Symmetric Cell Division in Pseudohyphae of the Yeast *Saccharomyces cerevisiae*

Stephen J. Kron, Cora A. Styles, and Gerald R. Fink

Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142

Submitted June 17, 1994; Accepted July 28, 1994
Monitoring Editor: David Botstein

Laboratory strains of *Saccharomyces cerevisiae* are dimorphic; in response to nitrogen starvation they switch from a yeast form (YF) to a filamentous pseudohyphal (PH) form. Time-lapse video microscopy of dividing cells reveals that YF and PH cells differ in their cell cycles and budding polarity. The YF cell cycle is controlled at the G1/S transition by the cell-size checkpoint Start. YF cells divide asymmetrically, producing small daughters from full-sized mothers. As a result, mothers and daughters bud asynchronously. Mothers bud immediately but daughters grow in G1 until they achieve a critical cell size. By contrast, PH cells divide symmetrically, restricting mitosis until the bud grows to the size of the mother. Thus, mother and daughter bud synchronously in the next cycle, without a G1 delay before Start. YF and PH cells also exhibit distinct bud-site selection patterns. YF cells are bipolar, producing their second and subsequent buds at either pole. PH cells are unipolar, producing their second and subsequent buds only from the end opposite the junction with their mother. We propose that in PH cells a G2 cell-size checkpoint delays mitosis until bud size reaches that of the mother cell. We conclude that yeast and PH forms are distinct cell types each with a unique cell cycle, budding pattern, and cell shape.

INTRODUCTION

Growth by budding distinguishes *Saccharomyces cerevisiae* and related fungi from most other eukaryotes. The bud is a protuberance that emerges from the surface of the mother cell at a time roughly coincident with duplication of the spindle pole body and onset of DNA synthesis (Byers, 1981). The bud grows continuously throughout S phase and until cytokinesis. The G2 and metaphase intervals are short and poorly defined because in *S. cerevisiae* the onset of mitosis is not marked by nuclear envelope breakdown or appreciable condensation of chromosomes. At anaphase, the spindle formed in S phase lengthens to distribute one daughter nucleus to the bud. A septum of cell wall then forms across the neck connecting bud to mother cell and the two cells, mother and daughter, separate from each other.

Budding confers a unique relationship between cell growth and the sequence of events that constitute the cell division cycle. Cell separation may occur long before the bud has grown to the size of the mother cell. The difference in size between mothers and daughters be-

comes pronounced when nutrients are limiting—the growth rate of the bud is reduced, whereas the duration of nuclear events and cell separation remain relatively constant (Hartwell and Unger, 1977). Small daughters remain in G1 proportionately longer than larger cells before initiating a cell cycle. This size-dependent delay led to the concept of Start, a G1 restriction point before bud emergence and DNA synthesis (Hartwell *et al.*, 1974), which limits entry into the cell cycle to daughters that have grown to a critical cell size. Bud emergence, DNA synthesis, nuclear division, and cytokinesis can proceed once the Start checkpoint is passed. In general, the daughter's cell cycle is considerably longer than that of the mother; the mother, already past Start, may enter a new budding cycle without delay. Under nutrient limitation, markedly asymmetric division results in a population consisting of a few full-sized cells with growing buds and a large contingent of unbudded daughter cells whose size varies over a wide range (Johnston, 1977). Although asymmetric cell division is unusual, regulation at the G1 to S transition is quite general. The restriction point where nutritional status, growth factors, and inhibitory signals control animal

cell proliferation is at the G1 to S boundary (reviewed by Pardee, 1989).

By contrast with the asymmetric division of *S. cerevisiae*, the fission yeast *Schizosaccharomyces pombe* proliferates by symmetric division (reviewed by Forsburg and Nurse, 1991). As in *S. cerevisiae*, in *S. pombe* DNA synthesis is completed well before the cytoplasm has doubled in volume. However, *S. pombe* cells remain in G2 until they double in size. Cell size is regulated by a G2 cell-size checkpoint that restricts anaphase and cytokinesis. Nascent daughter cells, already larger than the critical cell size for Start, bypass G1 and enter S phase promptly. *S. pombe* responds to moderate nutrient depletion by prolonging G2 rather than by increasing G1, maintaining a critical cell size at division (Fantès and Nurse, 1977). Only under severe nutrient stress is cryptic cell-size control at Start revealed (Nurse *et al.*, 1976).

In *S. cerevisiae*, peaks of distinct cyclins mark the G1/S transition, S phase, G2, and the G2/M transition. The molecular effectors of the Start cell-size checkpoint appear to be the G1 cyclins Cln1, 2, and 3 (Richardson *et al.*, 1989). A complex of the cyclin-dependent kinase Cdc28 (Wittenberg *et al.*, 1990) and a Cln cyclin is sufficient to initiate both bud emergence and DNA replication. Accumulation of critical levels of Cln1 and 2 at G1/S is driven by a positive feedback loop (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991) modulated by CLN3, a potential cell-size sensor (Tyers *et al.*, 1993). Cln/Cdc28 complexes are a target of physiological modulators of Start. Mating pheromone stimulation of a mitogen-activated protein (MAP) kinase cascade in haploid cells antagonizes entry into the cell cycle, whereas it promotes mating-specific gene expression and growth of an elongated mating projection. G1 arrest is a consequence of inhibition of Cln/Cdc28 by the cyclin-dependent kinase (CDK) inhibitor Far1 (Chang and Herskowitz, 1990) activated by the pheromone responsive MAP kinase Fus3 (Elion *et al.*, 1990). After Start, Cln1 and 2 levels decline. Three pairs of B-type cyclins appear in successive waves to associate with Cdc28, driving key cell cycle events required to complete cell division. Clb5 and 6 accelerate S phase, Clb3 and 4 promote spindle assembly, and Clb1 and 2 are required for mitotic anaphase (reviewed by Nasmyth, 1993). Conditions that antagonize accumulation of Clb1,2/Cdc28, such as overexpression of CLN1 (Hadwiger *et al.*, 1989) or deletion of CLB2 (Surana *et al.*, 1991), extend G2 and result in a division pattern similar to that of *S. pombe*.

In *S. pombe*, direct regulation of the cyclin-dependent kinase cdc2 (Nurse and Bissett, 1981) by phosphorylation mediates G2/M cell-size checkpoint control (reviewed by Feilottter *et al.*, 1992). Upon completion of DNA synthesis, phosphorylation of a single tyrosine (Y15) in the cdc2 protein (Gould and Nurse, 1989) by the kinase wee1 (Russell and Nurse, 1987) inhibits cell

cycle progression; dephosphorylation by the cdc25 phosphatase relieves this inhibition to promote mitosis (Millar *et al.*, 1991). This regulation is tied directly to cell size (Nurse and Thuriaux, 1980). Mutants lacking wee1⁺ have a diminished G2 and decreased cell size at cell division, causing a delay in G1, whereas those lacking cdc25⁺ arrest in G2. *S. pombe* expressing an activated cdc2 protein lacking the phosphorylation site have a phenotype like the wee1⁻ mutant (Gould and Nurse, 1989). In *S. cerevisiae*, CDC28 is phosphorylated in G2 on residue Y19 by the wee1⁺ homologue SWE1 (Booher *et al.*, 1993) and dephosphorylated before mitosis by the cdc25⁺ homologue MIH1 (Russell *et al.*, 1989). Mutation of the CDC28 phosphorylation site confers no detectable cell cycle phenotypes (Amon *et al.*, 1992; Sorger and Murray, 1992).

Recently, it has been discovered that laboratory strains of *S. cerevisiae* are dimorphic, capable of growing as separate ellipsoidal yeast cells or as chains of cells in a filament (Gimeno *et al.*, 1992). The signal for the switch from the yeast to the filamentous form is starvation for nitrogen. Upon nitrogen limitation, an ellipsoidal diploid cell gives rise to a long thin daughter. This thin cell in turn produces a long thin daughter that remains connected to the mother. Reiteration of this pattern gives rise to a visible filament called a pseudohypha (PH) that "moves" away from the founding mother. PH growth may be restricted to diploids because they divide in a polar manner, the bud emerging from each daughter away from its mother. Cells that divide in an axial pattern (haploids or MATa/MATa diploids) or random pattern (Bud⁻ mutants) fail to form pseudohyphae when starved (Gimeno *et al.*, 1992; Gimeno and Fink, 1994). A number of genes that function as inducers or inhibitors of PH growth have been reported (Ljungdahl *et al.*, 1992; Blacketer *et al.*, 1993). Strains that overexpress the PHD1 gene show a dramatic increase in the proportion of PH cells (Gimeno and Fink, 1994). Some elements of the pheromone-response MAP kinase cascade are required for the switch from yeast to PH form. Diploid strains homozygous for mutations in the STE20, STE11, STE7, or STE12 genes remain in the yeast form (YF) when starved for nitrogen (Liu *et al.*, 1993).

The existence of a new morphological cell type in *S. cerevisiae* prompted an examination of the PH cell cycle. We have observed multiple cycles of bud emergence and septation in growing YF and PH cell lineages with time-lapse video microscopy to determine the timing of cell cycle events. Our analysis reveals that each of the two cell types has a distinct geometry and a different mode of cell division. We propose that a key element of the developmental switch from YF to PH growth is a change in the regulation of the cell cycle.

MATERIALS AND METHODS

Media

Standard yeast media were prepared as described (Sherman, 1991). SLAD medium (synthetic minimal media with low ammonia and glu-

cose) was prepared as described (Gimeno and Fink, 1994). Cycloheximide-containing media supported PH growth when raffinose was used as the carbon source (SLAR, 6.7 g/l Yeast Nitrogen Base without amino acids and ammonium sulfate [Difco, Wilmington, DE], 0.25 mM ammonium sulfate, 2% raffinose $\pm$ 0.06 μ g/ml cycloheximide). YP is liquid YPD without glucose.

PH Growth on Solid and Liquid Media

Components of solid growth media were examined to define factors affecting PH induction. In general, PH growth was stimulated by media containing a low concentration of ammonium ion and an adequate carbon source. Of sugars examined, raffinose was the strongest inducer of PH growth. On SLAR, PH growth was enhanced and PH cells appeared both longer and thinner than cells grown on glucose. By contrast, carbon source restriction in the presence of high ammonium ion concentrations favored YF growth.

It is difficult to obtain pseudohyphae when cells are grown in liquid media. However, pseudohyphae can be obtained from dilute inoculation of liquid SLAD layered over SLAD agar in Petri plates (SLAD/LA). Typically, $1-5 \times 10^5$ cells of strain L5365 were inoculated into 10 ml SLAD liquid media layered over 10 ml SLAD 2% agar in Petri dishes and incubated at 30°C. After 10–15 h, cultures consisted of small PH filaments, short chains of cells radiating out from a founder cell. Many filaments adhered to and grew upon the agar surface. Cells were suspended by gently wiping the agar surface with a bent glass rod. The suspensions were consolidated in conical polystyrene tubes containing a small amount of YP liquid (<1/10 vol/vol) and pelleted in a swinging bucket rotor. These pellets were resuspended and treated as described for immunofluorescence, flow cytometry, and so on. YF cell cultures of L5535 and L5439 were prepared similarly. The clusters of PH cells and the YF cells were dispersed by 25 passes through a 26-gauge needle with a tuberculin syringe.

Characterization of the growth of strains L5365 (PH) and L5439 (YF) was carried out to measure the proportion of cells growing as pseudohyphae and to determine growth kinetics (Figure 1A). Cells were collected, formaldehyde fixed, and resuspended to a final volume of 250 μ l in SLAD liquid. Cell counts were performed using a Petroff-Hausser (Hausser Scientific Partnership, Horsham, PA) bacteria counting chamber. Budded cells were counted as single cells. Cells were examined for PH or YF morphology during counting. Under these conditions, L5439 YF cells grew exponentially between 8 and 16 h after inoculation, with a doubling time of 2.1 h and an apparent lag time of $\sim$ 6 h. During this period, the percentage of budded cells was 54–62% and few if any cells exhibited clear PH morphology. At the end of this phase of growth, budding index declined dramatically, reaching 3% by 22 h. The cell number at the end of exponential growth was $\sim 2.5 \times 10^6$ YF cells per plate. The growth of the PH strain L5365 was exponential between 6 and 18 h with a doubling time of 2.6 h and a percentage of budded cells ranging from 60 to 74%. The proportion of budded cells dropped to 13% at 22 h. During exponential phase, the morphology of cells was relatively constant only between 10 and 18 h, during which 45–50% of the cells were clearly elongated. Total cell number at 18 h was $\sim 1 \times 10^6$ cells per growth plate.

Yeast Strain Construction

Yeast strain manipulations and construction were performed essentially as described (Sherman, 1991). Yeast strains and transforming plasmids are listed in Tables 1 and 2. Strains used in these studies are isogenic to MB1000 (Brandriss and Magasanik, 1979), formerly Σ 1278b (Grenson *et al.*, 1966) and MB758-5B (Siddiqui and Brandriss, 1988). MB758-5B contains the *ura3-52* mutation. A *ura3-52/ura3-52* diploid strain obtained by mating spores derived from these parents was transformed to prototrophy with pCG38, a *URA3*-marked 2μ plasmid carrying a genomic clone of *PHD1* (Gimeno and Fink, 1994) to produce L5365. The vector-carrying control strain was L5439. For other genetic manipulations, additional auxotrophic mutations were

Figure 1. Growth kinetics of YF and PH cells in suspension culture and on agar media. (A) Plot of cell density of L5439 YF (○) and L5365 PH (●) cultures growing in SLAD liquid over agar media at 30°C as described in MATERIALS AND METHODS. The shown least-squares fits to exponential growth yield doubling times of 2.1 h for L5439 and 2.6 h for L5365. (B) Plot of increase in cell number in single L5439 YF (○) and L5365 PH (●) microcolonies on low-ammonia media agar at 30°C observed by time-lapse microscopy. The number of cells in the colonies was counted in frames recorded at 20-min intervals. The shown least-squares fits to exponential growth yield doubling times of 3.4 h for L5439 and 4.0 h for L5365.

introduced by transformation and crosses within the MB1000 lineage (Liu *et al.*, 1993).

Other strains were constructed by transformation of haploid or diploid segregants with the appropriate plasmids and integrating constructions, generally to confer prototrophy. Plasmids were constructed to convert chromosomal copies of *CDC28* to mutant alleles. The *Xho* I, *Pvu* II fragments containing two mutant alleles *CDC28* (Y19F) and *CDC28* (TY18AF) from plasmids p2957 and p3301 (Amon and Nasmyth, personal communication) were subcloned into the *Eco*RV and *Xho* I sites of the integrating vector pRS306 (Sikorski and Hieter, 1989). *CDC28* mutant alleles were introduced by integrative duplication. Spontaneous excision of the mutant or wild-type copies of *CDC28* was detected by selection of Ura⁻ segregants on 5-fluoro-orotic acid media. We distinguished mutant segregants from *CDC28* by replacing the *SWE1* locus with *GAL1::SWE1* as described (Booher *et al.*, 1993). Replica-printing to galactose media caused cells with the wild-type *CDC28* allele to become worm-like and cease to divide, whereas those lacking the *CDC28* Y19 phosphorylation site were able to grow. Pairs of *MATa* and *MATα* haploids identified by this method were mated to form homozygous diploids. The presence of the expected *CDC28* allele in each strain was confirmed by sequencing polymerase chain

Table 1. Yeast strain list

Name	Genotype	Plasmid	Source
MB1000	<i>MATα</i>		Σ 1278b, Brandriss and Magasanik, 1979
MB758-5B	<i>MATα ura3-52</i>		Siddiqui and Brandriss, 1988
L5365	<i>MATα/MATα ura3-52/ura3-52</i>	pCG38	This work
L5366	<i>MATα/MATα ura3-52/ura3-52</i>		This work
L5439	<i>MATα/MATα ura3-52/ura3-52</i>	pRS202	This work
L5486	<i>MATα ura3-52 leu2::hisG</i>		H. Liu, this laboratory
L5487	<i>MATα ura3-52 leu2::hisG</i>		H. Liu, this laboratory
L5502	<i>MATα/MATα CDC28/CDC28 ura3-52/ura3-52 swe1::GAL1-SWE1-LEU2/ SWE1 leu2::hisG/leu2::hisG</i>		This work
L5535	<i>MATα/MATα ste7::LEU2/ste7::LEU2 leu2::hisG/leu2::hisG ura3-52/ ura3-52</i>	pRS202	HLY351, Liu <i>et al.</i> , 1993
L5664	<i>MATα/MATα CDC28-AF18/CDC28-AF18 ura3-52/ura3-52 swe1::GAL1- SWE1-LEU2/swe1::GAL1-SWE1-LEU2 leu2::hisG/leu2::hisG</i>		This work
L5665	<i>MATα/MATα ura3-52/ura3-52 mih1::LEU2/mih1::LEU2 leu2::hisG/ leu2::hisG</i>		This work
L5666	<i>MATα/MATα CDC28-AF18/CDC28-AF18 ura3-52/ura3-52</i>		This work
L5667	<i>MATα/MATα CDC28-F19/CDC28-F19 ura3-52/ura3-52</i>		This work
EM93	<i>MATα/MATα ho1/ho1</i>		Mortimer and Johnston, 1986
CGX19	<i>MATα/MATα ura3-52/ura3-52 shr3-102/shr3-102</i>		Gimeno <i>et al.</i> , 1992

All strains are Σ 1278b derivatives except EM93 (Mortimer and Johnston, 1986).

reaction clones of the *CDC28* open reading frame to determine the amino-terminal sequence.

Strains Used for Video Microscopy

Documentation of cell growth and cell lineage by video microscopy required strains capable of forming completely YF or PH microcolonies from a single cell manipulated onto the appropriate medium. To study YF growth, we used either the Σ 1278b wild-type diploid strains L5439 or strain L5535, a homozygous *ste7* deletion mutant (*ste7::LEU2/ste7::LEU2*). The *ste7* mutation in L5535 blocks the switch to the PH form and yields a nearly pure population of YF cells on all media tested (Liu *et al.*, 1993). Although dimorphism can be observed in many diploid strains of the Σ 1278B background, these grow first as the YF cell type and only later switch to the PH form. After assessing many strains, we chose the Σ 1278b diploid strain, L5365, overexpressing the *PHD1* gene (Gimeno and Fink, 1994). L5365 cells enter the PH growth pattern immediately upon inoculation onto fresh SLAD media and remain PH for several generations.

A Chamber for High-Magnification Imaging of Yeast Growth

A sealed growth chamber was constructed by pouring 8–10 ml melted SLAD agar into a 15-cm plastic Petri dish containing a standard mi-

croscope slide. Cells were inoculated on the agar surface and an 18-mm square coverslip was placed directly over the cells. Excess agar surrounding the coverslip was trimmed away and the edges were sealed with valap (1:1:1 vaseline:lanolin:paraffin). Of the variety of methods examined for depositing cells on the agar surface, we preferred micromanipulating single cells to form a pattern marked by needle indentations in the agar. The marks facilitate locating the very small number of inoculated cells.

When individual small-budded wild-type cells (L5439 or EM93) are incubated in this growth chamber, most cells resume growth immediately, completing the first bud and initiating a second bud within 2 h at 30°C. The developing microcolony forms a roughly circular monolayer of mostly YF cells (Figure 2, left) that grows at a constant doubling time for ≥ 24 hours (Figure 1B). Although PH cells are observed in these microcolonies, they generally comprise a small minority of the cells. Chambers inoculated with ~ 10 cells support growth of colonies of up to several million cells, as estimated from colony size. The PH strain L5365 produces microcolonies of cells of various morphologies. A large proportion of these colonies grow for up to 24 h as exclusively PH cells (Figure 2, right). Such PH colonies also exhibited a constant doubling time for up to 24 h (Figure 1B). For filming, we preincubated slides for 3–5 h and chose those founder cells that produced a bud or two with well-developed cylindrical morphology characteristic of PH growth.

Table 2. Plasmid list

Plasmid	Markers	Source
pRS202	2 μ URA3	Connely and Hieter, unpublished data
pCG38	2 μ URA3 PHD1	Gimeno and Fink, 1994
p2957	CEN TRP1 CDC28-AF18	Amon and Nasmyth, personal communication
p3301	CEN TRP1 CDC28-F19	Amon and Nasmyth, personal communication
pRS306	URA3	Sikorski and Hieter, 1989
pRS306 CDC28-F19	URA3 CDC28-F19	This work
pRS306 CDC28-AF18	URA3 CDC28-AF18	This work
pSWE1-41	<i>swe1::GAL1-SWE1 LEU2</i>	Booher <i>et al.</i> , 1993
pSWE1-10g	<i>swe1::LEU2</i>	Booher <i>et al.</i> , 1993
pmih1::LEU2	<i>mih1::LEU2</i>	Russel <i>et al.</i> , 1989

Apparatus for Time-Lapse Autofocus Video Microscopy

A simple microcomputer-based system for time-lapse video recording was implemented. All optical components were mounted on an anti-vibration table. A Zeiss (Thornwood, NY) Axioscop equipped for Nomarski-differential-interference contrast (DIC) and epifluorescence microscopy was modified by addition of a video autofocus controller and stepping motor focus driver (Ludl Electronics Products MAC2000, Hawthorne, NY). A 12-V 100-W tungsten illuminator was used for transmitted light illumination. A 1.4-numerical aperture (N.A.) oiled DIC condenser (44 52 45) and high N.A. oil immersion objectives (Plan-Apochromat 63/1.4 and Plan-Neofluar 40/1.3) were used. Air flow from an air curtain incubator (model 279, Sage Instruments, Boston, MA) was directed to warm the microscope stage and the control thermocouple was located in the path of the air flowing toward the microscope objective. A Hamamatsu C2400-09 Newvicon video camera (Photronics Instruments, Edison, NJ) was coupled to the trinocular tube of the microscope via a 4X video adapter (45 29 85). Alternatively, the camera was coupled directly to the trinocular and an Optivar (45 19 04) magnification changer was used. The video signal was routed to the autofocus controller and to a Hamamatsu Argus 10 video processor to perform background subtraction and overlay a time base. The Argus 10 output was connected to the composite input of a TQ-3031F Optical Memory Disc Recorder (OMDR) (Panasonic, Secaucus, NJ). A 386-based DOS computer was connected via serial ports to the autofocus controller and the OMDR. Initialization and programming of the autofocus and OMDR as needed were performed via the serial ports. A BASIC program designed to activate the autofocus at intervals and record a single video frame on the OMDR was used to perform time-lapse recording.

A growth sequence was recorded by placing a growth chamber on the prewarmed microscope stage with condenser and objective oiled. The cells were brought into focus with eyepieces and the condenser adjusted for high N.A. Koehler illumination. The illuminator transformer was set close to lowest voltage and the adjustable Nomarski prism was set for moderate contrast. The video camera was then adjusted for optimum analog contrast. For real-time background subtraction, the microscope stage was moved to a region adjacent to the yeast cells and a reference frame recorded. The cells were brought back into the field of view to begin imaging and the BASIC program for time-lapse recording was activated.

The performance of the time-lapse recording system was evaluated for precision of timing, reliability of focus, and image quality. The minimum practical interval between successive frames was 10 s. The variability of the timing interval between successive frames as determined by the time base was found to be ± 0.2 s or better. Focus was relatively reliable, with the frame found to be in adequate focus >90% of frames examined.

Time-Lapse Observation of Yeast Growth

Preliminary studies demonstrated that both white light and monochromatic visible light inhibit growth. Attenuation of infrared light had little or no benefit. Shuttering was not evaluated. Low illumination was used, although momentary dips in line voltage resulted in significant changes in brightness of recorded frames. The magnitude of this "flicker" was attenuated by decreasing offset used for background subtraction. Use of an uninterruptible power supply (Unison/Tripp Lite, Chicago, IL) essentially eliminated the problem.

Observations of constitutive YF *ste7* mutants (L5535) and of the weakly dimorphic wild-type (L5439) were used to define characteristics of cell shape and budding pattern associated with YF growth. In general, the YF cells exhibit the classically described physical separation of immature daughter cell from mother after completion of the septum, accompanied by an abrupt change in positions of the mother and daughter pair when surface tension breaks their bond.

Quantitative Analysis of Video Images and Time-Lapse Recordings

All time intervals were derived from the time base recorded on each video frame. The mouse-driven linear measurement feature provided by the ARGUS 10 processor was used to determine distances between points on still frames displayed by the OMDR. Measurements were calibrated using video images of the 20- μ m divisions of a stage micrometer. In general, we used the highest magnification practical to reduce quantization error. Using the 63X Plan-Apochromat objective and 4X video adaptor, the video display was approximately a 40- μ m square field.

For classification as YF or PH, we examined video images to assay the size, shape, and axial ratio of each cell before quantitation. Cells with axial ratios that were less than ~ 2 were excluded from the PH group. Buds that failed to separate from the mother cell after septation were excluded from the YF group. In general, only cells that were members of lineages that maintained a consistent pattern were used for quantitation. Briefly, the long axis of the first daughter to bud from a mother cell lies roughly along the long axis of the mother. When this daughter buds, the bud appears at the pole opposite the birth junction with the mother. This "main lineage" of first daughter cells generally consists of cells with similar axial ratios. Occasionally, second daughters and subsequent buds from cells one or more generations back from the growing tip of the PH revert to YF growth. These YF second daughters may divide to form other YF cells. After three or more buds, a cell in the main lineage may fail to bud again. This dropout phenomenon limits the branching of PH strands. Thus, only the first bud from an apical cell appears reproducibly to both arise from and give rise to PH cells. Because of the variability in the lateral branches, most observations about PH growth reported in the following are restricted to the behavior of cells constituting a main lineage.

To construct pedigrees, a series of frames recorded at 10-min intervals was examined to determine the first time at which bud emergence could be discerned. Bud lengths were determined from measurements of distance from bud tip to bud neck, along the long axis of the cell. The length of the mother was determined similarly. The short axis length of a cell was determined by measuring the apparent diameter of the largest cross-section of a cell orthogonal to its long axis. Normalized cell volume (V_n) of mother cells and buds was determined for both YF and PH cells by measuring the long (l) and short (s) axes of the cells and applying the formula $V_n = l \times s^2$.

Slides of antitubulin stained PH cells were examined by DIC video microscopy to find budded PH cells. The long axis lengths of mothers and buds were determined as above, and the tubulin geometry was classified where possible into dot (single or unseparated spindle pole body), bar (short spindle), or anaphase (long spindle) by imaging in the rhodamine fluorescence channel.

Serial Section Transmission Electron Microscopy (TEM) of PH Colonies

PH colonies were grown in a monolayer facilitating fixation and serial section for imaging by electron microscopy as follows. A suspension of L5365 in distilled water was spread on SLAD 2% agar over a submerged slide prepared as described above. A 25-mm square film of agar 2 mm thick was cut free and placed cell-side down in an empty plastic 15-cm Petri plate and covered with additional 45–50°C SLAD agar as a source of nutrient. The overlay agar contained 0.5–2.5 mM ammonium sulfate (10–50 $\times$ "low ammonia"), which supports PH colonies growing in high density in this genotype. Most cells grew in the interface between agar and Petri plate. After overnight incubation at 30°C, the plate was processed for TEM. The initial fixation was by pouring 0.5% glutaraldehyde solution over the agar surface and incubating at room temperature for 30 min. A section of the agar was then excised and handled as a tissue block. Fixation, embedding, sectioning, and staining for TEM were performed essentially as described (Wright and Rine, 1989). All images were recorded on a Philips

410 microscope (Eindhoven, The Netherlands) with 4489 film (Kodak, Rochester, NY). Negatives were examined directly to detect the presence of pores in septa separating mother and daughter cells.

Fluorescence Microscopy

In general, cell growth and fixation for fluorescence microscopy were performed as follows. Cells were grown in SLAD/LA. Formaldehyde solution, 1/10 volume 37%, was added to cells resuspended in SLAD. Fixation was at 22°C for 1 h to overnight. After addition of 1/10 volume YP liquid medium, the cells were sedimented by centrifugation and washed with water. Imaging of nuclear DNA by 4',6-diamino-2-phenylindole (DAPI, Sigma, St. Louis, MO) staining, bud site chitin rings by calcofluor (Fluorescent Brightener F-6259, Sigma) staining (Cabib and Bowers, 1971), and actin cytoskeleton by tetramethylrhodamine phalloidin (Molecular Probes, Eugene, OR) staining were performed essentially as described (Pringle *et al.*, 1989; Adams and Pringle, 1991). To image calcofluor-stained cells, a suspension of stained YF or PH cells in glycerol/phenylenediamine photobleach inhibitor was placed on a slide and overlaid with a coverslip. Pressure was applied to flatten the cells, facilitating observation of bud scars in a single plane of focus. For actin distribution, a suspension of YF or PH cells stained with tetramethylrhodamine phalloidin and DAPI were placed on a slide and flattened as above. Cells were prepared for immunofluorescence essentially as described (Pringle *et al.*, 1989) and stained using YOL1/34 monoclonal antitubulin (Kilmartin *et al.*, 1982) culture supernatant (Accurate Chemical and Scientific Co., Westbury, NY) as primary antibody and stained with DAPI during final washes. Fluorescence images were recorded by photomicrography using T-MAX 400 film and T-MAX developer (Kodak) or by video microscopy using a Silicon Intensified Target camera (Hamamatsu C2400-08).

Budding Index, Mitotic Index, Bud Site Distribution, and Actin Distribution

To determine budding index, SLAD/LA cultures of L5365 and L5535 were inoculated and incubated at 30°C for 10 h. Cells were collected, fixed with formaldehyde, and the proportion of budded cells determined using phase microscopy. For mitotic index, DAPI-stained PH and YF cells were placed on a slide and the number of nuclei in each budded cell was counted. A cell was classified as anaphase mitotic only if its nucleus was very elongated and demonstrated a significant thinning of the stainable material in the bud neck or had resolved into two daughter nuclei. Bud site distribution was determined from samples prepared as described for photomicrography. Only budded cells were examined. Cells were imaged in the microscope eyepieces at 1250× nominal magnification using a Plan-Neofluar 100/1.3 NA objective. Mitotic index and bud site orientation of PH cells were scored only in cells with axial ratios of approximately two or greater.

Flow Cytometry

For measurement of DNA content by flow cytometry, YF and PH cells were grown in suspension culture in SLAD/LA, collected, sedimented by centrifugation, and prepared as described above. Aggressive mechanical disruption was used to disperse filaments into individual cells. Although relatively few cell fragments were produced by shearing cell suspensions through a 26-gauge needle, large buds may be lost by this treatment. The cell suspensions were fixed with 70% ethanol for 15–30 min at 23°C. Further preparation for propidium iodide flow cytometry of DNA content was essentially as described (Hutter and Eipel, 1979). Stained cells were analyzed on a FACSTAR (Becton-Dickinson, San Jose, CA) using LYSIS II Ver. 1.2. Cell sorting was performed by assigning windows of propidium iodide fluorescence per cell based upon flow cytometric profiles obtained from analysis of 10⁴ cells. Sorted cells were collected in phosphate-buffered saline buffer and then concentrated by centrifugation and/or filtering. These cells were restained with propidium iodide and examined for nuclear

morphology, cell shape, and budding index by epifluorescence (rhodamine channel) and Nomarski-DIC video microscopy.

RESULTS

PH Cell Division Is Symmetric

The growth of YF and PH microcolonies from single cells micromanipulated onto low ammonia agar media was recorded by time-lapse video microscopy (Figure 2). Two diploid strains, L5439 (wild-type, Figure 2, right) and L5535 (*ste7/ste7*) gave rise to YF colonies. Cells from both these strains divide asymmetrically. Before the bud reaches the size of the mother, a septum forms at the junction between the mother and daughter and the cells separate (Figure 3A). The mean volume of a

Figure 2. Time-lapse series of YF and PH colony growth. A single budded cell of L5439, the Σ 1278b wild-type strain (right), and of L5365, the *PHD1* overexpressing strain (left), were micromanipulated onto low-ammonia media and incubated on the microscope at 30°C as described in MATERIALS AND METHODS. At the indicated times after inoculation, single video frames were recorded using contrast-enhanced video Nomarski-DIC microscopy. (A) 3 h, (B) 6 h, (C) 9 h, and (D) 12 h. The scale bar indicates 10 μ m.

L5439 daughter cell at separation was 0.65 ± 0.25 (mean $\pm$ SD, $n = 13$) that of its mother. YF daughter cells grow for a period before budding (Figure 3A). Daughter cell size increases significantly during G1, the interval between cell separation and bud emergence. Bud emergence, marking onset of S phase, occurs once the cell size of the daughter approaches that of the mother cell. Daughters bud when their volume is 0.89 ± 0.14 -fold ($n = 7$) that of their mother. A characteristic cell size is maintained in each generation. These observations confirm the classically described pattern of YF growth (Hartwell and Unger, 1977): asymmetric cell division followed by a period of unbudded growth until a critical cell size is reached.

A second strain, L5365, gave rise to predominantly PH colonies (Figure 2, left). In most cases, when a single cell of this strain is inoculated in the growth chamber, it begins PH growth immediately, forming a PH microcolony over a number of hours. In some cases, a cell gives rise to a YF colony or a colony with sectors of YF and PH growth. We recorded the growth of many L5365 microcolonies and selected those for quantitative analysis that appeared most strongly induced toward PH growth. These records reveal that PH cells divide symmetrically (Figure 3B). A single cell of strain L5365 inoculated in the growth chamber begins PH growth immediately. Mother and daughter remain connected by a visible septum that forms along the boundary between the two cells. This septum serves as a visible marker for completion of cytokinesis. The PH budded period persists longer than that of YF cells. As a result, septation occurs when the volumes of mother and bud are equal. The mean volume of PH daughters was 1.03 ± 0.25 -fold ($n = 17$) that of the mother. The size of a PH daughter cell does not increase after emergence of the first bud. Thus, PH cells retain a characteristic size.

There Is No G1 Growth Period in PH Cells

We further characterized the budding of PH cells to examine growth in G1, the interval between septation and bud emergence. Growth of a primary lineage of PH cells can be represented by plotting the length of the most recent first bud, the cell at the tip of the filament, added to the sum of lengths of the cells of the previous generations (Figure 4). This plot reveals the following. 1) Extension of the length of the chain of first daughter cells is due solely to growth at the filament tip. 2) After septation, the length of each cell in the

Figure 3. Distinct cell cycles of YF and PH cells. Sequences of 16 single frames extracted from time-lapse video recordings of the growth of YF (A, L5439) and PH (B, L5365) colonies on low-ammonia media at 30°C. The frames in A are at 20-min intervals, whereas those in B are at 10-min intervals. D marks the nascent daughter cell, M the mother cell. The scale bar represents 10 μ m.

Figure 4. Growth of a PH filament over several cell cycles. The position of the tip of the main lineage of a growing L5365 PH strand was determined in video frames at 20-min intervals of a time-lapse recording. The length of the founder cell (O) and total length of the filament (●) are plotted vs. elapsed time. Carets (▼) indicate the first time point at which formation of a clear septum at the bud neck was observed, and arrows mark the first time point at which the cell wall showed changes characteristic of bud emergence.

filament remains constant. 3) Linear growth ceases for a short period each cell cycle. 4) This quiescent period is marked by septum formation, which compartmentalizes the mother and daughter and ends upon bud initiation by the daughter. 5) The interval between septation and bud emergence occupies only 5–10% of the daughter's cell cycle and is completely within the period marked by the absence of linear growth. 6) The interval from bud emergence to onset of the quiescent period before septation comprises about 85% of the first cell cycle of the daughter cell and is characterized by relatively constant linear growth. These data provide no evidence for a significant period of unbudded growth before bud emergence and are inconsistent with a cell cycle limited by a critical cell size at Start.

PH Mother and Daughter Cells Bud in Synchrony

The YF and PH budding cycles were examined using time-lapse video microscopy to observe multiple successive divisions in a single recording. Pedigrees were constructed using the times of each bud emergence from cells of a lineage derived from a single virgin daughter. In the pedigree of a YF lineage (Figure 5, YF), growth is characterized by asynchrony between bud emergence in mothers and their daughters. In the mothers, a second bud emerges soon after the separation of the first daughter and well before a new bud can be observed on the first daughter. In YF growth, daughter cells budded after mother cells, with a mean lag of 20 min ($\delta_{M-D} = 20 \pm 12$ min, mean $\pm$ SD, $n = 30$, Figure 6A). Because the rate of growth of buds on mother and daughter is similar, this delay is reflected in a proportional delay in cell division. Thus, asynchrony in cell division appears in the first cell cycle in YF growth.

By contrast, in the PH pedigree (Figure 5, PH), the first bud of the daughter cell and second bud of the mother cell grow and septate in near synchrony. This situation arises as buds emerge from both mother and daughter cells soon after septation. In contrast to the lag measured in YF budding, budding of PH daughter cells is synchronous with or leads that of the mother cell ($\delta_{M-D} = -3 \pm 6$ min, $n = 30$, Figure 6B). Lineages of PH cells may maintain budding synchrony for at least three generations. This persistent synchrony gives rise to the waves of budding shown in the PH pedigree in Figure 5.

Time-lapse video recordings of PH growth in three different strains show that mothers and daughters bud synchronously. In 30 PH cell divisions of the strain L5365, no mother cell budded before its daughter. In 20 of these divisions, mother and daughter budding was recorded in the same video frame, and in the remaining 10 divisions the daughter's division preceded that of the mother by 2–18 min. Of 25 PH divisions of strain CGX19 growing on SPHD (Gimeno *et al.*, 1992) observed at 10-min intervals, in 24, budding of mother and daughter was recorded in the same frame and 1 showed a daughter leading its mother by >10 min. We also examined PH growth in EM93 (Mortimer and Johnson, 1986), a dimorphic diploid ancestor of S288C, a standard laboratory strain incapable of dimorphic growth. Because EM93 grows first in the YF and only later as PH, observations of EM93 PH divisions were made on strands at the periphery of YF colony masses. Time-lapse records of 28 divisions of EM93 PH cells examined at 10-min intervals revealed that in 17 cases, bud emergence from mother and daughter was observed in a single frame and was therefore essentially simultaneous. In the remaining 11 cases, daughters led mothers by ≥ 10 min. Thus, in all strains we have studied, PH cell division is synchronous.

Figure 5. Pedigree analysis of YF and PH cell division. The times at which buds emerged on cells derived from a single founder were obtained from time-lapse records of L5439 YF and L5365 PH growth. A vertical line records the time that a bud was first observed. The horizontal line represents the lifetime of a cell. Incomplete lines in the PH record indicate that subsequent buds left the plane of focus.

We compared the generation times of first and second cell cycles in YF (L5439) and PH (L5365) growth (Figure 7). Although it takes longer for a virgin YF cell than a PH cell to produce a first daughter, this same YF cell produces a second daughter more rapidly than the PH cell produces its second daughter. As emphasized earlier, the YF mothers complete a cell cycle significantly faster (131 min) than their daughters (163 min), whereas PH mothers complete a second cell cycle in the same time as their daughters first budding cycle (147 vs. 142 min).

The Septum Divides Mother and Daughter into Two Distinct Cells

Video microscopy with Nomarski optics showed a visible septum forming between mother and daughter cells immediately before the appearance of the daughter's first bud. We used thin-section TEM to examine intact PH microcolonies of strain L5365 grown in a single plane (Figure 8). In many sections, the cell wall of a PH filament forms a continuous envelope surrounding each of the cellular segments. The septae that separate each cell appear as almost uniformly thick regions of lightly staining material continuous with and indistinguishable

Figure 6. Time differences between bud emergence from YF and PH mothers and daughters. Time-lapse records were examined to determine the time difference between first bud emergence from a daughter cell and second bud emergence from the mother cell as described in MATERIALS AND METHODS. The time of second bud emergence from the mother is assigned "0" time. Positive numbers indicate the daughter lags the mother. Negative numbers indicate the daughter leads or is synchronous with the mother. The distribution of lag and lead times for (A) 30 budding L5439 YF budding events and (B) 30 L5365 PH budding events are recorded on the respective histograms.

Figure 7. Division analysis of YF and PH growth after Hartwell and Unger (1977). (Top) A graphical representation of measurements taken to determine cell cycle parameters of budding in YF and PH growth. Interval A is defined as the time between emergence of cell A as a bud and the emergence of cell A's first bud, cell B. Interval B represents the time between emergence of B and emergence of a second bud on cell A. Interval A measures the time between emergence of B and the emergence of a first bud on B. (Bottom) A table showing the mean measured times in minutes and standard errors for cell cycle parameters A1, A2, and B1 during budding of L5439 YF and L5365 PH cells.

from the outer envelope of cell wall. Serial sections taken parallel to the long axis of mother-daughter pairs had sufficient resolution to detect cytoplasmic pores as small as 100 nm in diameter. In complete cell serial sections 90 nm thick of six junctions, no cytoplasmic connections were found. Thus, there is no evidence for an organelle or pore connecting the cytoplasm of mother and daughter.

Nuclear Division Occurs at the End of PH Bud Growth

The relationship between nuclear division and the extent of bud growth in PH cells was examined by fluorescence microscopy. PH cells from an exponential phase SLAD/LA culture of L5365 stained with DAPI were categorized with respect to representative stages in the mitotic cycle (Table 3). Cells designated as mitotic were those where the nucleus was clearly bilobed and/or distributed between a bud and its mother or a mother-daughter pair with two distinct nuclei but without a clear septum. Using these criteria, we found that mitotic cells constitute considerably <25% of the long cells. Examination of DAPI-stained PH filaments revealed that each segment of the filament contains a single nucleus (Figure 9). Cells in a filament shared a striking similarity in the appearance of the nucleus as if each cell in the filament were transiting steps in the cell cycle in synchrony. Upon completion of mitosis, the nucleus is centrally located and remains in this position through bud emergence. Before the bud grows to about one-third the length of the mother, the nucleus moves to the neck and remains

Figure 8. TEM of PH cells. Colonies of strain L5365 were grown in low ammonia agar, fixed in situ, and prepared for TEM as described in MATERIALS AND METHODS. (A) Typical section showing a cell, its mother, and their buds. (B) Higher magnification showing septum separating mother and first daughter cells. Ultrastructure observed by TEM confirmed the presence of one nucleus per cell and the expected cytoplasmic continuity between mothers and their nonnucleated buds. Vesicles were noted at the tips of small buds and along the mother's side of forming septae. A distinct polarity of organelle localization predominated in budded PH cells, with the nucleus in the cell's apical portion, vacuole basal, and the nucleus sectorized into light staining apical and dark staining basally oriented regions. Scale bar, 1 μm .

there until the daughter becomes nearly equal in size to the mother. Visual signs of mitosis, long extensions of the DNA mass out of the bud neck and into the cell

Table 3. Characterization of SLAD/LA growth of YF and PH cells

	No bud			All cells	
		Budded		Budded	Mitotic

YF	100% (163/163)	86% (204/237)	14% (33/237)	59% (237/400)	8% (33/400)
PH	100% (36/36)	78% (204/261)	22% (57/261)	88% (261/297)	19% (57/297)

Values are percents; with number of incidences per total number in parentheses. SLAD/LA liquid cultures were prepared as described in MATERIALS AND METHODS. Mitotic and budding indices of exponential L5535 YF and L5365 PH cultures were determined from exponential-phase cultures and each cell was classified as uninucleate or binucleate. Unbudded cells were always uninucleate. Cells with PH morphology (length $\sim 2 \times$ width) comprised 62% of the L5365 culture. The remaining cells had mitotic and budding indices intermediate between YF and PH.

body of both mother and daughter, are observed predominantly in cells where mother and daughter are of nearly equal size. Such mitotic nuclei are present in only a small proportion of cells with buds significantly smaller than the mother cell—such cells are almost uniformly marked by a single nuclear mass situated in the bud neck. In cells with buds of nearly equal size to the mother cell, we observed a distribution of nuclear morphologies consistent with S, G2, and M phases: a single nucleus localized in the mother cell, an elongated nucleus spanning the neck, or both mother and daughter each with a single centrally located nucleus. These data show that in PH growth, mitosis is restricted to cells that have doubled their size during the budded period.

The Majority of PH Cells Have Duplicated DNA

To identify the cell cycle parameters characteristic of YF and PH growth, we examined the distribution of

Figure 9. Nuclear morphology of PH cells during the budding cycle. PH florets from an exponential phase SLAD/LA suspension culture of strain L5365 were fixed and stained with the DNA dye DAPI as described in MATERIALS AND METHODS. Micrographs show Nomarski-DIC (top) and fluorescence (bottom) images of individual florets. (A) Postcytokinesis. (B) Bud emergence. (C) Small budded cells. (D) Large budded cells. (E) Karyokinesis. Note synchrony of timing of nuclear events among cells connected within a PH floret. Scale bar indicates 10 μm .

Figure 10. Flow cytometry of PH and YF cells to determine distribution of nuclear DNA content. Cells of the L5535 *ste7/ste7* constitutive YF strain and of L5365, the *PHD1*-overexpressing PH strain, were cultured, fixed, and prepared for flow cytometry by dispersing to single cells by shearing through a needle and staining with propidium iodide as described in MATERIALS AND METHODS. Histograms A and B show distributions of fluorescence intensity in 10 000 YF and PH cells, respectively. Abscissa in relative fluorescence units.

nuclear DNA in YF and PH cells by flow cytometry of propidium iodide stained cells grown in SLAD/LA. We compared the constitutively YF *ste7/ste7* mutant strain L5535 to the PH strain L5365 (Figure 10). The *ste7/ste7* diploid gives a homogeneous population of typical YF cells on SLAD/LA, whereas a wild-type strain (L5439) forms some PH cells on this medium. L5535 yields a flow cytometry distribution (Figure 10A) with an excess of 2N (G1) over 4N cells as is characteristic of *S. cerevisiae* growing under nutrient stress. Flow cytometry of the PH cells also reveals two peaks (Figure 10B), but their relative proportions are distinct from the YF cells. A significant excess of cells fall into the higher propidium iodide content peak that marks cells containing duplicated DNA. This distribution suggests that PH have a significantly increased proportion of cells with 4N G2/M DNA content and no obvious increase in S phase cells.

We further examined YF and PH cells by fluorescence-activated cell sorting of populations examined by flow cytometry to correlate nuclear DNA content to budding phenotype and nuclear position (Table 4). Samples sorted from the centers of the 2N and 4N peaks of fluorescence intensity were restained with propidium iodide and examined by both Nomarski DIC and epifluorescence microscopy. Nearly all the PH cells from the 2N population (all cells clearly members of the first peak) consist of unbudded cells. Similarly, the 4N population (all cells members of the second peak) consists almost exclusively of budded cells. The 4N population includes many long cells that carried buds with lengths less than one-fourth that of the mother. The PH cells

Table 4. Cell sorting of YF and PH cells by DNA content

Sorted window		Bud size				Budded	Mitotic
		G1 (%)	100	0	0	0	0% (0/77)	0% (0/77)
G2/M (%)	3	5	68	24	97% (92/95)	16% (11/70)

Fluorescence-activated cell sorted samples of PH (L5365) cells prepared as described in MATERIALS AND METHODS were examined to determine bud size and classified as uninucleate or binucleate. The data show that the central portion of the left peak (~15% of full range) contains exclusively unbudded uninucleate cells, consistent with G1 of the cell cycle. Cells from the center of the right peak (~15% of full range) are predominantly uninucleate with medium size buds. Only cells with length approximately two times width were counted. Their frequency in the culture was 63%.

Figure 11. Microtubule arrays in PH cells. PH cells were prepared for antitubulin immunofluorescence as described in MATERIALS AND METHODS. (Top) Nomarski-DIC image, (middle) DAPI imaging of nuclear DNA, and (bottom) anti-tubulin immunofluorescence. (A) Unbudded cell—unduplicated SPB. (B) Bud emergence—duplicated SPB. (C) Small bud—short complete spindle. (D) Medium bud—short complete spindle. (E) Large bud—short complete spindle. (F) Large bud—elongated spindle. Scale bar, 10 μ m.

in this population show a broad distribution of small, medium, and large buds, consistent with completion of DNA synthesis early in bud growth. These data suggest that the majority of YF cells have unduplicated DNA, whereas the majority of the PH cells are in a stage of the mitotic cell cycle that is post-DNA synthesis.

PH Growth Occurs Largely in G2

The distribution of nuclear DNA and the geometry of tubulin structures were used to estimate a mitotic index for PH growth. The classes of nuclear morphology observed in the DAPI staining correlated with tubulin distribution within the nucleus. In general, the organization of tubulin structures (Figure 11) was consistent with that previously described for the mitotic cycle of *Saccharomyces* (Adams and Pringle, 1984). Unbudded PH cells demonstrate a single centrally located nucleus with a tubulin distribution typical of G1 or early S phase—a single small aster of cytoplasmic microtubules. The majority of budded cells exhibit a short complete spindle characteristic of S, G2, and metaphase lying across the DAPI-staining region in the bud neck, connecting two origins of short cytoplasmic microtubules. In a proportion of small-budded cells, short spindles were not observed. Only mother–daughter pairs roughly equal in size had characteristic anaphase structures: a long extended spindle coincident with the DAPI-stained DNA separated into two masses.

The proportion of PH cells containing each type of tubulin structure was correlated with bud length. The G1/S tubulin distribution of a single or unseparated pair of spindle-pole bodies (SPBs) was present in 23% of the budded cells examined. Forty-nine percent of the budded cells displayed a short mitotic spindle separating duplicated SPBs characteristic of S/G2/metaphase and only 28% displayed a clearly extended spindle consistent with mitotic anaphase. A histogram of the bud length to mother length ratio for each class of tubulin organization (Figure 12) reveals a relatively consistent

progression of assembly of the mitotic apparatus through bud growth, with spindle pole separation and formation of a short spindle occurring early in bud for-

Figure 12. Distribution of tubulin structures in budded PH cells. Antitubulin-stained budded cells imaged by video microscopy were examined to determine bud length/mother cell length ratio and classified into one of three tubulin morphology classes: (A) unseparated spindle pole bodies or "undetermined," (B) short complete spindle, and (C) extended spindle. The distribution of bud/mother length ratios for each class is shown in the histograms for each class.

Figure 13. Actin arrays in PH cells. PH cells were prepared for rhodamine-conjugated phalloidin staining as described in MATERIALS AND METHODS. (Top) Nomarski-DIC, (middle) DAPI image of nuclear DNA, and (bottom) phalloidin fluorescence. (A) Bud emergence. (B) Large budded cells. (C) Cytokinesis. Scale bar is 10 μm .

mation, a long period of bud growth with a short complete spindle and extension of the spindle restricted to buds at least three-fourths the length of the mother cell. These results are consistent with a restriction on mitotic anaphase to PH cells with buds approaching the size of the mother cell.

The Actin Cytoskeleton of PH Cells Remains Polarized Throughout Bud Growth

Rhodamine phalloidin staining of the actin cytoskeleton of PH cells grown in SLAD/LA reveals significant differences in actin organization from that of YF cells. In both PH and YF cells, a cap of actin patches is associated with the site of bud emergence in the mother. In YF cells, the polarized actin patch disperses into small dots distributed over the bud surface and does not reappear in the daughter until just before bud emergence. By contrast in PH cells, an actin cap remains at the pole throughout bud growth (Figure 13). The cap disperses at cytokinesis then reappears before bud emergence.

Evidence for G₂/M Cell-Size Control in PH Growth

One mechanism for the long interval of growth before anaphase in PH cells is a cell-size checkpoint at G₂/M. Were this the case, the characteristic pattern of PH cell division should be maintained over a range of growth

rates. We tested whether retardation of growth with cycloheximide could release the apparent control of mitosis by bud size. Growth parameters measured from time-lapse recordings (Table 5) showed divergent responses of YF (L5535) and PH (L5365) cells to 0.06 $\mu\text{g}/\text{ml}$ cycloheximide. At this cycloheximide concentration, growth of YF microcolonies on low ammonia media (SLAR) is slowed 1.7-fold. As previously reported (Hartwell and Unger, 1977), YF cells exposed to growth inhibitory concentrations of cycloheximide produce smaller daughters. However, the critical cell size for Start is relatively unaffected. Thus, cycloheximide has the effect of further extending the period of unbudded growth in G₁. Cycloheximide has a marked inhibitory effect on PH development of L5365. Still, some PH microcolonies were observed. In such microcolonies, mitosis, septation, and synchrony of mother and daughter budding showed much the same pattern as cells multiplying in half the time (Figure 14). Examination of 15 PH mother-daughter pairs recorded by time-lapse microscopy showed that 6 pairs budded essentially simultaneously and in 7 of the remaining 9, the daughter budded with a lead of 4–28 min. In two pairs, the mother budded 36 and 44 min ahead of the daughter. Similarly, only a minority of PH cell divisions were markedly asymmetric—the mean mother-daughter ratio remained close to 1.0. These results suggest that in PH

Table 5. Characterization of YF and PH growth in the presence of cycloheximide

	YF		PH	
	SLAR	SLAR/cyh	SLAR	SLAR/cyh
δ_{M-D}	37 $\pm$ 5 (9)	65 $\pm$ 6 (11)	−6 $\pm$ 3 (8)	−2 $\pm$ 4 (15)
A1	198 $\pm$ 7 (9)	250 $\pm$ 6 (8)	133 $\pm$ 3 (6)	277 $\pm$ 5 (9)
B1	170 $\pm$ 6 (9)	175 $\pm$ 6 (8)	137 $\pm$ 4 (6)	327 $\pm$ 8 (9)
D/M	0.66 $\pm$ 0.03 (10)	0.52 $\pm$ 0.03 (12)	1.11 $\pm$ 0.08 (16)	0.92 $\pm$ 0.06 (11)

Values are shown as mean $\pm$ SD (δ_{M-D}) or mean $\pm$ SEM (A1, B1, and D/M) with sample number in parentheses. Mother-daughter budding interval (δ_{M-D}), generation times (A1 and B1, see Figure 7), and daughter to mother cell volume ratio (D/M) are tabulated for YF growth of L5535 and PH growth of L5365. SLAR/cyh indicates media containing 0.06 $\mu\text{g}/\text{ml}$ cycloheximide. Cells were cultured, recorded, and analyzed as described in MATERIALS AND METHODS. Time is recorded in minutes.

Figure 14. Growth of PH cells in the presence of cycloheximide. Pairs of single frames separated by a 4-h interval from time-lapse records of growth of the PH strain L5365 on low ammonia raffinose media with 0.06 µg/ml cycloheximide (A and B) and low ammonia raffinose media alone (C and D). Scale bar is 20 µm.

growth in the presence of cycloheximide, the reduced growth rate reflects increased time spent in G2 or metaphase.

Tyrosine Phosphorylation of Cdc28 Is Not Required for PH Growth

Cell size control at G2/M in *S. pombe* is mediated by regulation of the activity of *cdc2* via inhibitory tyrosine phosphorylation. We examined whether tyrosine phosphorylation of *CDC28* is involved in the proposed G2/M cell size control in PH cell division. To this end, we constructed strains in the Σ 1278b background where nonphosphorylatable alleles of *CDC28* (*CDC28* [Y19F] in strain L5667 and *CDC28* [TY18AF] in strain L5666) replaced the wild-type *CDC28* gene. The mutant strains form pseudohyphae indistinguishable from those of strains expressing wild-type phosphorylatable Cdc28. No difference could be observed in the extent of PH development in colonies or the size and shape of PH cells. These mutant PH cells exhibit the same symmetric cell division and synchronous budding pattern of cells carrying the wild-type *CDC28* gene. To confirm these results, we constructed strains lacking the *SWE1* kinase and/or the *MIH1* phosphatase that regulate tyrosine phosphorylation of *CDC28*. Strains carrying disruptions of *SWE1* or *MIH1* showed no dramatic effects on PH development.

PH Budding Is Unipolar

Previous observations of bud site preference in YF and PH cells growing under nitrogen starvation (Gimeno *et*

al., 1992) were consistent with the well-described budding pattern of *S. cerevisiae* growing on rich media (Chant and Pringle, 1991). Although the first bud of a virgin daughter cell is always at the pole opposite to the birth end of the cell, subsequent buds may distribute between either pole. Time-lapse microscopy allows observation of sites of bud emergence in both YF and PH cells. Because there is a visual record of the origin of each cell, the position of each bud on the mother can be oriented with respect to the mother's birth end. In both YF (>200 cells examined) and PH (>200 cells examined) virgin daughter cells, the first bud always emerged opposite the birth end. Subsequent buds of L5439 YF cells on SLAD distributed according to the diploid bipolar budding pattern. The second and third buds of YF cells emerged at either pole, although the second bud tended to appear opposite the birth end. The first three bud sites were recorded in 23 YF cells. In nine, all three buds were opposite the birth end. In seven, the third bud emerged at the birth end. In six, the second emerged at the birth end and the third appeared at the opposite pole. In one, the second and third buds were at the birth end. Subsequent buds continued to be distributed between apical and birth-end sites.

By contrast, the distribution of bud sites in PH cells is consistent with a unipolar budding pattern. All second buds on >100 PH cells examined in video records emerged at the pole opposite the birth end. In 20 PH cells where the first three buddings were observed, none

Figure 15. Fluorescence imaging of bud scar distribution of YF and PH cells. Calcofluor staining (see MATERIALS AND METHODS) of chitin rings revealing bud scars in L5439 YF (A–C) and L5365 PH (D–F) cells. (C, E, and F) Cells from cultures inoculated with few cells and allowed to grow for 48 h before calcofluor staining. Note single basal bud scar in F. Scale bar is 5 μ m.

emerged from the birth end. Not all budding events were resolvable in the time-lapse recordings. Because PH cells invade the agar medium, some buds were not observed that left the plane of focus.

A second method for analyzing bud site selection yielded results consistent with the time-lapse observa-

tions. We used suspension culture in SLAD/LA to obtain large numbers of cells from exponential phase cultures of L5535 (YF) and L5365 (PH). The cells were fixed, dispersed to single cells, and stained with calcofluor to reveal the chitin rings marking sites of bud emergence (Figure 15). This method does not permit reliable assignment of birth ends of cells, but all budding events are accurately recorded in the distribution of chitin rings. The bud pattern was determined by examining budded cells with two and three chitin rings (Table 6). Of budded YF cells with two chitin rings, 71% had both rings present at the same pole; the remaining 29% had one ring at each pole. Of YF cells with three rings, only 26% had all three on the same pole. These data confirm that YF growth of dimorphic yeast manifests the bipolar budding pattern described for other strains.

PH growth in suspension culture recapitulates the unipolar budding pattern observed in time-lapse video recordings. In all PH cells examined with two chitin rings, both were located at the same end, and in 97% of PH cells with three rings, they were localized to the same end. In SLAD/LA cultures inoculated at very low cell concentrations to extend the exponential phase of growth to >24 h, PH cells with as many as eight bud scars all at one end were readily observed. These data confirm that the PH budding pattern is unipolar rather than bipolar.

DISCUSSION

A Distinct Cell Cycle in PH Growth of S. cerevisiae

Our observations using video microscopy define a PH cell cycle in dimorphic strains of *S. cerevisiae* fundamentally different from the cell cycle of YF growth (Figure 16). The most striking distinction revealed in the

Table 6. Bud site selection revealed by calcofluor staining

Bud scar distribution	Two scars		Three scars		
YF	71% (125/177)	29% (52/177)	26% (25/97)	43% (42/97)	31% (30/97)
PH	100% (203/203)	0% (0/203)	97% (72/74)	3% (2/74)	0% (0/74)

Values are percents; with number of incidences per total number in parentheses. Exponential cultures of YF (L5535) and PH (L5365) cells were grown in SLAD/LA, fixed, and calcofluor stained as described in MATERIALS AND METHODS. Budded cells were classified by distribution and number of bud scars. Bud scars of diploid cells show a bipolar distribution in YF cells and a unipolar distribution in PH cells. Drawings depict each cell with its first bud scar on the left.

Figure 16. A model comparing the cell cycles of YF and PH cells. Although YF cells have a period of growth in G1 as well as during the budded period, all PH growth occurs during the budded period.

video records is that PH cells divide synchronously, whereas YF cells divide asynchronously. The continuous documentation of individual pedigrees provides a unique tool for discerning the underlying basis for the two cell cycles. Consider the fates of two nascent daughter cells just separated from their mothers. The newborn YF cell grows for a significant interval before initiating a new cell cycle. The period between cytokinesis and the initiation of a new division cycle may vary from a few minutes to greater than an hour. Once this cell has grown to a critical size, a bud emerges and matures and the cell divides to produce a new daughter. The period of growth after cytokinesis applies only to the first cell division. This cell enters subsequent division cycles without delay. Each newborn daughter is delayed by a period of growth before initiating a new cell cycle. YF mothers and daughters have distinct cell cycles imposed by the period of growth before budding in daughter cells. This difference in cycles between mothers and daughters results in the asynchrony observed in YF cultures.

By contrast, the newborn PH daughter, already equal to the mother cell in size, does not undergo a period of growth before budding. This cell buds and the bud in turn grows to the same size as the daughter. Within minutes of formation of the septum separating the cells, both mother and daughter cells reenter the cell cycle by budding. Each subsequent generation reiterates this pattern. PH mothers and daughters share the same cell cycle. Thus, in PH growth mothers and daughters bud synchronously.

Is There a G1 Delay in PH Growth?

The most obvious feature of the PH growth pattern we have established is that the G1 phase of the cell cycle

is dramatically shortened. Does this mean that there are G1-specific events present in YF cells but absent or accelerated in PH cells? The major cell cycle event specific to G1 in YF cells is growth of the newly formed cell. In PH cells the new daughter is as large as its mother and therefore has achieved the critical cell size required for initiation of a new cycle. If growth to a critical size were the only requirement for spindle pole body duplication, bud emergence, and entry into S, then it is possible that PH cells lack a G1 phase and pass Start immediately after the septum is formed. According to this interpretation, size alone and no other G1-specific event is required for the onset of DNA synthesis in PH cells. Indeed, YF mother cells appear to enter into S almost immediately after cytokinesis; it is only in YF daughters that an extended G1 phase intercedes before S phase.

The results of the cycloheximide experiment suggest that an essential feature of PH growth is a bypass of the unbudded growth interval characteristic of YF growth. Cycloheximide slows PH cell growth without altering the characteristic PH pattern of division. These PH cells exhibited an elongated budded interval by comparison with PH cells grown without cycloheximide. In the presence of cycloheximide, the proportion of PH cells was much lower than that in cultures grown without cycloheximide. Presumably, cycloheximide somehow blocks the conversion of YF to PH cells. Those YF cells that grow in the presence of cycloheximide are characterized by an elongated unbudded interval. The divergent responses of the two cell types in the presence of cycloheximide—extended unbudded period in YF cells and extended budded period in the PH cells—emphasizes the two different restriction points in the two cell types.

These considerations suggest that in the absence of an extended G1, the imposition of a new rate-limiting step after Start responsive to cell size would have the effect of inducing synchronous cell division. Alternatively, activation of an already characterized cell cycle checkpoint could serve as the “new” rate-limiting step. For example, limiting completion of DNA synthesis by growth in hydroxyurea or inhibition of spindle assembly by methyl-benzimidazole-2-ylcarbamate results in synchronous budding of mother and daughters (Johnston and Singer, 1983). Similarly, growth at semipermissive temperature of temperature sensitive *cdc* mutants, such as the essential DNA ligase gene *CDC9*, results in enlarged cells and synchronous budding of mother and daughter (Johnston and Nasmyth, 1978). In each of these examples, the synchrony between the mother and the daughter is the result of a temporal delay in completion of an event required for cell cycle progression coupled with continued metabolism and growth of the daughter. By the time of cytokinesis and cell separation, the size of the bud exceeds the critical cell size for Start; without size control, mother and daughter initiate cycles at the same time.

The Restriction Point in the PH Cell Cycle

The synchrony of cell division in PH cells appears to result from the imposition of a restriction point in the PH cell cycle that prevents the mother from initiating cell division before the bud has grown to full size. Our results place boundaries on where in the cell cycle this restriction point may lie. When a population of PH cells is examined for DNA content, the proportion of cells with duplicated DNA relative to those with unduplicated DNA appears to be much greater for PH cells than for YF cells. The simplest interpretation is that the majority of PH cells have completed DNA synthesis. These data suggest that the restriction point lies after S phase.

Analysis of nuclear and cytoskeletal events in the mother reveals a short bipolar spindle that appears soon after bud emergence. This short complete spindle separating duplicated spindle pole bodies is observed until the bud is nearly the same size as the mother. Only then do the microtubules elongate into a full-length spindle that spans both mother and daughter. Mitotic anaphase is therefore a very late event in the PH cell cycle; mitotic figures are rarely observed between a mother and daughter that are significantly unequal in size. These observations suggest that PH cells proceed rapidly from bud emergence through S to G2 or metaphase and arrest until the daughter grows to approximately the same size as the mother, whereupon mitotic anaphase and nuclear division can take place. These data place the restriction point between completion of DNA synthesis and onset of chromosome segregation.

The cell shape and distribution of the actin cytoskeleton of PH cells are consistent with an extended G2 period. Buds are elongated and sausage shaped and the actin distribution remains polarized at the growing end of the bud throughout most of the growth period. The elongated shape of the PH cells and the polarized actin pattern is reminiscent of the situation described for YF cells in which one of the G1 cyclins is overexpressed (Lew and Reed, 1993); such cells complete S phase but fail to leave G2 efficiently (Richardson *et al.*, 1989). A similar pattern of elongated bud shape is characteristic of *clb2* deletion mutants (Surana *et al.*, 1991). These comparisons between PH cells and cells carrying mutations in cell cycle components suggest that the restriction point for PH growth is present in G2. It should be noted that PH growth is not the consequence of a mutation; these cells exhibit an elongated bud and polarized actin cytoskeleton as regular features of their cell cycle.

Although these data place the restriction point within the cell cycle, they cannot distinguish between activation of a previously described checkpoint and the presence of a new cell-size checkpoint required for PH growth. Previously described mitotic checkpoints in *S. cerevisiae* include requirements for completion of S phase and spindle assembly. That a complete spindle is present

through much of PH bud growth is not consistent with activation of the MAD (Li and Murray, 1991) or BUB (Hoyt *et al.*, 1991) spindle-function checkpoints. In turn, preliminary studies suggest that DNA damage checkpoint control is not required for PH growth; the *rad9* disruption mutant has completely normal PH development (Franssen, Kron, and Fink, unpublished observations).

Critique of the Approach

A number of practical and theoretical issues impinge on the interpretation of our experiments. First, much of the time-lapse data and all of the flow cytometry, bud orientation, and nuclear DNA and tubulin imaging data have been collected with a strain overexpressing the *PHD1* PH inducer. This strain gave more homogeneous cultures of PH cells; wild-type strains were too sporadic in switching to the filamentous form to permit extended video recordings and failed to form a high proportion of PH cells in our liquid cultures. Conversely, we used the *ste7/ste7* diploid where we needed homogeneous cultures of YF cells grown on the same medium as the PH cells. On low ammonia medium, wild-type produces some PH cells, confounding the analysis. We believe these genetically altered strains give a faithful reflection of the two forms of growth because, in those cases where it was possible, comparisons with PH and YF growth in wild-type strains yielded similar data. The key observation: asynchronous growth of the YF and the synchronous growth of the PH cells is a feature of all wild-type and mutant strains tested.

We also used a novel type of suspension culture, liquid media over agar, to obtain large quantities of PH cells for cell sorting and cell polarity studies. This approach was necessitated by the inaccessibility of the PH cells grown on solid agar surfaces. Cells grown in suspension appear to recapitulate the PH growth pattern accurately. They have the characteristic elongated morphology of PH, remain attached in filaments and display the synchronous bud emergence and symmetrical cell divisions noted in the video records of cells grown on a solid substrate. Note that *PHD1*-overexpressing strains grown in suspension culture do not yield a homogeneous population of PH cells. As many as half of the cells appeared to be YF (axial ratios closer to 1:1 than 2:1) and many of these apparently YF cells were unbudded. These contaminating YF cells may have contributed a large component of the G1 peak seen in the flow cytometry data. The flow cytometry measurements of the PH cells could be biased in favor of the G2/M component by the presence of cell pairs that failed to be dispersed by our disruption procedure. However, our observations of sorted cells from the 4N content peak indicate that the disruption was effective. Also, attempts to further disperse the cells by shearing did not change the flow cytometry profile.

Comparison of *S. cerevisiae* PH and *S. pombe* Cell Cycles

There are striking similarities between the division pattern of fission yeast and the budding yeast PH growth pattern. In both *S. pombe* and filamentous *S. cerevisiae*, 1) division is symmetric, 2) the two cells formed after division each initiate a new cycle without appreciable delay, 3) DNA synthesis is completed relatively early in the cell cycle, 4) cells reside in G2 during the greater part of the cell cycle, and 5) mitosis and cytokinesis are restricted to a period of the cell cycle after cell mass has nearly doubled.

Nonetheless, *S. pombe* and PH cell divisions differ in the geometry of cell growth. In PH budding yeast, the bud emerges from the sausage-shaped mother cell only at the pole opposite the birth end. All growth is via enlargement of the bud, yet nuclear events up to mitotic anaphase take place within the mother cell. Once the bud grows to the size of the mother, a septum occludes the bud neck, dividing the cell into two distinct entities. Cells in a lineage remain attached to form filaments. In fission yeast, once the sausage-shaped cell grows to twice the length of the starting cell, the nucleus divides and a septum forms down the middle of the cell. The daughter cells separate, reenter the cell cycle, and begin to elongate at both ends. Fission yeast do not maintain a distinct orientation of growth end and septal end and do not form filaments.

A second difference between *S. cerevisiae* and *S. pombe* concerns the role of tyrosine phosphorylation of the cyclin-dependent kinase (cdc2/Cdc28) in controlling the G2/M transition. In *S. pombe*, the inactivating tyrosine phosphorylation of cdc2 by the wee1 kinase and the reactivating dephosphorylation by the cdc25 phosphatase are tied to cell growth via a network of kinases and phosphatases (reviewed by Feilottter *et al.*, 1992). We find no evidence for an essential role for an analogous pathway controlling Cdc28 tyrosine phosphorylation in G2/M cell-size control in *S. cerevisiae* PH growth. In fact, the nonphosphorylatable CDC28 allele lacking the tyrosine equivalent to the wee1 phosphorylation site on cdc2 conferred no discernible PH phenotypes, ruling out direct analogy between regulation of G2/M in *S. pombe* and *S. cerevisiae* PH cells. The mechanism of G2/M cell-size control in filamentous growth remains obscure.

The answer may lie in the observation that the PH cell cycle is under control of a signal transduction pathway. Mutations in the STE pathway block the PH growth pattern in a manner analogous to the mutations in the wee1 cell-size checkpoint gene in *S. pombe*. Ste⁻ *S. cerevisiae* and wee1⁻ *S. pombe* each perform a cell cycle regulated by cell-size control at Start. Extending this parallel, we propose that in PH growth, the STE pathway may inhibit the G2/M transition at the level of the cyclin-dependent kinase Cdc28. Perhaps, the STE

pathway is capable of inhibiting Clb/Cdc28 kinase in nitrogen-starved diploid cells in a manner analogous to the inhibition of Cln/Cdc28 by Fus3 and Far1 in pheromone-stimulated haploid cells. A candidate MAP kinase and CDK inhibitor have yet to be identified. Subsequent work may determine whether mitotic cyclins are indeed targets of the STE or other pathways responsible for induction of PH growth.

PH Cells Are a Distinct Cell Type

Previous studies of dimorphic *S. cerevisiae* established that PH cells have a characteristic cell shape unlike that of YF cells. We have extended these studies to show that pseudohyphae differ from YF cells in two other respects: pseudohyphae have a different cell cycle and exhibit a novel budding pattern. We propose that elongated cell shape, long budded period, and unipolar budding are essential features of PH growth; PH cells are not simply a chain of long YF cells. Rather, we conclude that the PH cell should join haploid and diploid vegetative YF cells, mating pheromone-arrested haploids, diploid zygotes, meiotic cells, and ascospores as another one of the distinct cell types in *S. cerevisiae*. As such, PH growth is a new developmental pathway in budding yeast responsive to environmental stimuli.

Altered cell cycles associated with developmental shifts are being uncovered in many organisms. In the formation of *Drosophila* and other animal embryos, initial embryonic cell cycles have no G1 or G2. These embellishments of the cell cycle and their differential control in distinct cell types are added only later in development of the embryo (Orr-Weaver, 1994). Dimorphism in budding yeast offers a genetically tractable system with which to dissect the association between a change in cell type and a change in the cell cycle. Of course, the key question is whether changes in developmental outcome are a cause or consequence of changes in the cell cycle. Elucidation of the interaction between the physiological signals and the genes that control the cell cycle in PH *S. cerevisiae* should contribute to answering this question.

ACKNOWLEDGMENTS

We thank Ya-Huei Tui for electron microscopy and Glenn Paradis and Linda Vaught for flow cytometry and cell sorting. We thank A. Amon, R. Booher, F. Cross, B. Futcher, M. Hoekstra, D. Lew, K. Nasmyth, J. Pringle, S. Reed, P. Russel, P. Sorger, B. Stillman, M. Tyers, C. Gimeno, H. Liu, and the rest of the Fink laboratory for generously providing reagents and for stimulating discussions. This work was supported by a Helen Hay Whitney Foundation fellowship to S.J.K. and NIH grants GM-35010 and GM-40266 to G.R.F. G.R.F. is an American Cancer Society Professor of Genetics.

REFERENCES

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

- Adams, A.E., and Pringle, J.R. (1991). Staining of actin with fluoro-chrome-conjugated phalloidin. *Methods Enzymol.* 194, 729–731.
- Amon, A., Surana, U., Muroff, I., and Nasmyth, K. (1992). Regulation of p34CDC28 tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. *Nature* 355, 368–371.
- Blacketer, M.J., Koehler, C.M., Coats, S.G., Myers, A.M., and Madaule, P. (1993). Regulation of dimorphism in *Saccharomyces cerevisiae*: involvement of the novel protein kinase homolog Elm1p and protein phosphatase 2A. *Mol. Cell. Biol.* 13, 5567–5581.
- Booher, R.N., Deshaies, R.J., and Kirschner, M.W. (1993). Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of p34CDC28 in response to G1 and G2 cyclins. *EMBO J.* 12, 3417–3426.
- Brandriss, M.C., and Magasanik, B. (1979). Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: enzyme induction by proline. *J. Bacteriol.* 140, 498–503.
- Byers, B. (1981). Cytology of the yeast life cycle. In: *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, eds. J.N. Strathern, E.W. Jones, and J.R. Broach, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 59–96.
- Cabib, E., and Bowers, B. (1971). Chitin and yeast budding. Localization of chitin in yeast bud scars. *J. Biol. Chem.* 246, 152–159.
- Chang, F., and Herskowitz, I. (1990). Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: *FAR1* is an inhibitor of a G1 cyclin, *CLN2*. *Cell* 63, 999–1011.
- Chant, J., and Pringle, J.R. (1991). Budding and cell polarity in *Saccharomyces cerevisiae*. *Curr. Opin. Genet. Dev.* 1, 342–350.
- Cross, F.R., and Tinkelenberg, A.H. (1991). A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. *Cell* 65, 875–883.
- Dirick, L., and Nasmyth, K. (1991). Positive feedback in the activation of G1 cyclins in yeast. *Nature* 351, 754–757.
- Elion, E.A., Grisafi, P.L., and Fink, G.R. (1990). *FUS3* encodes a *cdc2+*/CDC28-related kinase required for the transition from mitosis into conjugation. *Cell* 60, 649–664.
- Fantes, P., and Nurse, P. (1977). Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division. *Exp. Cell Res.* 107, 377–386.
- Feilotter, H., Lingner, C., Rowley, R., and Young, P.G. (1992). Regulation of the G2-mitosis transition. *Biochem. Cell Biol.* 70, 954–971.
- Forsburg, S.L., and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Annu. Rev. Cell Biol.* 7, 227–256.
- Gimeno, C.J., and Fink, G.R. (1994). Induction of pseudohyphal growth by overexpression of *PHD1*, a *Saccharomyces cerevisiae* gene related to transcriptional regulators of fungal development. *Mol. Cell. Biol.* 14, 2100–2112.
- Gimeno, C.J., Ljungdahl, P.O., Styles, C.A., and Fink, G.R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* 68, 1077–1090.
- Gould, K.L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast *cdc2+* protein kinase regulates entry into mitosis. *Nature* 342, 39–45.
- Grenson, M., Mousset, M., Wiame, J.M., and Bechet, J. (1966). Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. I. Evidence for a specific arginine-transporting system. *Biochim. Biophys. Acta* 127, 325–338.
- Hadwiger, J.A., Wittenberg, C., Richardson, H.E., de Barros Lopes, M., and Reed, S.I. (1989). A family of cyclin homologs that control the G1 phase in yeast. *Proc. Natl. Acad. Sci. USA* 86, 6255–6259.
- Hartwell, L.H., Culotti, J., Pringle, J.R., and Reid, B.J. (1974). Genetic control of the cell division cycle in yeast. *Science* 183, 46–51.
- Hartwell, L.H., and Unger, M.W. (1977). Unequal division in *Saccharomyces cerevisiae* and its implications for the control of cell division. *J. Cell Biol.* 75, 422–435.
- Hoyt, M.A., Totis, L., and Roberts, B.T. (1991). *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell* 66, 507–517.
- Hutter, K.J., and Eipel, H.E. (1979). Microbial determinations by flow cytometry. *J. Gen. Microbiol.* 113, 369–375.
- Johnston, G.C. (1977). Cell size and budding during starvation of the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* 132, 738–739.
- Johnston, G.C., and Singer, R.A. (1983). Growth and the cell cycle of the yeast *Saccharomyces cerevisiae*. I. Slowing S phase or nuclear division decreases the G1 cell cycle period. *Exp. Cell Res.* 149, 1–13.
- Johnston, L.H., and Nasmyth, K.A. (1978). *Saccharomyces cerevisiae* cell cycle mutant *cdc9* is defective in DNA ligase. *Nature* 274, 891–893.
- Kilmartin, J.V., Wright, B., and Milstein, C. (1982). Rat monoclonal antitubulin antibodies derived by using a new nonsecreting rat cell line. *J. Cell Biol.* 93, 576–582.
- Lew, D.J., and Reed, S.I. (1993). Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J. Cell Biol.* 120, 1305–1320.
- Li, R., and Murray, A.W. (1991). Feedback control of mitosis in budding yeast. *Cell* 66, 519–531.
- Liu, H., Styles, C.A., and Fink, G.R. (1993). Elements of the yeast pheromone response pathway required for filamentous growth of diploids. *Science* 262, 1741–1744.
- Ljungdahl, P.O., Gimeno, C.J., Styles, C.A., and Fink, G.R. (1992). *SHR3*: a novel component of the secretory pathway specifically required for localization of amino acid permeases in yeast. *Cell* 71, 463–478.
- Millar, J.B., McGowan, C.H., Lenaers, G., Jones, R., and Russell, P. (1991). p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast. *EMBO J.* 10, 4301–4309.
- Mortimer, R.K., and Johnston, J.R. (1986). Genealogy of principal strains of the yeast genetic stock center. *Genetics* 113, 35–45.
- Nasmyth, K. (1993). Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.* 5, 166–179.
- Nurse, P., and Bissett, Y. (1981). Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature* 292, 558–560.
- Nurse, P., and Thuriaux, P. (1980). Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics* 96, 627–637.
- Nurse, P., Thuriaux, P., and Nasmyth, K. (1976). Genetic control of the cell division cycle in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* 146, 167–178.
- Orr-Weaver, T.L. (1994). Developmental modification of the cell cycle. *Trends Genet.* 10, (in press).
- Pardee, A.B. (1989). G1 events and regulation of cell proliferation. *Science* 246, 603–608.
- Pringle, J.R., Preston, R.A., Adams, A.E.M., Stearns, T., Drubin, D.G., Haarer, B.K., and Jones, E.W. (1989). Fluorescence microscopy methods for yeast. *Methods Cell Biol.* 31, 357–435.
- Richardson, H.E., Wittenberg, C., Cross, F., and Reed, S.I. (1989). An essential G1 function for cyclin-like proteins in yeast. *Cell* 59, 1127–1133.
- Russell, P., Moreno, S., and Reed, S.I. (1989). Conservation of mitotic controls in fission and budding yeasts. *Cell* 57, 295–303.

- Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by *wee1⁺*, a gene encoding a protein kinase homolog. *Cell* 49, 559–567.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* 194, 3–21.
- Siddiqui, A.H., and Brandriss, M.C. (1988). A regulatory region responsible for proline-specific induction of the yeast *PUT2* gene is adjacent to its TATA box. *Mol. Cell. Biol.* 8, 4634–4641.
- Sikorski, R.S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- Sorger, P.K., and Murray, A.W. (1992). S-phase feedback control in budding yeast independent of tyrosine phosphorylation of p34cdc28. *Nature* 355, 365–368.
- Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A.B., and Nasmyth, K. (1991). The role of *CDC28* and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* 65, 145–161.
- Tyers, M., Tokiwa, G., and Futcher, B. (1993). Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J.* 12, 1955–1968.
- Wittenberg, C., Sugimoto, K., and Reed, S.I. (1990). G1-specific cyclins of *S. cerevisiae*: cell cycle periodicity, regulation by mating pheromone, and association with the p34CDC28 protein kinase. *Cell* 62, 225–237.
- Wright, R., and Rine, J. (1989). Transmission electron microscopy and immunocytochemical studies of yeast: analysis of HMG-CoA reductase overproduction by electron microscopy. *Methods Cell Biol.* 31, 473–512.