

Regulation of the start of DNA replication in *Schizosaccharomyces pombe*

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Accepted 8 January; published on WWW 25 February 1999

SUMMARY

Cells of *Schizosaccharomyces pombe* were grown in minimal medium with different nitrogen sources under steady-state conditions, with doubling times ranging from 2.5 to 14 hours. Flow cytometry and fluorescence microscopy confirmed earlier findings that at rapid growth rates, the G₁ phase was short and cell separation occurred at the end of S phase. For some nitrogen sources, the growth rate was greatly decreased, the G₁ phase occupied 30-50% of the cell cycle, and cell separation occurred in early G₁. In contrast, other nitrogen sources supported low growth rates without any significant increase in G₁ duration. The method described allows manipulation of the length of G₁ and the relative cell cycle position of S phase

in wild-type cells. Cell mass was measured by flow cytometry as scattered light and as protein-associated fluorescence. The extensions of G₁ were not related to cell mass at entry into S phase. Our data do not support the hypothesis that the cells must reach a certain fixed, critical mass before entry into S. We suggest that cell mass at the G₁/S transition point is variable and determined by a set of molecular parameters. In the present experiments, these parameters were influenced by the different nitrogen sources in a way that was independent of the actual growth rate.

Key words: Fission yeast, DNA replication, Cell cycle, Growth rate

INTRODUCTION

When a culture of cells is grown under steady-state conditions, each cell goes through the same cell cycle over and over again, within certain limits of variation. The occurrence of DNA replication, mitosis and cell separation at defined times and at a defined mass under a given set of conditions reflect the tight regulation of these processes and that they are in some way coupled to cell growth. Since cell mass is a monotonically increasing function of time, it is not surprising that any cell cycle dependent event occurs at a certain cell mass, under a given set of growth conditions.

Current models for growth control in the fission yeast *Schizosaccharomyces pombe* involves cell mass as the critical parameter. It is a long standing observation that mitosis occurs at a certain mass in a given growth medium, and never at lower masses, unless mutations are introduced (Nurse, 1975; Nurse and Thuriaux, 1977). Before passing from G₁ to S phase, *S. pombe* cells must also attain a certain mass, but here the situation is a little more complex. Under standard laboratory conditions, i.e. in broth medium or in minimal medium with good nutrient supply, the cells pass through mitosis with a cell mass that is higher than that required for further passage into S (Nurse and Thuriaux, 1977; Fantès and Nurse, 1978; Sveiczzer et al., 1996). Therefore, the cells spend a minimum of time in G₁ and the control point at the G₁/S border is silent. Only under special conditions, such as in the *wee1* mutant, is this control expressed: the *wee1* mutation allows the cells to go through mitosis at a much reduced mass so that they arrive

at the G₁/S border with a mass that is lower than required for passage into S. In this case, the cells must delay further cell cycle progression until the critical mass is attained, which results in an extension of the G₁ phase.

The above model for S phase control is supported by experiments where the protein content of mutant cells was measured (Nasmyth et al., 1979). Mutants that passed into mitosis with a reduced protein content were observed to extend their G₁ phase and continue into S only when a minimal protein content was achieved. As the protein content in mitosis was reduced beyond a certain level, by the different mutations, the G₁ phase started to extend. The conclusion was that DNA replication cannot be initiated until a critical cell mass is obtained.

In the present experiments, we have taken a different approach to investigate the obligatory coupling between DNA replication and cell growth. Cells of wild-type *S. pombe* were grown in minimal medium supplemented with different nitrogen sources to obtain a wide range of growth rates. This approach allows us to study the G₁ duration and entrance into S of cells that have arrived into G₁ with different cell masses.

MATERIALS AND METHODS

Strain, media and growth conditions

Wild-type *Schizosaccharomyces pombe* *h*⁺ and the otherwise isogenic *rum1Δ* (Moreno and Nurse, 1994b) were grown under steady-state conditions in shaker flasks at 32°C in minimal medium, EMM2

(Moreno et al., 1991) or in EMM2 where NH_4Cl was replaced by 20 mM of either L-cysteine, glycine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-serine, DL-threonine, L-tryptophan or uracil as nitrogen source. Growth was monitored by measuring the optical density (OD) at 595 nm and samples were withdrawn for flow cytometry at an OD_{595} of 0.2.

Fixation, staining and flow cytometry

The cells were initially fixed in 70% ethanol as described (Skarstad et al., 1985). Before staining with mithramycin/ethidium bromide (MEB) the cells were fixed in methanol-acetic acid (3:1) for at least 2 hours. For DNA staining, rehydrated cells were mixed with an equal volume of MEB in 10 mM Tris-HCl, pH 7.4, containing 10 mM MgCl_2 (Skarstad et al., 1985). Fluorescence and light scatter signals from individual MEB stained cells were measured with an Argus 100 machine (Skatron, Lier, Norway) as described earlier (Skarstad et al., 1985). Alternatively, for simultaneous protein and DNA measurements, the ethanol-fixed cells were rehydrated in 0.1 M phosphate buffer, pH 9.0, and diluted to the same protein concentration (10 $\mu\text{g}/\text{ml}$) before staining with FITC as described before (Wold et al., 1994). Afterwards, the DNA was stained with Hoechst 33258 (1.41 $\mu\text{g}/\text{ml}$) in a 20 mM phosphate buffer, pH 7.4, containing 0.13 M NaCl, and DNA-associated fluorescence was measured in a FACStar+ flow cytometer (Becton Dickinson).

RNase treatment

The cells were incubated with 0.1 mg/ml RNaseA (Sigma) either before MEB staining or after FITC staining.

Fluorescence microscopy

The cells were stained with DAPI (4',6-diamidino-2-phenylindole) in the presence of antifade (*p*-phenylenediamine) or with Calcofluor (fluorescence brightener 28, all from Sigma) as described (Moreno et al., 1991) and examined with a fluorescence microscope (Zeiss Axioskop).

Analysis of DNA distributions

The fractions of cells in the different cell cycle phases were determined with a standard program (Modfit, supplied by Skatron). Alternatively, the cells in two-parameter histograms were separated into three populations, containing 1C, 2C or between 1C and 2C DNA content. The borders between the populations were set by eye.

Conversion from population fractions to cell cycle period durations was performed by assuming an exponential age distribution (Wold et al., 1994).

RESULTS

Flow cytometry analysis of slowly growing cells

Cultures of *S. pombe* were maintained at generation times of between 2.5 and 14 hours (growth rates between 0.40 and 0.07 doublings/hour), by varying the nitrogen source (Table 1, column 2). Four independent series of experiments were performed, each involving cultures grown with the eleven different nitrogen sources. During steady-state growth, samples were withdrawn for analysis by flow cytometry to determine the DNA and mass of individual cells in the nonsynchronous population. In the present experiments, we observed two classes of DNA histograms. In one, the histogram contained one major peak of 2C DNA and a shoulder stretching towards 4C DNA, as shown schematically in Fig. 1A. In such cultures both the G_1 and the G_2 cells are in the 2C population since cell separation occurs close to the S/ G_2 border. Therefore, cells in S phase increase their DNA

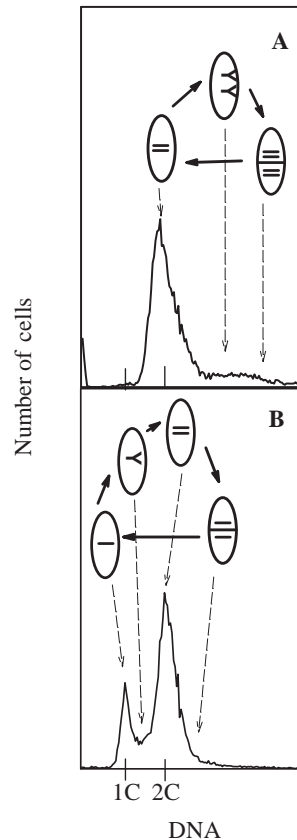


Fig. 1. Schematic DNA histograms of cells with a short G_1 period and cell separation after S (A) and with a long G_1 and cell separation before S (B). The ovals represent cells where each internal line represents a unit of 1C DNA and the Y-shape indicates the process of DNA replication. The stippled arrows refer to where in the histogram each cell form will be found.

content from 2C to 4C before cell separation occurs. Alternatively, the DNA histogram contained two distinct peaks, representing 1C and 2C cells (Fig. 1B). In this case cell separation occurs in G_1 . The 1C cells are in late G_1 , after cell separation has occurred, and the 2C cells are either in G_2/M or in early G_1 . The S phase cells are represented by the ridge connecting the 1C and 2C peaks.

With NH_4Cl as the nitrogen source the growth rate was 0.40 doublings/hour and the vast majority of the cells contained 2C DNA (Fig. 2A). The fraction of cells in S phase is represented by the shoulder in the DNA histogram stretching from 2C towards 4C DNA content. This experimental histogram is representative of cells in rich media and corresponds to the schematic histogram in Fig. 1A.

As a measure of cell mass we first used scattered light (see below). The light scatter signal from the S phase cells was higher than from the other cells in the population (Fig. 2B), confirming that S phase occurred in the largest cells and therefore towards the end of the division cycle.

In contrast, DNA histograms of cells in some of the more slowly growing cultures contain a distinct 1C peak (Fig. 2E-P), corresponding to the schematic histogram of Fig. 1B. The 1C cells can only occur because cell separation occurs in G_1 . Consequently, at slow growth rates cells in late G_1 and in S

were the smallest in the population, in contrast to what was found at the highest growth rates (Fig. 2, right column).

It should be noted that the fraction of cells with a given DNA content reflects the time spent with that particular DNA content. Therefore, when a large fraction of the cells contains 1C DNA the time from cell separation to G₁/S occupies a large fraction of the cell cycle. The general trend in the present experiments is that the frequency of 1C cells increases with the doubling time.

Cell cycle distribution at different growth rates

The fractions of cells in G₁, S, and G₂+M were estimated from the DNA histograms by two methods, computer simulation and by visual inspection of the histograms (see Materials and Methods). In a typical example, an isoleucine-grown culture was, by the simulation method, estimated to contain 16% cells with 1C DNA, 65% with 2C DNA and 19% with between 1C and 2C DNA. By the alternative, more direct method, the same histograms were estimated to contain 17% of the cells with 1C DNA, 70% with 2C DNA, and 13% with between 1C and 2C DNA. Also for the other nitrogen sources the two methods gave essentially the same results. For histograms where there was no 1C peak present the simulation method could not be used. Neither method could be used to distinguish between cells containing 2C DNA in G₁ and in G₂+M, and measurements of the septation index were used to estimate these fractions (see below).

For growth with NH₄Cl, the septation index was 13%. Thus, at any one time 13% of the cells are placed between the start of septation (end of mitosis) and cell separation. The fraction of cells undergoing septation varied more than twofold when the growth rate was varied (Table 1, column 3), showing that septation did not occupy a constant fraction of the cell division cycle.

The fraction of cells in G₁ phase was determined as follows. For NH₄Cl and uracil, all G₁ and S phase cells contain a septum (since the cells divide at the end of S), and the difference between the fractions of septated cells (Table 1, column 3) and S phase cells (column 4), yielded the fraction of G₁ cells (column 8). For cells grown with proline and cysteine, cell separation occurred during S phase. A similar procedure could be used here, i.e. subtracting the fraction of S phase cells with more than 2C DNA to obtain the fraction of G₁ cells. For the other nitrogen sources, the fraction of cells in G₁ before cell separation is equal to the septation index. The remainder of cells in G₁ are the cells with 1C DNA content (Table 1, column 5), and this fraction could be determined from the DNA histograms.

The fraction of cells in G₂+M (Table 1, column 7) could be found by subtracting the fraction of septated G₁ cells from the total fraction of cells with 2C DNA (Table 1, column 6). We have made no attempt to estimate separately the fractions of cells in G₂ and in M.

Cell cycle phase durations vary with growth rate

The fractions of cells in the different cell cycle phases were used to calculate the duration of G₁, S, G₂+M, and the septation period. The duration of all four was strongly dependent on the nitrogen source used (Fig. 3A). Extensions of all cell cycle phases could be observed when the nitrogen source was changed, but which phases that were extended varied with the

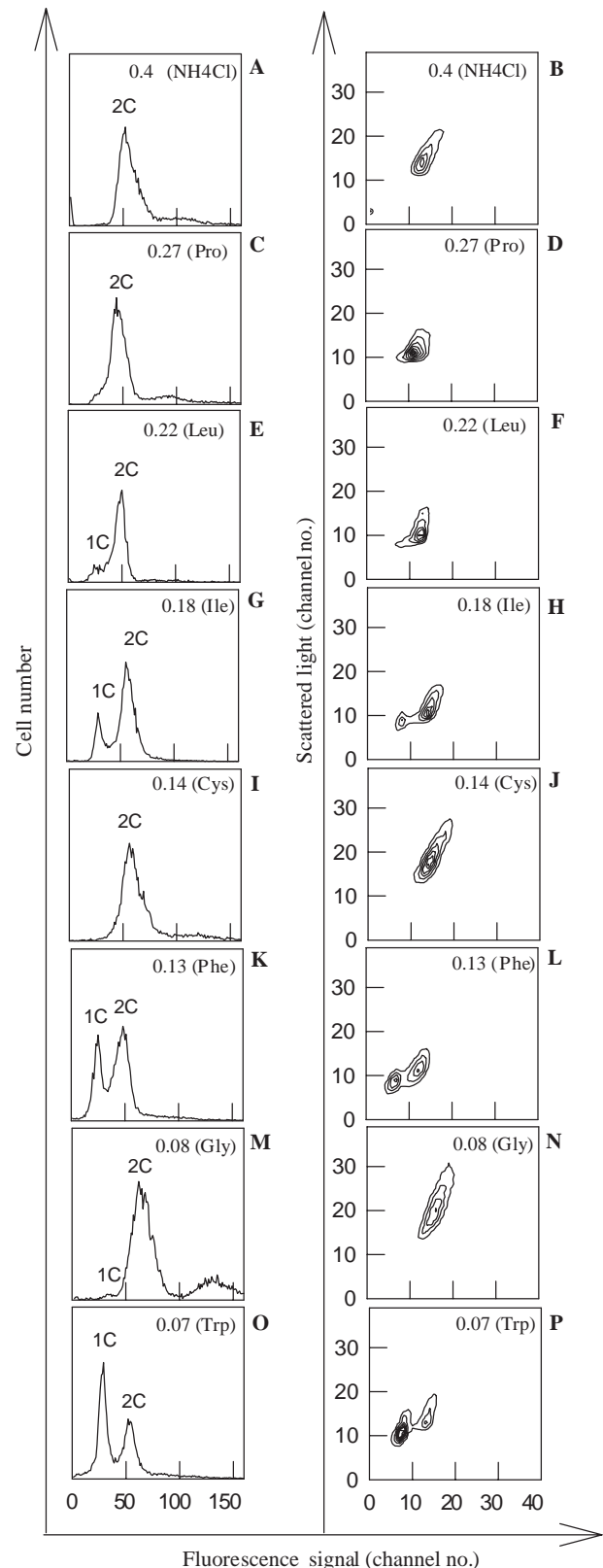


Fig. 2. Flow cytometry histograms of individual *S. pombe* cells grown at different growth rates and with different nitrogen sources, as indicated in each panel. The left column shows DNA histograms while the right column displays the two-parameter DNA contents versus scattered light histograms.

Table 1. The fractions of cells in the different cell cycle phases as determined by flow cytometry and fluorescence microscopy

1. Nitrogen source	2. Growth rate (doublings/hours)	3. Septated cells	4. S phase cells	5. Cells with 1C DNA	6. Cells with 2C DNA	7. G ₂ +M cells	8. G ₁ cells
NH ₄ Cl	0.40	0.13	0.09	0.00	0.91	0.87	0.04
Uracil	0.33	0.11	0.09	0.00	0.91	0.89	0.02
L-proline	0.27	0.11	0.13	0.00	0.87	0.76	0.11
L-leucine	0.22	0.11	0.19	0.06	0.75	0.64	0.17
L-isoleucine	0.18	0.10	0.13	0.17	0.70	0.60	0.27
L-serine	0.15	0.12	0.08	0.09	0.83	0.71	0.21
L-cysteine	0.14	0.05	0.05	0.00	0.95	0.90	0.05
L-phenylalanine	0.13	0.06	0.11	0.29	0.60	0.54	0.35
DL-threonine	0.11	0.05	0.09	0.13	0.78	0.73	0.18
Glycine	0.08	0.05	0.04	0.01	0.95	0.90	0.06
L-tryptophan	0.07	0.06	0.10	0.54	0.36	0.30	0.60

nitrogen source. There was no clear correlation between increase in the doubling time and extension of any of the different cell cycle phases. The length of G₁ varied from about 10 minutes to 7.2 hours and G₂+M varied from about 2 hours to 11 hours. The period of septation varied by a factor of two. Thus, although cell separation is probably coupled to mitosis in some way, it is not coupled by a mechanism involving a constant timer. The duration of S phase varied from around 20 minutes for the most rapidly growing cultures to almost 2 hours for the tryptophan-grown culture. There was no good correlation between growth rate and duration of S phase. Also for the relative duration of the cell cycle phases there was no correlation with the doubling time (Fig. 3B).

The main conclusion from the above data is that none of the cell cycle periods occupy a fixed length of time nor a fixed fraction of the cell cycle. This is in contrast to earlier data (Nasmyth, 1979), where the growth rate of *S. pombe* cells was limited by nitrogen supply in a chemostat. The duration of S+G₂ was found to be independent of growth rate and all increases in generation time could be accounted for by a lengthened G₁. Also, our data are different from those reported for *S. cerevisiae*, where there is a proportional expansion of all cell cycle phases as the doubling time is increased (Rivin and Fangman, 1980).

The cell cycle is not determined by growth rate

The correlation between longer generation times and a larger fraction of cells with a 1C DNA content does not hold for two of the nitrogen sources used. First, in cysteine the cells grew very slowly (0.14 doublings/hour), but their DNA histogram reveals no cells with 1C DNA (data not shown) and their DNA histogram (Fig. 2I) is similar to that of cells grown much more rapidly. Second, in glycine, at a growth rate of 0.08 doublings/hour, the DNA histogram (Fig. 2M) is similar to that found for leucine-grown cells (Fig. 2E), which gave a growth rate of 0.22 doublings/hour. The small 1C-peak in these histograms (glycine and leucine) is evidence that the cells divided near the entrance to S phase. Thus, slower growth and poorer growth conditions does not necessarily lead to G₁ extension and a concomitant movement of cell separation into G₁. These exceptions to the general trend are significant and give us important information: first, the temporal placement of cell separation and S phase relative to one another and to M phase is not constant and depends on the growth conditions. Second, this pattern, reflecting the cell cycle kinetics, is not determined by the growth rate alone but is strongly influenced

by the nitrogen source, independent of the actual growth rate obtained.

Pre-Start G₁ is extended during slow growth

During rapid growth the G₁ period is very short and commitment to another mitotic cycle is made in a narrow time window called Start, located close to the G₁/S border. The rum1 protein is an important regulator in G₁ and it is required for an extended pre-Start G₁ (Moreno et al., 1994a; Moreno and Nurse, 1994b). To investigate whether the extension of G₁ observed in the present experiments occurs before or after Start, or both, we compared *rum1Δ* and wild-type cells, grown with isoleucine as nitrogen source at the same growth rate (0.18 doublings/hour). In contrast to wild-type cells (Fig. 2G), the DNA histogram from *rum1Δ* mutant cells did not show evidence of any 1C cells (data not shown). There are two alternative explanations for this finding. First, the rum1 protein might be instrumental in the extension of G₁ under the present conditions, and this extension carries with it a relocation of cell separation to G₁, resulting in 1C cells. Without rum1p the cells are unable to extend their G₁ and cell separation occurs in or after S phase, yielding DNA histograms with no 1C cells and mainly 2C cells. Second, G₁ is extended even in the absence of rum1p, but in the *rum1Δ* mutant cell separation is delayed so that it occurs in S phase. However, the fraction of septated cells was the same in the two cases, showing that duration of septation was the same, which argues against the second alternative. We conclude that rum1p is required for the extension of G₁ in the present experiments. Given the role of rum1p in pre-Start regulation (Moreno et al., 1994a; Moreno and Nurse, 1994b), it is likely that the extension occurs before Start, presumably by a mechanism involving cdc2p phosphorylation.

Cell mass at G₁/S

By flow cytometry, we have obtained estimates of the cell mass at entrance to S. As measures of cell mass we have used scattered light and FITC fluorescence. Scattered light is a parameter commonly used to measure cell mass, e.g. in measuring optical density to monitor cell growth in suspension. There is good evidence that scattered light gives a good measure of cell mass in bacteria (Wold et al., 1994). FITC is a compound which binds quantitatively to protein and its fluorescence per cell is a good measure of total cell protein (Freeman and Crissman, 1975).

The G₁/S mass could be found from the two-parameter

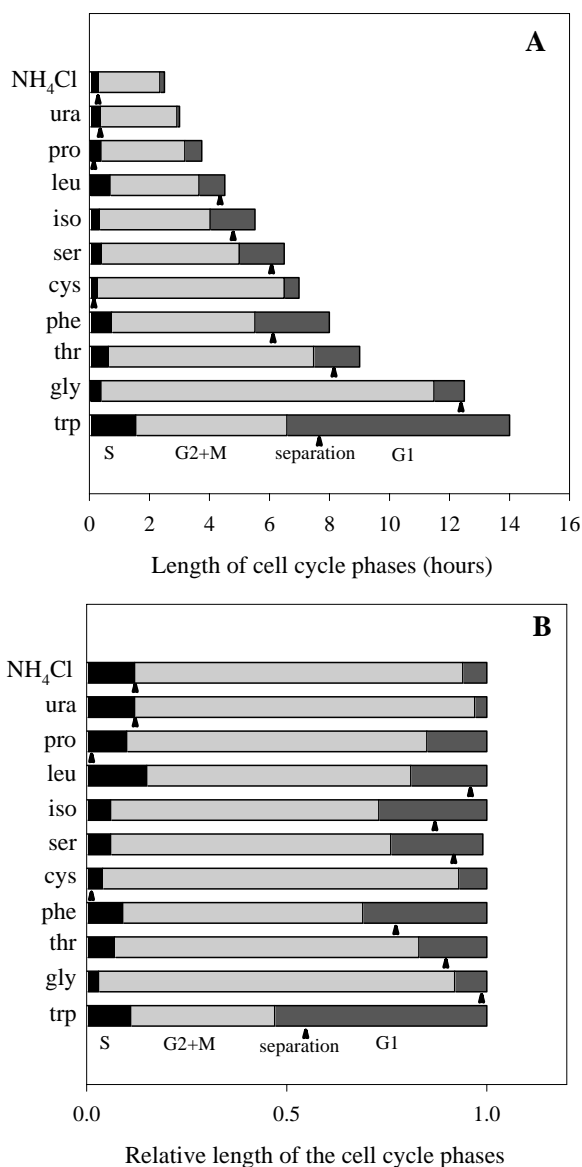


Fig. 3. Durations of the different cell cycle phases of *S. pombe* cells when grown with different nitrogen sources. (A) Absolute duration; (B) relative duration. All cell cycles have been aligned at the start of S phase, so that the black box at the left margin represents S phase. Then comes G₂+M (light grey), followed by G₁ (dark grey). Relative age at cell separation is indicated by an arrow.

histograms where the DNA content and scattered light (Fig. 2) or FITC fluorescence was measured. The point in the two-parameter histogram where S phase starts can be conveniently found as the point where a ridge of cells leaves the 2C population (or 1C, in some cases) towards higher DNA values. The channel number on the ordinate gives the value of scattered light or FITC fluorescence for individual cells as they enter S. For cells that entered S with two nuclei (2C DNA), the scattered light and FITC fluorescence values were divided by two, for comparison with cells that had gone through cytokinesis before S and entered S with 1C DNA.

The light scatter signal at entrance to S decreased slightly

with decreasing growth rate until it reached a lowest value at about 0.22 doublings/hour (Fig. 4A). Thereafter, the light scatter signal again increased. There did not seem to be a monotonic increase in G₁/S mass on either side of the lowest point. On the contrary, the variation observed between cultures grown on different nitrogen sources but with almost the same growth rate was in some cases much larger than the scatter of the four data points for one and the same medium, see e.g. the difference between cysteine and phenylalanine (0.14 and 0.13 doublings/hour, respectively). No straight line could be drawn through the data with a reasonable correlation coefficient. The data suggest that there is no simple relationship between growth rate and G₁/S mass, when measured as scattered light.

The FITC signal at the entrance to S also varied greatly with growth rate, and not in a monotonic fashion (Fig. 4B). Again, the variability between the results of four parallel experiments with the same nitrogen source was sometimes relatively large, but the variability between the different nitrogen sources was considerably larger. The data were analyzed by one way analysis of variance (ANOVA) to find out whether the FITC fluorescence in fact was independent of growth rate. The results show that the deviations are much greater than would be expected by chance ($P=0.001$). As was found for the light scatter data the best straight line through the data has a very low correlation coefficient ($R^2=0.37$), suggesting that there is no simple relationship between protein content at G₁/S and growth rate.

There was one major discrepancy between the data from measurements of FITC fluorescence and scattered light, namely for tryptophan-grown cells. Whereas these cells had the lowest FITC fluorescence (Fig. 4B), they had almost the highest light scatter value (Fig. 4A). In control experiments, average size of NH₄Cl-grown cells, measured by phase contrast microscopy, was about 40% larger than tryptophan-grown cells. This means that there is about a threefold difference between the sizes of NH₄Cl- and tryptophan-grown cells, at the time of entrance to S phase, since NH₄Cl-cells enter S at the end of the division cycle, when they are largest, whereas tryptophan-cells enter S at the start of the division cycle, when they are at their smallest. This threefold difference compares reasonably well with the 3.6-fold difference in FITC fluorescence at G₁/S (Fig. 4B) but not at all with the similar light scatter values obtained (Fig. 4A). The reason for the lack of correlation between size and scattered light is not clear, but presumably the shape or intracellular structure of tryptophan-grown cells make them scatter light to a much larger extent than expected from their size or protein content. We have independent evidence (not shown) that scattered light is not a good measure of yeast cell mass (see Discussion).

The relative duration of G₁ was highly variable (Fig. 4C) and might conceivably reflect that the cells were spending different lengths of time in G₁ in order to reach a certain critical mass. If this were true, all the nitrogen sources giving an extended G₁ should have the same G₁/S mass (the critical mass), while the other nitrogen sources, giving a short G₁, should give higher G₁/S mass. However, a plot of the G₁/S mass versus relative length of G₁ does not show any evidence for a minimum mass independent of G₁ duration (Fig. 4C).

Variability of mass at G₁/S

In each individual culture we could measure the variability of

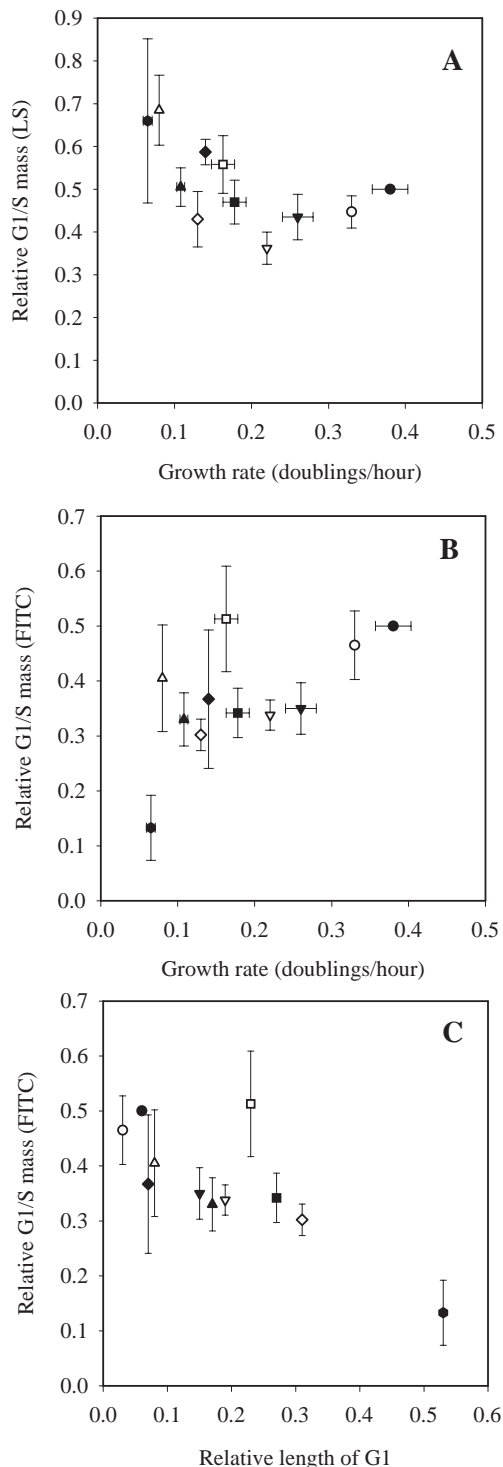


Fig. 4. Relationships between the mass at G₁/S and growth rate or G₁ length. Cell mass was measured as scattered light (A) or as FITC fluorescence (B) for cells grown with different nitrogen sources and plotted against growth rate. In both A and B the value for NH₄Cl, at about 0.4 doublings/hour, is normalized to 0.5. The mean and standard deviation of four measurements are shown. Also, FITC fluorescence is plotted against the fraction of the cell cycle occupied by G₁ (C). The symbols for the nitrogen sources are, in order of decreasing growth rate, NH₄Cl (●), uracil (○), proline (▼), leucine (▽), isoleucine (■), serine (□), cysteine (◆), phenylalanine (◇), threonine (▲), glycine (△), tryptophan (●).

cell mass at entry into S phase. In each two-parameter histogram (Fig. 2, right column) S phase cells were clearly separated from cells in G₁ or G₂, and the variability in scattered light or FITC-signal could be determined at different DNA contents through S phase. For most of the cultures, the coefficient of variation (CV) of this distribution is in the range 10-15%, except for tryptophan, where the CV is about 20%. The low CV for most nitrogen sources most likely reflects a tight coupling between cell mass and start of DNA replication. Such a tight coupling is a necessary consequence of steady-state growth conditions (see Introduction) and should not be interpreted as a control by cell mass over DNA replication.

DISCUSSION

Cells of *S. pombe* were grown under steady-state conditions at widely different growth rates by varying the source of nitrogen. The durations of G₁, S, G₂+M, and the septation period varied as the nitrogen source was varied, but there was no discernible pattern in how the phases depended on growth rate. Presumably, another spectrum of variations could be obtained by using alternative sources of nitrogen, carbon, and phosphorus, but this was not attempted in the present work. For some of the nitrogen sources a considerable extension of the G₁ phase was observed. These growth conditions may be exploited to unravel the order and kinetics of G₁-specific events, since the G₁ phase of *S. pombe* is, in standard laboratory media, extremely short.

A parameter related to mass determines entrance to S

The main purpose of this work was to investigate whether cell mass at entrance to S is constant or variable. We found a large variability of the G₁/S mass with growth rate and our data do not support a constancy of the G₁/S mass. We will emphasize the important finding that there are large and significant differences in G₁/S mass between cells grown at almost the same growth rate, but with different nitrogen sources. This argues for another and more likely hypothesis, namely that the G₁/S mass is variable and determined by the nutritional status of the cells. More specifically, this means that the G₁/S mass is determined by the status of a set of molecular determinants, e.g. their concentration, activity, and/or localization. The components supplied by the growth medium feed into the intracellular biochemical pathways in different ways and fill up the pools of intermediates in ways that are unique to each and every medium supplement. Thus, the intracellular composition is defined by the medium in which the cells are grown. Since, presumably, one or a set of intracellular metabolites determine the mass at entrance to S, it is reasonable that this mass should vary not only with growth rate but also with the actual nutrients supplied. Implicit in this idea is that the use of growth rate as a parameter in plots like those shown in Fig. 4 is misleading: the parameter along the abscissa, in effect the nitrogen source, is not meaningful since it affects the relative G₁/S mass in a discontinuous way. Our conclusion is that the G₁/S mass is not a continuous function of the growth rate.

A similar conclusion was suggested from earlier experiments (Fantès and Nurse, 1977), where the protein content in G₂/M was shown to decrease after a shift to nitrogen sources giving lower growth rates. The exception was serine,

which gave a lower growth rate but no concomitant decrease in cellular protein content.

Our explanation is similar to that proposed for the G₂/M control (Fantes and Nurse, 1977; Nurse and Thuriaux, 1977). In a given culture the observation is that the cells reach a minimal, defined mass before entrance to S. The minimum mass is dependent upon the growth conditions, suggesting that the critical parameter is not mass itself but rather a parameter related to mass. The use of mass as a parameter in this context is, in effect, misleading, since it implies that mass is an important parameter in cell cycle control. Rather, mass should be considered a composite parameter only indirectly related to cell cycle control.

The fact that cells in a given culture reach a certain mass before passing through a certain point is not evidence that mass is the parameter regulating this event (see Introduction), but only that the crucial parameter is in some way coupled to cell growth. The constant mass rule that appears to be valid when the growth rate is fixed, does not apply when the growth medium is varied, since the composition of the growth medium changes the relationship between cell mass and the critical parameter(s). The nature of the molecular mechanisms that regulate entry into S are not known, although several factors that are required for this event have been identified, such as G₁-specific transcriptional activity (Aves et al., 1985; Tanaka et al., 1992; Lowndes et al., 1992; Caligiuri and Beach, 1993; Zhu et al., 1994; Miyamoto et al., 1994; Nakashima et al., 1995; Koch et al., 1996), establishment of a pre-replication complex (Diffley, 1996; Rowles and Blow, 1997) and cyclin-dependent kinase activity (Jallepalli and Kelly, 1997).

In the present work, the mass of cells coming out of mitosis was varied by varying the growth medium, in contrast to an earlier report, where the mass was varied by using different mutations (Nasmyth et al., 1979). The mutant cells were grown in the same medium and the cells with the lowest masses were observed to extend their G₁ and only pass into S when a minimum mass was achieved. Since all the mutant strains were grown in the same medium the relationship between cell mass and the G₁/S-determining parameter was probably the same in all experiments, and the mass at G₁/S should be constant. Analysis of cells grown at different growth rates in a nitrogen-limited chemostat (Nasmyth, 1979) concluded that the G₁/S protein content is independent of growth rate. However, the authors note that there is no perfect correlation between the execution of the limiting G₁-event and protein content. And, again, it can be argued that the medium was the same in all these experiments, except for the concentration of the nitrogen source. Therefore, we feel that there is no discrepancy between our model and the earlier data. Our data may also be viewed in a different way. When mutant cells leave mitosis with a reduced mass, G₁ is prolonged only when the mass at mitosis has been reduced by about 50% (Nasmyth et al., 1979). It could be argued that, in the present experiments, mass at mitosis was not reduced to the proposed minimum mass necessary for G₁/S passage. From Fig. 2, right column, it seems that the highest mass in each panel does not vary very much, and certainly less than twofold. It may be that we have not achieved the mass reduction predicted to be necessary in order for the G₁/S control to be operative. However, in spite of this, we observed that the G₁ phase is significantly extended. This is a further

argument that cell mass (or protein content) is not a relevant parameter for G₁/S control.

The variability in cell mass at G₁/S is significantly lower than the variation in growth rate that we applied. In fact, within certain limits of growth rate variations, the G₁/S mass appears to be fairly constant (Fig. 4). This is not a valid argument for mass as the controlling parameter, but it emphasizes that the controlling parameter is tightly coupled to cell mass.

The relationship between G₁/S mass and G₁ duration

It has been presumed that, in rich media, G₁ is short because the cells are so large when passing through mitosis that they can pass into S without any mass increase in G₁ (Nurse and Thuriaux, 1977; Fantes and Nurse, 1978; Svecizer et al., 1996). This has led to the further assumption that when G₁ is extended under certain conditions it is because the cells pass mitosis with a low mass and must grow in G₁ before they are allowed to pass through to S (Nasmyth et al., 1979). Therefore, when the cells are growing in media giving progressively lower growth rates G₁ should remain short and constant in length until the G₂/M mass reaches a certain minimum, whereafter a G₁ extension is required to accommodate a mass increase before exit from G₁. The present data (Fig. 4C) are not consistent with the above model. There is no evidence of a minimum cell mass at entrance to S. Fig. 4C also does not support the idea that there is a minimum cell size which varies continuously and monotonically with growth rate. In particular, cells grown with serine has a G₁/S mass about as large as cells grown with NH₄Cl and yet the G₁ phase is extended in these cells. This is in agreement with earlier data (Fantes and Nurse, 1977) showing that serine-grown cells have an unexpectedly high mass at exit from mitosis. Thus, under certain conditions cell mass is sufficiently large but the cells are still unable to enter S. Presumably, growth in serine affects the critical parameter(s) in such a way that the G₁ phase must be extended before the criteria for S entry are fulfilled. This is further evidence that cell mass is not the parameter determining entry into S.

Why cell mass?

Over the last 30 years, experiments with bacterial, yeast and mammalian cells, have been claimed to support the existence of a mass control over certain cell cycle events. This can be explained as follows. First, mass or size or protein content are convenient parameters to measure. It is much more complicated to measure the relative occurrence of an mRNA species, a protein, or an enzymatic activity. Second, under a certain set of steady-state growth conditions, cell size/mass/protein content at a certain cell cycle event is necessarily determined by the growth conditions, giving the impression that mass is controlling the cell cycle event. Only when the growth parameters are changed is it possible to test the hypothesis that mass is the controlling parameter.

It is important to make a distinction between the observation that all cells in a steady-state population carry out a certain cell cycle event with well defined mass and the conclusion that mass is the parameter the cell can 'measure' and use to regulate this event. We show here that in a given culture of *S. pombe* the cells enter S phase when they have attained a certain protein content, and the cell-to-cell variation in protein content is very small. However, this protein content is not a universal constant

but varies with the growth conditions. Similar conclusions have been reached for the constant 'initiation mass' of *Escherichia coli* (Donachie, 1968), which was later shown to vary with growth rate (Churchward et al., 1981; Wold et al., 1994; Bipatnath et al., 1998).

Scattered light versus FITC measurements

The present data for mass measured as scattered light is not very similar to parallel data for FITC-measurements (compare Fig. 4A and B). A plot of average scattered light versus FITC fluorescence for all the different growth rates show no correlation between the two parameters (not shown). Based on several control experiments, e.g. the data for tryptophan-grown cells discussed above, we conclude that scattered light is not a good measure for cell mass for fission yeast cells. Scattered light is a very complex parameter and, in addition to cell mass, parameters such as cell shape, internal structure, angle of collected light, and wavelength of the incident light are important. Within one culture, scattered light is reasonably good, and the largest cells have twice the scattered light of the smallest cells. However, in comparing different cultures, the cells must be of about the same size and shape and have the same internal structure. These problems are considerably bigger when scattered light is measured in a laser-based instrument (e.g. the FACStar+). In these machines forward light scatter or side scatter is measured within a narrow angle, and in that case light scattering cannot be used as a measure of cell mass even within the same culture (T. Stokke, unpublished). For these reasons, we believe that protein content (FITC fluorescence) is a better measure for the mass of fission yeast cells.

This work was supported by The Norwegian Cancer Society and the Norwegian Research Council.

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