

Patterns of Bud-Site Selection in the Yeast *Saccharomyces cerevisiae*

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Abstract. Cells of the yeast *Saccharomyces cerevisiae* select bud sites in either of two distinct spatial patterns, known as axial (expressed by *a* and α cells) and bipolar (expressed by *a*/ α cells). Fluorescence, time-lapse, and scanning electron microscopy have been used to obtain more precise descriptions of these patterns. From these descriptions, we conclude that in the axial pattern, the new bud forms directly adjacent to the division site in daughter cells and directly adjacent to the immediately preceding division site (bud site) in mother cells, with little influence from earlier sites. Thus, the division site appears to be marked by a spatial signal(s) that specifies the location of the new bud site and is transient in that it only lasts from one budding event to the next. Consistent with this conclusion, starvation and refeeding of axially budding cells results in the formation of new buds at nonaxial sites. In contrast, in bipolar budding cells, both poles are specified persistently as potential bud sites, as shown by the observations that a pole remains competent for budding even after several generations of nonuse and

that the poles continue to be used for budding after starvation and refeeding. It appears that the specification of the two poles as potential bud sites occurs before a daughter cell forms its first bud, as a daughter can form this bud near either pole. However, there is a bias towards use of the pole distal to the division site. The strength of this bias varies from strain to strain, is affected by growth conditions, and diminishes in successive cell cycles. The first bud that forms near the distal pole appears to form at the very tip of the cell, whereas the first bud that forms near the pole proximal to the original division site (as marked by the birth scar) is generally somewhat offset from the tip and adjacent to (or overlapping) the birth scar. Subsequent buds can form near either pole and appear almost always to be adjacent either to the birth scar or to a previous bud site. These observations suggest that the distal tip of the cell and each division site carry persistent signals that can direct the selection of a bud site in any subsequent cell cycle.

SPATIALLY ordered and/or asymmetric cell divisions are crucial to the development of many multicellular organisms. Examples include the spiral cleavage mechanism by which snails develop left-handed or right-handed body plans (Freeman and Lundelius, 1982), the intricate patterns of divisions during the growth of plants (Gunning, 1982), and the early embryonic cell divisions of the nematode *Caenorhabditis elegans* (Hyman and White, 1987).

Some unicellular organisms also have ordered patterns of cell division, the study of which may provide paradigms for understanding the more complicated patterns observed in multicellular organisms. For example, the budding yeast *Saccharomyces cerevisiae* can select bud sites (and thus determine its patterns of division) in either of two distinct spatial patterns (Winge, 1935; Freifelder, 1960; Streiblová, 1970; Hicks et al., 1977; Sloat et al., 1981; Chant, 1994).

In the axial pattern, expressed by typical *a* or α strains, a mother cell and its daughter both form new buds near the preceding division site (i.e., near the bud scar on the mother cell and the birth scar on the daughter cell). In the bipolar pattern, expressed by typical *a*/ α strains, the mother cell can bud either near the preceding division site or near the opposite pole, and the daughter cell generally forms its first bud near the pole distal to the birth scar. These budding patterns have been observed both by growing cells on agar surfaces and by fluorescent staining of bud scars in the cell wall. With the latter approach, axially budding cells display a row or cluster of bud scars near the pole proximal to the birth scar, whereas bipolar-budding cells display clusters of scars near one or both poles.

We are studying the mechanisms of bud-site selection and polarity establishment in yeast (Drubin, 1991; Chant and Pringle, 1991; Chant, 1994). In the course of these studies, it became clear that the development of mechanistic models required more precise and detailed descriptions of the budding patterns than had been made previously. Accordingly, we have used fluorescence, time-lapse, and scanning electron microscopy to obtain such descriptions. The rules of ax-

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ial budding that we develop here appear to be explained, in part, by the behavior of the protein Bud3p, as described in the companion paper (Chant et al., 1995). The rules of bipolar budding make some interesting predictions about the behavior of the proteins that are presumably involved in determining this pattern.

Materials and Methods

Strains and Growth Conditions

Several *S. cerevisiae* strains were used in these studies. C276 and 52 are α/α diploids that bud in the bipolar pattern (Wilkinson and Pringle, 1974; Sloat et al., 1981; Chant et al., 1991). CPIAB-IBB is an axially budding α/α diploid (Paquin and Adams, 1982). 51 is an axially budding α/α diploid (Chant and Herskowitz, 1991). 51 and 52 were constructed to be completely isogenic except for the difference at *MAT* (Chant and Herskowitz, 1991). 489 and 490 are α/α *bud3 Δ /bud3 Δ* and *BUD3/BUD3* diploids, respectively (Chant and Herskowitz, 1995); 491 and 492 were constructed in the same way as 489 and 490, respectively.

Except as noted, all strains were grown in the rich, glucose-containing liquid medium YM-P (Lillie and Pringle, 1980) or on YPD agar solid medium (Rose et al., 1990) at 30°C. Except as noted, cells in liquid cultures had been growing exponentially (at densities less than 2×10^7 cells/ml) for ≥ 10 generations before observation or other experimental manipulation. For starvation experiments, exponentially growing cells (as just described) were harvested by filtration, washed with water, and resuspended in starvation medium comprised of 2% glucose and 7 g/l Difco yeast nitrogen base lacking ammonium sulfate and amino acids. After 8 h incubation at 30°C, cells were sonicated lightly to disperse clumps (Pringle and Mor, 1975), stained with FITC-conjugated concanavalin A (FITC-Con A)¹ (see below) for 5 min, washed by filtration, and returned to YM-P medium to allow resumption of growth. Samples were harvested, fixed with formaldehyde, and stained with Calcofluor (see below) before starvation, after 8 h of starvation, and then at hourly intervals after resuspension in YM-P.

Staining with Calcofluor and FITC-Con A

Bud scars and birth scars were visualized by fluorescence microscopy after staining with Calcofluor or FITC-Con A (both from Sigma Chem. Co., St. Louis, MO). For staining with Calcofluor, cells were fixed by the addition of formaldehyde to 3.7% and incubation for ~4 h with occasional agitation. Cells were then stained with Calcofluor as described previously (Pringle, 1991), using 0.1% Calcofluor in water. To stain living cells with FITC-Con A (Tkacz and Lampen, 1972; Sloat et al., 1981) with a minimal perturbation of growth, cells were harvested by filtration, resuspended in growth medium with 0.1 mg/ml FITC-Con A, sonicated lightly, and agitated for 5 min. Cells were then collected by filtration, washed with medium to remove unbound FITC-Con A, and returned to medium without FITC-Con A. All steps of FITC-Con A staining were performed at 30°C.

Stained cells were observed by standard epifluorescence methods using a Nikon Microphot SA microscope. Slides were scanned in an orderly fashion to prevent recounting of cells. All cells in every field were counted except when only a particular class of cells was of interest, in which case all cells of that class were counted and other cells were ignored. Where numbers (*n*) of cells counted are given in the text, reference is to the number of cells of the particular class being considered. In some cases, these numbers are rather low because the class of cells being considered was rare.

Scanning Electron Microscopy

Cells were fixed by the addition of formaldehyde (to 3%) and glutaraldehyde (to 2.5%) to the medium and incubation at 23°C with occasional agitation for 4 h. Cells were then harvested on filters, dehydrated in a graded ethanol series, critical point-dried in liquid CO₂, sputter coated with a 10-nm layer of gold/palladium (60:40), and observed using a Cambridge S-200 scanning electron microscope (SEM) (Cambridge Instruments, Cambridge, UK). Cells were randomly selected for analysis as follows. Fields were scanned

at low magnification for regions where cells were at a manageable density. A low magnification photograph of the chosen field was taken as a reference, and every cell upon which a bud scar was visible was then photographed at higher magnification. Analysis was performed from the higher magnification photographs.

Time-lapse Observations

Cells from liquid culture were diluted 100X in water, sonicated lightly, and spread on a YPD plate. Cells were observed and photographed approximately every hour using a Nikon Labophot microscope. Negatives were printed, and budding patterns were analyzed from the prints.

General Features of the Analysis

Apparent inconsistencies in previous descriptions of the axial and bipolar budding patterns and our own preliminary observations suggested that some features of these patterns might vary among strains and/or be sensitive to growth conditions; these points are confirmed by the data presented below. Thus, we were careful to standardize these conditions for our studies. First, except where the response of cells to other conditions was specifically at issue, we worked only with cells that had been growing exponentially in rich medium at 30°C for at least 10 generations at the time when observations were made. Second, we restricted our observations to a small number of strains (see above) that grew well under the conditions used and represented two different backgrounds. C276 and CPIAB-IBB are of the widely used S288C/X2180 background (Mortimer and Johnston, 1986; Sloat et al., 1981; Adams and Pringle, 1984; Adams et al., 1990; Sherman, 1991). 51 and 52 are of the 1237-13C background and have been used in genetic studies of budding pattern (Chant and Herskowitz, 1991; Chant et al., 1991; our unpublished studies).

Although the axial and bipolar budding patterns are characteristic of normal haploid (*MAT α* or *MAT α*) and diploid (*MAT α /MAT α*) strains, respectively, these budding patterns are actually determined by cell type (mating type) rather than by ploidy per se (Hicks et al., 1977; Hartwell, 1980; Chant and Herskowitz, 1991; Herskowitz et al., 1992). Thus, we could use the diploid strains CPIAB-IBB (α/α) and 51 (α/α), both of which behave phenotypically as α cells (Herskowitz, 1988), to analyze the axial budding pattern. This offered two advantages over the use of haploid cells. First, the larger size of diploid cells facilitated both observation and photomicroscopy. Second, the more elongated shape of diploid cells facilitated recognition of the cell poles, which was important for various aspects of our analysis. Comparison of our observations on the diploid α strains to previous, less detailed observations by ourselves and others on normal haploid strains indicates that the rules of axial budding are in fact the same in both cases.

Some aspects of our analysis depended on determining the precise geometry of bud-site positions relative to the cell poles. To a first approximation, the radially symmetrical growth of the bud results in a newborn daughter cell that is a prolate ellipsoid with two precisely defined poles, namely the geometric points at which the major axis of the ellipsoid intersects the cell surface. One such point (defining the "proximal pole") is at the center of the birth scar, whereas the other (defining the "distal pole") is directly opposite to (and at the point on the cell surface most distant from) this first point. For convenience (and in keeping with much previous usage), we use the terms "proximal pole" and "distal pole" loosely, to refer to the regions around the precise geometric poles; to refer to the precise geometric poles themselves, we speak of the cell "tips."

Much of our analysis also depended on distinguishing the proximal from the distal pole on cells that were no longer attached to their mothers; several methods were used to do this. In principle, the proximal pole can be recognized by the presence of the birth scar. In practice, this criterion worked well in the SEM experiments, in which the birth scar could usually be discerned in appropriately positioned cells (see Figs. 2 and 6), but was not always reliable in the fluorescence-microscopy experiments. In cells that were stained with FITC-Con A, the birth scar could usually be detected as a bright ring or patch, larger than a bud scar, at one pole of the cell. Birth scars could also be detected by staining with Calcofluor, as a stained ring at one pole of the cell that was larger in diameter and fainter than a bud scar, or as a dark patch surrounded by a faintly stained border. However, the success of discerning birth scars by Calcofluor staining was quite variable: in most preparations of strains 51 and 52, the birth scars were readily discerned on almost all cells, whereas with strains C276 and CPIAB-IBB, the birth scars were sometimes difficult or impossible to discern. In such cases, we were usually able to circumvent this difficulty by using cell shape as a criterion. Phase contrast observations showed that one pole of the cell is usually slightly protruded from the outline of the ellipsoid, whereas the

1. Abbreviations used in this paper: FITC-Con A, FITC-conjugated concanavalin A; SEM, scanning electron microscope; SPB, spindle pole body.

other pole smoothly follows the ellipsoidal contour. In every cell in which a birth scar could be detected, it was at the protruded pole, establishing that the cell shape criterion could accurately define the proximal pole. Finally, in some observations of the bipolar pattern, we could distinguish the cell poles using a criterion based on bud position. Once we had established that the first bud at the distal pole is always at the very tip of the cell, we could use this rule to recognize the distal pole on certain cells (those with a bud or bud scar(s) at both poles, but only at one tip) in subsequent experiments. Using the several criteria separately and (when possible) together, we were able to distinguish the proximal and distal poles unambiguously on nearly all cells.

The principal techniques used in this study, fluorescence microscopy and SEM, both had some advantages and limitations. With SEM, the birth scars could usually be discerned and easily distinguished from bud scars, and the precise spatial relationships of the bud sites could be observed and documented photographically with high resolution. However, only a limited number of cells could be observed, and the interpretations were sometimes weakened by the possibility that additional bud scars were hidden from view on the back sides of the cells. With fluorescence microscopy, we could examine large numbers of cells and be certain that all bud sites were seen (because of the transparency of the cells and the possibility of focusing up and down while making observations). However, as noted above, visualization of birth scars was sometimes a problem, and the spatial relationships of bud scars could not be observed with such high resolution as with SEM.

Results

Axial Pattern

Previous observations have shown that in *a* and α strains, both mother and daughter cells typically form new buds near the preceding division site (see the Introduction). Several models might explain these observations. First, the cells might be constrained to bud repeatedly from the proximal pole ("unipolar budding" model). Second, the cells might be constrained to form each new bud adjacent to some previous bud site (division site). Third, the cells might be constrained to form each new bud adjacent to the immediately preceding bud site (division site). The observation that some cells have lines of bud scars that leave the proximal-pole region (Chant and Herskowitz, 1991; Fig. 1 *D*, cell 1; 1 *E*; 1 *G*, cell 4; Fig. 2, *D* and *G*) argues against the first model but is compatible with either of the other models. To discriminate unambiguously among these models, we examined in detail the positioning of the first few bud sites on the axially budding diploid strains 51 and CP1AB-1BB (see Materials and Methods).

Position of the First Bud Site. Observation of Calcofluor-stained cells from exponentially growing populations of either strain showed that the first bud almost always formed close to, but slightly offset from, one tip of the cell. For cells on which the birth scar could also be seen, it was clear that the first bud site immediately abutted the birth scar (Fig. 1 *A*). For cells on which the birth scar was not clearly visualized by Calcofluor staining, use of the cell shape criterion (see Materials and Methods) showed that the first bud site was close to, but slightly offset from, the tip of the proximal pole (Fig. 1 *B*). Using the two criteria for identification of the proximal pole, we conclude that $\geq 99\%$ of axially budding daughter cells form their first buds immediately adjacent to the birth scar (strain 51, $n = 103$; strain CP1AB-1BB, $n = 183$); rare exceptions were seen in which the first bud was not in this region.

SEM observations on strain CP1AB-1BB were consistent with this conclusion (Fig. 2 *A*, cell 1; 2 *B*); no apparent exception was seen in 34 cells observed in which the presumed

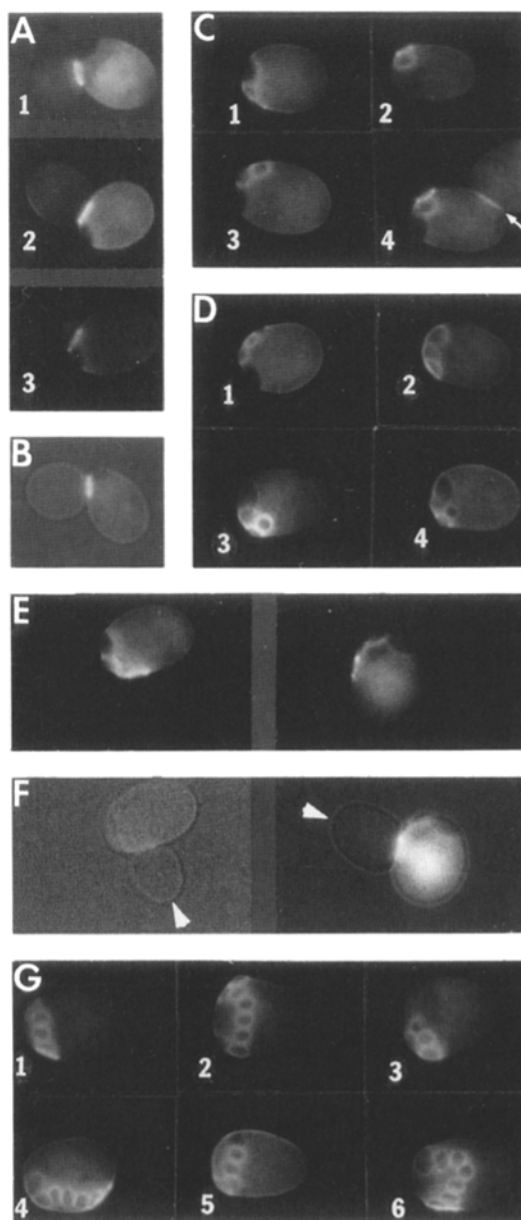


Figure 1. Aspects of the axial budding pattern. Cells of strain 51 were stained with Calcofluor and observed by fluorescence microscopy; for panels *B* and *F*, fluorescence and phase-contrast microscopy were combined to illustrate cell shape and the positions of buds more clearly. (*A* and *B*) Cells that have budded once; (*C*) cells that have budded twice (the arrow indicates a patch of fluorescence produced by two cells having been pressed together, not a bud scar); (*D*) cells that have budded three times; (*E*) cells with two bud scars plus a growing bud in which the order of the bud sites can be inferred (see text); (*F*) different images of the cells shown in panel *E*, to allow visualization of the buds (arrowheads); (*G*) cells that have budded multiple times. Some cells are numbered for reference in the text.

first bud site could be discerned (i.e., cells forming what appeared to be their first buds, cells apparently with a single bud scar, and cells forming what appeared to be a second bud in which the presumed first bud scar was also visible).

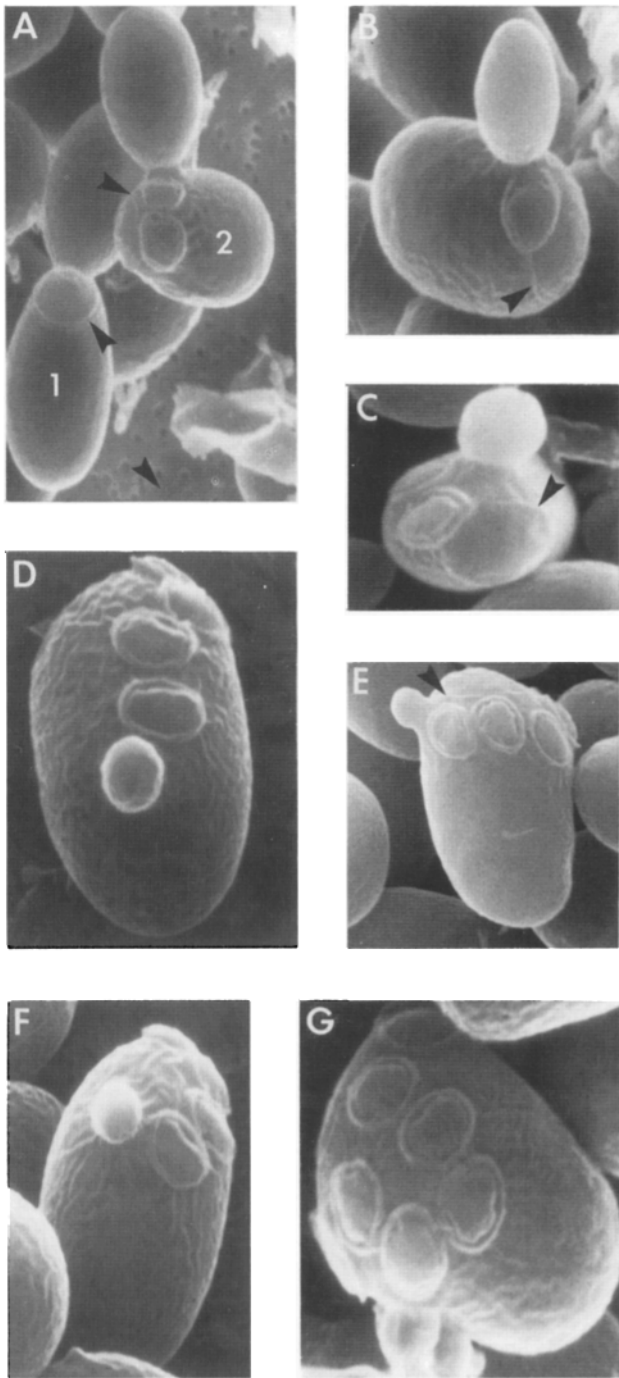


Figure 2. Aspects of the axial budding pattern. Cells of strain CPIAB-IBB were observed by scanning electron microscopy. (A–C) Cells apparently making their first (A, cell 1), second (B), and third (A, cell 2; C) buds. (D–G) Cells that have budded multiple times. Arrowheads indicate birth scars on cells where these can be discerned.

The first bud scar generally did not overlap the birth scar (Fig. 1 A, cell 1; 1 C, cell 1; Fig. 2 A, cell 1; 2 C). In a few cases (~1%), the first bud scar did significantly overlap the birth scar (Fig. 1 A, cells 2 and 3; 1 C, cell 3; Fig. 2 B); however, no case was observed in which the first bud scar was completely within the birth scar.

Positions of the Second and Third Bud Sites. Examination of Calcofluor-stained cells with two bud scars or one bud scar plus a growing bud revealed that the second bud almost invariably formed directly adjacent to the first bud site (Fig. 1 C); such an arrangement was seen in 154/154 cells scored for strain 51 and in 186/187 cells scored for strain CPIAB-IBB. (In the one exceptional case, the two bud scars were clearly separated although still very close to each other.) Similar observations were made on strain CPIAB-IBB by SEM: 40/40 cells on which the presumed first two bud sites could be discerned had these bud sites directly adjacent to each other (Fig. 2 A, cell 2; Fig. 2, B and C). In some cases, both bud sites were adjacent to the birth scar (Fig. 1 C, cell 4; Fig. 2 A, cell 2); in the remaining cases, one of the bud sites (known to be the second from the presence of a bud or inferred to be so from the rule that first bud sites are always adjacent to the birth scar) was separated from the birth scar (Fig. 1 C, cells 1 and 3; Fig. 2, B and C).

Examination of Calcofluor-stained cells with three bud scars or two bud scars plus a growing bud revealed that almost always each bud site was directly adjacent to one or both of the other two sites (Fig. 1 D); no exception was observed in 163 such cells examined for strain 51, and just two cases of separated bud sites were observed in 115 such cells examined for strain CPIAB-IBB. This conclusion was also supported by SEM observations on strain CPIAB-IBB: no separated buds or bud scars were seen on 16 cells examined that appeared to have three bud scars or two bud scars plus a growing bud (Fig. 2 A, cell 2; 2 C).

Ordering of Bud Sites. To ask whether the new bud site was always adjacent to the immediately preceding one, we used two approaches. First, on a subset of cells with two bud scars and a growing bud, the bud sites can be ordered unambiguously as follows: if only one of the bud scars touches the birth scar, it must mark the first bud site (see above), while the remaining bud scar marks the second bud site and the bud marks the third bud site. In examining Calcofluor-stained cells of strain 51, we analyzed 50 cells to which this logic could be applied (Fig. 1, E and F). In every case, the third bud site was adjacent to the second bud site. The third bud site could also be adjacent to the first bud site, the birth scar, or both, but no case was observed in which the third bud site was adjacent to the first bud site or the birth scar without also being adjacent to the second bud site. Similar observations were made on strain CPIAB-IBB by SEM: in each of several cells in which the order of bud sites could be determined, the third bud site was adjacent to the second bud site (Fig. 2 C).

The second approach allowed the ordering of bud sites on all budded cells. This approach was based on the observation that bud scars and birth scars on living cells can be stained with FITC–Con A (see also Lew and Reed, 1993). Thus, double-labeling experiments were performed in which cells were labeled with FITC–Con A in culture medium, washed, and returned to culture medium without FITC–Con A, all with little or no interruption of growth (see Materials and Methods). The cells were then grown for ~1.5 additional generations, fixed, and stained with Calcofluor. Under these conditions, the birth scar and bud scars present at the time of FITC–Con A staining were labeled with both dyes, whereas scars formed after FITC–Con A staining (including the chitin ring encircling the base of a growing bud) were la-

beled only with Calcofluor. With the two most recent bud sites distinguishable from each other (by the presence of a bud on the most recent one) and from earlier sites (by the FITC-Con A labeling of the earlier sites), we could ask whether the new bud always formed directly adjacent to the immediately preceding bud site. Using strain 51, we examined 78 cells with three bud sites (Fig. 3, cell 1) and 103 cells with multiple bud sites (Fig. 3, cells 2 and 3); in every case, the new bud was adjacent to the most recent previous bud site, even when the set of previous bud sites formed a large cluster rather than a simple chain (Fig. 3, cell 2). In some cells, the new bud was also adjacent to one or more older bud sites or to the birth scar (see also below). From these results and those described above, we conclude that in the axial pattern, the principal constraint is that each new bud forms directly adjacent to the immediately preceding bud site (or division site in daughter cells).

Pathways Taken by Chains of Bud Sites. According to the analysis presented above, successive bud sites should always form a chain. Although it is clear that these chains can take a variety of configurations (Fig. 1, *D* and *G*; Fig. 2 *A*, cell 2; Fig. 2, *C–G*; Fig. 3, cells 2 and 3), examination of fields of cells suggests that the chains have a tendency to remain near the proximal pole of the cell. We attempted to evaluate objectively whether such a bias exists by examining strain CPIAB-IBB by SEM. Among 40 cells that appeared to have one bud scar plus a growing bud or two bud scars plus a growing bud, the second bud site abutted the birth scar (as well as the first bud site) in 25 cells. In the 16 cells with two bud scars plus a growing bud, the third bud site was adjacent to the birth scar in 6 cells. These data suggest that there is a significant (although not absolute) bias causing successive bud sites to remain near the proximal pole of the cell.

To ask whether the position of the newest bud site is affected by the positions of bud sites other than the immediately preceding one, we examined by Calcofluor staining the arrangement of bud sites on cells having three bud scars or two bud scars plus a growing bud. In such cells, the three bud sites could be arranged in a triangular cluster in which each bud site was directly adjacent to both of the others (Fig. 1 *D*, cells 3 and 4; 38/225 [17%] and 14/104 [13%] cells for strains CPIAB-IBB and 51, respectively) or in a chain (straight or crooked) with bud site 2 lying between bud sites 1 and 3 (Fig. 1 *D*, cells 1 and 2; 1 *E*; Fig. 3, cell 1; 187/225 [83%] and 90/104 [87%] cells for strains CPIAB-IBB and 51, respectively). Similarly, among the 16 cells of strain CPIAB-IBB observed by SEM that had two bud scars plus a growing bud, just one had bud site 3 directly adjacent to bud site 1 (as well as to bud site 2). Thus, there appears to be little or no influence upon bud-site selection by bud sites previous to the immediately preceding one.

Transience of the Signal Marking the Preceding Division Site. The observations described above suggested that the spatial signal(s) recognized by the axial-budding machinery are transient, lasting no more than one cell cycle. Further support for this hypothesis came from experiments in which axially budding cells were starved, and then refed (see Materials and Methods). In brief, exponentially growing cells of strain 51 were sampled, and then shifted to nitrogen-free medium for 8 h, at which point the population was uniformly arrested in G1/G0 (97–98% unbudded cells) after an increase in cell number of approximately threefold (Pringle

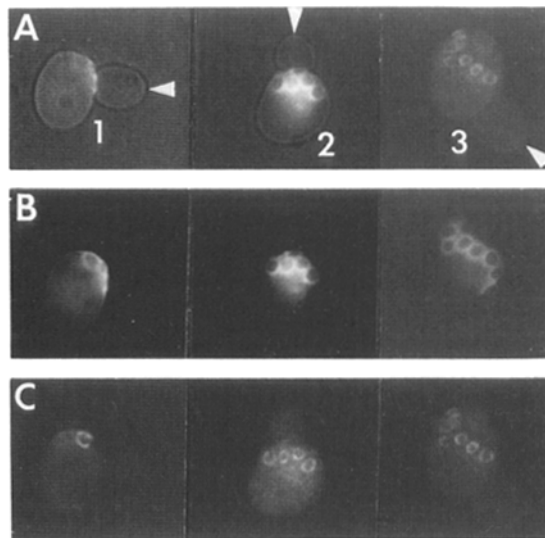


Figure 3. Ordering of bud sites in the axial pattern by double labeling with FITC-Con A and Calcofluor. Exponentially growing cells of strain 51 were stained with FITC-Con A, washed, grown for ~2 h in the absence of FITC-Con A, fixed, and stained with Calcofluor. (*A*) Phase-contrast micrographs to show the positions of growing buds. (Fluorescence from either the Calcofluor [cells 1 and 2] or FITC-Con A [cell 3] label is also visible.) Arrowheads indicate the positions of bud tips. (*B* and *C*) Fluorescence micrographs showing the Calcofluor staining of all bud scars (*B*) and the FITC-Con A staining of the older bud scars (*C*). Cell 1 is making its third bud; cells 2 and 3 have budded multiple times.

and Mor, 1975). The cells were then stained with FITC-Con A, returned to growth medium, harvested at various times, fixed, and stained with Calcofluor. In this procedure, the birth scar and bud scars formed before refeeding were stained with both FITC-Con A and Calcofluor, whereas the bud scars (and chitin rings at the bases of growing buds) formed after refeeding were stained only with Calcofluor.

The onset of starvation partially disrupted the axial budding pattern: no cell was observed in the exponentially growing population that had a bud or bud scar at the distal pole, but such cells were readily observed in the starved population. 12/100 cells with a single bud scar had that scar at the distal pole, while 27/100 cells with two bud scars and 26/76 (34%) cells with three bud scars had one scar (presumably the most recent one) at the distal pole. In addition, in a separate count, 11/54 cells (20%) with three bud scars, all of which were at the proximal pole, had one of these scars separated from the other two, an arrangement never observed in exponentially growing cells (see above). It is important to note that the nonaxial sites used for budding during the onset of starvation were not random in location: as just described, isolated bud scars at both poles were common, but very few cells (<1%) were observed that had bud scars in their mid-sections.

During release from starvation, the use of bud sites at the distal pole increased. One hour after refeeding, only 10–15% of the cells had formed new buds. Among 77 budded cells that were observed, 69 (90%) had their buds at their distal poles. Two hours after refeeding, most cells had resumed budding, and the majority of buds (187/252, or 74%) were

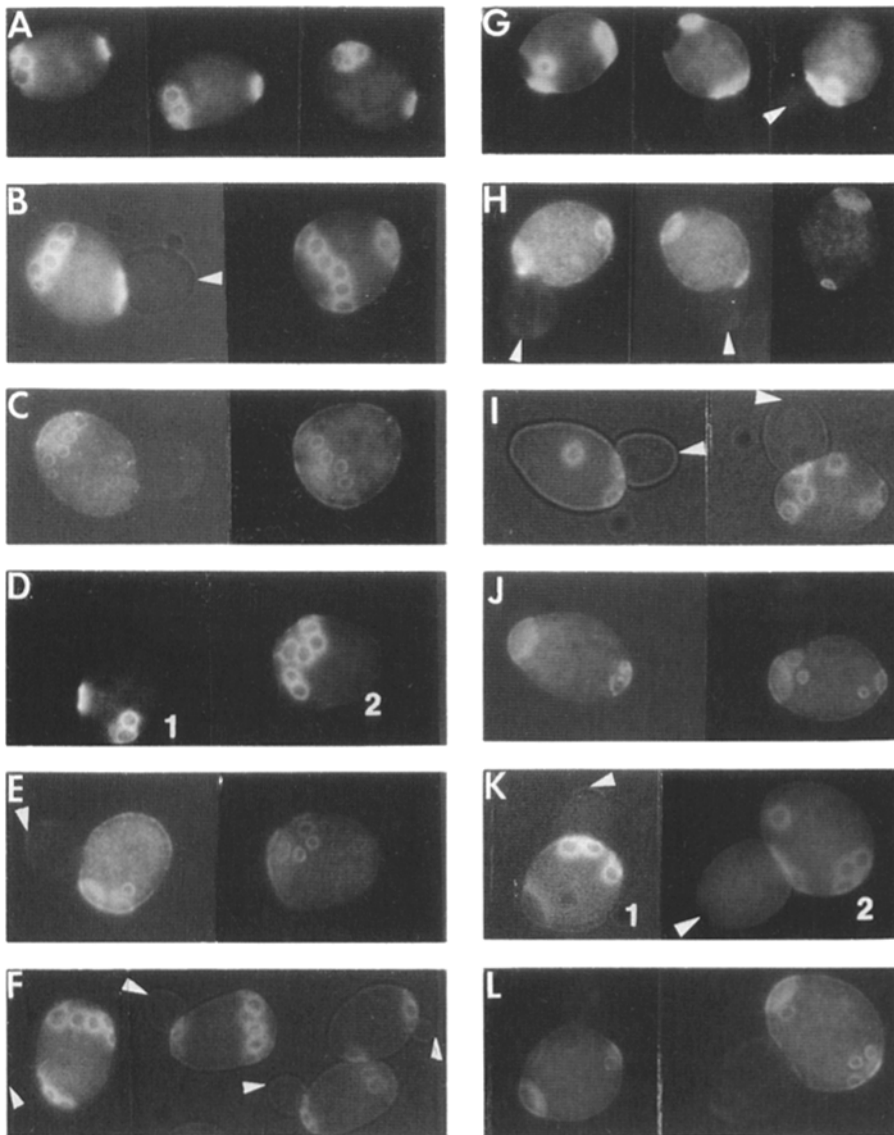


Figure 4. Effects on bud position of transiently arresting the cell cycle by starvation. Exponentially growing cells were starved, stained with FITC-Con A, re-fed, harvested at various times, stained with Calcofluor, and examined by fluorescence microscopy as described in the text. Some images combine fluorescence and phase-contrast microscopy to illustrate cell shape and the positions of buds more clearly. Arrowheads mark the tips of buds as visible in the original photomicrographs. Some cells are numbered to facilitate reference in the text. (A-F) Effects of transient arrest upon the axial pattern of strain 51. (A) Calcofluor-stained cells harvested 2 h after refeeding. Each cell displays one bud site at the distal pole. (B and C) Calcofluor (B) and FITC-Con A (C) staining of cells harvested 3 or 4 h after refeeding; new bud sites are at the distal pole. (D and E) Calcofluor (D) and FITC-Con A (E) staining of cells harvested 2 (cell 1) or 4 (cell 2) h after refeeding; new bud sites are at the proximal pole. (F) Calcofluor staining of cells harvested 4 h after refeeding; the new bud sites at the distal poles form chains as seen during axial budding (cf. Fig. 1, D-G). (G-L) Effects of transient arrest upon the bipolar pattern of strain 52. (G and H) Calcofluor (G) and FITC-Con A (H) staining of cells harvested 3 h after refeeding; each cell has one new bud scar plus a growing bud, all at the cell poles. (I and J) Calcofluor (I) and FITC-Con A (J) staining of cells harvested 3 or 4 h after refeeding; the newest bud sites are at the poles even though each cell had used a nonpolar bud site in the first cell cycle after refeeding. (K and L) Calcofluor (K) and FITC-Con A (L) staining of cells harvested 3 or 4 h after refeeding; new bud sites have formed in the vicinity of previous nonpolar bud sites used after refeeding.

again at the distal poles (Fig. 4 A). 3 and 4 h after refeeding, both new buds and bud scars formed after refeeding (stained by Calcofluor but not by FITC-Con A) were commonly seen at the distal poles (Fig. 4, B and C), as discussed further below. As in the case of the bud sites used during the onset of starvation, the bud sites used after refeeding were not random in location: nearly all of the bud sites that were not at the distal poles were at the proximal poles, with very few (<1%) in the midsections of the cells. Moreover, the first bud site used at the distal pole was generally at the very tip of the cell (Fig. 4 A), as is the case for the first distal-pole bud of bipolar-budding cells (see below).

For the minority of cells that used the proximal pole for budding during release from starvation, it was important to

ask whether they were continuing to use axial spatial information or merely budding in the vicinity of the proximal pole, as in the bipolar-budding pattern (see below). In the former case, the new buds should be directly adjacent to the most recent previous bud sites, whereas in the latter case, some cells should exhibit gaps between the new bud sites and the previous bud scars at the proximal pole. In fact, such gaps were readily observed (Fig. 4, D and E; note that in cell 2, the two bud sites used after refeeding [stained by Calcofluor but not by FITC-Con A] are directly adjacent to each other [as discussed further below] but separated from the earlier bud scars).

Resumption of Axial Budding Upon Release from Starvation. The data just described suggest that a transient signal

marking the preceding division site is lost during the prolonged unbudded phase produced by starvation; as a result, the first buds produced upon refeeding form in nonaxial positions (probably by using bipolar information, as discussed below). On this model, the resumption of rapid growth (and hence of short unbudded phases) after refeeding should result in a resumption of axial budding. If such axial budding follows the usual rules (as described above for exponentially growing cells), then the second and subsequent new buds produced after refeeding should form directly adjacent to the immediately preceding bud sites, regardless of whether the first buds produced upon refeeding were at the proximal or distal poles. Given that most first buds formed upon refeeding were at the distal poles (see above), one prediction of this model is that the total number of bud sites (bud scars or growing buds) observed at the distal poles should increase progressively during the first few generations after refeeding. Counts of cells with three total bud sites confirmed this prediction (Table I). As expected, comparison of the Calcofluor and FITC-Con A signals on cells harvested 3 or 4 h after refeeding showed that the great majority of the bud sites at the distal poles had formed after refeeding (Fig. 4, *B-C*); the exceptions presumably represented bud sites that had been used during the onset of starvation (see above).

A second prediction of the model outlined above is that cells that initially budded at their distal poles following refeeding should not then revert to budding at their proximal poles. Indeed, among 177 cells observed at 2, 3, or 4 h after refeeding that had one or more bud scars at their distal poles, plus growing buds, only five had those buds at their proximal poles. The five exceptional cells presumably had budded at their distal poles during the onset of starvation, lost the axial information at this site during the subsequent prolonged unbudded phase, and then budded at their proximal poles upon refeeding. Consistent with this interpretation, each of these cells that was examined had only a single bud scar at its distal pole, and that bud scar was stained by FITC-Con A.

As exponentially growing, axially budding cells do not display bud sites that are separated by gaps from other bud sites (see above), a third prediction of the model outlined above is that this same rule should apply to the groups of bud sites observed at the distal poles after refeeding. Indeed, among 209 cells observed with two or three bud sites at their distal poles, no separated bud scars were observed. This observation does not by itself distinguish between budding at the distal pole by an axial-budding mechanism and budding at the distal pole by the use of bipolar information, as gaps are only rarely observed between bud sites at the distal pole on bipolar-budding cells (see below). However, the common observation of cells with chains of scars at their distal poles after release from starvation (Fig. 4 *F*) suggests strongly that the mode of budding is indeed axial (compare Fig. 1, *D-G* to Fig. 5 *H*).

Finally, the model outlined above also predicts that axial budding (following the usual rules) should resume at the proximal pole when the first bud produced upon refeeding was at this pole. That is, even if the first bud site used upon refeeding was separated from earlier bud sites at the proximal pole, the subsequent bud sites used should be directly adjacent to their immediate predecessors. Although too few

Table I. Axial Budding at the Distal Pole

Time since refeeding	Number of cells counted	% cells with			
		0 distal bud sites	1 distal bud site	2 distal bud sites	3 distal bud sites
<i>h</i>					
1	99	57	42	1	0
2	146	8	48	40	4
3	99	14	13	67	6
4	99	23	4	34	38

At each time point, only cells with three visible bud sites (bud scars or growing buds) were scored. Note that this means that the samples scored at different times were derived from different subsets of the population present at the time of refeeding. For example, the cells scored at 2 h were mostly derived from cells that had two bud scars at the time of refeeding, whereas the cells scored at 4 h were mostly derived from cells that had zero or one bud scar at the time of refeeding. As cells in the starved population with single bud scars were more likely to have those scars near their proximal poles than cells with two bud scars were to have both of their scars near their proximal poles (see data in the text), this sampling effect presumably accounts for the increase in the percentage of cells scored with zero distal bud sites between 2 and 4 h.

cells were observed to allow a quantitative assessment, such patterns were readily observed (Fig. 4, *D* and *E*, cell 2).

Bipolar Pattern

Previous observations have shown that in *a/α* strains, the daughter cell typically forms its first bud at the distal pole, whereas the mother cell can bud at either pole (see Introduction). It has remained unclear whether the daughter cell ever forms its first bud at the proximal pole, whether the first bud at a given pole forms at the very tip of the cell or offset from it, whether successive bud sites at a given pole are adjacent to each other, and whether mother cells use the two poles in a regular alternation. To resolve these and other issues and provide a more precise description of the bipolar-budding pattern, we examined the *a/α* strains 52 and C276 using the same approaches as used to characterize the axial-budding pattern.

Position of the First Bud Site. Calcofluor staining of cells growing exponentially in liquid culture showed that the first bud sites on daughter cells were usually, but not always, at their distal poles. In strain 52, 97% of first bud sites were at the distal poles (Fig. 5 *A*) and 3% were at the proximal poles (Fig. 5 *C*) ($n = 245$). In strain C276, 85% of first bud sites were at the distal poles (Fig. 5 *B*) and 15% were at the proximal poles ($n = 207$). In neither strain were any first bud sites observed in nonpolar positions. Thus, it appears that both poles of a newborn daughter cell are competent for budding but that there is a bias for the use of the distal pole; the strength of this bias varies between strains. The strength of the bias for the distal pole also appears to depend on the growth conditions: time-lapse observations on strain C276 growing on YPD agar revealed that 108/111 (97%) daughter cells budded at their distal poles. As described below, the bias for the use of the distal pole can persist for several generations.

Interestingly, when the first bud formed at the distal pole, it was almost always at the very tip of the cell (Fig. 5, *A* and *B*; 6 *A*; 6 *D*, cell 1). In contrast, when the first bud formed at the proximal pole, it was usually distinctly offset from the tip and could either abut the birth scar (Fig. 5 *C*, cell 1; Fig.

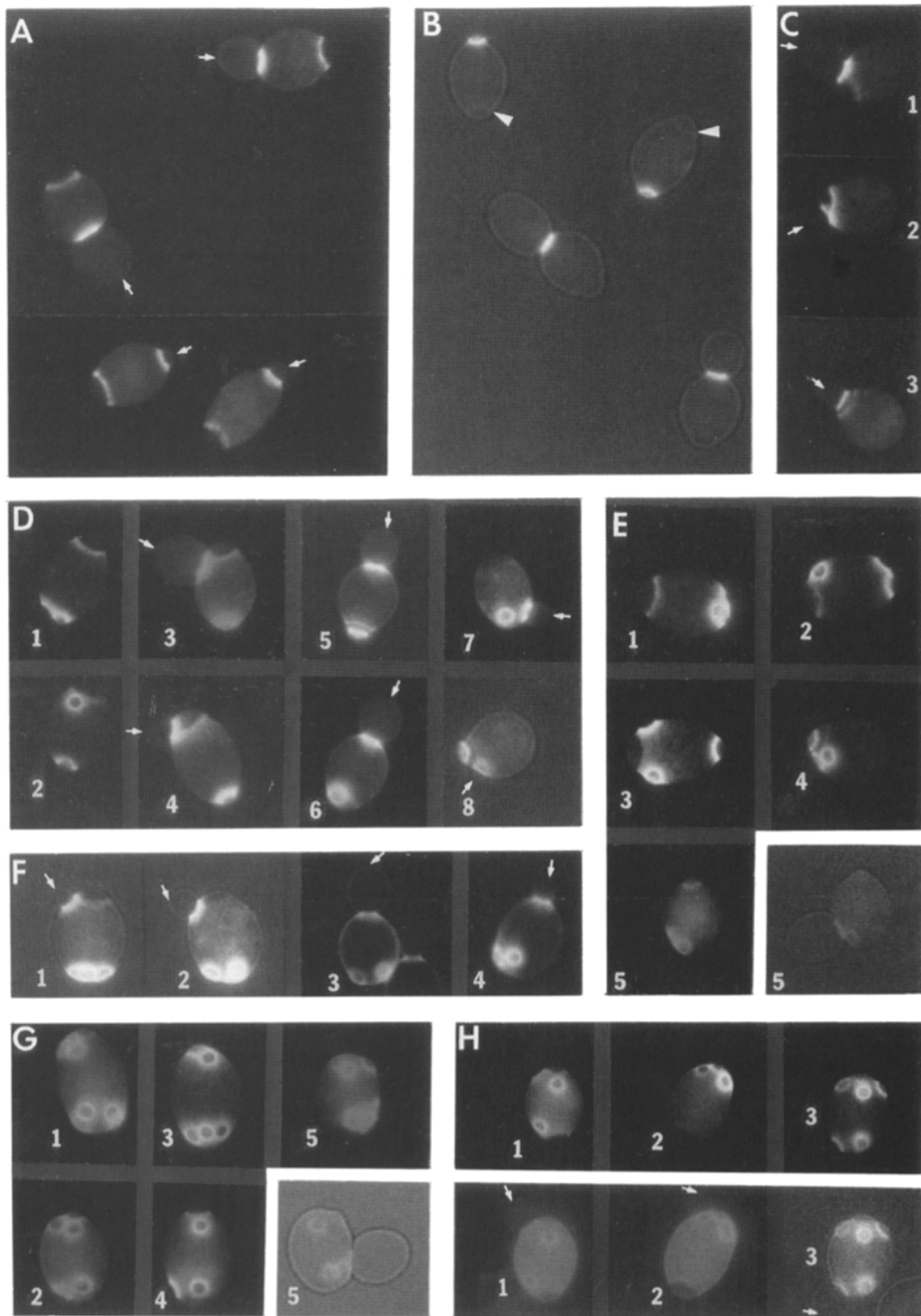


Figure 5. Aspects of the bipolar budding pattern. Cells of strain 52 (*A*; *C*; *D*, cells 1-6; *E*, cells 1-3; *G*; *H*) or C276 (*B*; *D*, cells 7 and 8; *E*, cells 4 and 5; *F*) were stained with Calcofluor and observed by fluorescence microscopy. For panel *B*, the insets in panels *E* and *G*, and the lower row of photomicrographs in panel *H*, fluorescence and phase-contrast microscopy were combined or exposures were adjusted during printing in order to illustrate cell shape and the positions of buds more clearly. Small arrows indicate the tips of buds as visible in the original photomicrographs. Some cells are numbered for reference in the text. Note that birth scars are conspicuous in strain 52 but much less prominent (e.g., arrowheads in *B*) in cells of strain C276. However, the proximal poles of cells of both strains are recognizable by their slightly protruded appearance. (*A-C*) Cells that had budded once at the distal (*A* and *B*) or proximal (*C*) pole. (*D*) Cells that had budded twice. The proximal poles are oriented up in cells 1-4, down in cells 5-7, and to the left in cell 8. (*E*) Cells that had budded three times. The proximal poles are oriented to the left in cells 1-4 and down in cell 5. The inset shows a different image of cell 5. (*F*) Cells that made their first several buds at the distal (cells 1 and 2) or proximal (cells 3 and 4) pole, and then produced a new bud at the opposite pole. (*G*) Cells that had budded multiple times. All cells are shown with their proximal poles down. The inset shows a different image of cell 5. (*H*)

Cells illustrating the patterns of budding at the distal pole. *Upper row*, micrographs printed to optimize visualization of the bud scars; *lower row*, different images of the same cells, printed to allow visualization of the buds.

6 *F*—note in this case that the growing bud appears to mark the position of the second bud site or overlap it (Fig. 5 *C*, cell 2). The fraction of cells with a first bud site at the proximal pole in which that bud site significantly overlapped the birth scar seemed higher in strain 52 (5/26 cells) than in strain C276 (10/95 cells), but was in both cases higher than the ~1% observed in axially budding cells (see above). In addition, the *a/α* strains displayed some cells (4/26 in strain

52; 1/95 in strain C276) in which the first bud sites at the proximal poles were entirely within the birth scars (Fig. 5 *C*, cell 3; 5 *D*, cells 5 and 8—note in these cases that the growing buds mark the positions of the second bud sites), a pattern never observed in the axially budding strains (see above).

These differences in the precise geometry of bud-site position suggest that the *a/α* daughter cells that make a first bud

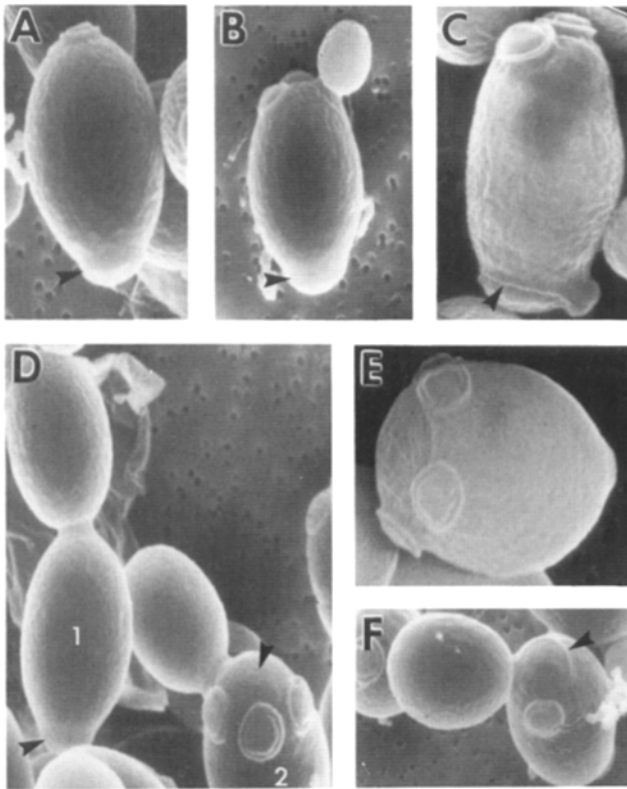


Figure 6. Aspects of the bipolar budding pattern. Cells of strain C276 were observed by scanning electron microscopy. (A) A cell that has apparently budded once at its distal pole. (B) A cell that has apparently budded three times at its distal pole. (C) A cell that has apparently budded twice at its distal pole and once at its proximal pole. (D) A cell that has apparently budded once at its distal pole (cell 1) and a cell that has apparently budded four times at its proximal pole (cell 2). (E and F) Cells that have apparently budded four times (E) or twice (F) at their proximal poles. Arrowheads indicate birth scars in all cells except in D, cell 2, where the birth scar cannot be clearly discerned. In this case, the arrowhead indicates the proximal pole as inferred from the arrangement of the bud scars.

at the proximal pole are not simply following the axial program. As a further test of this hypothesis, we examined *a/α* strains that should be defective in the axial program because of a loss of Bud3p (Chant and Herskowitz, 1991; Chant et al., 1995). Strains 489 and 491 (both *bud3/bud3*) and the closely related strains 490 and 492 (both *BUD3/BUD3*) were examined by Calcofluor staining after growth in liquid culture. The proportions of first buds at the proximal poles were ~20% for strains 489 and 491 ($n = 139$) and ~25% for strains 490 and 492 ($n = 120$). Thus, a defect in the axial budding program did not appear to affect the ability of *a/α* daughter cells to bud at their proximal poles.

Position of the Second Bud Site. Calcofluor staining of cells growing exponentially in liquid culture revealed that the second bud site was essentially always in a polar position and could be at either pole of the cell, regardless of the position of the first bud site. For strain 52, among cells exhibiting two bud scars or one bud scar plus a growing bud, 76% had both bud sites at the distal pole (Fig. 5 D, cell 1), 22% had

one bud site at each pole (Fig. 5 D, cells 2–6), and 1.5% had both bud sites at the proximal pole (not shown, but Fig. 5 D, cells 7 and 8, shows this pattern for strain C276) ($n = 332$). For strain C276, the corresponding numbers were 55%, 34%, and 11%, respectively ($n = 185$). For both strains, examination of cells with growing buds revealed that the cells with one bud site at each pole included both cells that had budded first at their distal poles (Fig. 5 D, cells 3 and 4) and cells that had budded first at their proximal poles (Fig. 5 D, cells 5 and 6). Moreover, the positioning of the first bud site to form at a particular pole appeared to be the same whether that bud site was the first or second to be formed by the cell. That is, when a cell whose first bud had been at the proximal pole produced its second bud at the distal pole, the second bud site was almost always at the very tip of the cell (Fig. 5 D, cells 5 and 6). Similarly, when a cell whose first bud had been at the distal pole produced a second bud at the proximal pole, that bud site was usually offset from the tip and either abutted or overlapped the birth scar (Fig. 5 D, cells 3 and 4). Thus, the competence of each pole for budding appears to be maintained through at least one cell cycle in which budding occurs at the opposite pole.

Taking the counts on cells exhibiting two bud sites together with those on cells exhibiting a single bud site (see above), we estimate that for strain 52, 78% of the cells whose first bud sites were at their distal poles also made their second buds at their distal poles; for strain C276, the corresponding number was 65%. Thus, among cells that budded first at their distal poles, the bias toward use of the distal pole persisted into the second cell cycle and was again stronger for strain 52. In contrast, approximately half (strain 52) or two thirds (strain C276) of cells that had made their first buds at their proximal poles also made their second buds at their proximal poles. Thus, cells that had budded first at their proximal poles showed no bias toward use of the distal pole for their second buds, and, indeed, a possible slight bias toward continued use of the proximal pole.

Examination of cells with both bud sites at the same pole revealed that the relative positioning of the two sites was different at the distal and proximal poles and, in the latter case, distinct from that observed in axially budding cells. At the distal pole, the two bud sites were almost always ($\geq 95\%$ of the time) directly adjacent (Fig. 5 D, cell 1); occasionally a small space between bud sites was observed. In contrast, a significant space between bud sites was observed in 29/69 cells of strain C276 that were observed with both bud sites at the proximal pole (Fig. 5 D, cells 7 and 8), and this is probably an underestimate, as it is difficult to see a space between bud sites that are separated from each other along the axis of view. Although strain 52 provided few examples of cells with their first two bud sites at the proximal pole, separation of these sites was common in the cases observed (not shown, but cf. Fig. 5 E, cell 3). Separation between two bud sites at the proximal pole was also readily observed by SEM (Fig. 6 F). These observations are in sharp contrast to those made on axially budding cells, in which the first two bud sites at the proximal pole were always directly adjacent to each other (see above). Interestingly, in every case observed in which two bud sites at the proximal pole were separated from each other, both were abutting or overlapping the birth scar (or one was totally within it) (Fig. 5 D, cells 7 and 8; Fig. 6 F; cf. also Fig. 5 E, cell 3; Fig. 6 D, cell 2; Fig. 6

E; and comments below about cells with >2 bud sites). Even among cells in which the two bud sites at the proximal pole were adjacent to each other, both were usually abutting or overlapping the birth scar: among 28 cells of strain C276 with two adjacent bud scars at the proximal pole, only five possibly had one bud site (presumably the second) separated from the birth scar, and in all of these cells the separation was so slight that scoring was uncertain. (In contrast, among cells that had budded more than twice, bud sites at the proximal pole that were separated from the birth scar were more common, as noted below.)

Positions of the Third and Subsequent Bud Sites. Examination of cells that had budded three or more times largely reinforced the conclusions drawn from observations on cells that had budded twice. First, it appeared that buds could form at either pole, regardless of the positions of previous bud sites. For example, among cells that had budded three times, all possible patterns of the three bud sites were observed (Fig. 5 *E*, cells 1–4); among cells with two bud sites at one pole and one at the other, the newest bud site (marked by a growing bud) could be at either pole (Fig. 5 *E*, cell 5; 5 *F*, cells 3 and 4; and data not shown). Particularly important is the observation that a cell whose first several buds had been at one pole could make its next bud at the previously unused opposite pole (Fig. 5 *F*). In these cases, the positioning of the new bud site was indistinguishable from that observed when the first bud site at a particular pole occurred earlier in the life history of the cell; that is, the first bud site at the distal pole was almost always at the very tip of the cell (Fig. 5 *F*, cells 3 and 4), whereas the first bud site at the proximal pole was almost always abutting or overlapping the birth scar (or occasionally entirely within it) (Fig. 5 *F*, cells 1 and 2). These observations suggest strongly that both poles are marked as potential regions for budding in some way that is preserved through multiple cell cycles in which the opposite pole is used.

Interestingly, the bias toward budding at the distal pole could, but did not invariably, persist into the third cell cycle. For strain 52, among 171 cells observed with three bud sites, ~75% had the most recent bud site at the distal pole. Moreover, 62% of the cells observed had all three bud sites at the distal pole; given the counts on cells observed with two bud sites (see above), we estimate that ~82% of the cells whose first two buds had been at the distal pole also produced their third buds at this pole. In contrast, among the cells that had budded once at each pole during their first two cell cycles, about half used each pole for budding during the third cell cycle. The results with strain C276 provided an even sharper contrast: among 245 cells observed with three bud sites, ~70% had the most recent bud site at the proximal pole. Moreover, only 23% of the cells observed had all three bud sites at the distal pole; thus, only ~36% of the cells whose first two buds had been at the distal pole also produced their third buds at this pole. Thus, in the third cell cycle, strain 52 continued to show an overall bias toward the use of the distal pole, although this bias was only manifest in the cells that had used the distal pole during both of the previous cycles. In contrast, strain C276 showed the opposite overall bias, even in cells that had used the distal pole during both of the first two cell cycles.

Finally, the relative positioning of successive sites at each pole maintained the patterns observed in cells with two bud

sites. At the proximal pole, individual bud sites were often separated from other bud sites, but were almost always directly adjacent to another bud site (Fig. 5 *E*, cell 5; 5 *G*, cell 5); abutting, overlapping, or (more rarely) entirely within the birth scar (Fig. 5 *E*, cells 3 and 4; 5 *F*, cells 3 and 4; 5 *G*, cells 2 and 4; Fig. 6 *C*; 6 *D*, cell 2; 6 *E*); or both (Fig. 5 *E*, cell 4; 5 *G*, cells 1, 3, and 5; Fig. 6 *D*, cell 2; 6 *E*). At the distal pole, the bud sites were almost always tightly clustered, with one site at the very tip of the cell (Fig. 5 *E*, cells 1 and 2; 5 *F*, cells 1 and 2; 5 *G*, cells 1–5; Fig. 6, *B* and *C*).

Given the tight clustering of bud sites at the distal pole, it was important to determine whether bud site *n* was always directly adjacent to bud site *n-1* (as described above for the axial pattern). To address this question, we examined cells with two bud scars plus a growing bud (marking the third bud site) at the distal pole; given the evidence described above, the bud scar at the very tip of the cell was presumed to represent the first bud site. On this basis, in both strains 52 and C276, we observed many cells in which bud site 3 was clearly adjacent to bud site 1 but not to bud site 2 (Table II; Fig. 5 *H*, cells 1 and 2; Fig. 6 *B* also appears to illustrate this arrangement, although, as noted above, in SEM observations we cannot eliminate the possibility that the visible bud sites are connected by a chain of additional bud sites around the back side of the cell). In cells with more than three bud sites at the distal pole, we also frequently observed arrangements that could not be explained on the hypothesis that each bud site forms next to the immediately preceding one (Fig. 5 *H*, cell 3). These arrangements are clearly distinct from the continuous chains of bud sites observed in axially budding cells, even when such cells are budding around their distal poles after starvation and refeeding (see above). In summary, it appears that each bud site (after the first) at the distal pole forms adjacent to one or more previous bud sites, but not necessarily adjacent to the immediately preceding bud site.

Persistence of Bipolar Budding during Starvation and Refeeding. Starvation and refeeding of axially budding cells produced an apparently complete disruption of the axial pattern (see above). In contrast, the same experimental conditions produced relatively little apparent perturbation of the bipolar pattern in the a/α strain 52. First, the cells appeared to be affected only slightly by the onset of starvation. Among 420 cells observed in the population before starvation that

Table II. Patterns of Budding at the Distal Pole in Bipolar Budding Cells

Strain	Arrangement of bud sites at the distal pole			
	Pattern 1*	Pattern 2†	Pattern 3‡	Pattern 4
52	9	8	25	12
C276	9	11	33	5

Cells with two bud scars plus a growing bud (marking the third bud site) at the distal pole were scored; the bud scar at the very tip of the cell was presumed to represent bud site 1 (see data in the text). To keep the geometry of the cells simple, and thus make the identification of bud site 1 unambiguous, only cells with no bud scars near the proximal pole were scored.

* Bud site 3 adjacent to bud site 1 but not to bud site 2.

† Bud site 3 adjacent to bud site 2 but not to bud site 1.

‡ Bud site 3 adjacent to both bud sites 1 and 2.

|| Ambiguous. Bud site 3 adjacent to both bud sites 1 and 2 or adjacent to bud site 2 but not to bud site 1.

had budded one to four times, only one displayed a nonpolar bud site. Similarly, among 438 cells with one to four bud scars observed after 8 h of starvation, just six displayed one (5 cases) or two (1 case) nonpolar bud scars. Cells producing their first buds after refeeding showed a somewhat greater perturbation. Among cells observed with growing buds at 1 and 2 h after refeeding, most had the growing bud at one or the other pole (not illustrated, but see Fig. 4, *G* and *H* for examples of cells whose first buds after refeeding had been in polar positions). Interestingly, even the bias of daughter cells for use of the distal pole persisted under these conditions: among 94 budded daughter cells observed at 1 and 2 h after refeeding, 89 had the bud at the distal pole, four had the bud at the proximal pole, and just one had a nonpolar bud. However, when all budded cells were scored, 25% at 1 h ($n = 173$) and 13% at 2 h ($n = 267$) had the growing bud in a nonpolar position (not illustrated, but see Fig. 4, *I-L* for examples of cells whose first buds after refeeding had been in nonpolar positions). This perturbation appears to be temporary: among 700 cells with growing buds observed at 3 or 4 h after refeeding, only seven (1%) had the bud in a nonpolar position (Fig. 4, *G-L*). Moreover, among 64 cells observed at 2, 3, or 4 h after refeeding that had one nonpolar bud scar plus a growing bud, 51 (80%) had the bud in a seemingly normal polar position (Fig. 4, *I* and *J*). These observations suggest strongly that the signals marking the poles as potential regions for budding persist through a period of starvation, even though they are not used with high fidelity during the period immediately after release from starvation.

It was also of interest to ask whether the formation of one bud at an atypical (nonpolar) site generated a new "pole equivalent," in the sense of a region marked as a potential site for subsequent budding events. In fact, among the 13 cells observed that had one nonpolar bud scar plus a growing bud at a nonpolar site, eight had the growing bud directly adjacent to the bud scar (Fig. 4, *K* and *L*, cell 2). In addition, clusters of adjacent bud sites were sometimes observed in nonpolar positions (Fig. 4, *K* and *L*, cell 1; note in this case that the cluster of new bud sites is not far distant from the pole, but is nonpolar in the sense that these sites are separated from the sites used at the distal pole before starvation). As such adjacency would be expected only rarely if the newest bud site were positioned randomly in the nonpolar regions, these data suggest that the original atypically positioned bud site is indeed marked in a way that can be recognized by the bipolar-budding machinery in subsequent generations.

Discussion

It has long been known that yeast cells can select bud sites in two different spatial patterns, known as axial and bipolar (Winge, 1935; Freifelder, 1960; Streiblová, 1970; Hicks et al., 1977; Sloat et al., 1981; Chant and Herskowitz, 1991). In this study, several different methods have been used to describe these patterns more precisely. As there appears to exist some differences in budding pattern among strains of different backgrounds, our descriptions refer, strictly speaking, only to the particular strains examined. However, as the differences observed here were subtle, and as our results seem generally consistent with previous, less detailed observations on other strains, we think it likely that the major fea-

tures of our descriptions are generally applicable. These descriptions allow us to predict some features of the mechanisms by which the two budding patterns are produced.

Nonoverlap of Bud Sites

Although bud sites sometimes overlap the birth scar, no case of overlapping bud sites (overlapping bud scars) has ever been observed in axially budding, bipolar-budding, or randomly budding (mutant) cells. The absolute nature of this prohibition suggests a basis in the structure of a previously used bud site. In particular, it seems likely that the chitin-rich cell wall of the bud scar (for review see Pringle et al., 1989; Bulawa, 1993) cannot be remodeled in such a way as to allow the growth of a bud. Consistent with this hypothesis is the observation that bud scars, once formed, appear immutable: they neither expand detectably nor change in their staining properties during subsequent cell cycles. It is not clear how the bud-site selection system (presumably operating at the cytoplasmic face of the plasma membrane) would recognize the regions occupied by the bud scars. Perhaps these regions are marked by transmembrane proteins that are anchored in the cell wall.

In contrast, the birth scar appears to be a more malleable structure, which expands as the cell grows. This behavior probably reflects its low or nonexistent chitin content (Beran et al., 1972; Roberts et al., 1983; Bulawa, 1993) and presumably explains how bud sites can overlap the birth scar. Nonetheless, such overlap is seen only in a minority of bipolar-budding cells and is rare in axially budding cells. The basis of this bias is currently obscure.

The Axial Pattern

The major features of the axial budding pattern as observed in exponentially growing cells can be described by two simple rules. (1) The first bud site on a daughter cell is directly adjacent to (or occasionally slightly overlapping) the original division site (birth scar). (2) Each subsequent bud site is directly adjacent to the immediately preceding bud site (bud scar). These rules suggest that the division site on both mother and daughter cells carries a signal that targets the next bud site to an adjacent position, as previously speculated by Chant and Herskowitz (1991) and Snyder et al. (1991). Moreover, rule 2 suggests that this signal is transient (as only the immediately preceding bud site is recognized), a suggestion that is also supported by the observation that cells that have been starved and refed typically form their next buds at novel locations (see also related observations by Thompson and Wheals, 1980; Madden and Snyder, 1992). The hypothesis of a transient signal is also consistent with the observation that treatment of a cells with low concentrations of α factor (which also extends the unbudded phase) alters the positioning of buds (Madden and Snyder, 1992). Rules 1 and 2 predict that successive bud scars will form chains (straight, curved, or crooked) that may encircle the proximal pole or wander away from it, as observed.

It should be emphasized that the observations that establish rule 2 also appear to eliminate other possible models for axial budding. In particular, both a unipolar model, in which cells are merely constrained to form buds near the proximal pole, and a model in which any previous division site can serve to position the next bud site predict patterns of budding

(such as bud site 3 next to bud site 1 but not bud site 2, or bud sites 1 and 2 separated from each other along the birth scar) that were essentially never observed in exponentially growing populations of axially budding cells.

The behavior predicted for the postulated signal that marks the division site matches the behavior of the *BUD3* gene product as described in the accompanying paper (Chant et al., 1995). As *bud3* mutations also specifically disrupt the axial budding pattern (Chant and Herskowitz, 1991; Chant et al., 1995), it is likely that Bud3p is a component of the spatial signal used for axial budding. There remain, of course, many open questions. For example, how is the Bud3p-containing signal recognized and acted upon? Why is only one new bud formed per cell cycle? (The perimeter of the preceding division site appears large enough to accommodate several new bud sites.) Why is the Bud3p signal expressed in a/α cells (Chant et al., 1995) yet not acted upon in such cells? Future studies should illuminate these issues (see additional discussion by Chant et al., 1995).

If the axial budding pattern were governed solely by the rules described above, the successive bud sites should form a random walk starting from the birth scar. Instead, it appears that there is a tendency (although not an absolute one) for the chains of bud scars to remain near the proximal pole (see Results). The basis for this tendency is not known. However, one possible model invokes the signal used to mark the birth scar region as a potential site for budding in bipolar-budding cells. As discussed below, it appears that this signal is both expressed in a and α cells and distinct from the Bud3p-containing signal used for axial budding. If the bipolar signal exerts a weak influence on the selection of sites for axial budding, it might explain the tendency for such sites to be nearer the proximal pole than expected from a random walk governed solely by rules 1 and 2 above.

The Bipolar Pattern

Although the bipolar budding pattern is more complicated than the axial pattern, its major features can also be described by a few simple rules. (a) The first bud site on a daughter cell can be at either pole of the ellipsoidal cell (although there is typically a strong bias for use of the distal pole). (b) The second and subsequent bud sites can also be at either pole, regardless of where the preceding bud site(s) have been (although there may again be biases for use of one or the other pole). (c) The first bud site to be used at the distal pole is at the very tip of the cell; the first bud site to be used at the proximal pole is adjacent to, overlapping, or entirely within the birth scar. (d) Each subsequent bud site at the distal pole is directly adjacent to (or occasionally very slightly separated from) one or more previous bud sites, but is not always adjacent to the immediately preceding bud site used at that pole. Each subsequent bud site near the proximal pole is directly adjacent to the birth scar or to one or more previous bud sites, but is not always adjacent to the immediately preceding bud site used at that pole.

These rules suggest that a newborn daughter cell carries signals at its distal tip and at its proximal pole (in the region defined by the birth scar) that can target the first bud site to either of these locations. In addition, rule *d* suggests that each previously used bud site also carries a signal that can target a subsequent bud site to an adjacent position; this sug-

gestion is supported by the observation that cells that have budded in a nonpolar position upon starvation and refeeding will often form a subsequent bud directly adjacent to the nonpolar bud scar. Moreover, rules *b-d* suggest that all of the postulated signals are persistent and perhaps even permanent, a suggestion that is supported by the observation that cells that have been starved and refeed usually form their next buds in polar locations or return to using polar locations after forming one or more nonpolar buds.

The above rules and inferences can be accommodated by a simple model (Fig. 7 A) that postulates that during the organization of the bud site before bud emergence, the patch of cell surface from which the bud will emerge is marked by some persistent or permanent signal molecule(s) (Fig. 7 A, cell 1). (For example, such a signal might be provided by a transmembrane protein that is anchored to a region of cell wall that does not get remodeled during bud growth.) When the bud emerges, the postulated signal molecules would be partitioned into a fraction that stays at the tip of the growing bud and a fraction that stays at the mother-bud neck (Fig. 7 A, cells 2-4). Although the bud grows predominantly by incorporation of new cell surface material around its tip (Tkacz and Lampen, 1972; Adams and Pringle, 1984; Lew and Reed, 1993; and references cited therein), the available data do not rule out the hypothesis that a patch of cell surface can remain intact at the very tip of the growing bud. Indeed, this hypothesis is supported by the observations that Spa2p, Cdc42p, and Bem1p can be seen as patches at the tip of the growing bud for substantial portions of the period of bud growth (Snyder, 1989; Snyder et al., 1991; Ziman et al., 1993; Corrado, K. and J. R. Pringle, manuscript submitted for publication). When the cell divides, the signal molecules that had remained at the neck would be partitioned into a fraction that marks the bud-scar region on the mother cell and a fraction that marks the birth-scar region on the daughter cell (Fig. 7 A, cell 5). Any of the regions marked by the signal molecules could then serve to target the selection of the bud site in any subsequent generation.

A related, but distinct, model (Fig. 7 B) that can also explain the available data is that the poles are marked as part of the process of concentrated cell surface reorganization and growth. During the early stages of bud growth, growth is concentrated at the bud tip (Fig. 7 B, cells 1-3), whereas later, insertion of cell surface materials is concentrated at the mother-bud neck in preparation for cytokinesis (Fig. 7 B, cell 4). If some remnant of the growth-targeting machinery were to persist at both locations, then the poles would be marked as predicted by the data described above (Fig. 7 B, cell 5).

Additional models that would also be consistent with the available data could perhaps be devised. However, one model that can apparently be ruled out is the proposal that an a/α mother cell can bud adjacent to any previous division site, while an a/α daughter cell buds at its site of recent tip growth (Chant and Herskowitz, 1991; Madden et al., 1992). A prediction of the second part of this model is that a daughter cell that made its first bud(s) at the proximal pole would never be able to use its distal pole in subsequent cell cycles, as this pole would neither be marked by a previous division site nor be a site of recent cell surface growth. However, cells that budded first at their proximal poles and only later at their distal poles were readily observed (see Results).

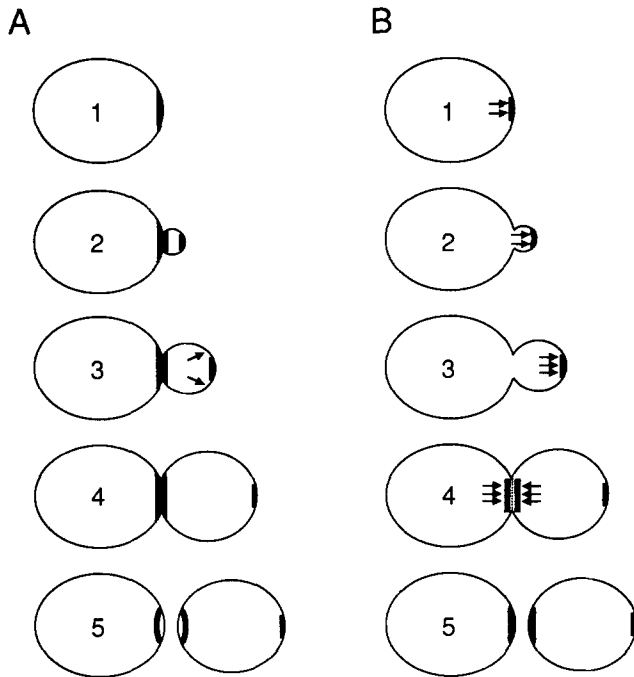


Figure 7. Two possible models to explain the bipolar budding pattern. Shading indicates the localization of the postulated positional signal(s); arrows indicate regions of active cell surface reorganization or growth. See text for details.

A Role for the Spindle Pole Body in Determining Bud Position?

Byers and Goetsch (1975; see also Byers, 1981) originally suggested that the spindle pole body (SPB) and/or cytoplasmic microtubules might play a role in determining the bud site. This suggestion was based on the observations that in vegetative wild-type cells, cell-cycle mutants, and zygotes, the duplicated SPB was always found to be facing the emerging bud. However, if the orientation of the SPB were the primary determinant of bud position, there should be a regular alternation of bud sites between the distal and proximal poles, in contrast to the patterns actually observed in *a*, α , and *a*/ α cells. In addition, in many individual cells, cortical markers of polarity establishment (namely actin, Spa2p, and proteins associated with the neck filaments) can be seen to be assembled at the presumptive bud site while the SPB is still on the opposite side of the nucleus from this site (Snyder et al., 1991; Chant, J., and J. Pringle, unpublished results). Subsequently, the nucleus apparently rotates so that the SPB faces the bud site.

To accommodate these observations, a more recent model (Snyder et al., 1991; Madden et al., 1992; Madden and Snyder, 1992; Flescher et al., 1993) suggests that the SPB functions only as a secondary system to direct budding when a primary system (based on an unstable marker at the preceding division site) has been lost or otherwise cannot be used. (For example, the model suggests that *a*/ α daughter cells usually bud at their distal poles, in response to SPB orientation, because the unstable marker at the division site is usually lost during their relatively long G1 phase.) However, this model has difficulty accounting for the observation that *a*/ α daughter cells still formed their first buds predomi-

nantly at their distal poles after disassembly of their cytoplasmic microtubules by nocodazole (Jacobs et al., 1988). In addition, some aspects of the starvation/refeeding experiments described above are difficult to explain on this model. In particular, the model would predict that *a* or α cells would always bud after refeeding at their distal poles (if the SPBs remain stationary during the period of starvation) or would be able to bud in any part of the cell (if the SPBs are able to move about during this period). Instead, new buds appeared only at the distal and proximal poles. Moreover, on an SPB-based model, it is difficult to explain the observation that after starvation and refeeding, most *a*/ α cells that have budded once or more in nonpolar positions return to using polar sites in subsequent cell cycles.

It might also be thought that SPB orientation could contribute to the bias of *a*/ α daughter cells for budding at their distal poles (see further discussion below). However, this hypothesis is unable to explain the continued bias for budding at the distal pole in a cell's second cell cycle, as, during its preceding (first) cell cycle, the SPB that remained in this cell would have been facing its proximal pole.

We conclude that there is no good evidence that the SPB and/or cytoplasmic microtubules play a role in determining bud position in *a*, α , or *a*/ α cells, and, indeed, that there is strong evidence against such a role. Instead, orientation of the SPB towards a previously established cortical site, in a process apparently mediated by the cytoplasmic microtubules and cytoplasmic dynein (Jacobs et al., 1988; Huffaker et al., 1988; Snyder et al., 1991; Sullivan and Huffaker, 1992; Palmer et al., 1992; Eshel et al., 1993; Li et al., 1993; McMillan and Tatchell, 1994), apparently explains the correlations that had originally suggested a role for the SPB in bud-site selection.

Remaining Questions about the Bipolar Pattern

The models of Fig. 7 leave many interesting questions open about the bipolar budding pattern. For example, if the cell can choose either the distal pole tip or any of its previous division sites to target the next budding event, then the phenomenon of budding singularity (i.e., the production of just one bud per cell cycle) is even more remarkable than in the case of the axial budding pattern (see above). Also requiring explanation is the bias toward budding at the distal pole during the first several cell cycles. At least two models might explain this bias. (I) The signal molecule(s) marking the division site might usually be partitioned unequally between the mother and daughter cells, so that the signal at the birth-scar region on the daughter is weak relative to the signals at the distal pole and at the bud-scar region on the mother. (II) The bipolar signal(s) might require some sort of maturation before they can target a subsequent bud site, and this maturation might typically be delayed at the proximal pole.

Both of these models can accommodate the observations that the strength of the distal-pole bias varies from strain to strain and is apparently affected by growth conditions (see below). Both models can also explain how a distal-pole bias could continue to be seen in the second and third cell cycles of cells that had previously budded only at their distal poles, but not of cells that had already budded once at their proximal poles (see Results). However, both models also have some apparent weaknesses. Model I has difficulty in explain-

ing the observation that strain C276 cells that had made their first two buds at the distal pole then showed a bias for the proximal pole in the third cell cycle, as well as the observation that cells that had made their first two buds at the proximal pole almost always had both bud sites abutting or overlapping the birth-scar. (That is, the rarity of cases in which bud site 2 was adjacent to bud site 1 but not to the birth scar suggests that the signal at the birth scar region is, if anything, stronger than that around the first bud site, in contrast to the prediction of the model.) Both models have difficulty in explaining the strong distal-pole bias of cells beginning to bud again after a period of starvation followed by refeeding. Clearly, more work is needed to discriminate among these and perhaps other models for the distal-pole bias.

Another interesting feature of the distal-pole bias is that its strength apparently varies with growth conditions. In addition to our data on strain C276 grown in liquid vs solid medium (see Results), Hayashibe (1975) has reported differences in budding patterns between cells of several bakers' yeast strains when grown in liquid culture or on agar, and Brewster and Gustin (1994) have noted an effect of high osmolarity on the relative frequencies with which the two poles are selected in a genetically defined *a/α* strain of different background. It is possible that the greater distal-pole bias of strain C276 when grown on agar reflects a response to nutrient limitation related to that which occurs during pseudohyphal growth, where an increased bias toward budding at the distal pole apparently contributes to the extended growth pattern of the pseudohyphal clone (Kron et al., 1994).

Relationship between the Axial and Bipolar Budding Patterns

Although both axially budding and bipolar-budding daughter cells can make their first buds at the proximal pole, several arguments indicate that they are responding to different spatial signals when they do so. First, the precise positioning of the first bud is different in the two cases: overlap of the bud site and the birth scar is more common in bipolar-budding cells than in axially budding cells, and bud sites entirely within the birth scars, which are observed regularly in bipolar-budding cells, are never seen in axially budding cells. Second, as argued above, the spatial signal recognized in axially budding cells appears to be transient, whereas that in bipolar-budding cells appears to be persistent or even permanent. Third, *bud3* mutants, which appear to be defective in the spatial signal for axial budding, display typical bipolar budding including the use of the proximal pole (Chant and Herskowitz, 1991; Chant et al., 1995; and see Results). Fourth, starved and refed *a* or *α* cells essentially never bud in nonpolar positions but can bud at the proximal pole in positions not adjacent to the preceding division site at that pole (suggesting that they are using a spatial signal other than the Bud3p-containing axial signal).

If the axial and bipolar patterns indeed use different spatial signals, a simple model for the control of budding pattern by cell type would be that the axial signal is only expressed in *a* and *α* cells, whereas the bipolar signal(s) is/are only expressed in *a/α* cells. However, this simple model does not appear to be correct. First, a *α bud3* mutant cells display typical bipolar budding (Chant and Herskowitz, 1991; Chant et al., 1995), and axially budding cells that have been starved and refed or exposed to mating factor produce new buds in what appear to be typical bipolar locations (see Results and

Madden and Snyder, 1992). Thus, remarkably, it appears that the postulated bipolar spatial signals are present also in *a* or *α* cells but are essentially always ignored unless the Bud3p-containing axial spatial signal is absent because of mutation or a delay in the unbudded phase. The mechanism by which this strict preferential use of the Bud3p-containing signal might be achieved is obscure. In addition, studies of Bud3p expression and localization (Chant et al., 1995) have shown that this component of the axial spatial signal is present in *a/α* cells, although it is usually, if not always, ignored. An attractive possibility to explain this behavior is that expression of some other component necessary for axial budding (perhaps another component of the axial spatial signal or a component necessary to recognize this signal) is repressed by the repressor *al-α2* in *a/α* cells (Chant and Herskowitz, 1991; Chant et al., 1995). If so, then an *a/α* cell would be unable to respond to the Bud3p-containing signal even if it were defective in producing or recognizing the bipolar spatial signals. The identification of a mutant that buds axially as an *a* or *α* cell but randomly (rather than axially) as an *a/α* cell (Bauer et al., 1993) suggests that this is indeed the case.

In any case, the arguments that the axial and bipolar-budding patterns depend on distinct spatial signals lead us to propose a modification of the model previously proposed (Chant and Herskowitz, 1991; Madden et al., 1992) for the morphogenetic hierarchy controlling bud-site selection. This earlier model suggested that the general bud-site selection functions (such as Rsr1p/Bud1p, Bud2p, and Bud5p) convert a default random budding pattern to a bipolar one, which can then be further modified to an axial pattern if the axial-specific functions (including Bud3p and Bud4p) are present. We now suggest that the general bud-site selection functions act upon positional information provided by either of two distinct types of spatial signals, the axial and the bipolar (Fig. 8). This model also leads to the prediction that it should be possible to isolate mutants defective in the bipolar positional signals and therefore specifically defective in bipolar budding. The complex phenotype (Bauer et al., 1993) of the

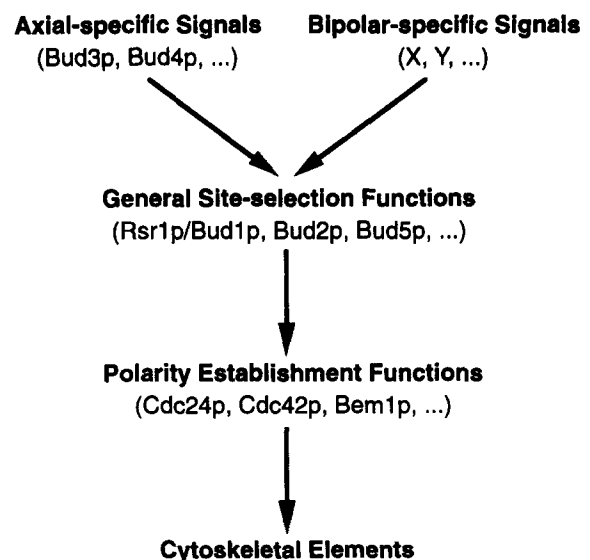


Figure 8. Proposed morphogenetic hierarchy for bud-site selection and polarity establishment. X and Y indicate the postulated bipolar spatial signals. See text for details.

previously reported mutant with a bipolar-specific defect in budding pattern suggests that it is unlikely to be defective simply in a bipolar spatial signal. Other mutants with bipolar-specific defects in budding pattern are currently being analyzed. This analysis should allow evaluation of the models presented in Figs. 7 and 8.

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