CDC42 and *CDC43*, Two Additional Genes Involved in Budding and the Establishment of Cell Polarity in the Yeast *Saccharomyces cerevisiae*

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Abstract. Budding in the yeast *Saccharomyces cerevisiae* involves a polarized deposition of new cell surface material that is associated with a highly asymmetric disposition of the actin cytoskeleton. Mutants defective in gene *CDC24*, which are unable to bud or establish cell polarity, have been of great interest with regard to both the mechanisms of cellular morphogenesis and the mechanisms that coordinate cell-cycle events. To gain further insights into these problems, we sought additional mutants with defects in budding. We report here that temperature-sensitive mutants defective in genes *CDC42* and *CDC43*, like *cdc24* mutants, fail to bud but continue growth at restrictive temperature, and thus arrest as large unbudded cells. Nearly all of the arrested cells appear to begin nuclear cycles (as judged by the occurrence of DNA replication and the formation and elongation of mitotic spindles), and many go on to complete nuclear division, supporting the hypothesis that the events associated with budding and those of the nuclear cycle represent two independent pathways within the cell cycle. The arrested mutant cells display delocalized cell-surface deposition associated with a loss of asymmetry of the actin cytoskeleton. *CDC42* maps distal to the rDNA on chromosome XII and *CDC43* maps near *lys5* on chromosome VII.

An important class of questions about the cell division cycle concerns the dependency relationships or other coordinating mechanisms that ensure that cell-cycle events occur in an appropriate sequence. Such questions have been investigated in the yeast *Saccharomyces cerevisiae* by using mutations and inhibitors that block specific cell-cycle events (Hartwell et al., 1974; Pringle, 1978; Pringle and Hartwell, 1981; Moir and Botstein, 1982; Wood and Hartwell, 1982; Jacobs et al., 1988; Hartwell and Weinert, 1989). In this context, temperature-sensitive (Ts^-)[1] mutants defective in gene *CDC24* have been of great interest. The observation that such mutants can continue DNA synthesis and nuclear division while bud emergence is blocked (Hartwell et al., 1973, 1974) suggests that the nuclear cycle is not dependent on the cytoplasmic processes involved in budding. Conversely, experiments with a variety of other mutations and inhibitors suggest that bud emergence is not dependent on the nuclear cycle (Hartwell et al., 1974; Pringle and Hartwell, 1981). Thus, it appears that many of the events of the yeast cell cycle are organized into two parallel and independent pathways. Integration of these pathways appears to be achieved by their common dependence on a cell cycle–initiating event known as Start and by their common necessity for cytokinesis (Pringle and Hartwell, 1981).

A weakness of this model has been its pivotal dependence on the properties of the *cdc24* mutants. In particular, it is possible that in these mutants, a control that normally links the nuclear cycle to budding has broken down (Pringle, 1978; Hartwell and Weinert, 1989). Thus, it is important to determine whether the nuclear cycle can also continue in other mutants with defects in bud emergence.

CDC24 is also of great interest in the context of studies of cellular morphogenesis. Under restrictive conditions, Ts^- *cdc24* mutants continue to grow but display delocalized secretion and cell-surface deposition (Sloat and Pringle, 1978; Field and Schekman, 1980; Sloat et al., 1981; Roberts et al., 1983). *cdc24* mutants are also defective in zygote formation (Reid and Hartwell, 1977) and are unable to localize either actin or the *CDC3* and *CDC10* gene products to presumptive budding sites (Adams and Pringle, 1984; Kim, H. B., B. K. Haarer, and J. R. Pringle, unpublished results). Thus, the inability of *cdc24* mutants to bud appears to be just the most conspicuous manifestation of a general inability to establish or maintain cell polarity. Several lines of evidence suggest that the *CDC24* gene product may interact with Ca^{2+} (Ohya et al., 1986*a*, *b*; Miyamoto et al., 1987), an intriguing possibility given the evidence that gradients or cur-

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1. *Abbreviations used in this paper*: DAPI, 4',6-diamidino-2-phenylindole; Ts^-, temperature sensitive; Ts^+, not temperature sensitive.

rents of calcium may be involved in the establishment and maintenance of cell polarity in other cell types (Jaffe et al., 1975; Kropf and Quatrano, 1987; Schmid and Harold, 1988). Isolation of other *cdc24*-like mutants and characterization of the corresponding genes and gene products should help to clarify further the nature of the cellular machinery involved in the establishment of cell polarity and the localization of growth and secretion in yeast.

For these reasons, we undertook an intensive search for additional Ts⁻, *cdc24*-like mutants. We report here the isolation of several such mutants, defining genes *CDC42* and *CDC43*, and a characterization of the *cdc42* and *cdc43* mutant phenotypes. In the companion paper (Johnson and Pringle, 1990), we report a molecular characterization of *CDC42*, which appears likely to encode a protein that binds and hydrolyzes GTP while bound to the inner surface of the plasma membrane.

Materials and Methods

Reagents

Most reagents were obtained from standard commercial sources and were of the highest purity available. Ethyl methanesulfonate, 4',6-diamidino-2-phenylindole (DAPI), *Streptomyces griseus* chitinase (cat. No. C1525), and FITC-labeled rabbit antiserum to rat IgG were obtained from Sigma Chemical Co. (St. Louis, MO). Giemsa stain was obtained from Sigma Chemical Co. or Fisher Scientific Co. (Liuonia, MI). Rhodamine phalloidin was obtained from Molecular Probes Inc. (Eugene, OR), and [2-^{14}C]uracil was obtained from Amersham Corp. (Arlington Heights, IL). Rat mAb to yeast tubulin (YOL1/ 34; Kilmartin et al., 1982) was a gift from John Kilmartin (Medical Research Council Laboratory of Molecular Biology, Cambridge, England), and Calcofluor White M2R New was a gift from American Cyanamid (Bound Brook, NJ).

Strains, Media, and Growth Conditions

The primary wild-type and Ts⁻ cell-division-cycle (*cdc*) mutant strains used are listed in Table I. Other strains derived from these are described where appropriate below.

Solid media used were the rich medium YEPD and minimal medium containing appropriate supplements (Sherman et al., 1986). Liquid cultures were grown in the rich, glucose-containing medium YM-P (Lillie and Pringle, 1980). Cultures used for physiological and morphological studies were grown in Erlenmeyer flasks with rotary shaking. Ts⁻ mutants were grown at appropriate permissive and restrictive temperatures (see below).

Mutant Isolation and Genetic Methods

Standard genetic methods (Sherman et al., 1986) were used for all crosses and complementation analyses in this study. Scoring of the segregation of Ts⁻ mutations in crosses was found to be more reliable when very light inocula were used during replica plating. To this end, dilute suspensions of cells from the spore clones were made in the wells of a microtiter dish, and droplets were then transferred to the appropriate replica plates using a 48-prong wire inoculator.

Mutants were isolated from strains C276-4A and C276-4B after mutagenesis with ethyl methanesulfonate (Fink, 1970) to ~1% survival. To assure the independent origin of the mutants analyzed, aliquots of the mutagenized culture were diluted with fresh medium to form numerous small subcultures immediately after mutagenesis, and no more than one mutant with a given phenotype was analyzed from each subculture. Ts⁻ lethal mutants identified by replica plating at 23–24°C and 36–37°C were screened microscopically (Hartwell et al., 1973) to identify mutants of interest. In the early part of the mutant hunt, each Ts⁻ clone was examined after temperature shift of an exponential-phase liquid culture from 23 to 36°C. Subsequently, however, the initial microscopic screening was done on cells scraped from the 36–37°C replica plate. Promising candidates were then reexamined after temperature shift of a liquid culture. To avoid problems of evolution of the mutant strains, deep frozen stocks (Pringle, 1975) were prepared after minimal subculturing, as soon as promising mutants were identified.

To evaluate dominance and segregation patterns, each mutant of interest was crossed to C276-4A or C276-4B and Ts⁻ segregants were again crossed to C276-4A or C276-4B. Because of the ostensible isogenicity of C276-4A and C276-4B (Wilkinson and Pringle, 1974), these crosses can be regarded as backcrosses. After various numbers of such backcrosses, appropriate pairs of segregants were mated to produce the congenic, heterozygous and homozygous diploid strains used for detailed physiological and morphological analyses.

Other Methods

Procedures for fixing cells with formaldehyde, sonicating to disperse clumps, determining total cell numbers turbidometrically or with the Coulter counter (model ZB; Coulter Electronics, Hialeah, FL), examining cell morphologies and determining the proportions of unbudded cells by phase-contrast microscopy, and measuring dry weights by filtration have been described previously (Pringle and Mor, 1975; Pringle et al., 1989). The volumes of individual cells were calculated from phase-contrast micrographs as described previously (Johnston et al., 1977). Time-lapse observations were performed essentially as described by Hartwell et al. (1970). Staining of nuclei with Giemsa or DAPI was performed as described (Adams and Pringle, 1984; Pringle et al., 1989), and incorporation of [2-^{14}C]uracil into DNA and RNA was determined as described by Wilkinson and Pringle (1974), except that experiments were carried out in YM-P rather than minimal medium. Antitubulin immunofluorescence, staining of cell wall chitin with Calcofluor, and staining of actin with rhodamine phalloidin were performed as described by Pringle et al. (1989); cell wall digestion before immunofluorescence used a mixture of glusulase and zymolyase at 37°C. Cell ghosts were prepared by sequential extractions as described by Cabib and Bowers (1971); these extractions remove cytoplasm and cell wall mannoproteins, leaving a glucan-chitin shell that is susceptible to chitinase digestion. Chitinase digestion used 0.25 mg/ml chitinase in 25 mM sodium phosphate buffer, pH 6.3, for 1.5 h at 37°C. The digested ghosts were collected by centrifugation at 20,000 *g* for 5 min and washed once with water by centrifugation before staining with Calcofluor.

Results

Isolation and Genetic Analysis of Mutants Defective in Budding

Of ~5,000 Ts⁻ lethal mutants that were screened microscopically, 35 satisfied the two criteria (*a*) that they arrested predominantly as large, unbudded cells of normal (i.e., ellipsoidal) or more nearly spherical shape at restrictive temperature and (*b*) that they were sufficiently tightly arrested at restrictive temperature and genetically tractable to be analyzed successfully. Of these, 23 carried *cdc24* mutations and 1 carried a *cdc41* mutation, as judged by complementation and linkage analyses, and were not studied further. Seven additional mutants (JPT163, JPT198, JPTR37, JPTR114, JPTR407, JPTR415, and JPTR592) are the subject of this report. (The remaining mutants will be described elsewhere.)

When JPT163 was crossed to C276-4B, temperature sensitivity segregated 0:4 (Ts⁺ [not temperature sensitive]:Ts⁻), 1:3, and 2:2 in 2, 15, and 4 tetrads, respectively, and only a fraction of the Ts⁻ segregants accumulated large, unbudded cells at 36°C. Thus, JPT163 contains two unlinked Ts⁻ mutations. When appropriate segregants were backcrossed further to C276-4A and C276-4B, temperature sensitivity segregated consistently 2:2 through >60 tetrads, and all Ts⁻ segregants that were examined accumulated large, unbudded cells at 36°C, indicating that a single nuclear mutation was responsible for the phenotype of interest. As diploids heterozygous for this mutation grew essentially normally at 36°C (see also below), the mutation is recessive. Segregants from

Table I. Yeast Strains Used in this Study

Strain	Relevant genotype*	Source/reference
C276	*a/α* (Ts^+, prototrophic)	Wilkinson and Pringle, 1974
C276-4A	*a* (Ts^+, prototrophic)	Wilkinson and Pringle, 1974
C276-4B	*α* (Ts^+, prototrophic)	Wilkinson and Pringle, 1974
104	*a cdc3-1 cdc43-1*	Hartwell et al., 1973
104BD1-4A	*a cdc3-1*	This study‡
104BD1-4C	*α cdc43-1*	This study‡
248	*a cdc22-1*	Hartwell et al., 1973
SR621-4	*a cdc28-4*	Reed, 1980
212	*a cdc32-1*	Hartwell et al., 1973
E17	*a cdc33-1*	Reid and Hartwell, 1977
E189	*a cdc41-1*	Pringle and Hartwell, 1981
DYA150B	*α tra3-1*	G. Fink (Whitehead Institute, Cambridge, MA)§
STX88-1D	*a lys5 aro2*	YGSC‖
XS144-S19	*a lys5 aro2*	YGSC‖
5011-D3	*a/α cdc24-1/cdc24-1*	Sloat et al., 1981
E187-JD1	*a/α cdc24-3/cdc24-3*	Sloat et al., 1981
JPT19	*a cdc24-4*	Sloat et al., 1981
JPT19α	*α cdc24-4*	Sloat et al., 1981
JPT19H01	*a/α cdc24-4/cdc24-4*	Sloat et al., 1981
JPT19H02	*a/α cdc24-4/cdc24-4*	Sloat et al., 1981
JPT163	*a cdc42-1 tsx*	See text
JPT163BD2-10A	*a cdc42-1*	This study¶
JPT163BD2-2B	*α cdc42-1*	This study¶
JPT163-H02	*a/α cdc42-1/cdc42-1*	This study**
JPT163-H04	*a/α cdc42-1/cdc42-1*	This study**
JPT198	*a cdc43-2*	See text
JPT198BD2-2B	*α cdc43-2*	This study‡‡
JPT198-H03	*a/α cdc43-2/cdc43-2*	This study§§
JPT198-H06	*a/α cdc43-2/cdc43-2*	This study§§
JPTR37	*α cdc43-3*	See text
JPTR114	*α cdc43-4*	See text
JPTR415	*α cdc43-5*	See text
JPTR592	*α cdc43-6*	See text
JPTR407	*α cdc43-7*	See text

Additional strains used in this study are described in Table II or in the text. Primary stocks of all strains were stored at −70°C (Pringle, 1975).
* Nutritional markers that were not used in this study are not listed.
‡ Segregant from a cross of 104 to C276-4B (see text).
§ See Pringle and Hartwell (1981). The *tra3-1* mutation is now known as *gcd1-101* (Hinnebusch, 1988).
‖ Yeast Genetics Stock Center, Berkeley, CA.
¶ Segregant from the second backcross of JPT163 to C276-4B.
** Diploid formed by mating segregants from the second (JPT163-H02) or seventh (JPT163-H04) backcross of JPT163 to C276-4B and C276-4A.
‡‡ Segregant from the second backcross of JPT198 to C276-4B.
§§ Diploid formed by mating segegants from the second (JPT198-H03) or fifth (JPT198-H06) backcross of JPT198 to C276-4B and C276-4A.

the second to sixth backcrosses were used in the analyses described below.

When JPT198, JPTR37, JPTR114, JPTR415, and JPTR592 were backcrossed to C276-4A and C276-4B, the heterozygous diploids grew essentially normally at 36°C (see also below), and temperature sensitivity segregated consistently 2:2 in ≥15 total tetrads from at least three successive backcrosses in each case. All Ts^- segregants that were examined accumulated large, unbudded cells at 36°C. Thus, each of these mutants harbors a single, recessive, nuclear mutation that is responsible for the phenotype of interest. In contrast, when JPTR407 was crossed to C276-4A, a mixture of 4:0 (Ts^+:Ts^-), 3:1, and 2:2 tetrads was obtained; when a Ts^- segregant was further backcrossed, this segregation pattern was repeated. Thus, at least two mutations are responsible for the Ts^- phenotype of JPTR407.

All seven of the new mutants complemented well with *cdc24* and other previously known Ts^- mutants showing accumulation of unbudded cells at restrictive temperature, and JPT163-derived strains complemented well with JPT198, JPTR37, JPTR114, JPTR415, JPTR592, and JPTR407 (Fig. 1 and data not shown). However, the last five mutants all failed to complement testers derived from JPT198 (Fig. 1 and data not shown; weak complementation was sometimes observed between JPTR407 and the JPT198-derived strains). In addition, *MATα* strains derived from JPT198 failed to complement strain 104, which is the original *cdc3* mutant but also contains a second, unlinked Ts^- mutation (Hartwell et al., 1973). When the two mutations were separated by picking appropriate segregants from a cross of 104 to C276-4B, the *cdc3-1* strains (which accumulated multibudded, multinucleate cells at restrictive temperature) complemented well with the JPT198-derived strains (Fig. 1), whereas the other Ts^- segregants (which produced morphologically heterogeneous populations at restrictive temperature) failed to complement the JPT198-derived strains. Thus,

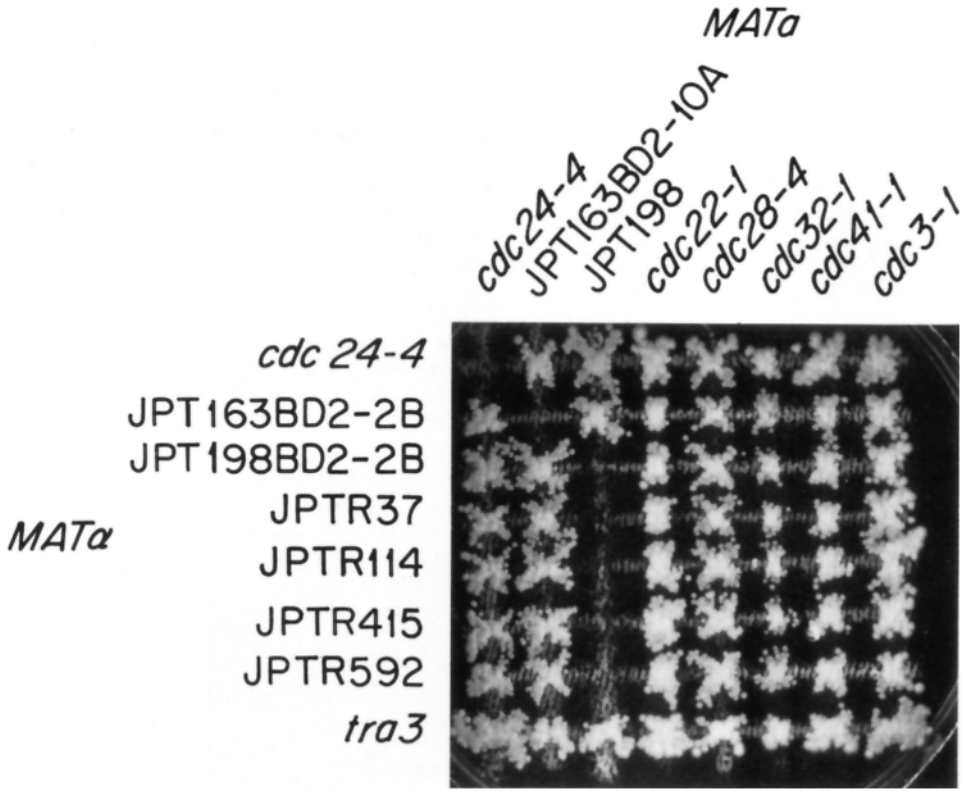

Figure 1. Complementation tests on the new and previously identified mutants. The testers are indicated by allele designations (see Table I for strain names); they represent previously known mutants that arrest as large unbudded cells (Hartwell et al., 1973; Pringle and Hartwell, 1981) plus the *cdc3-1* allele present in strain 104 (Hartwell et al., 1973; see text). The other strains define the new genes *CDC42* (JPT163 derivatives) and *CDC43* (JPT198, JPTR37, JPTR114, JPTR415, and JPTR592).

the complementation data suggested that the JPT163-derived strains carry an allele (*cdc42-1*) of a new gene, designated *CDC42*, whereas 104, JPT198, JPTR37, JPTR114, JPTR415, and JPTR592 carry alleles (*cdc43-1* through *cdc43-6*, respectively) of another new gene, designated *CDC43*. Although it has not been investigated further, the failure of the *cdc43-1* strains to share the morphological phenotype of the other *cdc43* mutants seems likely to reflect either the leakiness of the Ts^- mutation or the presence of background mutations that arose during the very heavy mutagenesis used (Hartwell, 1967) when strain 104 was isolated. Although it has also not been investigated further, JPTR407 presumably carries a weakly Ts^- allele of *CDC43* (designated *cdc43-7*) together with an additional mutation(s) that enhances its temperature sensitivity.

The interpretation of the complementation data was supported by the results of linkage analyses. Dissection of 42 tetrads from a cross between strains JPT163BD2-10A (*cdc42-1*) and JPT198BD2-2B (*cdc43-2*) yielded 119 viable spores (at 23°C), of which 44 were Ts^+, showing that the two Ts^- mutations are unlinked. Interestingly, complementation analyses showed that the remaining viable spores contained either the *cdc42-1* mutation or the *cdc43-2* mutation but not both, implying that the double mutant spores were inviable at 23°C (see Discussion). When Ts^- strains derived from 104BD1-4C, JPTR114, JPTR415, or JPTR592 were crossed with JPT198-derived strains, no Ts^+ segregants were obtained ($\geq$13 tetrads and $\geq$35 viable spores per cross). In contrast, a cross between strains carrying the Ts^- mutations from JPT198 and JPTR37 yielded at least 5 apparently Ts^+ segregants (plus several others that were $Ts^{+/-}$) among 88 viable spores from 22 tetrads. Although the reason for this high frequency of apparent recombination or reversion involving the *cdc43-2* and *cdc43-3* mutations is not known (see Discussion), the allelism of these mutations seems solidly established by (*a*) their essentially identical phenotypes, (*b*) their noncomplementation, and (*c*) the fact that each mutation showed tight linkage to *lys5* (next paragraph) in crosses in which each Ts^- mutation showed a clear 2:2 segregation.

The novelty of genes *CDC42* and *CDC43* was further established by determining their map positions. *CDC42* is on the right arm of chromosome XII, distal to the rDNA (Johnson et al., 1987). *CDC43* maps near *LYS5* on the left arm of chromosome VII. In a cross between a *cdc43-3* strain and XS144-S19, 16 of 16 tetrads were parental ditype for *cdc43* and *lys5*. When a *cdc43-2* strain was crossed to XS144-S19 and STX88-1D, 5 of 634 viable spores (from 225 tetrads) were recombinant for *cdc43* and *lys5*; of these, only one was recombinant between *lys5* and the centromere-proximal marker *aro2*. Thus, the gene order appears to be *CEN7-ARO2-LYS5-CDC43*, with *CDC43* ~1 cM from *LYS5*. Most other known genes whose mutants have phenotypes even roughly similar to those of *cdc42* and *cdc43* have previously been mapped to other locations (Hartwell et al., 1973; Mortimer et al., 1989). For the unmapped genes, the complementation analyses cited above were supplemented by tetrad analyses where desirable and practicable. Numerous Ts^+ recombinants were observed when *cdc33* or *cdc41* strains were crossed to *cdc42* or *cdc43* strains, supporting the distinctness of these genes. Attempts to do tetrad analysis with *cdc22* or *cdc32* strains were precluded by the same problems noted previously (Hartwell et al., 1973; Pringle and Hartwell, 1981), and the distinctness of the *cdc1* phenotype (Hartwell et al., 1973, 1974) seemed sufficient to obviate further analyses.

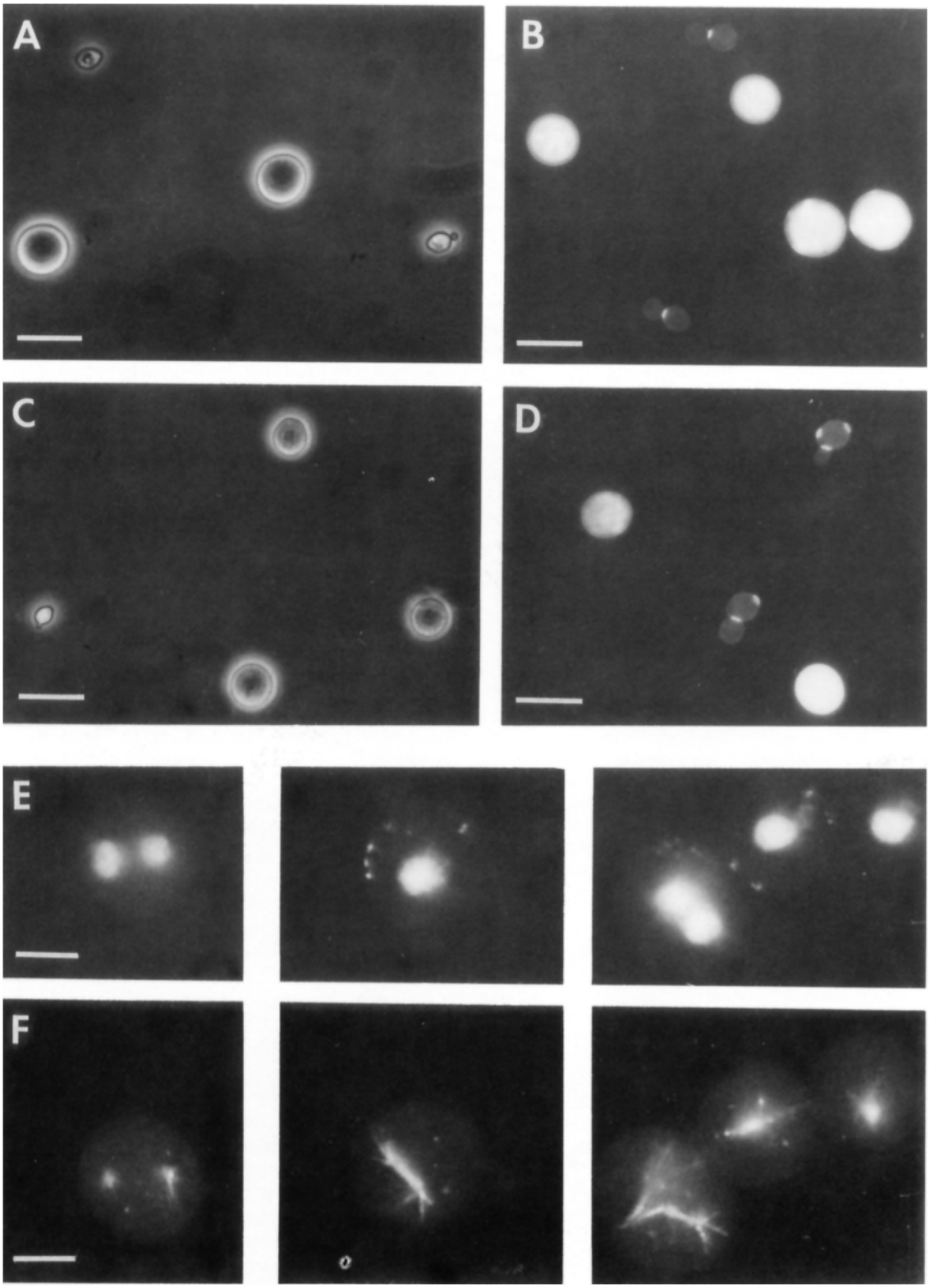

Figure 2. Morphological characterization of *cdc42* and *cdc43* mutant strains. (*A–D*) Cells of the *cdc42-1* strain JPT163-HO2 (*A* and *B*) and the *cdc43-2* strain JPT198-HO3 (*C* and *D*) were fixed during exponential growth at 19°C (permissive temperature) or 6–7 h after shift to 36.5°C (restrictive temperature; cf. Fig. 3). For each strain, cells from the 19 and 36.5°C cultures were mixed and observed by phase-contrast microscopy (*A* and *C*) or stained with Calcofluor and observed by fluorescence microscopy (*B* and *D*). The smaller cells from the 19°C cultures and larger cells from the 36.5°C cultures are readily distinguished. (*E* and *F*) Cells of strain JPT163-HO2 growing exponentially at 19°C were shifted to 36°C for 6 h, then fixed and stained both with DAPI to visualize nuclear DNA (*E*) and with antitubulin antibodies to visualize microtubules (*F*), as described in Materials and Methods. (*E* and *F* show the same cells.) Bars (*A–D*) 15 μm; (*E* and *F*) 4 μm.

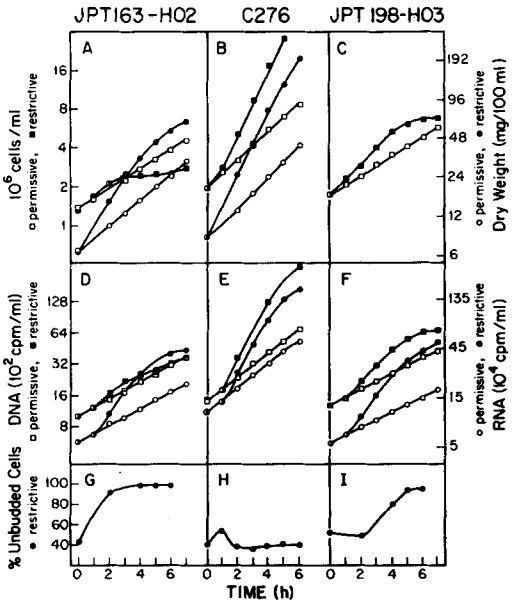

Figure 3. Growth characteristics of *cdc42* and *cdc43* mutant strains in relation to wild type at permissive (20°C) and restrictive (36°C) temperatures. Data for the *cdc42* strain JPT163-HO2 (*A*, *D*, and *G*), the wild-type strain C276 (*B*, *E*, and *H*), and the *cdc43* strain JPT198-HO3 (*C*, *F*, and *I*) are shown; data for strains JPT163-HO4 and JPT198-HO6 (see Table I) were very similar to those for JPT163-HO2 and JPT198-HO3, respectively. Cultures growing exponentially at 20°C were split into two portions, one of which received 0.9 μCi/ml of [2-^{14}C]uracil. After 12-h further incubation at 20°C, each labeled and unlabeled culture (at 1–2 × 10^6 cells/ml) was split into two portions, one of which was incubated further at 20°C (*open symbols*) while the other was shifted to 36°C (*solid symbols*). Samples removed from the unlabeled cultures were used to determine cell numbers with the Coulter counter (*squares*, *A–C*) and the proportions of unbudded cells (*G–I*), whereas samples from the labeled cultures were used to determine incorporation into DNA and RNA (*squares* and *circles* respectively, *D–F*). Dry weight data for strains JPT163-HO2 and C276 (*circles*, *A* and *B*) were collected in a separate experiment involving a shift from 22 to 36°C; data for cell numbers and percents unbudded cells in this experiment were very similar to those shown here, except that growth was somewhat faster at 22°C than at 20°C, as expected.

Growth Characteristics of cdc42 and cdc43 Strains

Detailed characterization of the mutant phenotypes was conducted using homozygous and heterozygous diploids constructed after backcrossing several times to C276-4A and C276-4B (see Materials and Methods). We first examined the mutants after shifting cultures from 23–24°C to 36–37°C (the temperatures used in the original mutant isolations). Under these conditions, both JPT163-HO2 and JPT198-HO3 increased ~1.9-fold in cell number before arresting as unbudded cells. Thus, both strains exhibited "first-cycle arrest" (Hartwell, 1974) with apparent "execution points" (Hartwell et al., 1970, 1973) between 0.05 and 0.1 of the cell cycle. This interpretation was consistent with the results of time-lapse observations (data not shown), which suggested execution points partway through the unbudded phase (but also indicated some leakiness of the mutants, from the presence of cells that apparently divided and budded again at 36–37°C).

However, closer examination of the cultures revealed that this interpretation was misleading, especially for *cdc43*, because 23–24°C was in fact only semipermissive for the mutants. For several *cdc43-2* strains, the generation time at 24°C was significantly longer than that of wild-type or heterozygous mutant strains, and microscopic examination revealed that up to 30% of the cells were distinctly abnormal morphologically. Presumably, these abnormalities reflected an abnormally low activity of the *CDC43* gene product during growth of the mutant at 23–24°C. Abnormal cells were also apparent in cultures of *cdc42-1* strains grown at 24°C, although the generation time was not significantly different from that of control strains. Reducing the permissive temperature to 18–20°C allowed *cdc43-2* strains to grow with a generation time not grossly different from that of wild type at this temperature, and reduced the proportion of morphologically abnormal cells to 2–10% (for various strains on various occasions). Shift of mutant cultures from 18–20°C to 36°C did not produce results substantially different from those of a 24-to-36°C shift for the *cdc42* strains, but the *cdc43-2* strains now underwent an approximate fourfold increase in cell number before arresting (see below). Strains carrying other *cdc43* alleles also underwent an increase in cell number of fourfold or more upon shift from permissive (20–24°C) to restrictive temperatures. These results illustrate the difficulty of extracting useful information from execution point data (see Discussion).

When cultures of *cdc42-1* and *cdc43-2* strains were shifted from 20 to 36°C, they underwent ~1.9-fold and about fourfold increases in cell number, respectively, before arresting uniformly as unbudded cells (Fig. 2, *A* and *C*; Fig. 3, *A*, *C*, *G*, and *I*). The slow increases in the apparent cell numbers at the later times in these experiments may reflect some leakiness of the mutants (see above), inadvertent counting (with the Coulter counter) of particles released by cell lysis (which was apparent by microscopic examination in the cultures that had been 5–7 h at 36°C), or both. Cells heterozygous for the *cdc42* or *cdc43* mutations grew essentially as well as wild type at 36°C in such experiments (data not shown), confirming the recessiveness of the mutations. As growth (measured as cell volume, dry weight, or total RNA) of the mutant cells continued for some time after cell proliferation had ceased (Fig. 2, *A* and *C*; Fig. 3, *A*, *D*, and *F*), the cells became abnormally large. In the experiment of Fig. 3, the volumes of individual unbudded cells were determined during growth at 20°C and after 7 h at 36°C (n = 85–100 for each sample). The mean volume per cell increased ~12-fold for *cdc42* and about fivefold for *cdc43*. Similarly, the mean dry weight per cell increased about fourfold for *cdc42* (Fig. 3 *A*) and about threefold for *cdc43* (in a 24-to-36°C shift experiment; data not shown). (It should be noted that these data on mean dry weight per cell underestimate the increases undergone by individual arrested cells, for several reasons. First, the starting population includes budded as well as unbudded cells. Second, the cell lysis, noted above, presumably causes a loss of measurable dry weight in the samples from the later time points. Third, for *cdc43* particularly, the use of a semipermissive temperature in these experiments meant that the cells were already larger than normal at the time of the shift to 36°C.) In similar experiments, *cdc43-3* strains yielded data very similar to those shown here for *cdc43-2*.

Table II. Formation of Multinucleate Cells by Mutants Defective in Budding

Mutant allele	Strain*	Number of backcrosses*	Number of cell bodies counted	Percent of cell bodies with		
				One nucleus	Two nuclei	Three or more
cdc24-1	5011-D3	NA‡	417	27	34	39
	5011-JH01	5	569	28	65	7
	5011-JH03	5	412	43	56	1
cdc24-3	E187-JD1	1	278	71	27	2
	E187-JH01	7	582	26	62	12
cdc24-4	JPT19H01	2	265	43	57	0
	JPT19H02	2	548	82	18	0
	JPT19H05	5	288	29	63	8
cdc42-1	JPT163-H01	2	598	47	53	0
	JPT163-H02	2	955	79	21	0
	JPT163-H04	7	1,501	40	56	4
cdc43-2	JPT198-H03	2	1,381	76	24	0
	JPT198-H04	5	633	83	17	0
cdc43-3	JPTR37-H04	3	400	66	33	1
cdc43-4	JPTR114-H01	5	729	93	7	0
cdc43-5	JPTR415-H01	3	241	78	22	0
cdc43-6	JPTR592-H01	3	310	68	32	0

Cultures of each strain growing exponentially at 22–24°C were adjusted to ~2–3 × 10^6 cells/ml and shifted to 36°C. After 7–8 h at 36°C, cells were fixed and their nuclei stained with Giemsa (see Materials and Methods), and counts were made of the number of nuclei per cell. Because it was not always possible to distinguish a budded cell from a pair of adjacent unbudded cells, counts were done on the basis of "cell bodies"; thus, for example, a budded cell with a nucleus in the mother and another in the bud would have been counted as two cell bodies, each with one nucleus. Cell bodies containing no detectable nuclei (e.g., immature buds, or unstained or lysed cells) were excluded from the counts.

* Strains not described in Table I were constructed by backcrossing the appropriate original haploid mutant (Hartwell et al., 1973; Sloat et al., 1981; or this study) the indicated number of times into the C276-4A/C276-4B background, then constructing homozygous diploids by mating appropriate pairs of segregants.

‡ 5011-D3 is a diploid derived from mutant 5011 (Hartwell et al., 1973) without backcrossing into the C276-4A/C276-4B background (Sloat et al., 1981).

Continuation of the Nuclear Cycle in cdc24, cdc42, and cdc43 Mutants

We used several assays to examine the extent to which the nuclear cycle could continue in mutants that were blocked in bud emergence. One assay was to stain the arrested cells with DAPI or Giemsa and determine the proportions that contained two or more nuclei (or, at least, two or more separated sets of chromosomes: note that these DNA stains do not necessarily reveal the status of the nuclear envelope [Pringle et al., 1989]). As reported previously (Hartwell et al., 1973, 1974), the *cdc24-1* strain 5011-D3 arrested with most cells containing two or more nuclei (Table II). Surprisingly, however, the *cdc24-3* strain E187-JD1 and the *cdc24-4* strains JPT19-HO1 and JPT19-HO2 were much less successful in continuing nuclear division in the arrested cells (Table II). To determine whether these differences were due to the *cdc24* alleles themselves or to the genetic backgrounds of the strains (the different *cdc24* mutants were all of different origins and were isolated after heavy mutagenesis), we backcrossed all three *cdc24* alleles extensively into the C276-4A/C276-4B genetic background and tested several of the resulting diploids for each allele. As the representative data in Table II show, these strains were rather similar in their behavior, but none was as successful in completing multiple nuclear cycles as 5011-D3. Similarly, strains harboring *cdc42* or *cdc43* mutations also arrested development with significant numbers of binucleate and (in some cases) multinucleate cells, but none was as successful in completing nuclear cycles as 5011-D3 (see representative data in Table II). Thus, the results suggested that the high proportion of binucleate and multinucleate cells achieved by 5011-D3 was a special property of this strain, and left some uncertainty as to the normal dependence or independence of the nuclear cycle upon bud emergence.

To shed more light on this issue, we asked if cells that failed to complete nuclear division could at least begin the nuclear cycle, as judged by their ability to replicate DNA and to form mitotic spindles. (Previous analyses have indicated that DNA replication and spindle formation are themselves independent events [Pringle and Hartwell, 1981].) During

Table III. Formation and Elongation of Mitotic Spindles by Arrested cdc42 Mutant Cells

Time at 36°C	Number of cells displaying			
	One nucleus, spindle-pole body staining only*	One nucleus, short spindle	One nucleus, longer spindle	Two nuclei
h				
4	28	48	19	0
5	17	17	53	8‡
6	6	20	62	6§

Cells of strain JPT163-H02 growing exponentially at 19°C were shifted to 36°C for various times, then fixed and stained both with DAPI to visualize nuclear DNA and with tubulin antibodies to visualize microtubules, as described in Materials and Methods (cf. Fig. 2, *E* and *F*).

* This staining pattern could represent either a single spindle-pole body (SPB) or a duplicated, but unseparated, pair of SPBs.

‡ In five cases, each nucleus displayed SPB staining only (compare with previous footnote); in three cases, each nucleus displayed a spindle.

§ In two cases, each nucleus displayed SPB staining only (compare with first footnote); in four cases, each nucleus displayed a spindle.

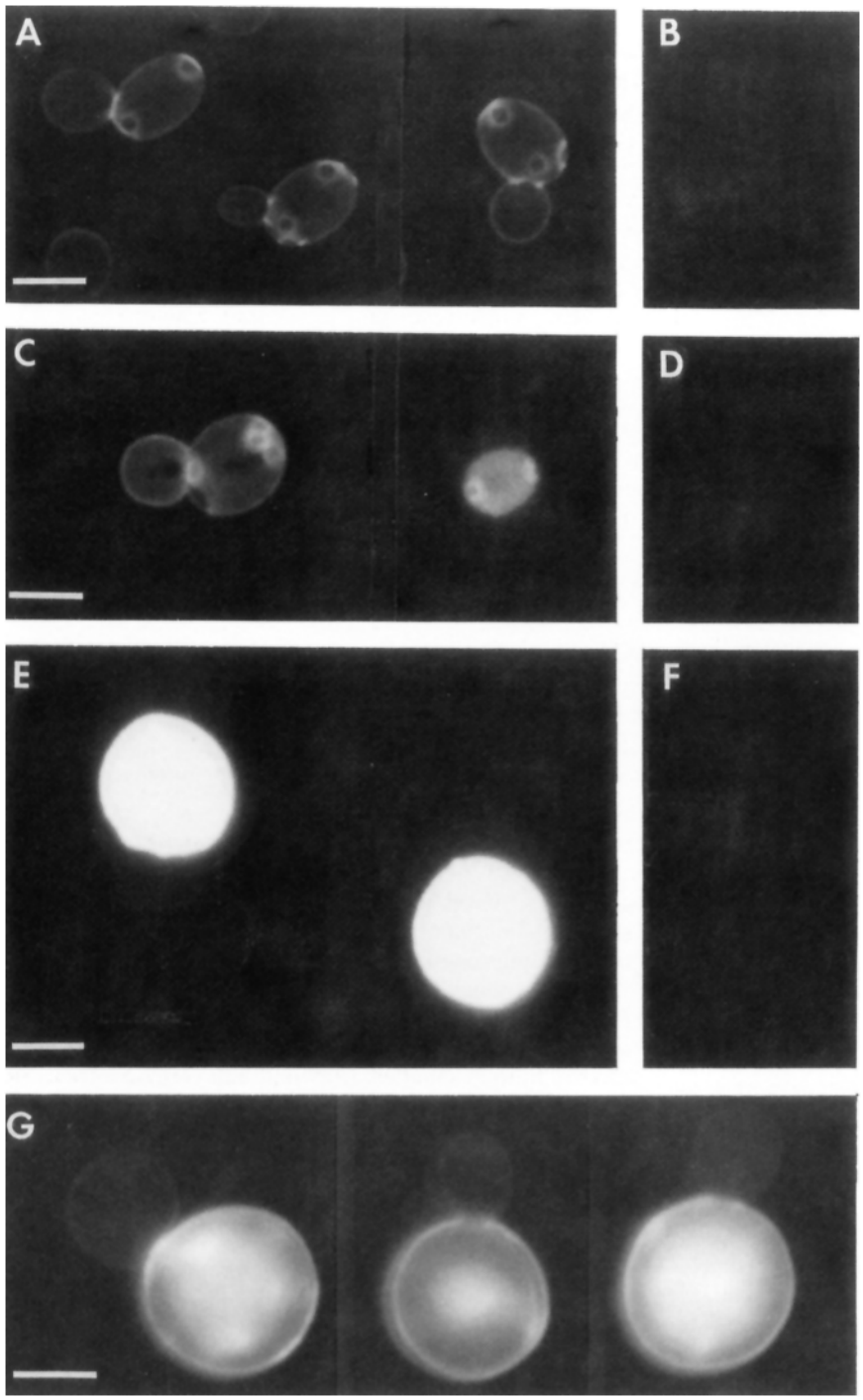

Figure 4. (*A–F*) Fluorescence micrographs of cell "ghosts" of strain C276 (*A* and *B*) or JPT163-HO2 (*C–F*) stained with Calcofluor before or after chitinase treatment. Cells growing exponentially at 23 (*A* and *B*) or 19°C (*C* and *D*) or cells that had been shifted from 19 to 36°C for 6 h (*E* and *F*) were fixed with formaldehyde and extracted to prepare cell ghosts (see Materials and Methods). The ghosts were then either stained directly with Calcofluor (*A*, *C*, and *E*) or treated with chitinase (see Materials and Methods) and then stained with Calcofluor (*B*, *D*, and *F*). Calcofluor staining used 0.005% Calcofluor in water for 3 min at 23°C followed by two washes with water. *A–F* were all photographed and printed using identical magnifications and exposure times. Although not visible in *B*, *D*, and *F*, the general outlines of the ghosts were not detectably affected by the chitinase treatments. (*G*) Fluorescence micrograph of JPT163-HO2 cells during renewed budding after "shift-down" from restrictive to permissive temperature. Cells growing exponentially at 23°C were shifted to 36°C for 4 h, then returned to 23°C for 6 h, then fixed with formaldehyde and stained with 0.1% Calcofluor for 10 min followed by two washes with water. Bars, 4 μm.

the 6–7 h after a shift of *cdc42* strains from 20 to 36°C, the amount of DNA increased ~3.5-fold while the cell number increased only ~1.9-fold (Fig. 3, *A* and *D*). Given that ~50% of the cells in the population at 20°C would already have been in G2, these data suggest that most cells in the arrested population had replicated their DNA even when nuclear division was unsuccessful. Similarly, the amount of DNA in arresting *cdc43* populations increased ~5.5-fold while the cell number increased only ~4-fold (Fig. 3, *C* and *F*), again suggesting that most cells in the arrested populations had replicated their DNA. However, it must be noted that interpretation of these experiments was complicated by several factors, including (*a*) the leakiness of the mutants, (*b*) the unknown contribution of mitochondrial DNA synthesis to the total, (*c*) the appreciable amount of cell lysis at later times, and (*d*) the possibility that the binucleate cells might have replicated their DNA again while some of the uninucleate cells remained in G1.

The analysis of spindle formation appeared to be more straightforward. Immunofluorescence with antitubulin antibodies showed clearly that most arrested *cdc42* cells had progressed into the nuclear cycle at least to the extent of

forming complete spindles (Fig. 2, *E* and *F*; Table III). Moreover, most of these spindles went on to elongate (Table III); as spindle elongation appears to be dependent on prior DNA replication (Pringle and Hartwell, 1981), this observation provided further evidence that DNA replication had occurred in these cells.

Thus, taking the various results together, we conclude that when bud emergence is arrested, nearly all cells can begin the nuclear cycle, even though not all may be able to complete it (see Discussion).

Abnormal Morphogenesis in the cdc42 and cdc43 Mutants

The fact that the *cdc42* and *cdc43* mutants continued to grow, but failed to bud, suggested that they might be defective in establishing the polarization of secretion and cell-surface deposition that is necessary for budding (see Introduction). Further evidence in support of this hypothesis was provided by the shape of the arrested cells, which were nearly spherical (Fig. 2, *A* and *C*), suggesting that they had grown by an isotropic incorporation of new cell-surface material. Indeed, when the arrested cells were stained with Calcofluor, they appeared uniformly bright (Fig. 2, *B* and *D*; the *cdc43* cells were more variable in this regard than were the *cdc42* cells). As Calcofluor appears to stain chitin specifically within the *Saccharomyces cerevisiae* cell wall (Pringle et al., 1989), the results suggested that chitin had been incorporated into the cell wall in a delocalized manner, rather than specifically at the budding sites (compare the Calcofluor-staining patterns of the cells grown at permissive temperature in Fig. 2, *B* and *D*). This interpretation was supported by the finding that chitinase treatment could remove the Calcofluor-positive material from "ghosts" of normal or arrested *cdc42* cells without detectably altering the overall outlines of the ghosts (Fig. 4, *A–F*). Moreover, the brightly stained cells were not simply cells whose staining properties had changed because they were dead: return of such cells to permissive temperature allowed the formation of new buds, whose walls were Calcofluor dark like those of normal cells (Fig. 4 *G*).

As the cellular actin network appears to be directly involved in the polarization of secretion and cell-surface deposition in yeast (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985; Drubin et al., 1988), we anticipated that this network might lose its normal asymmetric distribution in *cdc42* and *cdc43* cells at restrictive temperature. Staining with rhodamine phalloidin showed that this was indeed the case for the vast majority of arrested *cdc42* cells (Fig. 5). (*cdc43* was not examined because of the partial abnormality of the cells at permissive temperature and their slow arrest at restrictive temperature.)

Certain nonlethal mutations in *CDC24* (Sloat et al., 1981) and several other genes (Bender and Pringle, 1989; Chant, J., and I. Herskowitz, personal communication) can affect the pattern of bud-site selection, suggesting that the products of these genes are involved in the initial decision as to where the bud will form. Examination of the bud-scar patterns of strains carrying the available *cdc42* and *cdc43* alleles, after growth at permissive temperatures, did not reveal any obvious abnormalities (see Discussion).

Discussion

Isolation and Analysis of Mutants Defective in Budding

Mutants defective in gene *CDC24*, which continue growth but are unable to bud or establish cell polarity, have been of great interest with regard both to the mechanisms of cellular morphogenesis and to the mechanisms that coordinate cell-cycle events. To gain further insight into these problems, we sought additional mutants with defects in budding. To this end, a collection of ~5,000 Ts^- lethal mutants was screened microscopically to identify mutants that arrested uniformly as large, unbudded cells of normal or rounder-than-normal shape at restrictive temperature. We have reported here the characterization of seven such mutants, which define the genes *CDC42* and *CDC43*. The genetic and phenotypic analyses presented several features of interest.

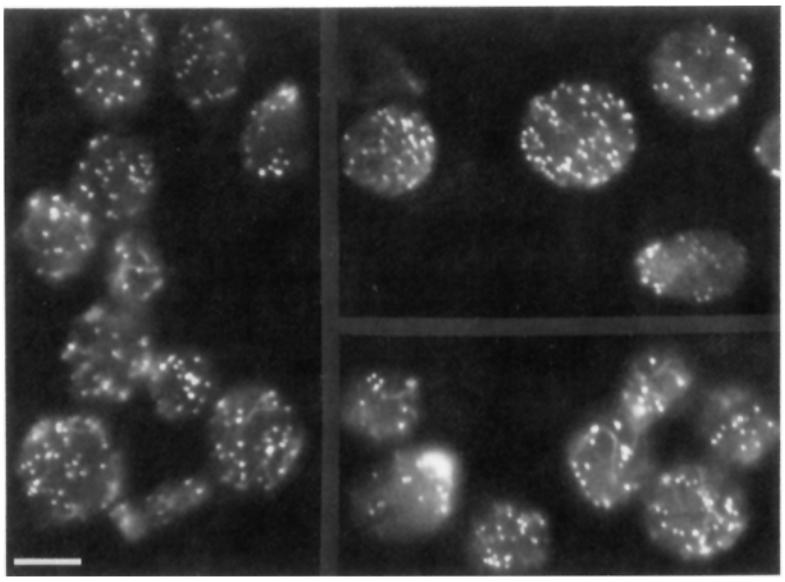

Figure 5. Cells of strain JPT163-HO1 (see Table II) growing exponentially at 23°C were shifted to 36°C for 2 h, then fixed and stained with rhodamine phalloidin as described in Materials and Methods. Bar, 4 μm.

First, the distribution of mutations among genes was far from random: 23 additional mutations in *CDC24* were isolated in comparison to six in *CDC43* and just one in *CDC42*. These results provide further illustration (see also Hartwell et al., 1973; Pringle, 1981; Kaback et al., 1984; Pringle, 1987) of the markedly different susceptibilities of different genes to Ts⁻ mutations (at least during in vivo mutant hunts), and thus emphasize the impracticality of relying solely on Ts⁻ mutations if all genes involved in particular cellular processes are to be identified. As expected, genetic strategies not relying on Ts⁻ mutations have begun to identify numerous additional genes whose products appear to be involved in bud emergence (Bender and Pringle, 1989; Corrado, K., A. Farewell, and J. R. Pringle, unpublished results; Chant, J., and I. Herskowitz, personal communication). A possibly related point is that all of the *cdc43* mutants obtained arrested only gradually after shift from an appropriately permissive (see below) to restrictive temperature. It is possible that this behavior reflects an intrinsic aspect of *CDC43* function (for example, the *CDC43* product could be involved in the synthesis of some component that is itself normally present in excess). However, it seems as likely that we simply failed to identify any *cdc43* alleles in which the gene product was sufficiently thermolabile (at least at 36–37°C) to give a more rapid arrest.

Second, the results illustrate a variety of other important but often neglected points about the handling of Ts⁻ cell-cycle mutants. For example, the markedly different success of different *cdc24-1* strains in completing the nuclear cycle (Table II) illustrates the potentially profound effect of "genetic background" on mutant phenotype, especially when heavy mutagenesis has been used, and thus emphasizes the importance of backcrossing to achieve isogenicity if conclusions about the effects of particular mutations are to be drawn. Moreover, achieving the desired isogenicity may not be trivial, as illustrated here by the rather different behavior of the twice-backcrossed strains JPT163-HO1 and JPT163-HO2 (Table II; the differences between the five times–backcrossed 5011-JHO1 and 5011-JHO3 are probably also significant). However, it should also be noted that the results obtained with "messier" strains may sometimes actually be more informative: for example, the success of strain 5011-D3 in completing nuclear division seems in fact to be the best guide to the actual relationship between bud emergence and the nuclear cycle (see further discussion below). (Similarly, it seems that the nonlethality of clathrin-deletion mutations in genetic backgrounds that may involve suppressors is as informative as is their lethality in other genetic backgrounds [Payne et al., 1987; Lemmon and Jones, 1987].) Finally, the behavior of the *cdc43* mutants illustrates dramatically that the "permissive temperature" at which a Ts⁻ mutant was isolated may not in fact be fully permissive. A corollary of this conclusion is that the observation of "first-cycle arrest" (Hartwell, 1974), the correlated determination of an "execution point" (Hartwell et al., 1970, 1973), and (especially) inferences from such an execution point about the time of gene product function may be quite misleading (Pringle, 1978; Pringle and Hartwell, 1981; Richmond and Williamson, 1983).

Third, the inviability of *cdc42 cdc43* double mutants at 23°C is one of a rapidly proliferating number of examples (see also Hinnebusch and Fink, 1983; Salminen and Novick, 1987; Wallis et al., 1989) indicating that such "synthetic lethality" (Dobzhansky, 1946; Sturtevant, 1956) may constitute a highly specific test for genetic interaction and perhaps a basis for novel genetic screens. In the present case, it should also be noted that overexpression of *CDC42* can suppress certain Ts⁻ *cdc24* mutations (Bender and Pringle, 1989). Thus, it is likely that *CDC24*, *CDC42*, and *CDC43* or their products all interact in some common pathway, as indeed already suggested by their very similar mutant phenotypes.

Finally, the large number of Ts⁺ (or Ts$^{+/-}$) segregants observed in crosses between strains carrying the *cdc43-2* and *cdc43-3* alleles was surprising, especially as each allele displayed a simple 2:2 segregation in other crosses. Similar results have been obtained in crosses involving certain pairs of *cdc24* alleles (McAllister, M., R. A. Preston, and J. R. Pringle, unpublished results) and certain pairs of *cdc28* alleles (Dutcher, 1980). Such results may reflect the presence of recombinational hotspots (Coleman et al., 1986; Symington and Petes, 1988), perhaps involving allele-specific effects on gene conversion frequencies (Borts and Haber, 1987; Ponticelli et al., 1988; Schuchert and Kohli, 1988). However, in the case of *cdc24*, most of the apparently Ts⁺ segregants that were analyzed appeared to harbor suppressors rather than being bona fide wild-type recombinants. It is not clear what sort of mechanism would generate suppressors at high frequencies in particular crosses.

The Functional Organization of Cell-cycle Events

The discovery that *cdc24* mutants could continue DNA replication and nuclear division while bud emergence was blocked (Hartwell et al., 1974) provided an antidote to extreme "domino theories" (Mitchison, 1971; Murray and Kirschner, 1989) in which the events of the cell cycle were viewed as comprising a single dependent sequence. Instead, confirming Mitchison's (1971) prediction, these events appeared to be organized as a set of dependent pathways that were independent of each other but were integrated by certain key regulatory events. Although this general view has received support from other sources and is now widely accepted (Pringle and Hartwell, 1981; Murray and Kirschner, 1989; Hartwell and Weinert, 1989), its original experimental justification had always been weakened by the possibility that *cdc24* mutations might eliminate a control mechanism that would normally make the nuclear cycle dependent on bud emergence (Pringle, 1978; Hartwell and Weinert, 1989). Thus, it was important to ask whether the nuclear cycle could also continue in other mutants in which bud emergence was blocked.

The results of monitoring DNA replication, spindle formation and elongation, and nuclear division in *cdc42* and *cdc43* mutants suggest that nearly all arrested cells begin and progress into the nuclear cycle, even though not all are able to complete it and only a few manage to complete a second cycle. Moreover, studies of various *cdc24* mutant strains indicate that the ability of strain 5011-D3 to complete multiple nuclear cycles (Table II; Hartwell et al., 1974) is a special property of this strain (presumably dependent on unidentified elements in its genetic background) and does not reflect a general disruption of a normal control by *cdc24* mutations. Although we do not know why the various arrested mutants

do not uniformly complete multiple nuclear cycles, we suspect that interactions between the nucleus and the budding site (as normally mediated by the cytoplasmic microtubules [Huffaker et al., 1988; Jacobs et al., 1988]) may be necessary for efficient nuclear division. Despite this remaining puzzle, the observation that mutations in three different genes, which encode very different kinds of proteins (Miyamoto et al., 1987; Johnson and Pringle, 1990; Johnson et al., 1990), can all block bud emergence without blocking the nuclear cycle considerably strengthens an important conclusion about the functional organization of cell-cycle events.

The Generation of Cell Polarity in Yeast

The abnormal morphogenesis of *cdc42* and *cdc43* mutants has not yet been characterized as fully as that of *cdc24* mutants. However, from the available evidence it appears that the inability of *cdc42* and *cdc43* mutants to bud, like that of *cdc24* mutants, reflects a general inability to establish normal spatial organization and cell polarity in cells in which macromolecule synthesis continues unabated. This interpretation is supported by the lack of asymmetry of the actin network, the delocalized chitin deposition, and the essentially spherical shape (presumably reflecting an isotropic incorporation of glucan, the shape-determining component of the cell wall [Cabib and Bowers, 1971; Zlotnik et al., 1984]) seen in the arrested cells. It was interesting that the loss of polarity at restrictive temperature was not irreversible: cells that had been at 36°C for 4 h were able to resume budding (and make seemingly normal buds) upon return to 23°C. Although examination of the bud-scar patterns of mutant cells grown at permissive temperature revealed no abnormalities, studies of *CDC42* overexpression suggest that this gene, like *CDC24*, is also involved in the initial selection of the budding site (Johnson and Pringle, 1990).

Thus, it can be anticipated that further studies of *CDC24*, *CDC42*, *CDC43*, and other genes that interact with them will provide important insights into the mechanisms of morphogenesis in yeast, and thus, by extension, in other eukaryotic cells. Already, the predicted sequences of the *CDC24*, *CDC42*, and *CDC43* products have provided provocative clues to the nature of these mechanisms: the *CDC24* product appears to interact with Ca^{2+} (Miyamoto et al., 1987); the *CDC42* product probably binds and hydrolyzes GTP while bound to the inner surface of the plasma membrane (Johnson and Pringle, 1990); and the *CDC43* product may be a nuclear protein (Johnson et al., 1990). It seems likely that the generation of antibodies specific for these gene products and use of these antibodies in immunolocalization experiments will provide further insights.

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References

Adams, A. E. M., and J. R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* 98:934–945.

Bender, A., and J. R. Pringle. 1989. Multicopy suppression of the *cdc24* budding defect in yeast by *CDC42* and three newly identified genes including the *ras*-related *RSR1* gene. *Proc. Natl. Acad. Sci. USA.* 86:9976–9980.

Borts, R. H., and J. E. Haber. 1987. Meiotic recombination in yeast: alteration by multiple heterozygosities. *Science (Wash. DC).* 237:1459–1465.

Cabib, E., and B. Bowers. 1971. Chitin and yeast budding. Localization of chitin in yeast bud scars. *J. Biol. Chem.* 246:152–159.

Coleman, K. G., H. Y. Steensma, D. B. Kaback, and J. R. Pringle. 1986. Molecular cloning of chromosome I DNA from *Saccharomyces cerevisiae*: isolation and characterization of the *CDC24* gene and adjacent regions of the chromosome. *Mol. Cell. Biol.* 6:4516–4525.

Dobzhansky, T. 1946. Genetics of natural populations. XIII. Recombination and variability in populations of *Drosophila pseudoobscura*. *Genetics.* 31: 269–290.

Drubin, D. G., K. G. Miller, and D. Botstein. 1988. Yeast actin-binding proteins: evidence for a role in morphogenesis. *J. Cell Biol.* 107:2551–2561.

Dutcher, S. K. 1980. Genetic control of karyogamy in *Saccharomyces cerevisiae*. Ph.D. thesis, University of Washington, Seattle, WA.

Field, C., and R. Schekman. 1980. Localized secretion of acid phosphatase reflects the pattern of cell surface growth in *Saccharomyces cerevisiae*. *J. Cell Biol.* 86:123–128.

Fink, G. R. 1970. The biochemical genetics of yeast. *Methods Enzymol.* 17A:59–78.

Hartwell, L. H. 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. *J. Bacteriol.* 93:1662–1670.

Hartwell, L. H. 1974. *Saccharomyces cerevisiae* cell cycle. *Bacteriol. Rev.* 38:164–198.

Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: controls that ensure the order of cell-cycle events. *Science (Wash. DC).* 246:629–634.

Hartwell, L. H., J. Culotti, and B. Reid. 1970. Genetic control of the cell-division cycle in yeast. I. Detection of mutants. *Proc. Natl. Acad. Sci. USA.* 66:352–359.

Hartwell, L. H., R. K. Mortimer, J. Culotti, and M. Culotti. 1973. Genetic control of the cell-division cycle in yeast. V. Genetic analysis of *cdc* mutants. *Genetics.* 74:267–286.

Hartwell, L. H., J. Culotti, J. R. Pringle, and B. J. Reid. 1974. Genetic control of the cell-division cycle in yeast. *Science (Wash. DC).* 183:46–51.

Hinnebusch, A. G. 1988. Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol. Rev.* 52:248–273.

Hinnebusch, A. G., and G. R. Fink. 1983. Positive regulation in the general amino acid control of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* 80:5374–5378.

Huffaker, T. C., J. H. Thomas, and D. Botstein. 1988. Diverse effects of β-tubulin mutations on microtubule formation and function. *J. Cell Biol.* 106: 1997–2010.

Jacobs, C. W., A. E. M. Adams, P. J. Szaniszlo, and J. R. Pringle. 1988. Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* 107:1409–1426.

Jaffe, L. A., M. H. Weisenseel, and L. F. Jaffe. 1975. Calcium accumulations within the growing tips of pollen tubes. *J. Cell Biol.* 67:488–492.

Johnson, D. I., and J. R. Pringle. 1990. Molecular characterization of *CDC42*, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. *J. Cell Biol.* 111:143–152.

Johnson, D. I., C. W. Jacobs, J. R. Pringle, L. C. Robinson, G. F. Carle, and M. Olson. 1987. Mapping of the *Saccharomyces cerevisiae CDC3*, *CDC25*, and *CDC42* genes to chromosome XII by chromosome blotting and tetrad analysis. *Yeast.* 3:243–253.

Johnson, D. I., J. M. O'Brien, and C. W. Jacobs. 1990. Isolation and sequence analysis of *CDC43*, a gene involved in the control of cell polarity in *Saccharomyces cerevisiae*. *Gene.* In press.

Johnston, G. C., J. R. Pringle, and L. H. Hartwell. 1977. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* 105:79–98.

Kaback, D. B., P. W. Oeller, H. Y. Steensma, J. Hirschman, D. Ruezinsky, K. G. Coleman, and J. R. Pringle. 1984. Temperature-sensitive lethal mutations on yeast chromosome I appear to define only a small number of genes. *Genetics.* 108:67–90.

Kilmartin, J. V., and A. E. M. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. *J. Cell Biol.* 98:922–933.

Kilmartin, J. V., B. Wright, and C. Milstein. 1982. Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93:576–582.

Kropf, D. L., and R. S. Quatrano. 1987. Localization of membrane-associated calcium during development of fucoid algae using chlorotetracycline. *Planta (Berl.).* 171:158–170.

Lemmon, S. K., and E. W. Jones. 1987. Clathrin requirement for normal growth of yeast. *Science (Wash. DC).* 238:504–509.

Lillie, S. H., and J. R. Pringle. 1980. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. *J. Bacteriol.* 143: 1384–1394.

Mitchison, J. M. 1971. The Biology of the Cell Cycle. Cambridge University

Press, Cambridge, England. 313 pp.
Miyamoto, S., Y. Ohya, Y. Ohsumi, and Y. Anraku. 1987. Nucleotide sequence of the *CLS4* (*CDC24*) gene of *Saccharomyces cerevisiae*. *Gene*. 54:125–132.
Moir, D., and D. Botstein. 1982. Determination of the order of gene function in the yeast nuclear division pathway using *cs* and *ts* mutants. *Genetics*. 100:565–577.
Mortimer, R. K., D. Schild, C. R. Contopoulou, and J. A. Kans. 1989. Genetic map of *Saccharomyces cerevisiae*, edition 10. *Yeast*. 5:321–403.
Murray, A. W., and M. W. Kirschner. 1989. Dominoes and clocks: the union of two views of the cell cycle. *Science (Wash. DC)*. 246:614–621.
Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell*. 40:405–416.
Ohya, Y., Y. Ohsumi, and Y. Anraku. 1986*a*. Isolation and characterization of Ca^{2+}-sensitive mutants of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* 132:979–988.
Ohya, Y., S. Miyamoto, Y. Ohsumi, and Y. Anraku. 1986*b*. Calcium-sensitive *cls4* mutant of *Saccharomyces cerevisiae* with a defect in bud formation. *J. Bacteriol.* 165:28–33.
Payne, G. S., T. B. Hasson, M. S. Hasson, and R. Schekman. 1987. Genetic and biochemical characterization of clathrin-deficient *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 7:3888–3898.
Ponticelli, A. S., E. P. Sena, and G. R. Smith. 1988. Genetic and physical analysis of the *M26* recombination hotspot of *Schizosaccharomyces pombe*. *Genetics*. 119:491–497.
Pringle, J. R. 1975. Induction, selection, and experimental uses of temperature-sensitive and other conditional mutants of yeast. *Methods Cell Biol.* 12: 233–272.
Pringle, J. R. 1978. The use of conditional lethal cell cycle mutants for temporal and functional sequence mapping of cell cycle events. *J. Cell. Physiol.* 95:393–406.
Pringle, J. R. 1981. The genetic approach to the study of the cell cycle. *In* Mitosis/Cytokinesis. A. M. Zimmerman and A. Forer, editors. Academic Press, Inc., New York. 3–28.
Pringle, J. R. 1987. The gene-number paradox and the complexity of the cell-division cycle and other cellular processes. *In* Modern Cell Biology. Vol. 5. Molecular Mechanisms in the Regulation of Cell Behavior. C. Waymouth, editor. Alan R. Liss, Inc., New York. 299–306.
Pringle, J. R., and J.-R. Mor. 1975. Methods for monitoring the growth of yeast cultures and for dealing with the clumping problem. *Methods Cell Biol.* 11:131–168.
Pringle, J. R., and L. H. Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. *In* The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance. J. N. Strathern, E. W. Jones, and J. R. Broach, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 97–142.
Pringle, J. R., R. A. Preston, A. E. M. Adams, T. Stearns, D. G. Drubin, B. K. Haarer, and E. W. Jones. 1989. Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31:357–435.
Reed, S. I. 1980. The selection of *S. cerevisiae* mutants defective in the Start event of cell division. *Genetics*. 95:561–577.
Reid, B. J., and L. H. Hartwell. 1977. Regulation of mating in the cell cycle of *Saccharomyces cerevisiae*. *J. Cell Biol.* 75:355–365.
Richmond, K. M. V., and D. H. Williamson. 1983. Residual cell division measurements are unreliable as indicators of the timing of events in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Sci.* 64:307–322.
Roberts, R. L., B. Bowers, M. L. Slater, and E. Cabib. 1983. Chitin synthesis and localization in cell division cycle mutants of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 3:922–930.
Salminen, A., and P. J. Novick. 1987. A *ras*-like protein is required for a post-Golgi event in yeast secretion. *Cell*. 49:527–538.
Schmid, J., and F. M. Harold. 1988. Dual roles for calcium ions in apical growth of *Neurospora crassa*. *J. Gen. Microbiol.* 134:2623–2631.
Schuchert, P., and J. Kohli. 1988. The *ade6-M26* mutation of *Schizosaccharomyces pombe* increases the frequency of crossing over. *Genetics*. 119:507–515.
Sherman, F., G. R. Fink, and J. B. Hicks. 1986. Methods in Yeast Genetics: Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 186 pp.
Sloat, B. F., and J. R. Pringle. 1978. A mutant of yeast defective in cellular morphogenesis. *Science (Wash. DC)*. 200:1171–1173.
Sloat, B. F., A. Adams, and J. R. Pringle. 1981. Roles of the *CDC24* gene product in cellular morphogenesis during the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* 89:395–405.
Sturtevant, A. H. 1956. A highly specific complementary lethal system in *Drosophila melanogaster*. *Genetics*. 41:118–123.
Symington, L. S., and T. D. Petes. 1988. Expansions and contractions of the genetic map relative to the physical map of yeast chromosome III. *Mol. Cell. Biol.* 8:595–604.
Wallis, J. W., G. Chrebet, G. Brodsky, M. Rolfe, and R. Rothstein. 1989. A hyper-recombination mutation in *S. cerevisiae* identifies a novel eukaryotic topoisomerase. *Cell*. 58:409–419.
Wilkinson, L. E., and J. R. Pringle. 1974. Transient G1 arrest of *S. cerevisiae* cells of mating type α by a factor produced by cells of mating type *a*. *Exp. Cell Res.* 89:175–187.
Wood, J. S., and L. H. Hartwell. 1982. A dependent pathway of gene functions leading to chromosome segregation in *Saccharomyces cerevisiae*. *J. Cell Biol.* 94:718–726.
Zlotnik, H., M. P. Fernandez, B. Bowers, and E. Cabib. 1984. *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. *J. Bacteriol.* 159:1018–1026.