CELL CYCLE PHASE EXPANSION IN NITROGEN-LIMITED CULTURES OF *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

The time and coordination of cell cycle events were examined in the budding yeast *Saccharomyces cerevisiae*. Whole-cell autoradiographic techniques and time-lapse photography were used to measure the duration of the S, G1, and G2 phases, and the cell cycle positions of "start" and bud emergence, in cells whose growth rates were determined by the source of nitrogen. It was observed that the G1, S, and G2 phases underwent a proportional expansion with increasing cell cycle length, with the S phase occupying the middle half of the cell cycle. In each growth condition, start appeared to correspond to the G1 phase/S phase boundary. Bud emergence did not occur until mid S phase. These results show that the rate of transit through all phases of the cell cycle can vary considerably when cell cycle length changes.

When cells growing at different rates were arrested in G1, the following synchronous S phases were of the duration expected from the length of S in each asynchronous population. Cells transferred from a poor nitrogen source to a good one after arrest in G1 went through the subsequent S phase at a rate characteristic of the better medium, indicating that cells are not committed in G1 to an S phase of a particular duration.

The budding yeast, *Saccharomyces cerevisiae*, has been a particularly favorable eukaryotic organism for cell cycle studies (9). Visible landmarks which signal progress through the cell cycle are provided by the emergence and growth of the bud, the position and morphology of the nucleus, and the sensitivity of bud formation to mating factors. The analysis of cell cycle mutants has shown that the emergence and development of the bud can occur in the absence of nuclear replication and vice versa, demonstrating that these processes are largely independent of one another. However, they are both dependent on a common control event that is mediated by gene *cdc*28. Temperature-sensitive *cdc*28 mutants will neither bud nor initiate DNA synthesis at restrictive temperature (11). α-Factor, a mating pheromone, also arrests cells at this point in the cycle (12) which has been termed "start" (11).

Inferences about the coordination of events in the yeast cell cycle have been drawn from experiments in which the rate of progress through the cycle is changed. Alterations in cell cycle times have been accomplished by varying the rate of supply or kind of carbon source (2, 14, 5), or by the presence of low concentrations of cycloheximide in the medium (10). At low growth rates the prestart and unbudded intervals of the cell cycle are increased in length, while the durations of the poststart and budded periods are unchanged. In addition, daughter cells are produced which are much smaller than the mother cells from which

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they bud. These small cells then have a prestart period which is longer than that of the mother cell, presumably because cells must reach a critical size before proliferative events ensue (15). The S phase has also been monitored in cultures growing at different rates set by the source of carbon (22, 1). It was found that the S phase began concomitant with bud emergence and was of the same duration in slow-growing cultures as in fast-growing ones. All of these results have led to the idea that slow-growing cultures are rate limited primarily in the completion of the prestart interval.

To initiate a study on regulation of the S phase, we have examined the organization of the nuclear cycle (G1, S, and G2 phases) and the cell growth cycle (unbudded and budded intervals) in *S. cerevisiae* cultures whose growth rates were varied by the source of nitrogen. The results presented here show that coordination of cell cycle events in nitrogen-limited cells differs considerably from that seen in the previous studies, and that the length of the S phase can vary at least fourfold. A preliminary account of this work has been presented (18). Experiments on the molecular basis of the S phase variation are presented in the following paper (19).

MATERIALS AND METHODS

Strains and Steady-State Growth

The haploid strain (A364A (*a*, *ade*-1, *ade*-2, *ura*-1, *tyr*-1, *his*-7, *lys*-2, *gal*-1) was used for all experiments except those involving synchronization. A364A was grown at 30°C. For synchronization experiments a spontaneous homozygous diploid derivative of strain 4008 was used. Strain 4008 contains the temperature-sensitive *cdc*7-4 allele (7) as well as the markers found in A364A; the permissive temperature is 23°C. Both strains were supplied by L. H. Hartwell. The medium used for all experiments was Y-N which contained, per liter, 1.45 g of Difco yeast nitrogen base (Difco Laboratories, Detroit, Mich.) without amino acids and without ammonium sulfate, 10 g of succinic acid, 6 g of NaOH, 30 mg of lysine, and 50 mg each of tyrosine, histidine, and adenine. The medium was buffered to pH 5.8, and 20 g/liter glucose was added. For nitrogen sources, glutamine, methionine, threonine, or proline was added to a concentration of 0.5 g/liter and ammonium sulfate to 1 g/liter. The amount of uracil or uridine added to the medium is given for each experiment. Cultures were judged to be in steady-state growth if two cell cycle parameters, population doubling time, and percent of unbudded cells remained constant over a wide range of cell densities (5×10^5 to 2×10^7 cells/ml). It has been reported that some yeast strains growing in media containing a poor nitrogen source are inhibited by the addition of lysine (23). Because the strains used in this work are lysine auxotrophs, it was necessary to supply this amino acid at a low concentration (30 μg/ml) in all media. To check for possible inhibitory effects of lysine, spontaneous lys^+ revertants of strain A354A were isolated and grown in the proline nitrogen source medium in the presence of various concentrations of lysine (0–250 μg/ml). No effect was observed on growth rate, cell size, or the fraction of unbudded cells.

α-Factor Arrest and Cell Volume

The α-factor used was a gift of R. Chan. It was used at a concentration sufficient to keep 10^8 cells/ml arrested for two doubling times in media buffered to pH 5.8. YEPD agar used for time-lapse photography experiments contained, per liter, 20 g of glucose, 20 g of Difco Bacto Peptone, 10 g of Difco yeast extract, 15 g of Difco nutrient agar. Cell volume distributions were obtained with a Coulter channelyzer (Coulter Electronics, Hialeah, Fla.). The particle size distribution analyzer had been calibrated with 22.26- and 73.62-μm^3 polystyrene beads (10). Greater than 10^4 cells were scored for each distribution.

Autoradiography

Whole-cell autoradiography experiments employed [6-^{3}H]uracil, 25 Ci/mM (New England Nuclear, Boston, Mass.). Whole-cell autoradiograms were prepared by a modification of method of Williamson (24). A 1-ml sample (~10^7 cells) was added to 4 ml of ice-cold 0.2 M perchloric acid and held on ice for 30 min. The cells were then collected on Millipore filters (Millipore Corp., Bedford, Mass.), washed with 3 vol of water, and resuspended in 0.5 ml of 0.05 M phosphate buffer, pH 7.6. 250 μg of RNase B (Worthington Biochemical Corp., Freehold, N. J.) was added and the solution was incubated for 1 h at 37°C. After filtering and washing as before, the cells were placed in 5 ml of 0.3% (wt/vol) HCHO in 0.05 M phosphate buffer, pH 7.0, for at least 30 min at room temperature. The fixed cells were then filtered, washed, and resuspended in 5 ml of 1N NaOH and incubated for 30 min at 37°C. Cells were collected by centrifuging for 10 min at 2×10^3 rpm in a Sorvall GLC-1 centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.), resuspended in water, and re-pelleted. The pellet was suspended in 2 ml of water containing 0.05 ml of Glusulase (Endo Laboratories Inc., Garden City, N. Y.) and incubated for 30 min at 37°C. After centrifuging and washing, the pellet of fixed spheroplasts was suspended in 0.1 ml of water. One drop of the cell suspension was placed on a clean microscope slide that had been subbed in 0.5% gelatin-0.05% chrome alum. The drop was spread across the slide with a glass rod, and completely dry slides (~1.5 h) were dipped into Kodak NTB-2 nuclear track emulsion diluted 2:1 with water and warmed to 37°C, dried, then stored with a dessicant at 5°C. After exposure for 1 mo or more, the slides were developed for 2 min in Dektol (Kodak), diluted 1:2 with water at 20°C, fixed in Kodak Rapid Fixer, and washed in water. In control experiments, cells were treated with DNAse before the preparation of autoradiograms. Only background label was observed.

After whole-cell autoradiograms were developed, they were stained with either Giemsa or the fluorescent dye mithramycin to reveal the nuclear morphology of the cells. For Giemsa staining, slides were immersed for 20 min in a 1:10 dilution of Giemsa stain in Gurr buffer, pH 6.9 (both from G. D. Searle & Co., Des Plaines, Ill.). They were rinsed in two washes of Gurr buffer and, if necessary, destained with dilute acetic acid. The autoradiograms were allowed to dry thoroughly and viewed under oil immersion. Mithramycin provides superior contrast between nucleus and cytoplasm, as it is specific for DNA. Mithramycin (Pfizer Chemicals Div., New York) was used as a 0.4 mg/ml solution in 20 mM $MgCl_2$-0.6 M NaCl (21). Slides were washed with 70% cold ethanol, air dried completely, and spread with 0.05 ml of the stain solution. The wet slides were kept flat

in light-tight boxes at 5° for 3 h to stain, and then the solution was allowed to dry on the slides in the light. The autoradiograms were viewed under UV light with occasional transmission of a small amount of visible light permitted in order to view both grains and stained nuclei for the same cell.

RESULTS

Bud Emergence, α-Factor Sensitivity, and Cell Size

Cultures of strain A364A growing at different rates with various nitrogen sources were examined to determine the cell age[1] at which the bud emerges and at which sensitivity to α-factor is lost. Previous experiments in which low growth rates were generated by other nutritional conditions (especially carbon source limitation) or sublethal amounts of cycloheximide have shown that the α-factor-sensitive and unbudded portions of the cell cycle expanded greatly, whereas the α-factor-insensitive and budded portions of the cycle changed little in length (10, 14). This alteration in cell cycle organization was accompanied by the appearance of a subpopulation of cells of small size. Our results obtained with nitrogen source-limited cells contrast with these previous observations. Table I shows the duration of the unbudded and budded phases of cells growing at 30° with the various nitrogen sources. To obtain the fraction of the cell cycle in which cells are unbudded, a correction must be made for the relative number of cells at different ages. We used the equation[2] derived by Puck and Steffen (16) for this purpose. The data in Table I show that the emergence of the bud occurs progressively later in the cell cycle at slower growth rates, as was seen in the previous studies. However, the increased length of the unbudded period did not account completely for the increase in cell cycle time. Rather, the unbudded and budded intervals both increased in a roughly linear fashion with a greater proportional increase in the unbudded interval.

The relative age at which cells become insensitive to inhibition of bud formation by α-factor was determined by the method of Hartwell and Unger (10). Cell samples were removed from the steady-state cultures, sonicated to remove mature buds, and placed on agar slabs containing α-factor. The fraction of unbudded cells which formed buds was scored from time-lapse photomicrographs. The data in Table II show that the relative cell age at which cells became insensitive to α-factor was essentially invariant for cells growing at different rates. Therefore, unlike what has been observed for other growth conditions, the periods of the cycle that precede and follow the α-factor-sensitive point vary proportionately when the growth rate is controlled by the nitrogen source. Moreover, buds emerged at a cell age significantly later than the age at which insensitivity to α-factor was acquired.

Fig. 1 shows the distribution of cell volumes for cultures growing with glutamine or proline as a nitrogen source. Cultures in which growth has been limited in other ways exhibit a marked bimodal distribution of cell sizes (10, 6). This cell size heterogeneity was not observed in our experiments with the proline-limited culture. The range of cell sizes was only slightly greater at the lower growth rate (proline) than at the higher one (glutamine); the modal cell size in each culture was very similar.

Measurements of G1, S, and G2 Phases

The lengths of cell cycle phases were measured in asynchronous populations so that steady-state growth would be unperturbed. Determinations of the length of G1, S, and G2 phases were made by adapting the percent labeled mitoses technique (13): An exponentially growing population is exposed briefly to a radioactive DNA precursor and then chased with an excess of unlabeled precursor for two generations. Throughout the chase period, samples are taken for whole-cell autoradiography. The samples are stained to reveal nuclear morphology and scored for the percentage of cells undergoing mitosis (nuclear division in yeast) which contain radioactive DNA, as indicated by silver grains in the photographic emulsion. As cells which were in S phase at the time of the pulse progress through the cycle, they enter nuclear division in a wave which is described by plotting the percentage of labeled nuclear division figures as a function of the time of sampling.

Three conditions must be met to obtain reliable measurements by this method: (*a*) the population

[1] In all culture conditions, cell age immediately after cell division is taken as 0, and cell age immediately before cell division is taken as 1.0. Halfway through the cell cycle a cell has an age of 0.5.

[2] The fraction of cells (n) in an asynchronous population which occupies a terminal fraction (q) of the total cell cycle is given by $n = 2^q - 1$; and $q = \log(1 + n)/\log 2$. The fraction of cells ($N = 1 - n$) in all the earlier fractions of the cell cycle ($Q = 1 - q$) is, therefore, given by $N = 2 - 2^{(1-Q)}$; and $Q = 1 - \log(2 - N)/\log 2$.

TABLE I

Duration of Unbudded and Budded Phases in Nitrogen-Source-Limited Cultures

Nitrogen source	Population doubling time	Fraction of unbudded cells	Relative cell age at bud emergence	Unbudded period	Budded period
	min			*min*	*min*
Ammonia	105	0.54	0.45	47	58
Glutamine	105	0.55	0.46	48	57
Methionine	195	0.59	0.50	98	98
Threonine	255	0.62	0.54	138	117
Proline	400	0.71	0.63	252	148

The growth rate of A364A in medium supplemented with 500 μg/ml amino acid or 1 mg/ml ammonium sulfate as nitrogen source and 20 μg/ml uracil was measured by the increase in optical density at 0.66 mm. To obtain the percentage of unbudded cells, a sample of an exponential phase population was sonicated at a low power setting to remove mature buds. At least 300 cells were scored for the presence of a bud or small protrusion corresponding to an emerging bud. The fraction of the cycle that is unbudded was calculated as 1 − log(2 − fraction of unbudded cells)/log2.

TABLE II

Age at Which Cells Growing with Various Nitrogen Sources Become Insensitive to α-Factor

Nitrogen source	Doubling time	Fraction of unbudded cells (A)	Fraction of unbudded cells that bud in the presence of α-factor (B)	Relative cell age	
				When insensitive to α-factor	At bud emergence
	min				
Ammonia	100	0.50	0.43	0.23	0.42
Glutamine	105	0.52	0.44	0.23	0.43
Proline	400	0.69	0.48	0.28	0.61

The fraction of unbudded cells which form buds in the presence of α-factor (B) was determined by monitering 100–250 unbudded cells by time-lapse photography after they were placed on agar medium along with excess α-factor. The fraction of the cell cycle that is sensitive to α-factor (and, therefore, the age of acquisition of insensitivity) was calculated as 1 − log(1 + fraction of budded cells + fraction of total cells which are unbudded and form a bud in the presence of α-factor)/log2, or 1 − log[1 + (1 − A) + AB]/log2 = log(2 − A + AB)/log2, where A and B are the values in columns A and B. Growth media were as for Table I.

must be asynchronous; (*b*) the pulse time must be much shorter than the length of S phase; (*c*) the cytological stage at which the cells are scored must be unique in appearance and occupy only a small fraction of the cell cycle time. The requirement for a rapid pulse and effective chase was met in the following way: Cells were grown with 25 μg/ml uridine (20), pulsed for 15 min with [6-^{3}H]uracil (20 μCi/ml, 0.1 μg/ml), and chased by the addition of 1.67 mg/ml unlabeled uracil. This procedure gave an effective labeling period of ~10 min (Fig. 2). The high concentration of uracil present during the chase did not affect the growth rate or the percent of unbudded cells. The cell cycle stage of medial nuclear division which occupies 6–8% of the cell cycle (11) was used as the equivalent of mitosis (Fig. 3). The yeast strain used in these experiments contains in addition to chromosomal DNA two multiple copy extrachromosomal DNAs, mitochondrial DNA, and 2-μm plasmid DNA. As these molecules constitute only ~10 and 2%, respectively, of the total DNA, incorporation of radioisotope into them will not be appreciably reflected in the autoradiograms. Moreover, mitochondrial DNA replicates throughout the cell cycle (25), and its small contribution will be distributed over cells of all ages. 2-μm DNA replicates during the S phase (26).

Data from pulse-chase experiments on cells growing with ammonia, glutamine, or proline as nitrogen sources are shown in Fig. 4. The first ascending curve of the percent labeled nuclear

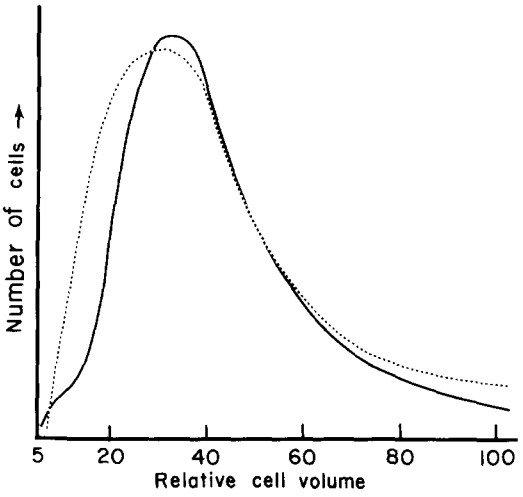

FIGURE 1 Distribution of cell volumes. Exponentially growing cultures using glutamine (—) or proline (- - -) as a nitrogen source were analyzed with a Coulter Channelyzer, scoring $>10^4$ cells for each culture. The abscissa scale is arbitrary but directly proportional to cell volume.

division (%LND) plot can be understood as the transit of cells labeled at the end of S phase into nuclear division, and is thereby a measure of the G2 phase. The time interval between the first rise and fall of the %LND curve is a measure of the length of S phase. As labeled cells pass through another cell cycle a second rise and fall of labeled nuclear division figures will occur, although the shape of this curve may be distorted by the variation in the time required for individual cells to traverse the cycle. The interval between successive %LND peaks is a measure of the average cell cycle time. The half-heights of the ascending and descending curves mark G2/S and S/G1 phase boundaries, respectively, giving the average lengths of the G2 and S phases. Subtracting these values from the total cycle time gives an estimate of the length of G1. Note that under these definitions the interval from nuclear division to cytokinesis is included in the G1 phase. Microscope analysis of stained cells showed that only 5–10% of the cell cycle time is in this interval for all the culture conditions.

The data are summarized in Table III. For each of the growth conditions, it can be seen that the population doubling time was about the same as the sum of the cell cycle phases determined in the %LND experiment. When cells are grown on glutamine the length of each phase is similar to that of cells grown in standard media where ammonia is the nitrogen source, whereas cells using proline as a nitrogen source require much more time for each phase. What is striking, however, is that the fraction of the cycle time devoted to each interval is not influenced by the growth rate, or source of nitrogen, but is a fixed value. The G1, S, and G2 phases occupied ~0.27, 0.48, and 0.26, respectively, of the cell cycle in all three media.

Relationship of Budding Cycles and Nuclear Cycles

Our results have shown that while there is a proportional expansion of the G1, S, and G2 phases, the unbudded portion of the cell cycle occupies a larger fraction of the cell cycle time as the growth rate is decreased. That is, changing growth rate by the nitrogen source alters the temporal relationship of the budding cycle to the nuclear cycle. This relationship was examined di-

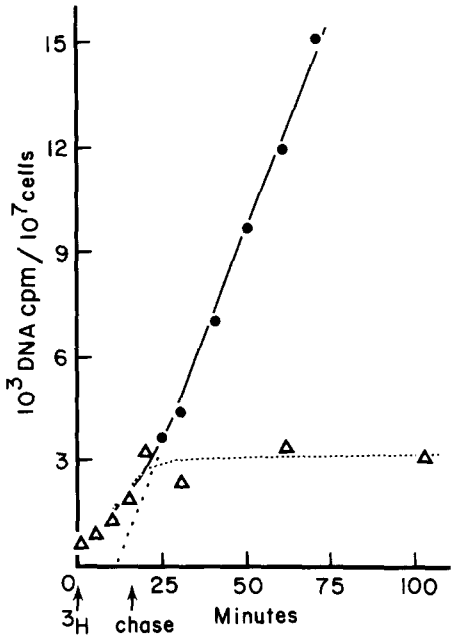

FIGURE 2 Kinetics of radiolabel incorporation in a pulse-chase experiment. 20 μCi/ml (0.1 μg/ml) of [6-^{3}H]uracil was added to a log phase ammonia-supplemented culture growing with 25 μg/ml with unlabeled uridine. After 15 min of labeling, the culture was split in two and 1.67 mg/ml unlabeled uracil was added to one part. The amount of label incorporated into DNA was followed by sampling the cultures at intervals for the number of alkali-stable, acid-precipitable counts. The incorporation curve (—) can be extrapolated (· · ·) to 10 min on the abscissa. When the unlabeled uridine is added, incorporation (- - -) stops within 10 min.

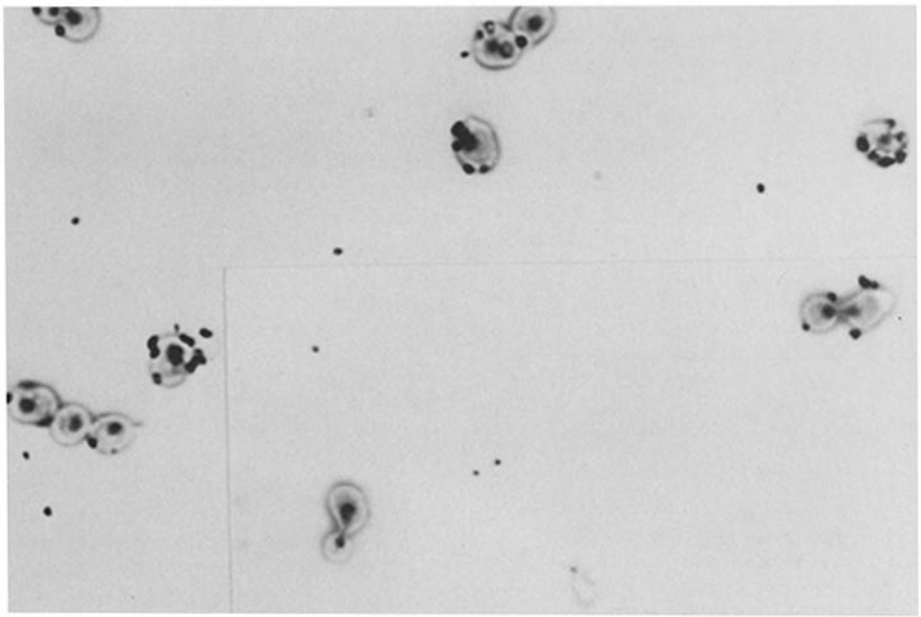

FIGURE 3 Giemsa stained whole cell autoradiograms. Giemsa stained cells revealing nuclear morphology in an autoradiogram prepared during a pulse-chase (%LND) experiment. Labeled and unlabeled nuclear division cells can be seen.

rectly by preparing whole-cell autoradiograms immediately after pulse-labeling the cultures. Cells were scored for both the incorporation of label into DNA and the presence or absence of buds. It was observed that grains appeared over unbudded as well as budded cells, indicating that the S-phase starts before bud emergence. By scoring the fraction of cells that were both unbudded and labeled in a pulse, the time of initiation of DNA synthesis was fixed relative to bud emergence and cytokinesis.

An accurate estimate of the percent of labeled cells and labeled unbudded cells must take into account the number of grains per cell produced in the photographic emulsion. Assuming that all labeled cells contain about the same amount of radioactivity, the number of grains per labeled cell will exhibit a Poisson distribution:

$$P(x) = \frac{e^{-m}m^x}{x!},$$

where x is the number of grains associated with a given cell and m is the average number of grains per cell. If m is large, almost all labeled cells will have more grains than background and the error in scoring labeled cells will be negligible. However, at high m values (>10) the grains can obscure the presence of small buds. For this reason, labeling was conducted at low values of m, by reducing the specific activity of [^{3}H]uracil (10 μCi/ml, 0.1 μg/ml) in the pulse and using shorter exposures with the photographic emulsion. In this case, however, a significant number of labeled cells will show no more than the background number of grains. To obtain the true fraction of labeled cells, all cells were scored for the number of associated grains without regard for the background, and the frequency of different grain counts, $P(x)$, was plotted as log $P(x)x!$ vs. grain counts, x (Fig. 5). In this form a Poisson distribution graphs as a straight line. The higher grain counts were used to determine the slope of this line for the data, and an extrapolation was made to obtain the actual percentage of labeled cells (total and unbudded) with no or only a few grains (4).

Table IV summarizes the data from pulse experiments with cultures grown in five nitrogen sources, and gives the calculated lengths of the G1, S, and G2 phases. Because these experiments use cytokinesis as a phase boundary, the time from nuclear division to that point (~7% of the cycle) is included in the measure of G2 phase. These data confirm the conclusion from the %LND experiments that nitrogen source limitation resulted in a proportional expansion of each nuclear cycle phase. The trend toward a disproportionate in-

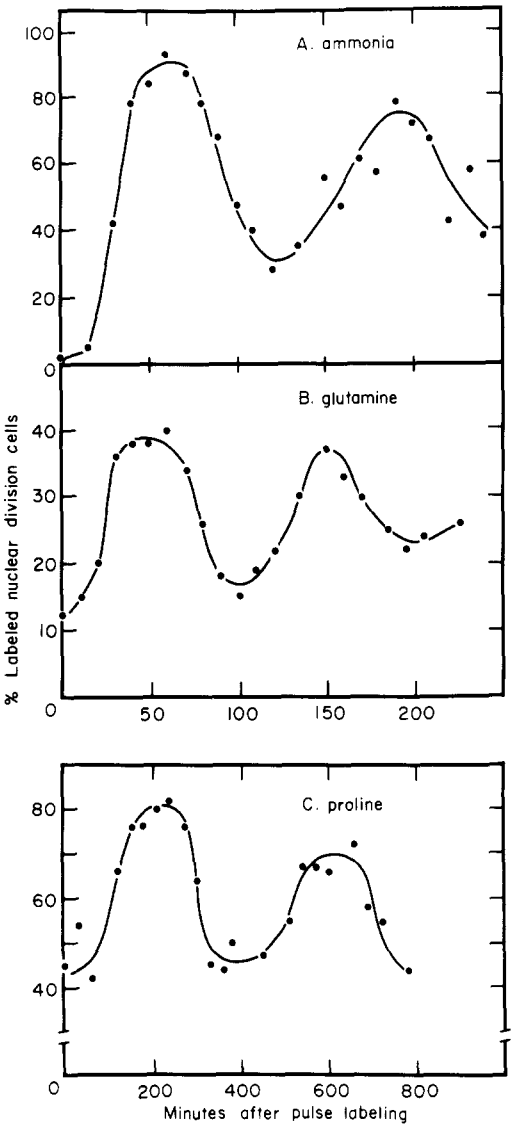

FIGURE 4 Percent of labeled nuclear division cells. Exponentially growing cultures were pulse labeled and chased as explained in the legend to Fig. 2, and sampled for two doubling times. Cells growing with three different nitrogen sources were examined. (*A*) Ammonia. Doubling time was 125 min; at least 150 nuclear division figures were scored for each time point. (*B*) Glutamine. Doubling time was 105 min; 50–100 nuclear divisions were scored per time point. (*C*) Proline. Doubling time was 390 min; effective pulse time was 10 min; 100–200 nuclear division figures were scored for each point. The curves in *B* and *C* are damped because the average number of grains/cell was low in these experiments. Because grain number follows a Poisson distribution, the low labeling results in a large fraction of labeled cells that do not exhibit grains (diminishing the curve height) and many cells that have no more than the background number of grains. Inclusion of background labeled cells in the scored sample raises the curve base line but does not affect its shape.

crease in the unbudded period is evident, though not so marked as that seen in the data in Tables II and III. The values for the fractional length of the G1 phase which are shown in Table IV also represent the relative cell age at the beginning of S phase. Unlike what has been reported by others (24, 1, 22), this age does not correspond to the cell age at which the bud emerges. In all nitrogen sources, the bud emerged while cells were in the middle of the S phase (Table IV).

S Phase Variation in Synchronous Cultures

Synchronized cultures are useful for answering a variety of questions about the cell cycle. However, data from cultured animal cells have suggested that synchrony techniques which arrest cells in G1 phase shorten the subsequent S phase (3). Because yeast cells can be tightly blocked in G1, an experiment was performed to determine whether a prolonged arrest in G1 has any effect on the length of S phase. Cells were arrested in G1 by treating asynchronous cultures of a mutant temperature-sensitive for gene *cdc7* with α-factor for one generation time at permissive temperature (23°C), then washing out the α-factor and transferring the culture to 35°C for 2 h. Both treatment with α-factor and incubation of the *cdc7* mutant at 35°C result in the arrest of cells in the G1-phase (12). Better synchronization is obtained when the two treatments are applied sequentially than when one treatment is used alone. Transfer of the cultures to 23°C initiates a synchronous round of DNA synthesis (Fig. 6*A* and *B*).

The data from experiments with cells growing at different rates are summarized in Table V. For each culture condition, the time required for a synchronous S phase is half the length of the population doubling time at 23°C. Therefore, the duration of the S phase in cells grown with the different nitrogen sources is unaffected by the synchronization procedure. (It is assumed that steady-state asynchronous populations of cells at 23°C have the same relative lengths of cell cycle phases as cells growing in the same media at 30°C.) We also tested whether cells become committed to an S phase of a given length during G1 phase, before the S phase actually begins. Temperature-sensitive *cdc7* cells grown in proline me-

TABLE III

Cell Cycle Phases Derived from %LND Experiments

Nitrogen source	Doubling time	Cell cycle time	Cell cycle phases					
			G1	S	G2	G1	S	G2
	min	*min*	*min*			*fraction of total*		
Ammonia	125	130	35	61	34	0.27	0.47	0.27
Glutamine	110	105	30	50	25	0.28	0.48	0.24
Proline	400	390	95	190	105	0.24	0.49	0.27

Cell cycle time is the time between the midpoints of sucessive %LND peaks. The boundaries of the cycle phases were taken from the half-heights of the curves in Fig. 4. G1 includes the interval from nuclear division to cytokinesis, ~0.07 the cell cycle.

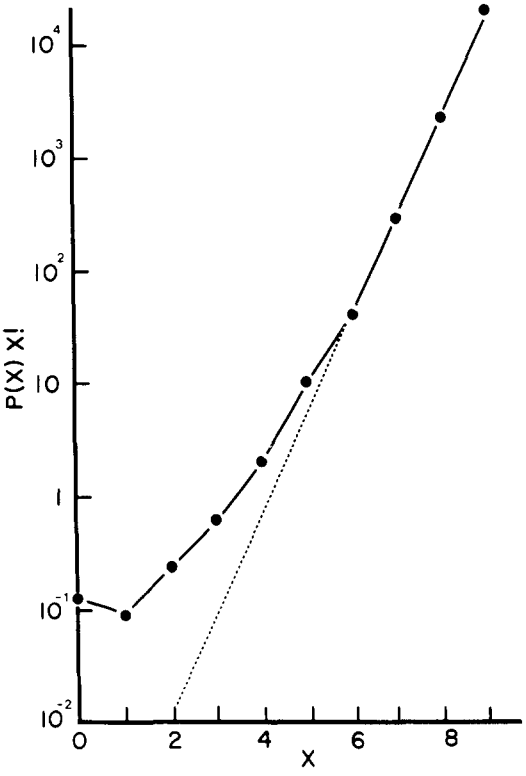

FIGURE 5 Poisson distribution of the number of grains per cell. Cells growing in glutamine medium containing 25 μg/ml uridine were pulse labeled by adding [^{3}H]uracil (10 μCi/ml, 0.1 μg/ml) for 15 min. The true fraction of labeled cells among those cells with no more than background numbers of grains was found by assuming a Poisson distribution of grains over labeled cells. At least 500 cells from the whole cell autoradiogram were scored for the number of silver grains (x). The plot is such that a Poisson distribution gives a straight line. Higher grain numbers are used to extrapolate the line to lower values of x to obtain the true fraction of labeled cells among those with low grain numbers.

dium were blocked as before, first with α-factor and then by transfer to restrictive temperature without α-factor. While arrested at the restrictive temperature, the cells were transferred into 35°C medium containing glutamine as nitrogen source for 30 min, then returned to permissive temperature (23°C). The length of the subsequent S phase, shown in Fig. 6 *C*, is characteristic of glutamine-grown cells.

DISCUSSION

Length and Variation of the S Phase

Cells growing in good nitrogen sources, such as ammonia or glutamine, have population doubling times as short as cells growing in enriched broth, ~100 min at 30°C. Three independent methods, percent of labeled nuclear divisions, percent of cells labeled in a pulse, and synchronous release from a G1 phase arrest, have been used to measure S phase in cells growing with ammonia or glutamine. The results of all three methods are consistent in showing that the S phase occupies ~50% of the cell cycle time (Tables III–V). This can be compared with other measurements of S phase in rapidly dividing cells. Barford and Hall (1) also found 50% of the cell cycle to be devoted to S phase, whereas Williamson (24) and Slater et al. (22) found S phase to occupy only about 25% of the cell cycle.

Barford and Hall (1) and Slater et al. (22) also measured the length of S phase in cells whose cycle times were substantially increased by growth with different carbon sources. Both groups found that the S phase occupied a constant amount of time regardless of the doubling rate of the culture. We found that when the growth rate is systematically limited by the source of nitrogen, there is a considerable effect on the length of the S phase

TABLE IV

Cell Cycle Phases Derived from Pulse-labeling Experiments

Nitrogen source	Doubling time	Fraction of cells			Fractional lengths of cell cycle phases			Relative cell age at	
		Unbudded (A)	Labeled (B)	Unbudded and labeled (C)	G1*	S‡	G2§	Bud emergence	Mid S phase‖
	min								
Ammonia	120	0.48	0.50	0.20	0.22	0.49	0.29	0.40	0.46
Glutamine	100	0.58	0.48	0.32	0.20	0.47	0.33	0.49	0.44
Methionine	195	0.57	0.52	0.26	0.24	0.53	0.23	0.48	0.50
Threonine	255	0.62	0.41	0.25	0.30	0.41	0.29	0.54	0.50
Proline	380	0.62	0.52	0.34	0.22	0.52	0.26	0.54	0.48

The values for B and C were obtained from pulse-labeled cells and are corrected for the Poisson distribution of grains (see text and legend to Fig. 5); the m values ranged from 3.2 to 8.5, and the P(o) values from 0 to 0.04. At least 500 cells were scored for each culture.

* The fraction of cells in G1 phase = N_{G1} = (fraction unbudded) − (fraction both labeled and unbudded) = (A) − (C), where A and C are the values in columns A and C. The fraction of the cell cycle in G1 = $Q_{G1} = 1 - \log(2 - N_{G1})/\log 2$.

‡ The fraction of the cell cycle in S phase, Q_S, was calculated as follows: The fraction of cells in S = N_S = fraction of labeled cells. The fraction of the cell cylce in G1 + S = $Q_{G1+S} = 1 - \log(2 - N_{G1+S})/\log 2$, and $Q_S = Q_{G1+S} - Q_{G1}$.

§ The fraction of the cell cycle in G2 phase = $Q_{G2} = 1 - Q_{G1+S}$. The ~7% of the cycle between nuclear division and cytokinesis is included in the G2 phase in these calculations.

‖ G1 plus one-half of S phase.

(Tables III–V). Strikingly, regardless of the nitrogen source used or the method of measurement employed, the S phase occupies close to 50% of the cell cycle. A DNA fiber autoradiographic analysis of these cells has shown that the longer S phases can be accounted for by a reduction in replication fork rate (19). That cells do not become committed early in the cell cycle to an S phase of a given length was demonstrated by the observation that cells shifted from a poor nitrogen source (proline) to a good one (glutamine) during a G1 phase arrest complete the subsequent synchronous S phase in a time that is typical of cells grown with glutamine (Fig. 6 *C* and Table V).

Phase Coordination in Nitrogen-Source-Limited Cell Cycles

G1 phase and G2 phase were also measured in %LND and pulse-labeled cell experiments. Like S phase, they are maintained as a constant fraction of the cell cycle when cells are grown with the different nitrogen sources (Tables III and IV). The definitions of these intervals are somewhat different in the two types of experiments. In the analysis of a %LND curve, G2 phase is taken as ending with nuclear division. Consequently, the G1 phase determined from %LND experiments should be slightly longer than G1 phase determined from pulse-labeled cell experiments, and G2 phase should be slightly shorter by the same amount. When the interval between nuclear division and cytokinesis (~7% of the cell cycle) is taken into account, the two methods give a consistent picture of the proportional expansion of the cell cycle phases.

The α-factor-sensitive period also occupies a fairly constant proportion of the cycle. Measurements of the α-factor block point and the initiation of DNA synthesis were not done in the same experiment, so their precise relationship cannot be deduced. However, the data indicate that cells become insensitive to α-factor at about the time that S phase begins in cells growing in the three nitrogen sources (Tables II and IV). α-Factor arrests *a* haploid cells at the same point in the cell cycle, start, as do temperature-sensitive mutations in gene *cdc*28 (12). From this point, several different life cycle activities can ensue: starting a new vegetative cell cycle, mating to form a diploid zygote (17), or entering meiosis to form haploid spores (see reference 8). Our data indicate that in the three cultures examined the start point is at or very close to the G1 phase/S phase boundary.

In culture conditions that give longer doubling times, bud emergence takes place at later relative

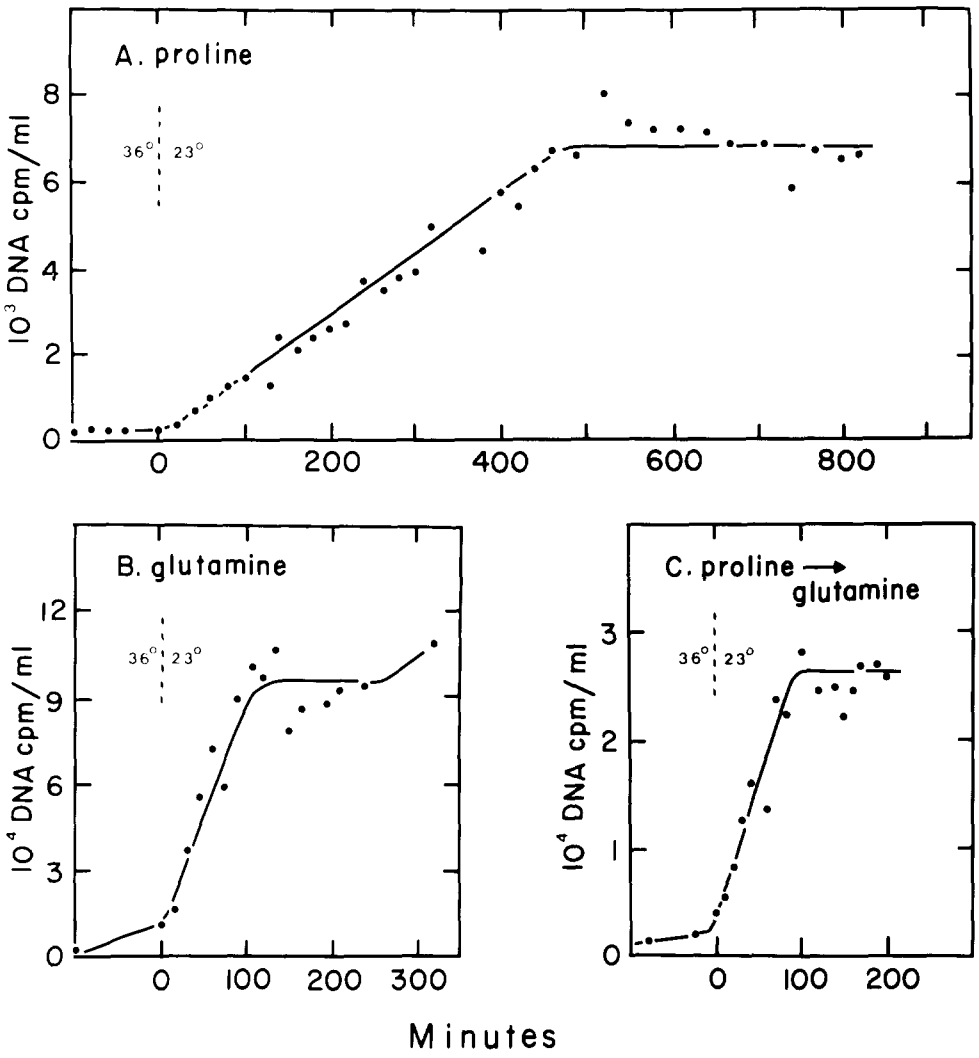

FIGURE 6 Synchronous S phases. Cells of strain 4008 (*cdc7-4*) grown with (*A*) proline medium (doubling time 915 min at 23°C) or (*B*) glutamine medium (doubling time 230 min at 23°C) containing 25 μg/ml uridine were treated for one doubling time with α-factor at 23°C. α-Factor was removed by filtration and the cells were placed in fresh media warmed to 36°C. 10 μCi/ml (0.1 μg/ml) [^{3}H]uracil was added after 100 min; 20 min later, the culture was shifted to 23°C and the subsequent synchronous S phase was monitored by the incorporation of ^{3}H into DNA. The length of S phase (from the start of the rise to the plateau of incorporation) is equal to about half the doubling time of the respective cultures. (*C*) Strain 4008 grown in proline medium was synchronized by α-factor and a temperature shift as above. After 2 h at 36°C the cells were transferred to glutamine medium containing 10 μCi/ml (0.1 μg/ml) [^{3}H]uracil at 36°C. 30 min later, the culture was transferred to 23°C and the S phase was determined.

cell ages (Tables I, II and IV). Thus, while both the unbudded and budded portions of the cycle increase in absolute length with increasing doubling time, the unbudded interval increases by a larger factor. With all growth conditions employed here, including growth with ammonia as the nitrogen source, the daughter cell bud emerges at about the middle of the S phase (Table IV). It is clear, therefore, that bud emergence does not necessarily signal initiation of the S phase as is often assumed. The cell age at bud emergence and, indeed, the organization of the cell cycle into phases can vary considerably in *S. cerevisiae*. In the first studies of cell cycle phases in *S. cerevisiae*, Williamson (24)

TABLE V

Duration of Synchronous S Phases

Nitrogen source	Asynchronous doubling time at 23°C	Length of the synchronous S phase of 23°C	S phase length/doubling time
	min	*min*	
Ammonia	210	100	0.48
Glutamine*	170, 195	85, 95	0.50, 0.45
Threonine	500	260	0.52
Proline	915	500	0.55
Proline → Glutamine‡	915	100	—

Asynchronous cells of strain 4008 (*cdc*7-4) were arrested in G1 phase, first with α-factor and then by transfer to 36°C (Materials and Methods). The synchronous S phases were measured as the time over which [^{3}H]uracil is incorporated at a linear rate upon transfer back to 23°C (see Fig. 6 for examples).

* Two experiments.

‡ See the text and Fig. 6 *C*.

found a very short G1 phase; both bud emergence and initiation of the S phase occurred at a very early cell age (~0.1). The relative lengths of cell cycle phases may be strain specific as well as being dependent on the conditions of growth.

The size distributions of cells grown with a poor nitrogen source (proline) and a good one (glutamine) are largely overlapping (Fig. 1). However, a small fraction of cells in the poor nitrogen source is smaller than any in the glutamine population. A pronounced bimodality in cell size was observed by Hartwell and Unger (10) for cultures whose slow growth rate was caused by limiting the rate of protein synthesis. This variation is caused by the presence of smaller than normal daughter cells. Similar observations were made by Carter and Jagadish (6) using cultures limited by the rate of supply of glucose or by different carbon sources. It was proposed that the small daughter cells require a longer period than the mother cells before initiating the S phase. Because the slow-growing nitrogen-limited cultures examined here exhibit only a modest increase in cell size distribution, inequality between daughter cell and mother cell cycles does not seriously affect our estimates of the lengths of cell cycle phases.

CONCLUSION

Our results show that bud growth, DNA synthesis, and cell division rates are all affected to about the same degree in cells whose growth rates are limited with poor nitrogen sources. Cell cycle expansion for four growth rates is diagramed in Fig. 7. It is clear that the rate of transit through all phases of the cell cycle can vary considerably, contributing to changes in total cycle time. Thus, cell cycle phases other than the prestart interval can be rate-limiting.

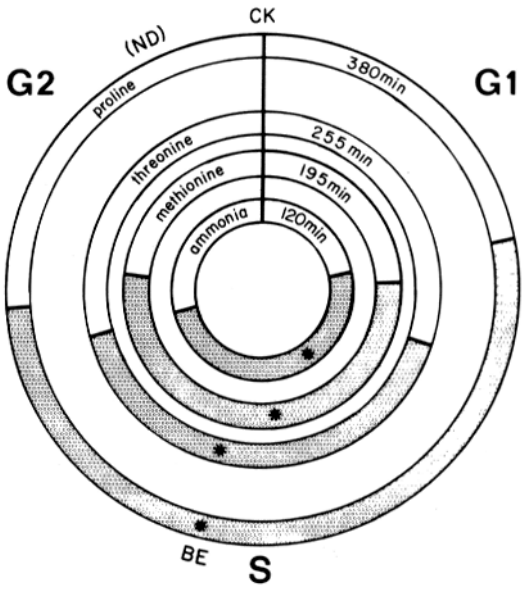

FIGURE 7 Scale diagram of cell cycle phases. The data are taken from Table IV. Cytokinesis (CK) was used to align the different cycles. Bud emergence (BE) is indicated by an asterisk in each cycle. Nuclear division (ND) as scored in the percent labeled nuclear division experiments occurs at ~7% of the cycle before cytokinesis at all growth conditions.

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