A Mutant of Yeast Defective in Cellular Morphogenesis

Abstract. *In the budding yeast* Saccharomyces cerevisiae, *each bud appears within a ring of chitin formed in the cell wall of the mother cell. Temperature-sensitive mutants defective in gene* cdc24 *synthesize chitin at restrictive temperatures, but do not organize it into the discrete rings found in normal cells, nor do they form buds. The chitin ring or an annular precursor structure may play an essential role in reinforcing the region of the cell wall involved in budding.*

Each type of living cell has a characteristic shape and three-dimensional spatial organization. The processes of cellular morphogenesis that generate this spatial organization play an essential role in cell reproduction and differentiation. In some cases, the appropriate structures self-assemble after their constituent macromolecules have been synthesized (*1*); in others, some of the information for assembling micromolecules into three-dimensional structures appears to derive from already existing patterns of organization (*2*). In general, the mechanisms involved in cellular morphogenesis are poorly understood (*2–4*).

The cell division cycle of the yeast *Saccharomyces cerevisiae* provides an experimental system for approaching the problem of cellular morphogenesis (*4–6*). At a specific time early in the cycle, the future daughter cell appears as a small bud at a discrete and nonrandom site on the surface of the mother cell (*5, 7, 8*). Coincident with or shortly before the appearance of the bud, a ring of chitin appears in the largely nonchitinous cell wall of the mother cell (*9–12*). The bud emerges within the confines of this ring, which may serve as a structural reinforcement for the region of the mother cell wall involved in budding (*6, 9, 10*). Subsequent growth of the bud involves a highly localized addition of new cell wall material (*13*); its ultimate scission from the mother cell involves the localized formation of septal membrane and cell wall (*4, 14, 15*). The chitin ring, seen by fluorescence microscopy after staining with the dyes primulin or Calcofluor (*9, 10, 16*), is visible throughout bud growth (*9–11*) (Fig. 1, b and c); it remains visible after cell division as a bud scar on the surface of the mother cell (*7, 9–11, 17, 18*) (Fig. 1d). We report here that a temperature-sensitive mutant of yeast, defective in bud formation, seems able to synthesize chitin at the restrictive temperature but is unable to organize this chitin into a normal ring.

Temperature-sensitive mutants defective in gene *cdc24* were isolated by Hartwell and his colleagues (*5, 19*). The mutant cells reproduce normally at the permissive temperature (23°C), but at the restrictive temperature (36°C), although DNA synthesis and nuclear division continue, they are unable to form buds, producing large, round, multinucleate cells. To test whether *cdc24* mutants were defective in budding due to an inability to form a normal chitin ring, mutant cells that had been grown at 36°C were examined by fluorescence microscopy.

A culture of diploid strain 5011D3, homozygous for the *cdc24-1* mutation, was grown at 23°C in YM-1 medium (*20*) with supplementary adenine and uracil (each at 20 μg/ml) until stationary phase was reached. In this type of cell population, the smallest cells were newly formed daughter cells that had never produced buds and had no bud scars (*20, 21*). A homogeneous population of small, scar-free cells was isolated by centrifuging the cells through density gradients of 5 to 20 percent sucrose or 2 to 10 percent Ficoll (Sigma, type 400) in water. Cells from 10 ml of stationary phase culture were centrifuged and resuspended in half the original volume of medium; the cells were layered onto prepared gradients (44 ml total volume) in 50-ml plastic tubes and centrifuged at 1000 rev/min (183*g*) for 5 minutes in a Sorvall GLC-2 centrifuge at 23°C. Successive 3-ml fractions were removed from the top of each gradient with a Pasteur pipet and examined microscopically. Fractions containing predominantly small cells, generally the second and third fraction from each gradient, were pooled and recentrifuged. Portions of the isolated cells were resuspended in fresh YM-1 medium for growth at either 23° or 36°C. Hourly samples were removed from each culture, and the cells were fixed with 3 percent formaldehyde (*22*). The samples were examined for the appearance of buds by phase contrast microscopy, and for the appearance of chitin rings by fluorescence microscopy. Prior to fluorescence microscopy, the fixed cells were suspended in 0.1 percent Calcofluor White M2R New (American Cyanamid) in water for 5 minutes at 23°C, centrifuged, and washed once with water. The cells were observed with a standard Zeiss fluorescence microscope with exciter filters BG-12 and UG-1, a bright field condenser, a 100× oil-immersion phase objective, and barrier filter 44. Photographs were obtained with high-speed Ektachrome daylight film (ASA 160) at a 90-second exposure.

The cells isolated by density gradient centrifugation were small, devoid of buds, and free of bud scars or other conspicuous fluorescence (Fig. 1a; Table 1). After transfer to fresh growth medium at 23°C, the small cells increased in size, and began to bud after a lag of approximately 7 hours. The appearance of a bud was always accompanied by a distinct fluorescent ring at the mother-bud junction (Fig. 1, b and c). No appreciable fluorescence was detectable elsewhere on the surface of the budding cell. Since the percentage of cells with fluorescent rings was higher at 8 and at 10 hours than was the percentage of cells with detectable buds (Table 1), it seemed as if the ap-

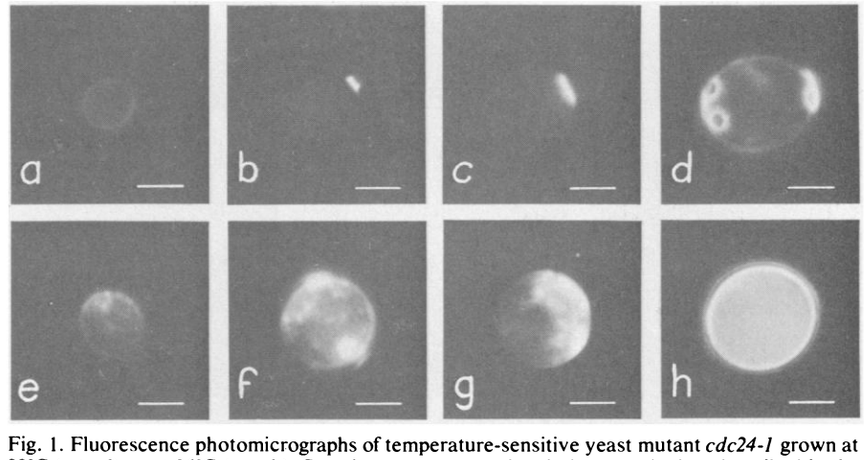

Fig. 1. Fluorescence photomicrographs of temperature-sensitive yeast mutant *cdc24-1* grown at 23°C (a to d) or at 36°C (e to h). Samples were prepared and photographed as described in the text. All prints were made at the same enlarger and exposure settings. (a) Isolated small, unbudded cell, devoid of fluorescent regions. (b and c) Budded cells after 7 to 8 hours growth at 23°C with highly localized fluorescence at the mother-bud junctions. (d) Multiparous mother cell with multiple bud scars after continued growth at 23°C. (e to h) Large unbudded cells with disorganized fluorescent regions or a generalized fluorescence after growth at 36°C for 2 hours (e), 4 hours (f), 6 hours (g), or 8 hours (h). Scale bar, 4 μm.

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pearance of the ring normally preceded that of the bud, as reported by Hayashibe and Katohda (*9*). The cultures grown at 23°C reattained stationary phase within 60 hours; the cell populations from these cultures consisted of multiparous cells with numerous bud scars (Fig. 1d).

When the isolated small cells were transferred to fresh medium at 36°C, they increased greatly in size, but formed neither buds nor distinct fluorescent rings (Table 1). Disorganized fluorescent areas (Fig. 1, e and g) and brightly fluorescent patches (Fig. 1f) appeared beginning as early as 2 hours after the transfer to fresh medium (Table 1). Although the detailed appearance of the fluorescence was variable from cell to cell, cultures grown for eight or more hours at 36°C resulted in nearly all the cells becoming generally and brightly fluorescent (Fig. 1h).

The uniform bright fluorescence of such cells might be due to either an extensive incorporation of Calcofluor-binding material, presumably chitin, over the entire surface of the growing cell or to a change in staining properties upon cell death. To distinguish between these possibilities, cells that had been grown at 36°C for 8 hours were shifted to 23°C. After 1 to 2 hours, samples were removed, prepared for fluorescence microscopy, and photographed. Many uniformly bright cells with dark buds (Fig. 2, a and b) were observed. Thus, many of the brightly fluorescent cells (Fig. 1h) are alive and capable of forming a normal bud after returning to a permissive temperature; the cell wall of the bud, like that of a normal cell, does not bind Calcofluor extensively.

Table 1. Appearance of buds and fluorescent regions during incubation of isolated small cells in fresh growth medium at 23°C or 36°C. All percentages are based on counts of at least 150 cells, except as noted.

Time (hour)	Temperature (°C)	Percentage of cells showing			
		Buds	Fluorescent rings	Other fluorescent regions	No fluoresence
0	23 or 36	<1	<1	<1	>99
2	23	<1	<1	<1	>99
	36	<1	<1	12	88
4	23	<1	<1	<1	>99
	36	<1	<1	71	29
8	23	8.3*	11.7*	<1*	88*
	36	<1	1	81	18
10	23	16*	21.5*	<1*	78*
	36	<1		>99†	<1

*Based on counts of 400 cells. †Cells are very large and exhibit generalized fluorescence (Fig. 1h).

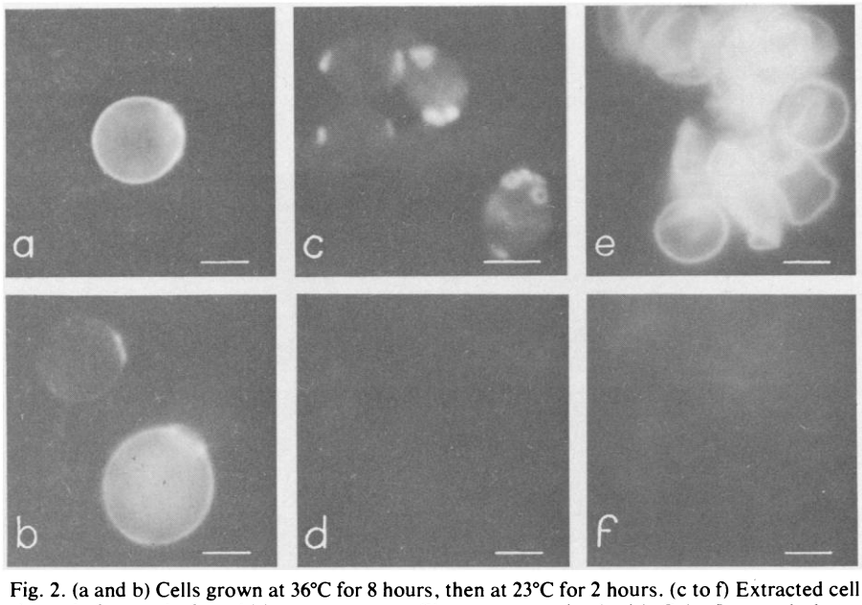

Fig. 2. (a and b) Cells grown at 36°C for 8 hours, then at 23°C for 2 hours. (c to f) Extracted cell ghosts before and after chitinase treatment. Ghosts were stained with Calcofluor and photographed as described in the text; all prints were made at the same exposure settings. (c) Extracted ghosts of cells grown to stationary phase at 23°C. (d) Ghosts from the same preparation after incubation for 8 hours at 37°C in 0.025*M* sodium phosphate buffer, *p*H 6.3, containing 0.25 mg of chitinase per milliliter [ICN 100466, partially purified from *Streptomyces griseus* (*24*)]. After incubation, chitinase was removed by centrifugation, and the pellet was treated with Calcofluor. (e) Extracted ghosts of isolated small cells that were grown for 8 hours at 36°C. (f) Ghosts as in (e) after chitinase treatment. Scale bar, 4 μm.

Previous studies (*9*, *12*) have provided evidence that Calcofluor binds preferentially to chitin in the yeast cell wall. The other possibility suggested for the localized fluorescence is that fluorescent dyes bind preferentially or fluoresce maximally in regions with an orderly circular arrangement of polysaccharide microfibrils (*10*, *23*). Our results (Fig. 1, e to h) do not support this hypothesis. To test further the hypothesis that the disorganized fluorescent areas (Fig. 1, e to h) are disorganized patches of chitin, we examined the effect of chitinase (*24*) treatment on Calcofluor binding. Chitinase was ineffective on intact cells; however, two cycles of alkali and acid extraction of cells (*18*) yielded cell ghosts whose appearance after staining with Calcofluor was similar to that of the intact cells (Fig. 2, c and e). Treatment of ghosts with chitinase did not affect their general outlines, but almost eliminated the Calcofluor binding both in rings and in the disorganized patches (Fig. 2, d and f).

Yeast cells homozygous for the temperature-sensitive *cdc24-1* mutation synthesize chitin at restrictive temperatures, but fail to organize this chitin into normal rings. Normal chitin rings do form at appropriate times during growth at permissive temperatures. Delocalized chitin deposition has also been observed (*25*) following osmotic shock or cycloheximide treatment of *Aspergillus* hyphae, and a partial delocalization of chitin deposition has been observed (*26*) following treatment of yeast cells of mating type a with α mating factor.

The disorganized pattern of chitin synthesis in the mutant cells may be due to a primary defect in the organization or function of the extranuclear microtubules. Byers and Goetsch (*27*) have shown that microtubules normally extend from the spindle plaque in the nuclear envelope to the site of the growing bud, and have suggested that they are involved in directing vesicles with new wall material and appropriate enzymes (*28*) to the appropriate cell wall site. Another possibility is that the ring of microfilaments, found in the cytoplasm underlying the mother-bud junction (*15*), plays an essential role in the normal localization of chitin synthesis, and that the organization or function of this micro-

filament ring is defective in the mutant. A third possibility derives from evidence that inactive chitin synthetase zymogen is present at numerous sites on the plasma membrane (*29*), and must undergo a localized activation by an activating factor during budding (*4*). The membrane-bound zymogen of the mutant strain may experience a spontaneous and delocalized activation when shifted to the restrictive temperature.

Results obtained with the *cdc24-1* mutant are consistent with the necessity for a reinforcing ring of chitin if cell wall expansion is to result in bud formation, rather than in generalized cell expansion. It is difficult to reconcile this idea with the report of Cabib and Bowers (*12*) that treatment of yeast cells with polyoxin D, an inhibitor of chitin synthetase, can prevent chitin synthesis and formation of the Calcofluor-binding ring, while allowing normal buds to appear. Although their experiment used very high concentrations of polyoxin D (*30*) at a temperature near the upper limit of the normal growth range, it supported the view (*4*) that, although most chitin is synthesized early in the cell cycle, it functions only at the time of cytokinesis. A hypothesis that would explain both results is that the *cdc24-1* mutant is defective in forming an annular structure, such as the microfilament ring (*15*) or the "collar-like circular zone" described by Seichertová *et al.* (*31*), that is necessary both for localized deposition of chitin and for budding.

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Potent Antidopaminergic Activity of Estradiol at the Pituitary Level on Prolactin Release

Abstract. *Prior incubation of rat anterior pituitary cells with 17β-estradiol led to an almost complete reversal of the inhibitory effect of two dopamine agonists, dihydroergocornine and RU 24213, on both basal prolactin release and thyrotropin releasing hormone–induced prolactin release. These experiments thus demonstrate a direct interference of dopamine action by a peripheral hormone. Prolactin secretion by pituitary cells in primary culture could possibly serve as an easily accessible model of a system under dopaminergic control.*

Recent studies indicate that dopamine may be the main or even the only inhibitory substance of hypothalamic origin controlling prolactin secretion. In fact, the prolactin inhibiting activity contained in hypothalamic extracts could be accounted for by their catecholamine content (*1*), and prior incubation of hypothalamic extracts with aluminum oxide or monoamine oxidase led to complete loss of prolactin release inhibiting activity (*2*).

Estrogens are potent stimulators of prolactin secretion in both man (*3*) and rat (*4*, *5*). Moreover, the increased rate of prolactin secretion in the afternoon of proestrus in the rat is presumably under estrogenic influence (*6*, *7*). These effects of estrogens in vivo could, however, be exerted at the hypothalamic or pituitary level, or both. Our observation that, in

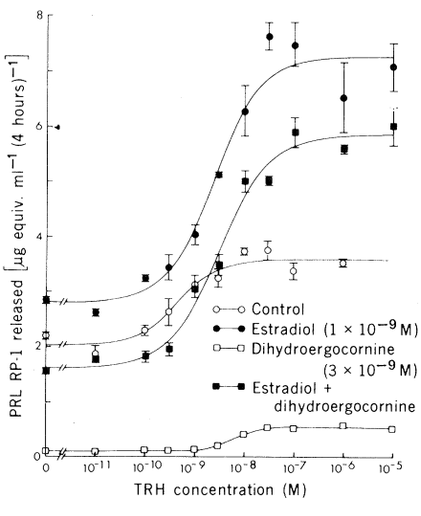

Fig. 1. Effect of 1 n*M* 17β-estradiol (E_2), 3 n*M* dihydroergocornine, or the vehicle alone (control) on the prolactin response to increasing concentrations of TRH in female rat anterior pituitary cells in primary culture. Cells were first incubated for 120 hours in the presence or absence of E_2, and then incubated for 4 hours in the presence or absence of the dopamine agonist DHE and the indicated concentrations of TRH. Data are expressed as means ± standard error of duplicate measurements of triplicate petri dishes. Note that E_2 led to an almost complete reversal of the inhibitory effect of DHE on prolactin release.

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