

REPLICATION FORK RATE AND ORIGIN ACTIVATION DURING THE S PHASE OF *SACCHAROMYCES CEREVISIAE*

CAROL J. RIVIN and WALTON L. FANGMAN

From the Department of Genetics, University of Washington, Seattle, Washington 98195. Dr. Rivin's present address is the Department of Biology, Washington University, St. Louis, Missouri 63130.

ABSTRACT

When the growth rate of the yeast *Saccharomyces cerevisiae* is limited with various nitrogen sources, the duration of the S phase is proportional to cell cycle length over a fourfold range of growth rates (C. J. Rivin and W. L. Fangman, 1980, *J. Cell Biol.* **85**:96–107). Molecular parameters of the S phases of these cells were examined by DNA fiber autoradiography. Changes in replication fork rate account completely for the changes in S-phase duration. No changes in origin-to-origin distances were detected. In addition, it was found that while most adjacent replication origins are activated within a few minutes of each other, new activations occur throughout the S phase.

Chromosome replication occurs during an interval which can change in length at different stages of development. This variation is especially dramatic from early to late embryogenesis in some organisms (19). The duration of the S phase can be thought of as the product of three potentially rate-limiting parameters: the rate of DNA chain elongation (fork rate), the distance between replication origins, and the time in S when different origins become active.

There have been two detailed studies of the basis of S-phase length variation. In the newt *Triturus* the S phase of spermatocytes is one hundred times as long as that of neurula cells, and four times as long as the S phase of somatic cells (3, 4). Fork rate does not vary in the different cell types, and the changes in S-phase length could be accounted for by changes in inter-origin spacing. Even greater S-phase variation has been observed in *Drosophila* in which cultured cells have a 600-min S phase while the cleavage nuclei of early embryos complete replication in <3.5 min (1). Again, the replication fork rate does not vary, and the observed inter-origin distance was only four-

fold shorter in the early embryos. The change in S-phase duration, therefore, may reflect a change in the temporal pattern of activation of origins (or clusters of origins). The observations that the inactive mammalian X chromosome replicates later than the active one and that the times of replication of some mammalian chromosomes exhibit tissue specificity (6, 25) also suggest that changes in the temporal pattern of origin activation can occur. In these cases, however, no change in the length of S phase has been noted.

We have begun a study of the relationships of the molecular parameters of the S phase in the budding yeast *Saccharomyces cerevisiae*. As in other eukaryotes, DNA synthesis in yeast proceeds bidirectionally from multiple replication origins (20, 21). Estimates of the replication fork rate (21, 15) are similar to those for mammalian cells. In addition, there is evidence that the replication of specific genes and, therefore, the activation of origins occurs at specific times in the S phase (2, 17). However, it is not known whether replication origins are activated throughout the S phase of yeast as they are in the cells of higher eukaryotes

(8), or only at the beginning of the S phase. In the preceding paper, we showed that S-phase duration increases as the cell cycle length increases for yeast cells growing with different sources of nitrogen (22). The experiments reported here show that changes in replication fork rate can account quantitatively for variations in S-phase duration, and that origins are activated throughout the yeast S phase.

MATERIALS AND METHODS

Strains, Media, and Radiolabeling

Strains and media are given in the preceding paper (22). For DNA fiber autoradiography, cells were grown in medium containing 5 $\mu\text{g/ml}$ uridine (23), pulse labeled with low specific activity [5,6- ^3H]uracil (8.9 Ci/mM, 100 $\mu\text{Ci/ml}$; Amersham Corp., Arlington Heights, Ill.), washed quickly by filtration, and incubated in the same medium with fivefold higher specific activity [5,6- ^3H]uracil (44.5 Ci/mM, 100 $\mu\text{Ci/ml}$).

Preparation of Fiber Autoradiograms

Fiber autoradiograms were made following the procedure of Petes and Williamson (21) with some modifications. Labeling was stopped by adding 0.5 ml of ice-cold, 10-fold concentrated carrier cells to 0.5 ml of labeled cells and chilling in an ice slurry. The cells were then collected on a Millipore filter (Millipore Corp., Bedford, Mass.), washed twice with ice-cold glass-distilled water, resuspended in 1 ml of pretreatment buffer (0.2 M Tris, 0.1 M EDTA, 1.2 M sorbitol, pH 9.1) with 7 μl 2-mercaptoethanol, and allowed to stand for 15 min at room temperature. The cells were again collected on a filter prewashed with SCE-60 (1 M sorbitol, 0.1 M Na citrate, 0.06 M EDTA, pH 8.0) and washed twice with that solution. They were then suspended in 2 ml of SCE-60 containing 50 μl Glusulase (Endo) and 0.1 ml of 10 mg/ml RNase for 45 min at 37°C or until spheroplasts were formed. Spheroplasts were collected by centrifugation for 10 min at 2×10^3 rpm. The spheroplasts were twice resuspended in SCE-60 and pelleted, the supernate was drained, and the pellet was dispersed in the little remaining liquid. 5 μl of the RNase solution and 5 μl of 5% sodium dodecylsarkosinate were placed in a drop on a subbed slide, and 5 μl of the spheroplast suspension was added to it. After lysis, the drop was slowly and smoothly spread across the slide with a glass rod, using a sinusoidal motion. 10 slides could be made from the original 0.5-ml culture sample in this way. The slides were air dried thoroughly and then immersed for 5 min in ice-cold 5% TCA. They were then washed twice with 5% TCA and twice in ice-cold 95% ethanol. When dry, the slides were coated with Kodak NTB-2 nuclear track emulsion diluted 1:1 with water. The autoradiograms were stored at 5°C over desiccant in light-tight boxes for 6–7 mo. The slides were developed in 1:2 Dektol (Kodak) and fixed in Kodak Rapid Fixer. Tracks of silver grains were measured with an ocular micrometer at $\times 800$.

RESULTS

Fork Rate Changes and Origin Spacing

A comparison was made of the fork rates in cultures of *S. cerevisiae* strain A364A using am-

monia, glutamine, threonine, or proline as the sole source of nitrogen at 30°C. The cultures had population doubling times of 105, 105, 325, and 380 min, respectively, and in each culture S phase occupies about half of the cell cycle time (22). The fork rate was measured by pulse labeling the asynchronous cultures with [^3H]uracil, preparing DNA fiber autoradiograms, and measuring the length of tracks of silver grains (12). When labeling was conducted in the presence of the DNA synthesis inhibitor hydroxyurea, such tracks were absent. To ensure that measurements were made on tracks from forks which were active throughout the labeling period, cultures were pulsed with a low specific activity radiolabel followed by a pulse at fivefold higher specific activity (Materials and Methods). The autoradiographic tracks of higher grain density (Fig. 1a) were measured to determine fork rates. Several different lengths of the two pulses were used for each growth condition, to increase the chance of finding dense grain tracks long enough to measure but not so long as to have resulted from the fusion of forks from adjacent origins (Fig. 2).

To be acceptable for a fork rate measurement, a densely labeled track had to be contiguous with a track of lighter density on one side but not the other. Dense tracks that terminated a linear array were not scored. These criteria minimize the possibility of including measurements of ambiguous autoradiographic patterns in the data (13). The length distributions of high specific activity tracks from ammonia, glutamine, threonine, and proline cultures are presented in Fig. 3. For cultures grown in ammonia or glutamine, the track length is proportional to the pulse time for labeling periods from 2 to 10 min, indicating that the uptake of the high specific activity label occurred without appreciable lag. When the mean track length is divided by the pulse time, these experiments give a fork rate of ~ 2.1 $\mu\text{m}/\text{min}$ for both culture conditions. Cells using threonine as a nitrogen source had a growth rate one half that of the cells in

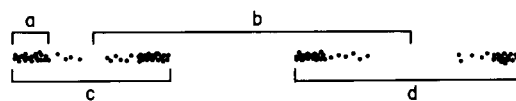


FIGURE 1 Diagram of DNA fiber autoradiographic patterns. The dots represent silver grains produced in the photographic emulsion by exposure to DNA labeled with a low specific activity then a high specific activity [^3H]uracil pulse. See text for explanations of intervals a, b, c, and d.

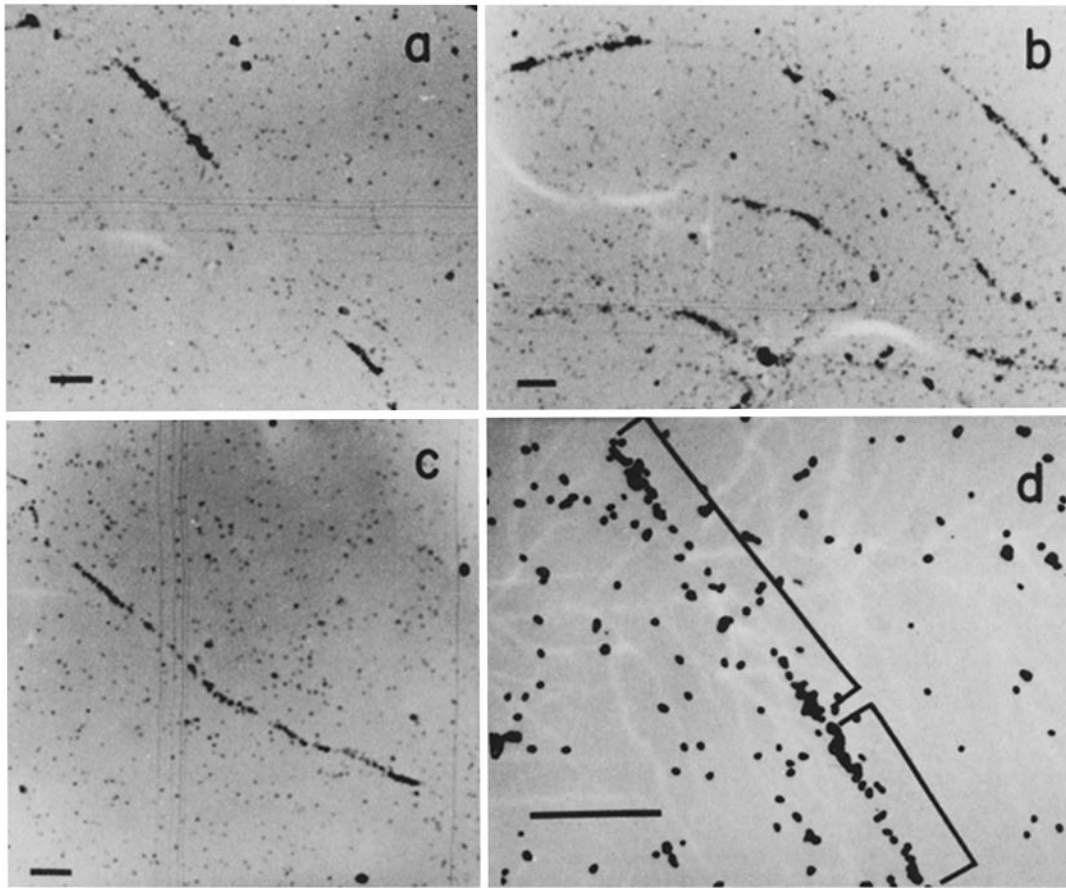


FIGURE 2 DNA fiber autoradiograms prepared from cultures exposed to a pulse of low specific activity [^3H]uracil followed by a pulse at high specific activity. The nitrogen source of the culture and the length of the high specific activity pulse are given. (a) Ammonia medium, 5-min pulse; (b) glutamine medium, 5-min pulse; (c) proline medium, 20-min pulse; (d) adjacent replicons (glutamine medium, 2-min pulse). The brackets *c* and *d* indicate lengths of DNA synthesized from two adjacent origins (see text). In each case, the horizontal bar represents $10\ \mu\text{m}$.

ammonia or glutamine medium. Data from DNA molecules pulse labeled for 8 and 12 min in threonine medium revealed that the fork rate ($0.98\ \mu\text{m}/\text{min}$) was also about one half that of cells in the better nitrogen sources. Experiments on cells growing in proline medium showed that both growth rate and fork rate ($0.56\ \mu\text{m}/\text{min}$) are about fourfold lower than in the best media. These results are summarized in Table I.

To determine the distance between origins, a more complex autoradiographic pattern was scored. Fig. 2 shows the pattern formed by adjacent bidirectional replicons during a low to high specific activity pulse sequence. A replication origin is taken to be the center of the symmetrical

light-dense labeled figures which reveal bidirectional replication. The distance between the centers of such patterns (Fig. 1*b*) was measured for replicating DNA from the fastest (glutamine medium) and slowest (proline medium) growing cultures. The data (Fig. 4) show that inter-origin distances cover a broad range, but that in both media most center-to-center distances are between 25 and $45\ \mu\text{m}$.

Activation of Adjacent Origins

The relative times of activation of adjacent origins can be measured if we assume that chain elongation from an origin occurs at a constant

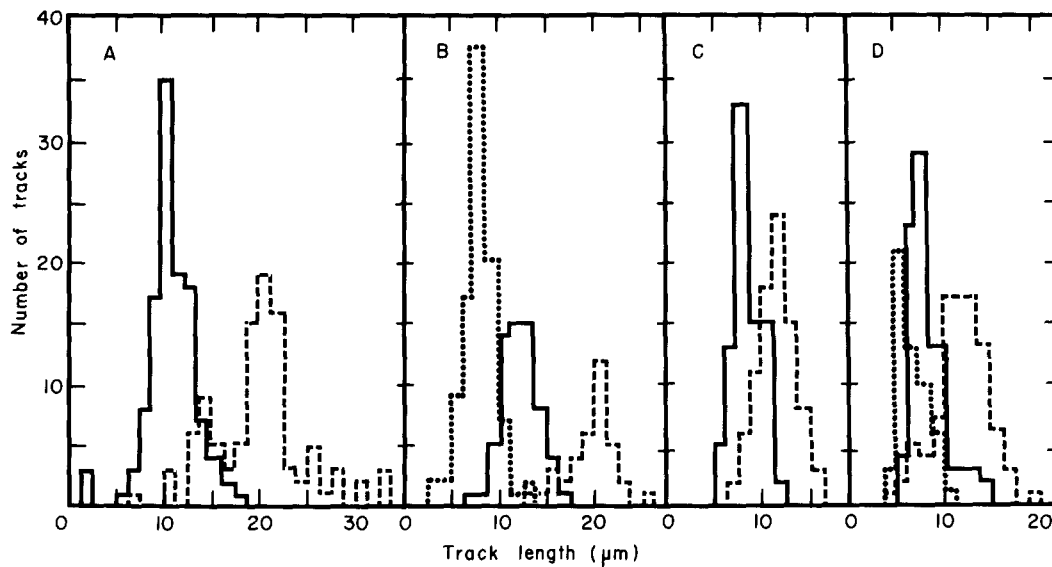


FIGURE 3 Track length distributions from pulse labeling of cells growing in four media. A364A cells grown in four nitrogen sources at 30°C were given a pulse of low specific activity [³H]uracil, followed by high specific activity pulses of various lengths. The high-density grain tracks from each pulse length were measured to obtain the fork rate in the different growth conditions. The track length measurements are plotted in 2.5- μ m intervals. (A) Ammonia medium (doubling time 105 min); pulse lengths: 5 min (—) and 10 min (---). (B) Glutamine medium (doubling time 105 min); pulse lengths: 4 min (···), 6 min (—), and 10 min (---). (C) Threonine medium (doubling time 235 min); pulse lengths: 8 min (—) and 12 min (---). (D) Proline medium (doubling time 380 min); pulse lengths: 10 min (···), 15 min (—), and 20 min (---).

TABLE I
Replication Fork Rates for Cells Growing with Various Nitrogen Sources

Nitrogen source	Doubling time	No. of tracks measured	Fork rate	Relative	
				Fork rate*	Growth rate‡
	<i>min</i>		μ m/ <i>min</i>		
Ammonia	105	254	2.13	1	1
Glutamine	105	393	2.04	0.96	1
Threonine	235	176	0.98	0.46	0.45
Proline	380	317	0.56	0.26	0.28

The mean fork rate was determined by dividing the average track length by the length of the pulse (see Fig. 2).

* Fork rate relative to that of cells in ammonia medium.

‡ Doubling time of cells in ammonia medium divided by doubling time of cells in media containing the other nitrogen sources.

rate, and that the size of each bidirectional pattern reflects how long replication forks have been active (7). Such measurements were made with asynchronous populations of cells growing in both glutamine and proline media. The length of DNA synthesized from each origin was measured for pairs of adjacent origins (Fig. 1 *c* and *d*, and Fig.

2 *d*). In Fig. 5 *A* the pairs of values are plotted, with *c* being the shorter length for each pair, and *d* the longer one. As the measurements are precise to about $\pm 1 \mu$ m, about two-thirds of the pairs of values differ significantly from equality. When a length value is divided by twice the fork rate, the time elapsed since activation of an origin is ob-

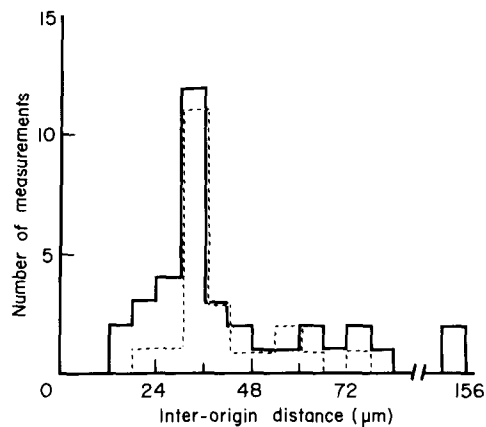


FIGURE 4 Inter-origin spacing for cells growing with glutamine or proline. The distances between adjacent origins were measured in autoradiograms prepared from cells grown in glutamine (—) or proline (---). The center of symmetry of a bidirectional replication pattern was taken to represent an origin. The measurements are plotted in 6- μ m intervals.

tained. The difference between activation times for adjacent origins is shown in Fig. 5 B. The data show that less than a few minutes elapse between activations of most adjacent origins. Nevertheless, in both cultures activation of adjacent origins may be separated by as much as 20 min. Because the S phase is a total of 50 min for cells growing with glutamine at 30°C (22), the data suggest that origin activation can take place over about half of the S phase. The data are not sufficient to determine whether glutamine- and proline-grown cells differ in the temporal pattern of origin activation.

Constancy of the Fork Rate during S Phase

We showed in the preceding paper that when yeast cells growing with a particular nitrogen source are synchronized at the G1/S phase boundary, they complete the subsequent synchronous S phase in the same length of time as cells in steady-state growth in the same nitrogen source (22). This apparent equivalence makes it possible to examine the temporal structure of the S phase. Fiber autoradiograms were prepared from glutamine-grown cells undergoing a synchronous S phase to learn (a) whether changes in the fork rate occur during the S phase, and (b) whether new origin activations take place throughout the S phase. Synchrony was achieved by blocking a temperature-sensitive DNA synthesis initiation mutant (strain 4008, *cdc7-4*) first with α -factor, a mating pheromone,

at 23°C, and then by exposure of the restrictive temperature, 36°C (22). S phase was then allowed to take place in the presence of low specific activity [³H]uracil by transferring the cells back to 23°C. At times corresponding to early, middle, and late S phase (Fig. 6), samples of cells were placed in medium containing high specific activity [³H]uracil for 10 min and then processed for autoradiography.

The track length distributions for the three samples (Fig. 7) are very similar with a mean value of ~1.2 μ m/min, indicating that the fork rate does not change appreciably during the course of S

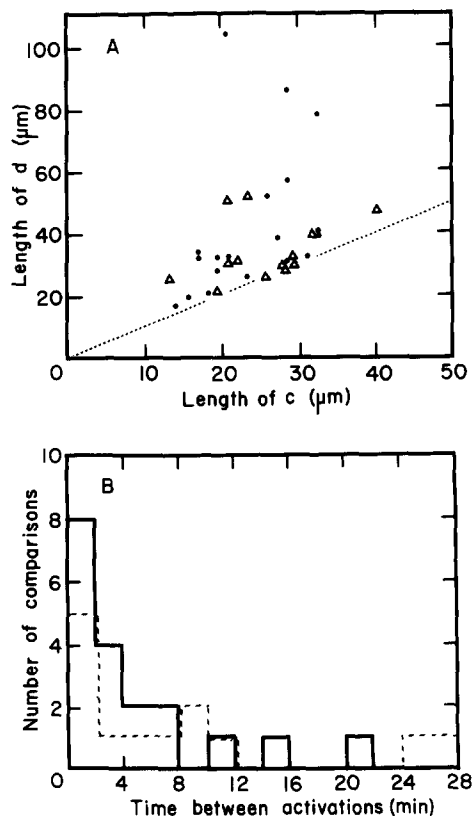


FIGURE 5 Difference in activation time of adjacent origins. (A) The lengths of pairs of adjacent bidirectional replication patterns are plotted such that *c* is always the shorter length of the pair, and *d* is the longer one (see Fig. 1). Pairs with equal lengths would fall on the dashed line. (●) Glutamine-grown cells; (Δ) Proline-grown cells. (B) The minutes elapsed between activation of one origin (*d*) and the adjacent one (*c*) are given in 2-min intervals for glutamine- (—) and proline-grown (---) cells. The length of time that an origin had been active was determined by measuring the length of the bidirectional pattern and dividing this by twice the fork rate.

phase. Allowing for the difference in temperature, the fork rate in synchronized cells (23°C) is about the same as that in unsynchronized cells (2.1 $\mu\text{m}/\text{min}$ at 30°C). Although the new origin activation patterns observed in these experiments were ambiguous because of the aggregation of labeled molecules, the constancy of the fork rate and the constancy of the rate of replication (Fig. 6, and Fig. 6 of reference 22) lead to the conclusion that origins are activated throughout the S phase (see Discussion).

DISCUSSION

Replication fork rate has been observed to change as much as two- to 3fold during the S phase in some mammalian cells growing in culture but not in others (e.g., references 16 and 11). Measurements made with synchronized yeast cells grown in glutamine medium showed that the fork rate stays constant throughout the S phase (Figs. 6 and 7). However, when cells are grown at different rates using various nitrogen sources, the rate of fork movement is characteristic of the growth rate (Fig. 3 and Table I). The best nitrogen sources, glutamine and ammonia, gave fork rates of $\sim 2.1 \mu\text{m}/\text{min}$ at 30°C. Threonine medium gave a fork rate of $\sim 1 \mu\text{m}/\text{min}$, and proline medium gave a fork rate of $0.56 \mu\text{m}/\text{min}$. As the fork rates are

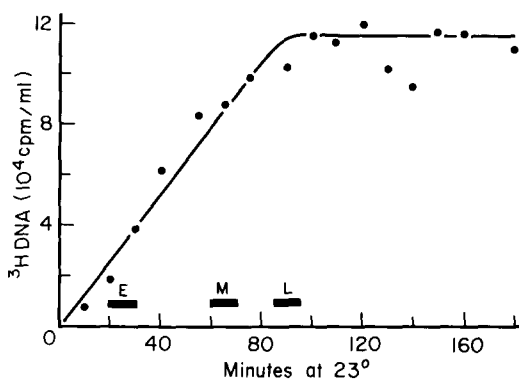


FIGURE 6 Incorporation of radiolabel into DNA during a synchronous S phase. Cells of strain 4008 (*cdc 7-4*) growing in glutamine medium (doubling time 190 min at 23°C) were synchronized by exposure to α -factor for one generation time at 23°C, then transferred to medium without α -factor at 36°C (22). After 100 min at 36°C, low specific activity [^3H]uracil was added. 10 min later, the cells were shifted to 23°C, allowing S phase to begin. At three times during the synchronized S phase, samples were removed (bars in the figure), labeled at high specific activity for 10 min, and DNA fiber autoradiograms were prepared.

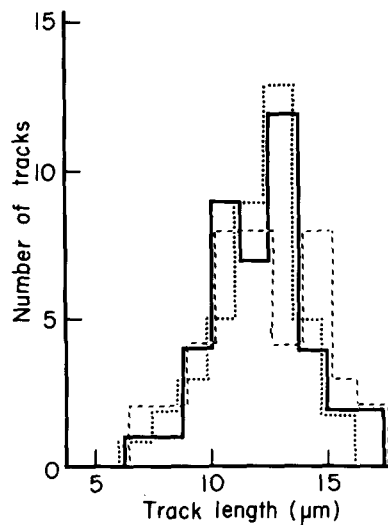


FIGURE 7 Constancy of fork rate during a synchronous S phase. Measurements were made of high-density grain tracks resulting from 10-min high specific activity pulses at different times during a synchronous S phase of strain 4008 in glutamine medium (Fig. 6). Track length distributions from early (\cdots), mid ($-$) and late ($---$) S phase are plotted in 2.5- μm intervals.

inversely proportional to the length of S in these cultures (22), fork rate variation can account entirely for the S-phase variation observed during nitrogen-limited growth. These results contrast with earlier studies of S-phase length variation where only changes in origin spacing were observed (3, 4, 1). Therefore, the length of the S phase can be changed in at least two different ways.

We are unable to say how the nitrogen-limited growth affects fork movement. This complex process involves a large number of nitrogenous compounds including many proteins, the deoxyribonucleotide triphosphates, and ATP. It seems unlikely that changes in the rate of protein synthesis directly cause fork rate changes since the cell cycle data for yeast growing slowly because of sublethal amounts of cycloheximide are not consistent with the occurrence of S-phase expansion (9). One possible explanation is that both ATP and deoxyribonucleoside triphosphate pool sizes are decreased in the nitrogen-limited cells, as this condition induces purine-degrading enzymes in *S. cerevisiae* (5).

Differences in replication origin spacings were not observed for yeast cells grown in glutamine or proline (Fig. 4), although the S-phase length dif-

fers fourfold between these culture conditions. In both cultures, the distribution was broad with a maximum at 30 μm , a value in good agreement with other autoradiographic measurements of inter-origin spacing in yeast (21). We did not observe the two clusters of origin spacings, at ~ 15 and 30 μm , which have been seen by electron microscopy (20; C. Newlon, personal communication).

The extreme ends of the inter-origin distance distribution may be under-represented in the data. Closely spaced origins are difficult to distinguish by autoradiography; converging forks from origins which are only a few microns apart would fuse during the labeling sequence. Very closely spaced origins ($\sim 0.3 \mu\text{m}$) have been observed for insect (1, 26) and sea urchin embryos (18) by electron microscopy. Electron microscope analysis of replicating yeast DNA has revealed that a few percent of inter-origin distances are 2–3 μm (C. Newlon, personal communication). Distantly spaced origins are likely to be separated by strand breakage. However, the distribution of spacings up to 70 μm cannot be greatly underestimated because only 15% of the origin pairs measured were spaced $>70 \mu\text{m}$ apart, while 45% of DNA fibers spread from fully labeled cells were greater than this length. As only 3% of fully labeled DNA fibers were $>180 \mu\text{m}$, very widely spaced origins, or clusters of origins, would not be detected.

Based on observed inter-origin spacings, there are at least 150 origins in the *S. cerevisiae* genome (20, 21). It has not been demonstrated whether, as in higher eukaryotes, different yeast origins initiate replication throughout the S phase. Because protein synthesis is not required to complete the S phase in yeast (24, 10), it has been suggested that most origins may initiate early (21). We measured the size of the autoradiographic patterns produced by pairs of adjacent origins as an index of the relative times of activation. As in mammalian DNA (7, 14), most adjacent origins start synthesis within a brief period. However, activation of some adjacent origins appears to be separated by 10–20 min (Fig. 5), or up to almost one-half the duration of the S phase of glutamine-grown cells.

Although alternative explanations of the autoradiographic patterns are possible (12, 13), the existence of temporally staggered origin activations is supported by other observations on glutamine-grown cells: (a) Most origin spacings are $<40 \mu\text{m}$ (Fig. 4). This value is in agreement with data for cells grown on ammonia (20, 21) and on proline (Fig. 4). (b) The average fork rate is 1.2

$\mu\text{m}/\text{min}$ at 23°C (Fig. 7). With bidirectional replication, most early activated forks should meet and cease replication in ~ 15 min. (c) At 23°C, the synchronous S phase is ~ 100 min in length (Fig. 6) and appears to be equivalent to the asynchronous S phase (22). The total rate of DNA synthesis is approximately constant throughout the synchronous S phase (Fig. 6, and Fig. 6 of reference 22). This rate must be maintained (after 15 min) either by a large increase in the replication fork rate or by new origin activations. Because the replication fork rate does not change from early to mid to late S phase in the synchronized cells (Fig. 7), we conclude that there are new activations throughout the yeast S phase.

Two features of origin activation follow from this conclusion and existing data. First, because specific genes are known to replicate in an ordered sequence (2, 17), origins must be activated according to a temporal program. Second, because S phase is completed in the presence of cycloheximide (24, 10), activation of origins late in S phase does not require protein synthesis. These late activations must be accomplished by proteins synthesized before the S phase. The temporal program itself may be provided by some other molecules, perhaps origin-specific RNAs synthesized in response to the replication of “early” DNA sequences.

If origins activated in late S phase are located between closely spaced origins ($<40 \mu\text{m}$) which are activated early, then specific termination sites for early replication forks must exist. So far, early fork termination has not been detected by DNA fiber autoradiography experiments, or by sedimentation analysis of nascent DNA (15). Alternatively, the late activations may occur in regions of chromosomes different from those regions that are activated early.

In summary, changes in replication fork rate can account quantitatively for changes in the duration of the S phase in yeast cells growing with different nitrogen sources. Nevertheless, because origins are activated throughout the S phase, the possibility that there are also changes in the number of active origins or in the temporal pattern of origin activation cannot be eliminated.

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