

Localized Secretion of Acid Phosphatase Reflects the Pattern of Cell Surface Growth in *Saccharomyces cerevisiae*

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ABSTRACT Secretion of cell wall-bound acid phosphatase by *Saccharomyces cerevisiae* occurs along a restricted portion of the cell surface. Acid phosphatase activity produced during derepressed synthesis on a phosphate-limited growth medium is detected with an enzyme-specific stain and is localized initially to the bud portion of a dividing cell. After two to three generations of phosphate-limited growth, most of the cells can be stained; if further phosphatase synthesis is repressed by growth in excess phosphate, dividing cells are produced in which the parent but not the bud can be stained.

Budding growth is interrupted in *a*-mating-type cells by a pheromone (α -factor) secreted by the opposite mating type; cell surface growth continues in the presence of α -factor and produces a characteristic cell tip. When acid phosphatase synthesis is initiated during α -factor treatment, only the cell tip can be stained; when phosphatase synthesis is repressed during α -factor treatment, the cell body but not the tip can be stained. A mixture of derepressed *a* cells and phosphatase-negative α cells form zygotes in which mainly one parent cell surface can be stained.

The cell cycle mutant, *cdc 24* (Hartwell, L. H. 1971. *Exp. Cell Res.* 69:265–276), fails to bud and, instead, expands symmetrically as a sphere at a nonpermissive temperature (37°C). This mutant does not form a cell tip during α -factor treatment at 37°C, and although acid phosphatase secretion occurs at this temperature, it is not localized. These results suggest that secretion reflects a polar mode of yeast cell-surface growth, and that this organization requires the *cdc 24* gene product.

Yeast cell-surface growth is restricted to the bud portion of a dividing cell. A bud develops on the parent cell and grows at a constant rate until cell division. Asymmetric surface growth is seen in the incorporation of glucan (19, 20) and mannan (14) into the cell wall. The assembly of surface determinants recognized by concanavalin A (31) or antibody (32) is also known to be bud-localized. However, the site of protein and lipid insertion into the plasmalemma is not known.

Two mechanisms for yeast cell-surface growth have been proposed (13). One is that polymers such as glucan, mannan, and chitin are assembled at the plasmalemma and directly inserted into the wall. This theory is supported by the fact that partially purified plasmalemma fractions are enriched in mannosyltransferase (8) and chitin synthetase activities (11). An alternative proposal is that mannan and other secretory molecules are assembled on intracellular membranes and transported to the cell surface by membrane-bounded vesicles,

where secretion is achieved by exocytosis. The latter model is supported by autoradiographic studies, which demonstrate an internal site for the initial incorporation of [³H]mannose, followed by enrichment of label along the bud surface (14).

Mannan synthesis is coupled to protein synthesis (12), and the secreted enzymes acid phosphatase and invertase are representative cell wall mannoproteins (1, 21). A number of intracellular organelles including the vacuole, nuclear membrane, endoplasmic reticulum, a Golgi-like structure, and a group of small bud-localized vesicles, are labeled when Gomori stain is applied to acid phosphatase-secreting cells (34). Freeze-fragment and thin-section electron microscopic evidence (24) suggests that the bud-localized vesicles fuse with the plasmalemma. These findings support the exocytosis theory of cell-surface growth and suggest that assembly of the plasmalemma as well as of the cell wall is accomplished by this process.

In this report we describe a technique for the localization of

newly secreted acid phosphatase. We demonstrate that under both normal and aberrant conditions of growth, cell-surface assembly is reflected in the pattern of secretion of acid phosphatase.

MATERIALS AND METHODS

Strains and Media

E1a (*a acp 1-1*) and A279 [*a/α(II ACP 1-2/II ACP 1-2)/(U ACP 102, P/U ACP 1-2, P)*] were obtained from Dr. P. Hansche (University of California, Davis, Calif.), who has described their characteristics (16). X2180-1A and -1B were obtained from the Yeast Genetic Stock Center (Berkeley, Calif.). A279-2c (*a II ACP 102/U ACP 1-2, P*) was obtained by sporulation of A279. SF 78-13 A (*a cdc 24-1 ade 2 acp 1-1/U ACP 1-2, P*) was derived from the above strains by standard genetic methods.

YPD medium contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose. Phosphate-free minimal medium was a modification of Wickerham's synthetic dextrose (SD) medium (35) and contained 5.5 g/liter of KCl (instead of KH_2PO_4), 0.1 M succinic acid at pH 5, 30 mg/liter of adenine, 2% glucose, and KH_2PO_4 at the indicated concentrations.

Reagents

α -Factor was purified from the culture fluid of X2180-1B cells as described by Ciejek et al. (7), and the phosphocellulose fraction (15,000 U/mg; 1 U will arrest budding in 10^5 X2180-1A cells for 3 h) was used without further purification. Basic fuchsin, α -naphthylphosphate, and *p*-nitrophenylphosphate were obtained from Sigma Chemical Co., St. Louis, Mo.; Bacto-yeast extract and Bacto-peptone were from Difco Laboratories, Detroit, Mich.; Oxoid ion agar no. 2, from Colab Laboratories, Chicago Heights, Ill.; sterile nitrocellulose filter units (0.2 μm), from the Millipore Corp., Bedford, Mass.

Growth Conditions

Derepressed synthesis of acid phosphate depended upon the cell density and the concentration of phosphate in the medium. Sodium phosphate at 1 mM caused repression of cultures with an A_{600} (measured in a 1-cm quartz cuvette in a Zeiss PMQ II spectrophotometer; an A_{600} unit corresponds to $\sim 10^7$ cells) of 3 or less; at an A_{600} above 3, a higher phosphate concentration (7 mM) was necessary for full repression. Rapid derepression of phosphatase synthesis occurred at 0.1 mM phosphate for cultures above an A_{600} of 3. For most derepression experiments, cells were grown in 1 mM phosphate-SD medium to an A_{600} of 3 or less. Cells were filtered, washed with sterile water, and resuspended in phosphate-free SD medium to an A_{600} of 0.2–0.3. For most repression experiments, cells were grown in 0.1 mM phosphate-SD medium to an A_{600} of 3 or more and were diluted into fresh 7 mM phosphate-SD medium to an A_{600} of 0.2–0.3. Most strains were grown in flasks at 30°C in a shaking water bath; *cdc 24* strains were grown at 24°C (permissive) or 37°C (restrictive). α -Factor was used at 350 U/ 10^7 cells; cultures were supplemented with an equal amount of α -factor after 3 h of growth.

Mating was performed by mixing $1-3 \times 10^7$ cells of each mating type in a conical tube. The cells were centrifuged, the supernatant fluid was replaced with 7 mM phosphate-SD medium, and the pellet was incubated without mixing for 30 min at 25°C. The pellet was then gently resuspended and the mixture was incubated further with gentle swirling.

Acid Phosphatase Assay and Stain

Acid phosphatase was measured in whole cells as described by van Rijn et al. (33); 1 U of activity releases 1 μmol of *p*-nitrophenol per minute. Protein concentrations were measured in cell lysates produced by extraction at alkaline pH (30). The acid phosphatase stain was based on the α -naphthylphosphate-hexazonium pararosanilin technique of Barka (2, 3). Basic fuchsin was dissolved at 8 mg/ml in 2 N HCl; 8 ml of this solution was mixed with 12 ml of 4% sodium nitrite and left at room temperature for 3 min. The mixture was titrated to pH 6 with 1 N NaOH; and 8 ml was mixed with 7 ml of α -naphthylphosphate (8 mg/ml; in 0.1 M sodium acetate, pH 6, and 0.01% Merthiolate buffer). This solution was warmed to 50°C in a water bath and mixed with 15 ml of molten Oxoid ion agar (2%; in acetate-Merthiolate buffer), and the mixture was applied in 3-ml aliquots to glass microscope slides. Agar-coated slides were kept in a moist chamber at 4°C until needed.

Cells to be stained were first killed by treatment with 0.01% Merthiolate and kept at 0°C where acid phosphatase activity decayed <10% in 30 h. Aliquots (50 μl) of the treated cultures were spotted onto the agar surface (2–4 spots/slide), and after 5–10 min the excess fluid was drained. The slides were then placed in

a moist chamber, and incubation at room temperature was continued for 4–6 h. Cell staining patterns were examined with a Zeiss WL research microscope, and photomicrographs were taken with a Polaroid camera attachment and type 667 black and white film. Staining patterns were unchanged for up to 2 d at 4°C.

RESULTS

Acid Phosphatase Secretion during Budding Growth

Acid phosphatase activity was measured in whole cells (strain A279-2c) during induction or repression (Fig. 1 and Table I) of enzyme synthesis. Enzyme activity first appeared at 2 h after the cells were suspended in a phosphate-free minimal medium. Depletion of an intracellular pool of phosphate probably accounted for this time lag. Repression of phosphatase synthesis occurred 1–2 h after the addition of 7 mM phosphate to a derepressed culture.

Strain A279-2c contains a duplicated acid phosphatase gene and produces 20- to 40-fold more enzyme than does a normal strain, such as X2180, growing on a phosphate-limited medium. The duplicated genes are of the *ACP 1-2* allele, which produces

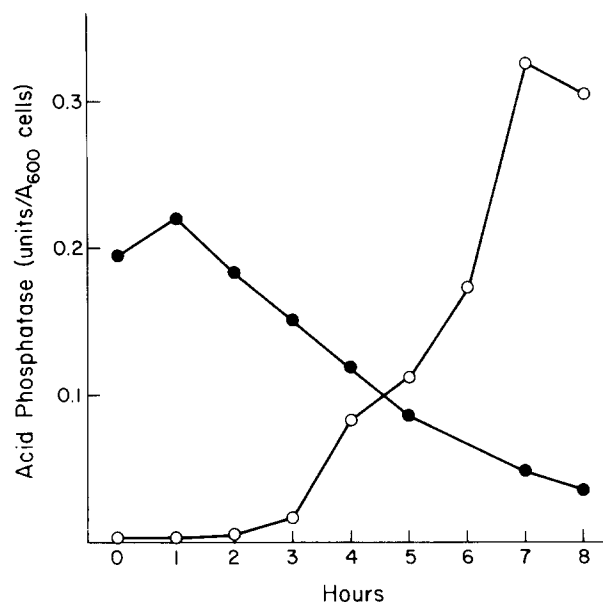


FIGURE 1 Time-course of acid phosphatase repression and derepression. Strain A279-2c was repressed (●) or derepressed (○) on 7 mM phosphate-SD medium or phosphate-free medium, respectively. Aliquots were removed at 1-h intervals and assayed.

TABLE I
Acid Phosphatase Content of Budding, α -Factor-treated and *cdc 24* Mutant Cells

Strain and treatment	Temperature °C	Repressed*	Derepressed†
		U/mg protein	
A279-2c	30	0.03	0.72
	37	0.03	0.61
A279-2c + α -factor	30	0.01	0.69
	37	0.03	0.68
SF 78-13A	24	0.03	1.44
	37	0.02	1.44

* Cells were grown at 24° or 30°C in 7 mM phosphate-SD medium to an A_{600} of 0.5. After a 7-h incubation at the indicated temperatures, cells were collected and assayed.

† Repressed cells were transferred to phosphate-free medium and incubated for 7 h at the indicated temperature, after which cells were collected and assayed.

an enzyme with a higher pH optimum (5.2 instead of the normal 4.2; see reference 15). Both the high level of the enzyme and an elevated pH optimum (which favors rapid precipitation of the naphthyl-pararosanilin complex) allow rapid and selective histochemical staining of secreting cells.

The cells in Fig. 2*a* and 2*b* were from fully repressed (7 mM phosphate) or induced (no phosphate) cultures of A279-2c, respectively. When repressed and derepressed cells were mixed and treated together, the number of cells that stained was proportional to the number of derepressed cells present (data not shown). Strain Ela (*acp 1-1*) was not stained under any growth conditions. These results demonstrated the enzyme and cell specificity of our staining procedure.

The emergence of stainable cells, when a repressed culture was shifted to a phosphate-free medium, paralleled the appearance of phosphatase activity. Fig. 2*c* shows cells grown for 2 h in a phosphate-free medium and then stained. In dividing cells, only the buds showed staining, although some unbudded cells were stained and the staining intensity varied. The proportions of various cell staining patterns were quantified at hourly intervals after removal of phosphate from repressed cultures (Fig. 3*A*). Bud-localized staining was seen as a major pattern at 2–3 h, but from 3 h on most of the cells were completely stained. No preferential staining of the parent cell was seen, and staining was never restricted to only a portion of a bud or parent cell surface. When fully-induced cells were repressed by the addition of phosphate, unstained cells appeared as the phosphatase specific activity dropped. Fig. 2*d* shows induced cells grown for 2 h in a phosphate-rich medium and then stained. In this experiment, only the parent portions of dividing cells were stained. The appearance of cell staining patterns was quantified (Fig. 3*B*) as in the above-described experiment. Parent cell-localized staining was a dominant cell type from 3–5 h and unstained cells were the majority after 5 h. Stainable cells persisted for four to five generations in this

experiment, although the staining intensity was not as strong as at the earlier time points.

Acid Phosphatase Localization during Pheromone Treatment and Mating

During the yeast mating response, α -mating-type cells secrete a peptide pheromone (α -factor) that interrupts the cell cycle of *a* cells at G₁ (5). Although budding growth is arrested in *a* cells by α -factor, surface growth continues and results in the formation of a cell tip (10). We examined the pattern of acid phosphatase secretion during α -factor treatment in experiments analogous to those described for budding growth. *a* Cells, grown in phosphate-rich medium, were shifted to phosphate-free medium and incubated with α -factor during the derepression of phosphatase synthesis. In this experiment, most of the cells were in G₁-arrest when phosphatase activity first appeared. Pheromone-treated cells secreted nearly as much phosphatase as budding cells did (Table I).

Fully repressed and derepressed α -factor-treated cells showed no staining and uniform staining, respectively (Fig. 4*a* and *b*). Phosphatase-activity was restricted to the cell tip when derepression occurred during G₁-arrested growth (Fig. 4*c*). Conversely, the cell body and not the tip contained phosphatase activity when derepressed cells were repressed during pheromone treatment (Fig. 4*d*). The enzyme localization during these conditions of growth was followed with time (Fig. 5*a* and *b*). The only exception to the patterns in Fig. 4*c* and *d* occurred when derepression was continued for 6–7 h of pheromone treatment with the result that >50% of the cells showed stain covering the cell surface.

G₁-arrested cells of the opposite mating type fuse to form diploid zygotes, and a new cell cycle begins with the emergence of a bud from the middle of the zygote. We examined the distribution of phosphatase activity on the surface of zygotes

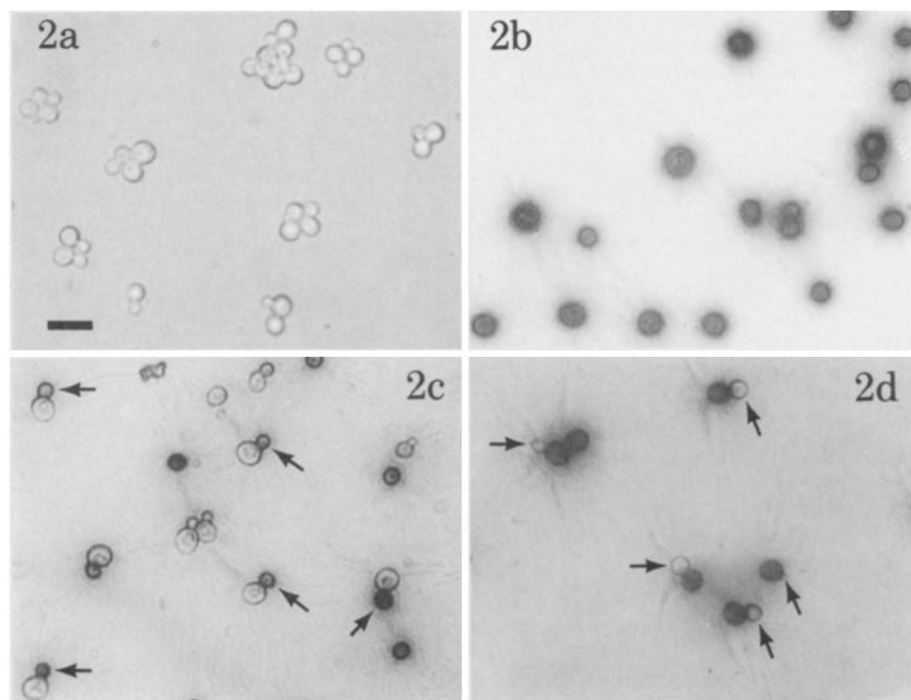


FIGURE 2 Staining pattern of repressed and derepressed budding cells. (a) Repressed A279-2c cells. (b) Derepressed cells. (c) Repressed cells were shifted to phosphate-free medium for 5 h at 30°C. (d) Derepressed cells were shifted to 7 mM phosphate-SD medium for 2 h at 30°C. Arrows indicate buds. Bar, 10 μ m.

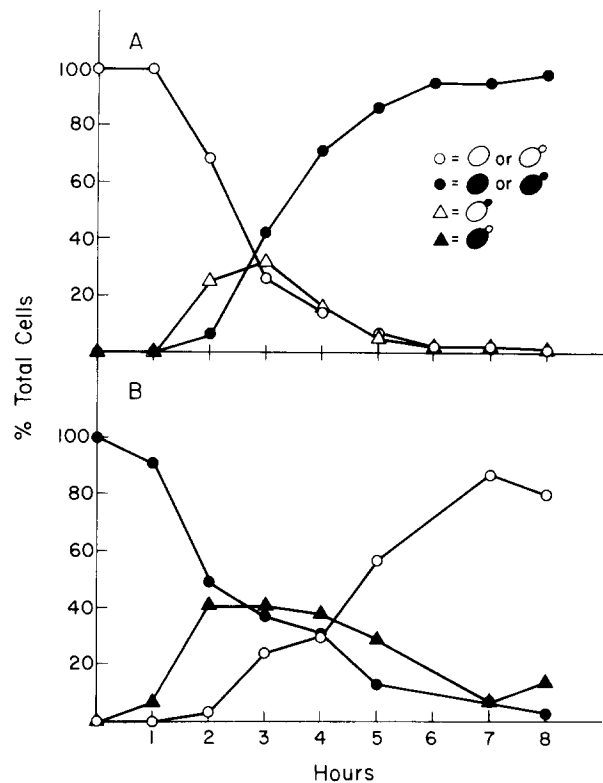


FIGURE 3 Time-course of staining pattern during derepression and repression. (A) Cells were treated as in Fig. 2 *c*, except that samples were taken at 1-h intervals. (B) Cells were treated as in Fig. 2 *d*, with the above exception. At least 100 cells were counted for each time point.

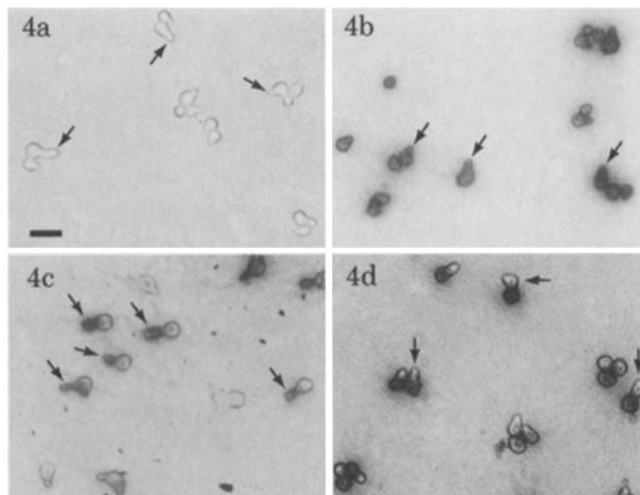


FIGURE 4 Staining pattern of α -factor-treated repressed and derepressed cells. (a) Repressed A279-2c cells were treated with α -factor for 4 h in 7 mM phosphate-SD medium. (b) Repressed cells were treated with α -factor for 5 h in 0.1 mM phosphate-SD medium. (c) Repressed cells were transferred to phosphate-free SD medium and incubated with α -factor for 5 h. (d) Derepressed cells were transferred to 7 mM phosphate-SD medium and incubated with α -factor for 4 h. Arrows indicate cell tips. Bar, 10 μ m.

formed when derepressed *a* cells were mated with phosphatase-negative α cells. Examples of budded zygotes stained for phosphatase activity are shown in Fig. 6 *a-e*. Although a spectrum of phosphatase distribution is seen in these examples, the hybrid staining pattern predominated. Zygotes (279 total) were

counted in two experiments, and 175 were like Fig. 6 *a* and *b*, 50 were like *c* and *d*, and 4 were like *e*.

Delocalized Growth in a Cell Cycle Mutant

The conditional cell cycle mutant, *cdc 24*, fails to initiate bud formation at a restrictive temperature during G_1 ; instead, it shows delocalized growth resulting in much larger cells that contain up to four nuclei (17). Sloat and Pringle (29) found that chitin, which is restricted to an annulus between the mother and bud portion of a dividing cell, was deposited uniformly over the expanding *cdc 24* cell surface at the restric-

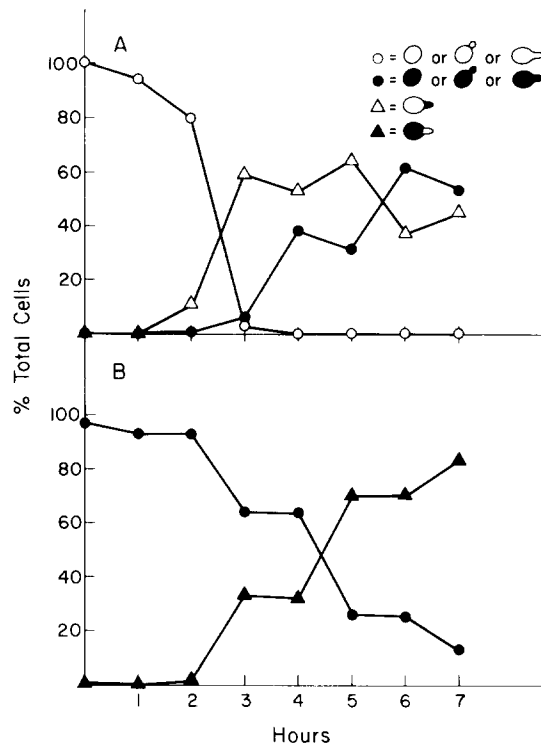


FIGURE 5 Time-course of staining pattern for α -factor-treated depressed and repressed cells. (A) Cells were treated as in Fig. 4 *c*, except that samples were taken at 1-h intervals. (B) Cells were treated as in Fig. 4 *d*, with the above exception. At least 100 cells were counted for each time point.

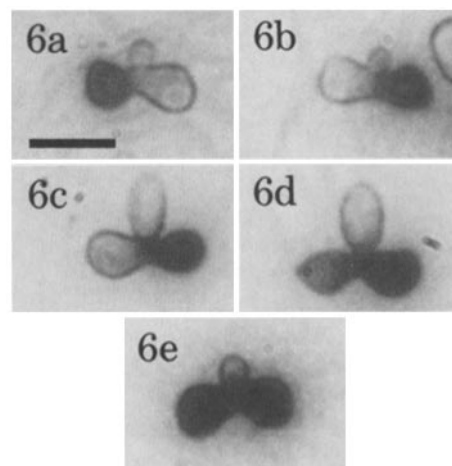


FIGURE 6 Staining pattern of zygotes made from phosphatase-containing and phosphatase mutant parent cells. Zygotes were examined after 4.5 h of mating. Zygotes are shown with the new bud pointing up. Bar, 10 μ m.

tive temperature. We examined the surface distribution of phosphatase secreted by *cdc 24* cells during growth at the restrictive temperature and tested the effect of α -factor on this pattern of growth.

Acid phosphatase synthesis and secretion were not reduced in a *cdc 24* strain grown at 37°C when compared with a wild-type strain grown at the same temperature (Table I). When a *cdc 24* strain was derepressed for phosphatase synthesis at the permissive temperature (24°C), the subsequent staining pattern was indistinguishable from the wild-type distribution (Fig. 2c). However, when the *cdc 24* strain was derepressed during incubation at 37°C, no localized staining pattern developed; phosphatase was deposited uniformly over the mutant cell surface. Fig. 7a is a representative field of *cdc 24* cells derepressed at 37°C; Fig. 8A is a time-course of the appearance of various cell types at 37°C. Similarly, when the *cdc 24* strain was fully derepressed at 24°C and then repressed at 37°C, the staining pattern showed an even dilution of phosphatase as the mutant cell surface expanded (Fig. 7b, Fig. 8B).

α -Factor did not induce the formation of cell tips in an *a*-mating-type *cdc 24* strain at 37°C (the cells had the same morphology as those in Fig. 7a and b), although the pheromone effect was seen in the mutant at 24°C and in a wild-type strain at 37°C. Mutant cells treated with α -factor at 24°C retained a small cell tip when subsequently incubated at 37°C; phosphatase secretion was delocalized when derepression occurred during the 37°C treatment (Fig. 7c). Conversely, when mutant cells were derepressed during α -factor treatment at 24°C (as in Fig. 4c) and then grown at 37°C in a repressing medium, phosphatase activity remained associated with the residual cell tip (Fig. 7d).

DISCUSSION

The localized secretion of acid phosphatase by *S. cerevisiae* appears to follow the pattern of cell-surface growth. During

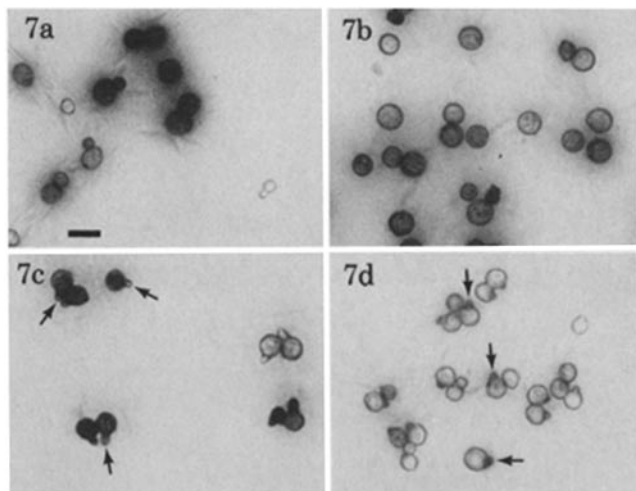


FIGURE 7 Staining pattern of *cdc 24* mutant cells. (a) Repressed SF 78-13a cells were transferred from 24°C into 0.05 mM phosphate-SD medium at 37°C and incubated for 7 h. (b) Derepressed cells were transferred from 24°C into 7 mM phosphate-SD medium at 37°C and incubated for 6 h. (c) Repressed cells were incubated in 7 mM phosphate-SD medium at 24°C with α -factor for 4 h and then transferred into phosphate-free medium at 37°C for 5 h. (d) Repressed cells were transferred into phosphate-free SD medium at 24°C with α -factor for 5 h, and then 7 mM phosphate was added and incubation was continued at 37°C for 4 h. Arrows indicate cell tips. Bar, 10 μ m.

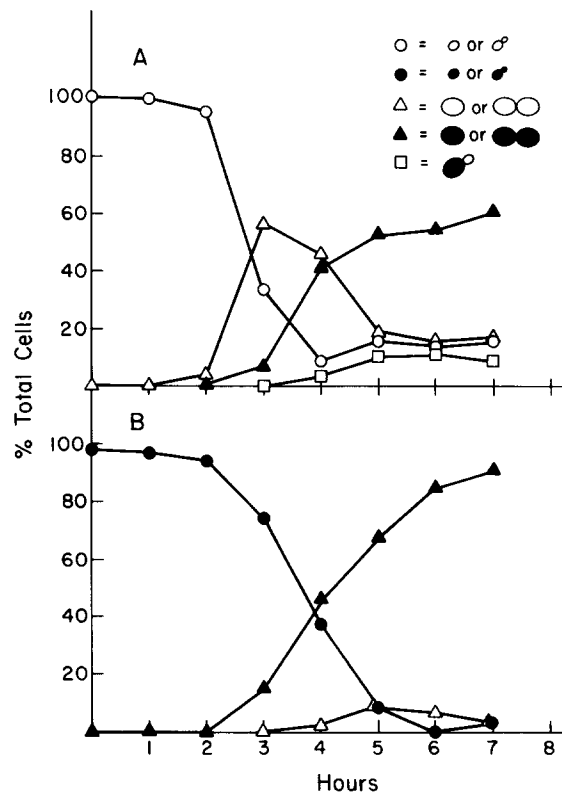


FIGURE 8 Time-course of staining pattern for *cdc 24* mutant cells. (A) Cells were treated as in Fig. 7a, except that samples were taken at 1-h intervals. (B) Cells were treated as in Fig. 7b, with the above exception. At least 100 cells were counted for each time point.

budding growth, and in preparation for mating, newly secreted acid phosphatase appears on the new cell surface area. This asymmetric distribution speaks both to the mechanism of secretion and to the structure of the yeast cell wall. Although the exocytosis model for secretion is clearly consistent with our observations, the results do not exclude a secretory process involving direct extrusion of secretory proteins along a restricted portion of the plasmalemma. Both mechanisms require that recognition sites (for protein extrusion or for secretory vesicle fusion) that promote local growth will be available only on the bud portion of a dividing cell, or the tip portion of a pheromone-treated cell, and that these sites will be inactive during subsequent cell cycles. It is likely that the recognition sites, as components of a fluid plasmalemma (23), are confined to the growing portion of a cell by some mechanism that resists the tendency to diffuse laterally. The defect that results in delocalized growth of *cdc 24* cells at 37°C may affect the mechanism that organizes the secretory recognition sites during budding or pheromone-directed growth.

Recent independent evidence from our laboratory strongly supports the exocytosis model of secretion. We have isolated a conditional yeast mutant blocked in secretion, cell-surface growth, and division, that thermoreversibly accumulates an intracellular pool of vesicle-bound acid phosphatase (25). The mutant also affects the secretion of invertase and is blocked in the incorporation of a plasmalemma sulfate permease activity. The behavior of this mutant suggests that cell-surface growth and secretion are achieved by the same exocytotic process. From the results reported here, we infer that secretory and plasmalemma components are incorporated primarily into the bud surface during the cell division cycle.

The bud-localized incorporation of cell-surface components

was seen with fluorescent-concanavalin A (31) and with fluorescent-anti-invertase antibody (32). However, the yeast cell wall excludes polyethylene glycol molecules larger than ~700 daltons (28), thus it is likely that lectin and antibody molecules will bind only the outermost structures on the cell surface. Histochemical staining has shown that in fully derepressed cells, acid phosphatase activity is located in two cell wall layers: one is directly outside of the plasmalemma, and the other is on the cell wall surface (22). The inner layer stains more densely on the parent than on the bud portion of a dividing cell. Thus, reagents that fail to penetrate the outer wall layer may not detect newly secreted enzymes in the inner layer. We have eliminated this objection by the use of a low-molecular-weight histochemical stain. Secreted acid phosphatase appears to be fixed in the cell wall. Localized staining of phosphatase was possible with cells kept at 0°C for up to 5 d, and at room temperature under nongrowing conditions for up to 8 h; newly secreted phosphatase does not appear to diffuse back to the older part of a cell (Figs. 2c, 3A, 4a and 5A), and previously secreted phosphatase does not move into the area of new growth (Figs. 2d, 3B, 4d, and 5B). Restricted diffusion of secreted phosphatase is also apparent when zygotes are formed between phosphatase-containing and phosphatase-mutant cells (Fig. 6). The hybrid staining pattern of the zygotes formed in this experiment suggests that cell fusion does not result in extensive mixing of secreted enzymes.

Although secretion is largely restricted to the bud portion of a dividing cell, the experiment in Figs. 2c and 3A shows that >95% of the cells are stainable within two generations after the appearance of phosphatase activity; this result is not expected for the simple case of bud-limited secretion. Because of the stability of the localized staining pattern, we suggest that limited growth and secretion by the parent portion of a dividing cell, or growth between cell cycles, may account for the rapid disappearance of phosphatase-free cells during derepression. Growth during the unbudded phase of the cell cycle has been described elsewhere (18).

Localized incorporation of cell wall enzymes may have important consequences in budding and in mating. A glucanase activity is secreted at the beginning of the cell cycle (9) and increases during mating (4). Glucan hydrolysis may facilitate the budding process by softening the cell wall in a restricted area. During zygosis, mating cells contact each other at the point of growth. Localized secretion and cell wall modification may promote the adhesion and subsequent fusion of two cells. Delocalized secretion in *cdc 24* cells may explain the inability of mutant cells to mate at 37°C (26).

To act locally, the diffusion of secreted proteins must be restricted. Although this report documents a constraint on the movement of secreted acid phosphatase, the available evidence does not support a strong association of this enzyme with a cell wall structure. Simple mechanical breakage of cells releases most of the secreted acid phosphatase (33) and invertase (21) into a soluble fraction. Alternatively, secreted enzymes may be trapped in a network of cell wall polymers. The distribution of chitin on the cell surface suggests a possible role in compartmentalization of the wall. Chitin is enriched in the junction between the parent and bud portion of a dividing cell (6); this position also defines the point of separation between newly and previously secreted acid phosphatase. During pheromone treatment, chitin is deposited along the cell tip (27), an area that separates new and old secreted phosphatase. Finally, *cdc 24* cells show a delocalized pattern of chitin at 37°C (29), thus

the mechanism that organizes secretion may also regulate the distribution of chitin.

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