Control of Cell Size at Bud Initiation in *Saccharomyces cerevisiae*

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The cell volume at bud initiation for both haploid and diploid cells of *Saccharomyces cerevisiae* is dependent on growth rate within the mass doubling time range 2.1 to 3.7 h. At slower growth rates, the volume at bud initiation is independent of growth rate. At all growth rates, the volume at bud initiation for diploids is 1.7-fold larger than that for haploids. When unbudded cells from synchronous cultures growing in poor media are shifted to rich medium, all except those very close to the size characteristic of bud production on poor medium go on to produce a bud at the larger size characteristic of the richer medium. Cells do not become committed to producing a bud at the size characteristic of bud initiation on ethanol medium (a poor medium) until they are within about $3\ \mu\text{m}^3$ of that size. When unbudded cells are shifted from rich medium to poor medium, cells that are smaller than the size of bud initiation on the poor medium produce a bud at the size characteristic of the poor medium. If they are larger than the commitment size for the poor medium at the time of the shift, they produce a bud without appreciable growth. These results are examined in relation to the unstable inhibitor model for the control of cell division.

INTRODUCTION

There has been considerable interest recently in how cells coordinate the processes of growth and cell division. Much of this interest centres on three eukaryotes – *Saccharomyces cerevisiae* (Hartwell & Unger, 1977; Jagadish *et al.*, 1977; Johnston *et al.*, 1977; Carter, 1978; Carter & Jagadish, 1978), *Schizosaccharomyces pombe* (Nurse, 1975; Mitchison, 1977) and *Physarum polycephalum* (Sudbery & Grant, 1975).

Johnston *et al.* (1977) have proposed that coordination between these two processes in *Saccharomyces cerevisiae* is achieved by the necessity to attain a critical cell size before an event at, or just before, the cell cycle step identified by the *cdc 28* mutation. It is difficult to determine accurately the critical size at the *cdc 28* step because the *cdc 28* mutation is slightly leaky. We have investigated the critical size by measuring the cell volume at bud initiation (an event which occurs very shortly after the *cdc 28* step) at different growth rates. Our results show that the cell size at bud initiation varies inversely with generation time over the mass doubling time range 2.1 to 3.7 h. Thus the critical size necessary for initiating the DNA division cycle is different in different environments. We have investigated the point in the unbudded phase of the cycle at which cells become committed to producing a bud at a particular cell size, even if they are shifted to a richer medium in which a larger volume is normally required before bud initiation can occur. These results are examined in detail in relation to the unstable inhibitor model for the control of cell division.

METHODS

Strains and growth conditions. Haploid cells of *Saccharomyces cerevisiae* strain C4,2 (derived from the diploid C276, kindly provided by J. R. Pringle) and the temperature-sensitive mutant *cdc 7* (4008) derived from A364a (Hartwell *et al.*, 1973) were used. The batch culture media employed were YEPD medium, YEPG medium, proline medium and ethanol medium, all of which have been described previously (Jagadish & Carter, 1977). All experiments were carried out at 24°C. Yeast cultures were also grown in a chemostat as described previously (Jagadish & Carter, 1977).

Shift-up experiments. Cells of C4,2 were grown in ethanol medium, harvested by centrifuging and separated according to size by zonal centrifugation in a 10 to 40% (w/v) sorbitol gradient made up in ethanol medium (Carter *et al.*, 1971; Sebastian *et al.*, 1971; Halvorson *et al.*, 1971). A fraction containing the smallest cells was isolated and the cells were removed from the sorbitol by centrifuging. These cells were then inoculated into fresh ethanol medium at 24°C. At time 0 a sample was removed, cells were concentrated by centrifugation and suspended for 5 min in the presence of 2% Calcofluor (American Cyanamid). They were then examined by fluorescence microscopy. All cells were unbudded and considered to be age 0 cells because of the absence of bud scars.

At intervals, samples (5 ml) were removed from the culture and cells were collected on a Millipore filter (0.45 µm pore diam.). The cells on the filter were washed once with YEPD medium (10 ml) pre-warmed to 24°C, and then resuspended in fresh YEPD medium at 24°C. The culture left in ethanol medium and the cultures shifted to YEPD medium were examined microscopically at intervals to detect bud formation. Samples shifted to YEPD medium were photographed for cell volume measurements both at the time of shift and again when approximately 50% of the cells in the population had budded.

Shift-down experiments. Cells of C4,2 were grown in YEPD and then separated according to size by zonal centrifugation in a 10 to 40% (w/v) sorbitol gradient made up in YEPD medium. Small cells were selected, incubated in YEPD medium, and at intervals samples were shifted to ethanol medium after Millipore filtration. Cells were washed three times with ethanol medium (10 ml) before resuspending in ethanol medium.

Photography. Samples were centrifuged and placed on a microscope slide, and photographs were taken with a Leitz Ortholux photomicroscope, using Kodak fine-grain positive film. A graticule was incorporated into the eyepiece objective to correct for slight variations in magnification on subsequent projection. Photographs were also taken of a graticule for calibration purposes.

Bud scar determinations were carried out after cells were resuspended in 2% Calcofluor (American Cyanamid) and photographed using a Zeiss fluorescence microscope.

Cell size measurement. Photographs of cells were projected on to a screen and the lengths of the long and short axes of the parent cells were measured. Cell volumes were calculated from these measurements assuming that yeast cells approximate to a prolate spheroid.

RESULTS

The growth rate of yeast cells is dependent on nutritional conditions. In media supporting fast growth, bud initiation occurred at a larger size than in media capable of supporting only slow growth (Table 1). The cell volume at bud initiation increased with each generation by about 17% and appeared to be independent of the growth rate (Table 1).

In YEPD medium (mass doubling time 2.4 h), the cell volume at bud initiation for age 0 cells of strain C4,2 was 38.7 µm³. In ethanol medium (mass doubling time 6.84 h), the corresponding value was 28.5 µm³. There must therefore be a point in the cycle at which cells growing on ethanol medium become committed to producing a bud at the size characteristic of ethanol medium even when they are shifted to YEPD medium. To determine this point, cells were grown in ethanol medium and separated according to size by zonal centrifugation; then small unbudded age 0 cells were placed in fresh ethanol medium at 24°C, and at intervals samples were transferred to YEPD medium. When the culture remaining in ethanol medium was 50% budded, the mean parent cell volume of cells with buds was 26.2 µm³ (Table 2). When cells within the size range 13.7 to 23.5 µm³ were shifted to YEPD medium, they produced a bud at the size characteristic of YEPD medium. However, when cells with a mean volume of 23.8 µm³ from an ethanol-grown culture showing 17% budding were shifted to YEPD medium, some unbudded cells

Table 1. *Effect of nutritional conditions on growth rate and cell volume at bud initiation*

Strain	Medium	Mass doubling time (h)	Mean cell volume at bud initiation (μm^3) ± standard deviation			
			0 bud scar	1 bud scar	2 bud scars	≥ 3 bud scars
C4,2	YEPD	2.40	38.7 ± 4.5	43.4 ± 4.0	47.6 ± 6.0	62.1 ± 11
C4,2	Ethanol	6.84	28.5 ± 3.4	36.0 ± 2.4	41.5 ± 5.9	57.0 ± 8.7
4008	YEPD	3.16	41.0 ± 5.4	48.9 ± 4.4	60.7 ± 7.2	75.0 ± 10
4008	Ethanol	7.75	29.9 ± 3.0	35.0 ± 3.3	41.0 ± 5.7	59.6 ± 8.9

Table 2. *Effect on cell volume at budding of shifting samples from a synchronous culture grown in ethanol medium to YEPD medium*

Percentage of budded cells at time of shift	Ratio of 'YEPD-sized' to 'ethanol-sized' cells at 50% budding*	Mean volume ± standard deviation of unbudded cells at time of shift (μm^3)	Mean volume ± standard deviation of 'YEPD-sized' budding cells when the total population shows 50% budding
0	100:0	13.7 ± 1.3	41.3 ± 3.5
0	100:0	14.4 ± 1.25	41.8 ± 2.7
0	100:0	15.1 ± 1.2	41.0 ± 4.3
0	100:0	15.9 ± 1.8	41.7 ± 3.8
0	100:0	16.3 ± 1.7	41.7 ± 3.3
0	100:0	17.2 ± 1.6	41.8 ± 3.1
0	100:0	17.6 ± 1.7	42.5 ± 3.6
0	100:0	19.4 ± 2.6	41.2 ± 2.2
1	95:5	22.2 ± 3.5	42.3 ± 2.5
5	86:14	23.5 ± 3.6	42.2 ± 4.3
17	75:25	23.8 ± 3.1	41.0 ± 3.5
21	66:34	24.2 ± 2.6	48.5 ± 8.3

The volume of parent cells at the first budding event on ethanol medium was $26.2 \pm 2.4 \mu\text{m}^3$ at 50% budding.

* Since all unbudded cells in the 50% budded culture go on to produce buds when they reach the 'YEPD size', they have been included with the 'YEPD-sized' cells.

produced a bud at the size characteristic of ethanol medium and others produced buds at the size characteristic of YEPD medium (Fig. 1). The size distribution of parent cells at bud initiation when 50% of the cells had formed a bud after a shift to rich medium is shown in Fig. 2. Only the mean volumes of cells in the largest peak (YEPD) are included in the data for Table 2. The results indicate that cells grown in ethanol medium are not committed to producing a bud at the size characteristic of ethanol medium until they have almost reached the size at which bud initiation occurs. Cells growing on ethanol medium initiate a bud when they reach a particular volume and the bud then grows throughout the cell cycle; however, at division the bud is smaller than the parent cell (Hartwell & Unger, 1977; Jagadish *et al.*, 1977; Carter & Jagadish, 1978). When cells are shifted from ethanol medium to YEPD medium, buds are initiated at the size characteristic of either the pre-shift medium or the post-shift medium. In those cells which initiated a bud at the size characteristic of the pre-shift medium, time-lapse photomicrographs revealed buds that grew to a larger size than the parent cell. The length of the budded phase of the cycle varies only slightly over a wide range of growth rates (Von Meyenburg, 1968; Hartwell & Unger, 1977), and at 24°C it is approximately 2 h. Thus, on ethanol medium which supports mass doubling times of 6.84 h, the bud will not reach the same size at cell separation as it will in YEPD medium (mass doubling time 2.4 h). The size at bud initiation for age 0 cells on

Fig. 1. (a) Photomicrograph of cells from a synchronous culture shifted from ethanol medium to YEPD medium when 17% of the cells were budded and remaining in YEPD medium until 50% of the cells were budded. The three encircled areas show clearly that in budded cells the parent cell is either of the 'ethanol size' (cells with buds and parent cells of about the same size) or of the 'YEPD size' (cells in which the bud is much smaller than the parent cell). For comparison, photomicrographs of synchronous cultures in ethanol medium (b) and YEPD medium (c) are also shown. Bar marker represents $10\text{ }\mu\text{m}$.

YEPD medium is $41.6\text{ }\mu\text{m}^3$ and the bud reaches almost the size of the parent cell. The bud can therefore reach a volume of almost $41.6\text{ }\mu\text{m}^3$ in 2 h. The size at bud initiation for age 0 cells on ethanol medium is $26.2\text{ }\mu\text{m}^3$. If such a cell is placed in YEPD medium the bud can grow to more than $26.2\text{ }\mu\text{m}^3$ in 2 h, i.e. larger than the parent cell.

We have also grown cells in YEPD medium, isolated the smallest cells in the culture by zonal centrifugation and inoculated them into fresh YEPD medium. (In a YEPD-grown culture there are always some unbudded cells of a smaller size than that at which ethanol-grown cells produce a bud, and it is these cells which were used as the inoculum.) At intervals before synchronous cell division occurred, cells were shifted to ethanol medium. The shifted samples were monitored for the appearance of budded cells and were photographed to determine the size at which bud initiation occurred. The culture remaining in

Fig. 2. Size distribution of the parent cell volume of budded cells for cells from a synchronous culture shifted from ethanol medium to YEPD medium when 17% of the cells were budded and remaining in YEPD medium until 50% of the cells were budded.

Table 3. *Effect on cell volume at budding of shifting samples from a synchronous culture grown in YEPD medium to ethanol medium*

Percentage of budded cells at time of shift	Mean volume $\pm$ standard deviation* of cells on YEPD medium at time of shift (μm^3)	Mean volume $\pm$ standard deviation of parent portion of cells at % budding indicated	
		μm^3	% budding
0	23.6 ± 2.6	28.9 ± 2.7	41
1	29.3 ± 2.9	32.6 ± 2.8	48
5	30.9 ± 3.3	33.4 ± 3.3	49
18	33.7 ± 3.7	34.7 ± 4.9	49
49	34.1 ± 3.2	35.7 ± 5.2	70

The volume of parent cells at the first budding event on YEPD medium was $37.5 \pm 3.6 \mu\text{m}^3$ at 50% budding.

* Parent portions of budded cells are also included.

The increase in volume after a shift from, for instance, $29.3 \mu\text{m}^3$ to $32.6 \mu\text{m}^3$ is more apparent than real. At the time of the shift there is a distribution of cells around a mean volume. All cells, including the smallest are counted. After the shift, when the culture was 48% budded the volumes of parent portions of budded cells were measured. The small unbudded cells at the time of the shift were not budded when the culture was analysed after the shift. Their inclusion in the first measurement and exclusion from the second results in an apparent volume increase.

YEPD medium produced buds when the parent cell size reached $37.5 \mu\text{m}^3$. There was a slight variation in cell size at budding from culture to culture which reflected variation in mass doubling times; for cultures in which cells grew slightly faster on YEPD, the cell size at bud initiation was enhanced.

When cells were smaller at the time of shift to ethanol medium than the size characteristic of bud initiation in ethanol medium, they produced a bud when they reached the appropriate size for ethanol medium (Table 3). Unbudded cells which, when shifted, were intermediate between the sizes at bud initiation for ethanol medium and YEPD medium produced buds

Fig. 3. Cell volumes at bud initiation for 0 bud scar haploids (●) and diploids (○) grown at different rates in a glucose-limited chemostat.

without appreciable increase in size although there was a considerable lag before bud initiation.

The size of the haploid parent cell at bud initiation was dependent on growth rate within the mass doubling time range 2.1 to 3.7 h (Fig. 3). However, at slow growth rates a minimum cell size was reached which was independent of growth rate over the mass doubling time range 3.7 to 9 h. We also measured diploid cells grown at different rates in the chemostat to see if they reached a minimum cell size equal to that of the haploids at slow growth rates, as suggested by the results of Adams (1977). There was, indeed, a minimum size for diploid cells at slow growth rates, but this minimum was larger than the minimum size characteristic of the haploid (Fig. 3). At all growth rates the ratio of diploid to haploid parent cell size at budding was 1.75:1.

DISCUSSION

The size at which an age 0 yeast cell produces a bud is related to growth rate within the mass doubling time range 2.1 to 3.7 h; the faster the growth rate the larger the cell. When unbudded cells growing on poor medium are shifted to rich medium, all except those very close to the size characteristic of bud initiation on the poor medium go on to produce a bud at the size characteristic of the rich medium. Thus cells do not become committed to producing a bud until they are within about $3 \mu\text{m}^3$ of the size characteristic of bud initiation on the poor medium. In addition, upon shift to a new medium cells quickly 'sense' their altered circumstances. All cells that are below the commitment size for the poor medium at the time of shift to a richer medium initiate their bud at the size characteristic of rich medium. This suggests that any progress cells make towards bud initiation on poor medium is irrelevant when they are shifted to rich medium unless they have reached the commitment point.

When cells that are almost at the point of commitment are shifted to rich medium, some initiate a bud at a size which is somewhat larger than that characteristic of the rich medium. We do not understand the reason for this phenomenon.

When cells are shifted from rich to poor medium, unbudded cells that are smaller than the size for bud initiation on the poor medium produce a bud at the size characteristic of the poor medium. If they are larger than the commitment size for poor medium at the time of shift, they produce a bud without appreciable growth. Again they rapidly sense their changed circumstances.

It has been suggested (Johnston *et al.*, 1977; Hartwell & Unger, 1977; Jagadish *et al.*, 1977; Carter & Jagadish, 1978) that cells must attain a critical cell size before they progress beyond the cdc 28 step and initiate a bud. We have found that this critical size is growth-rate dependent within the doubling time range 2.1 to 3.7 h. Our results may be explained on the basis of the unstable inhibitor model of cell division (Ycas *et al.*, 1965; Fantes *et al.*, 1975). According to this model, an inhibitory substance is produced at a rate proportional to the number of genome equivalents. Thus the rate of production doubles once per cycle when the genome is duplicated. However, the inhibitor is unstable, each molecule having a constant half-life independent of concentration (rather like the decay of messenger RNA molecules). The amount of inhibitor (A) in the cell will depend on the rates of production and decay, such that $A = kN/k'$, where A is the amount of inhibitor, N is the number of genome equivalents, k is the production constant and k' is the decay constant. The decay of inhibitor balances synthesis and so the amount of inhibitor is proportional to the number of genome equivalents. While the amount remains constant until there is a change in the number of genome equivalents, the concentration per cell will fall due to growth. We propose that when a critically low concentration is reached the cdc 28 event and bud initiation occur.

The observation that the size at bud initiation is dependent on the growth rate can be explained if the production constant for the inhibitor is dependent on the growth rate. Thus the amount of inhibitor will be greater in fast growing cells and consequently they will have to grow to a correspondingly larger size before the concentration of inhibitor becomes critical.

This model also provides an explanation for the results of the shift-up and shift-down experiments. If cells on poor medium (where the amount of inhibitor is comparatively low) are shifted to rich medium then the amount of inhibitor will very rapidly reach a new equilibrium amount which will be higher than before the shift. The equilibrium amount of inhibitor after the shift will be independent of the size of unbudded cells at the time of shift and thus all cells will need to grow to the same size to dilute out the inhibitor.

When unbudded cells grown on rich medium are shifted to poor medium, they divide at whatever size they have reached provided this size is larger than that characteristic of bud initiation on poor medium. This is predicted by the model. When cells are shifted down, the rate of production of inhibitor declines because of the growth-rate dependent production constant. The amount of inhibitor per cell falls rapidly to the lower equilibrium value characteristic of the poor medium.

At fast growth rates, cell division occurs when the bud is almost the size of the parent cell and both produce another bud in an almost equivalent unit of time. At slow growth rates, the bud is much smaller than the parent cell (Hartwell & Unger, 1977; Jagadish *et al.*, 1977; Carter & Jagadish, 1978). The parent cell produces another bud in an interval of time much shorter than the mass doubling time of the culture before budding (Jagadish *et al.*, 1977; Carter & Jagadish, 1978). These observations are also consistent with the model. At fast growth rates, the bud and parent cell have almost the same amount of inhibitor at division. After division, the amount of inhibitor is rapidly established at an identical level in both the bud and the parent cells. The latter can initiate the next cell cycle because its size is sufficient for the concentration of inhibitor to be below the critical value. The bud, which at division is almost the same size as the parent, needs only to grow a small amount to reach an equivalent position to the parent cell. At slow growth rates, the bud is appreciably smaller than the parent cell at division. Immediately after cytokinesis, the parent and daughter cells each have one genome equivalent and the high constants for production and decay of inhibitor quickly result in an equal amount of inhibitor in both cells. The amount of inhibitor in each cell will be identical to that just prior to bud initiation in the previous cycle and since the parent cell is no smaller its low concentration of inhibitor should permit the next bud initiation provided any other necessary requirements are met.

The daughter cell, however, is much smaller and will need to grow before it has diluted out the inhibitor sufficiently to permit completion of the *cdc 28* event and bud initiation.

If yeast cells are grown on poor media but treated with 0.2 M-hydroxyurea prior to DNA synthesis, bud initiation occurs but cell separation is prevented and the bud continues to grow until the bud and parent cell are the same size. If hydroxyurea is then removed, cell division occurs with the production of equal-sized parent and daughter cells. These each produce a bud in the same interval of time as expected if the *cdc 28* event requires the unstable inhibitor to fall to a critical concentration (M. N. Jagadish, unpublished results).

Sudbery & Grant (1975) have indicated that the unstable inhibitor model fits their experimental observations concerning the control of cell division in the slime mould *Physarum polycephalum*. They have also suggested that the experiments of Hartmann (1928) and Prescott (1956) on amoeba and of Frazier (1973) on the ciliate *Stentor coeruleus* can be understood in terms of this model.

However, the inhibitor-dilution model is not the only one which can explain our results. Pritchard *et al.* (1969) suggested a model for the control of the initiation of DNA synthesis in *Escherichia coli* in which a stable inhibitor is synthesized once per cycle and is subsequently diluted out until its concentration falls to a value which permits initiation of DNA synthesis. This model will fit our results provided it is modified such that the interaction of inhibitor with its binding site is growth-rate dependent, resulting in a higher binding constant at faster growth rates. In addition, one must propose that the inhibitor is destroyed during each cell cycle and that the pulse of inhibitor production occurs immediately after cell division.

Although the unstable inhibitor-dilution model provides a plausible explanation of the growth rate dependence of cell size at fast growth rates, it is inadequate to explain the fact that at slow growth rates the size at bud initiation is independent of the growth rate. It is possible, however, that an alternative mechanism not expressed during fast growth is responsible for bud initiation at slow growth rates.

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