing and mating at 30°C.

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 and J. C., 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, Yagawa and Anraku 1990) and in cells defective in calcineurin, a Ca^{++}/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^{++} 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, Gehrung and Snyder 1992). Mutations in this gene were identified as being synthetically lethal with *spa2* mutations (Costigan, Gehrung and Snyder 1992) and independently as dominant suppressors of a defect in protein kinase

C (Lee and Levin 1992). *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, 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 leads to arest of shmoo tip growth when productive mating is not achieved.

We have recently cloned the *PEA1* gene and obtained partial sequence information (N.V., 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 an identical mutant phenotype 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.

CHAPTER 3

A YEAST GENE (BEM1) NECESSARY FOR CELL POLARIZATION IN RESPONSE TO INTRACELLULAR AND EXTRACELLULAR SIGNALS WHOSE PRODUCT CONTAINS TWO SH3 DOMAINS

ABSTRACT

Polarization of a cell requires that a cellular axis or cell-surface site be chosen and that the cytoskeleton be organized with respect to it. The molecular nature of the link between the cytoskeleton and the chosen axis or site is not well understood (Luna 1991). Cells of the yeast Saccharomyces cerevisiae exhibit cell polarization in two phases of their life cycle, during vegetative growth and during mating, which reflects responses to intracellular and extracellular signals, respectively. Here we describe the isolation of two mutants defective specifically in cell polarization in response to peptide mating pheromones. The mutants prove to harbor special alleles (denoted bem1-s) of the BEM1 gene recently shown to be required for cell polarization during vegetative growth (Bender and Pringle 1991; Chant *et al.* 1991). Unlike other bem1 mutants, the bem1-s mutants are normal for vegetative growth. Complete deletion of BEM1 leads to the defect in polarization of vegetative cells observed for previously identified bem1 mutants (Bender and Pringle 1991; Chant *et al.* 1991). The predicted sequence of the BEM1 protein (Bem1p) reveals two copies of a domain (denoted SH3) that has been found in many proteins associated with the cortical cytoskeleton and that may mediate binding to actin or some other component of the cell cortex (Drubin *et al.* 1990; Rodaway, Sternberg and Bentley 1990). The sequence of Bem1p and the properties of mutants defective in this protein indicate that it may be an element that links the cytoskeleton to morphogenetic determinants on the cell surface.

MATERIAL AND METHODS

Yeast strains, media and genetic manipulations. Yeast strains are listed in Table 2-1 and Appendix 4. Crosses, sporulation and tetrad dissection were performed as described previously (Sherman, Fink and Hicks 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).

Isolation of mutants. Mutants were isolated in a two-step screen. First, mutants were isolated that were unable to mate with strains that themselves have reduced mating ability (defective in FAR1 (Chang and Herskowitz 1990) or in FUS1 and FUS2 (Trueheart, Boeke and Fink 1987). These mutants were then examined for their morphological response to mating pheromone, which in wild-type cells induces the formation of shmoos (Figure 3-1g).

Assay of morphology. For viewing under the microscope, yeast strains were grown in YEPD medium to early log phase at 30°C, except for the bem1-deletion strain, which was grown at 25°C. Where appropriate, pheromone (α-factor; Sigma) was added to a final concentation of 10^{-6} M, and the cells were grown for a further 2-3 hours. To observe morphology, live cells were viewed by DIC (Differential Interference Contrast) microscopy. Cells were fixed and stained with phalloidin to visualize actin or with Calcofluor to visualize chitin as described previously (Pringle *et al.* 1989).

Identical results were obtained when an anti-actin antibody was used to visualize actin (data not shown).

Mating tests. Patches of **a** strains isogenic except at the BEM1 locus were replica-plated to lawns of wild-type or mutant α strains on YEPD (permissive) medium and incubated at 30°C for approximately 5 hours to allow mating. These plates were then replica-plated to minimal (restrictive) medium on which only diploid cells were able to grow and incubated for a further 36 hours. MAT**a** bem1-s and MATα bem1-s strains exhibited similar mating defects.

Cloning and sequencing of BEM1. BEM1 was cloned using a genomic DNA library in a low-copy number vector (YCp50) (Rose *et al.* 1987) by complementation of the mating defect of a bem1-s1 strain. The complementing gene was confirmed to be the wild-type allele of the bem1-s mutation and not a suppressor by segregation analysis using the original bem1-s mutation and a mutation constructed in vitro in the cloned gene which was integrated by homologous recombination (data not shown). The Sanger dideoxy chain termination method was used for sequencing M13 clones generated using restriction sites or a series of ExoIII-generated deletions in pBluescript SK+ and KS+. The nucleotide sequences of both strands were determined independently in both the Ann Arbor and San Francisco laboratories and were in exact agreement. The predicted protein sequence was compared to the PIR/Dayhoff database using the FASTA program (Pearson and Lipman 1988); significant homologies were detected only for proteins containing an SH3 domain (see Figure3-4).

Deletion of BEM1. The strain deleted for BEM1 contains the

LEU2 gene in place of the entire BEM1 coding sequence and was constructed by a scheme suggested by Tim Stearns, as follows: A fragment containing BEM1 and several hundred base pairs of flanking DNA was cloned into a pUC vector. A portion of this plasmid was amplified by the Polymerase Chain Reaction using divergent primers that hybridized just upstream of the BEM1 start codon and just downstream of the stop codon. The resulting linear fragment was cleaved at a site introduced by the primers and circularized, yielding a plasmid that contained just the BEM1 flanking regions joined by a restriction site. A selectable marker (LEU2) was then introduced into this site. A linear fragment containing the marker gene surrounded by the BEM1 flanking regions was used to replace the chromosomal BEM1 gene by the one-step gene replacement method (Rothstein 1983). Southern blotting confirmed that gene replacement had occurred by homologous recombination (data not shown).

RESULTS

The bem1-s mutants were identified in a screen designed to detect nonmating mutants specifically defective in localizing cell-surface growth towards a mating partner (details to be described elsewhere; see Figure 3-1 legend). In contrast to wild-type strains, which form polarized cells called shmoos when treated with mating pheromone (Figure 3-1g), two mutants were found which enlarged in a uniform manner (the Shmoo$^-$ phenotype; Figure 3-1j). The mutations in these strains, designated bem1-s1 and bem1-s2, were

Figure 3-1. Morphology (left column), chitin distribution (middle column), and actin distribution (right column) in wild-type and *bem1* mutant cells of a mating type.

A, B, C: wild-type strain KO2-5A

D, E, F: *bem1-s1* mutant strain JC-G11

G, H, I: wild-type strain JC2-1B treated with α-factor

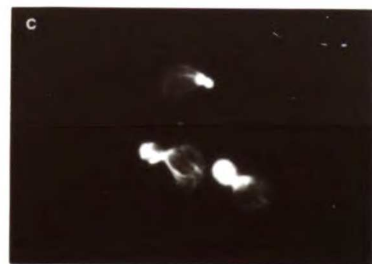
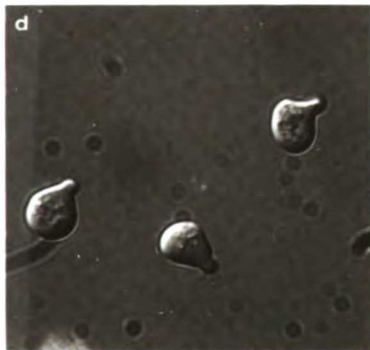
J, K, L: *bem1-s1* mutant strain JC-G11 treated with α-factor

M: *bem1-s2* mutant strain JC-F5 treated with α-factor

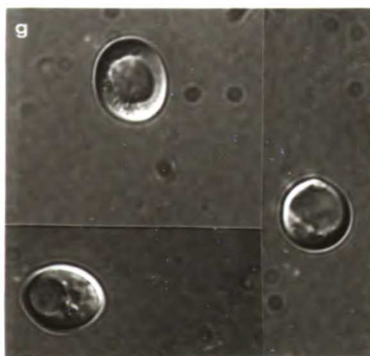
N, O, P: *bem1*-deletion strain KO2-5C

Final magnifications are the same in all panels. Representative cells are shown.

a

b

c

d

e

f

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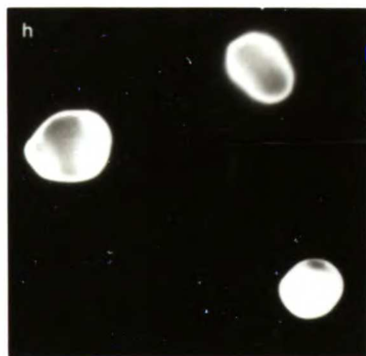
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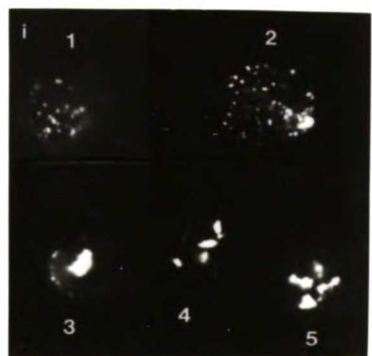
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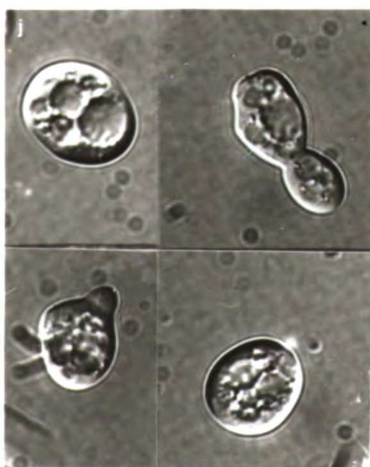
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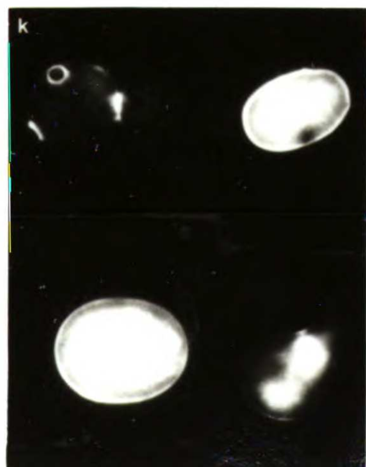

k

l

shown to affect the same gene by segregation and complementation tests (data not shown). These mutants exhibited a weak mating defect when mated to wild-type cells and a strong mating defect when mated with other bem1-s mutants (Figure 3-2). The bem1-s mutants grew normally at all temperatures on rich and minimal media. Their morphologies and budding patterns were similar to those of wild-type cells (Figure 3-1a, b, d, e).

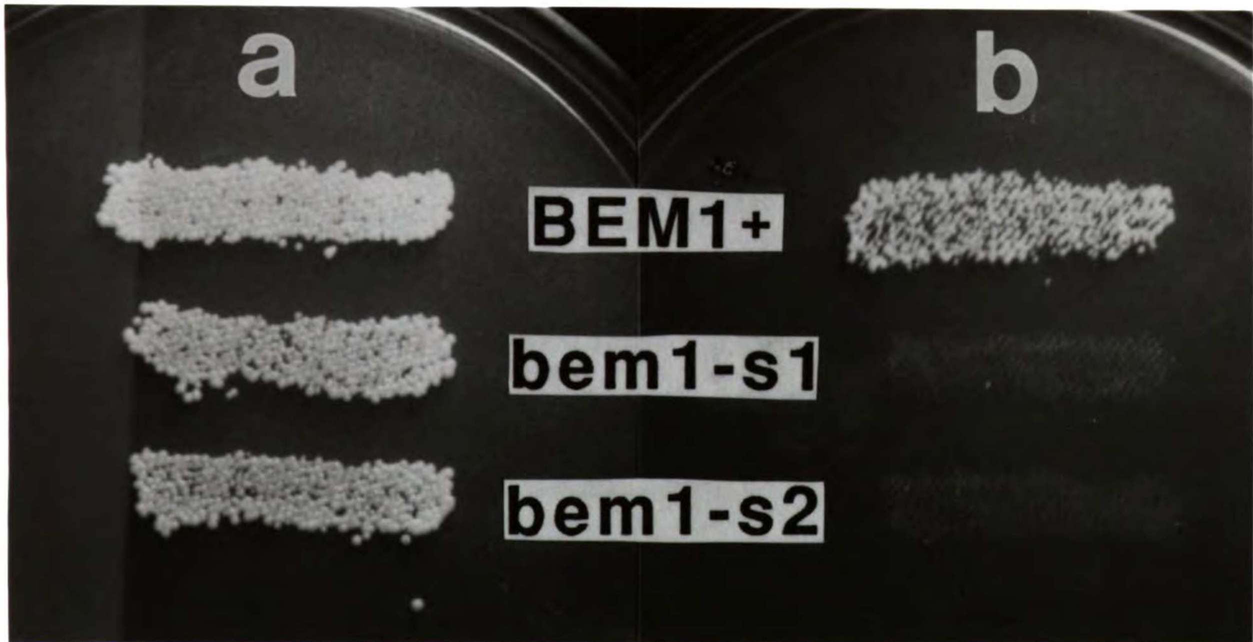
To examine the polarization defect of the bem1-s mutants in more detail, we determined the distribution of cell-wall chitin by staining with Calcofluor and the distribution of actin by staining with rhodamine-phalloidin and by immunofluorescence using an actin-specific antibody (Pringle *et al.* 1989). Chitin is localized predominantly to the shmoo neck in wild-type cells exposed to pheromone (Figure 3-1h). In contrast, bem1-s mutants deposited chitin throughout the enlarging cell surface (Figure 3-1k). The actin organization in vegetatively growing bem1-s cells generally appeared normal: the actin patches were concentrated in the buds, and actin cables ran through the mother cells into the buds (compare Figure 3-1f and 3-1c). Wild-type cells exhibited a similarly polarized actin distribution during shmoo formation (Figure 3-1i). In contrast, the bem1-s cells exhibited a delocalized actin distribution after addition of pheromone (Figure 3-1l). Approximately one-third of bem1-s2 mutant cells showed an additional, more aberrant actin distribution: 1-10 large clumps of actin per cell (Figure 3-1m). The bem1-s mutations did not affect other aspects of pheromone response, notably cell-cycle arrest and induction of FUS1-lacZ (see Chang and Herskowitz 1990).

Figure 3-2. Mating defect of *bem1-s* mutant cells.

A: Mating to *BEM1*$^{+}$ α strain IH1793.

B: Mating to *bem1-s1* α strain JC107.

The **a** strains are *BEM1*$^{+}$, JC2-1B; *bem1-s1*, JC-G11; *bem1-s2*, JC-F5.


a
b
BEM1+
bem1-s1
bem1-s2

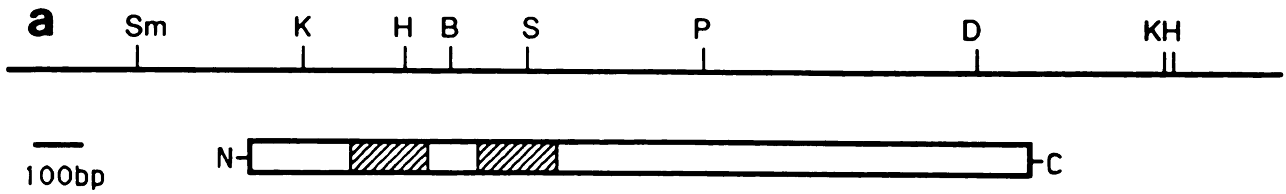
BEM1 was cloned by complementation of the mating defect of a bem1-s1 strain (see Figure 3-3 legend) and had been cloned independently by complementation of the vegetative growth defect of a bem1-3 mutant (Bender and Pringle 1991). The nucleotide sequences of both BEM1 isolates were identical. The predicted BEM1 protein contains 551 amino acids (Figure 3-3) with a calculated molecular weight of 62 kD. The amino-terminal half contains two regions similar to the "SH3 domain" (Figure 3-4), a motif of 50 amino-acid residues that has been found in a variety of disparate proteins, including nonreceptor tyrosine kinases, myosin-I, phospholipase C, and the yeast actin-associated protein Abp1p (Drubin *et al.* 1990). The function of the SH3 domain is unknown, but many of the proteins containing this motif associate with the cortical cytoskeleton or localize to the plasma membrane (see Drubin *et al.* 1990; Rodaway, Sternberg and Bentley 1990).

To investigate further the role of Bem1p during vegetative growth, a strain deleted for BEM1 was constructed. The chromosomal BEM1 coding sequence was replaced precisely by a fragment containing LEU2 (see legend to Figure 3-1). Cells deleted for BEM1 were able to germinate but grew slowly at room temperature or at 30°C and not at all at 14°C or 37°C. Cells grown at 25°C or 30°C displayed heterogeneous, odd morphologies (Fiure 3-1n): some were either triangular or shmoo-like; others were very large and rounded and usually unbudded. Many cells were multinucleate (as seen by DAPI staining; not shown). Chitin deposition was delocalized (Figure 3-1o) and the asymmetric actin distribution seen in wild-type cells was disrupted (Figures 3-1f, 1p).

Figure 3-3. <u>BEM1</u> map and sequence.

A: Restriction map of sequenced region and location of the open reading frame. Hatched boxes indicate the positions of the SH3 domains. Restriction sites are as follows: B, *Bam*HI; D, *Dra*I; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

B: Nucleotide and predicted amino-acid sequences of <u>BEM1</u>. The SH3 domains are boxed.



b

```
   1: acggcatcacatctggggttttatgatttaatttaggacgcacatagatagatatatattatggaattaagaaaagtttataatgtagcaacctaaaaatgttacaggtgaacattcatggaaaat

 127: gaaatgtttctcagccagtagatagtattgttgaaggtattattggtttgagaaagctactcctcaaaaatgtcacttgataatatggaattataatgtcgttatattttcaatcatttcaataca

 253: atatggtaaaccgaaatccaaaaactttacaatatctcttatctattgttattcgacattcttcccgggccttaacggcttcttaactaatattaccctaaacggacaaatggcgaagggccccat

 379: tcatttacgaacaggaaaaaaagaaagtgacaatacagccataatttgatgtaccatcttaatcagcatatgttttcactaatatactaaacccatatggatgcacgttgaaagcactgtgtgaaa

 505: agaaattgtcaagaaagccatataaatgctgaaaaacttcaaactctcaaaaagagatagtaatgggtcgaagggcagaattacatccgcagatatttccacaccttctcatgataatgggagtgt
                                M  L  K  N  F  K  L  S  K  R  D  S  N  G  S  K  G  R  I  T  S  A  D  I  S  T  P  S  H  D  N  G  S  V

 631: tataaagcatattaagacagtaccagtgaggtacctttcttcatcttccaccccagtgaaaagccaacgggactcttcaccaaaaacagacataattctaaagatattacttctccagagaaagt
       I  K  H  I  K  T  V  P  V  R  Y  L  S  S  S  S  T  P  V  K  S  Q  R  D  S  S  P  K  N  R  H  N  S  K  D  I  T  S  P  E  K  V

 757: tataaaagccaaatacagttatcaggcacaaacttcaaaggaactatctttcatggaaggtgaatttttttatgtatctggagatgagaaggattggtataaagcttcaaatccttctactggaaa
       I  K [A  K  Y  S  Y  Q  A  Q  T  S  K  E  L  S  F  M  E  G  E  F  F  Y  V  S  G  D  E  K  D  W  Y  K  A  S  N  P  S  T  G  K

 883: agagggtgttgtccccaaaacctatttttgaagtgtttgatagaactaaaccttcctctgtgaatggatccaatagttcaagtcggaaggttacaaatgattcattaaacatgggatcgttatacgc
       E  G  V  V  P  K  T  Y  F] E  V  F  D  R  T  K  P  S  S  V  N  G  S  N  S  S  S  R  K  V  T  N  D  S  L  N  M  G  S  L  Y  A

1009: cattgttttatatgactttaaggcagaaaaagctgatgagttgacaacttatgtgggagagaatttgtttatttgcgcccaccataactgtgaatggttcattgctaagccaattggtcgacttgg
       I [V  L  Y  D  F  K  A  E  K  A  D  E  L  T  T  Y  V  G  E  N  L  F  I  C  A  H  H  N  C  E  W  F  I  A  K  P  I  G  R  L  G

1135: agggcccggccttgttcctgttgggtttgttagcatcatagatattgccacggggtacgcaacaggtaacgacgtaatagaagacatcaagtcagttaacctaccaactgttcaagaatggaaaag
       G  P  G  L  V  P  V  G  F  V] S  I  I  D  I  A  T  G  Y  A  T  G  N  D  V  I  E  D  I  K  S  V  N  L  P  T  V  Q  E  W  K  S

1261: caacattgcaagatacaaagctagtaatattagccttggttctgtggagcagcagcagcagcaatccattacaaaacctcagaacaaaagcgcaaagctggttgacggtgaattattagtgaaagc
       N  I  A  R  Y  K  A  S  N  I  S  L  G  S  V  E  Q  Q  Q  Q  Q  S  I  T  K  P  Q  N  K  S  A  K  L  V  D  G  E  L  L  V  K  A

1387: atccgttgaatcctttgggttagaagatgagaagtattggtttttggtttgctgtgaattatctaacggtaagacgaggcaactgaaaagatactatcaagatttctacgatctgcaggttcaact
       S  V  E  S  F  G  L  E  D  E  K  Y  W  F  L  V  C  C  E  L  S  N  G  K  T  R  Q  L  K  R  Y  Y  Q  D  F  Y  D  L  Q  V  Q  L

1513: tttggatgcgttcccagcagaggccggtaagttgagggatgcaggtggacaatggtccaaacgtataatgccctatatccctggacccgttccatacgttaccaatagcataacaaaaaaaagaaa
       L  D  A  F  P  A  E  A  G  K  L  R  D  A  G  G  Q  W  S  K  R  I  M  P  Y  I  P  G  P  V  P  Y  V  T  N  S  I  T  K  K  R  K

1639: ggaggacttgaatatatatgtggcagacctagtgaatcttcccgattatatatctcgctcagaaatggtgcattccttattcgttgttttgaataatggtttcgacagagaatttgaaagagacga
       E  D  L  N  I  Y  V  A  D  L  V  N  L  P  D  Y  I  S  R  S  E  M  V  H  S  L  F  V  V  L  N  N  G  F  D  R  E  F  E  R  D  E

1765: aaatcaaaacaatattaaaacattacaagaaaacgatacagcaacctttgcaacagcatctcaaacttctaatttgcctcaactaaccaagacaacactttaactggcgaagacttgaagttaaa
       N  Q  N  N  I  K  T  L  Q  E  N  D  T  A  T  F  A  T  A  S  Q  T  S  N  F  A  S  T  N  Q  D  N  T  L  T  G  E  D  L  K  L  N

1891: caagaaactctcagatttatctttatcgggctccaagcaggctccagcccaatcaacttctggattgaaaactactaaaattaaattttattacaaagacgatattttgctttaatgctgaaggg
       K  K  L  S  D  L  S  L  S  G  S  K  Q  A  P  A  Q  S  T  S  G  L  K  T  T  K  I  K  F  Y  Y  K  D  D  I  F  A  L  M  L  K  G

2017: tgacacaacctataaagaacttcgtagtaaaatcgctccaaggattgatacagataatttaaattacaaaccaaattatttgatggtagcggggaggaaattaagactgattcacaagtcagtaa
       D  T  T  Y  K  E  L  R  S  K  I  A  P  R  I  D  T  D  N  F  K  L  Q  T  K  L  F  D  G  S  G  E  E  I  K  T  D  S  Q  V  S  N

2143: cataatccaagccaaactgaaaatttccgttcacgatatttgaatctagtgttagaagacgaagcatttttcttctttacttggcagatgcacataggttgaacattttaatcaactttatgatga
       I  I  Q  A  K  L  K  I  S  V  H  D  I  @

2269: tttgcgagagtaataagattctcccgaacagtaataatattaaggtatccaattcttccgaaacagcgtgatttgtaataaaaataaataaggacatatatgtatatagaaagttgcttaaacttt

2395: ttagaccacttggaaatatttttatttatatttttcttcaaatgtaaagttgggtacctaagcttaaattcacctgaaaatgaattatatgaaagattttacaccacatgtaaaaaaaaacgtac

2521: aaaaagccaaatatttccactcaatcataatgcatgaattataggtattttaagttatattgctgacgtaaaattcagaagagactacaagaaaattctaacatatttattctt
```

Figure 3-4. Amino-acid alignment of homologous regions of Bem1p and representative SH3 domain-containing proteins. The amino-acid of the first residue of each sequence is given in parentheses. Φ signifies hydrophobic residues. Boxed residues denote conservation with either BEM1 domain; residues that are identical are boxed and shaded. References to amino-acid sequences: *Acanthamoeba* myosin-IB (Jung, Korn and Hammer 1987), v-yes (Kitamura *et al.* 1982), c-src (Takeya and Hanafusa 1983), α-spectrin (Wasenius *et al.* 1989), yeast ABP1 (Drubin *et al.* 1990), consensus (Rodaway, Sternberg and Bentley 1990).

```
BEM1 (162)        V L Y D F K A E K A D E L T T Y V G E N L F I C A H H N C E W F I A K P I G R L G G P G L V P V G F V
BEM1 (79)         A K Y S Y Q A Q T S K E L S F M E G E F F Y V S G - D E K D W Y K A S N - P S T G K E G V V P K T Y F
MYOSIN 1B (983)   A L Y D F A A E N P D E L T F N E G A V V T V I N K S N P D W W E - G E L N - - G Q R G V F P A S Y V
v-YES (372)       A L Y D Y E A R T T D D L S F K K G E R F Q I I N N T E G D W W E A R S I - A T G K T G Y I P S N Y V
c-SRC (88)        A L Y D Y E S R T E T D L S F K K G E R L Q I V N N T E G D W W L A H S L - T T G Q T G Y I P S N Y V
SPECTRIN (974)    A L Y D Y Q E K S P R E V T M K K G D I L T L L N S T N K D W W K V - E V N - D R Q - G F V P A A Y V
ABP1 (539)        A E Y D Y D A A E D N E L T F V E N D K I I N I E F V D D D W W - L G E L E K D G S K G L F P S N Y V

CONSENSUS         A L Y D Y           E Φ   Φ     G E   Φ   Φ Φ           W W                     G Φ Φ P     Y Φ
```

Shift of a culture to 37°C resulted in a population with heterogeneous terminal morphologies and an increased fraction of unbudded cells (70% vs. 45% at lower temperatures). The phenotypes of the bem1 null mutant are similar to those of bem1-3 and bem1-4 mutants identified previously (Bender and Pringle 1991).

DISCUSSION

Three observations thus indicate that Bem1p plays a direct role in organizing the actin cytoskeleton. First, bem1 null mutants display a disorganized actin cytoskeleton. Second, bem1-s2 mutants produce distinctive clumps ("bars") of actin resembling those that accumulate in certain actin (Novick and Botstein 1985) or profilin (Haarer *et al.* 1990) mutants. Third, Bem1p contains two SH3 motifs. We propose that Bem1p has two functional domains, one (containing the SH3 domains) concerned with binding actin or some other component of the cortical cytoskeleton, the second involved with recognizing the site for cell polarization during vegetative growth or mating. In vegetative cells, the morphological landmark may be a structure that is adjacent to a site of prior budding (see Chant and Herskowitz 1991). The BUD gene products are proposed to direct the positioning of Cdc24p, Cdc42p, and Bem1p to this site (Chant *et al.* 1991; Chant and Herskowitz 1991; Ruggieri *et al.* 1992). During mating, a cell polarizes towards a signal from the mating partner which is likely a gradient of secreted pheromone (Jackson and Hartwell 1990a). This process requires, first, an override of the vegetative bud-site-selection program and, second, establishment of

polarity towards the mating partner. Erasing the bud-site-selection program may be accomplished by pheromone-induced inactivation of one or more of the BUD gene products. The site-recognition domain of Bem1p would then be free to interact with the new site; bem1-s mutants might be unable to interact with this site (but capable of interacting with the site in vegetative cells). Several observations suggest that the morphogenetic marker in mating cells might be the pheromone receptor itself. First, cells lacking the carboxy-terminal segment of the pheromone receptor exhibit the same polarization defect as bem1-s mutants (Konopka, Jenness and Hartwell 1988). Second, receptors cluster at the site of pheromone-induced morphogenesis (Jackson, Konopka and Hartwell 1991). They apparently can play a role in cell polarization that is independent of their coupled G protein and associated signalling pathway (Jackson, Konopka and Hartwell 1991). Spa2p is also necessary for polarization in response to mating factors (Gehrung and Snyder 1990) and may interact with Bem1p or the morphogenetic determinant. By determining the location of Bem1p and molecules with which it interacts, we may begin to understand how the cytoskeleton recognizes signals on the cell surface to form a polarized cell.

CHAPTER 4

CHARACTERIZATION OF MUTATIONS IN *BEM1* WHICH DISRUPT CELL POLARIZATION IN RESPONSE TO MATING PHEROMONES

ABSTRACT

Yeast cells can polarize either to form a bud (during vegetative growth) or a shmoo (during conjugation). BEM1 is a gene required for both types of polarization: a yeast strain deleted for bem1 is impaired in budding and shmooing. Two special alleles of BEM1 exist (bem1-s1 and bem1-s2) which are proficient for budding but defective in mating and shmooing. We analyzed the bem1-s alleles and find that they are different from wild-type BEM1 in two ways: the bem1-s proteins are truncated and are also present in reduced amount. When the levels of the mutant bem1-s proteins are elevated, normal mating is restored. Thus the phenotypes of the bem1-s alleles are apparently not due to a defect in a specific protein domain, but rather to a reduced level of Bem1p. By varying the expression level of a nontruncated BEM1 fusion protein, we show that less Bem1p is required for the budding function than for the mating function. Bem1p itself is not induced in reponse to pheromone. During these studies another bem1 mutant, bem1-s3, was generated which encodes a BEM1 protein lacking an internal segment of 191 amino acids. This internally deleted bem1 allele confers a bem1-s-like phenotype on yeast cells: they bud properly but are defective in mating. Some of the mutants affect one or both of the SH3 domains of BEM1. We propose that Bem1p performs essentially the same function in polarizing the cell for budding or for mating and that more Bem1p is required to perform this function during mating than during budding.

INTRODUCTION

Yeast cells polarize during two phases of their life cycle: during cell division and during mating. In both cases new membrane and cell wall material is inserted at a defined site on the cell surface so that the cell grows in a polarized manner (Tkacz and Lampen 1972; Tkacz and Lampen 1973; Lipke, Taylor and Ballou 1976; Tkacz and MacKay 1979; Field and Schekman 1980). The cytoskeleton orients toward the growth site: actin dots localize at this site and actin cables run along the cell surface towards this site (Adams and Pringle 1984; Ford and Pringle 1986; Hasek *et al.* 1987; Gehrung and Snyder 1990). The spindle pole body, the yeast analog of the centrosome, orients towards the site and cytoplasmic microtubules which emanate from the spindle pole body point toward the bud site or the shmoo site (Byers 1981; Rose and Fink 1987; Gehrung and Snyder 1990; Meluh and Rose 1990). Eventually the nucleus and other organelles migrate towards the site and accumulate there (Byers and Goetsch 1975; Tkacz and MacKay 1979; Hasek *et al.* 1987; Rose and Fink 1987; Segev, Mulholland and Botstein 1988; Baba *et al.* 1989; Gehrung and Snyder 1990). During vegetative growth, this localized morphogenesis results in bud growth and the eventual formation of a daughter cell. During mating, the localized morphogenesis of one cell toward another cell opposite mating type results in cell fusion and the formation of a diploid zygote cell (reviewed in Cross *et al.* 1988).

The site at which a yeast cell forms a bud is chosen according

to defined spatial patterns: MATa or MATα cells, for example, bud near previous bud sites (Friefelder 1960; Hicks, Strathern and Herskowitz 1977). Mutants which form normal buds but at improper positions identify the bud-site selection genes (BUD1 -5) (Chant and Herskowitz 1991; Chant *et al.* 1991). During mating, however, the intrinsic bud site selection program is overridden, and a cell chooses a site based on an extracellular signal from its mating partner. This signal is apparently a high concentration of mating pheromone (Kurjan 1985; Michaelis and Herskowitz 1988; Jackson and Hartwell 1990a; Jackson and Hartwell 1990b; Segall 1993). Purified pheromone from one cell type causes yeast cells of the opposite cell type to develop a polarized morphology known as a shmoo. The cytoskeleton of a shmoo is oriented toward the cell's growth tip in a similar manner as in a budding cell (Ford and Pringle 1986; Hasek *et al.* 1987; Rose and Fink 1987; Gehrung and Snyder 1990; Meluh and Rose 1990; Barnes, Drubin and Stearns 1990; Read, Okamura and Drubin 1992), and proteins important in mating are localized to the shmoo tip (Watzele, Klis and Tanner 1988; Trueheart, Boeke and Fink 1987; Gehrung and Snyder 1990; Jackson, Konopka and Hartwell 1991; Kuchler, Dohlman and Thorner 1993; E. Elion, personal communication).

Polarity establishment genes BEM1, CDC24, and CDC42 are required to polarize the yeast cell; mutants defective in any of these genes are unable to polarize growth and instead display delocalized actin deposition and uniform cell surface growth (Sloat and Pringle 1978; Sloat, Adams and Pringle 1981; Adams *et al.* 1990; Johnson and Pringle 1990; Bender and Pringle 1991; Chant *et al.* 1991;

Chenevert *et al.* 1992). Thus the products of these genes are responsible, directly or indirectly, for the localization of the cytoskeleton and the growth machinery of the cell to a single site on the cell surface. Genetic studies of BUD genes and polarity establishment genes indicate that they interact in order to form a bud (Bender and Pringle 1989; Chant *et al.* 1991). BUD gene products are thought to recognize some intracellular landmark, perhaps a remnant of a previous cell division, and direct the positioning of polarity establishment gene products so that growth occurs at the proper site (Chant and Herskowitz 1991; Snyder, Gehrung and Page 1991). Proteins may exist which perform a similar role during mating. Such "mating site selection" proteins would direct the positioning of polarity establishment gene products away from a bud site and toward the pheromone signal.

The existence of special alleles of BEM1 (bem1-s) which are defective in mating and shmoo formation but not in bud formation (Chenevert *et al.* 1992) indicates that there is a different or additional role of BEM1 in shmooing as compared to its role in budding. This difference may be related to the different site-specificities required for mating versus budding. The bem1-s mutants might be defective in responding to information from mating site selection proteins but not bud site selection proteins. If this were the case, the bem1-s alleles might contain missense mutations clustered in a common protein domain. Alternatively, the mating defect in bem1-s mutants could be unrelated to site specificity but may be due to a more rigorous requirement for BEM1 in mating cells than in budding cells, a requirement that bem1-s

mutant proteins are unable to fulfill. If this were the case, the bem1-s mutations might be expected to generally decrease BEM1 function and may not be clustered in a specific protein domain. In order to understand the nature of the role of Bem1p in mating and shmooing, we analyzed the molecular defects in the bem1-s mutant genes and proteins.

MATERIALS AND METHODS

Yeast strains, media and genetic manipulations. Yeast strains are listed in Table 2-1 and Appendix 4. Crosses, sporulation and tetrad dissection were performed as described previously (Sherman, Fink and Hicks 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).

Mating assays. All matings described are of MAT**a** cells against an enfeebled partner, the MATα far1 strain JC31-7D. Mating assays were routinely done in a qualitative way on colonies and patches essentially as described (Chenevert, Valtz and Herskowitz submitted). A lawn of far1 mating tester was spread on a YEPD plate and the transformants were replica-plated to this lawn. After incubation at 30°C for 4-6 hours, the mating plates were replica-plated to minimal medium (SD) to select for diploids.

Morphology assays. For strains not containing plasmids,

cultures were grown in YEPD to mid-log phase and α-factor was added to a concentration of 1 uM. After further incubation for 1.5-2 hr, cells were sonicated briefly and observed microscopically to determine cell shape. Phenotypes conferred by plasmids are typically assayed in minimal media selecting for the plasmid, but the bem1-s shmooless phenotype is most dramatic in rich media (J. C., unpublished observations), so the following protocol was adopted. Strains containing plasmids were grown overnight in minimal medium lacking uracil to select for the plasmid. They were then diluted in YEPD, grown for 3 hr and treated with α-factor as described above. For each sample, 200 cells were counted and results from three independent experiments were averaged.

BEM1 plasmid constructions. The plasmid used for gap repair was pCENBEM, a 2.1kb SmaI-KpnI fragment containing BEM1 cloned into pRS316 (Sikorski and Hieter 1989). pCENBEM contains two PstI sites, one in BEM1 and one in the vector. In order to construct a plasmid in which the PstI site in BEM1 was converted to a unique EcoRI site, pCENBEM was digested with PstI for a limited time, treated with calf intestinal alkaline phosphatase (Boehringer Mannheim), and run on an agarose gel. Linear molecules which had been cut at only one of the PstI sites were isolated from the gel. Linker oligonucleotides which contain an EcoRI site and a stop codon flanked by PstI cohesive ends were synthesized (sequences: 5'-CTAAGAATTCGTGCA-3' and 5'-ACGTGATTCTTAAGC-3'). These oligonucleotides were ligated to the PstI cleaved pCENBEM, and the ligation mix was transformed into bacteria. A plasmid containing a unique EcoRI site in the BEM1 gene and a unique PstI site in the

vector was recovered and named pBEM324. pbem1-s1 and pbem1-s2 were generated by recovering the bem1-s alleles onto pBEM324, as described in Results ("Recovery and sequence of bem1-s1 and bem1-s2").

pBEMΔN110: A 1.6 kb HindIII fragment containing most of the BEM1 coding region and some downstream sequence was cloned into the HindIII site of yCp50. This construct lacks the amino-terminal 110 amino acids and the promotor of BEM1 and it complements bem1 mutant strains in one orientation with respect to the vector but not the other orientation.

p2uBEM: A high copy BEM1 plasmid was constructed by cloning the 2.1kb SmaI-KpnI fragment containing BEM1 into pRS426 (Sikorski and Hieter 1989). p2ubem1-s1 and p2ubem1-s2 were then constructed by replacing the 1.8 KpnI fragment of p2uBEM with the 1.8 kb KpnI fragment from either pbem1-s1 or pbem1-s2. The bem1-s1 and bem1-s2 mutations lie withing this 1.8 kb KpnI fragment (see Results). Restriction analysis identified clones with the KpnI fragment in the original orientation.

pHABEM: The hemaglutinin (HA) epitope tag (Kolodziej and Young 1991) was introduced into the BEM1 coding sequence by site-directed mutagenesis. An oligonucleotide "oJC-HA" was synthesized which introduces nine amino acids after the first 3 amino acids of BEM1. The sequence of oJC-HA is 5'-GCC ATA TAA **ATG** CTG AAA TAC CCA TAC GAC GTC CCA GAC TAC GCT AAC TTC AAA CTC TCA-3'. The ATG in bold is the BEM1 start codon. The underlined portion of oJC-HA encodes nine amino acids which constitute the HA epitope, and the remaining nucleotides comprise a linear sequence from the

5' region of BEM1. A restriction site for AatII (GACGTC) is introduced by the HA epitope; this facilitates identification of the clones which have successfully incorporated the tag. oJC-HA was phosphorylated with T4 polynucleotide kinase, annealed to a single-stranded M13 phage containing a SmaI-HindIII fragment from the 5' region of BEM1, and used to prime polymerization with Klenow polymerase. Following ligation, the reaction mix was transformed into *E. coli* strain JM101. Infected colonies were transferred to nitrocellulose filters and probed with oJC-HA which had been radioactively labelled with γ^{32}P-ATP and T4 polynucleotide kinase. Double-stranded phage from positively hybridizing clones were prepared and tested for the presence of the diagnostic AatII site. Single-stranded phage from a clone which contained the AatII site was sequenced to verify the presence of the HA epitope. The HA-tagged BEM1 sequence from this clone was then used to replace the corresponding region in pCENBEM in two steps: an EcoRI-HindIII fragment containing the tagged portion of BEM1 was cloned into pRS316 and then cut out on a XbaI-HindIII fragment which was used to replace the XbaI-HindIII fragment of BEM1 in pCENBEM. The resulting plasmid, pHABEM, was transformed into bem1 mutant strains JC-G11 and KO2-5C and wild-type strains JC2-1B and KO2-5A, and the effects on bem1 mutant phenotypes were tested. pHABEM completely complements the mating and growth defects of bem1 mutants and does not interfere with growth or mating of BEM1$^+$ strains (data not shown).

pGAL-GST-BEM: A plasmid containing most of BEM1 fused to the 5' end of the glutathione-S-transferase gene under the control of

the inducible GAL1 promoter was generated by cloning a 1.5 kb BamHI-KpnI fragment from pCENBEM containing BEM1 into pRD56 (from R. DeShaies). This plasmid, named pGAL-GST-BEM, encodes a fusion protein in which the initial 140 amino acids of Bem1p have been replaced by 220 amino acids of glutathione-S-transferase.

Fortuitous generation of a new BEM1 allele during first attempt at gap repair. The mutant bem1-s alleles were recovered by the method of gap repair (Orr-Weaver and Szostak 1983; Guthrie and Fink 1991). A plasmid containing BEM1 (pCENBEM) was cut at two unique restriction sites (HindIII and SalI) which cut within the BEM1 gene (Figure 4-1A). This gapped plasmid (Figure 4-1A, pCENBEMΔHS) was then purified on an agarose gel, recut and transformed into bem1-s1 strain JC-G11 and bem1-s2 strain JC-F5. The transformants were replica-plated to the far1 mating tester JC31-7D to determine if they were able to mate (and had thus repaired the BEM1 gene on the plasmid to a wild-type gene) or mating defective (and had healed the gap with the mutant portion of the bem1-s allele from the chromosome). For the bem1-s1 strain, 64 out of 137 total transformants were mating defective; and for the bem1-s2 strain 50 out of 152 total transformants were mating defective. Plasmids from two mating-defective bem1-s1 transformants and from two mating-defective bem1-s2 transformants were recovered. Surprisingly, restriction analysis revealed that all four plasmids lacked 500-600 base pairs between the KpnI and PstI sites in BEM1; they were missing the HindIII, BamI, and SalI sites completely (Figure 4-1A, pbem1-s3). The region in question was sequenced from two of the recovered

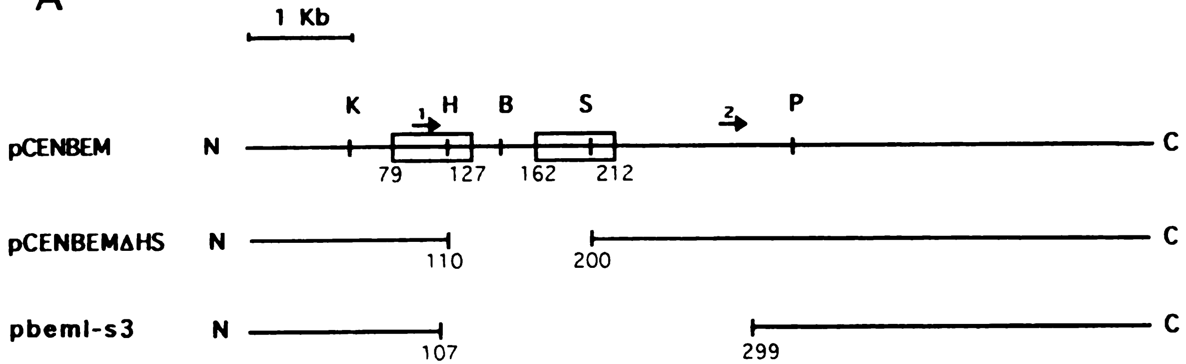
Figure 4-1. Generation of an internal deletion of BEM1 by homologous recombination at direct repeats.

A: Schematics of BEM1 (as found in pCENBEM), the gapped BEM1 construct which was transformed into yeast (pCENBEMΔHS) and the further deleted BEM1 mutant which was recovered from yeast (pbem1-s3). Numbers refer to amino acid positions. Bold areas indicate SH3 domains. Restriction sites discussed in the text are indicated: K=KpnI, H=HindIII, B=BamHI, S=SalI, P=PstI. Arrows indicate positions of the small direct repeats at which recombination occurred.

B: Protein and nucleotide sequences of the two repeats at which recombination occurred and the sequence at the junction of the resultant recombined protein. The homologous region is underlined. Recombination occurred within the five bases which are italicized.

C: Possible cryptic SH3 domain generated by recombination event.

A



B

	position	DNA sequence	amino acid sequence
1 → first repeat	104	g<u>ga gat gag aag gat tgg t</u>at	S DEKDW Y
2 → second repeat	295	a<u>ga gat gag aag tat tgg t</u>tt	E DEKYW F
recombined	107-299	*gga gat gag aag gat tgg* ttt	S DEKDW F

C

consensus	ALYDY-A----EΦ-Φ--GE-Φ-Φ----E-DWW-Φ---------GΦΦP--YΦ
BEM1(79)	AKYSY-A----EL-F--GE-F-Y----E-DWY-A---------GVVP--YF
bem1-s3	AKYSY-A----EL-F--GE-F-Y----E-DWF-V---(53)--GPVP YV

plasmids and in both cases revealed a 573 bp (191 aa) deletion between two small direct repeats at positions 104 and 295 in the BEM1 coding sequence (Figure 4-1B). Since the BEM1 gene harboring this 191 aa deletion confers a bem1-s like phenotype on bem1Δ strains (see text), we refer to it as bem1-s3, and to the plasmid bearing the internally deleted BEM1 gene as pbem1-s3.

Recovery and sequence of bem1-s1 and bem1-s2. In the second attempt at gap repair, a larger gap was created which eliminated one of the recombinogenic repeats. A plasmid with two unique restriction sites which were well-spaced within the BEM1 gene was made, by converting the PstI site in BEM1 to an EcoRI site (see Plasmid Constructions). The resulting plasmid, pBEM324, allowed a molecular diagnosis of successful gap repair: repair of the BEM1 gene on the plasmid from the BEM1 chromosomal locus should lead to reconversion of this EcoRI site to PstI (see Figure 4-3A). pBEM324 was cut with HindIII and EcoRI, removing 640 bp of BEM1, gel-purified and transformed into bem1-s1 (JC-G11) and bem1-s2 (JC-F5) strains, and the transformants were scored for ability to mate to far1 strain JC31-7D. All transformants of the bem1-s1 strain, but only 1/4 transformants of the bem1-s2 strain, were mating defective. This observation indicates that that the bem1-s1 mutation lies within the gap and that the bem1-s2 mutation lies outside of but near the gap (as turned out to be the case). Plasmids were recovered from the mating-defective bem1-s1 and bem1-s2 transformants and found to have a restriction map identical to that of the original ungapped pCENBEM and, indeed, had lost the EcoRI site and regained the original PstI site. These plasmids were named

pbem1-s1 and pbem1-s2. Primers homologous to regions of BEM1 inside the gap and surrounding it were synthesized and double-stranded sequencing was carried out on pbem1-s1 and pbem1-s2 in order to locate the bem1-s1 and bem1-s2 mutations.

Protein extracts and western blots: Total yeast cell protein extracts were prepared as described (Peter *et al.* 1993). Cells were centrifuged and resuspended in 2X sample buffer and an equal volume of acid-washed glass beads was added. Samples were vortexed 3 times for 30 sec each with 1 min cooling on ice in between. The proteins were then boiled for 5 min, and centrifuged to pellet beads and cell debris. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electro-blotted to nitrocellulose (Schleicher & Schuell, Inc.) using the BioRad Mini-gel system as recommended by the manufacturer. Blots were blocked by incubation for > 1 hr in Tris-buffered saline-Triton X-100 (TBST; 50 mM Tris pH7.5, 500 mM NaCl, 0.1% Triton X-100) containing 10% fat-free milk powder (Carnation). Primary antibodies were affinity-purified rabbit anti-Bem1p (a generous gift of K. Corrado), affinity-purified polyclonal anti-GST (from D. Kellogg via T. Peter), or monoclonal mouse anti-HA 12CA5 (company). Antibodies were incubated in TBST in 2% milk for 2-4 hr and the blots were washed 3 times for 10 min with TBST. Secondary-antibodies were phosphatase-coupled conjugates of either goat anti-rabbit immunoglobulin G (IgG) (Boehringer Mannheim) or goat-anti-mouse IgG (BioRad Laboratories) diluted 1:3000 in TBST containing 2% milk. After 60 min incubation the blots were washed three times for 10 min with TBST. Blots were developed using epichemiluminescence

detection reagents (Amersham Corporation) as recommended by the manufacturer.

RESULTS

Generation and characterization of bem1-s3. A mutation in a gene on a yeast chromosome may be recovered onto a plasmid by a strategy called gap repair (Orr-Weaver and Szostak 1983, Rothstein 1991). If a plasmid containing homology to the region of interest is gapped, the plasmid will repair the missing information using the resident chromosomal allele as template. In our initial attempt at gap repair of the bem1-s alleles, only plasmids in which 573 bp internal to BEM1 had been deleted were recovered (pbem1-s3, Figure 4-1A). When the sequence of the deleted region was examined in the wild-type BEM1 gene, we noted a 17 base pair sequence which was repeated almost identically at amino acid positions 104 (GAGATGAGAAGgATTGGT) and 295 (GAGATGAGAAGtATTGGT) (Figure 4-1B). Apparently, this amount of homology was sufficient to allow intramolecular homologous recombination, and the gap in BEM1 was healed by deleting the information between the repeats.

pCENBEM contains over 2 kb homology with the chromosomal BEM1 locus, which could have participated in homologous recombination, so it is surprising that recombination between sites with only 16 bp homology of occurred preferentially. Perhaps the preference for the intramolecular event reflects the advantage of concentration of sites in a unimolecular reaction over the difficulty

in locating or gaining access to a relatively small region of the entire genome. We note that, while one entire SH3 domain and one-third of the other SH3 domain have been removed by this internal deletion, most of the consensus residues of the first SH3 domain remain. In fact, if an allowance is made for a spacing gap, the in-frame fusion generates a fairly respectable cryptic SH3 domain (Figure 4-1C).

In order to determine whether the BEM1 gene lacking this 191 amino acid segment was partially functional or behaved as a null allele, pbem1-s3 was transformed into bem1 deletion strains KO2-5B (MATα) and KO2-5C (MATa). The resultant transformants were able to grow at high temperature, but not as well as $BEM1^+$ strain or bem1-s strains (Figure 4-2). The phenotypes conferred by the recovered plasmids on bem1Δ strains were intermediate between those of the bem1Δ and the bem1-s mutants (Table 4-1): they were mating defective but also had a growth defect at high temperature (forming small colonies after three days), although not as severe a growth defect as the bem1 deletion strain (no colonies after three days).

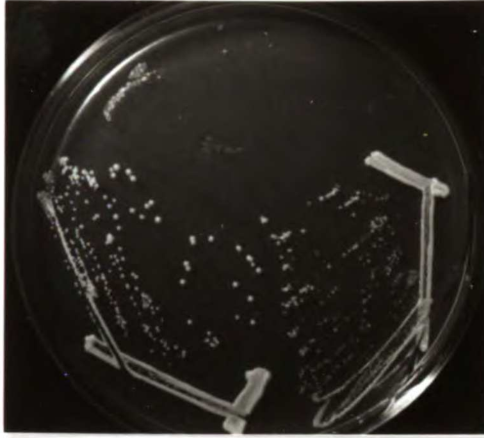
Characterization of bem1-s1 and bem1-s2 mutant genes and proteins. In a second attempt to recover the bem1-s1 and bem1-s2 alleles, a 640 bp gap, encompassing one of the recombinogenic repeats, was created in pCENBEM, and this gapped plasmid was introduced into mutant strains JC-G11 (bem1-s1) and JC-F5 (bem1-s2). The bem1-s alleles were recovered onto plasmids pbem1-s1 and pbem1-s2 and the sequences of the mutations were obtained (see Materials and Methods). One difference was found

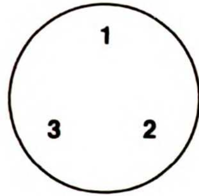
Figure 4-2. pbem1-s3 confers a _bem1-s_-like phenotype on a _bem1Δ_ strain.

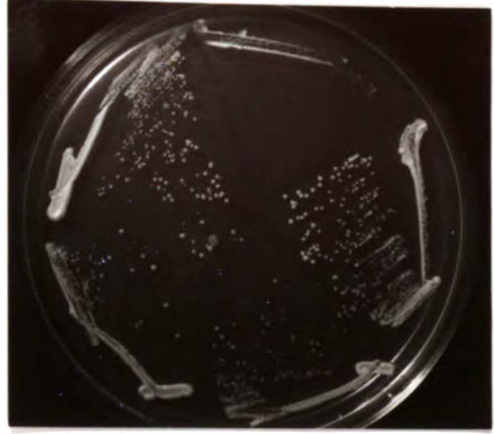
A: Growth at high temperature of _bem1Δ_ strain KO2-5C containing (1) vector alone, (2) pbem1-s3, (3) pCENBEM, (4) pbem1-s1, or (5) pbem1-s2.

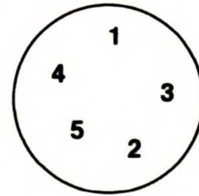
B: Mating to _far1_ of KO2-5C containing plasmids numbered as in Panel A.

A

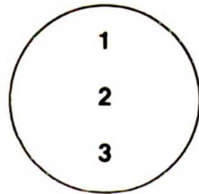


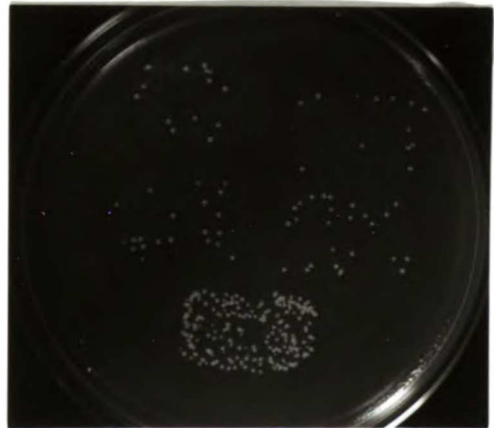






B





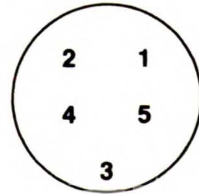


Table 4-1

pbem1-s3 confers a bem1-s like phenotype to strains lacking BEM1

Phenotype	strain: plasmid:	bem1Δ[a] vector	bem1Δ pCENBEM	bem1Δ pbem1-s3	bem1-s1[b] none
growth at 30°C		-[f]	+	+	+
budding morphology		-	+	+/-	+
growth at 37°C		-	+	+/-	+
a-factor production[c]		-	+	+/-	+
α-factor production[c]		-	+	+/-	+
ability to settle[d]		-	+	+	+
color[e]		-	+	-	+
mating		-	+	-	-
shmooing morphology		-	+	-	-

[a]both MATa (KO2-5C) and MATα (KO2-5B) bem1Δ strains were tested.

[b]strain JC-G11

[c]bem1Δ strains show a slight defect in production of a-factor and a strong defect in production of α-factor.

[d]liquid cultures of bem1Δ cells do not settle as rapidly as BEM1+ cells, probably due to their large size and abnormal shapes.

[e]BEM1+ strains are white; bem1Δ strains are yellow.

[f]+ indicates wild-type phenotype; - indicates defective or abnormal for a given phenotype.

between the bem1-s1 allele and the wild-type BEM1 gene (Figure 4-3): codon 299 (TGG, or W) was changed to TAG, a stop codon. No differences were found within the gapped region for the bem1-s2 allele, as was expected, since 3/4 of the bem1-s2 transformants were able to recover wild-type BEM1 function. Sequencing was continued from pbem1-s2 downstream and upstream of the gapped region, and one change was found at codon 519, just 32 amino acids from the C terminus: CAA (Q) was changed to TAA, another stop codon (Figure 4-3). Thus both of the bem1-s mutant alleles are expected to generate truncated proteins, in the bem1-s2 case deleting only 32 amino acids from the C terminus and in the bem1-s1 case deleting nearly half the coding sequence.

In order to verify that the proteins expressed in the bem1-s mutants were the truncated ones predicted from the sequencing, we performed western blots on extracts from these strains using an anti-Bem1p polyclonal antibody. The BEM1 protein from the wild type strain runs at approximately 60 kD, which is comparable to the predicted molecular weight of 61 kD. The protein from the bem1-s1 strain is much smaller, near its predicted size of 33 kD (Figure 4-3C). The bem1-s2 protein appears to be slightly reduced in mobility relative to wild-type (predicted molecular weight is 58 kD). We also noted that the levels of these mutant proteins are much reduced compared to the level of Bem1p in a wild-type strain. This reduction could result from a lack of C-terminal sequences necessary for protein stability or from instability of the RNA or both.

Phenotype of overexpression of bem1-s alleles. In order to address whether the bem1-s mutant phenotype was due to

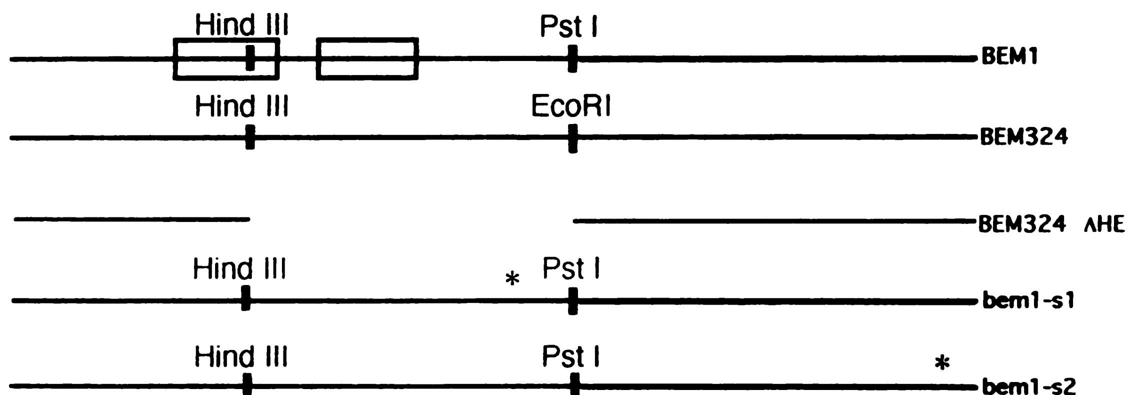
Figure 4-3. The *bem1-s1* and *bem1-s2* mutant alleles encode truncated Bem1 proteins.

A: Strategy for recovery of mutant alleles. Bold areas indicate SH3 domains; * indicates positions of mutations.

B: Base changes in *bem1-s* mutant genes and predicted changes in the gene products.

C: Western blot with anti-Bem1p antibody to wild-type yeast strain JC2-1B (lane 1), *bem1-s1* mutant strain JC-G11 (lane 2), *bem1-s2* mutant strain JC-F5 (lane 3), and *bem1*Δ strain KO2-5C (lanes 4-6) containing pbem1-s1 (lane 4), pbem1-s2 (lane 5), or pbem1-s3 (lane 6).

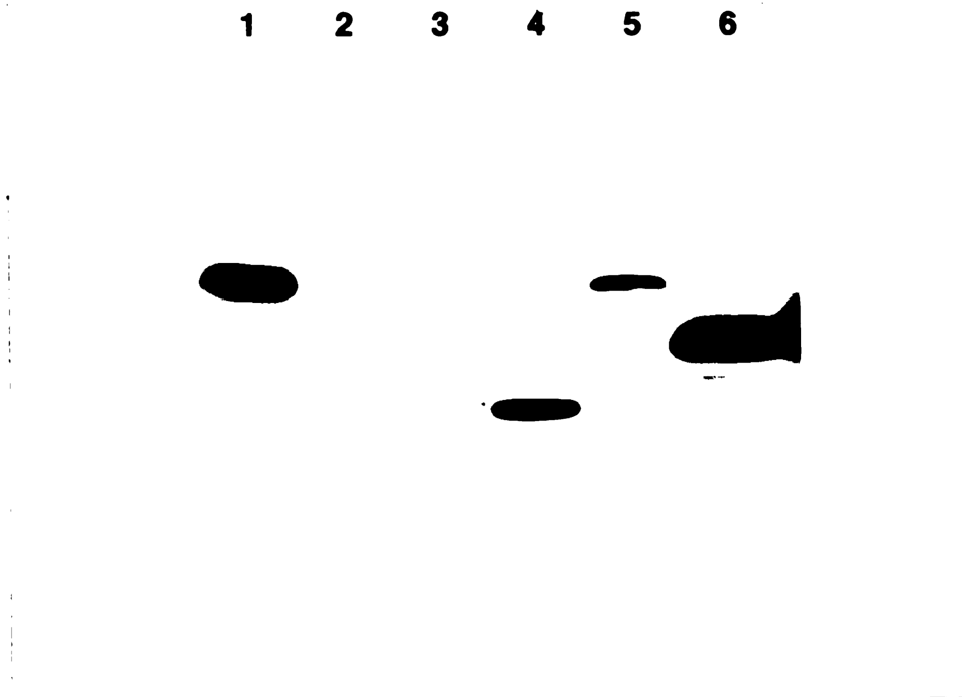
A



B

mutation	position	change	MW of predicted gene product
bem1-s1	299	TGG ->TAG	33kD
bem1-s2	518	CAA ->TAA	58kD

C

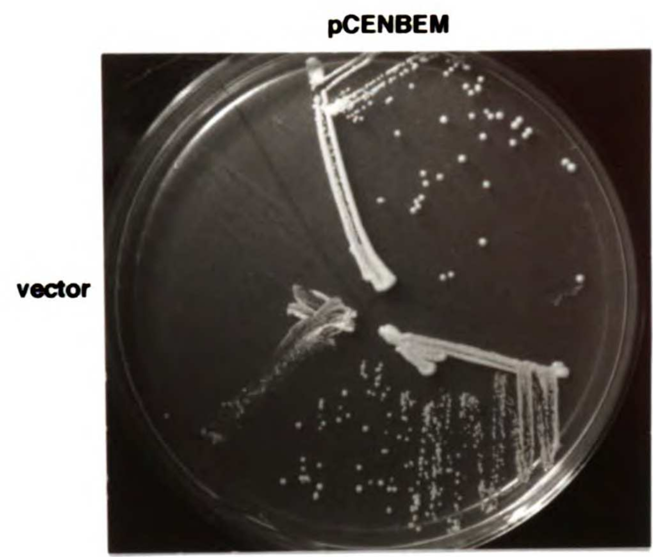


the lack of specific sequences from the protein or to the reduced level of protein, we transferred these mutant alleles to high copy number (2u-based) plasmids to increase the gene copy and presumably the protein amount in the cell. If the bem1-s phenotype is due to a reduced amount of protein, then overproduction of the mutant bem1-s proteins is expected to restore wild-type function. However, if the bem1-s phenotype is due to the lack of specific sequences from the protein, then overproduction of the mutant bem1-s proteins would not be expected to restore function. When introduced into bem1 deletion strain KO2-5C, p2ubem1-s1 and p2ubem1-s2 restore growth at high temperature like the CEN-based bem1-s plasmids (Figure 4-4A). Unlike the single-copy CEN plasmids, however, the 2u plasmids also complement the mating defect of the bem1 deletion strain (Figure 4-4B). p2ubem1-s2 restores mating to near wild-type levels and p2ubem1-s1 restores mating partially, indicating that the carboxy-terminal half of the BEM1 protein is important for efficient mating.

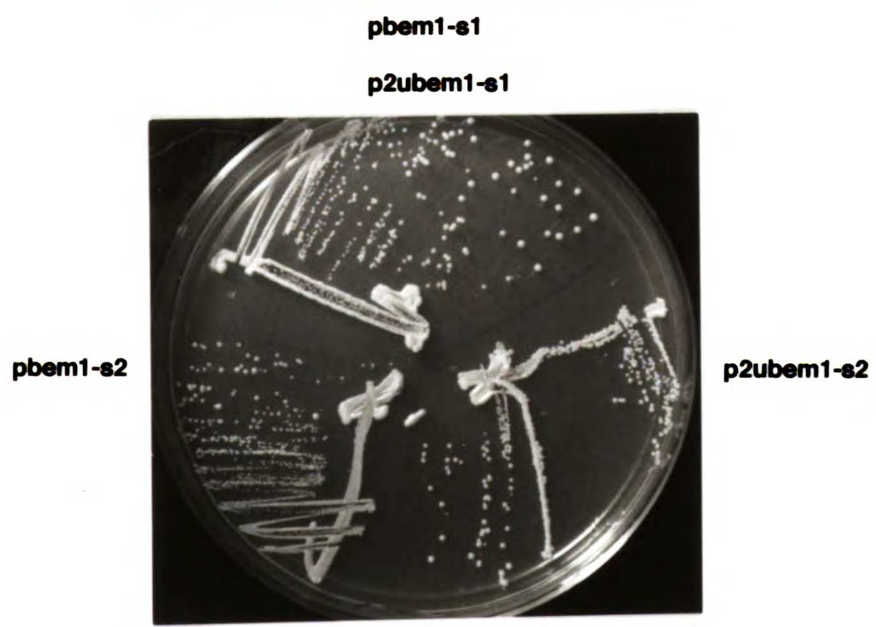
The expression level of BEM1 under an inducible promoter correlates with extent of complementation. In order to test whether a different level of Bem1p is required during mating as opposed to budding, we constructed yeast strains which expressed different amounts of BEM1 and determined whether there was a correlation between BEM1 expression level and the ability to complement mutant phenotypes. A plasmid containing a GST-BEM1 protein fusion under control of the inducible GAL1 promoter (pGAL-GST-BEM) was introduced into bem1 deletion strain KO2-5C. A western blot with anti-GST antibody verified that a fusion protein of

Figure 4-4. Effect of overproduction of <u>bem1-s</u> alleles.
A: Growth at high temperature of <u>bem1</u> deletion strain KO2-5C carrying various plasmids.
B: Mating of KO2-5C carrying various plasmids to <u>far1</u> strain JC37-7D.

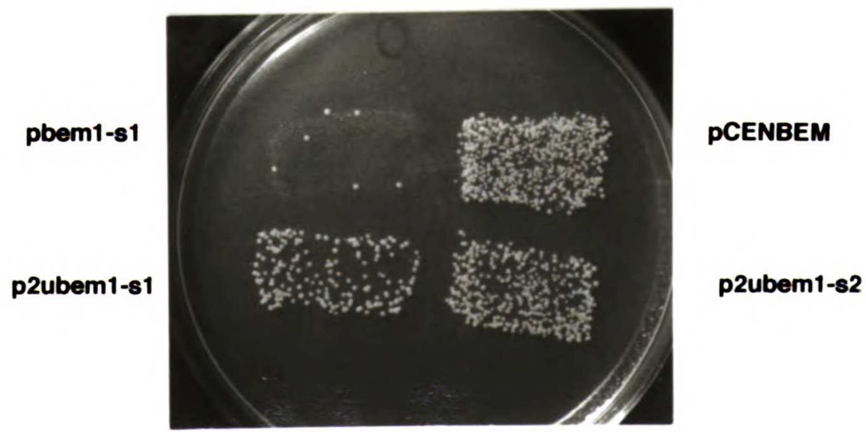
A



pbem1-s1



B



the expected size was produced when the strain was grown under inducing conditions (in galactose); this protein was absent when the strain was grown under noninducing conditions (in glucose) (Figure 4-5A). Complementation of mutant phenotypes was tested on glucose and galactose. The temperature-sensitive defect of KO2-5C was complemented by pGAL-GST-BEM when the cells were grown on glucose as well as galactose (Figure 4-5B). This observation indicates that, although undetectable by western blot, there must be a small amount of BEM1 fusion protein expressed even in the absence of induction and that this amount is sufficient to support growth of a bem1 deletion strain at the nonpermissive temperature. Mating of KO2-5C containing pGAL-GST-BEM was defective on glucose and restored on galactose (Figure 4-5C). A similar result was found for a bem1-s1 strain (JC-G11) containing pGAL-GST-BEM (data not shown). These results indicate that a small amount of BEM1 protein is sufficient for the vegetative function of BEM1 and that greater levels of BEM1 protein are required for the mating function of BEM1.

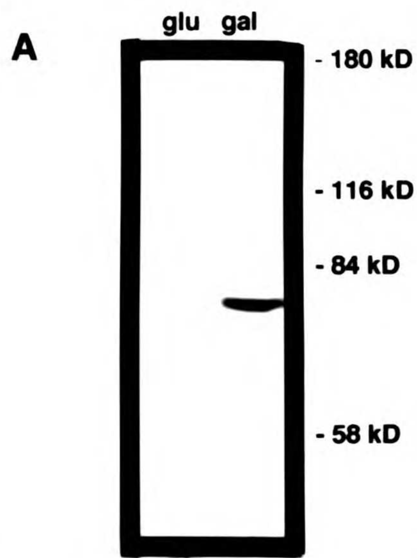
Effect of pheromone treatment on level of Bem1 protein. The results presented thus far indicate that a greater level of Bem1p is required during mating than during budding. The BEM1 gene thus might have been expected to be induced in cells treated with mating pheromone, as are many genes with important roles in mating (Marsh, Neiman and Herskowitz 1991; Kurjan 1992). However this does not appear to be the case. Extracts were prepared from MAT**a** cells of two different wild-type strains containing pHABEM1 treated for varying lengths of time with α-factor. Western blots on these extracts with the anti-HA 12CA5 antibody

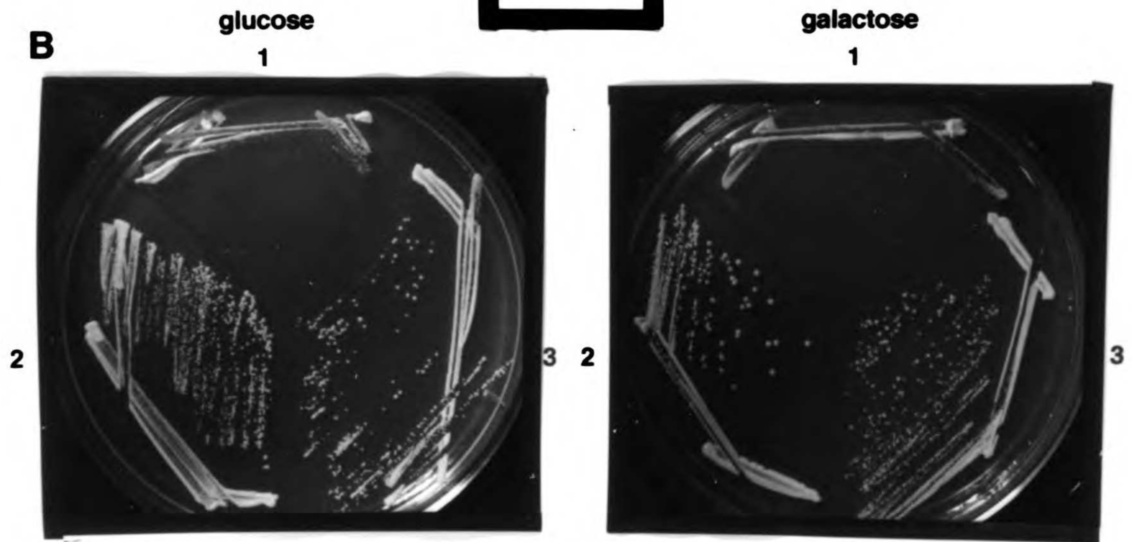
Figure 4-5. Effect of expression of BEM1 from an inducible promoter on bem1 mutant phenotypes.

A: Western blot of bem1Δ strain KO2-5C containint pGALGSTBEM grown in glucose (left lane) or galactose (right lane) and probed with anti-GST antibody. The same amount of total protein extract was loaded in each lane.

B: Growth at high temperature of KO2-5C containing (1) vector alone, (2) pGALGSTBEM, or (3) pCENBEM on either glucose (left) or galactose (right).

C: Mating to far1 of KO2-5C carrying (1) vector alone, (2) pGALGSTBEM, or (3) pCENBEM on either glucose (left) or galactose (right).


A
glu
gal
- 180 kD
- 116 kD
- 84 kD
- 58 kD


B
glucose
1
2
3
galactose
1
2
3

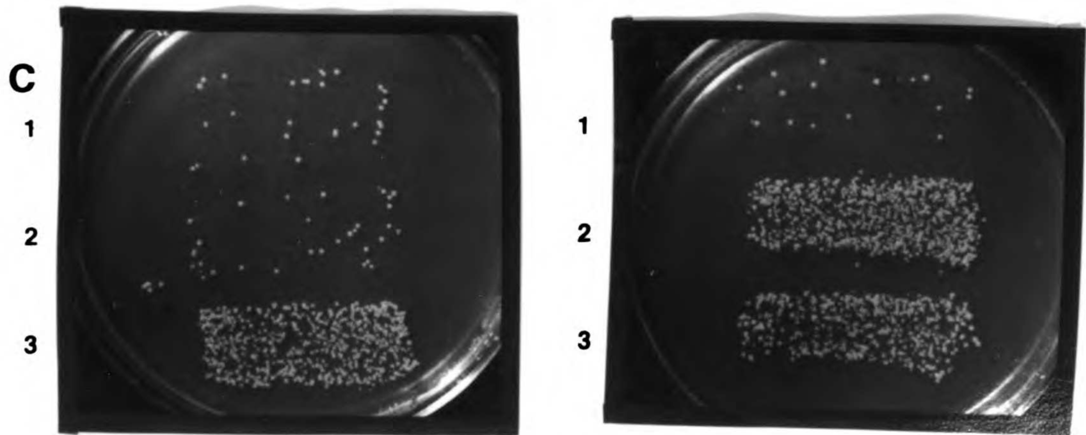

C
1
2
3
1
2
3

Figure 4-6. Pheromone treatment does not alter the amount or mobility of the BEM1 protein.

A: Western blot on extracts from bar1$^-$ yeast strain JC2-1B containing pHABEM1 or BAR1$^+$ strain IH1783 containing pHABEM1 treated with α-factor for 0, 10, 30, or 90 minutes. The filter was immunoblotted with the anti-HA antibody 12CA5. bar1$^-$ yeast strains are supersensitive to α-factor.

B: The same extracts as in panel A were immunoblotted with an anti-Far1p antibody (gift of Matthias Peter). Far1p is known to be induced and phosphorylated in response to mating factor (Chang & Herskowitz 1990; Peter et al., 1993).

BAR1	-	-	-	-	+	+	+	+
min α-factor treatment	0	10	30	90	0	10	30	90

A

B

revealed that Bem1p did not change in abundance or mobility upon pheromone treatment (Figure 4-6). The same result was obtained with the anti-Bem1p polyclonal antibody (data not shown).

DISCUSSION

BEM1 is required for establishing polarity during both vegetative growth and mating (Bender and Pringle 1991; Chant *et al.* 1991; Chenevert *et al.* 1992). The existence of bem1-s mutants which are defective only in the mating function implies that BEM1 may have a different or additional role in mating as opposed to budding. We wanted to distinguish between two possible explanations for bem1-s mutant phenotypes: According to explanation 1, bem1-s mutants are unable to respond to mating site selection information. This model proposes that BEM1 has a novel activity or specificity in pheromone treated cells. According to explanation 2, bem1-s mutants are crippled in a way that allows only partial BEM1 function and this level of BEM1 activity is sufficient for budding but not for mating. In this scenario, Bem1p performs the same activity in budding and mating cells and does not have any added specificity in pheromone-treated cells.

Bem1p: one function or two?: Two independent arguments suggest that a greater level of BEM1 protein is required in mating cells than in budding cells. First, we found that the BEM1 protein produced by bem1-s mutants is reduced in amount and that expressing the mutant proteins on high copy number plasmids

restores mating ability. If bem1-s mutants were defective in some function unique to mating cells, then producing more of the mutant proteins would not necessarily be expected to restore mating. Rather, the defect in bem1-s mutants is likely due to a reduced amount of protein leading to compromised activity. Second, the level of expression of BEM1 from an inducible promoter correlated with extent of complementation: low levels of Bempl were sufficient to provide the vegetative function, but high levels were necessary to complement the mating defect. Both observations demonstrate that the vegetative function of Bem1p can be performed by a low amount of the protein, much less than is actually present in budding cells (compare Figure 4-3C lanes 2 and 3 with lane 1).

This model raises two interesting questions regarding the role of BEM1 in budding and mating. First, why is there an excess of BEM1 protein in budding cells? One possibility for the excess of Bem1p in vegetative cells is that it is present in anticipation of receiving a pheromone signal. It is likely that proper orientation of the cytoskeleton must occur quickly during mating. If the localized spatial signal which initiates cell polarization is subject to eventual diffusion or degradation, then there may not be time for completion of the pheromone response pathway and induction of BEM1. The recruitment of preexisting Bem1p to the proper site could be more rapid than synthesis and placement of new Bem1p. This explanation leads to the second question raised by this model: why is more Bem1p necessary during mating than during budding? Upon pheromone treatement, the bud-site selection program is overridden, but it may be that the budding complex is never actually

dismantled. Some Bem1p may remain complexed with the BUD gene products. Thus excess Bem1p would be necessary to be available to respond to the pheromone-initiated polarization signal. It would be interesting to know whether other polarity establishment genes such as CDC24 and CDC42 are also required in greater quantities during mating than during budding.

bem1-s3, which contains an intact C terminus, is expressed at normal levels from pbem1-s3, and yet pbem1-s3 does not complement the bem1Δ mating defect. The behavior of the bem1-s3 mutant could be interpreted as evidence for a model in which BEM1 has an additional function or specificity in mating cells. In this scenario, the 191 amino acid segment lacking in bem1-s3 is dispensible in budding cells but required in mating cells for a mating-specific function. However the behavior of the bem1-s3 mutant could also be interpreted as consistent with our model, in which the function of BEM1 in mating and budding is the same. The production of a protein lacking over one-third of the BEM1 coding sequence may effectively be the same as producing a reduced amount of functional protein, like the bem1-s1 and bem1-s2 alleles. An experiment in which bem1-s3 is overproduced, from the GAL1 promoter for example, could address this issue: if elevating the amount of bem1-s3 could increase mating ability, this would be evidence for the one-function interpretation; if not, it may favor the two-function model.

The simplest model is that the functions performed by Bem1p, and probably the other polarity establishment proteins Cdc24p and Cdc42p, are the same in mating and budding cells. This idea is

satisfying because it appears that roles of the cytoskeletal elements which manifest polarity are the same in both budding and mating cells (Field and Schekman 1980; Barnes, Drubin and Stearns 1990; Byers 1981; Read, Okamura and Drubin 1992), so a common polarity establishment activity to organize the cytoskeleton in both cases would seem to be the most efficient arrangement. Of course, the polarity establishment complex must ultimately respond to different site selection information in budding and mating cells. Site specificity in budding cells is provided by the products of the BUD site selection genes. The molecules which mediate site specificity in mating cells are not known. An approach at identifying genes involved in mating site selection is the focus of the next chapter.

CHAPTER 5

IDENTIFICATION OF TWO GENES, *DRT1* AND *FUS2*, WHICH CAN PARTIALLY SUPPRESS *bem1-s* MUTATIONS WHEN OVERPRODUCED

ABSTRACT

During conjugation, yeast cells polarize towards a mating partner. The molecules responsible for properly orienting the cell's polarity during mating are not known, but a gradient of mating pheromone secreted by the mating partner appears to be the signal which guides the localization of the polarization response. BEM1 is required for polarization both in response to mating pheromones and during vegetative growth (budding). Special alleles of BEM1 exist (bem1-s) which are proficient for budding but defective in mating and shmooing. In order to identify genes which interact with BEM1 during mating, we isolated high copy plasmids which can suppress the mating defect of a bem1-s strain. Two genes were isolated which when overexpressed can partially restore mating and shmooing to the bem1-s alleles. One is the previously identified gene FUS2, which is required for cell fusion. The FUS2 transcript is highly pheromone-inducible and not detectable in vegetative cells. The second multicopy suppressor is a new gene, DRT1, which is expressed vegetatively and not affected by pheromone. DRT1 encodes a 366 amino acid protein with a small region of similarity to BUD3 protein. DRT1 and FUS2 do not suppress cdc24 mutant phenotypes. Overproduction of DRT1 in wild-type cells causes a hyperpolarized morphology. The DRT1 and FUS2 gene products may be involved in recruiting Bem1p or other polarity establishment gene products towards the region of the cell surface which is most near the source of pheromone during shmooing and mating.

INTRODUCTION

Yeast cells polarize during two phases of their life cycle: during cell division and during mating. In both cases new membrane and cell wall material is inserted at a defined site on the cell surface so that the cell grows in a polarized manner (Tkacz and Lampen 1972; Tkacz and Lampen 1973; Lipke, Taylor and Ballou 1976; Tkacz and MacKay 1979; Field and Schekman 1980). The cytoskeleton polarizes toward this cell-surface site, and the nucleus and other organelles migrate toward and accumulate at the site (Byers 1981; Adams and Pringle 1984; Ford and Pringle 1986; Byers and Goetsch 1975; Tkacz and MacKay 1979; Hasek *et al.* 1987; Rose and Fink 1987; Segev, Mulholland and Botstein 1988; Baba *et al.* 1989; Gehrung and Snyder 1990; Meluh and Rose 1990). During vegetative growth, this localized morphogenesis results in bud growth and the eventual formation of a daughter cell. During mating, yeast cells do not bud. Rather, two cells of opposite cell type (MATa and MATα) grow toward one another and fuse at the site of cell contact, resulting in the formation of a diploid zygote cell (Cross *et al.* 1988).

The site at which a yeast cell buds during vegetative growth is chosen according to defined spatial patterns: MATa or MATα cells, for example, bud near previous bud sites (Friefelder 1960; Hicks, Strathern and Herskowitz 1977). Mutants which form normal buds but at improper positions identify the bud-site selection genes (BUD1-5) (Chant and Herskowitz 1991; Chant *et al.* 1991). Polarity establishment genes BEM1, CDC24, and CDC42 are required to

polarize the yeast cell; mutants defective in any of these genes are unable to polarize growth and instead display delocalized actin deposition and uniform cell surface growth (Sloat and Pringle 1978; Sloat, Adams and Pringle 1981; Adams *et al.* 1990; Johnson and Pringle 1990; Bender and Pringle 1991; Chant *et al.* 1991; Chenevert *et al.* 1992). Genetic studies of BUD genes and polarity establishment genes indicate that they interact in order to form a bud (Bender and Pringle 1989; Chant *et al.* 1991). BUD gene products are thought to recognize some intracellular landmark, perhaps a remnant of a previous cell division, and direct the positioning of polarity establishment gene products so that growth occurs at the proper site (Chant and Herskowitz 1991; Snyder, Gehrung and Page 1991).

The site at which a yeast cell polarizes during mating is determined by an external cue, the location of the mating partner. Mating cells signal one another with cell-type specific secreted peptide mating pheromones: **a** cells secrete **a**-factor to which α cells have the receptor and α cells secrete α-factor to which **a** cells have the receptor. The pheromone receptors, encoded by STE2 in **a** cells and STE3 in α cells, belong to the large family of seven transmembrane domain G-protein-coupled receptors (reviewed in Marsh, Neiman and Herskowitz 1991; Kurjan 1992). Much evidence has accumulated to support the idea that the signal emanating from the mating partner to which a mating cell orients is a high concentration of secreted mating pheromone. Exogenously added pheromone cannot restore mating to mutant strains unable to produce pheromone (Kurjan 1985; Michaelis and Herskowitz 1988;

Bender and Sprague 1989), perhaps indicating that the mating pheromone must be presented by a cell in a spatially restricted manner. Given a choice of different mating partners, yeast cells choose to mate with the partner producing the most pheromone, a phenomenon termed mating partner discrimination (Jackson and Hartwell 1990a; Jackson and Hartwell 1990b; Jackson, Konopka and Hartwell 1991). This ability of yeast cells to discriminate among mating partners requires the pheromone receptors. When purified pheromone from one cell type is added to cells of the opposite cell type, the cells adopt a polarized morphology known as a shmoo which contains a cell surface projection in which the cytoskeleton (Ford and Pringle 1986; Hasek *et al.* 1987; Rose and Fink 1987; Gehrung and Snyder 1990; Meluh and Rose 1990; Barnes, Drubin and Stearns 1990; Read, Okamura and Drubin 1992) and proteins important for mating (Watzele, Klis and Tanner 1988; Trueheart, Boeke and Fink 1987; Gehrung and Snyder 1990; Jackson, Konopka and Hartwell 1991; Kuchler, Dohlman and Thorner 1993; E. Elion, personal communication) are localized. Finally, if the pheromone is supplied in a micropipet, cells on a semisolid support will emit mating projections towards the source of pheromone (Segall 1993).

Polarity establishment genes (BEM1, CDC24, and CDC42) are required to polarize yeast cells; mutants defective in any of these genes are unable to polarize growth and instead display delocalized actin deposition and uniform cell surface growth (Sloat and Pringle 1978; Sloat, Adams and Pringle 1981; Adams *et al.* 1990; Johnson and Pringle 1990; Bender and Pringle 1991; Chant *et al.* 1991; Chenevert *et al.* 1992). Mutants in BEM1 or CDC24 are defective in

shmoo formation and display delocalized growth when treated with pheromone (Field and Schekman 1980; Chenevert *et al.* 1992). Thus Bem1p and Cdc24p appear to be responsible, directly or indirectly for the localization of the cytoskeleton and the growth machinery of the cell to a single site on the cell surface in shmooing as well as in budding. During mating, the function of the polarity establishment proteins is required not at the budding site but at the mating site. The mating site presumably corresponds to the area of greatest pheromone receptor stimulation.

A reasonable hypothesis to explain how mating partners polarize toward one another is that interaction of pheromone with receptors causes an intracellular alteration which results in recruitment of the polarity establishment gene products and subsequent organization of the cytoskeleton at the site of pheromone stimulation. The nature of this localized intracellular change, for example whether it is G-protein mediated or if receptor capping is involved, is not known. A mutant form of the receptor which lacks the intracellular C-terminus is proficient for signalling but produces uniformly enlarged cells rather than polarized shmoos (Konopka, Jenness and Hartwell 1988). We used special alleles of polarity establishment gene BEM1 which are defective in shmoo formation but not in bud formation in order to isolate genes which interact genetically with BEM1 in mating cells with the goal of identifying molecules which are involved in positioning the polarity establishment proteins towards a source of pheromone.

MATERIALS AND METHODS

Yeast strains, media and genetic manipulations. Yeast strains are listed in Table 2-1 and Appendix 4. Crosses, sporulation and tetrad dissection were performed as described previously (Sherman, Fink and Hicks 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).

Mating assays. All matings described are of *MATa* cells against an enfeebled partner, *MATα far1* strain JC31-7D. Mating assays were routinely done in a qualitative way on colonies and patches essentially as described (Chenevert, Valtz and Herskowitz submitted). A lawn of *far1* mating tester was spread on a YEPD plate and the transformants were replica-plated to this lawn. After incubation at 30°C for 4-6 hours, the mating plates were replicaplated to minimal medium (SD) to select for diploids. Putative maters were picked from the original transformant plate, patched, and retested for mating. For quantitative mating tests, 1.5 x10^6 cells of each parent were collected on a Millipore filter, allowed to mate for 4-6 hours, and analyzed for zygote production by resuspending cells off the filter and plating dilutions on plates that select for the growth of diploid cells as previously described (Neiman *et al.* 1992).

Morphology assays. For strains not containing plasmids, cultures were grown in YEPD to mid-log phase and α-factor was added to a concentration of 1 uM. After further incubation for 1.5-2

hr, cells were sonicated briefly and observed microscopically to determine cell shape. Phenotypes conferred by plasmids are typically assayed in minimal media selecting for the plasmid, but the _bem1-s_ shmooless phenotype is most dramatic in rich media (J. C., unpublished observations), so the following protocol was adopted. Strains containing plasmids were grown overnight in minimal medium lacking uracil to select for the plasmid. They were then diluted in YEPD, grown for 3 hr and treated with α-factor as described above. For each sample, 200 cells were counted and results from three independent experiments were averaged.

Protein extracts and western blots: Preparation of protein extracts, electrophoresis of protein samples, and Western blotting with antibodies were performed as described in Chapter 4.

Isolation of high-copy number plasmids that confer mating to a _bem1-s_ strain. Yeast strains JC-G11 and JC109 were transformed with a genomic plasmid library in vector YEp24 (Carlson and Botstein, 1982) to a density of 100-200 colonies per plate. Mating to _far1_ strain JC31-7D was tested as described above. Following growth on 5FOA (5-fluoroorotic acid) to select for loss of the plasmid, mating was retested and colonies were eliminated which were not plasmid dependent. The plasmids were recovered from yeast and reintroduced into _bem1-s_ strains to verify that they carried a gene which conferred mating. In an initial screen of 6000 colonies, we analyzed only the transformants which mated as well as a wild-type strain. Three plasmids were recovered which completely complemented the _bem1-s_ mutant phenotype, of which two were identical. Restriction mapping and Southern hybridization

revealed that all three contained the *BEM1* gene.

In a second round of screening, 21,000 colonies were tested and transformants which exhibited an intermediate level of mating between a wild type strain and a *bem1-s* strain were analyzed. Again, all the strongest maters contained the *BEM1* gene. Three transformants contained plasmids which partially suppressed the mating defect when reintroduced into a *bem1-s* strain. Restriction analysis showed that they did not contain the *BEM1* gene; two plasmids (p35 and p36) contained the same insert and the third plasmid (p19) was unique.

Subcloning of high-copy suppressors. Portions of the original inserts were deleted by cleaving with restriction enzymes which leave cohesive ends, diluting and religating (constructs in Figures 5-1 and 5-2 with dashed ends). Other subclones were made by ligating fragments from the plasmid inserts into high copy number vectors as follows: p2uDRT (Figure 5-1) was generated by cloning the 3.2 kb *Bam*HI fragment into YEp24. The *DRT1* gene in this plasmid was disrupted by deleting 420 bp from the *Kpn*I site to the *Bgl*II site to generate p2uDRTΔKB in the following way: p2uDRT was cut with *Kpn*I and the 3' overhang was chewed back with enzyme to generate a blunt end. *Bam*HI linkers were added and the plasmid was cut with *Bam*HI and *Bgl*II and religated. p2uFUS2 (Figure 5-2) was constructed by cloning the 4 kb *Sph*I fragment from p35 into p2uSph, a derivative of pRS426 in which an *Sph*I site replaces the *Bam*HI site, the *Sal*I site, and all sites in the polylinker between *Bam*HI and *Sal*I. Cleavage of p2uFUS2 by *Hin*dIII or *Bgl*II followed by religation removed the 1.3 kb *Hin*dIII fragment or the

1.3 kb BglII fragment and resulted in p2uFUS2ΔH and p2uFUS2ΔB, respectively.

Sequencing of DRT1. Portions of the 3.2 kb BamH I fragment from p2uDRT were subcloned into pUC19 and sequenced by double-stranded sequencing using fluorescence methods at the BRC-UCSF. Two strategies were used: sequencing from primers which hybridize to the pUC polylinker region on several different subclones, and synthesis of primers homologous to DRT1 sequence in order to sequence far into larger subclones. Sequence information was obtained from a total of 2.5 kb (Figure 5-6A). A 1.1 kb open reading frame was detected.

Deletion of DRT1. The 3.2 kb BamHI fragment containing DRT1 was cloned into pUC, generating pUCDRT. The DRT1 gene in pUCDRT was precisely replaced by LEU2 using the PCR technique described previously (Chenevert *et al.* 1992) to generate pKODRT. Divergent primers which hybridized just upstream and downstream of the DRT1 coding sequence and which introduced a restriction site (XhoI) were synthesized (upstream primer: 5'- GCC CTC GAG TCT AGA AAC ACT TTC CCT - 3'; downstream primer: 5'- GCC CTC GAG TGA AAA CAA TCA GGA GAA - 3'). PCR with these primers on pUCDRT followed by cleavage with XhoI and ligation generated a pUC plasmid containing 1 kb sequence upstream of DRT1 joined by a XhoI site to 1.1 kb sequence downstream of DRT1. LEU2 was cloned into this XhoI site on a 2.2 kb SalI-XhoI fragment, yielding pKODRT. The 4.5 kb BamHI fragment from pKODRT was gel isolated and transformed into diploid strain IH1788. Southern hybridization on Leu^+ transformants identified diploids in which one copy of the DRT1

locus was disrupted. These DRT1+/drt1::LEU2 diploids were sporulated and dissected, generating MATa drt1::LEU2 and MATα drt1::LEU2 segregants.

Construction of double mutants. To construct a drt1 bem1 double mutant, strain KO2-5C (MATa bem1::LEU2) was crossed with strain KOD-1B (MATα drt1::LEU2). Leu+ segregants from the non-parental ditypes are drt1 bem1 double mutants. A drt1 bar1 double mutant was generated by crossing KOD-1B with AN37-4C-s. MATa Leu+ segregants were tested for supersensitivity to a factor, an indicator of the bar1 mutation. The drt1 bud3 mutant was constructed by crossing strain KOD-1B (drt1::LEU2) with JC262 (bud3::TRP1); the Leu+ Trp+ segregants are drt1 bud3 double mutants. Similarly, a drt1 bud1 mutant was constructed by crossing KOD-1A (drt1::LEU2) to HPY36 (bud1::TRP1); Leu+ Trp+ segregants are the double mutants. fus2 drt1 and fus2 bem1 double mutants were generated by transforming KOD-? (drt1::LEU2) and KO2-5C (bem1::LEU2) with SphI-cut pKOFUS2 (fus2::URA3). Ura+ transformants were tested for the ability to mate to a fus1 fus2 strain (IH2351). Inability to mate to fus1 fus2 is a Fus- phenotype and indicates that FUS2 has been disrupted.

Molecular mapping of DRT1 and FUS2. DNA fragments obtained from the plasmid inserts were used to probe yeast chromosome blots and nylon filters on which lambda clones that represent approximately 82% of the yeast genome had been immobilized (Riles *et al.* 1993). The 4.5 kb BglII fragment for p19(DRT1) and the 1.8 kb BamHI fragment for p35(FUS2) were labelled with ^{32}P dCTP using the random-primed DNA labelling kit

(Boehringer Mannheim). These probes were hybridized to chromosome blots using the conditions for Southern hybrization (Maniatis, Fritsch and Sanbrook 1982) at 65°C. Probing of the lambda clone filters was performed as recommended by the supplier (L. Riles, personal communication).

Northern analysis. Total RNA was isolated from cells essentially as described (Lindquist 1981). 6-10 OD_{600} of yeast cells were collected and resuspended in 0.3 ml RNA lysis buffer (0.1 M Tris pH 7.5, 0.1 M LiCl, 0.01 M DTT). Resuspended cells were transferred to prechilled Eppendorf tubes containing 0.6 ml phenol:$CHCl_3$, 60 ul 10% SDS, and 0.5 ml glass beads. The tubes were vortexed 6 times for 30 sec and chilled on ice in between times. After 1 min centrifugation, the aqueous phase was recovered and the phenol:$CHCl_3$ extraction was repeated. The aqueous phase was removed into 40 ul of 3 M NaOAc and 800 ul cold EtOH was added. The tubes were spun 20 min, the supernatants were discarded, and the pellets were dried and reuspended in 50 ul diethly pyrocarbonate-treated H_2O. The quantity and purity of RNA was determined by measuring the OD_{260}/OD_{280} of a 1:100 dilution in H_2O. The RNA was separated by electrophoresis through a 1.5% agarose denaturing gel and transferred to the nylon membrane Hybond according to the instructions of the manufacturer (Amersham) and hybridized to DNA probes prepared with the Random Primed DNA Labelling Kit (Boehringer-Mannheim). Hybridization and washing conditions were as described in Ausubel *et al.* 1991. Probes for the transcripts were a 680 base pair XbaI fragment internal to the DRT1 coding sequence and a 1.3 kb HindIII

fragment internal to the FUS2 coding sequence. A control probe was made from the 450bp PstI-StuI fragment of URA3. Values for the intensity of the bands were quantitated on a Molecular Dynamics phosphorimager using the Image Quant program.

RESULTS

Identification of high-copy number plasmids which can partially suppress the mating defect of bem1-s mutants. Two high-copy number plasmids, p19 and p35, were isolated which could partially suppress the mating defect of a bem1-s1 strain (see Materials & Methods). In order to determine if these plasmids contained known genes, probes from the insert DNA were prepared and hybridized to yeast chromosome blots and to a set of filters on which lambda clones containing yeast DNA were immobilized. The probe from p19 hybridized to a band which identified either chromosome V or VIII. No signal was detected on the lambda clone grids, so the insert in p19 may be among the 18% of the yeast genome which is not represented on these grids. The probe from p35 hybridized to a band on the chromosome blot which identified either chromosome XVI or XIII and to a lambda clone containing a 10 kb region of chromosome XIII. This 10 kb clone contains FUS2, a gene required for yeast cell fusion (Trueheart, Boeke and Fink 1987) and at least two other genes, RNA1 and RNH1.

Portions of the inserts from p19 and p35 were subcloned into 2u vectors and assayed for ability to suppress mating of bem1-s strains (Figures 5-1, 5-2). A 3.2 kb fragment of p19 was identified

Figure 5-1. Map of bem1-s suppressing plasmid p19 and determination of location of suppressing gene.

A: Restriction map of p19 and location of DRT1 gene. Broken lines indicate vector sequences and the dark line represents the insert in the plasmid. Relevant restriction sites are indicated; B: BamHI, Bg: BglII, K: KpnI, S: SalI, Sm: SmaI, Sp: SphI.

B: Phenotypes conferred by various subclones of p19. + indicates suppression of bem1-s mating defect; - indicates failure to suppress.

C: Plasmid used to delete DRT1 from the chromosome. DRT1 was replaced by LEU2 as described in the text.

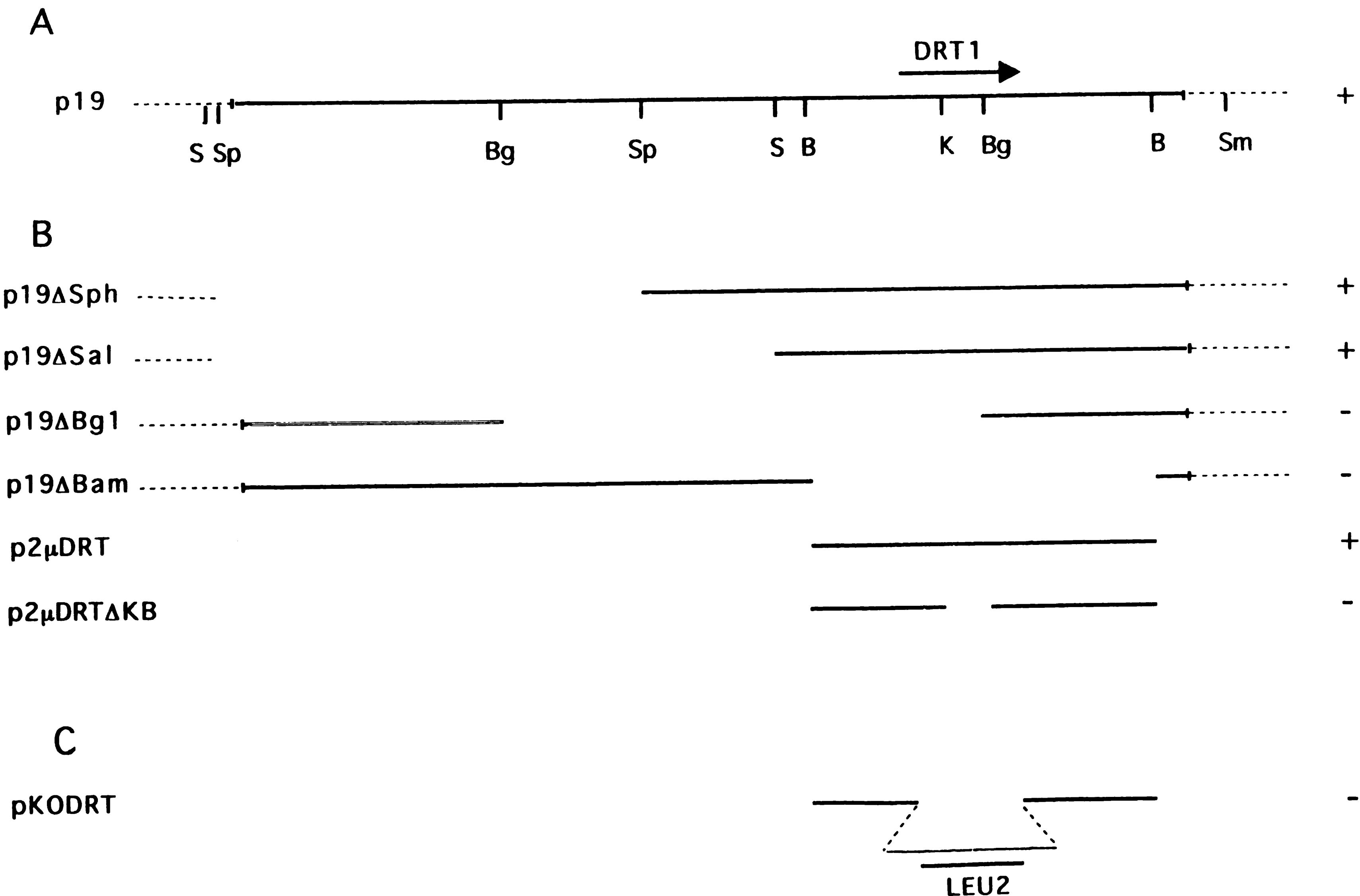
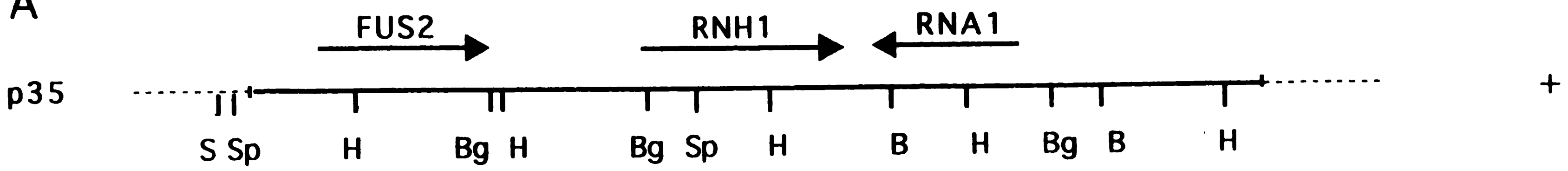
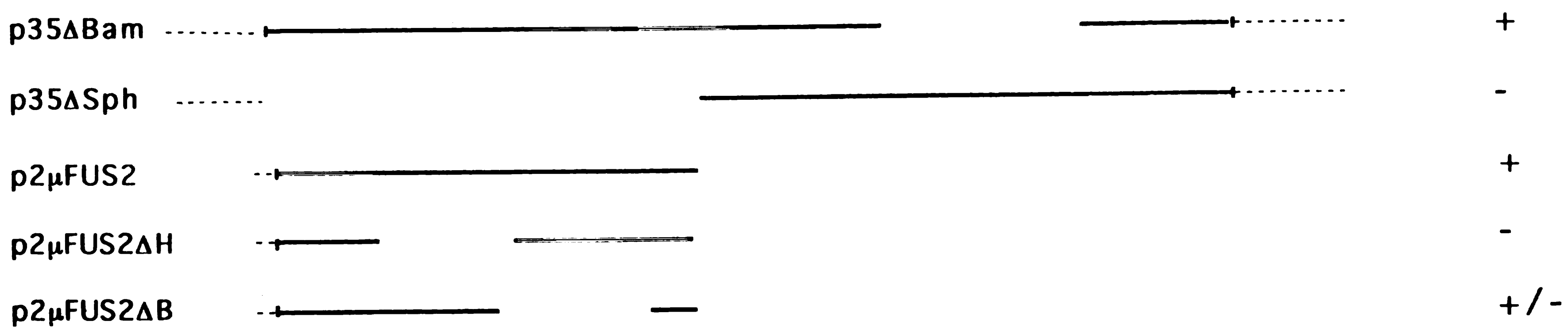
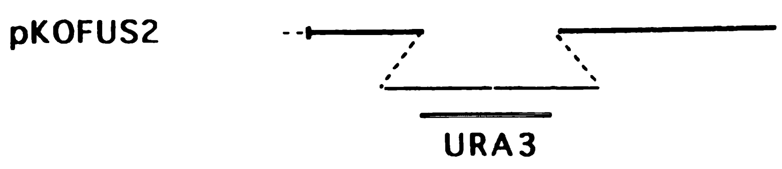

1 Kb
A
DRT1
p19
+
S Sp
Bg
Sp
S B
K Bg
B
Sm
B
p19ΔSph
+
p19ΔSal
+
p19ΔBg1
-
p19ΔBam
-
p2μDRT
+
p2μDRTΔKB
-
C
pKODRT
-
LEU2

Figure 5-2. Map of bem1-s suppressing plasmid p35 and determination of location of suppressing gene.

A: Restriction map of p35 and location of FUS2 gene. Broken lines indicate vector sequences and the dark line represents the insert in the plasmid. Relevent restriction sites are indicated; B: BamHI, Bg: BglII, H: HindIII, S: SalI, Sp: SphI.

B: Phenotypes conferred by various subclones of p35. + indicates suppression of bem1-s mating defect; - indicates failure to suppress.

C: Plasmid used to delete FUS2 from the chromosome. FUS2 was replaced by URA3 as described in the text.




1 Kb
A
FUS2
RNH1
RNA1
p35
S Sp H Bg H Bg Sp H B H Bg B H
+
B
p35ΔBam
+
p35ΔSph
-
p2μFUS2
+
p2μFUS2ΔH
-
p2μFUS2ΔB
+/-
C
pKOFUS2
URA3
-

[illegible] LIBRARY

which was sufficient to confer mating to a bem1-s1 strain (Figure 5-1). Most of this fragment was sequenced and a 1.1 kb open reading frame was detected. Deleting 400 bp internal to this ORF (p2uDRTΔKB) eliminated ability to suppress, so we conclude that this open reading frame is the gene in p19 (named DRT1) responsible for suppression. A 4 kb fragment from p35 which contained the FUS2 gene was sufficient to confer mating on a bem1-s1 strain (Figure 5-2). An internal deletion of the FUS2 gene (p2uFUS2ΔH) disrupted suppressing ability, confirming that the gene responsible for suppression in p35 is FUS2.

Characterization of suppression of bem1 mutant phenotypes by FUS2 and DRT1. p19(DRT1) and p35(FUS2) increase the mating ability of a bem1-s1 strain (Figure 5-3A). They could do this either by restoring pheromone-induced cell polarization or by some other means. To determine if p19 and p35 are able to restore shmooing, bem1-s strains containing the suppressing plasmids were treated with pheromone and observed microscopically. 47% of bem1-s1 cells containing p19, and 35% of bem1-s1 cells containing p35 are able to form shmoos, as compared to only 3% of bem1-s1 cells containing a vector control (Figure 5-3B). Similar results were found for a bem1-s2 strain (data not shown). In these experiments cells are grown in rich media, which does not select for the plasmids (a condition necessary to maximize the bem1-s phenotype, see Materials and Methods), so some plasmid loss may obscure the full effects of the plasmids. Plasmid loss may account for the observation that only 70% of bem1-s1 cells containing a BEM1 plasmid form shmoos, as compared to 95% $BEM1^+$ cells (Figure

Figure 5-3. Ability of p19(DRT1) and p35(FUS2) to confer mating and pheromone induced polarization to *bem1-s* strains.

A: Mating of *bem1-s1* strain JC-G11 containing YEp24, p19, p35, or pCENBEM.

B: Percentage of shmoos formed after two hr treatment with α-factor.

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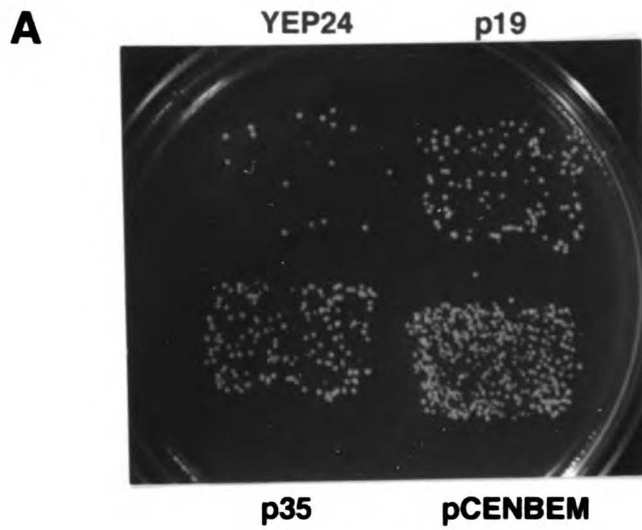
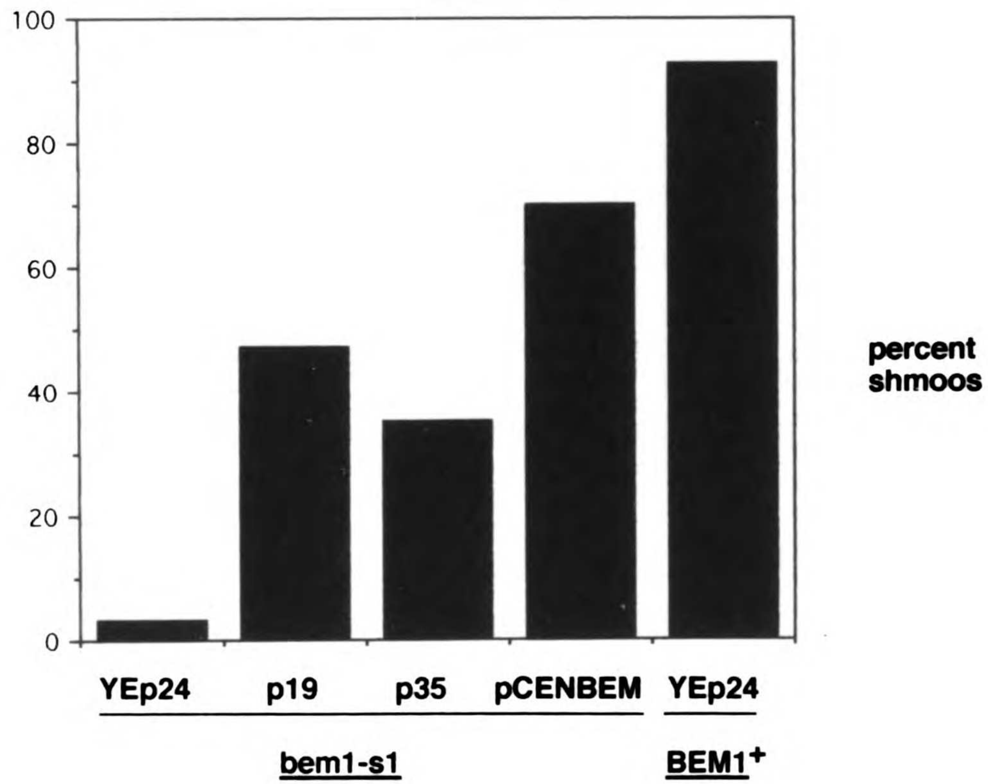
A



B



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5-3B).

Since the bem1-s alleles generate truncated products and are reduced in level, we tested whether p19 or p35 allowed full-length BEM1 protein to be produced or increased the stability of Bem1p in the presence of pheromone. Extracts from the bem1-s strains containing p19 and p35 were prepared and immunoblotted with an anti-Bem1p antibody. As shown in Figure 5-4, the sizes and the levels of the mutant proteins produced in a bem1-s1 strain or a bem1-s2 strain are not altered by the presence of p19 or p35. This is true in the absence and presence of pheromone. The experiment shown in Figure 5-4 also addresses the possibility that the bem1-s1 and bem1-s2 alleles become null alleles when cells are treated with pheromone. The same amount of mutant bem1-s1 or bem1-s2 protein is present before and after pheromone treatment (Figures 5-4A and 5-4B, lane 1 versus lane 4). Thus a further destabilization of the BEM1 protein cannot account for the pheromone-specific mutant phenotype of the bem1-s alleles.

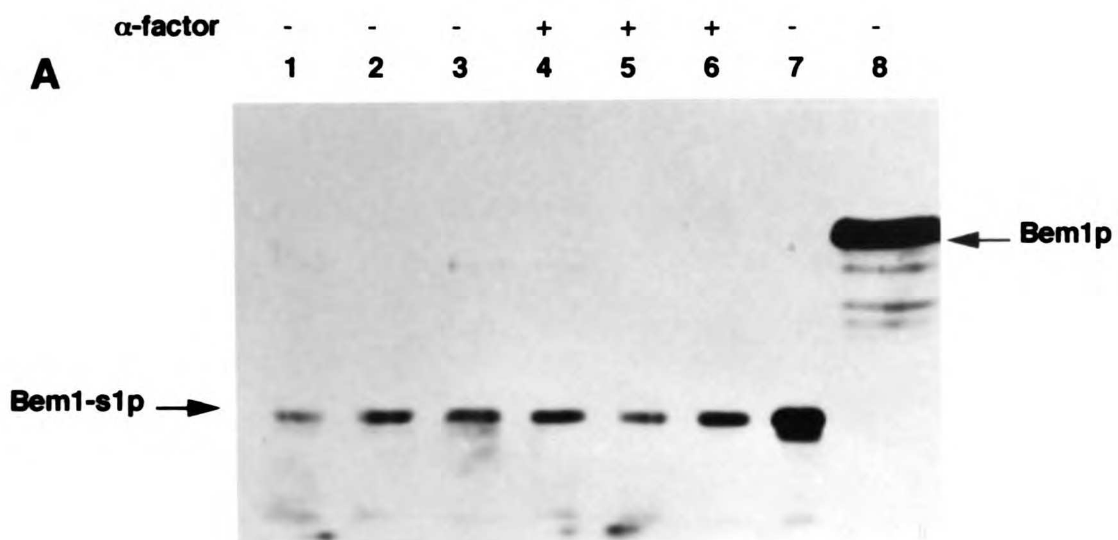
p19 and p35 were transformed into a strain deleted for BEM1 in order to determine, first, if DRT1 or FUS2 were able to improve mating in a strain completely lacking BEM1 and, second, if overexpression of DRT1 or FUS2 had any effect on the vegetative defect of a bem1 mutant. Patch mating tests indicated that p19(DRT1) and p35(FUS2) did suppress the mating defect of the bem1 deletion strain (Figure 5-5A). A strain deleted for BEM1 does not form colonies at 37°C (Chant *et al.* 1991; Bender and Pringle 1991; Chenevert *et al.* 1992; Figure 5-5B). A bem1Δ strain containing p19(DRT1) or p2uDRT can eventually form colonies at

Figure 5-4. p19(DRT1) and p35(FUS2) do not alter the sizes or amounts of mutant bem1-s proteins.

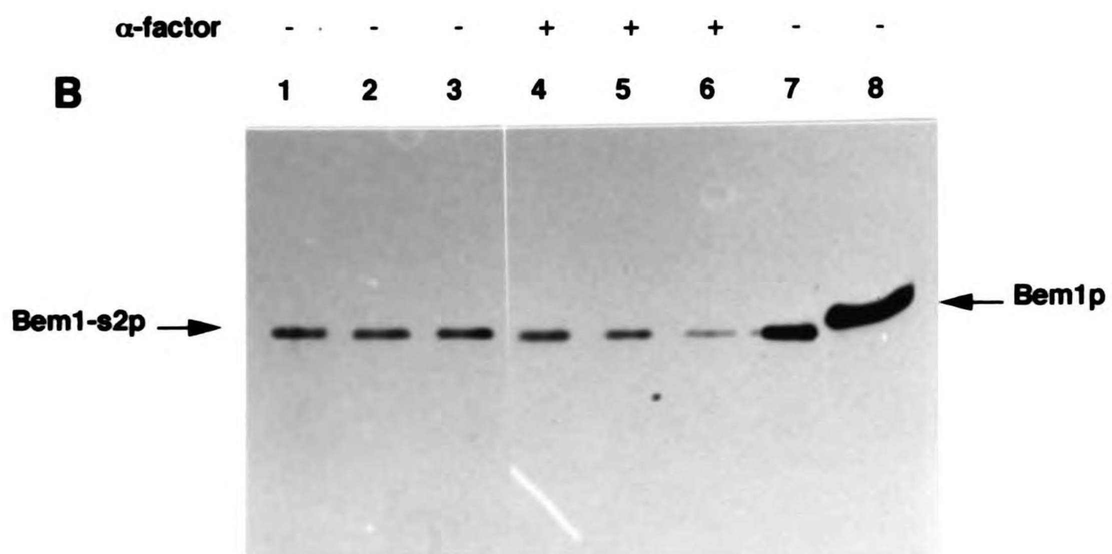
A: Western blot of bem1-s1 strain JC-G11.

B: Western blot of bem1-s2 strain JC-F5.

In both panels, the yeast strains contained the following plasmids: YEp24 (lanes 1, 4), p19 (lanes 2, 5), p35 (lanes 3, 6), or pCENBEM (lane 7). Lane 8 contains an extract from $BEM1^{+}$ strain JC2-1B. Extracts were prepared from cells either untreated (lanes 1, 2, 3, 7, 8) or treated (lanes 4, 5, 6) with α-factor and immunobloted with anti-Bem1p antibody.



α-factor - - - + + + - -
A
1 2 3 4 5 6 7 8
Bem1p
Bem1-s1p



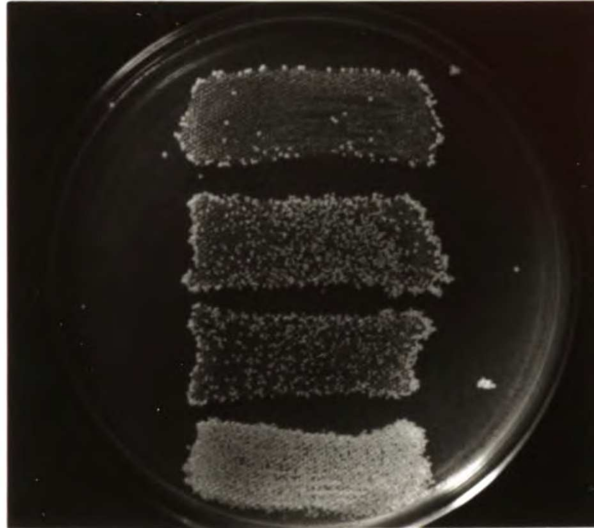
α-factor - - - + + + - -
B
1 2 3 4 5 6 7 8
Bem1p
Bem1-s2p

Figure 5-5. Effect of p19 and p35 on phenotyes of bem1 deletion strain.

A: Mating of bem1 deletion strain KO2-5C containing YEp24, p19, p35, or pCENBEM.

B: Growth at 37°C. Strain KO2-5C containing p19, p35, or pCENBEM were streaked on plates lacking uracil and incubated at 37°C for three days.

A



YEp24

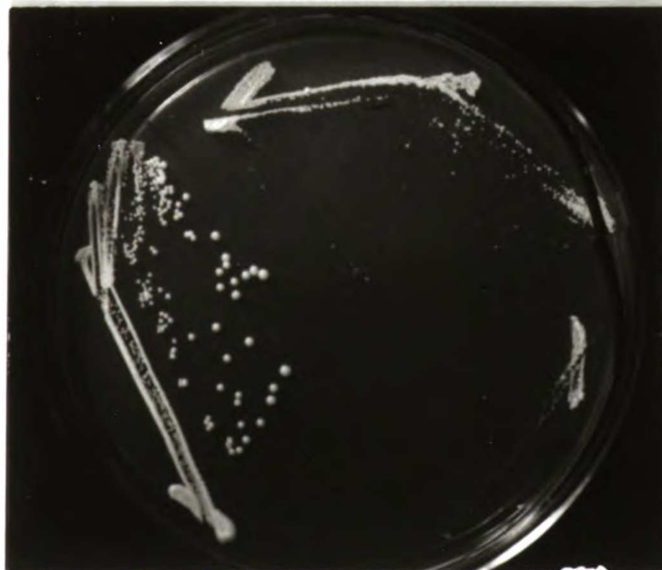
p19(DRT1)

p35(FUS2)

pCENBEM

B

p19(DRT1)



pCENBEM

p35(FUS2)

37°C, although they are much smaller than the colonies formed by a bem1 deletion strain containing a BEM1 plasmid (Figure 5-5B). p35(FUS2) had no effect on growth of the bem1 deletion strain, as expected, since FUS2 is not expressed vegetatively (see below).

Sequence and deletion of DRT1. Sequence information was obtained from the insert of p2uDRT, beginning in the region which was necessary for complementation (Figure 5-6A). One major open reading frame of 1.1 kb was detected. Figure 5-6B shows the sequence of the predicted DRT1 gene product. A computer search revealed no strong homology to sequences in the Genbank data base, but a small region of homology to BUD3 was detected (30% identity over 80 amino acids, Figure 5-6C). This homology lead us to investigate the effect of p2uDRT on budding pattern. p2uDRT does not alter the budding pattern of a wild-type strain or of bud3 or bud4 mutants (data not shown).

A deletion of the DRT1 gene was constructed in both a BAR^+ (wild-type) and a bar^- (pheromone supersensitive) background. A battery of phenotypes was investigated, including budding pattern, growth at 16°,17°, 25°, 30°, 33°, 35°, 37°, and 38°C, mating to wild-type strains and to the enfeebled mating testers defective in far1 or fus1 and fus2, shmooing, arrest in response to pheromone, pheromone production, and mating partner discrimination. No defect associated with the drt1 deletion was detected. The following double mutants were constructed: drt1 bem1, drt1 bud3, drt1 bud1, drt1 fus2. The drt1 mutation did not exacerbate or suppress the growth or mating defects of any of these mutant strains.

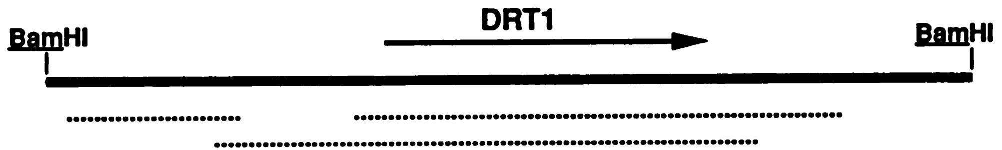
Figure 5-6. Nucleotide and predicted amino acid sequence of DRT1.

A: Region of p19 insert sequenced on either one (...) or both (:::::) strands.

B: Sequence of DRT1 and predicted sequence of DRT1 protein.

C: Homology between DRT1 and BUD3. Identical residues are noted; * indicates a conservative change.

A



B

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                    CTTTCTAAGCATTATAAGATAATATTAAAGGGAAAGTGTTTCTAGA ATG CAA TCG AGC TTA
                                                                   M   Q   S   S   L

CCT CAA TTT ACG TTC AAA TGG CCC AAA GGA CCC GAA GCA ATT ATT CTG ACA GGC ACG TTC
P   Q   F   T   F   K   W   P   K   G   P   E   A   I   I   L   T   G   T   F

GAC GAC TGG AAA GGT ACT TTG CCG ATG GTG AAG GAC CCC AGT GGC GCC TTC GAA ATA ACG
D   D   W   K   G   T   L   P   M   V   K   D   P   S   G   A   F   E   I   T

CTG CCA GTA ACG TTT GAT AGC CCT AGC AGC AAG TTT TAT TTC AAG TTT ATT GTT GAT GGC
L   P   V   T   F   D   S   P   S   S   K   F   Y   F   K   F   I   V   D   G

CAA TGG CTG CCA AGC AAA GAC TAC AAG GTG AAC ATC GAC GAG GGA GTG GAA AAC AAC TTT
Q   W   L   P   S   K   D   Y   K   V   N   I   D   E   G   V   E   N   N   F

ATT ACC GAG GAA GAC GTA ATA AAG CAA CGC GAA AAT GGC TCT AGC ACG CTG GTA CCT GAA
I   T   E   E   D   V   I   K   Q   R   E   N   G   S   S   T   L   V   P   E

AGT GCC GGA TTA GCT GTT TCA AAG AAT GCC CCT CTT ATC GAA CCA GAA GCT GAA AAA CGT
S   A   G   L   A   V   S   K   N   A   P   L   I   E   P   E   A   E   K   R

GCA AAA AAA TTA AGA AAG TTC AAG ATC AAG AGA GTG ATC AAG ACA AAT AAA CAA ACC GGA
A   K   K   L   R   K   F   K   I   K   R   V   I   K   T   N   K   Q   T   G

GAA AGG TCG ATA TTT TCC CAA GAA GTG GTT GAA TTG CCC GAT AGC GAG GAT GAA ACC CAG
E   R   S   I   F   S   Q   E   V   V   E   L   P   D   S   E   D   E   T   Q

CAG GTG AAC AAA ACG GGC AAG AAT GCG GAT GGC TTA AGC GGT ACT ACA ACG ATA ATT GAG
Q   V   N   K   T   G   K   N   A   D   G   L   S   G   T   T   T   I   I   E

AAT AAT GTT GGT GTA AAC GAG GAA AAA GCA ATC AAG CCG TAT GAA GAG AAT CAC CCC AAA
N   N   V   G   V   N   E   E   K   A   I   K   P   Y   E   E   N   H   P   K

GTT AAT CTA GTT AAG AGT GAA GGA TAT GTT ACG GAC GGT TTG GGT AAG ACG CAA TCT TCT
V   N   L   V   K   S   E   G   Y   V   T   D   G   L   G   K   T   Q   S   S

GAG TCT AGA TTA TAT GAA CTA TCG GCC GAA GAT CTT GAA AAG GAA GAA GAG GAA GAG GAC
E   S   R   L   Y   E   L   S   A   E   D   L   E   K   E   E   E   E   E   D

GAA GAT AAA GGC GGC GGT AAG GAC ACC AGT ACA AGT GCA GAC GCT GAA GCT TCA GAA GAT
E   D   K   G   G   G   K   D   T   S   T   S   A   D   A   E   A   S   E   D

CAA AAT AAG GAA CCA TTA AGT AAA TCC GCT AAA TTT GAA AAA CCG GAA GAA AAA GTA CCT
Q   N   K   E   P   L   S   K   S   A   K   F   E   K   P   E   E   K   V   P

GTA AGC TCA ATT ACA AGC CAT GCT AAA GAG ACT TCT GTG AAA CCA ACC GGC AAG GTT GCG
V   S   S   I   T   S   H   A   K   E   T   S   V   K   P   T   G   K   V   A

ACT GAG ACT CAA ACT TAC GAG ACG AAA CAG GGC GCT CCT ACC GCT GCC GCA AAA AAA ATC
T   E   T   Q   T   Y   E   T   K   Q   G   A   P   T   A   A   A   K   K   I

GAA GCT AAG AAA GCT ACT AGA CCT TCG AAA CCT AAG GGC ACG AAA GAA ACA CCA AAT AAA
E   A   K   K   A   T   R   P   S   K   P   K   G   T   K   E   T   P   N   K

GGT GTC CAA AAG AAC CCT GCT AAG AAT GGA GGG TTC TTT AAA AAG TTG GCC CAG CTT TTG
G   V   Q   K   N   P   A   K   N   G   G   F   F   K   K   L   A   Q   L   L

AAG TGA AAACAATCAGGAGAATACCTAAATAT
K   *
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C

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Drt1p 288 SITSHAKETS-VKPTGKVATETQTYETKQGAPTAAAKKIEAKKATRPSKPKGTKETPNKGVQKNPAKNGGFFKKLAQLL
          *IT*    TS *K *         T ETK* *P    KK  K      K K              K P K  GFF  L  **
Bud3p 840 TITTFRSYTSDLKDSPSGDNSNVTKETKEILPVKPTKKSSKKPREIQKKTKTNASKAEHIEKKKPNKGKGFFGVLKNVF
```

Phenotype of overproduction of DRT1 in wild-type cells. A weak phenotype associated with the overproduction of DRT1 in wild-type strains was detected. p2uDRT alters the vegetative morphology of 15-30% of the cells in a population, giving them a longer, more oval shape than cells containing no plasmid or control plasmid p2uDRTΔKB (in which 420 bp internal to the DRT1 coding sequence are deleted). The extent of this shape change is strain-dependent and is exaggerated in JC2-1B which is $bar1^-$ (Figure 5-7). $bar1^-$ strains are supersensitive to mating pheromone and maintain a higher basal level of the pheromone response pathway than BAR^+ strains (Marsh, Neiman and Herskowitz 1991; Kurjan 1992). Thus the unusual vegetative morphology conferred by p2uDRT may be enhanced be a gene which is induced by pheromone.

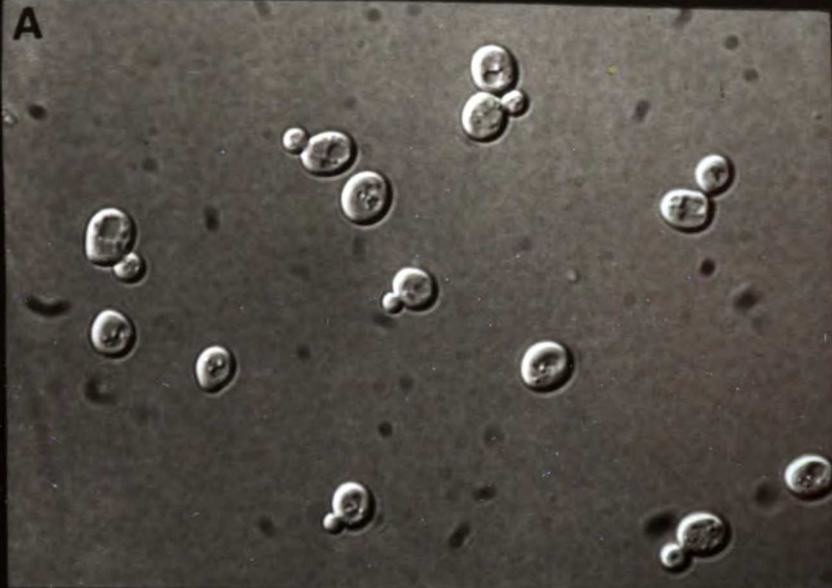
Expression of DRT1 and FUS2. Northern analysis revealed that DRT1 mRNA is expressed in all cell types and unaffected by addition of pheromone (Figure 5-8). A slight decrease in DRT1 signal was found in RNA from pheromone-treated cultures, but this decrease also occurred for the control transcript URA3. When converted to quantitative measurements, the slight decrease in DRT1 mRNA levels is not significantly different from the decrease in URA3 mRNA (Table 5-1). The reduction of message levels may reflect a nonspecific consequence of pheromone treatment. In contrast to DRT1, FUS2 mRNA is undetectable in vegetative cells and greatly induced by mating pheromone (Figure 5-8), as previously noted (E. Elion, personal communication).

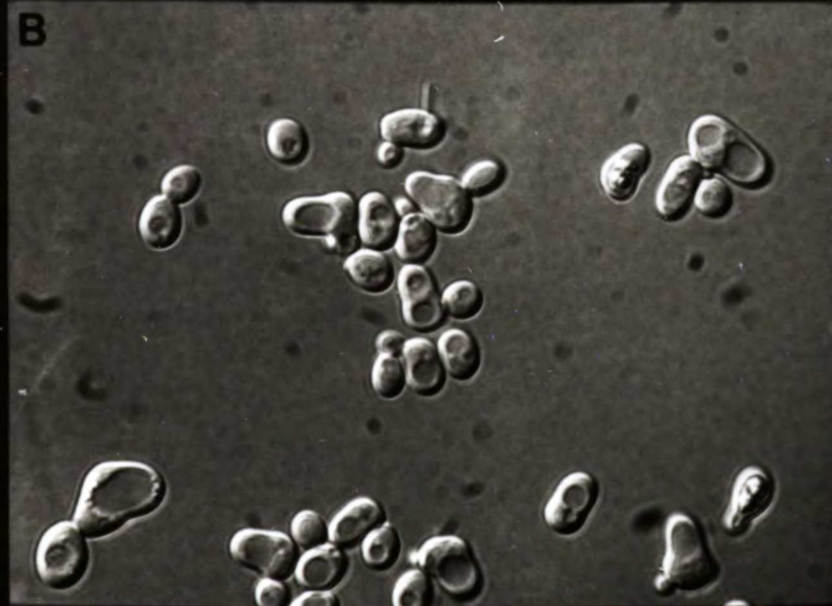
Figure 5-7. Phenotype of overproduction of DRT1 in vegetative cells.

A: Strain JC2-1B containing p2uDRTΔKB. These cells contain a control plasmid in which a portion of the DRT1 coding sequence has been deleted. Their morphology is normal.

B: Strain JC2-1B containing p2uDRT. This photo depicts the most common shape alterations

C: Strain JC2-1B containing p2uDRT. This picture shows the rarer and most extreme morphological aberrations.


A


B

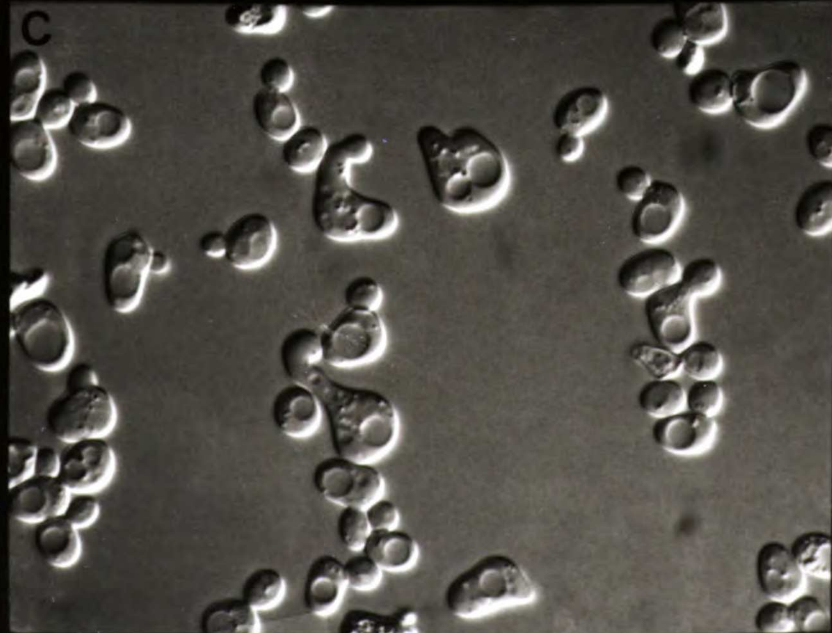

C

Figure 5-8. Effect of cell type and pheromone treatment on expression level of DRT1 and FUS2.

Northern hybridization was performed on total RNA prepared from a MATa strain (JO54), the same MATa strain treated with α-factor for 2 hours, a MATα strain (JO14), and a MATa/MATα strain (JO226). Probes for DRT1 and FUS2 were as described in Materials and Methods. The identity of the DRT1 transcript was determined by comparison with RNA prepared from a strain deleted for DRT1 (KOD-1A) (data not shown). The filter showing FUS2 (lower panel) was exposed for five hours; that showing DRT1 (upper panel) was exposed ten times as long.

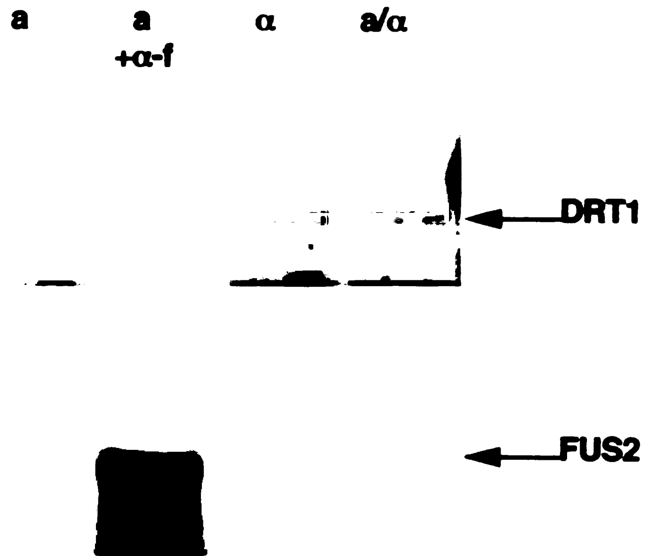

a
a
+α-f
α
a/α
DRT1
FUS2

Table 5-1

Effect of pheromone treatment on DRT1 expression

strain	min α-factor treatment	DRT1	URA3
JO54[b]	0	100[a]	100[a]
	120	61	85
JC2-1B[c]	0	100	100
	30	53	55
	60	29	26
	120	34	30

[a]values given are percent of untreated signal, normalized for each gene.

[b]MATa BAR1 strain; numbers result from an average of three experiments.

[c]MATa bar1 strain; numbers result from an average of two experiments.

Specificity of high copy suppression. To determine whether the suppressing acitivy of DRT1 and FUS2 was specific to bem1 mutants or if they would have the same effect on mutants in another gene involved in polarity establishment, p19 and p35 were transformed into strains containing temperature sensitive mutations in CDC24 (JC-I13 and HOPY147). p19 and p35 had no effect on the growth of cdc24 mutants at any temperature (data not shown). Thus the weak suppression by DRT1 of temperature sensitivity (Figure 5-5) appears to be specific for bem1 mutants. cdc24 mutants are defective in shmooing and in mating to far1 mutants (Field and Schekman 1980; Chenevert, Valtz and Herskowitz submitted, see Chapter 2). p19 and p35 appeared to have no effect on the mating of cdc24 strains, but the mating defect is weak and a subtle effect may have been missed. When cdc24 cells containing p19 or p35 were treated with pheromone and observed microscopically, it was clear that the presence of the plasmids did not restore shmoo formation.

DISCUSSION

In both budding and mating, polarity establishment proteins such as Bem1p act to recruit the cytoskeleton and secretory machinery to the site of cell polarization. The polarity establishment proteins must somehow respond to site selection information, from the BUD gene products in the case of budding or presumably from the pheromone receptor in the case of mating. The passage of information from the site selection molecules to the polarity

establishment molecules may be direct, for example via a BUD1-CDC24 or STE2-BEM1 link, or indirect, with an unknown number of molecules in between. Using the knowledge that BEM1 is involved in cell polarization and that bem1-s mutants are defective only in mating-specific polarization, we developed a relatively unbiased approach to identify potential mating site selection molecules. We have identified two genes, DRT1 and FUS2, which could restore mating to bem1 mutants when present on multicopy plasmids.

Possible mechanisms for multicopy suppression. There are several possibilities to explain the action of high copy suppressors. (1) Extra copies of proteins which directly interact with Bem1p could compensate for an interaction weakened by the bem1-s mutation, allowing the interaction to occur by mass action. (2) Another type of high copy suppressor could restore function to a bem1-s mutant by increasing the input signal in a pathway of components which ultimately impinges on the function of BEM1. This sort of suppressor would be "upstream" of BEM1 and would not interact directly with Bem1p. (3) Finally, another type is a bypass suppressor, which is able to restore a wild-type phenotype to bem1 mutants through a mechanism unrelated to the normal function of BEM1.

Clearly DRT1 and FUS2 can affect cell polarity without directly interacting with BEM1, since suppression can occur in a strain completely lacking BEM1 for both suppressors (Figure 5-5). Thus a mass action explanation in which a feeble interaction with a bem1-s protein is overcome seems unlikely. It is possible that overexpression of DRT1 or FUS2 increases a signal to polarity

establishment proteins, for example, by marking an appropriate site for polarization on the cell surface. If this were the case, suppression could occur in both a BEM1-dependent and BEM1-independent manner: In bem1-s mutants, an increased signal at the cell surface could allow a stronger recruitment or attachment of bem1-s protein molecules to the shmoo tip site. Suppression in the bem1Δ strain could occur via an increased signal to other polarity establishment proteins, which would then recruit the cytoskeleton and so compensate for the lack of BEM1. Alternatively, DRT1 or FUS2 may be able to restore cell polarity in a manner completely independent of polarity establishment gene products, for example, by organizing the cytoskeleton directly.

Role of DRT1. Several effects of overexpression of DRT1 indicate that it may be involved in cell morphogenesis. First, p2uDRT can restore shmooing to 40-50 % of the cells in bem1-s1 and bem1-s2 shmooless mutant strains. Second, p2uDRT partially reverses the slow growth and aberrant morphology displayed by a bem1Δ strain. Third, p2uDRT causes wild-type cells to form hyperpolarized shapes during vegetative growth. If Drt1p were part of a complex important for polarizing the cell, hyperpolarization of wild-type cells might be an exaggerated manifestation of the normal role of DRT1 during budding. The suppression of bem1 mutants may reflect a role in polarizing the cell that DRT1 normally performs during shmooing, for example, by providing a signal for polarity establishment proteins. If however, the primary role of Drt1p is in vegetative polarity, the suppression by DRT1 of morphological defects in pheromone-treated cells may be an artificial effect of

overproduction. The lack of stimulation of DRT1 transcript by mating factor is consistent with this idea.

If DRT1 does have an important role in morphogenesis, the lack of phenotype associated with deleting DRT1 from yeast strains is puzzling. One explanation is that DRT1 homologues or other proteins which are able to perform Drt1p's function may exist in the yeast cell. There are two possible experiments which would address whether DRT1 is directly involved in yeast cell polarity. (1) A drt1 mutant should be tested in the recently developed assay for growth towards a source of mating pheromone (Segall 1993); a strong defect in this assay would implicate DRT1 as important in mating site selection. (2) Immunolocalization with antibodies to DRT1 protein may reveal a distribution relevant to cell polarization such as concentration at the mother-bud neck (like Bud3p) or staining at the site of bud and shmoo tip emergence (like Bem1p).

An independent identification of DRT1 supports the idea that it may interact with BEM1 to promote mating. DRT1 was found in a screen for high copy suppressors of a dominant negative mutation in the β subunit of the pheromone receptor-coupled G protein (E. Leberer, personal communication). Strains carrying a particular mutation in Gβ (Ste4p-D62N) mate poorly, at a frequency of about 10^{-5} (Leberer *et al.* 1992) A high copy number plasmid containing DRT1 significantly improves mating of the Ste4p-D62N mutant (to a frequency of about 10^{-2}). Interestingly, BEM1 was also isolated in this screen as a gene which can restore mating to the Gβ mutant. It was also found that a drt1 mutation exacerbates the mating defect of ste4 mutants, a phenomenon known as "synthetic sterility". These

results, in combination with our observation that DRT1 can suppress the shmooing defect of bem1 mutants, strongly indicate that DRT1 and BEM1 interact to bring about cell polarization in response to mating pheromones and suggest a branch from the G protein to molecules which promote morphogenesis.

Role of FUS2. Unlike DRT1, FUS2 mRNA is undetectable in vegetative cells and is highly induced by pheromone treatment. This is one prediction for genes which may be involved in mating site selection and recruitment of the polarity establishment proteins. The localization of Fus2p to the shmoo tip (E. Elion, personal communication) is also consistent with a possible role in mating site selection. The defect in cell fusion displayed by fus2 mutants could result from a primary defect in cell polarity leading to failure to properly localize fusion proteins (such as Fus1p). Mutants defective in FUS2 should be tested in the pheromone-orientation assay (Segall 1993). It would also be interesting to know whether overproduction of FUS2 , like DRT1, can suppress mutations in Gβ.

The sequence of FUS2 (E. Elion, personal communication) shows that its upstream region contains pheromone response elements, which confer pheromone inducibility on many genes in yeast (Marsh, Neiman and Herskowitz 1991; Kurjan 1992). Otherwise, the sequence is uninformative. We note that the lack of suppression by p2uFUS2 of vegetative defects of the bem1Δ strain is not meaningful in light of the fact that FUS2 is not expressed under these conditions. It would be interesting to determine whether forced expression of FUS2 in vegetative cells had any effect on cell polarity or bud site selection in wild-type yeast cells or in mutants defective in polarity

CHAPTER 6

CONCLUSION

The goal of this work was to learn about how mating factors influence cell polarity in yeast. A genetic screen for mating defects followed by a microscopic screen for morphology defects identified genes required for pheromone-induced cell polarization. One of these genes, BEM1, was studied in depth, and several others are being cloned and analyzed by Nicole Valtz. A second genetic screen identified other genes which interact with BEM1. Some of the genes and proteins identified in this study appear to comprise components of the link between mating factors and the cell polarization machinery.

The design of the screen to identify genes involved in pheromone-induced cell polarization was partly rational thinking, partly fantasy. We imagined that for an event such as morphogenesis, a normal function in one partner would be sufficient for mating. Therefore mutants were isolated which were defective in mating to enfeebled partners but not defective in mating to wild-type partners. Other primary screens were considered, such as isolation of mutants that exhibit reduced mating when both mating partners are defective in the same gene (bilateral steriles) or mutants which mate to more than one partner. However, these other ideas generally required greater preparation and sophisticated strain constructions. The hunt for mutants which fail to mate to enfeebled partners (far1 or fus1 fus2 strains) was very simple to perform and produced an embarassment of riches. To date, we have analyzed scarcely half of the interesting mutants generated in the screen. Equally important to the original idea for the primary

genetic screen were the powerful secondary screens which eliminated many mutants and focussed interest on others.

At the outset of this work, there was a suspicion that the genes which controlled the establishment of cell polarity in mating might be the same as those in budding. Our unbiased identification of mutants in BEM1 and CDC24 which are mating-defective and which enlarge in an unpolarized manner in response to mating pheromone lends support to the notion that common elements of the polarity machinery are used in budding and mating. BEM1 was simultaneously identified by Kathy Corrado, through a genetic interaction with BUD5, and by Alan Bender, through a genetic chain of interactions beginning with CDC24. The numerous independent identifications of BEM1 and genetic interactions with BEM1 (see Table 6-1) underscore its importance, but with so many interactions it is difficult to know which correspond to meaningful protein-protein interactions.

Bem1p as part of a polarity establishment complex. There is evidence that Bem1p may physically interact with Cdc24p. The C-terminal half of Bem1p (a portion which lacks the SH3 domains) was used as a probe in a two-hybrid screen (Song *et al.* 1991) and shown to interact specifically with Cdc24p (Alan Bender, personal communication). In addition, both Bem1p and Cdc24p are found in the same high molecular weight complex in yeast cells (Lauryl Bender, personal communication).

The similar intracellular localizations of Bem1p and Cdc42p to sites of cell surface growth is consistent with their possible physical interaction. An interaction between Bem1p and Cdc42p is further

Table 6-1

Genetic interactions with BEM1

INTERACTION	GENE	REFERENCE
synthetic lethality	BUD5	Chant *et al.* 1991
	BUD1	H-O. Park[a]
	BUD2	"
	MSB1	Bender and Pringle 1991
	BEM2	A. Bender[a]
	CDC24	"
	CDC42	"
	AKR1	"
	SPA2	M. Snyder[a]
high copy suppression of bem1		
growth defect	CDC42	A. Bender[a]
	DRT1	J. Chenevert[b]
mating defect	FUS2	"
	DRT1	"
high copy suppression by BEM1		
of mutant growth defect	rho3	Matsui and Toh-e 1992
	rho3 rho4	Matsui and Toh-e 1992
of mutant mating defect	cdc24	J. Chenevert[b]
	ste4	E. Leberer[a]
of mutant arrest defect	fus3	E. Elion[a]

a personal communication
b unpublished observations

suggested by an analogy with two interacting components of the NADPH oxidase system of neutrophils, p47 and rac2. rac2 is a small GTP-binding protein 70% identical to yeast Cdc42p (Mizuno *et al.* 1992; Rotrosen *et al.* 1992). Like Bem1p, p47 contains two SH3 domains. In addition, there is a short but striking region of homology (30% identity or 50% similarity over 50 amino acids) between Bem1p and p47 outside of the SH3 domains (A. Neiman, personal communication; Figure 6-1). This sequence is likely to represent a discrete functional domain, as this precise motif is present in at least two additional proteins (A. Neiman, personal communication). An intriguing possibility is that the region of homology between Bem1p and p47 represents a recognition domain for Cdc42p. Alternatively, this domain may specify an interaction with an as yet unidentified molecule which is part of a complex involving Cdc42p.

Given these arguments and the similarity between the phenotypes of mutants in BEM1, CDC24, and CDC42, it seems likely that the genetic interaction between BEM1 and CDC24 and that between BEM1 and CDC42 correspond to meaningful protein-protein interactions. We propose that Bem1p acts as part of a protein complex involving Cdc24p and Cdc42p to recruit the cytoskeleton and secretory machinery to the site of cell polarization.

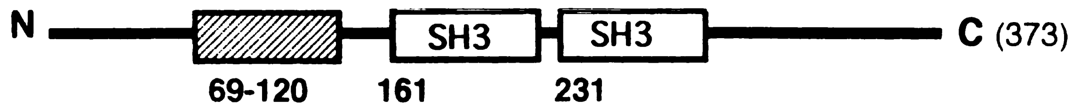
Importance of SH3 domains in BEM1. We have not carried out any experiments to address specifically the role of the SH3 domains in the BEM1 protein. However, the phenotypes of various bem1 mutants and BEM1 constructs are informative in this respect (Figure 6-2). First, it is clear that both SH3 domains are not

Figure 6-1. Homology between Bem1p and human p47.

A: Schematics of Bem1p and p47. The numbers refer to the number of amino acids in the proteins. The hatched region indicates the area of homology between the two proteins in addition to the SH3 domains.

B: Sequence of homologous region. Identical residues are noted; + indicates a conservative change.

A



B

```
Bem1p   KRIMPYIPGPVPYVTNSITKKRKEDLNIYVADLVNLPDYISRSEMVHSLFVV
        +RI+P +P+P  +      + +R   L  Y + L++LP  ISR   +   F V
  p47   NRIIPHLPAPKWFDGQRAAENRQGTLTEYCSTLMSLPTKISRCPHLLDFFKV
```

necessary for function of Bem1p, since removal of the first SH3 domain (as in pBEMΔN110 or pGALGSTBEM) does not impair complementation of bem1 mutant phenotypes. Other results indicate that one SH3 domain may be a requirement for proper Bem1p function. Different constructs with an altered amino terminus (pBEMΔN110), carboxy terminus (pbem1-s1), or internal sequences (pbem1-s3) all complement the vegetative growth defect of a bem1Δ strain. These observations seem puzzling, because these three constructs lack complementary segments of BEM1. All three constructs, however, have in common a possible functional SH3 domain: pBEMΔN110 retains the second SH3 domain; pbem1-s1 retains the first SH3 domain; and the protein fusion created in pbem1-s3 generates a novel SH3 domain (see Figure 4-1). For mating ability, a greater level of this SH3-mediated function may be required. SH3 domains have been shown to bind peptides rich in proline (Cicchetti *et al.* 1992; Ren *et al.* 1993). As yet no yeast candidate proteins with the consensus binding sequence are known.

Bem1p may interact with the G protein coupled to pheromone receptors. The polarity establishment complex is proposed to be responsive to the pheromone signal during mating. How might polarity establishment proteins such as Bem1p receive information from activated pheromone receptors? Two recent identifications of BEM1 shed new light on its role in mating and contribute to a model for the establishment of cell polarity in response to pheromones.

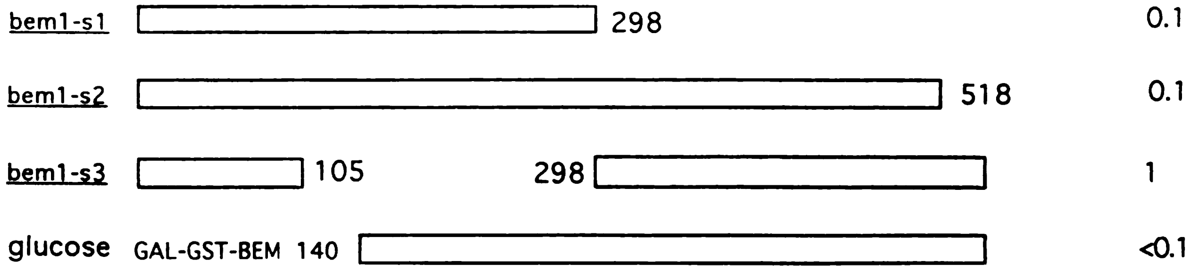
BEM1 was identified as a high copy suppressor of a mutation in STE4, the β subunit of the pheromone receptor-coupled G protein

Figure 6-2. Summary of phenotypes associated with mutant versions of BEM1.

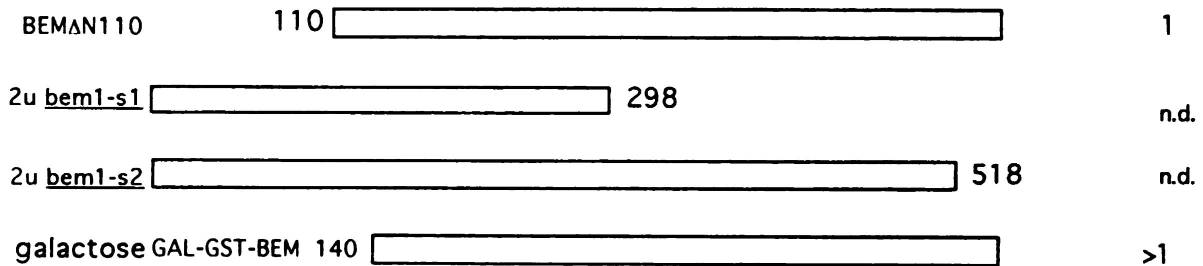
Plasmid constructions are described in Chapter 4. "GAL-GST-BEM" is a construct in which BEM1 is under the control of the inducible GAL promoter; in glucose this promoter is repressed and in galactose this promoter is induced. Numbers near the bars indicate amino acid residues in the BEM1 protein. Numbers to the right of the figure indicate approximate BEM1 protein level relative to that present in wild-type. In general, fully complementing proteins are present at wild-type levels; complementation of the budding defect but not the mating defect occurs when proteins are present at less than wild-type levels, and no complementation occurs when the proteins are absent. Although bem1-s1, bem1-s3, and GAL-GST-BEM each lack different portions of the protein, they all retain a possible functional SH3 domain (see text). The approximate relative protein amounts were determined by comparing signals from immunoblots. n.d.: protein level not determined.

approximate relative protein amount

shmooless alleles (bud+ shmoo-)



fully complementing alleles (bud+ shmoo+)



null alleles (bud- shmoo-)

(E. Leberer, personal communcation; see Discussion, Chapter 5). A possible explanation for this observation is that there may be two STE4-dependent pathways, one leading to the kinase cascade and arrest and induction, and another leading to BEM1 and cytoskeletal organization. The possibility of a branchpoint at the G protein leading to morphogenesis is consistent with the genetic requirements for mating partner discrimination (K. Schrick, personal communication; see Chapter 1). Further evidence for a branch at the G protein could be obtained by determining which pheromone responses are suppressed by BEM1 in the ste4 mutant. If BEM1 suppresses mating by restoration of shmooing but not cell cycle arrest or gene induction, it would be the complement to a bem1 mutant which is defective in shmooing but not in arrest or induction and would support a branch from the G protein to molecules which promote morphogenesis. To address further this model, one could try to generate shmooless alleles of STE4, that is, mutations in Gβ which are defective in shmooing but not in other pheromone responses.

BEM1 was also found in a high copy suppressor hunt as a gene which can restore pheromone-induced cell cycle arrest to a fus3 mutant (E. Elion, personal communication). At face value, this observation seems to indicate that BEM1 could be involved in inhibiting cell division in response to pheromone. However, bem1 mutants are not defective in arrest in reponse to pheromone (J. C., unpublished observations; see Chapter 2). An alternative explanation is that there is some coordination or checkpoint between cell morphology and cell cycle controls. For example, some BEM1-

dependent structure may have to be built in order for the pathway leading to cell cycle arrest to proceed at full activity. A third explanation for the suppression of fus3 by BEM1 involves the G protein branchpoint model. Different pheromone responses may require different threshholds of G protein activity. BEM1 might be essential for the morphogenesis pathway but required for full activity of STE4 through both pathways. Interestingly, a CEN plasmid containing STE4 induces the pheromone response pathway and dramatically slows the growth of wild-type yeast cells but does not slow the growth of cells containing a bem1-s1 mutation (J. C., unpublished observation), further indicating that BEM1 is required for full potency of Gβ.

DRT1 was also identified in the screen for high copy suppressors of the mating defect of a Gβ mutation (E. Leberer, personal communication). Furthermore, a drt1 mutation exacerbates the mating defect of ste4 mutants, a phenomenon known as "synthetic sterility" (E. Leberer, personal communication). These results, in combination with our observation that DRT1 can suppress the shmooing defect of bem1 mutants, strongly indicate that DRT1 and BEM1 interact to bring about cell polarization in response to mating pheromone. Whether DRT1 restores all aspects of mating response or just shmooing should be determined. It is known that in a wild-type strain, high copy DRT1 does not induce the expression of a pheromone-inducible gene, FUS1-lacZ (E. Leberer, personal communication; J. C., unpublished observations) so it seems unlikely that DRT1 functions by turning up all aspects of the pathway.

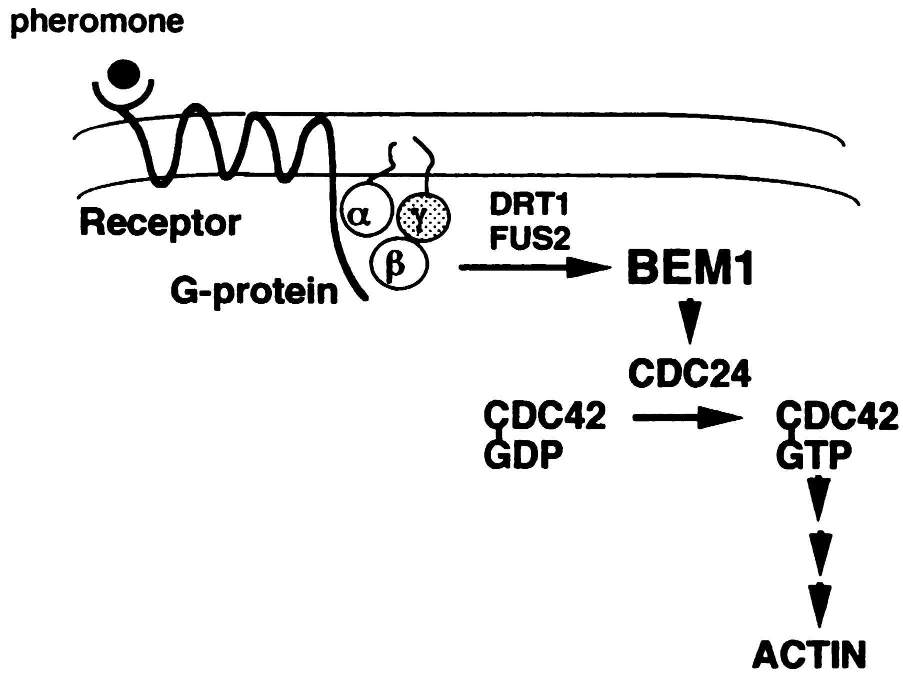
As these results suggest a branch from the G protein to molecules which promote morphogenesis, we propose that BEM receives information from Gβγ through DRT1 (see below and Figure 6-3). FUS2 may also act in this capacity and will be tested for the ability to suppress Gβ mutants. Thus the role of DRT1 may be similar to the proposed role of the BUD proteins, which is to recruit BEM1 or other polarity establishment proteins to the site of cell-surface growth. In this regard, the region of homology between DRT1 and BUD3 (Figure 5-6) becomes intriguing.

A fanciful model for pheromone-induced cell polarization. At the outset of this work, there was little information concerning a molecular mechanism for the establishment of cell polarity in response to mating pheromones. During the past few years, work from a number of laboratories has contributed results bearing on this question. Combining these results with the findings presented in this work, the outline of a model can be formed (Figure 6-3). The purpose of such a model, which is unlikely to be correct in detail, is to provide a framework for thinking about the issues and to aid in the design of future experiments.

The central features of the model depicted in Figure 6-3 are as follows. Binding of pheromone to the pheromone receptor leads to release of Gβγ from the inhibitory Gα subunit. Gβγ signals to the polarity machinery through an effect on Bem1p. Drt1p (and possibly Fus2p) promote the interaction between Gβ and Bem1p. Bem1p binds to Cdc24p and activates it to catalyze nucleotide exchange on Cdc42p. GTP-bound Cdc42p stimulates actin

Figure 6-3. Speculative model for establishment of cell polarity in response to mating factors.

Binding of pheromone to the pheromone receptor leads to release of Gβγ from the inhibitory Gα subunit. Gβγ signals to the polarity machinery through an effect on Bem1p. Drt1p (and possibly Fus2p) promote the interaction between Gβ and Bem1p. Bem1p binds to Cdc24p and activates it to catalyze nucleotide exchange on Cdc42p. GTP-bound Cdc42p stimulates actin polymerization. Evidence for the proposed steps and a discussion of the model is presented in the text.


pheromone
Receptor
G-protein
α
γ
β
DRT1
FUS2
BEM1
CDC24
CDC42
GDP
CDC42
GTP
ACTIN

polymerization.

There is some evidence, albeit sketchy in certain cases, to support the different steps in the proposed pathway. The proposal that the G protein mediates a signal to proteins which cause the morphological response, rather than a signal coming directly from the receptor, is based on the requirement for the G protein for mating partner discrimination in yeast, and also on analogy to eucaryotic chemotaxis systems (see Chapter 1). A branch from the G protein to the polarity machinery away from the rest of the conventional signal transduction pathway is invoked because of the lack of requirement for protein kinases or other components of the pheromone response pathway for mating partner discrimination and because genes involved in polarity, BEM1 and DRT1, can suppress mutations in Gβ. Bem1p is a likely component of the polarization branch, since bem1 mutants are defective only in cell polarization and not in other pheromone responses. DRT1 is placed as a mediator between the G protein and BEM1 because it has genetic interactions with both Gβ and BEM1. Overexpression of DRT1 can suppress a Gβ mutation or a bem1 mutation, and a drt1 mutation exacerbates the mating defect of a Gβ mutation. The proposed interactions between BEM1, CDC24, and CDC42 are based on the observation that Bem1p can bind to Cdc24p and on the homology between Cdc24p and *dbl*, an exchange protein for a human homologue of Cdc42p (Hart *et al.* 1991). The stimulation of actin organization by GTP-bound Cdc42p is inspired by the effects of ras-related GTPases on actin organization in mammalian cells (Hall 1992).

A few modifications to this model could lead to a view of the

establishment of cell polarity during budding. The pheromone receptor would be replaced by a landmark such as the 10-nanometer filaments. The G protein would be replaced by BUD proteins, which recognize the landmark and communicate the site to the polarity establishment proteins. The actions of BEM1, CDC24, and CDC42 and the subsequent organization of the cytoskeleton would be the same.

Ways to identify mating site selection genes. Bud site selection genes are thought to encode connectors between a cell surface landmark and polarity establishment proteins. Analogous mating site selection genes would encode connectors between activated pheromone receptors and polarity establishment proteins. Genetic approaches to identify mating site selection genes are modeled on the genetic interactions that have been found between bud site selection genes and polarity establishment genes. BUD1 was identified as a high copy suppressor of the vegetative defect of a cdc24 mutant, and so we performed an analogous hunt for high copy suppressors of the mating defect of bem1-s mutants. A mutation in bud5 was identifed as enhancing the vegetative defect of a bem1 mutant (resulting in lethality). An appropriate analogous mutant hunt for mating site selection genes would be to search for mutants which enhance the mating defect of bem1 or cdc24 mutants ("synthetic steriles").

Mutants defective in mating site selection could also be sought in a direct microscopic hunt, as in the microscopic tour-de-force in which BUD1-BUD4 were isolated (Chant and Herskowitz 1991). This approach necessitates prediction of a phenotype and development of

an assay. Mutants defective specifically in mating site selection should be proficient for pheromone responses such as cell cycle arrest, gene induction, and projection formation, but only defective in the proper selection of shmoo site. Until recently, there was no direct assay for selection of shmoo site. Hopefully, the demonstration of a directional response of **a** cells on a solid surface to α-factor provided from a micropipet (Segall 1993) will be easily reproducible. It is doubtful, however, that this technique as reported could be used for a large scale mutant hunt, since only about a dozen cells around each micropipet can be scored, and only 50% of those show proper orientation.

I favor a combination approach for identifying mating site selection genes in which a genetic screen is followed by microscopic analysis. Isolation of mating defective mutants which are proficient for shmooing and other pheromone responses would provide an enrichment for mating site selection mutants. These mutants could then be tested individually in the shmoo site selection assay. An ideal group of mutants to test already exists, the class IV mutants with wild-type morphology which fail to mate to enfeebled mating partners (Chapter 2; Chenevert, Valtz and Herskowitz submitted). Other ways in which putative mating site selection mutants could be accumulated include the hunts for synthetic steriles with bem1 or cdc24 mutants as suggested above or a hunt for bilateral steriles which are mutants that exhibit reduced mating when both mating partners are defective in the same gene.

Some questions regarding the establishment of cell polarity in yeast have been answered, but many more have been raised: What

is the nature of the spatial cue which determines where mating cells polarize? What are the relevant targets of the pheromone receptor and the G protein? Do these targets act to position polarity establishment proteins such as Bem1p? Do BUD gene products recruit Bem1p, Cdc24p, or Cdc42p to the bud site during vegetative growth? How are the interactions between site selection molecules and polarity establishment proteins regulated? How do polarity establishment proteins influence actin organization? The future promises exciting answers to these and other urgent questions.

APPENDIX 1

LOCALIZATION OF BEM1 PROTEIN IN PHEROMONE-TREATED CELLS

Bem1p has an important role in polarizing the cell to make a bud or a shmoo (Bender and Pringle 1991; Chant *et al.* 1991; Chenevert *et al.* 1992). Bem1p localizes to the growth tip just before and during bud emergence (Corrado 1992). In order to determine whether Bem1p is also at the site of cell polarization during shmoo formation, I localized the BEM1 protein in pheromone-treated cells using an anti-Bem1p antibody.

METHODS

Affinity-purified anti-Bem1p antibody was a generous gift of Kathy Corrado. Before use, the antibody was further purified as follows. Cells from an exponentially growing culture of the bem1Δ strain KO2-5C were fixed and spheroplasted as if for immunofluorescence (Pringle *et al.* 1989). A small volume of these cells was then collected by centrifugation and resuspended in an equal volume of the affinity-purified antibody preparation. After incubation at room temperature for one hour, the mixture was centrifuged and the supernatant solution was collected. After three cycles of such treatment, the final supernatant solution was stored at 4°C for use in immunofluoresence. Yeast cells of the strain JC2-1B

were treated with 10^{-6} M α-factor for two hr and prepared for staining essentially as described (Pringle *et al.* 1989). Cells were incubated with the Bem1p antibody diluted 1:2 in PBS/BSA (40 mM K_2HPO_4 + KH_2PO_4, pH 6.5, 0.5 mM $MgCl_2$, 1% BSA) overnight at 4°C in small eppendorf tubes. Antibody incubation was carried out in tubes rather than on slides because the primary antibody tended to stick to the glass slides and cause a high background. The Bem1p antibody could then be recovered from the tubes and reused for another staining. After incubation with the primary antibody, cells were washed twice with PBS/BSA, placed on polylysine-coated slides, and washed five more times with PBS/BSA. Secondary antibody (Rhodamine-conjugated goat-anti rabbit from Cappel) was added at a 1:200 dilution for one hour. The wells were washed twice with PBS/BSA, once with PBS, and mounted in DAPI-containing mounting medium (gift of N. Valtz).

RESULTS AND DISCUSSION

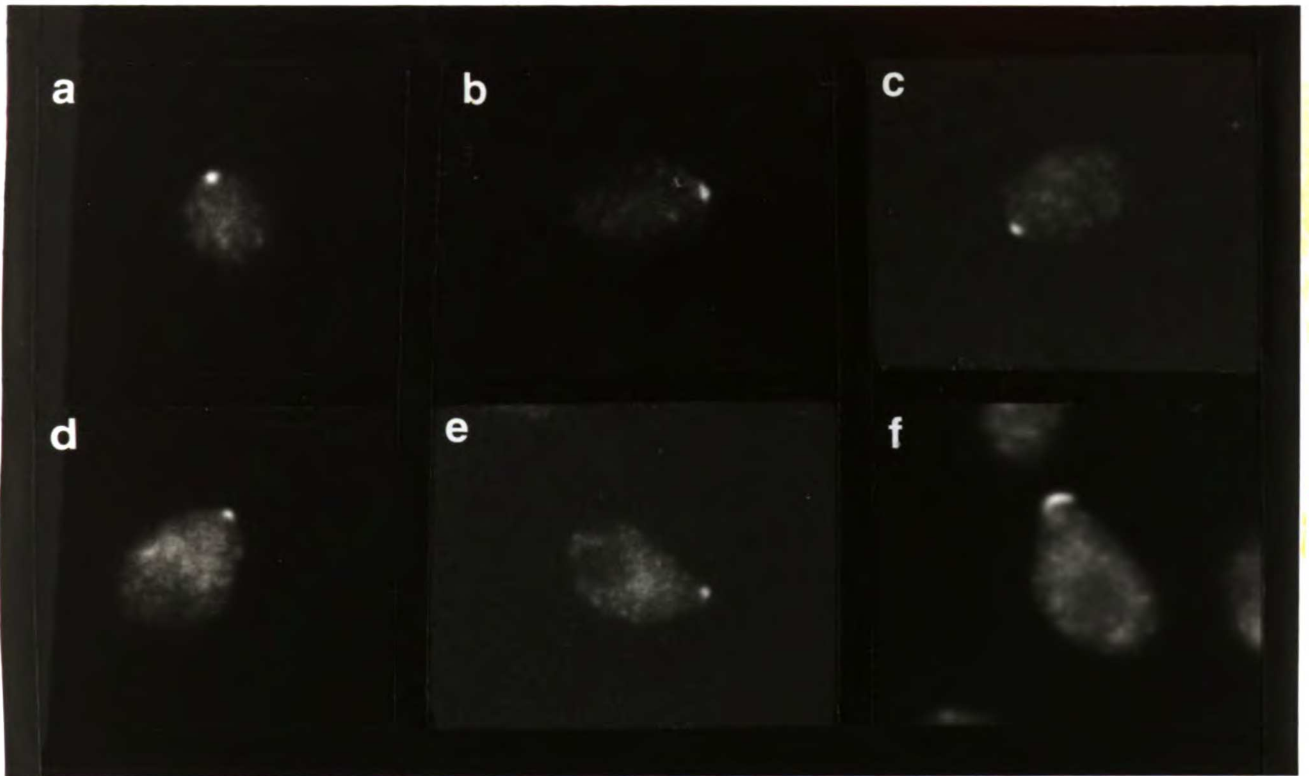
In all cells in which anti-Bem1p staining was observed, Bem1p localized to a patch on the surface of the cell. This was true for both cells in early stages of projection formation (Fig. A1-1, cells a,b,c) and for fully formed shmoos (Fig. A1-1, cells d,e,f). In fully formed shmoos, the Bem1p patch always coincided with the tip of the projection (Fig. A1-1, cells d,e,f). No staining of any cells was observed in control wells lacking the primary antibody.

Most of the cells (80%) did not stain with the Bem1p antibody. The fact that the majority of the cells did not stain with Bem1p

antibody is not due to problems with fixation or permeability of the cells to antibodies, since > 90% of the same cells stained with a control antibody which also stains the shmoo tip, anti-Spa2p (data not shown). Staining with anti-Bem1p is very weak, sensitive to experimental conditions, and fades quickly (J. C., unpublished observations; K. Corrado, personal communication) relative to other antibodies. I therefore believe that the Bem1p antigen is probably present in most shmoos but was not detected in all cells due to technical limitations of this experiment. I cannot however rule out the possibility that the localization of Bem1p to the shmoo tip does not occur in some cells or that this localization is transient and thus only observed in certain cells fixed at the appropriate stage.

Figure A1-1: Bem1p localizes to the tip of the shmoo projection.

Several cells of strain JC2-1B treated with α-factor for two hours are shown. Some cells are in the early stages of shmoo formation (cells a,b,c) and others exhibit pronounced projections (cells d,e,f).


a
b
c
d
e
f

APPENDIX 2

DISCRIMINATION ASSAYS

Mating partner discrimination is defined as the ability of wild-type yeast cells to discriminate pheromone-producing from non-producing mating partners (Jackson and Hartwell 1990a; Jackson and Hartwell 1990b; Jackson, Konopka and Hartwell 1991). Wild-type yeast cells are able to discriminate among possible mating partners and choose a mating partner that produces the highest level of pheromone. Discrimination is proposed to involve reorganization of the cytoskeleton and orientation of the nucleus and secretory pathways in the direction of the pheromone-producing partner. The fidelity of mating partner discrimination is such that less than one in 10^5 matings occurs with pheromone non-producing partners. Mutants which are completely defective in mating partner discrimination mate equally well with either pheromone-producing or pheromone non-producing partners when both are present in equal numbers. These mutants are typically supersensitive to mating pheromones (defective in SST1 or SST2) and thus presumably are unable to detect the source of the pheromone gradient properly. If mating partner discrimination reflects polarized morphogenesis towards a pheromone gradient, it may be considered an assay for mating site selection. It was therefore of interest to know if mutants in any of the genes identified in this work were defective in mating partner discrimination.

METHODS

Yeast strains were grown in YEPD (or minimal media lacking uracil when a plasmid was present) to mid-log phase (OD_{600} approximately 0.7). For each experiment three strains were used: the strain to be tested for ability to discriminate, the wild-type strain of mating type opposite to the strain to be tested, and the pheromoneless strain of mating type opposite to the strain to be tested. 10^7 cells of each of the three strains were mixed and filtered onto 0.45-um nitrocellulose filters (Millipore), giving 3×10^7 total cells per filter. The filters were rinsed with 5 ml YEPD and placed on YEPD plates (or a minimal plate lacking uracil if a plasmid was involved), and incubated at 25°C for 3.5 hours or 30°C for 2.5 hours. Cells were resuspended in 5 ml SD by 30 sec vigorous vortexing followed by sonication for 3 sec. Dilutions were plated on YEPD to determine total colony-forming units and on appropriate minimal medium plates to select for the two types of possible diploids.

We strived for a ratio of strain being tested: wild-type partner: pheromoneless partner of 1:1:1, since this ratio gives the highest mating efficiency (K. Schrick, personal commmunication). The ratio of pheromoneless mating partners to total mating partners was predicted to be 0.5 based on using equivalent OD amounts. In most experiments, this ratio was calculated by plating dilutions of the input strains and counting colonies and was between 0.44 and 0.57. The same ratio was used for all strains in a given experiment.

The percent total diploids was calculated as the percentage of

cells which formed diploids with either the wild-type or the pheromoneless strain (the latter was negligible in most cases) times 100. Percent matings with the pheromoneless strain was calculated as the number of diploids formed with the pheromoneless strain divided by the total number of diploids formed with either strain times 100. In some cases, genetic markers were such that the numbers of the two types of diploids could be determined separately on selective plates. In other cases, only the number of total diploids and the number of diploids formed with the pheromoneless strain could be determined.

RESULTS AND DISCUSSION

Mutants defective in BEM1, FAR1, DRT1, and FUS2 and strains which overproduce BEM1, DRT1, or FUS2 were tested in an assay for mating partner discrimination. In no case was the ability of yeast cells to choose the pheromone-producing mating partner properly altered significantly. In these assays, strains defective in BAR1 did display defects in mating partner discrimination: they mated with the pheromone non-producing strain up to one-fourth of the time. (The reported frequency of 50% mating with the pheromoneless strain was never obtained).

The lack of defect in mating partner discrimination was initially surprising, particularly for the bem1 mutants, which are defective in pheromone-induced polarization. A possible explanation is that in a bem1 mutant, polarity establishment is defective but mating site selection may be intact. Thus when bem1 cells do mate,

they mate at the proper site and with the wild-type partner. The same argument does not appear to hold true for budding, since cells deleted for BEM1 are defective in bud site selection (J. C., unpublished observations).

The ability of mutants defective in BEM1 or in the other genes tested to discriminate could also be attributed to redundancy in a mating site selection pathway. Perhaps the only single genes involved in mating site selection which are essential for mating partner discrimination (or mating site selection) are those that encode the receptor and the subunits of the G protein. Downstream of the G protein there may be numerous targets involved in mating partner discrimination, none of which is itself essential for the process.

Finally, it may be that the discrimination assay is in fact not a measure of polarized morphogenesis, or that the genes tested may have nothing to do with polarized morphogenesis. I do not favor this explanation. It is important to bear in mind that in this assay the event which is scored (mating) is a complicated and multi-faceted process. Mating partner discrimination may be an indirect assay for one aspect of the mating process, oriented growth. A negative result does not necessarily imply that a particular gene is not involved in morphogenesis during mating. The strains of interest should also be tested in a direct assay for polarized growth towards a source of pheromone which is independent of successful mating (Segall 1993).

Table A2-1: Mutants defective in BEM1 are not defective in mating partner discrimination.

Strain	Relevant Genotype	% Total Diploids	% Matings with Pheromoneless Strain
IH1783[a]	MATa	47	< 2.4 X 10^{-5}
JC109[a]	MATa bem1-s1	15	< 1.1 X10^{-5}
JC2-1B[a]	MATa bar1	5.1	0.16
JC-G11[a]	MATa bar1 bem1-s1	0.5	0.16
JC-F5[a]	MATa bar1 bem1-s2	2.1	0.13
JO14[b]	MATα	28	< 1.7 X 10^{-5}
JC111[b]	MATα bem1-s1	5.9	< 1.8 X 10^{-4}
IH2362[c]	MATa bar1	4.3	0.12
KO2-5C[c]	MATa bem1Δ	10	< 2.0 X 10^{-5}

[a] The α-factor producing strain was IH1784; the mfα1 mfα2 strain was IH1866.

[b] The **a**-factor producing strain was IH1783; the mfa1 mfa2 strain was IH2276.

[c] The α-factor producing strain was AN37-4C; the mfα1 mfα2 strain was IH1868.

Table A2-2: Mutants defective in FAR1 are not defective in mating partner discrimination.

Strain	Relevant Genotype	% Total Diploids	% Matings with Pheromoneless Strain
JO31-1A	MATa	26	< 1.4×10^{-6}
IH2487	MATa bar1	2.6	0.12
JC-B4	MATa bar1 far1	0.9	0.08
FC307	MATα	49	4×10^{-5}
IH2497	MATα far1	0.11	< 4.5×10^{-4}

For testing mating of MATa strains (first three lines), the α-factor producing strain used was IH1784 and the mfα1 mfα2 strain was IH1866. For testing mating of MATα strains (last two lines), the **a**-factor producing strain used was IH1783 and the mfa1 mfa2 strain was IH2276.

Table A2-3: Mutants defective in DRT1 or FUS2 are not defective in mating partner discrimination.

Strain	Relevant Genotype	% Total Diploids	% Matings with Pheromoneless Strain
IH1783	MATa	59	1.1×10^{-5}
KOD-1A	MATa drt1	63	2.7×10^{-5}
KOFUS2	MATa fus2	47	$< 1.2 \times 10^{-6}$
IH2362	MATa bar1	5.9	0.07

The α-factor producing strain used was AN37-4C and the mfα1 mfα2 strain was IH1868.

Table A2-4: High copy plasmids containing either BEM1, DRT1, or FUS2 do not cause a defect in mating partner discrimination.

Strain	Relevant Genotype	Plasmid	% Total Diploids	% Matings with Pheromoneless Strain
IH1783	MATa	YEP24	60	1.3 X 10-6
IH1783	MATa	p2uBEM	29	6.1 X 10-6
IH1783	MATa	p2uDRT	37	1.9 X 10-6
IH1783	MATa	p2uFUS2	27	5.4 X 10-6
IH2362	MATa bar1	none	4.3	0.27

The α-factor producing strain used was AN37-4C and the mfα1 mfα2 strain was IH1868.

APPENDIX 3

ARE BEM1 HOMOLOGUES PRESENT IN OTHER ORGANISMS?

Bem1p is important for organizing actin and establishing cell polarity in cells responding to intrinsic or extrinsic signals (see Chapters 3 and 4). Many molecules involved in organizing the cytoskeleton and cell polarity are conserved among diverse species. As a first step in determining whether a Bem1p-like protein might be present in other organisms, I performed a western blot on extracts from other eucaryotes with an affinity-purified Bem1p antibody.

METHODS

Protein extracts from a variety of sources were collected. The identities and providers of the extracts were: Drosophila oocyte extract, gift of Ken Schneider; human HeLa cell nuclear and cytoplasmic extracts, gifts of Beate Schwer; nematode extracts, gifts of Steve Salser; monkey kidney (COS cell) extract, gift of Michael Garabedian; Xenopus oocyte extract, gift of Tim Stearns. The yeast extract was prepared from strain "YMP05" (MATa bar1). Gel electrophoresis, electro-blotting and antibody incubations were performed as described in Chapter 4. The primary antibody was affinity-purified rabbit anti-Bem1p (a generous gift of K. Corrado) and the secondary antibody was phosphatase-coupled conjugate of

goat anti-rabbit immunoglobulin G (Boehringer Mannheim).

RESULTS AND DISCUSSION

A strong Bem1p signal is detected in the expected location in the lane containing yeast extract (Figure A3-1). Much more yeast protein was loaded than was necessary to detect Bem1p. Proteins of apparently larger molecular weights than Bem1p are clearly detected in the extracts from COS cells, Drosophila oocytes, and nematode. The band in lane N is half as wide as the others because of transfer-interference by a bubble in this region (data not shown); the same nematode band is apparent in lane N_m. A low-molecular weight band is detected in the HeLa cytoplasmic extract but not the HeLa nuclear extract. No signal is detected in the Xenopus oocyte extract.

In the cases where cross-reacting proteins were detected, a single discrete band is present in each lane. It is therefore unlikely that the anti-Bem1p antibody is simply recognizing the SH3 domains of SH3-domain containing proteins, because SH3-domain containing proteins are very numerous and many more bands would be expected. In HeLa cell nuclei, a band was detected in the cytoplasmic fraction but not in the nuclear fraction, as expected for a protein possibly involved in organizing the cortical cytoskeleton. The lack of signal in the Xenopus extract may not be meaningful, since this is a specialized cell type which is arrested in development. A band might have been detected in actively growing frog cells.

This "zoo blot" is merely an initial step in investigating Bem1p-

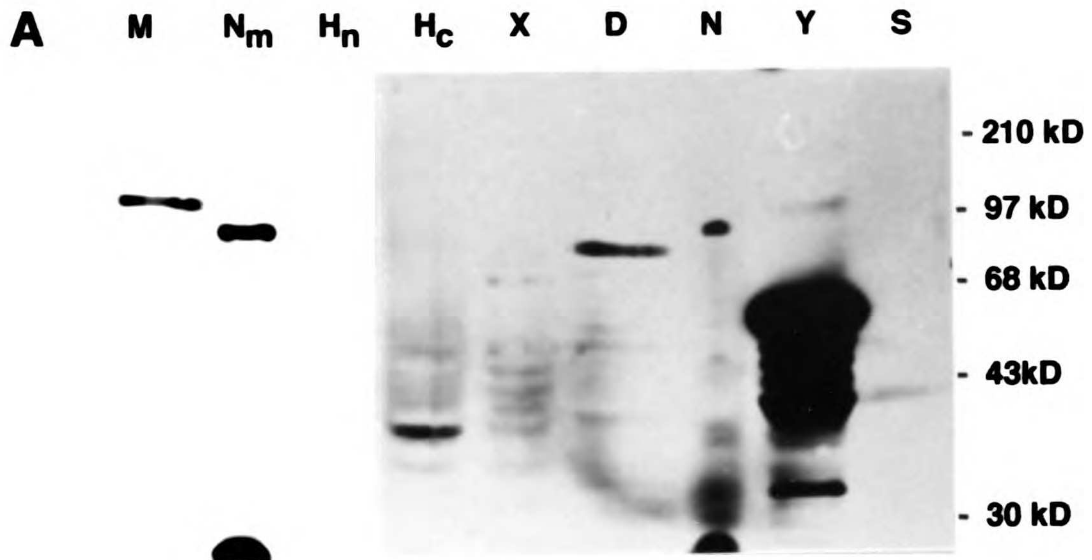
like proteins in other organisms. In order to pursue this line of investigation, the experiment should be repeated, and further characterization of the cross-reacting species would be required. Before bothering to clone a putative Bem1p-homologue, it would be prudent to stain the cells of interest with the anti-Bem1p antibody and look for a meaningful localization. An encouraging result was obtained in a preliminary atttempt to localize the Bem1p cross-reacting protein in Drosophila embryoes. Affinity-purified anti-Bem1p antibody stained a band along the cell cortex in the region where actin is found (G. Pruliere, personal communication). If several Bem1p-like proteins are eventually cloned, degenerate primers could be prepared to consensus regions and cloning by the polymerase chain reaction could be carried out directly from a given organism's nucleic acid. The identification and characterization of Bem1p homologues may enhance our understanding both of cell polarity in other organisms and of the role of BEM1 in yeast cell polarity.

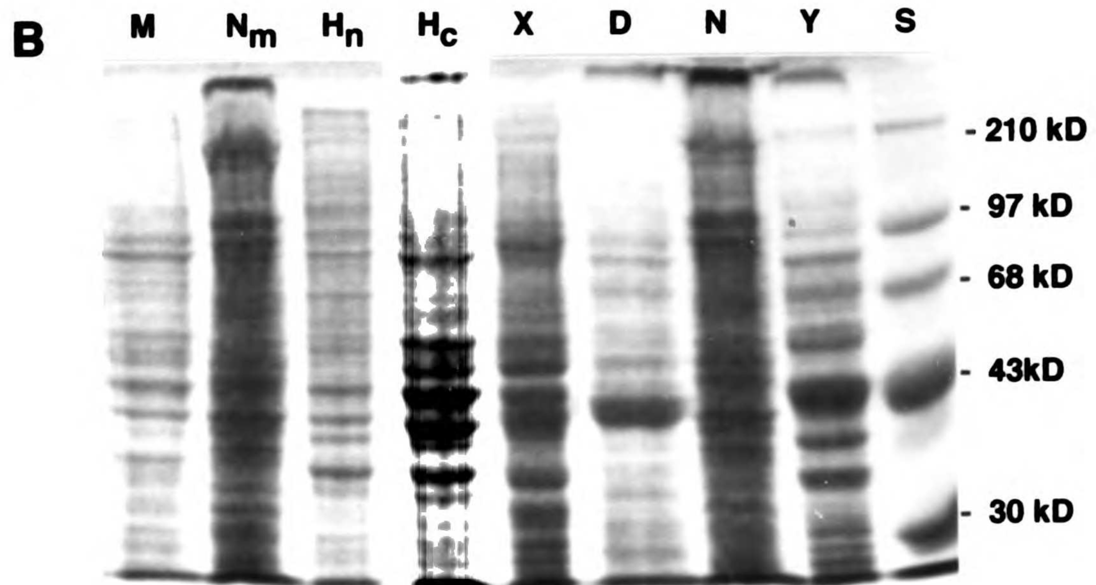
Figure A3-1: Immunoblot on extracts from various organisms using anti-Bem1p antibody.

A: Western blot on extracts from yeast and other eucaryotes with an affinity-purified Bem1p antibody.

B: Coomassie Blue staining of gel identical to that used for the immunoblot.

The identities of the lanes are as follows. M: Monkey kidney (COS cell) extract, N_m: extract from nematode mab-5 mutant, H_n: Human HeLa cell nuclear extract, H_c: Human HeLa cell cytoplasmic extract, X: Xenopus oocyte extract, D: Drosophila oocyte extract, N: nematode extract, Y: yeast strain YMP05, S: molecular weight standards.


A
M
N_m
H_n
H_c
X
D
N
Y
S
- 210 kD
- 97 kD
- 68 kD
- 43kD
- 30 kD


B
M
N_m
H_n
H_c
X
D
N
Y
S
- 210 kD
- 97 kD
- 68 kD
- 43kD
- 30 kD

APPENDIX 4:

YEAST STRAINS AND PLASMIDS

Following is a list of some of the strains and plasmids made during this work. This same list can be found in the listings of the Herskowitz laboratory strain collection, where these strains and plasmids are frozen. Other strain information can be found in Table 2-1.

Table A4-1

YEAST STRAINS

lab name/my name/genotype and comments

The following strains are isogenic with IH1783 (a), IH1784 (α), and IH1788 (a/α) (trp1 leu2 ura3 his4 can1) except as noted:

IH2591	KO1	MATa bem1::URA3* / MATα BEM1
IH2592	KO1-1A	MATα bem1::URA3*
IH2593	KO1-1B	MATa bem1::URA3* (*in the previous three strains, all of BEM1 is not deleted; the first 110 aa are remaining, but it behaves like a complete null.)
IH2594	KO2SB-1	MATa bem1::LEU2 / MATα BEM1
IH2595	KO2-5B	MATα bem1::LEU2
IH2596	KO2-5C	MATa bem1::LEU2
IH2597	KO2-1A	MATα bem1::LEU2
IH2598	KO2-1D	MATa bem1::LEU2
IH2599	KOD-1A	MATa drt1::LEU2
IH2600	KOD-1B	MAT α drt1::LEU2
IH2601	XD2-1A	MATa drt1::LEU2 bem1::LEU2
IH2602	XD2-1B	MATα drt1::LEU2 bem1::LEU2

IH2603 EGfus2 MATa fus2::URA3*

IH2604 EGdf2 MATa drt1::LEU2 fus2::URA3*

IH2605 EGbf2 MATa bem1::LEU2 fus2::URA3*
(*in the previous three strains, the fus2 disruption was not checked by Southern, but it's probably right since they fail to mate to fus1 fus2 strain IH2351.)

IH2606 AN1005 **a/a/a/a** Ura^+, probably contains pGALHO

IH2607 AN1009 **a/a/α/α**
(the previous two strains are tetraploids for use in immunofluorescence, made by Aaron Neiman)

<u>other strains (not isogenic with previous series):</u>

IH2608 JC262 MATa HMRα HMRα trp1 leu2 ura3 his4 bud3::TRP1
(strain from John Chant)

IH2609 XD1-8B MATa bar1-1 met1 his4 ura3 trp1 leu2 drt1::LEU2

IH2610 XD1-14C MATa bar1-1 met1 ade2 his4 ura3 trp1 leu2 drt1::LEU2

IH2611 XD3-8A MATa trp1 leu2 ura3 his4 bud3::TRP1 drt1::LEU2
(allele at HMR unknown)

IH2612 XD3-9B MATα trp1 leu2 ura3 his4 bud3::TRP1 drt1::LEU2
(allele at HMR unknown)

IH2613 XD4-5D MATa trp1 leu2 ura3* his4 drt1::LEU2 bud1::TRP1
(*<u>ura3</u> allele is pretty useless because it papillates to give <u>Ura</u>$^+$ colonies)

IH2614 XD4-2C MATα trp1 leu2 ura3* his4 drt1::LEU2

bud1::TRP1
(*ura3 allele is pretty useless because it papillates to give Ura+ colonies)

IH2615 wtafus2 MATa HMLa HMRa ura3-52 ade2-101 met1 bar1-1 fus2::URA3
(fus2 disruption was not checked by Southern, but it's probably right since they fail to mate to fus1 fus2 strain IH2351.)

cdc24 strains: (also see IH2431 and around there)

IH2616	HOPY147	MATa cdc24-4 ura3 leu2-3,112 his3
	(ref: Bender & Pringle, 1989 PNAS)	
(IH2630)	JC-I13	MATa bar1-1 cdc24-5 (ts) ura3-52 ade2-101
IH2617	XI13-7A	MATa bar1-1 cdc24-3 (ts) ade2-101 ura3-52
IH2618	XI13-8B	MATa bar1-1 cdc24-5 (ts)
IH2619	XI13-2D	MATa bar1-1 cdc24-3 (ts) ura3
IH2620	XI13-6C	MATa bar1-1 cdc24-5 (ts) ura3 met1

(Note - both cdc24-3 and cdc24-5 mutants are fine at 25°C and dead at 33°C, but they can be distinguished at 30°C: cdc24-3 strains form normal sized colonies at 30°C; cdc24-5 strains form smaller-than-wild-type colonies at 30°C)

mating testers:

normal ones:

IH2621	IH1792	MATa cry1 lys1 mating tester
IH2622	IH1793	MATα lys1

halo testers:

IH2623	IH414	MATa sst1 arg9 ilv3 ura1 sst1 leaky his4?
IH2624	IH993	MATα sst2-1 met1 his6 can1 cyh2

far1 mating testers:

IH2625	JC31-7D*	MATα far1-c lys1 (allele at HML unknown)
IH2626	JC31-1D*	MATa far1-c lys1 (allele at HML unknown)

parent strain for mutant hunt (JC2-1B) and 36 original mutants derived from it:

IH2627	JC2-1B	MATa HMLa HMRa ura3-52 ade2-101 met1 bar1-1
IH2628	JC-G11	bem1-s1
IH2629	JC-F5	bem1-s2
IH2630	JC-I13	cdc24-5
IH2631	JC-J9	pea1-1 (spa2)
IH2632	JC-D6	pea1-2 (spa2)
IH2633	JC-A10	pea2-1
IH2634	JC-I14	pea2-2
IH2635	JC-G16	tny1-1
IH2636	JC-H9	tny1-2
IH2637	JC-B4	far1
IH2638	JC-D1	far1
IH2639	JC-G18	far1
IH2640	JC-H7	far1
IH2641	JC-F20	fus2

wild-type mutants:

IH2642	JC-A5	
IH2643	JC-B6	
IH2644	JC-E15	
IH2645	JC-F16	random budder, possibly bud2-
IH2646	JC-G3	
IH2647	JC-G17	
IH2648	JC-I5	
IH2649	JC-I11	
IH2650	JC-I15	bipolar budder
IH2651	JC-J3	
IH2652	JC-J10	
IH2653	JC-J21	
IH2654	JC-J26	

ts or vegetatively abnormal:

IH2655	JC-A1	"mickey mouse" phenotype
IH2656	JC-A2	cells are large, not ts
IH2657	JC-C5	**a**-specific sterile, reduced **a**-factor production
IH2658	JC-C6	
IH2659	JC-C8	**a**-specific sterile, reduced **a**-factor production
IH2660	JC-E13	looks like arrest in mitosis

IH2661	JC-I3	
(IH2630)	JC-I13	cdc24-5
IH2662	JC-J16	bipolar budder

<u>beml-s1 strains:</u> (allele at HML unknown for all of them)

IH2663	JC108	MATα ura3 trp1 ade2-101 beml-s1
IH2664	JC109	MATa met1 ura3 trp1 ade2-101 beml-s1
IH2665	JC111	MAYα leu2 his4 ade2-101 ura3 FUS1-lacz::URA3 beml-s1
IH2666	JC112	MATα ade2 leu2 his4 ura3 FUS1-lacz::URA3 beml-s1
IH2667	JC114	MATa met1 trp1 leu2 his4 ura3 FUS1-lacz::URA3 beml-s1
IH2668	JC117	MATα leu2 his4 ura3 FUS1-lacz::URA3 beml-s1
IH2669	JC123	MATa ura3 leu2 trp1 ade2 beml-s1
IH2670	JC54-3A	MAT a1- (23a50) beml-s1 trp1 leu2 his4 ura3 FUS1::lacZ (URA3)
IH2671	JC54-9C	MAT a1- (23a50) beml-s1 trp1 met1 ade2-101 leu2 his4 ura3 FUS1::lacz (URA3)

<u>Strains possibly containing mutations in genes which interact with BEM1:</u>

IH2678	JC-p25	mutant derived from JC-G11
IH2679	JC-p40	mutant derived from JC109
IH2680	JC-p43	mutant derived from JC109
IH2681	JC-p44	mutant derived from JC109
IH2682	JC-p45	mutant derived from JC109
IH2683	JC-p48	mutant derived from JC109

During the hunt for high-copy suppressors of <u>beml-s1</u> mating defect (Chapter 5), the previous six plasmid-independent mutants were identified in which mating was partially suppressed. These strains may contain extragenic suppressing mutations of <u>beml-s1</u> (spontaneously generated), or they may be intragenic revertants of <u>beml-s1</u> (these two possibilities could be distinguished by a western blot.)

Table A4-2

PLASMIDS

BEM1 plasmids are described in Chapter 4; DRT1 and FUS2 plasmids are described in Chapter 5.

CY name my name bacterial strain if known

CY361 pCENBEM JM101
2.1 kb SmaI-KpnI fragment containing BEM1 cloned into pRS316 (Sikorski and Hieter, 1989).

CY362 p2uBEM JM101
2.1 kb SmaI-KpnI fragment containing BEM1 cloned into pRS426 (a URA3 2u vector, Sikorski and Hieter, 1989).

CY363 pbem1-s1 JM109
bem1-s1 mutation recovered from chromosome onto pCENBEM by gap repair.

CY364 pbem1-s2 MH6
bem1-s2 mutation recovered from chromosome onto pCENBEM by gap repair.

CY365 pbem1-s3 DH5α
pCENBEM in which 573 bp internal to BEM1 have been deleted. Amino acids 107-299 are removed and an in-frame fusion is generated; confers a bem1-s like phenotype to bem1Δ strains.

CY366 p2ubem1-s1 DH5α
BEM1 gene in p2uBEM1 replaced by bem1-s1 gene (see Chapter 4). 1.8 kb Kpn1 fragment in p2uBEM1 was replaced by 1.8 kb KpnI fragment from pbem1-s1 in original orientation.

CY367 p2ubem1-s2 DH5α
BEM1 gene in p2uBEM1 replaced by bem1-s2 gene (see Chapter 4) 1.8 kb Kpn1 fragment in p2uBEM1 was replaced by 1.8 kb KpnI fragment from pbem1-s2 in original orientation.

CY368 pKO1 DG98
BEM1 knock out with URA3
The 1.6 kb HindIII fragment in the BEM1 in pEB4.2 was replaced with the 1.2 kb HindIII fragment containing URA3. Cut with EcoRI and BamHI; isolate 3.8 kb fragment; transform and select for Ura^+. (note - this deletion leaves about 110 amino acids of Bem1p amino terminus intact, but it behaves as a complete null)

CY369 pUCBEM (also called pUCSHM)
2.1 kb SmaI-HindIII fragment containing BEM1, filled in and ligated into the SmaI site of $pUCBgl^2$ (a derivative of pUC18 I believe; obtained from Joe Ogas).

CY370 pKO2 JM101
BEM1 knock out with LEU2
The BEM1 gene in pUCBEM was precisely replaced by LEU2.
Cut with SmaI and BamHI; transform and select for Leu^+.
(Cutting with SmaI and SalI or SmaI and PstI should work also.)
If you want to purify the fragment, it's a little tricky since it is 2.7 kb, the same size as pUC. I cut pUC with ScaI in addition to SmaI and BamHI. There is minimal homologous flanking sequence from the BEM1 locus (210 bp upstream, 270 bp downstream); only 2/16 transformants were bem1 deletions.

CY371 pHABEM MH6
pCENBEM containing a BEM1 gene in which nine amino acids comprising the HA epitope are introduced at the amino terminus of BEM1, just after the third codon. (warning - the epitope tends to be cleaved off in yeast extracts if care is not taken)

CY372 p2uHABEM
The 5' region of the BEM1 gene in p2uBEM was replaced with the same region (on a 550 bp SacI-HindIII fragment) containing the HA epitope introduced after the third codon.

CY373 pGALGSTBEM DH5α
Most of BEM1 was cloned into pRD56 (from R. DeShaies) on a BamHI-KpnI fragment. This plasmid produces a fusion protein in which the initial 140 amino acids of Bem1p have been replaced by 220 amino acids of glutathione-S-transferase.

CY374 placZ:BEM1 (Pringle lab pUR-91)
The 1.3 kb SalI-HindIII fragment from plasmid pPB321 was subcloned into SalI/HindIII-cut plasmid pUR289, yielding pUR-91. Directs the synthesis of a fusion protein of the expected size of -154 kDa. (made by Kathy Corrado, described in Chapter 2 of her thesis.)

CY375 ptrpE:BEM1 (Pringle lab pATH32)
The 1.5 kb BamHI-HindIII fragment from plasmid pPB321 was subcloned into BamHI/HindIII-cut plasmid pATH3, yielding pATH-32. Directs the synthesis of a fusion protein of the expected size of - 81 kDa. (made by Kathy Corrado, described in Chapter 2 of her thesis.)

CY376 pBEMΔN110 (also called pHind 1.6)
A 1.6 kb HindIII fragment containing most of the BEM1 coding region cloned into yCP50. This construct lacks the amino-terminal 110 amino acids and the promotor of BEM1 and it complements bem1 mutant strains in one orientation with respect to the vector but not the other orientation.

CY377 pEB4.2
A 4.2 kb EcoRI-BamHI fragment contiainig BEM1 cloned into YCp50 (basically another CEN-BEM construct).

CY378 p19 JM109
Plasmid isolated from Carlson YEp24 library during high-copy suppressor hunt. Suppresses bem1 mating defect and slightly suppresses bem1 ts defect. Contains DRT1.

CY379 pUCDRT
The 3.2 kb BamHI fragment containing DRT1 cloned into the BamHI site of pUC something (pUC19 I think).

CY380 pCENDRT
The 3.2 kb BamHI fragment containing DRT1 cloned into the BamHI site of YCp50.

CY381 p2uDRT
The 3.2 kb BamHI fragment containing DRT1 cloned into the BamHI site of YEp24.

CY382 p2uDRTΔKB
The DRT1 gene in p2uDRT was disrupted by deleting 420 bp from the

DRT1 coding sequence between the KpnI site and the BglII site.

CY383 pKODRT
DRT1 knock out with LEU2. The DRT1 gene in pUCDRT was precisely replaced by LEU2 using the PCR technique described previously (Chenevert et al., 1992) to generate pKODRT (see chapter 5).
To delete DRT1, cut with BamHI, isolate 4.5 kb fragment and transform, selecting Leu^+.

CY384 p35 JM109
Plasmid isolated from Carlson YEp24 library during high-copy suppressor hunt. Suppresses bem1 mating defect only. Contains FUS2.

CY385 p2uSph
The BamHI and SalI sites in pRS426 (and all sites in between) were destroyed and replaced with SphI site. pRS426 was cut with BamHI and SalI and filled in with Klenow. SphI linkers were added.

CY386 p2uFUS2
4.5 kb SphI fragment containing FUS2 cloned into p2uSph.

CY387 pCENSph
A SphI site was introduced into pAS109 (gift of Anita Sil), a derivative of pRS316 lacking HindIII sites) by cleavage at SmaI and ligation of SphI linkers to the blunt SmaI site.

CY388 pCENFUS2
4.5 kb SphI fragment containing FUS2 cloned into pCENSph.

CY389 pKOFUS2
FUS2 knock out with URA3
The 1.6 kb HindIII fragment in p2uFUS2 was replaced with the 1.1 kb HindIII fragment containing URA3. Cut with SphI; isolate 4 kb fragment and transform, selecting for Ura^+.

CY390 p39 JM109
Plasmid isolated from Carlson YEp24 library during high-copy suppressor hunt. Suppresses bem1 mating and ts defect well; does not appear to contain BEM1. May be very interesting. Was not studied further.

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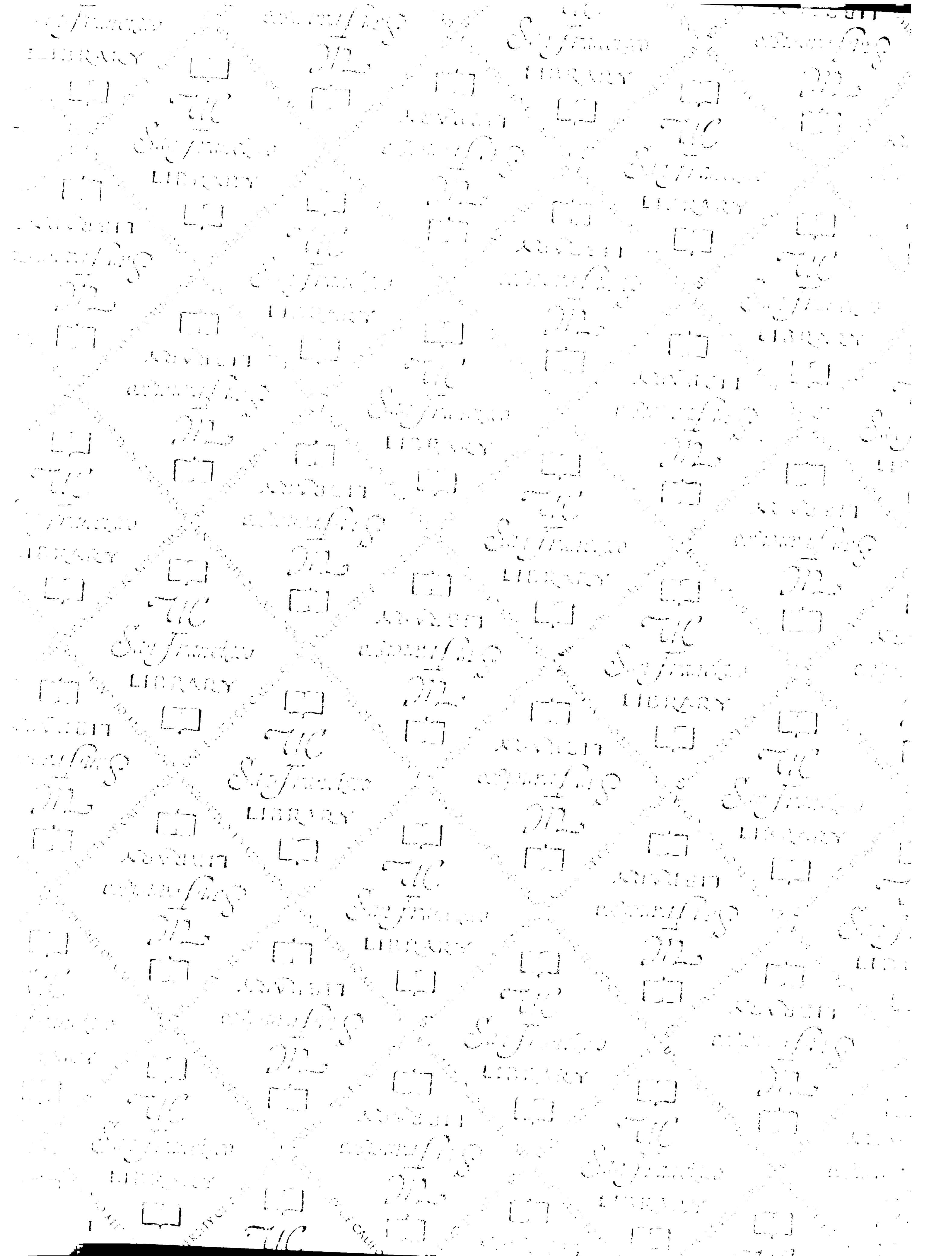

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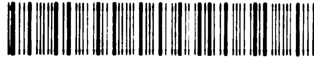

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