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FULL PAPER

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# Effects of growth temperature upshift on cell cycle progression in *Cryptococcus neoformans*

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**Abstract** Cell cycle progression of *Cryptococcus neoformans* was studied for cells grown exponentially at 15°, 24°, and 30°C. Except for speed, cell cycle progression was similar. In particular, budding occurred relatively soon after initiation of DNA synthesis at 15°, 24°, and 30°C. After growth temperature was shifted from 15° to 30°C, cells were transiently arrested before initiation of DNA synthesis. Thus, similar to *Saccharomyces cerevisiae*, "Start" was the main susceptible cell cycle controlling point in *C. neoformans.* However, after spontaneous release from arrest as above, cells were further arrested in the unbudded state. Thus, the timing of budding was delayed just before the $G_2$ phase, or even until after entering the $G_2$ phase, but it was also transient, and 5 h after the shift buds emerged relatively soon after initiation of DNA synthesis. Thus, *C. neoformans* cells can respond adaptively to mild stress by delaying budding. The existence of the second susceptible cell cycle control point, i.e., budding, appears to endow *C. neoformans* with a unique characteristic of stronger inhibition of multiplication than growth. A model of the *C. neoformans* cell cycle is also presented.

**Key words** Budding · Cell cycle · *Cryptococcus neoformans* · Growth temperature · Mild stress

## Introduction

"Start" in the late $G_1$ phase of the cell cycle is vitally important in cell cycle progression of the ascomycetous yeast *Saccharomyces cerevisiae* (reviewed in Lew et al. 1997). It triggers a series of events leading to the three landmarks of the mitotic cell cycle, i.e., initiation of budding, initiation of DNA synthesis, and the initial step of nuclear division by duplicating the spindle pole body. Usually the cell cycle runs until cytokinesis once the cell passes "Start," and thus the cells can enter the dormant $G_0$ phase only before "Start." "Start" also connects cell cycle progression with growth as the attainment of critical cell size is required before "Start" (Hartwell and Unger 1977; Lew et al. 1997). "Start" is usually the most sensitive point when the cells are subjected to various stresses. For example, sublethal heat shock elicits in *S. cerevisiae* transient growth arrest in the $G_1$ phase before "Start," in addition to thermotolerant reactions such as transcription of heat shock proteins (Barnes et al. 1990; Craig 1992; Piper 1993; Rowley et al. 1993; Li and Cai 1999; Raboy et al. 1999). Other stresses, such as osmotic shock, deprivation of nutrients, or oxidative agents, also elicit similar changes. In particular, growth arrest always occurs in the $G_1$ phase before "Start" (Lee et al. 1996; Wanke et al. 1999; Belli et al. 2001). The only exception to this is treatment that interferes with replication of DNA or nuclear division, resulting in $G_2$ arrest or arrest during mitosis (Jamieson 1992; Flattery-O'Brien and Dawes 1998).

We have reported that cell cycle control of the basidiomycetous pathogenic yeast *Cryptococcus neoformans* is different in several respects from that of *S. cerevisiae* (Takeo et al. 1995; Ohkusu et al. 2001a,b). Budding occurs relatively soon after the initiation of DNA synthesis when cells are rapidly growing as in *S. cerevisiae*. The timing of budding is delayed, however, into the $G_2$ phase of the cell cycle at transition to the stationary phase (Takeo et al. 1995; Ohkusu et al. 2001a), or under oxygen deficiency (Ohkusu et al. 2001b). The change in the timing of budding has not been definitely reported in any other yeast species, and how the delay occurs remains unclear. However, because growth conditions, and the succeeding cell cycle, change gradually in these two systems, time sequence analysis is difficult to perform. Furthermore, both these stresses become very strong with time, due to more severe deficit in either nutrients or oxygen. Thus, the cells deviate more and more from the usual progression of the mitotic cycle and are finally arrested either in the unbudded $G_1$ or unbudded $G_2$ phase (Takeo et al. 1995; Ohkusu et al. 2001a,b). Also, *C. neoformans* is a nonfermentative yeast, and therefore its

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responses to oxygen deficiency will be fundamentally different from those of fermentative yeasts, *S. cerevisiae* in particular.

The aim of the present work was to show the existence of an independent control mechanism for budding from that of "start" in *C. neoformans* under unperturbed exponential phase. For this purpose, we perturbed growth mildly under mild stress by shifting the growth temperature from 15°C to 30°C. Both temperatures were well within the growth limits of 10°C and 37°C. Here we present evidence that the timing of bud emergence in *C. neoformans* changes to later cell cycle points with a temperature shift from 15° to 30°C.

## Materials and methods

### Organisms and growth

Five IFM (Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University) strains of *C. neoformans* were used after single-colony isolation. Serotype, mating type, and ploidy are described in Table 1. Diploid strains were mostly used because their large cell size made it easy to discriminate the earliest stage of budding.

The cells were first incubated for 1 day to the exponential phase in YPG (1% each of glucose, yeast extract, and polypeptone). Then the culture was diluted with fresh medium and incubated overnight, to obtain $OD_{660\,nm}$ 0.1–0.5 cultivation, at various temperatures as described in the text. All cultivations were done under extensive aeration as described previously (Ohkusu et al. 2001b). In short, cultures with 30 ml YPG in 200-ml flasks plugged with cotton stoppers were shaken reciprocally at 150 rpm.

### Raising or lowering growth temperature

To raise or lower growth temperature, cells were grown at 15° or 30°C overnight to about OD 0.5. Then the flasks containing the cells were dipped into a water shaker at the growth temperature for 5 min and shaken vigorously. Next the flasks were transferred to an air shaker at the growth temperature for the time indicated.

### Fixation and morphological analysis of cell cycle phase

To avoid possible change of cells during fixation, 0.7 ml 100% ethanol precooled to 4°C was added to 0.3 ml cell culture solution. Both ethanol and culture solution were increased at the same rate when necessary. Cells were stained with 4′6-diamidino-2-phenyl-indole (DAPI; Sigma) solution (0.1 μg/ml), and observed under a fluorescence microscope. The proportion of cells having the morphological characteristics shown in Table 2 was scored among 100 cells. The same measurements were repeated five times with different cell samples and the average was scored.

### Analysis of cellular DNA content

After fixation of cells as above, cellular DNA was stained with 5 μg/ml propidium iodide and 0.5 mg/ml RNase from bovine pancreas (Sigma type 1-AS) as described previously (Ohkusu et al. 2001a).

To analyze DNA content in each cell with regard to cell morphology, cells stained with propidium iodide were examined with a fluorescence microscope (Olympus model BS2) equipped with a photomultiplier as described previously (Ohkusu et al. 2001a).

To examine the distribution of cells having a particular DNA content, large numbers of cells were measured using a laser scanning cytometer (Olympus LSC model 101) (Ohkusu et al. 2001a); this uses long-range laser light for good focusing, and thus is able to quantify more than 10000 cells for each measurement in a short time.

## Results

### *Cryptococcus neoformans* grew optimally at 30°C

*Cryptococcus neoformans* was grown at various temperatures between 15° and 34°C. Mass doubling time was assessed by measuring optical density of cultures time sequentially; it was 4 h 18 min ± 1 h 6 min at 15°C (averaged for five strains; see Table 1), 2 h 50 min ± 22 min at 20°C and 2 h 28 min ± 21 min at 24°C. Mass doubling time took the shortest time of 1 h 52 min ± 18 min at 30°C, but it was 2 h

**Table 1.** Mass doubling time of *Cryptococcus neoformans* at growth temperatures between 15° and 34°C

| Strains (IFM) | Sero-type | Mating type | Mass doubling time | | | | |
|---|---|---|---|---|---|---|---|
| | | | 15°C | 20°C | 24°C | 30°C | 34°C |
| 47848 | A | αα[a] | 4:45[b] | 2:25 | 2:20 | 2:20 | 2:20 |
| 5860 | AD | a/α[c] | 4:42 | 3:05 | 2:30 | 1:45 | 2:00 |
| 49144 | A | u[d] | 5:42 | 3:00 | 2:55 | 1:55 | 2:20 |
| 51642 | A | αα | 3:00 | | | 1:30 | |
| 51657 | A | αα | 3:24 | | 2:06 | 1:50 | |

[a] Heterothallic diploid (α-mating type)
[b] 4:45 should be read as 4 h 45 min
[c] Homothallic diploid showing both α- and a-mating types
[d] Haploid (untypable)

**Table 2.** Changes of the index of budding with tiny bud and of the mitotic index after temperature shiftup

| Strains | Unbudded index | | Index of budding with | | | Mitotic index |
|---|---|---|---|---|---|---|
| | Before separation | Existing singly | Tiny bud[a] | Small bud[b] | Medium[c] to large bud[d] | |
| 51657 | | | | | | |
| 15°C | 29.2 ± 6.1 | 22.6 ± 2.2 | 5.6 ± 1.8 | 6.4 ± 3.7 | 20.4 ± 3.6 | 15.8 ± 5.2 |
| 0.5 h[e] | 37.4 ± 4.3 | 35.4 ± 5.4 | 2.0 ± 1.0 | 2.2 ± 1.1 | 16.0 ± 4.9 | 7.0 ± 3.7 |
| 1 h | 33.0 ± 4.6 | 48.6 ± 6.1 | 2.2 ± 0.8 | 2.8 ± 2.5 | 9.8 ± 1.3 | 3.6 ± 1.5 |
| 2 h | 34.8 ± 4.2 | 36.6 ± 3.2 | 3.8 ± 1.1 | 2.6 ± 0.9 | 12.2 ± 2.2 | 10.0 ± 4.7 |
| 30°C | 23.6 ± 5.2 | 37.0 ± 5.7 | 3.2 ± 1.9 | 1.8 ± 1.9 | 16.0 ± 1.9 | 18.4 ± 3.6 |
| 5860 | | | | | | |
| 15°C | 18.0 ± 3.4 | 31.4 ± 7.3 | 7.4 ± 2.9 | 7.0 ± 2.4 | 12.6 ± 3.2 | 23.6 ± 6.0 |
| 0.5 h | 33.4 ± 7.1 | 39.4 ± 6.7 | 4.0 ± 1.9 | 5.4 ± 2.3 | 11.6 ± 3.4 | 6.2 ± 2.2 |
| 1 h | 48.2 ± 7.7 | 38.6 ± 7.4 | 1.2 ± 0.8 | 2.8 ± 1.3 | 6.0 ± 1.4 | 3.2 ± 1.1 |
| 30°C | 31.6 ± 11.1 | 31.0 ± 11.0 | 4.8 ± 1.3 | 6.4 ± 1.8 | 12.4 ± 3.3 | 13.8 ± 2.6 |

[a] Tiny bud taking a pimple form; see Fig. 2 in Ohkusu et al. 2001a
[b] Small bud with round tip having a diameter smaller than 1/8 that of the mother
[c] Medium bud having 1/8–1/4 of the diameter of the mother
[d] Large bud having 1/4–1/2 of the diameter of the mother; cells during mitosis were not counted here
[e] Number indicates hours after shift from 15° to 30°C

**Table 3.** The average duration time for landmark cell cycle stages

| Strains | Temperature | | Unbudded period | | Budded period having | | | Mitosis |
|---|---|---|---|---|---|---|---|---|
| | | | Before separation | Existing singly | Tiny bud[a] | Small bud[b] | Middle[c] to large bud[d] | |
| 51657 | 15°C | Cells found (%) | 29.2 ± 6.1 | 22.6 ± 2.2 | 5.6 ± 1.8 | 6.4 ± 3.7 | 20.4 ± 3.6 | 15.8 ± 5.2 |
| | | Duration time (min)[e] | 58 | 45[f] M[g]: 20 D[g]: 70 | 11[h] | 13[h] | 41[h] | 32[h] |
| | 30°C | Cells found (%) | 23.6 ± 5.2 | 37.0 ± 5.7 | 3.2 ± 1.9 | 1.8 ± 1.9 | 16.0 ± 1.9 | 18.4 ± 3.6 |
| | | Duration time (min) | 26 | 41 M: 10 D: 70 | 4 | 2 | 18 | 20 |

[a,b,c,d] See legend to Table 2
[e] Mass doubling time was shown to be 204 min by optical density (see Table 1); i.e., 1% cells means that the duration time is 2 min; thus, if cells of N % were found in a certain cell cycle, then its duration time is calculated to be 2N min
[f] Represents average for the mother and daughter cell
[g] M and D indicate the value for the mother and the daughter, respectively; see text for details
[h] The value for the mother and for the daughter is estimated to be similar; see text for details

13 min ± 12 min at 34°C, indicating that cells grew and divided at the highest rate at 30°C.

*C. neoformans* grown to OD 1–2 at the above temperatures was observed under the light microscope. In all the strains and at all temperatures, cells took a round or nearly round shape, typical of this species. The average cell size showed a tendency to become larger with temperature, although to only a small extent, and the size was 10% larger at 30° than at 15°C. In contrast to *S. cerevisiae*, the mother cells separated from the daughter cells before the next bud emerged at of 15°, 20°, 24°, and 30°C; this made observation of *C. neoformans* cells, especially the existence of the small buds, easier. Separation was delayed slightly at 34°C. Therefore, we studied the cells grown at 15° and 30°C in detail.

## Cell cycle proceeded similarly at 15° and 30°C except for duration

The time required to pass each phase of the cell cycle was estimated roughly. First, we describe strain IFM 51657 grown at 15°C. Cells stained with DAPI were grouped into each phase as described in the first row of Table 3, using the morphology of the bud and the nucleus as landmarks. Mass doubling time was 3 h 24 min, i.e., roughly 200 min (Table 1); this indicates that a certain cell cycle stage containing N % cells is estimated to continue for 2N min (Table 3).

In the unbudded period 1 (after mitosis to cell separation), the proportion of cells in doublet was roughly 30% (Table 3). Thus, both the mother and the daughter cells were estimated to take 1 h before separation. In unbudded period 2 (after separation to budding), it should be noted that mother cells of *C. neoformans* soon produced buds after they separated from their daughters, a character mak-

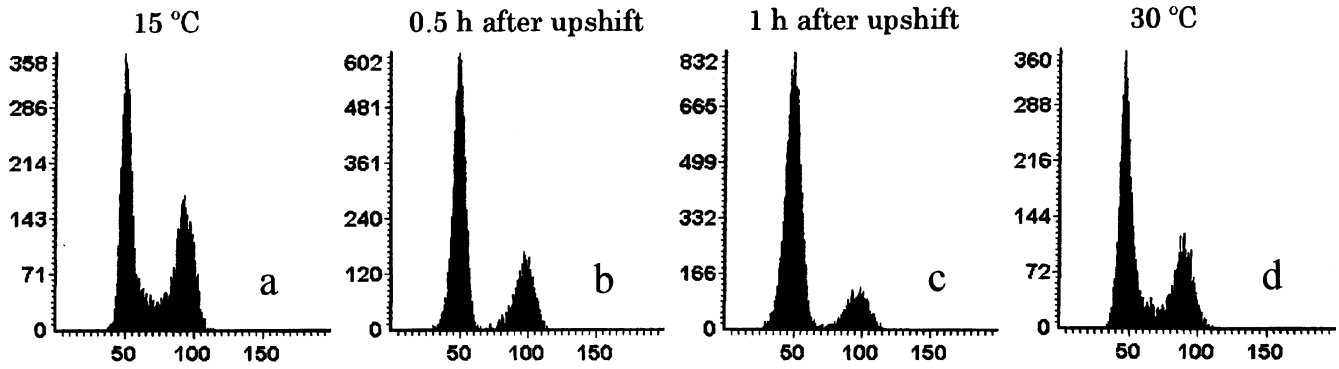


**Fig. 1.** Change in cell cycle distribution of *Cryptococcus neoformans* IFM 5860 after shift from 15° to 30°C as examined by laser scanning cytometer. Horizontal axis shows propidium iodide fluorescence value; vertical axis shows full scale count, i.e., number of cells showing a particular propidium iodide fluorescence value

ing time course estimate easy. In *C. neoformans* a new bud emerged exactly where the daughter cell had attached to the mother, thus physically expelling the preexisting daughter cell. The volume of the daughter cells at division was clearly smaller than that of the mother cells (figure not shown). It is generally accepted that prolongation of doubling time occurs in the $G_1$ phase before "Start" to attain critical cell size (Hartwell and Unger 1977; Lew et al. 1997), i.e., the daughter cells spend longer time than the mother in $G_1$ phase. Cells in unbudded period 2 were 23% (Table 3). From these results, the mother cells were estimated to have existed singly for roughly 20 min. For the purpose of making the easy estimate, the number of the mother was assumed to be not much different from that of the daughter. Then, the mother cells (20 min) contributed 5%, and thus the daughter cell contribution should be roughly 18%, i.e., the daughter should exist singly for 70 min. Thus, the mother cells were roughly estimated to spend 1 h 20 min in the unbudded period, and the daughter cells, 2 h 10 min.

In the tiny bud period, cells were estimated (from Table 3) to spend 11 min. In the small bud period, cells were estimated to spend 13 min. In the medium- to large-bud period, cells were estimated to spend 41 min. Taken together the budded period before mitosis can be estimated to be 1 h 5 min. In the final period the cells were engaged in mitosis. From Table 3, this period was estimated to be 32 min.

The cells grew and multiplied much quicker, mass doubling time being 110 min (see Table 1), when the cells were grown exponentially at 30°C. However, the proportion of the cells having the above morphological characteristics was similar (Tables 2, 3). The duration time for landmark cell cycle stages is shown in Table 3. The cells spent 67 min (the average of the mother and the daughter cells) during the unbudded period, 24 min during the budded period before mitosis, and 20 min during mitosis (Table 3). Mass doubling time of *S. cerevisiae* is known to increase in low-nutrition media. Prolongation of doubling time is generally accepted to occur in the $G_1$ phase before "Start" to attain critical cell size (Hartwell and Unger 1977; Lew et al. 1997). Thus, general prolongation, in both the unbudded and the budded periods, at 15°C compared with 30°C was unexpected, which may be explained by the fact that chemical reaction speed doubles when reaction temperature is raised by 10°C. Similar cell cycle progression was observed in the other strain examined, IFM 5860 (Table 2).

The distribution of DNA content per cell among cell samples collected at various ODs was examined by laser scanning cytometer. It revealed two peaks, the first corresponding to $G_1$ and the second to $G_2$ cells, the S phase between the two peaks (Fig. 1a,d).

To see whether the emergence of buds varies from initiation of DNA synthesis with growth temperature, the DNA content of each cell was measured with regard to cell morphology. The daughter cells which were still attached to the mother after cytokinesis always had $G_1$ DNA content, regardless of the growth temperature (data not shown). The mother cells that had large buds always had a DNA content near $G_2$, regardless of growth temperature (data not shown). In contrast, the DNA content of cells with tiny buds varied with strain and growth temperature (Table 4). Budding occurred at later cell cycle point in strain IFM 49144 compared with other strains examined (Table 4). The budding appeared to occur a little later at 30°C compared with that at 15°C, but the differences were not distinct. Thus, it was safe to say that budding followed relatively soon after initiation of DNA synthesis when cells were grown exponentially at 15°, 24°, or 30°C (Table 4).

## A mild rise in growth temperature transiently arrested cells before initiation of DNA synthesis, budding, and mitosis

Cells were first grown at 15°C until the exponential phase of OD 0.5–1 and then the growth temperature was shifted to 30°C. Cells grown at 30°C for 0.5–5 h were observed under the microscope. Morphology of the cells appeared normal (figures not shown), but cell cycle progression changed; 0.5–1 h after the shift, the cells in S phase became reduced almost to zero (Fig. 1b,c). The S value became normal 2 h after the shift (data not shown). These facts indicated that

the cells were transiently arrested before initiation of DNA synthesis, but soon recovered. The next perturbation was a more pronounced delay in budding (Tables 4, 5). Thus, buds emerged in the $G_2$ or near the $G_2$ phase. The timing of the budding also recovered 5 h after the shift. The delay of budding was also reflected by the fact that the index of budding having tiny buds decreased for 0.5–2 h after the shift (see Table 2).

Fluorescence microscopy revealed that the cells during mitosis decreased to less than half from 0.5 to 1 h after the mild heat shock (Table 2), indicating that arrest also occurred before mitosis.

We were also curious whether the timing of budding is delayed after lowering growth temperature from 30° to 15°C. No appreciable delay in the timing of budding was observed (data not shown).

A milder stress of raising growth temperature from 24° to 30°C did not delay the timing of budding to $G_2$ (data not shown), which is easy to understand. If the cell cycle is disturbed before $G_2$ budding even after a small "insult" or stress, the consistency of the cell cycle progression could not be retained. Probably there exists a threshold level of stress to cause delay of budding into the $G_2$ phase.

## Discussion

In the present study, *C. neoformans* cells were shown to be transiently arrested before initiation of DNA synthesis under mild heat shock, as expected from observations on *S. cerevisiae* (Barnes et al. 1990; Rowley et al. 1993; Lee et al. 1996; Li and Cai 1999; Belli et al. 2001). After spontaneous release from the arrest, however, *C. neoformans* cells were further arrested in an unbudded state, resulting in $G_2$ phase budding. Considered together with our observations reported previously (Takeo et al. 1995; Ohkusu et al. 2001a,b), the mechanism of *C. neoformans* cell cycle control is different in some important respects from that of *S. cerevisiae*. A model is shown schematically in Fig. 2.

The most important two events in the eukaryotic cell cycle are DNA synthesis and mitosis. The point that commits the cell to initiation of DNA synthesis is called the "$G_1$

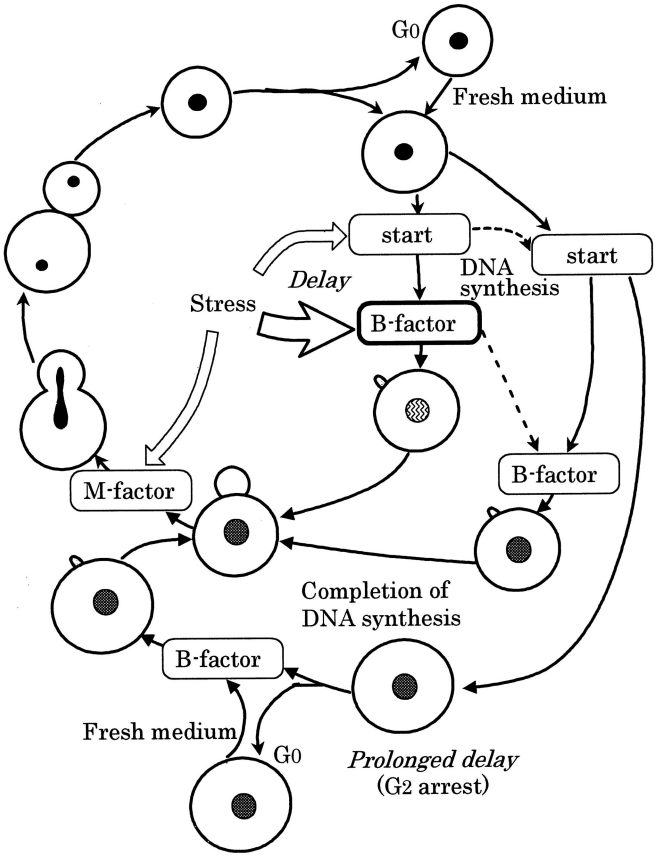


**Fig. 2.** A model of *C. neoformans* cell cycle control. Instead of strong control at "Start" as in *Saccharomyces cerevisiae*, after which the cell goes through the cell cycle to cytokinesis, *C. neoformans* has two controlling points, i.e., "*Start*" and *B-factor*. See Discussion for details. In addition to the delay after temperature upshift described here, oxygen deficiency and transition to the stationary phase greatly delay cell cycle progression (Takeo et al. 1995; Ohkusu et al. 2001a,b)

**Table 4.** Relative DNA content of mother cells with tiny buds at growth temperatures of 15°, 24°, and 30°C

| Strains (IFM) | 15°C | 24°C | 30°C |
|---|---|---|---|
| 47848 | 1.28[a] ± 0.18[b] (19[c]) | 1.28 ± 0.19 (12) | 1.47 ± 0.20 (14) |
| 5860 | 1.23 ± 0.10 (28) | 1.26 ± 0.13 (19) | 1.33 ± 0.21 (25) |
| 49144 | 1.55 ± 0.19 (21) | 1.54 ± 0.27 (28) | 1.69 ± 0.19 (26) |
| 51642 | 1.27 ± 0.15 (29) | 1.17 ± 0.12 (25) | 1.38 ± 0.16 (26) |
| 51657 | 1.31 ± 0.16 (24) | 1.23 ± 0.17 (16) | 1.40 ± 0.23 (19) |

[a] Normalized amount of DNA in cells having tiny buds (see Ohkusu et al. 2001a for details); DNA content of daughter cells was standardized as = 1
[b] Standard deviation
[c] Number of cells measured

**Table 5.** Delay of the timing of budding after temperature upshift

| Strains (IFM) | 0 h | 0.5 h | 1 h | 2 h | 5 h |
|---|---|---|---|---|---|
| 47848 | 1.28[a] ± 0.18[b] (19[c]) | 1.95 ± 0.17 (20) | 1.67 ± 0.25 (14) | 1.76 ± 0.24 (16) | 1.44 ± 0.12 (19) |
| 5860 | 1.23 ± 0.10 (28) | 1.86 ± 0.15 (25) | 1.71 ± 0.21 (19) | 1.66 ± 0.24 (26) | 1.43 ± 0.11 (14) |
| 49144 | 1.55 ± 0.19 (21) | 2.01 ± 0.16 (23) | 2.11 ± 0.17 (19) | 1.99 ± 0.21 (27) | 1.72 ± 0.23 (21) |
| 51642 | 1.27 ± 0.15 (29) | 1.88 ± 0.27 (27) | 1.81 ± 0.29 (39) | 1.37 ± 0.17 (14) | 1.41 ± 0.16 (16) |
| 51657 | 1.31 ± 0.16 (24) | 2.06 ± 0.17 (32) | 2.04 ± 0.17 (24) | 1.34 ± 0.16 (16) | 1.41 ± 0.11 (16) |

Time indicates hours after shift from 15° to 30°C
[a,b,c] See legend to Table 4

restriction point" in animal cells or "Start" in *S. cerevisiae*, i.e., the point is universal among eukaryotic cells (Reed 1996). Thus, the "Start" of *C. neoformans* should be, first of all, to commit the cell to initiation of DNA synthesis, in addition to general transcription of genes that set the cell cycle progress (Breeden 1996).

As to the timing of *C. neoformans* budding, we tried to find unreplicated mother cells that have middle-sized or large buds, but without success, in spite of our long experience of measuring DNA for 50 000 cells or more. The mothers with pimple-shaped buds, i.e., the earliest stage of bud formation discernible, had a DNA value clearly larger than that of $G_1$ phase cells (see Table 4). This fact strongly suggests that commitment to budding does not occur before "Start"; i.e., it occurs either at "Start" as in *S. cerevisiae* (Lew et al. 1997) or later. If we suppose that it occurs at "Start," i.e., concomitantly with initiation of DNA synthesis, then it is not easy to understand why the budding is easily delayed until the $G_2$ phase after mild heat shock (from 15° to 30°C), and cells even fell into the dormant $G_0$ phase after commitment to budding under oxygen deficiency (Ohkusu et al. 2001b). A more detailed discussion is as follows. The time from "start" or rather initiation of DNA synthesis to bud emergence can be estimated fairly exactly by the amount of DNA at bud emergence, the "bud-emerging DNA value." Budding is a very important event, directly leading the cell to multiplication. Thus, the time required for bud emergence after commitment, i.e., the "bud-emerging DNA value" should be relatively constant among strains, at least when cells are grown rapidly in a rich YPG medium at an optimal temperature of 30°C under extensive aeration. In fact, however, the value differed considerably among strains (see Table 4). Furthermore, even unbudded cells sometimes exhibited the $G_2$ DNA value or a value near it when the cells were exponentially grown (unpublished data). These lines of evidence strongly argue against the supposition that "Start" directly commits to budding. Thus, commitment to budding must occur later than "Start," i.e., execution of some hitherto unknown B-factor is needed for initiation of budding in *C. neoformans* (see Fig. 2). This discussion also strongly suggests that the B-factor is not related to initiation, completion, or any point of DNA synthesis.

Spindle pole body duplication can be regarded as the initial step of mitosis. As to its timing, there are three logical possibilities, i.e., at "Start" concomitantly with DNA synthesis as in the case of *S. cerevisiae* (Lew 1997), concomitantly with initiation of budding under the control of the B-factor, or concomitantly with the actual onset of mitosis as in *Schizosaccharomyces pombe* (Ding et al. 1997). It is relevant that *C. neoformans* cells exhibit a high probability of spontaneous diploidization (Hata et al. 2000), which is explained naturally if spindle pole body duplication is committed together with DNA synthesis at "Start" as follows. Execution of the B-factor is easily prolonged under several conditions until the $G_2$ phase, i.e., budding does not occur even after completion of DNA synthesis (legend to Fig. 2 and unpublished data). There may be exceptional cases when execution of a new "Start" followed by a new cell cycle occurs without execution of the old B-factor. Then, the cell naturally possesses a double amount of DNA and also two spindle pole bodies, i.e., it becomes diploid.

Cell cycle control may be variable among species within several restrictions for the sake of better survival. The eukaryote cells are apparently weak during the periods of DNA synthesis and mitosis followed by cytokinesis. Thus, these fall into the dormant $G_0$ state either from $G_1$ before the "restriction point" or $G_2$ before mitosis.

Budding is a unique mode of multiplication among the eukaryotic cells. The budded state is intrinsically physically weak. Also, the rather rigid mother cell wall is elasticized by wall lytic enzymes before bud emergence. The new bud cell wall is thin and chemically weak. For example, the cell wall in the bud part is more easily digested by snail and other lytic enzymes than that in the mother. Thus, yeast cells need to avoid a long budded period. The following characteristics of *S. cerevisiae* suit this purpose. Critical cell size requirement is attained before "Start," and during budding cell expansion occurs mostly in the bud part (Woldringh et al. 1993). Probably this is also true in most yeast species.

Cell cycle control of *S. cerevisiae* is further concentrated in "Start" (see Introduction and also literature cited there). Once "Start" is passed, the cell goes through the mitotic cell cycle to cytokinesis, and thus the cell cannot fall into dormant $G_0$ after "Start," which fulfills the need to avoid a long budded period.

In *S. pombe*, the cell is also committed to the mitotic cell cycle and initiation of DNA synthesis at "Start" (as a review of *S. pombe* cell cycle control, see Fantes 1989). However, *S. pombe*, which grows by elongation and multiplies by fission, eliminates the restriction posed on budding yeasts. Thus, *S. pombe* cells are enabled to perform critical cell size requirement and spindle pole body duplication in the $G_2$ phase just before onset of mitosis. Cell size at cytokinesis is usually larger than the critical cell size required for "Start," and thus the cell initiates DNA synthesis soon after cytokinesis, making "Start" control cryptic. "Start" control is not so strong that the cells can enter into $G_0$ phase either from the $G_1$ or the $G_2$ phase.

Although well suited to the need to avoid a long budded period, *C. neoformans* cell cycle control is not concentrated. In addition to "Start" when the cell is committed to initiation of DNA synthesis, there is another control point, the B-factor, that actually commits the cell to initiation of budding. Double control for DNA synthesis and budding appears to be suitable under adverse circumstances. After delayed "Start" the cell continues growth, although slowly due to adverse growth conditions, without budding (Fig. 2). The cell is large enough when the B-factor is eventually executed that the cell is able to complete bud formation relatively soon even under adverse conditions. Another route, to fall into dormancy, is also provided. Probably these are the reason why most *C. neoformans* cells become quickly unbudded during transition to the stationary phase (see Fig. 1 in Takeo et al. 1995), whereas in *S. cerevisiae* several cells persist in the budded state. The existence of the B-factor gives *C. neoformans* more flexible cell cycle control than there is in *S. cerevisiae*.

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