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Specification of Sites for Polarized Growth in *Saccharomyces cerevisiae* and the Influence of External Factors on Site Selection

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Many eucaryotic cell types exhibit polarized cell growth and polarized cell division at nonrandom sites. The sites of polarized growth were investigated in G1 arrested haploid *Saccharomyces cerevisiae* cells. When yeast cells are arrested during G1 either by treatment with α-factor or by shifting temperature-sensitive *cdc28-1* cells to the restrictive temperature, the cells form a projection. Staining with Calcofluor reveals that in both cases the projection usually forms at axial sites (i.e., next to the previous bud scar); these are the same sites where bud formation is expected to occur. These results indicate that sites of polarized growth are specified before the end of G1. Sites of polarized growth can be influenced by external conditions. Cells grown to stationary phase and diluted into fresh medium preferentially select sites for polarized growth opposite the previous bud scar (i.e., distal sites). Incubation of cells in a mating mixture results in projection formation at nonaxial sites: presumably cells form projections toward their mating partner. These observations have important implications in understanding three aspects of cell polarity in yeast: 1) how yeast cell shape is influenced by growth conditions 2) how sites of polarized growth are chosen, and 3) the pathway by which polarity is affected and redirected during the mating process.

INTRODUCTION

In most eucaryotes, polarized growth is a fundamental process that often begins at a nonrandom site. The molecular mechanisms by which polarized growth occurs are poorly understood, and very little is known about how sites of directional cell growth are selected.

The budding yeast *Saccharomyces cerevisiae* undergoes polarized cell growth at two times in its life cycle: during vegetative growth and mating (Drubin, 1991; Madden *et al.*, 1992). Vegetative yeast cells display asymmetric growth during the budding process when a daughter cell is produced as a bud from a mother cell. Bud formation initiates at a specific site on the cell surface late in the G1 phase of the cell cycle; the location of the emerging bud will also be the future site of cytokinesis (Byers, 1981). Yeast cells display different budding patterns dependent on the mating locus and pedigree of the cell (Freifelder, 1960). Haploid *MATa* and *MATα* mother cells bud adjacent to the previous bud site and daughter cells form buds next to the birth site (i.e., axial budding). Diploid *MATa/MATα* daughter cells usually form buds opposite the birth site (i.e., distal budding) whereas mother cells bud either adjacent or opposite to the previous site (Freifelder, 1960; Byers, 1981; Snyder, 1989; Chant and Herskowitz, 1991). Mutations have been found that alter the process of bud site selection (Sloat *et al.*, 1981; Snyder, 1989; Bender and Pringle, 1989; Chant *et al.*, 1991; Chant and Herskowitz, 1991; see DISCUSSION). For example, *bud1/rsr1*, *bud2*, and *bud5* cells bud randomly, whereas *bud3* and *bud4* mutations have been reported to cause haploid cells to bud in a bipolar (diploid) pattern rather than axially (Chant *et al.*, 1991; Chant and Herskowitz, 1991).

Yeast cells also exhibit polarized growth during mating. After exposure to pheromone from the opposite mating type, cells arrest late in G1. Detection and transduction of pheromone signal initates a cascade of events, including polarized growth in the form of an elongated projection (Cross *et al.*, 1988); the pear-shaped cells that result are called shmoos. Yeast cells preferentially mate with partners that secrete the highest level of pheromone (Jackson and Hartwell, 1990b; Jackson *et al.*, 1991). This evidence, along with the observation that zygote formation appears to occur by fusion at the tips of projections (Lipke *et al.*, 1976; Tkacz and MacKay, 1979; Hasek *et al.*, 1987), suggests that mating yeast cells also

exhibit polarized growth toward their partner, presumably by detection of the source of mating pheromone.

Polarized growth appears to be specified by cortical sites (Snyder *et al.*, 1991), and models have been proposed to explain the nonrandom positioning of bud sites in haploid and diploid cells (Chant and Herskowitz, 1991; Snyder *et al.*, 1991; Madden *et al.*, 1992). It has been suggested that axial budding results from positioning new growth at determinants that either localize to or are preserved from the previous site, i.e., the cytokinesis tag model. Distal budding that occurs in *MATa/MATα* diploids may be the result of formation of new growth opposite the microtubule organizing center of yeast (the spindle pole body) (Byers, 1981; Snyder *et al.*, 1991) or budding at the last site of cell surface growth (Chant and Herskowitz, 1991). Based on genetic evidence Chant and Herskowitz (1991) have suggested that the BUD1/RSR1, BUD2, and BUD5 proteins permit polarized growth at diploid bud sites; the BUD3 and BUD4 proteins further increase specificity of bud site selection to axial sites (Chant and Herskowitz, 1991).

Previous studies have suggested that components involved in bud formation begin assembling at the cortex during the G1 phase of the cell cycle (Snyder *et al.*, 1991). The SPA2 protein and actin localize to a discrete site at the cell periphery during G1, before the CDC28-dependent step (Snyder *et al.*, 1991; see below). Furthermore, the neck filament-associated proteins also assemble at the cortex before formation of a visible bud (Ford and Pringle, 1991; Kim *et al.*, 1991). It is presumed, though not directly demonstrated, that the newly polarized components are localized to the expected site of bud emergence (i.e., axial sites in haploid cells, axial, or distal sites in diploid cells). This suggests that intrinsic sites of polarized growth are established before emergence of a visible bud, which occurs at the end of G1 (Pringle and Hartwell, 1981). Because polarized growth during projection formation and budding utilizes many of the same components, arrest of cells during G1 by use of pheromone or other means that allows cell growth might be expected to result in polarized growth at the intrinsic sites. Furthermore yeast cells are expected to form projections toward their mating partner; presumably during the mating response yeast cells forego growth at predetermined sites and instead initiate growth toward their partner.

The experiments described later use a different assay to demonstrate that cell polarity and polarized growth are both specified before the end of G1. In addition, we found that the process of site selection can be altered in response to different growth or mating conditions. These observations have important implications for how cell morphogenesis occurs and how the selection of sites for polarized growth is regulated during both vegetative growth and the mating process.

MATERIALS AND METHODS

Yeast Strains, Medium, and Genetic Techniques

Yeast strains are listed in Table 1. Y762 and Y763 are derived from S288C, Y145 and Y168 are in a A364A background, and Y477, Y479, Y480, Y481, Y483 are derived from a yeast strain whose background is less well defined (Chant and Herskowitz, 1991; Chant, personal communication). Y799 is a wild-type haploid segregant from a cross between Y479 and Y481. Additional wild-type segregants yielded results identical to that of Y799; for presentation purposes only the Y799 results are shown. Growth medium and standard genetic manipulations are as described by Sherman *et al.* (1986).

Mating Projection Formation

Five-milliliter cultures of vegetative wild-type (Y762, Y145, Y799) and bud site selection mutants (Y477, Y480, Y481, Y483) were grown for ~12 h to mid- to late-log phase, diluted in YPD medium and grown 4 h at 30°C to a cell density of OD(600) = 0.3 (~4×10^6 cells/ml). α-factor (Sigma, St. Louis, MO) was added to a final concentration of 5.0 μg/ml. After incubation for 40 min at 30°C the treatment was repeated and cells were incubated for an additional 40 min. After this incubation >90% of the cells had formed small projections; the small size of the projection facilitated quantitation of the *initial* sites of polarized growth. Cells were fixed by addition of formaldehyde to 3.7% (vol/vol) and incubated at room temperature for 30 min. Cells were then washed three times with phosphate buffered saline (PBS) (150 mM NaCl, 50 mM $NaPO_4$, pH 7.2) and resuspended in PBS.

To examine projection formation in cells emerging from stationary phase, wild-type (Y762) cells were grown in YPD at 30°C for 65 h. Cells were diluted to OD(600) = 0.3 in fresh medium and α-factor was added to a concentration of 5.0 μg/ml. α-factor treatment was repeated after incubation at 30°C for 60, 120, and 150 min. After 3-h incubation, cells were fixed as previously described.

To examine projection formation in mating mixtures, wild-type *MATa* (Y762) and *MATα* (Y763) cells were grown to OD(600) = 0.3 at 30°C in YPD medium. Aliquots (0.5 ml) of each culture were combined in a 1.5 cm test tube and incubated at 30°C for 4 or 6 h without shaking. The mating mixture was fixed by addition of formaldehyde and washed as described above. Similar results regarding the position of projection formation in shmoos and the position of fusion in zygotes were observed for the 4 and 6 h incubations; only the 6 h results are presented.

Projection Formation in cdc28 Cells

Mutant *cdc28-1* (Y168) cells were grown at room temperature until OD(600) = 0.3. A 5-ml culture was shifted to the restrictive temperature of 37°C; 1-ml aliquots were fixed by addition of formaldehyde

Table 1. Yeast strains

Strain	Genotype
Y145	*MATa his7 ura1*
Y168	*MATa his7 ura1 cdc28-1*
Y477	*MATa hmr2 hml2 his4 trp1 ura3 CAN1 bud1-1*
Y479	*MATα hmr2 hml2 his4 trp1 ura3 CAN1 bud2-1*
Y480	*MATa hmr2 hml2 his4 trp1 ura3 CAN1 bud2-1*
Y481	*MATa hmr2 hml2 his4 trp1 ura3 CAN1 bud3-1*
Y483	*MATa hmr2 hml2 his4 trp1 ura3 CAN1 bud4-1*
Y762	*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200*
Y763	*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200*
Y799	*MATa hmr2 hml2 his4 trp1 ura3 CAN1*

to 3.7% (vol/vol) after incubation for 60, 75, 90, 120, and 135 min. Approximately 90% of *cdc28* cells had initiated polarized growth at the 75-min timepoint, and this population was used for quantitation.

Analysis of Cells Treated with Low Levels of Pheromone

To analyze polarity in cells treated with different levels of pheromone, 5-ml cultures of vegetative Y762 cells were grown to OD(600) = 0.3 and were treated with α-factor to a final concentration of either 0, 0.05, 0.1, 0.2, or 5.0 μg/ml (Moore, 1983; Read *et al.*, 1992). After incubation at 30°C for 85 min, the cells were fixed and stained with rhodamine-conjugated phalloidin.

To examine the effect of low levels of pheromone on bud site selection, Y762 cells (1-ml culture) were grown to OD(600) = 0.3 and incubated with 0.2 μg/ml of α-factor at 30°C for 80 min. The cells were washed twice with 1 ml of water and resuspended in 1 ml of fresh YPD medium. Cells were incubated at 30°C for 1 hr and fixed and stained with Calcofluor (see below).

Fluorescence Microscopy

Visualization of bud scars was performed similar to that described previously (Sherman, 1986). Yeast cells forming mating projections or temperature-sensitive *cdc28* cells undergoing polarized growth at the restrictive temperature were fixed, washed, and resuspended as described previously. Fluorescent Brightener 28 (Calcofluor; Sigma) was added to a final concentration of 1.7 μg/ml in PBS.

F-actin staining in *cdc28* cells and cells forming mating projections were performed with rhodamine-conjugated phalloidin (Molecular Probes, Eugene OR), in a method similar to that described previously (Adams and Pringle, 1984; Gehrung and Snyder, 1990). Fixed cells suspended in PBS were incubated with equal volume of 3.3 μM rhodamine-conjugated phalloidin (dissolved in methanol). Samples were incubated in the dark at room temperature for 2 h and washed two times with PBS. Cells were then either used for microscopy or double-stained with Calcofluor as previously described.

Quantitation of Selection of Sites for Polarized Growth

Quantitation of the distribution of sites selected for mating projection formation, or bud formation, was performed by dividing a cell into three distinct domains. These domains were determined relative to an x-axis running the length of the cell (specified by the projection or emerging bud) and a y-ordinate beginning at the center of the spherical or ellipsoidal portion of the cell (i.e., the portion not containing the projection). The dot shown in Fig. 2 marks the intersection of the x-axis and y-ordinate. Class I cells contained bud scars located −45 to +45° from the x-axis at the end of the cell containing the projection; Class III cells contained bud scars −45 to +45° from the horizontal axis on the other side of the cell. Class II cells contained bud scars in the center. Angles were estimated by visual inspection during microscopic observation. Cells containing single bud scars were then assigned to one of the three classes, depending on the site where projection initiation occurred. Cells containing single bud scars were scored to facilitate the accuracy of the analysis, although cells containing multiple scars are expected to yield similar results. In the rare cases where the bud scar lay on the boundary between two domains, one-half were scored in each class. Because cells displaying projections in mating mixtures were large relative to the size of the bud scars and few cells had scars that spanned more than one domain, cells containing one or more bud scars were scored.

Assuming the cells were spherical before projection formation, the relative surface areas of the Class I, II, and III domains would be 1, 4.8, and 1, respectively (calculated from Beyer, 1991). Although we believe the distributions of the *bud1*, *bud2*, and *bud3* strains are generally random, the numbers do not precisely match that predicted for a random distribution based on relative surface areas on a sphere (i.e., the number of cells with bud scars in the Class II domain is only 2.5–3-fold larger than those with bud scars in the Class I and III domains). This difference may reflect the fact that either the cells are not spherical or perhaps the estimated angles were actually slightly larger than 45°.

When haploid *cdc28-1* cells (which have a long morphology even at the permissive temperature) were shifted to the restrictive temperature, >95% of the cells initiated polarized growth at either of the poles along the long axis of the cell. Therefore, polarized growth sites were scored as either opposite or adjacent to the pole displaying previous bud scar(s).

The site of zygotic fusion was also quantitated relative to previous bud scars. Because the neck of the zygotes often had a thick diameter (relative to the diameter of the cell), it was difficult in many cases to precisely estimate the 45° angles; hence the location of single bud scars in each mating partner was examined and scored as either adjacent or distal to the site of fusion.

RESULTS

Projection Formation Following α-Factor Treatment Occurs at Axial Sites

As noted above, previous studies have suggested that components involved in bud formation begin assembling at the cell periphery during G1 in the cell cycle (Snyder *et al.*, 1991). To independently assess whether polarity is established before the end of G1, and if so, whether it is established at specific sites, we analyzed G1 arrested cells that exhibit polarized growth. When yeast cells are exposed to high concentrations of mating pheromone they arrest during G1 and form projections at one end of the cell. Because polarized growth during projection formation and budding utilizes many of the same components, projection formation in a haploid cell exposed to a uniform concentration of mating pheromone might be expected to occur at the same sites as those involved in budding i.e., next to the previous bud site (axial sites).

To test this hypothesis log phase *MATa* cells were incubated in the presence of 5 μg/ml α-factor for 80 min. At this time >90% of the cells had undergone cell-cycle arrest and formed short projections, thereby allowing analysis of the initial site for polarized growth (Fig. 1). Following fixation the cells were stained with Calcofluor, which stains bud scars (Hayashibe and Katohda, 1973), and the site of projection formation was analyzed relative to the bud scars. For yeast strains of two different backgrounds (A364A and S288C) plus a third wild-type strain whose original background is not well-defined (see MATERIALS AND METHODS), it was apparent that the vast majority of cells formed projections near bud scars (Fig. 1).

To quantitate the site of projection formation relative to bud scars, cells containing a single bud scar were scored according to the scheme shown in Fig. 2. In Class I cells the projection formed next to the bud scar. Class II cells contained a bud scar in the central region, and in Class III cells the projection formed on the opposite side of the cell. The relative cell surface area for the Class II domain is estimated to be several-fold larger

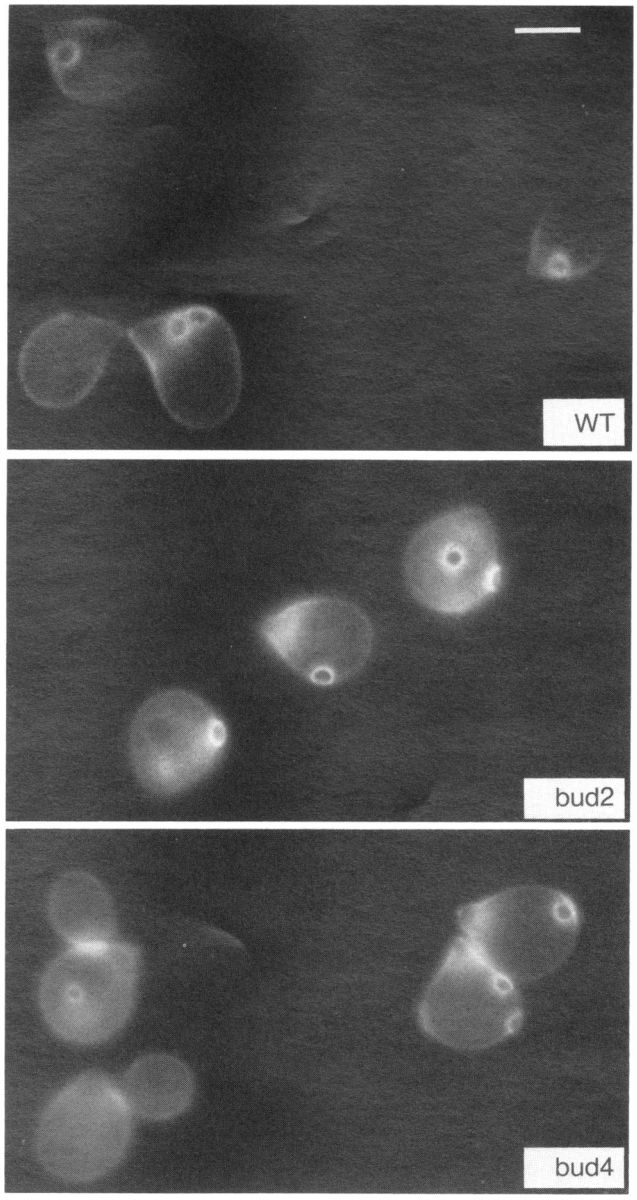

Figure 1. Calcofluor staining of wild-type and bud-site selection mutants after exposure to α-factor. Wild-type yeast cells (Y145) usually form projections near previous bud sites (top). Mutant *bud2* (Y480) and *bud4* (Y483) initiate projection formation in response to α-factor at random locations on the cell surface relative to bud scars (*middle and bottom*). Bar, ≈3 μm.

than that for Class I and III domains (see METHODS). In a wild-type A364A strain, 70% of the cells containing single bud scars were Class I, 30% were Class II, and only 0.5% of the cells were class III (n = 400). A similar result was observed for the yeast strain whose background is poorly defined (Fig. 2), and a slightly less polarized distribution of classes was observed for a wild-type S288C strain (shown in Fig. 5). These results indicate that projections usually form adjacent to the previous bud site and further suggest that sites of cell growth are specified before the end of the G1 growth phase.

Mutations in the *BUD1/RSR1*, *BUD2*, *BUD3*, and *BUD4* genes each alter selection of the proper site for bud formation (see INTRODUCTION; Chant *et al.*, 1991; Chant and Herskowitz, 1991). Studies were performed to determine whether mutations in these genes would also affect the selection of site for projection formation in response to α-factor. Log phase *rsr1/bud1*, *bud2*, *bud3*, and *bud4* mutant cells were exposed to 5 μg/ml α-factor, and in each case most cells were capable of initiating projection formation within 80 min (Fig. 1). As shown in Figs. 1 and 2, in contrast to wild-type strains, projection formation in each mutant occurred at randomly chosen sites on the cell surface. In *bud4* mutants a slight increase in axial budding was observed over that expected for a random pattern, but we note that for both *bud3* and *bud4*, the distribution appeared closer to random than to the bipolar pattern, which might be expected for these mutants (see DISCUSSION). In any event, we conclude that in the presence

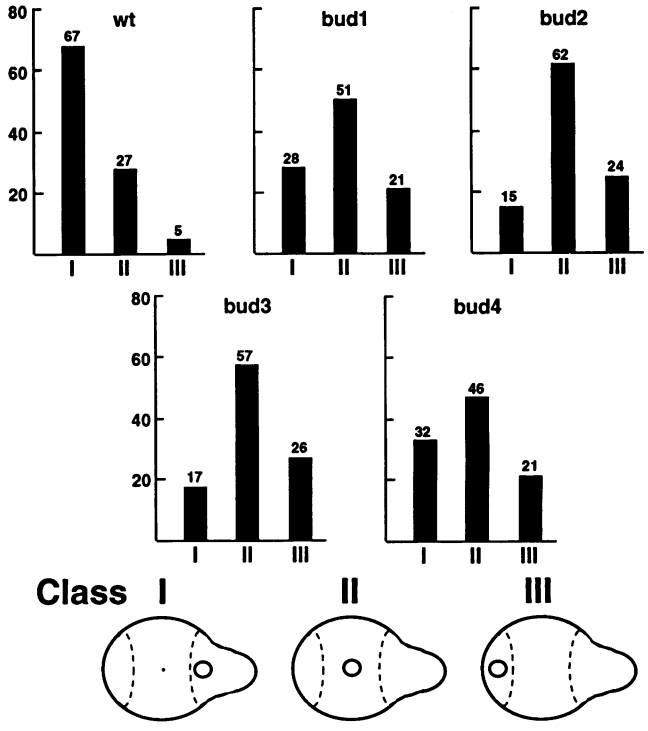

Figure 2. Distribution of sites selected for projection formation in wild-type and bud-site selection mutants exposed to 5.0 μg/ml α-factor for 80 min. Cells were assigned to either Class I, II, or III according to the scheme described in the text and MATERIALS AND METHODS. Wild-type cells show a predominance of Class I cells, whereas bud site selection mutants display a generally random distribution of cells among the three classes. The total number of cells counted were: Y799 (n = 353); Y477 (411); Y480 (452); Y481 (355); and Y483 (394).

of a homogenous concentration of α-factor, projection formation occurs preferentially at axial sites and that these sites are altered in the *bud* mutants.

Polarized Growth in cdc28-Arrested Cells

As an independent test for determining sites of polarized growth during G1, *cdc28* cells were analyzed. Temperature-sensitive *cdc28-1* cells are longer and thinner than wild-type cells at the permissive temperature; after shifting to the restrictive temperature these cells arrest during late G1 before progression through "START" (Pringle and Hartwell, 1981). The arrested cells enlarge and form a projection. To determine the position of projection formation relative to previous bud sites, temperature-sensitive haploid *cdc28* cells were shifted to the restrictive temperature (37°C) for 75 min and then stained with both rhodamine-conjugated phalloidin and Calcofluor. Rhodamine-conjugated phalloidin stains F-actin and allows a clear visualization of the sites of polarized growth. After 75 min ~90% of the cells contained a polarized distribution of F-actin; clusters of cortical actin spots are seen at the tips of the projections and actin cables extend along the axis of the cell and into the projection (Fig. 3). This distribution of actin is expected on the basis of observations of both budding and pheromone-treated cells (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985; Hasek *et al.*, 1987; Gehrung and Snyder, 1990; Read *et al.*, 1992). The position of the sites of projection formation were analyzed relative to the location of previous bud sites. In this analysis, because polarized growth always occurred at one end of the cell, projection formation was scored as either opposite or adjacent to bud scars, and all cells with one or more bud scars on the same side of the cell were scored. Eighty-three percent of the cells formed projections adjacent to previous bud sites (332 of 402 cells), and the remaining 17% formed projections opposite the bud scars. Thus, as with the α-factor studies, these results indicate that sites required for polarized growth are established before the end G1. They further indicate that these sites form before the CDC28-dependent step(s).

Selection of Site for Projection Formation After Entry into Stationary Phase

Wild-type cells grown for extensive periods of time under normal growth conditions eventually exhaust the available nutrient supply, cease cell growth, and arrest in a distinct physiological state known as stationary phase (Lillie and Pringle, 1980; Pringle and Hartwell, 1981). Previous immunofluorescence studies have shown that specific proteins that localize in a polarized manner in vegetatively growing cells are no longer polarized in stationary phase cells. For example, the SPA2 protein is absent in stationary phase cells (Snyder *et al.*, 1991), and actin spots appear randomly distributed around the cortex (Snyder, unpublished data). Thus, a cortical component or complex required for polarized growth may no longer localize to the cell surface.

To determine whether preferred sites of polarized growth are lost when cells enter stationary phase, a culture of cells grown in rich medium was incubated continuously for 65 h and then was diluted into fresh medium in the presence of 5 μg/ml α-factor. After 3 h, 75% of cells had initiated projection formation (Fig. 4). Cells with a single bud scar were assigned to one of the three classes described above (Fig. 5). Polarized growth does not usually occur at previous bud sites; only 5% of the cells form projections near bud scars. Instead, polarized growth usually occurs opposite the site of previous budding; 69% of the cells form projections at this location. Thus, the normal axial site appears lost after cells enter stationary phase.

These results suggest that after cells exit stationary phase the preferred site for polarized growth is distal to the normal axial site. To further test this hypothesis, stationary phase cells were diluted into fresh medium, and the position of bud formation was scored relative

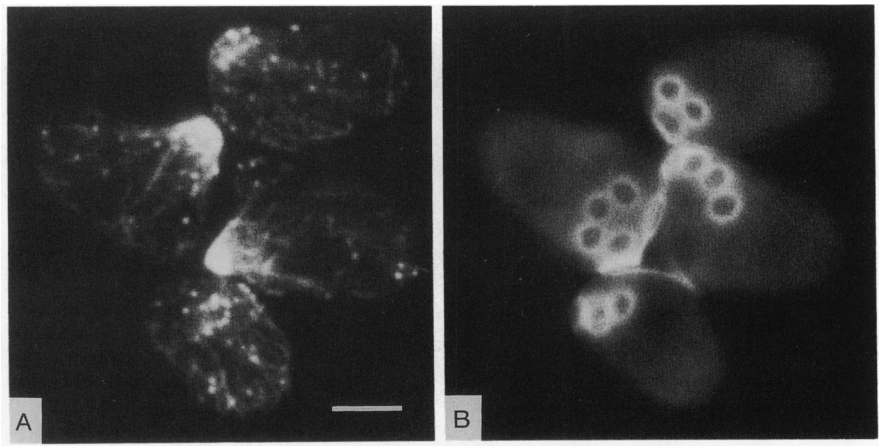

Figure 3. Rhodamine-conjugated phalloidin/Calcofluor double fluorescent staining of haploid *cdc28-1* cells after shift to the restrictive temperature for 75 min. The sites of polarized growth, as assayed by distribution of F-actin (A), in these elongated cells are located adjacent to bud scars (B). Bar, ≈3 μm.

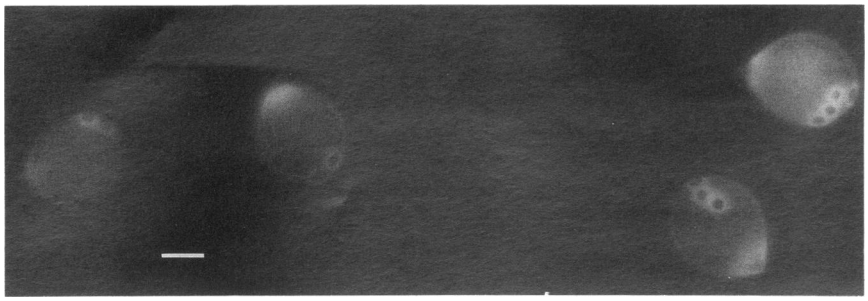

Figure 4. Calcofluor staining of stationary phase cells treated with 5 μg/ml α-factor after dilution into fresh medium. Wild-type cells (Y762) initiate projection formation distal to previous bud sites in contrast to log phase cells, which form projections adjacent to bud scars after pheromone treatment. Bar, ≈3 μm.

to the bud scar in cells that had a single scar (n = 402). The percentage of Class I, II, and III cells was 24.9, 19.9, and 55.2%, respectively, indicating that bud formation occurred preferentially on the distal side of the cell. Thus, the projection formation and budding results both indicate that once cells enter stationary phase, the normal site for polarized growth is often lost and another site for preferential growth is chosen (see DISCUSSION).

Sites of Polarized Growth and Zygote Formation in a Mating Mixture

As noted in the INTRODUCTION, during a normal mating process yeast cells are expected to grow toward their mating partners. We therefore would expect that in a mating mixture, yeast cells will undergo polarized growth at sites other than axial sites. To examine this possibility, early log-phase cells of opposite mating type (S288C) were mixed and incubated at 30°C for 4–6 h without shaking (see METHODS). After incubation the culture contained several cell types, including cells that contained projections and newly formed zygotes. Most noticeably, all cells displayed a pronounced increase in size (Fig. 6).

Cells with projections were scored for location of polarized growth relative to bud scars as previously described. The large size of cells allowed for accurate analysis of nearly all cells with bud scars (one or more), because a trail of bud scars rarely extended over the borders of adjacent scoring domains. In contrast to cells treated with α-factor, in the mating mixture, projections formed at random locations along the cell surface (Figures 5 and 6). These data are compatible with the hypothesis that mating cells form projections toward a mating partner rather than at the predetermined sites.

To examine the sites of cell fusion in a mating mixture, zygotes were analyzed (Fig. 6). Zygotes containing a single bud scar were scored as to whether conjugation occurred on the same or opposite half of a mating partner relative to the bud scar. Forty-nine percent of zygotes fused opposite the site of bud scars, whereas 51% of matings occurred on the same half of the cell as previous budding events. Therefore, consistent with the shmoo formation results shown above, in a mating mixture the sites of polarized growth and fusion do not occur at previously specified sites; these results support the hypothesis that a cell directs growth toward a mating partner.

Actin Distribution in Yeast Cells Exposed to Different Concentrations of Mating Pheromone

Mating cells seem capable of initiating polarized growth at any site on the cell surface. Furthermore, in a mating mixture the yeast cells are very large, not only in length but also in diameter. During their initial encounter, mating cells are probably exposed to low levels of pheromone (see DISCUSSION). Because low levels of pheromone cause cell cycle arrest without induction of projection formation (Moore, 1983; Read *et al.*, 1992), it is plausible that initially during the mating process yeast cells might arrest in a depolarized state and undergo uniform growth; subsequently, a localized stimulus at

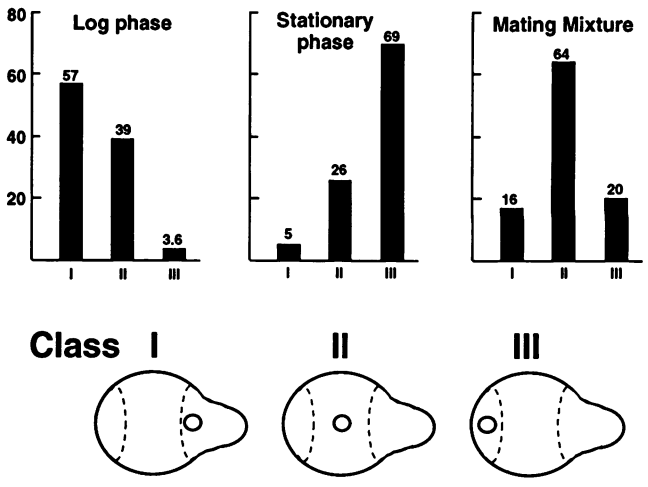

Figure 5. Sites selected for polarized growth in vegetative, stationary phase, and mating cells. Wild-type yeast cells (Y762) in an exponentially growing culture preferentially initiated projection formation near previous bud sites (n = 411). In comparison, stationary phase Y762 cells usually formed projections on the opposite side of the cell relative to bud scars (n = 400). In a 6 h mating mixture of wild-type *MATa* (Y762) and *MATα* (Y763) cells, sites of projection formation appeared random (n = 264). Similar results were observed for mating mixtures incubated for 4 h.

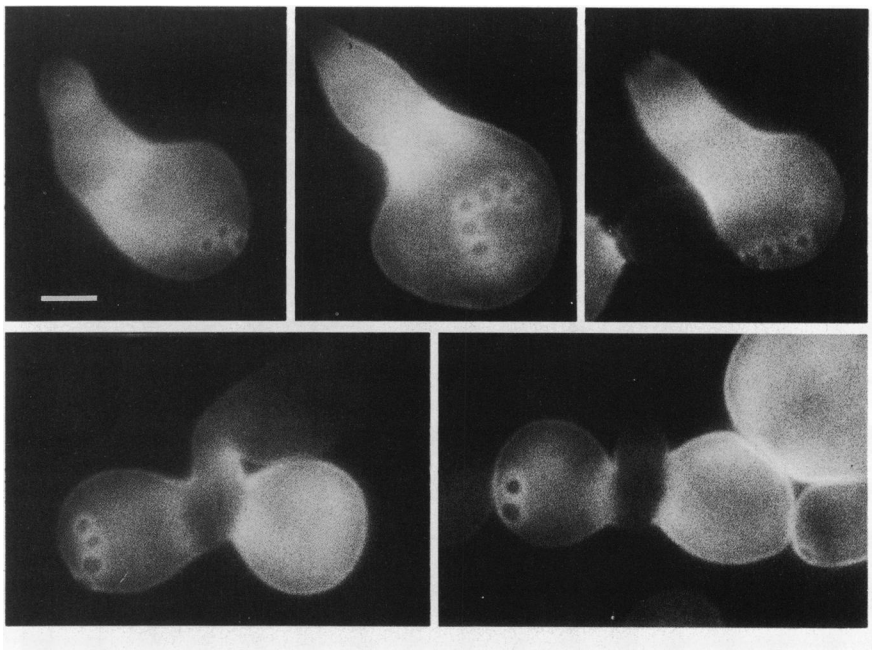

Figure 6. Calcofluor staining of shmoos and zygotes from mating mixture. Wild-type *MATa* (Y762) and *MATα* (Y763) cells were mixed and kept stationary for 6 h. The top series depicts cells containing elongated projections that form at random locations on the cell surface. Bottom series displays zygotes initiating formation of the first diploid daughter bud. Fusion occurred at random locations relative to previous bud sites. Note the profound increase in overall cell size in comparison to vegetative cells treated with α-factor (Fig. 1). Bar, ≈3 μm.

any site on the cell surface could elicit a response that results in polarized growth at that site (see DISCUSSION).

To test the hypothesis that low levels of pheromone causes a loss in cell polarity, *MATa* yeast cells were exposed to various concentrations of α-factor for 85 min and then stained with rhodamine-conjugated phalloidin. In the absence of α-factor, 40% of vegetatively growing cells were unbudded and 70% of the unbudded cells contained a polarized distribution of actin (n = 300). Treatment with 0.05 μg/ml pheromone increased the percentage of unbudded cells to 55%; no projections were observed (n = 300; see METHODS). Less than 5% of the arrested cells show a strongly polarized arrangement of F-actin, and approximately one-half of the unbudded population exhibits a partially polarized distribution of actin spots preferentially on one side of the cell (Fig. 7). The remaining cells show a random distribution of actin spots, and very few cables are seen. Treatment with 0.2 μg/ml α-factor resulted in 81% unbudded cells (n = 300); 5% of the population had formed projections. Less than 2% of the unbudded cells contained a polarized distribution of actin. In contrast, cells treated with 5 μg/ml α-factor initiated projection formation, and >95% of the cells show a strong polarized array of both actin spots and cables. Thus, low levels of α-factor results in an accumulation of cells with a depolarized or partially depolarized distribution of actin.

To further test the effect of low concentrations of α-factor on loss of cell polarity, *MATa* cells were incubated with 0.2 μg/ml α-factor for 80 min, washed, and transferred for 60 min to fresh medium lacking pheromone. Eighty percent of the cells had budded, and for cells that contained a single bud scar, the position of the bud was scored relative to the bud scar (n = 310). The percentage of Class I, II, and III cells was 62, 18, and 20, respectively. In an asynchronous culture of the same strain, >99% of cells exhibit axial budding (n = 300). Thus, exposure to low levels of pheromone reduces the fidelity of axial budding and significantly increases the number of cells that bud at distal sites.

DISCUSSION

The studies presented above indicate that when haploid yeast are arrested in G1 either by treatment with α-factor or by shifting temperature-sensitive *cdc28* cells to the restrictive temperature, cells undergo polarized growth preferentially at axial sites, the sites where bud formation normally occurs. Selection of the site can be altered in at least three ways: 1) mutations in *RSR/BUD1* and the *BUD* genes, 2) growth of cells to stationary phase, or 3) incubation of cells with the opposite mating type. The implications of these observations are described below.

Sites of Polarized Growth are Chosen Before the End of G1

The studies presented above clearly demonstrate that sites of polarized growth are chosen before the end of G1, and before the *CDC28*-dependent step of the cell cycle (START). Previous studies have examined the site of projection formation in cells incubated in a uniform concentration of α-factor. Ford and Pringle found that projection formation occurred at all possible sites relative to the position of the bud scar (Ford and Pringle, 1986). The distribution in their studies appeared closer to ran-

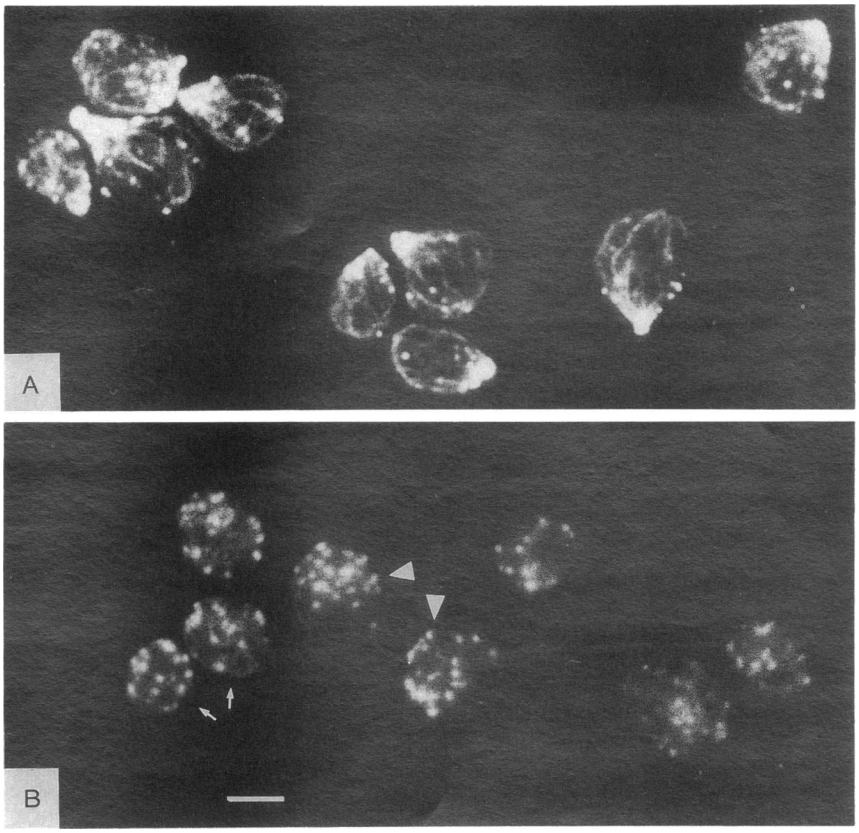

Figure 7. Rhodamine-conjugated phalloidin staining of wild-type cells (Y762) exposed to either 5.0 μg/ml (A) or 0.05 μg/ml (B) of α-factor for ~85 min. (A) Cells incubated with 5.0 μg/ml α-factor have a strongly polarized distribution of F-actin. (B) Cells treated with 0.05 μg/ml of α-factor have not initiated projection formation within ~85 min. The F-actin staining pattern in these cells varies from a depolarized (→) to a partially polarized (◗) distribution of actin spots.

dom than the results reported above. This is probably because these workers used lower concentrations of pheromone (0.17 μg/ml; Ford and Pringle, unpublished data), which is expected to cause cells to arrest in a partially depolarized state (see information above/below). Baba *et al.*, (1989) and Thorner (1980) performed electron microscopic experiments of shmoos and mentioned that projections form adjacent to bud scars, but these were not quantitative studies. Baba *et al.* (1989) speculated that projection emergence may occur at bud sites because of a greater accessibility to α-factor at this location. We prefer the hypothesis that determinants that specify sites of polarized growth are established before the end of G1 (perhaps even from the previous cell cycle, see below). When cells are arrested in a growth phase during G1, growth will occur at the specified sites (i.e., "default sites"). Our data is consistent with the cytokinesis tag model in which determinants from the previous site of cytokinesis mark the position where polarized growth will next occur (Chant and Herskowitz, 1991; Snyder *et al.*, 1991). During vegetative growth, polarized growth will ultimately produce a bud, whereas in the presence of α-factor the end product will be a projection.

Analysis of mutants suggests that components involved in selection of sites for projection formation in α-factor treated cells are the same as those involved in selection of sites for bud formation. *bud1/rsr1*, *bud2*, *bud3*, and *bud4* cells exhibit altered budding patterns (Chant *et al.*, 1991; Chant and Herskowitz, 1991); all formed projections at random locations, although the *bud4* strain still contained a slight increase in cells with projections at axial sites. For *bud3* and *bud4* mutants this result is somewhat surprising because these strains have been reported to yield a bipolar pattern of budding; hence projection formation would have been expected at both ends (i.e., a high proportion of Class I and Class III cells). In our experiments, Calcofluor staining suggested that *bud4* cells usually exhibit axial buds, although many cells containing bud scars at random locations can be observed (Madden and Snyder, unpublished data). This overall pattern is generally consistent with that observed in the projection formation experiments, although the distribution in the projection experiments appeared more random. Calcofluor staining of *bud3* cells generally revealed bipolar budding, as previously reported (Chant *et al.*, 1991; Chant and Herskowitz, 1991). The formation of projections at random sites rather than bipolar sites in these cells might reflect differences either in the timing of events during the initiation of projection formation and bud formation or in the relative importance of different components involved in specification of sites for these two processes. The use of axial sites for polarized growth in wild-type

G1 arrested cells is inherently weaker than for vegetatively growing cells (~60–70% vs. 99%); this lower fidelity might be accentuated in the *bud3* and *bud4* mutants. In any event, it is clear that the *RSR/BUD* genes affect selection of the site of projection formation, and this result strongly suggests that many of the same components participate in selection of default sites for projection formation as well as bud site selection.

Establishment of Cell Polarity During G1 can Affect Cell Shape

The observation that polarity is established during G1 has important implications regarding cell shape. Recently, Gimeno *et al.* (1992) have described a pseudohyphal growth phase for diploid *S. cerevisiae* cells grown in a nitrogen limiting source and sufficient glucose. In this phase, cells no longer follow the normal budding process; instead, cells become long and thin and fail to detach from mother cells. These changes, along with the distal budding pattern exhibited by daughter cells, result in the formation of pseudohyphae. Early specification of sites for polarized growth would help ensure that proper bud sites are selected. For diploids, the budding pattern may help provide access to scarce nutrients (Gimeno *et al.*, 1992; Madden *et al.*, 1992).

The establishment of polarity during early G1 (Snyder *et al.*, 1991) coupled with the polarized growth of cells during G1 can also explain the dramatic elongated shapes of these diploid cells. Nitrogen limiting conditions are expected to promote an elongated G1 phase (Pringle and Hartwell, 1981); in the presence of sufficient glucose, yeast cells are thought to remain in the cell cycle and undergo many growth events (Granot and Snyder, 1991). Thus, a longer G1 growth phase in a polarized cell would be expected to result in an elongated shape. Although the general long shape of the pseudohyphae cells is reminiscent of *cdc28* cells, differences do exist; thus growth in nitrogen limiting medium itself must directly or indirectly contribute to changes in cell morphology. In any event, establishment of polarized growth at specific sites during early G1 can partially explain the resulting cell shape after growth in modified medium.

Sites of Polarized Growth in Cells Exiting Stationary Phase

Previous studies have shown that specific cortical components involved in polarized growth are either absent (SPA2 protein) or delocalize (actin) when cells enter stationary phase (Snyder *et al.*, 1991; M. Snyder, unpublished data). Stationary phase cells diluted into fresh medium containing high levels of α-factor preferentially initiate polarized growth opposite previous sites of budding. Furthermore, cells diluted into fresh medium (without pheromone) usually bud at the distal site. Thus, we speculate that the preferred sites of axial budding are no longer present, and a new site is selected. There are several possibilities as to why polarized growth occurs opposite the previous bud site. One possibility is that once the axial sites are lost, the new sites of polarized growth occur proximal to the spindle pole body (Byers, 1981; Snyder *et al.*, 1991). It is expected that many cells under nutrient limiting conditions will enter stationary phase directly following cytokinesis; under these circumstances the spindle pole body (SPB) will reside distal from the site of cytokinesis. After recovery from stationary phase, new growth might occur proximal to the SPB and/or microtubules that emanate from the SPB (Byers, 1981; Snyder *et al.*, 1991). It is unlikely that projection formation after growth to stationary phase occurs at the last site of cell surface growth (as proposed for diploid daughter cells; Chant and Herskowitz, 1991), because the cells that we are scoring contain bud scars and therefore are mother cells that exhibited growth at the axial sites.

The observations that projection and bud formation can occur at distal sites, similar to diploid daughter cells, indicates that the distal pattern can take place in haploid cells; these observations are consistent with the hypothesis that there is a hierarchy of preferred sites for budding (Chant and Herskowitz, 1991; Snyder *et al.*, 1991; Madden *et al.*, 1992). Axial sites (formed in the presence of the *BUD3* and perhaps *BUD4* gene products) might be the preferred sites. Loss or modification of these primary sites in diploid daughter cells or by growth to stationary phase might result in the selection of secondary sites e.g., opposite the spindle pole body or last site of cell surface growth. It is interesting to note that in cells grown to stationary phase cells or under nutrient limiting conditions, polar budding is expected to be advantageous over axial budding to maximize access to nutrients.

Establishing Sites of Polarized Growth During the Mating Process

The studies presented earlier demonstrate that in a uniform concentration of mating pheromone, yeast cells exhibit polarized growth at "default" axial sites. Compelling evidence from mating partner selection and from examination of zygotes suggests that cells grow towards their mating partner (Jackson and Hartwell, 1990a; Jackson *et al.*, 1991). Indeed in this study we found that shmoos in a mating mixture contain projections at random locations on the cell surface relative to previous bud sites. Likewise, zygote formation did not occur adjacent to bud scars, consistent with the observations of Ford and Pringle (1986). These results suggest that mating partners are capable of abandoning previous patterns of polarized growth and are consistent with the hypothesis that they initiate growth toward each other.

Exposure of vegetative cells to low levels of mating pheromone promotes cell-cycle arrest and partial or complete depolarization of the F-actin distribution. Be-

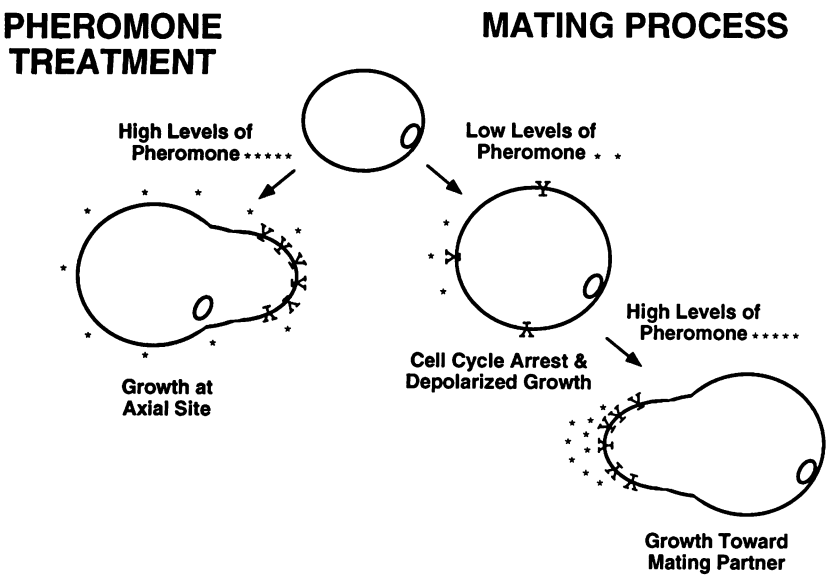

Figure 8. Model depicting cellular responses to mating pheromone. (Left) Exposure to a uniform distribution of high concentration of α-factor results in cell cycle arrest and projection formation at axial sites i.e., sites previously chosen for polarized growth in the budding process. (Right) In contrast, during a natural mating process, yeast cells are expected to first encounter a relatively low level of mating pheromone constitutively expressed from a mating partner. Cell cycle arrest may result in both the depolarization of certain cellular components (such as actin) as well as the uniform growth of the cell. After the initial exposure, cells increase the expression of the pheromone that they produce (Strazdis and MacKay, 1983; Jackson and Hartwell, 1990a). The subsequent high concentration of pheromone (potentially in combination with cell agglutination) is expected to stimulate projection formation that would occur towards the mating pheromone source. Polarized secretion from the projection would also increase the concentration of pheromone nearest the mating partner; polarization of the receptor in the partner is expected to facilitate detection of that signal.

cause cells in the mating mixture are very large, depolarized growth probably occurs during the mating process, and this depolarization may be important in redirecting the projection formation. The depolarization of cells exposed to low levels of pheromone may occur by one of two means. Either cells traverse the cell cycle and often accumulate in a depolarized window in the cell cycle, or alternatively, cells that have arrested in G1 may gradually lose their polarity (of at least some cortical components such as actin). Regardless of which possibility is correct, directional growth may not proceed from this depolarized state until a cell is exposed to sufficient levels of pheromone to induce mating projection.

Exposure to low levels of pheromone does not completely disrupt polarity, because after transfer to medium lacking pheromone the cells still usually bud at axial sites albeit at a much lower frequency than normal vegetative cells. Thus, after treatment with pheromone, either some small amount of cortical actin is still polarized, but difficult to detect with our staining procedures, or another component remains polarized at the cortex and can facilitate the axial budding process.

Based on the observations cited previously, we propose that a temporal series of events occurs during the mating process (Fig. 8). This scheme is modified from that proposed previously (Moore, 1983; Jackson and Hartwell, 1990a,b; Jackson *et al.*, 1991). When cells of opposite mating types first encounter one another, the concentration of mating pheromone is low. This low level is expected to cause cell cycle arrest and agglutination, and the arrest may result in a state in which critical components for polarized growth are partially or completely depolarized. After exposure to pheromone from the opposite mating type, cells increase the level of expression of the pheromone that they produce (Strazdis and MacKay, 1983; Jackson and Hartwell, 1990a). The higher concentrations of pheromone that result might then induce projection formation, which presumably would be directed toward cells producing the most pheromone. Agglutination is expected to dramatically increase the local concentration of pheromone as well and would further help direct polarized growth and fusion.

Recent localization of the STE2, α-factor receptor to the tip of mating projections, suggest that this protein serves a critical function both in the perception of the pheromone signal and in the propagation of a response (Jackson *et al.*, 1991). It is likely that exposure to pheromone results in production and localization of additional receptors as well as the production and polarized secretion of the opposite mating pheromone. The pheromone receptor may also directly or indirectly participate in cytoskeletal rearrangement (Jackson *et al.*, 1991) and thereby help direct the mating response.

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