# Roles of the *CDC24* Gene Product in Cellular Morphogenesis during the *Saccharomyces cerevisiae* Cell Cycle

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ABSTRACT Temperature-sensitive yeast mutants defective in gene *CDC24* continued to grow (i.e., increase in cell mass and cell volume) at restrictive temperature (36°C) but were unable to form buds. Staining with the fluorescent dye Calcofluor showed that the mutants were also unable to form normal bud scars (the discrete chitin rings formed in the cell wall at budding sites) at 36°C; instead, large amounts of chitin were deposited randomly over the surfaces of the growing unbudded cells. Labeling of cell-wall mannan with fluorescein isothiocyanate-conjugated concanavalin A suggested that mannan incorporation was also delocalized in mutant cells grown at 36°C. Although the mutants have well-defined execution points just before bud emergence, inactivation of the *CDC24* gene product in budded cells led both to selective growth of mother cells rather than of buds and to delocalized chitin deposition, indicating that the *CDC24* gene product functions in the normal localization of growth in budded cells as well as in unbudded cells.

Growth of the mutant strains at temperatures <36°C revealed allele-specific differences in behavior. Two strains produced buds of abnormal shape during growth at 33°C. Moreover, these same strains displayed abnormal localization of budding sites when grown at 24°C (the normal permissive temperature for the mutants); in each case, the abnormal pattern of budding sites segregated with the temperature sensitivity in crosses.

Thus, the *CDC24* gene product seems to be involved in selection of the budding site, formation of the chitin ring at that site, the subsequent localization of new cell wall growth to the budding site and the growing bud, and the balance between tip growth and uniform growth of the bud that leads to the normal cell shape.

Cellular morphogenesis is the process by which cellular form and spatial organization are generated. During the cell division cycle of the yeast *Saccharomyces cerevisiae*, cellular morphogenesis includes the following sequential events: (*a*) selection of a nonrandom site at which budding will occur (1, 14, 23, 25, 41, 46); (*b*) formation of a ring of chitin (the "bud scar") in the largely nonchitinous cell wall at that site (24, 34); (*c*) localization of new cell wall growth to the region bounded by the chitin ring, resulting in the appearance and selective growth of a bud (10, 11, 22, 27, 28); (*d*) localization of new cell wall growth to the tip of the growing bud (10, 11, 27, 43); (*e*) cytokinesis and the formation of septal cell wall (6, 43). In addition, it seems that periods of uniform growth of the bud cell wall precede and follow the period of tip growth (11); presumably, the relative amounts of tip growth and of uniform growth are adjusted to yield the normal ellipsoidal shape of the daughter cell (10, 11).

The yeast cell cycle is a convenient object for studies of cellular morphogenesis, in part because the morphogenetic processes are relatively simple and well defined, and in part because many temperature-sensitive mutations affecting cell cycle events are available (18, 19, 21). In one class of such mutants, defective in the gene *CDC24*, budding is blocked at the restrictive temperature, although growth (i.e., increase in cell volume, mass, and protein content), DNA synthesis, and nuclear division apparently continue (19, 21, 28). We reported previously (39) that, in strains carrying the *cdc24-1* mutant allele, the failure to bud at 36°C was accompanied by a failure to form a normal chitin ring. Instead, large amounts of chitin seemed to be deposited randomly, and eventually more-or-less uniformly, over the cell wall of the expanding cell. These conclusions were based on fluorescence microscope observations after staining with Calcofluor, a dye that seems to stain chitin selectively in the yeast cell wall (8, 24). We now report

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direct measurements of cell wall polysaccharides that confirm the synthesis of large amounts of chitin by *cdc24-1* cells at 36°C. We also report studies of various aspects of cellular morphogenesis in strains carrying other mutant alleles of gene *CDC24*. The results suggest that the *CDC24* gene product is involved in most, if not all, of the morphogenetic processes described above.

# MATERIALS AND METHODS

## Chemicals

Calcofluor White M2R New was a gift from American Cyanamid (Bound Brook, N. J.). Chitinase (from *Streptomyces griseus*; 40 U/mg, where 1 U releases 1 μmol of *N*-acetylglucosamine/min from colloidal chitin at 37°C) and β-glucosidase (from almonds; 2.5 U/mg, where 1 U releases 1 μmol glucose/min from salicin at 37°C) were purchased from ICN Pharmaceuticals, Life Sciences Group (Cleveland, Ohio). Colloidal chitin was prepared from crab shell chitin (Sigma Chemical Co., St. Louis, Mo.) as described by Berger and Reynolds (2). Fluorescein isothiocyanate-conjugated concanavalin A (FITC-Con A) was purchased from Miles Laboratories (Elkhart, Ind.), hydroxyurea from Sigma Chemical Co., and methyl-α-D-mannopyranoside from Pfanstiehl Laboratories (Waukegon, Ill.).

## Genetic Methods, Yeast Strains, and Culture Conditions

Standard genetic methods (37) were used for all crosses and complementation analyses in this study. The wild-type and temperature-sensitive (*ts*) cell division cycle (*cdc*) mutant strains used are described in Table I. Strains 5011-D6-J2D and 5011-D6-J2A were isolated as *a ts* and *α ts* segregants from strain 5011-D6. Strain E187Jα was isolated as an *α ts* segregant from a cross of E187 with C276-4B. Strain E187-JD1 was isolated after mating E187Jα to an *a ts* segregant from the same cross. Thus, the genetic background of E187-JD1 was derived both from the S288C family (C276, C276-4A, C276-4B, and related strains) and from the A364A family (A364A, A364A-D5, 5011-D3, 5011-D6, E187, and related strains).

Mutant strain JPT19 was isolated from strain C276-4A after mutagenesis with ethylmethane sulfonate (13) to ~2% survival, using a screening procedure similar to that of Hartwell et al. (21). To construct homozygous and heterozygous mutant diploids, JPT19 was crossed with C276-4B, and a *ts* segregant of mating type *a* was then crossed again with C276-4B. (Because of the presumed isogenicity of C276-4A and C276-4B (45), these two crosses can be regarded as backcrosses.) The four homozygous diploids (JPT19H01–JPT19H04) and the two heterozygous diploids (JPT19HE1 and JPT19HE2) were then isolated after mating segregants from this second backcross in various combinations. An *α ts* segregant from the first backcross was designated JPT19α.

To construct strains JPT19-428-D1 and JPT19-281-D1, ts428 and ts281 were crossed with JPT19α. In each cross, tetrads showing a 2 wild type: 2 *ts* segregation pattern were identified. In such tetrads, the *ts* segregants are expected to be double mutants. This was confirmed for each segregant used in diploid construction by demonstrating the segregation of both mutations after crossing with C276-4A or C276-4B. JPT19-428-D1 and JPT19-281-D1 were then isolated after mating appropriate pairs of double-mutant haploids.

Cultures were grown in YM-1 medium containing 20 g/liter glucose (28), with supplementary adenine and uracil (each at 20 μg/ml) added for strains A364A-D5, 5011-D3, and 5011-D6. All cultures were incubated in Erlenmeyer flasks with rotary shaking (~170 rpm), either at room temperature (24 ± 1°C) or in a water bath held at 33° or 36°C.

## Quantitative Chitin Measurements

Cell ghosts were prepared from ~150–600 mg (dry weight) of cellular material (see Table II) by alkaline extraction as described by Cabib and Bowers (7). Chitin in the ghosts was hydrolyzed according to Jeuniaux (26) in a mixture containing one-fourth of the ghosts from a particular sample, 40 U chitinase, 0.75 U β-glucosidase, and 50 mM sodium phosphate buffer, pH 6.3 (2), in a total volume of 4 ml. The mixtures were incubated for 12 h at 37°C before terminating the reactions by boiling for 3 min. Control experiments established (*a*) that the chitinase remained highly active after 12 h at 37°C (as judged by its activity against colloidal chitin) and (*b*) that incubation for >12 h gave little, if any, further hydrolysis of chitin, even if fresh chitinase and β-glucosidase were added. The hydrolysates were centrifuged, and *N*-acetylglucosamine (GlcNAc) in the supernates was determined by the method of Reissig et al. (32). The chitin values obtained were compared with measurements of total carbohydrate made on other portions of the same samples. Total carbohydrate was determined by the anthrone reaction (40) and is expressed as glucose equivalents.

TABLE I

*Yeast Strains Used in this Study*

| Strain* | Relevant genotype | References |
|---|---|---|
| C276 | *a/α CDC24⁺/CDC24⁺* | 45 |
| C276-4A | *a CDC24⁺* | 45 |
| C276-4B | *α CDC24⁺* | 45 |
| A364A | *a CDC24⁺* | 15 |
| A364A-D5 | *a/α CDC24⁺/CDC24⁺* | 16 |
| 5011-D3 | *a/α cdc24-1/cdc24-1* | 21‡ |
| 5011-D6 | *a/α cdc24-1/cdc24-1* | 21‡ |
| 5011-D6-J2D | *a cdc24-1* | See text |
| 5011-D6-J2A | *α cdc24-1* | See text |
| E187 | *a cdc24-3* | 21 |
| E187Jα | *α cdc24-3* | See text |
| E187-JD1 | *a/α cdc24-3/cdc24-3* | See text |
| JPT19 | *a cdc24-4* | See text |
| JPT19H01 | *a/α cdc24-4/cdc24-4* | See text |
| JPT19H02 | *a/α cdc24-4/cdc24-4* | See text |
| JPT19H03 | *a/α cdc24-4/cdc24-4* | See text |
| JPT19H04 | *a/α cdc24-4/cdc24-4* | See text |
| JPT19HE1 | *a/α CDC24⁺/cdc24-4* | See text |
| JPT19HE2 | *a/α CDC24⁺/cdc24-4* | See text |
| JPT19α | *α cdc24-4* | See text |
| ts428 | *a cdc13-1 CDC24⁺* | 9, 21 |
| 428D1 | *a/α cdc13-1/cdc13-1 CDC24⁺/CDC24⁺* | 9 |
| ts281 | *a cdc16-1 CDC24⁺* | 18, 19, 21 |
| JPT19-428-D1 | *a/α cdc13-1/cdc13-1 cdc24-4/cdc24-4* | See text |
| JPT19-281-D1 | *a/α cdc16-1/cdc16-1 cdc24-4/cdc24-4* | See text |

* All strains were maintained as deep-frozen stocks as described previously (29).

‡ Strains 5011-D3 and 5011-D6 were constructed by L. Hartwell, University of Washington (personal communication), from mutant ts5011, an A364A derivative (21), using a procedure analogous to that used to construct A364A-D5 from A364A (16). 5011-D3, 5011-D6, and A364A-D5 all are auxotrophic for adenine and uracil.

## Staining with FITC-Con A

Cells were stained with FITC-Con A, washed free of excess lectin, and grown in new medium according to the procedures of Tkacz et al. (42) and Tkacz and Lampen (43), with the following modifications: both staining and wash solutions were buffered with 10 mM sodium phosphate, pH 7.2; the concentration of NaCl in these solutions was reduced to 0.15 M (44); for staining, ~$2 \times 10^7$ cells were suspended in 1.5 ml FITC-Con A solution (92 μg/ml) at the temperature at which the cells had been grown; for viewing, cells were applied to slides as wet mounts in distilled water.

Cells were examined with a Leitz Sm-Lux microscope equipped with epi-illumination, a 50 W mercury lamp, and Leitz filter system "H-broad band blue." Photographs were made with Kodak high-speed Ektachrome daylight film (ASA 200), at a camera ASA setting of 18/50, using a 60-s exposure.

## Other Methods

Procedures for fixing culture samples with formaldehyde, sonicating to disperse clumps, determining total cell numbers turbidometrically or with the Coulter Counter model ZB (Coulter Electronics Inc., Hialeah, Fla.), determining proportions of budded and unbudded cells, and measuring dry weights by filtration have been described previously (31). Determination of mutants' execution points by time-lapse photography was performed as described by Hartwell et al. (20). Methods for isolating small, bud scar-free cells (i.e., newborn cells that have never budded), and for observing the appearance and pattern of cell wall chitin deposition by fluorescence microscopy after staining with Calcofluor, have been described previously (39). Exponentially growing cultures were treated with hydroxyurea as described by Slater (38), with the final concentration of hydroxyurea altered to 0.2 M. The volumes of individual cells were calculated from phase-contrast photomicrographs as described previously (28).

## RESULTS

### Chitin Synthesis by cdc24-1 Cells

Fluorescence microscopy after staining with Calcofluor suggested that cells carrying the *cdc24-1* allele accumulate abundant chitin when grown at the restrictive temperature, although they cannot organize this chitin into normal rings (39). We have now confirmed this conclusion by direct chitin measurements on cell wall preparations. When quantitative carbohydrate measurements were made on cell ghosts (see Materials and Methods) prepared from strain 5011-D3 grown at permissive or restrictive temperature, it was found that cells grown for 8 h at 36°C accumulated at least threefold as much chitin per unit weight of total ghost carbohydrate as did cells grown at 23°C (Table II). In contrast, wild-type cells (strain A364A-D5) yielded approximately the same ratio of chitin to total ghost carbohydrate whether grown at 23° or 36°C (data not shown).[1]

TABLE II

*Chitin Synthesis by cdc24-1 Cells at Permissive and Restrictive Temperatures*

| Time | Temperature | Cellular dry weight* | Chitin in extracted ghosts‡ | |
|---|---|---|---|---|
| | | | exp 1 | exp 2 |
| *h* | *°C* | *mg* | | |
| 0 | 23 | 180 | 290/24 (1.0) | 290/42 (1.0) |
| 4 | 23 | 180 | 430/26 (1.4) | 370/42 (1.2) |
| 8 | 23 | 420 | 800/62 (1.1) | 960/110 (1.3) |
| 4 | 36 | 280 | 910/30 (2.5) | 560/53 (1.5) |
| 8 | 36 | 620 | 1900/48 (3.3) | 1800/83 (3.1) |

For each experiment, a single stationary-phase culture of strain 5011-D3 was divided into five 50-ml portions. One portion (0 h sample) was used directly for preparation of extracted cell ghosts and carbohydrate measurements (see Materials and Methods). Cells were collected from the remaining portions by centrifugation and inoculated into separate 500-ml portions of fresh medium at the desired temperature. At 4 and 8 h after inoculation, cells were collected by centrifugation (at 23°C) from one entire culture at each growth temperature and extracted and assayed as before.

* Values are expressed as total cellular dry weight per culture. The values given are from exp 2; values for exp 1 were somewhat smaller.

‡ Values are expressed as μg GlcNAc per culture/mg total ghost carbohydrate per culture. The ratios, normalized to the 0 h values, are shown in parentheses.

### Characterization of Additional cdc24 Mutants

ISOLATION AND GENETIC ANALYSIS OF *cdc24-4* MUTANTS: In the hopes (*a*) of learning more about the functions of the *CDC24* gene product by examining the morphogenetic consequences of various mutations in this gene, and (*b*) of identifying other gene products involved in budding, we undertook a search for additional mutants blocked specifically in budding (see Materials and Methods). Mutant JPT19 was detected during this search. It grew well at 24°C but at 36°C displayed first-cycle arrest (18), yielding a homogeneous population of large, unbudded cells (Fig. 1*a*). When JPT19 was backcrossed to C276-4B (see Materials and Methods), 22 of 24 tetrads from the first backcross and 19 of 19 tetrads from the second backcross showed a clear 2:2 segregation of temperature sensitivity, indicating that JPT19 carries a single, nuclear *ts* mutation. (The two odd tetrads, a 3 *ts*:1 wild type and a 1 *ts*:3 wild type, are presumably either "false tetrads" or tetrads in which gene conversion has occurred.) Because diploids heterozygous for this mutation grew and divided normally at 36°C (Fig. 2), the mutation is recessive.

JPT19 and JPT19α showed no detectable complementation with tester strains carrying *cdc24* mutations (strains 5011-D6-J2A, 5011-D6-J2D, and E187) but complemented well with testers carrying mutations in other *CDC* genes (including *CDC3, CDC4, CDC10, CDC11, CDC12, CDC14, CDC18, CDC27, CDC28, CDC31, CDC33,* and *CDC34*). Moreover, dissection of 20 tetrads from a cross of JPT19α with 5011-D6-J2D showed no wild-type segregants among 70 viable spores (10 asci with four viable spores, 10 asci with three viable spores). Thus, both complementation and linkage analysis show that JPT19 carries a mutant allele of gene *CDC24*; since this mutant allele was isolated independently of the three alleles reported by Hartwell et al. (21), it is designated *cdc24-4*.

Before detailed characterization of the effects of the *cdc24-4* mutation, JPT19 was backcrossed twice to wild type, and homozygous mutant diploids were constructed (see Materials and Methods).

[1] Similar results have been obtained by R. L. Roberts and E. Cabib, National Institutes of Health, Bethesda, Md. (personal communication), who measured chitin in cell walls isolated from cells broken with glass beads and not extracted with alkali, in an attempt to avoid possible losses of chitin during the preparation of cell ghosts. They found a sixfold higher ratio of chitin to total carbohydrate in *cdc24-1* cells grown at 36°C than in cells grown at 24°C.

GROWTH CHARACTERISTICS OF *cdc24-4* STRAINS: The growth and cell division of diploids homozygous for the *cdc24-4* allele were compared to those of homozygous wild-type and heterozygous diploids. The strains tested proliferated at similar rates at 24°C (Fig. 2*a* and *b*). At 36°C, the homozygous mutant strains continued to grow at nearly the same rate as wild type (Fig. 2*d*) but ceased to divide after an ~1.6-fold increase in cell number (Fig. 2*e*). The arrested population consisted almost exclusively of large, unbudded cells (Figs. 1*a* and 2*f*). The extent of increase in cell number at 36°C was slightly greater than the proportion of budded cells in the culture at the time of the shift to 36°C. This fact, taken together with the uniform termination of development in the unbudded phase, suggests an execution point (18, 30) for the *cdc24-4* allele that is shortly before the time of bud emergence, or at ~0.32 in the cell cycle in this strain. This value is consistent with those reported previously for *cdc24-1* and *cdc24-2* strains (21). That the execution point for *cdc24-4* strains is somewhat earlier than the time of bud emergence has been confirmed by time-lapse photography (42 of 51 unbudded cells arrested in the first cycle, i.e., remained unbudded, after a shift to 36°C).

The continued growth of the mutant cells after budding had ceased led to the production of very large cells (Fig. 1*a*); in the experiment of Fig. 2, the mean volume of unbudded cells (calculated from photomicrographs) and the mean dry weight per cell both increased about sixfold during a 7-h incubation at 36°C. These increases are similar to, but somewhat greater than, those reported by Johnston et al. (28) for the *cdc24-1* strain 5011-D6; in both experiments, the values reported are underestimates of the increases undergone by individual cells, as 10–40% of the cells have lysed by 7 h at 36°C.

PATTERNS OF CHITIN DEPOSITION BY *cdc24-4* AND *cdc24-3* STRAINS AT 36°C: As do *cdc24-1* cells (39), cells homozygous for the *cdc24-4* or *cdc24-3* allele displayed delocalized, and eventually more-or-less uniform, deposition of chitin in the enlarging cell walls during growth at 36°C (Fig. 1*b* and *c*; cf. the normal tight localization of Calcofluor-binding material to the bud-scar sites evident in Fig. 3). The disorganization of the pattern of chitin deposition was apparent within 2 h after a shift of stationary-phase cells to fresh medium at

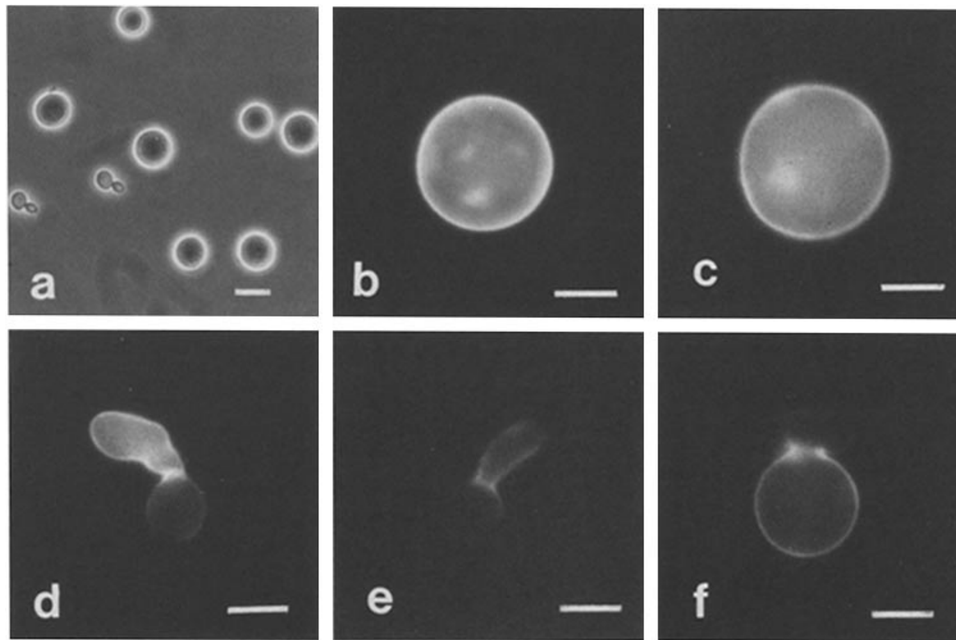


FIGURE 1 Phase-contrast and fluorescence photomicrographs of *cdc24-4* and *cdc24-3* cells grown at various temperatures. Samples were prepared and photographed as described in Materials and Methods. Fluorescence micrographs in this figure, in Fig. 3, and in Fig. 5 *a–e* were photographed with the same lenses and exposure times, and were printed at identical enlarger and exposure settings. (*a*) Phase-contrast photomicrograph of *cdc24-4* cells (strain JPT19) fixed during exponential growth at 24°C (the two smaller, budded cells), or 8 h after shifting an exponentially growing culture to 36°C (the larger, unbudded cells). Cells from the two cultures were mixed on the slide before photomicroscopy. Bar, 10 μm. (*b* and *c*) Fluorescence photomicrographs of (*b*) typical *cdc24-4* cell (strain JPT19H01) and (*c*) typical *cdc24-3* cell (strain E187-JD1) stained with Calcofluor 8 h after inoculation of stationary-phase cells into fresh medium at 36°C. The cells are large, unbudded, and display delocalized chitin deposition. (*d* and *e*) Fluorescence photomicrographs of (*d*) *cdc24-4* cell (strain JPT19H01) and (*e*) *cdc24-3* cell (strain E187-JD1) stained with Calcofluor 4 h after inoculating a population of small, bud-scar-free stationary-phase cells into fresh medium at 33°C. Buds are abnormally elongated and display delocalized chitin deposition. (*f*) Fluorescence photomicrograph of typical *cdc24-4* cell (strain JPT19H01) stained with Calcofluor after 6 h of growth at 33°C followed by 2 h of growth at 24°C. The mother cell is large and displays delocalized chitin deposition, while the bud is bipolar, apparently having grown in two directions from the mother:bud junction. Bars (*b–f*), 6 μm.

36°C. Shifting populations of bud-scar-free cells to fresh medium at 36°C made it possible to see that no normal rings were formed by *cdc24-3* or *cdc24-4* cells at this temperature.

## *Formation of Abnormal Buds by cdc24-4 and cdc24-3 Cells*

Strains homozygous for the *cdc24-4* or *cdc24-3* mutation were grown at temperatures intermediate between 24° and 36°C to explore the morphogenetic consequences of a presumed partial reduction in *CDC24* gene-product activity. With a *cdc24-4* strain, budding, bud-scar formation, and cell division seemed normal at 27° and 30°C. However, growth at 33°C yielded a strikingly different phenotype. 4 h after inoculating a population of small, bud-scar-free cells into fresh medium at 33°C, two distinct cell types were observed. Most cells were large, unbudded, and generally fluorescent after staining with Calcofluor, as would be observed after growth at 36°C. In contrast, 10-13% of the cells consisted of abnormally elongated buds, whose walls apparently contained abundant, delocalized chitin, attached to normal-sized mother cells whose walls bound little Calcofluor (Fig. 1 *d*). Similar results were obtained with a *cdc24-3* strain (Fig. 1 *e*).

Even stranger cell morphologies were observed when cells that had been grown for 4 to 6 h at 33° or 36°C were returned to 24°C for further growth. Such temperature shifts had no discernible effect on the morphologies of wild-type cells. Moreover, when *cdc24-1* cells were treated in this way, they typically produced normal-looking buds and bud scars within 15 min after the return to 24°C (39). *cdc24-4* Cells also budded soon after the return to 24°C, but shortly after emergence the buds could be seen to be abnormally elongated and not orthogonal to the surface of the mother cell. By 1 h after the temperature shift, bud shapes and abnormal patterns of chitin deposition at the mother-bud junction similar to those shown in Fig. 1 *f* were observed on nearly all cells. Such cells divided to yield normal-shaped mother cells and abnormal-shaped daughter cells, both of which then produced new buds, and chitin rings, of apparently normal shape.

## *Abnormal Bud-Site Selection by cdc24-3 and cdc24-4 Cells*

It is known that the pattern of budding sites in wild type *S. cerevisiae* is not random (1, 14, 23, 25, 41, 46). Moreover, there seems to be a systematic difference in this pattern between haploid strains (or strains homozygous at the mating-type locus) and strains heterozygous at the mating-type locus (including normal *MATa/MATα* diploid strains) (14, 25, 41). Typical patterns of budding sites, revealed as the patterns of bud scars, observed with haploid and *MATa/MATα* diploid wild-type strains in this study are shown in Fig. 3 *a–d*. The bud scars of multiparous cells are clustered at the poles of the cells,

generally at one pole on haploid cells and at both poles on diploid cells.

Cells carrying the *cdc24-1* allele also displayed a typical polar clustering of bud scars after growth at 24°C (Fig. 3 *e*). In contrast, haploids carrying the *cdc24-3* and *cdc24-4* alleles, and *MATa/MATα* diploids homozygous for these alleles, showed bud scars scattered apparently randomly over the surfaces of the cells after growth at 24°C (Fig. 3 *f–i*). To establish that the abnormal scar patterns observed at "permissive" temperature really were caused by the mutant *cdc24* alleles, strains JPT19α and E187Jα were crossed with C276-4A, and the segregation of the abnormal scar pattern was compared to that of temperature sensitivity. In addition, the scar patterns of several diploids homozygous for the *cdc24-4* allele were compared to those of two related diploids heterozygous for this allele. Because the scar patterns vary from cell to cell in all strains, there seemed to be some danger of subjectivity in scoring a particular strain as "typically showing scattered scars" or "typically showing clustered scars." Thus, all scoring of scar pattern was done by presenting the strains in random order to an observer ignorant of the genotypes of those strains at the *CDC24* locus. As a further check on the fidelity of scoring, several other wild-type and mutant strains were included at random places in the sequence of strains presented for scoring. The results (Table III) are fully consistent with the hypothesis that the abnormal pattern of bud-site localization is a recessive effect of the *cdc24-3* and *cdc24-4* mutant alleles. Further support for this conclusion was provided by the isolation of spontaneous revertants to temperature insensitivity of a haploid *cdc24-4* strain. Of the nine revertants isolated, two showed a full recovery of the normal polar scar pattern after growth at 24°C, and two showed a partial recovery of the normal pattern.

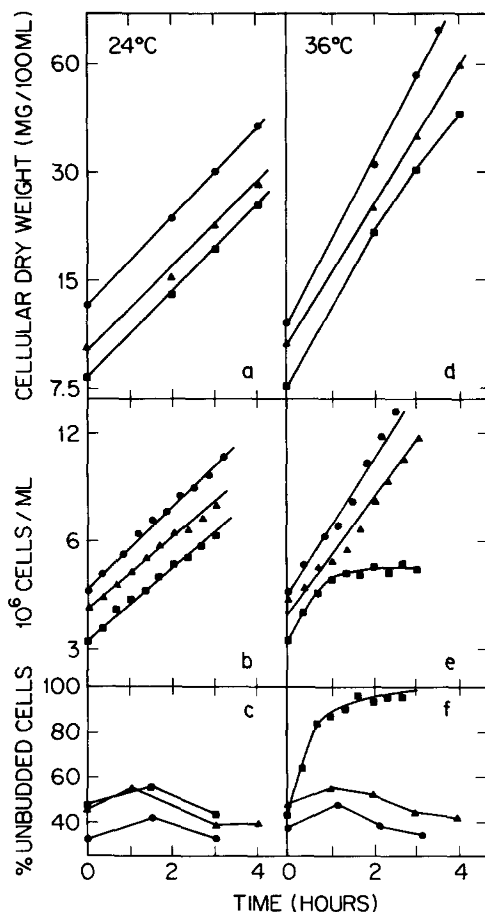


FIGURE 2 Growth characteristics of strains homozygous and heterozygous for the *cdc24-4* mutation. Exponentially growing precultures of strains JPT19H01, JPT19H02, JPT19HE1, and C276 were used to inoculate overnight cultures of each strain. When the overnight cultures reached late exponential phase (~4 × $10^7$ cells/ml), 50-ml samples of each culture were mixed with 450 ml portions of fresh medium equilibrated at 24° or 36°C. The diluted cultures were incubated further at 24°C (*a–c*) or 36°C (*d–f*), and sampled at intervals for cellular dry weight (*a* and *d*), cell number (*b* and *e*), and the proportion of budded cells (*c* and *f*). ●, C276; ▲, JPT19HE1; ■, JPT19H02. Data for JPT19H01 (not shown) were very similar to those for JPT19H02.

## *Delocalization of Mannan Deposition in cdc24-4 Cells*

The α-mannan of the yeast cell wall can be stained specifically with FITC-Con A (42). Tkacz and Lampen (43) used this procedure to demonstrate the localized deposition of new mannan in the cell wall at the tips of growing buds. We used staining with FITC-Con A to ask whether mannan deposition, like chitin deposition, is delocalized in *cdc24-4* mutant cells growing at restrictive temperature. First, we confirmed that when exponentially growing cells (wild-type cells growing at 24° or 36°C; *cdc24-4* cells growing at 24°C) were treated with FITC-Con A at the temperature at which the cells had been grown, each cell exhibited uniform fluorescence (Fig. 4 *a*). Here and in subsequent experiments with FITC-Con A, fluorescence of the cells was abolished by preincubation of the lectin with 0.1 M methyl-α-D-mannopyranoside (42). Moreover, when such stained cells were washed free of excess lectin and allowed to continue growth in lectin-free medium at the original temperature, localized deposition of new (hence unstained) mannan was observed (Fig. 4 *b*), as reported previously (43). The intensity of fluorescence exhibited by the mother cells did not decrease appreciably during growth in lectin-free medium for up to 3 h at 24°C (wild-type or mutant cells) or 36°C (wild-type cells) (Fig. 4 *c*; note also the unstained cells and buds formed during growth in lectin-free medium).

Very different results were obtained when *cdc24-4* cells were preincubated at 36°C for 1 h to allow budded cells to divide (Fig. 2 *f*), then treated with FITC-Con A and washed at 36°C, then allowed to continue growth in lectin-free medium at this same temperature. As before, staining left such cells uniformly and brightly fluorescent (Fig. 4 *d*). However, no discrete unstained patches or regions were observed on such cells as they grew in lectin-free medium. Instead, the intensity of fluorescence exhibited by the enlarging cells gradually diminished and became less uniform as incubation continued at 36°C (Fig. 4 *e* and *f*). These observations suggest that the deposition of new mannan in the expanding cell walls of the mutant cells was not localized.

## *Functions of the CDC24 Gene Product in Budded Cells*

In the course of the normal yeast cell cycle, there seem to be at least two periods of localized chitin synthesis, one shortly before bud emergence (24), and one at the time of cytokinesis and septum formation (6). When growing *cdc24* cells are shifted to 36°C, the arrested, unbudded cells synthesize abundant delocalized chitin (Table I; Fig. 1 *b* and *c*). We wished to ask whether inactivating the *CDC24* gene product (by shifting mutant cells to 36°C, see Discussion) would also induce delo-

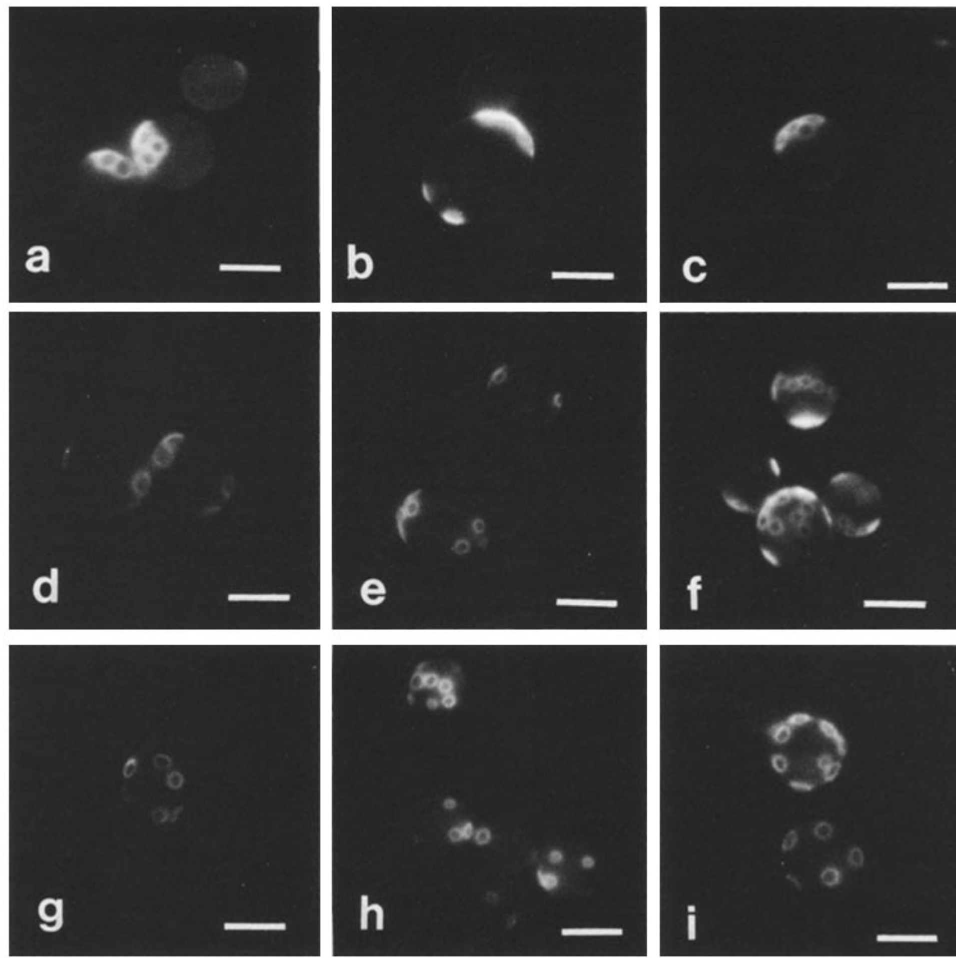


FIGURE 3 Fluorescence photomicrographs showing budding-site patterns of wild type and *cdc24* mutant cells grown at 24°C. In all cases, cultures were grown to stationary phase at 24°C; cells were then fixed, stained with Calcofluor, and photographed as described in Materials and Methods and in the legend to Fig. 1. (*a* and *c*) Wild-type haploid strains (*a*) A364A and (*c*) C276-4B, showing bud scars clustered at one pole of each cell. (*b* and *d*) Wild-type diploid strains (*b*) A364A-D5 and (*d*) C276, showing bud scars clustered at the two poles of each cell. (*e*) Mutant strain 5011-D3 (*cdc24-1/cdc24-1*), showing no deviation from the normal diploid pattern. (*f–i*) Mutant strains (*f*) E187 (*cdc24-3*), (*g*) E187-JD1 (*cdc24-3/cdc24-3*), (*h*) JPT19 (*cdc24-4*), and (*i*) JPT19H01 (*cdc24-4/cdc24-4*), all showing bud scars apparently positioned randomly over the cell surface. Bars, 6 μm.

TABLE III

*Dependence of Bud-Scar Pattern on the CDC24 Gene Product*

| Strain | Bud-Scar Pattern* |
|---|---|
| Cross of JPT19α with C276-4A: | |
| *cdc24-4* segregants | 10/10 with "scattered scars" |
| *CDC24*$^+$ segregants | 10/10 with "clustered scars" |
| Cross of E187Jα with C276-4A: | |
| *cdc24-3* segregants | 10/10 with "scattered scars" |
| *CDC24*$^+$ segregants | 10/10 with "clustered scars" |
| C276, C276-4A, 5011-D3, JPT19HE1, JPT19HE2 | All with "clustered scars"‡ |
| D187, JPT19H01, JPT19H02, JPT19H03, JPT19H04 | All with "scattered scars"§ |

* Calcofluor-stained samples of the 50 strains listed were presented in random order to an observer ignorant of the genotypes of the strains. The observer then characterized each strain as "typically showing clustered scars" (e.g., Fig. 3 *a–e*) or "typically showing scattered scars" (e.g., Fig. 3 *f–i*). See also notes ‡ and §.

‡ Among 500 multiparous C276 cells, 81% had scars clustered at both poles of the cell, 13% had scars clustered at one pole, and 6% had one or more scars not in the polar regions.

§ Among 600 multiparous JPT19H01 cells, 1% had scars clustered at both poles, 7% had scars clustered at one pole, and 92% had one or more scars not in the polar region.

calized chitin synthesis in cells at positions in the cell cycle at which chitin synthesis may not normally occur. To this end, we used the inhibitor hydroxyurea to block cells in DNA synthesis, and temperature-sensitive cell cycle mutations to block cells in nuclear division. These experiments also provided an opportunity to ask whether a normal level of *CDC24* gene-product activity is necessary for the normal selective growth of the bud, relative to the mother cell.

As a preliminary experiment, we shifted stationary-phase *cdc24* cells to 36°C without a change of medium, to ask whether extensive, delocalized chitin deposition is an invariable consequence of shifting the mutant cells to restrictive temperature. The result (Fig. 5 *a*) was clearly negative; in the nongrowing cells, no delocalized chitin deposition was observed even after 24 h at 36°C.

HYDROXYUREA ARREST: Hydroxyurea reversibly inhibits DNA synthesis, and hence nuclear division and cytokinesis, in *S. cerevisiae* (19, 38). Exponentially growing homozygous diploid *cdc24-4* cells were treated with 0.2 M hydroxyurea for 1.5 h at 24°C; half the culture was then shifted to 36°C. As expected from studies of wild-type cells (38), the cells left at 24°C continued to grow without dividing, with most of

the new growth being directed to the bud. Thus, by 7.5 h, the population contained 95% budded cells, with buds generally comparable in size to their mother cells (Fig. 5 *b*; Table IV). Similar results were obtained in a parallel experiment with a wild-type diploid (Table IV). When stained with Calcofluor, the arrested wild-type or mutant cells looked like normal budded cells: 80–90% showed only the localized fluorescence of the bud scars and mother-bud junctions (Fig. 5 *b*), while the remaining 10–20% showed some weak general fluorescence of mother cell wall, or bud cell wall, or both.

The mutant culture incubated at 36°C presented a very different picture. The proportion of budded cells declined from 75 to 38% during 6 h of incubation at 36°C (Table IV). This decline probably had two bases. First, some cells that were past the hydroxyurea execution point (18, 30) when the hydroxyurea was added were probably still budded, although about to divide, at the time of the shift to 36°C. Second, the fact that most of the observed decline in percent budded cells occurred between 3 and 6 h at 36°C (data not shown) indicates that cells were escaping from the hydroxyurea block under these conditions. In either case, the daughter cells produced by division encountered the *cdc24* block, and thus accumulated as large, unbudded cells, whose walls were generally fluorescent after staining with Calcofluor (*cf.* Fig. 1 *b* and *c*). Escape from the hydroxyurea block was also observed in the wild-type culture incubated at 36°C; this was reflected in a 20–30% increase in cell number, but the proportion of budded cells did not decrease because the cells that divided promptly budded again.

In contrast to the budded cells observed in the presence of hydroxyurea at 24°C, the mutant cells that remained budded at 36°C showed preferential growth of the mother cells rather than the buds (Fig. 5 *c* [compare the size of the mother cell to that shown in Fig. 5 *b*]; Table IV). The wild-type cells incubated at 36°C still showed preferential growth of the buds (Table IV), despite the fact that the measured mean bud volume/mother-cell volume ratio has almost certainly been reduced by the inclusion of cells that have escaped the hydroxyurea block, then divided, then produced new (and initially small) buds. In addition, the arrested mutant cells typically showed extensive, delocalized chitin deposition, primarily in the wall of the mother cell. After staining with Calcofluor, 77% of these budded cells showed mother cells markedly more fluorescent than

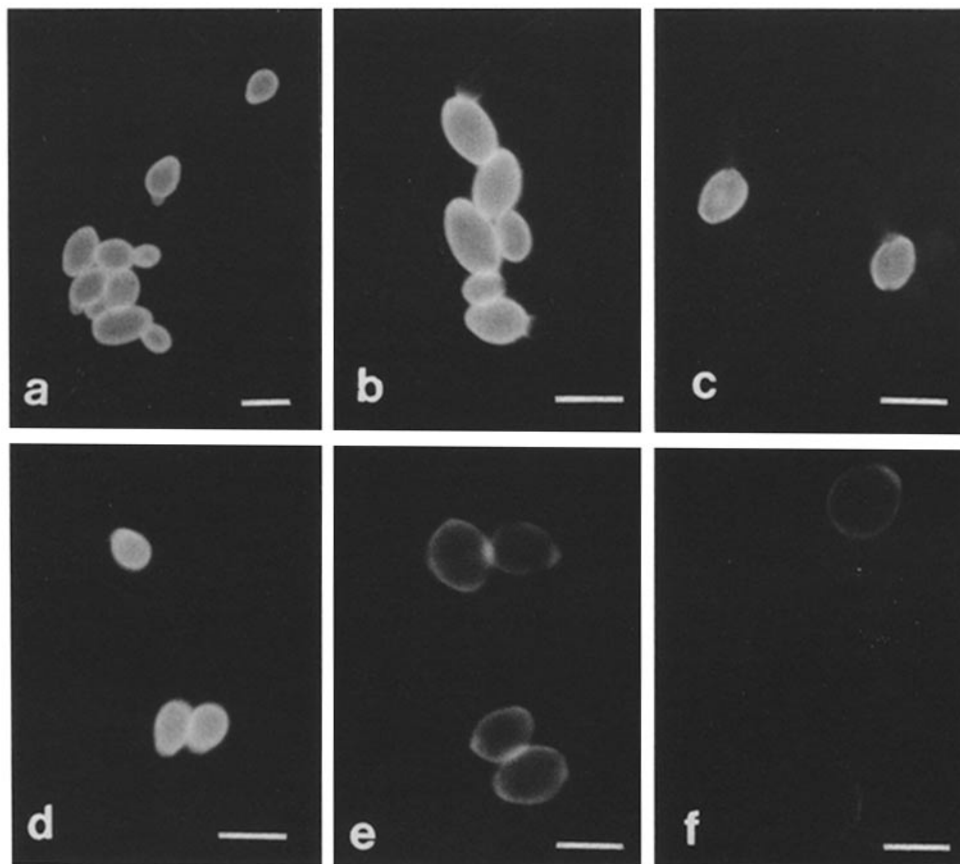


FIGURE 4 Fluorescence photomicrographs of wild-type (*a–c*) and *cdc24* mutant (*d–f*) cells stained with FITC-Con A to localize cell wall mannan (see Materials and Methods). Prints *a–f* were made at the same enlarger and exposure settings, but *a* was photographed using a × 40 objective rather than the × 54 objective used in *b–f*. (*a*) Wild-type diploid cells (strain C276) growing exponentially at 24°C, showing uniform fluorescence of mother cells and buds after staining with FITC-Con A at 24°C. Bar, 10 μm. (*b*) Cells from the same culture washed free of excess lectin and grown in lectin-free medium for 20 min at 24°C, showing the localized incorporation of new (hence unstained) mannan at the tips of growing buds. (*c*) Cells from the same culture after 3 h of growth in lectin-free medium at 24°C, showing the undiminished fluorescence of the originally stained cells. Newly formed cells and buds are nonfluorescent. (*d*) *cdc24* mutant cells (homozygous diploid strain JPT19H01) after 1 h of growth at 36°C, showing uniform fluorescence after staining with FITC-Con A at 36°C. (*e*) Cells from the same culture washed free of excess lectin and grown in lectin-free medium for 30 min at 36°C. In comparison to *d*, the cells are larger and the intensity of fluorescence is diminished, but no discrete dark patches are visible. (*f*) Cells from the same culture after 2 h of growth in lectin-free medium at 36°C. The cells are larger than those in *e*, and the intensity of fluorescence is further diminished. Bars (*b–f*), 10 μm.

their buds (Fig. 5*c*), while in the remaining cells no clear difference in general fluorescence was seen. In both groups, about one-third of the cells showed one or more bright patches of fluorescence in their buds.

DOUBLE-MUTANT STUDIES: Temperature-sensitive mutations in genes *CDC13* and *CDC16* prevent nuclear division and cytokinesis, but not DNA synthesis, at restrictive temperatures (9, 21). We constructed diploid strains homozygous for *cdc24-4* and either *cdc13-1* or *cdc16-1* (see Materials and Methods). When an exponentially growing population of the *cdc24 cdc13* double mutant was shifted from 24° to 36°C, the percent budded cells declined abruptly during the first 1.5 h (Table V). This was expected, because if neither the *cdc24-4* block nor the *cdc13-1* block is leaky (as seems the case from studies of the single mutants), the only cells in the population at time 0 that can terminate development as budded cells are those cells lying between the *cdc24-4* execution point and the *cdc13-1* execution point, or ~20–30% of the population. The further decline in percent budded cells between 1.5 and 7.5 h at 36°C (Table V) probably reflects primarily continued division by cells that were past the *cdc13-1* execution point at the time of the shift to 36°C, although it is also possible that some cells escape from the *cdc13* block. In any case, the cells that remained budded behaved as did the hydroxyurea-arrested

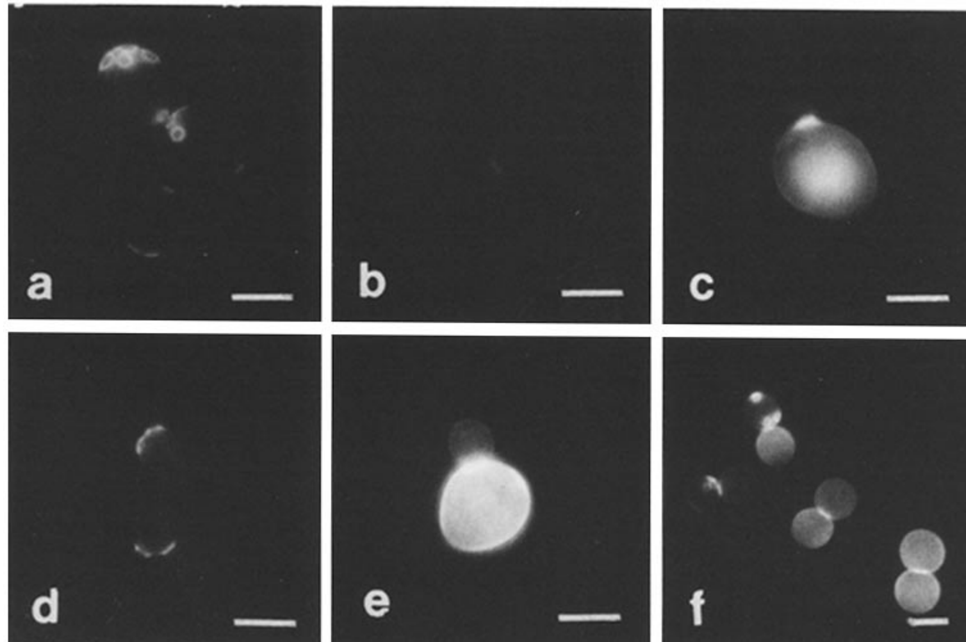


FIGURE 5 Fluorescence photomicrographs showing the effects of inactivating the *CDC24* gene product in cells blocked in DNA synthesis or in nuclear division (see text). Cells were fixed, stained with Calcofluor, and photographed as described in Materials and Methods and in the legend to Fig. 1. Bars (*a–e*), 6 μm. (*a*) Homozygous diploid *cdc24-1* cells (strain 5011-D3) grown to stationary phase at 24°C, then shifted to 36°C for 24 h without a change of growth medium. Similar results were obtained with homozygous diploid *cdc24-4* cells (strain JPT19H01). (*b*) Typical *cdc24-4* cell (strain JPT19H01) observed after treatment of an exponentially growing culture with 0.2 M hydroxyurea for 7.5 h at 24°C. (*c*) Typical *cdc24-4* cell (strain JPT19H01) observed after treatment of an exponentially growing culture with 0.2 M hydroxyurea for 1.5 h at 24°C followed by 6 h at 36°C. (*d*) Typical *cdc24 cdc13* double-mutant cells (strain JPT19-428-D1) observed in a culture growing exponentially at 24°C. (*e*) Typical *cdc24 cdc13* cell (strain JPT19-428-D1) observed 7.5 h after shift of an exponentially growing culture from 24° to 36°C. (*f*) Typical *cdc13* cells (strain 428D1) observed 7 h after shift of an exponentially growing population from 24°C to 36°C. These cells were photographed and printed at lower magnification than *a–e*, so that cell sizes and brightness are not directly comparable. Bar, 10 μm.

TABLE IV

*Growth of Mother Cells and of Buds in cdc24-4 and Wild-type Cells Exposed to Hydroxyurea*

| Time | Treatment | Temperature | Budded cells | No. of cells measured | Mother-cell volume* | Bud volume* | Volume ratio (bud/mother cell)‡ |
|---|---|---|---|---|---|---|---|
| *h* | | °C | *% of total* | | | | |
| 0 | None | 24 | 71 | 52 | — | — | 0.33 (0.38) |
| 1.5 | HU | 24 | 75 | 69 | 1.0 | 1.0 | 0.22 (0.25) |
| 7.5 | HU | 24 | 95 | 71 | 1.6 | 5.1 | 0.71 (0.68) |
| 7.5§ | HU | 36 | 38 | 57 | 3.2 | 1.5 | 0.12 (0.48) |

At time 0, hydroxyurea (HU) was added (final concentration = 0.2 M) to a culture of strain JPT19H01 growing exponentially at ~2 × $10^6$ cells/ml at 24°C. Immediately before the addition of HU, and again 1.5 h later, samples were removed from the culture for counts of budded cells, cell volume measurements, and staining with Calcofluor (see Fig. 5). Immediately after the second sampling, half the culture was shifted to 36°C, while half was incubated further at 24°C. 6 h later, both cultures were sampled as before. A parallel experiment was run with strain C276. All data in the table are from the experiment with JPT19H01 except those in parentheses in the right-hand column.

* The mean volume of mother cells and the mean volume of buds at each time of sampling are normalized to the values obtained at 1.5 h. Only budded cells were included in the analysis.

‡ For each budded cell, the ratio of bud volume to mother-cell volume was determined. The mean values of these ratios were then calculated for each time of sampling. Ratios in parentheses are from the experiment with strain C276.

§ 1.5 h in 0.2 M HU at 24°C plus 6 h in 0.2 M HU at 36°C.

TABLE V
*Growth of Mother Cells and of Buds in cdc24 cdc13 Double-Mutant Cells at 36°C*

| Time | Budded cells | No. of cells measured | Mother-cell volume* | Bud volume* | Volume ratio (bud/mother cell)‡ |
|---|---|---|---|---|---|
| *h* | *% of total* | | | | |
| 0 | 75 | 68 | 1.0 | 1.0 | 0.28 |
| 1.5 | 42 | 55 | 2.6 | 1.3 | 0.16 |
| 7.5 | 25 | 51 | 2.8 | 1.3 | 0.17 |

At time 0, a culture of strain JPT19-428-D1 growing exponentially at 24°C at ~$2 \times 10^6$ cells/ml was shifted to 36°C. Immediately before the shift, and at intervals thereafter, samples were removed from the culture for counts of budded cells, cell volume measurements, and staining with Calcofluor (see Fig. 5).

* The mean volume of mother cells and the mean volume of buds at each time of sampling are normalized to the values obtained at the time of the shift to 36°C. Only budded cells were included in the analysis.

‡ For each budded cell, the ratio of bud volume to mother-cell volume was determined. The mean values of these ratios were then calculated for each time of sampling.

*cdc24-4* cells described above. They showed preferential growth of the mother cells rather than the buds (Fig. 5 *e* [compare the size of the mother cell to those shown in Fig. 5 *d*]; Table V), in marked contrast to the behavior of cells mutant only in gene *CDC13*, in which the buds grow preferentially at 36°C until somewhat greater than the mother cells in volume (Fig. 5*f*; see also reference 9). In addition, delocalized chitin deposition was evident in the mother-cell walls but not in the bud walls. After staining with Calcofluor, 88% of the cells showed mothers markedly more fluorescent than their buds (Fig. 5 *e*), while in the remaining cells no clear difference in general fluorescence was seen. Control cells grown at 24°C showed only the normal localized fluorescence of bud scars and mother-bud junctions after staining with Calcofluor (Fig. 5 *d*). Curiously, cells mutant only in gene *CDC13* were not uniform in appearance when stained with Calcofluor after incubation at 36°C (Fig. 5*f*). In some cases, both mother and bud displayed general fluorescence, in other cases only the bud, and in other cases neither. However, no cells with a generally fluorescent mother and nonfluorescent bud were observed.

Results similar to those just described were also obtained with the *cdc24 cdc16* double mutant.

## DISCUSSION

The present and previous studies (19, 21, 39) have described yeast strains carrying four independently isolated temperature-sensitive mutant alleles of gene *CDC24*. The mutations have little effect on cell growth at restrictive temperatures (Fig. 2 *d*; see also reference 28), and do not prevent DNA synthesis and nuclear division (19, 21). However, in addition to blocking bud emergence, the mutations cause a variety of other morphological abnormalities. The abnormalities observed differ among the mutants; this illustrates the importance, in cellular and developmental studies, of comparing the phenotypes of several different mutant alleles of a gene of interest (36).

A constraint on the interpretation of our results is that we do not know the molecular nature of the *CDC24* gene product. Moreover, we cannot be certain whether the mutant alleles studied are temperature-sensitive for synthesis or for function of this gene product (18, 29, 30), or whether the lesions are tight or leaky at restrictive temperatures (29, 30). However, the following arguments suggest that the alleles studied are temperature-sensitive for function, and reasonably tight, at 36°C. First, the four mutations all are recessive in heterozygous diploids. Thus, their phenotype probably results from a loss in activity by the *CDC24* gene product, rather than from the acquisition of an abnormal activity. This conclusion is supported by the fact that the four independently isolated mutants (and 20 other independent *cdc24* mutants isolated in our laboratory but not yet studied in detail) all have essentially the same phenotype at 36°C, while extensive complementation screening of other *cdc* mutants has not revealed *cdc24* mutants with other phenotypes (21; our unpublished results). It would be surprising to isolate so many mutants in which the gene product had acquired an abnormal function while simple loss-of-function mutants remained cryptic, although the possibility cannot be ruled out that the latter mutants occur in high frequency among *ts* mutants that do not give *cdc* phenotypes. Second, the mutants studied have well-defined execution points (18, 30) just before bud emergence (Fig. 2 *e* and *f*, and associated text; reference 21). Although it is possible to hypothesize that the coincidence of execution points results from the mutants' all being temperature-sensitive for synthesis, or leaky to just the same degree (30), this is unlikely. Moreover, because a shift to 36°C just before budding can prevent budding, if the mutants are temperature-sensitive for synthesis, or leaky, then even a modest reduction in activity of the gene product must lead to a total inability to bud. This conclusion is difficult to reconcile with the formation of buds (albeit abnormal ones) by *cdc24-3* and *cdc24-4* strains at 33°C (Fig. 1 *d* and *e*). Thus, it seems likely that the *CDC24* gene product quickly becomes nonfunctional when the mutants in question are shifted to 36°C.

Whether or not this argument is correct, it seems possible to draw several conclusions about the roles of the *CDC24* gene product in the morphogenetic events of the *S. cerevisiae* cell cycle. First, the data of Fig. 3*f–i* and Table III suggest that this gene product functions in the selection of the budding site. Presumably, the *cdc24-3* and *cdc24-4* alleles lead either to a decrease or to an alteration in the activity of the *CDC24* gene product at the nominally permissive temperature of 24°C; given the nature of temperature-sensitive mutations (29), neither effect would be surprising.

Byers and Goetsch (3) have shown that microtubules normally extend from the spindle pole body in the nuclear envelope to the sites of incipient and growing buds, and have suggested that these microtubules are involved in directing vesicles containing cell-wall precursors to the appropriate site on the cell surface. Thus, the orientation of the spindle pole body, and hence of the extranuclear microtubules that emanate from it, may be the proximal determinant of bud position. However, given that the pattern of budding sites is not random (Fig. 3 *a–e*; references 1, 14, 23, 25, 41, 46), it is necessary to suppose also that the spindle pole body or extranuclear microtubules interact with some other system of positional information in the cell. If these hypotheses are correct, then the *CDC24* gene product might be involved in the function either of the spindle pole body and extranuclear microtubules, or of the other system of positional information with which these structures interact.

Second, the *CDC24* gene product seems to function in the organization of the budding site before bud emergence. When the activity of this gene product is reduced or eliminated (see arguments above) by incubating the mutant strains at 36°C,

normal chitin rings are not formed. Instead, the substantial amounts of chitin synthesized (Table II) are deposited in the cell wall in a delocalized fashion (Fig. 1 *b* and *c*; reference 39). The *CDC24* gene product may normally function directly in guiding chitin synthetase (33), or a chitin-synthetase-activating factor (6), to the appropriate sites in the cell wall. Alternatively, the *CDC24* gene product may normally function directly in the formation at the budding site of the ring of microfilaments described by Byers and Goetsch (4), or of the "collar-like circular zone" of the cell wall described by Seichertová et al. (35). Formation of the chitin ring may then be dependent on the prior formation of the appropriate precursor structure. Although the mechanism of formation of annular structures at the budding site is not clear, involvement of the extranuclear microtubules seems likely. Thus, the behavior of the mutants again seems consistent with an involvement of the *CDC24* gene product in the function of the extranuclear microtubules.

Third, the *CDC24* gene product seems to function in the localization of new growth to the budding site and the growing bud. It seems clear that the normal localization of chitin deposition to the base of the bud is disrupted by inactivation of the *CDC24* gene product, both in cells that have failed to bud (Fig. 1 *b* and *c*; Table II; reference 39), and in budded cells (Fig. 5 *c* and e). (See below for further discussion of the latter result.) Moreover, the Con A experiments (Fig. 4) suggest that mannan deposition is also delocalized in cells that have failed to bud. This conclusion is subject to the caveats (*a*) that we cannot be sure we would have detected the pattern of fluorescence resulting from localized incorporation of mannan in these cells; and (*b*) that we have no direct evidence that any mannan was actually being incorporated into the walls of such cells, although their retention of viability (39) makes it unlikely that cell wall structure is too grossly perturbed. Our conclusion is supported by the finding of Field and Schekman (12) that a *cdc24-1* strain is unable to achieve a normal localized secretion of acid phosphatase (a manno-protein) at 37°C.

The hypothesis that inactivation of the *CDC24* gene product leads to a general breakdown in the ability to localize new cell wall growth to the budding site is supported by the results of the hydroxyurea and double-mutant experiments (Fig. 5 *b*, *c*, *e*, and *f*; Tables IV and V). Although the quantitative aspects of these experiments are difficult to interpret in detail (because of both the progressive loss of budded cells from the arrested populations and the limited growth observed after 1.5 h in the double-mutant experiment), it seems clear that the overall effect of inactivating the *CDC24* gene product in budded cells is to substitute preferential growth of the mother cell for the normal preferential growth of the bud. Presumably, both new mannan and new glucan are incorporated in a delocalized fashion into the growing wall of the mother cell, although this has not been tested directly. These results also imply that the delocalized incorporation of new wall material in *cdc24* cells that have failed to bud cannot be interpreted simply as a consequence of the failure to organize the budding site properly. This view seems consistent with the finding of Field and Schekman (12) that a *cdc24-1* mutation prevents the localized tip growth normally observed in *a* cells responding to $\alpha$ factor. Thus, it seems that the *CDC24* gene product may function either in the system (perhaps the extranuclear microtubules) that guides vesicles with cell wall precursors to the appropriate site, or in the ability of the vesicles to interact normally with the guidance system.

These conclusions vitiate a previous interpretation of the behavior of the *cdc24* mutants. We had suggested (39) that the correlation between the failure to bud and the failure to form a chitin ring supported the hypothesis that the chitin ring, or an annular precursor structure, serves as an essential reinforcement for the region of the cell wall involved in budding. This hypothesis may well be correct, but it seems clear that the *cdc24* mutants, in which the localization of growth is directly disrupted, cannot be used to ask whether localized growth can lead to formation of a bud only if an annular reinforcement is present.

Fourth, the *CDC24* gene product seems to function in determining the pattern of growth within the growing bud, and hence the eventual shape of the daughter cell. When incubated at 33°C, some *cdc24-3* and *cdc24-4* cells formed abnormally elongated buds (Fig. 1 *d* and *e*), presumably reflecting a distortion of the normal pattern of growth in favor of tip growth. This distortion may result from a reduction in *CDC24* gene-product activity (recall that most cells in such populations were unbudded, the phenotype associated with an absence of *CDC24* gene-product activity), although the hydroxyurea and double-mutant experiments suggest that such a decrease should yield an excess of uniform growth. Thus, the abnormal buds may result from an abnormal function of the gene product, and hence of the intracellular guidance system. The delocalized chitin deposition in the walls of these buds may be a further indication of such an abnormal activity. It is also possible that the abnormal buds do not result directly from effects on the guidance system per se, but rather are consequences of some abnormality in the organization of the budding site. It may be relevant that several mutants with defects in cytokinesis have early execution points (17, 21), lack the microfilament ring that normally forms at the budding site (5), and form abnormally elongated buds at restrictive temperatures (17). Either abnormal function of the guidance system or an abnormal organization of the budding site seems a plausible explanation for the strange buds formed when *cdc24-3* or *cdc24-4* cells grown at restrictive temperatures were returned to 24°C (Fig. 1 *f*). In accord with the latter possibility, the chitin rings observed at the bases of such buds often appeared abnormal (Fig. 1 *f*).

Thus, the *CDC24* gene product seems to play a key role in many of the morphogenetic events of the *S. cerevisiae* cell cycle. Of the major events detectable at the cell surface, the only one in which this gene product is not directly implicated is cytokinesis. Although the mutant strains fail to divide at 36°C, the failure to form buds is probably an adequate explanation for the subsequent failure of cytokinesis (19).

Most of the abnormalities associated with *cdc24* mutations seem readily explained on the hypothesis that the gene product is involved in the function of the extranuclear microtubules. One observation that may be more difficult to explain on this basis is the synthesis of chitin by cells blocked in DNA synthesis (Fig. 5 *c*) or in nuclear division (Fig. 5 *e*), as it has not been clear that chitin synthesis normally occurs during these periods of the cell cycle (6, 8, 24). It is conceivable that inactivation of the *CDC24* gene product by the shift of temperature leads directly to an activation of chitin synthetase, but this hypothesis seems at odds both with the failure of nonproliferating mutant cells to synthesize chitin at 36°C (Fig. 5 *a*), and with the confinement of chitin deposition to the mother-cell portions of the budded cells (Fig. 5 *c* and *e*). Thus, it seems more likely that chitin is normally added to the ring (perhaps at a low rate) during the growth of the bud, and that the chitin that appears in the mother-cell wall (Fig. 5 *c* and *e*) represents merely a

delocalization of this normal chitin synthesis. The rather puzzling chitin accumulation in the walls of some *cdc13* single-mutant cells (Fig. 5*f*) may also represent a delocalization of chitin synthesis that normally occurs during the budded phase. The continued synthesis of chitin in mutant cells apparently arrested in nuclear division (8) is consistent with this interpretation.

It should be noted that both the abnormal chitin deposition and the selective growth of mother cells observed in the hydroxyurea and double-mutant experiments indicate that the *CDC24* gene product, although essential for bud emergence, also continues to function during the budded phase of the cell cycle. Thus, the execution point of a tight temperature-sensitive-for-function mutant does not necessarily represent the last point in the cell cycle at which the gene product functions (30), but only the last point at which such function is necessary for the subsequent division.

In summary, it seems clear that identification of the *CDC24* gene product, and similar studies of other mutants with defects in budding, will illuminate the mechanisms of morphogenesis during the yeast cell cycle.

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