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Effects of Different Carbon Fluxes on G₁ Phase Duration, Cyclin Expression, and Reserve Carbohydrate Metabolism in Saccharomyces cerevisiae


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Cell cycle progression of eukaryotic cells is primarily controlled at two checkpoints: just before the G₁/S transition and at the G₂/M transition. The main control point in cell cycle progression of the yeast Saccharomyces cerevisiae is situated just before the G₁/S transition and is called Start (12). While this control point in higher eukaryotes is mainly regulated by hormones and growth factors, progression over Start in yeast cells is strongly affected by environmental conditions like nutrient supply. The yeast cell must integrate environmental signals in order to decide whether to begin a new cell cycle, go into a differentiation pathway like mating (haploid cells) or sporulation (diploid cells), or enter a quiescent state. Thus, the cells require two closely integrated systems, one sensing growth conditions and regulating metabolism and another for carrying out the cell division processes.

Progression through Start is controlled by a complex molecular mechanism in which the Cdc28 protein kinase plays a central role (reviewed in references 9, 16, 23, and 24). Cells pass Start when a certain level of G₁ cyclin-associated Cdc28 kinase activity has been reached (39). The G₁ cyclins Cln1 and Cln2 are 75% homologous and are expressed in late G₁ (39). Cln3 is 20 to 25% homologous to Cln1 and Cln2 and has been shown to be constitutively expressed during the cell cycle (10, 22, 39). The Cln proteins are highly unstable, with half-lives of about 10 min, mainly due to PEST sequences in their carboxy-terminal domains (28). Upon entrance into S phase, these cyclins are rapidly degraded due to Cdc28-mediated phosphorylation and subsequent ubiquitination (19, 41). Transcription of CLN1 and CLN2 is regulated by the Swi4/Swi6 transcriptional activation complex (Swi cell cycle box [SCB]-binding factor [SBF]) which binds to the SCB in the CLN2 promoter and to MCB (Mlu cell cycle box) in the CLN1 promoter (4, 25). Also the expression of SWI4 is cell cycle regulated and has been shown to occur 5 to 10 min before S phase and to be regulated by SCB- and MCB-mediated transcription (3). Although the SWI4 promoter itself contains SCB and MCB elements, other elements within its promoter are also involved in the regulation of SWI4 transcription (8). It has been shown that although Swi4/Swi6 is bound to the CLN2 promoter in G₁, CLN2 was not transcribed, suggesting that the Swi4/Swi6 complex has to be activated (11, 17). The Cln3 protein is a potent activator of CLN1, CLN2, and SWI4 expression (38). The amount of Cln3 protein per unit of cell protein is constant, and since Cln3 appears to be nuclear, this means that the Cln3 concentration in the nucleus increases with increasing cell size (9). It is therefore suggested that once Cln3 has reached a certain concentration, it activates the expression of other G₁ cyclins in association with Cdc28 (9). In this way, G₁ cyclin expression is coupled to cell size. The activation of G₁ cyclin expression by Cdc28/Cln3 is probably mediated by activation of SWI4 expression and/or via activation of the Swi4/Swi6 complex (6, 17, 32, 38).

Although the conditions of poor growth are common under natural conditions, cell cycle progression is usually investigated in cells growing on rich media at high growth rates. From asynchronous cultures grown at different growth rates, it has been calculated that the duration of the S, G₂, and M phases is asynchronous cultures grown at different growth rates, it has been calculated that the duration of the S, G₂, and M phases is relatively constant, while duration of the G₁ phase may vary widely (5, 15). The effects of growth rate on cell cycle progression have never been investigated in synchronized cultures, and
hence it is not known how growth rate affects the cell cycle machinery. Therefore, we studied the effects of growth rate on cell cycle progression by controlled addition of galactose to a galactose-limited culture of synchronized cells, which were obtained by elutriation (in analogy to a fed-batch system with a linear feed [31]). By using different galactose addition rates, the G1 phase duration could be modulated. Under all conditions, the expression of CLN1 and CLN2 was initiated always just before bud emergence. Although maximal SWI4 expression preceded maximal CLN1 and CLN2 expression under all growth conditions, the time span between these events strongly increased below a particular consumption rate. Below this consumption rate, there was a marked increase in G1-phase duration, accompanied by an increase in reserve carbohydrates levels. Thus, reserve carbohydrates were metabolized just before budding, thereby transcending increasing the energy flux under these low growth rates.

MATERIALS AND METHODS

Strain and growth conditions. In all experiments, S. cerevisiae SU32 (30, 33) was used. Unless otherwise stated, this strain was grown at 30°C on yeast nitrogen base without amino acids (YNB; Difco Laboratories Ltd., East Molesey, England) and with 2% galactose as the carbon source. Growth under galactose limitation was performed on YNB with a residual galactose concentration of 0.4 mM. Galactose solution was added to the cultures at different rates resulting in galactose consumption rates ranging from 71 to 25 fmol h
-1 cell
-1. The highest consumption rate of 110 fmol h
-1 cell
-1 was reached on medium containing 2% galactose. Galactose concentrations were measured as specified in reference 30. An initial galactose concentration of 0.4 mM was used, since at lower starting concentrations the yeast did not directly initiate galactose consumption, while at higher concentrations this galactose was partly consumed. Each culture contained about 6 × 106 cells ml
-1, determined in an electronic particle counter.

Elutriation. Elutriation was carried out with a 5-ml elutriator rotor (JE-6B; Beckman Instruments BV, Mijdrecht, The Netherlands). For elutriation, cells were grown in batch cultures on YNB with 2% galactose as the carbon source. Elutriation was performed essentially as described previously (40). At an optical density at 600 nm of about 0.2, cells were spun down and reloaded into the elutriation chamber in the same medium. These cells were subsequently cultivated in the elutriation chamber in the same medium at 30°C. At a centrifugal force of 2,500 rpm and a flow rate of about 12 ml h
-1, newborn daughter cells were washed out and collected on ice, while parent cells and budded cells remained in the elutriation chamber.

Analysis of cell cycle parameters. Cell sizes and cell numbers were determined with a Coulter electronic particle counter. Cell sizes were calculated by calibration with latex beads of known sizes. This method was validated by microscopic size determination (40). For determination of the percentage of budded cells, at least 200 cells were analyzed microscopically.

Determination of galactose consumption rates. Samples (2 ml) for the determination of residual galactose concentrations were taken at regular time intervals from the cultures and were directly filtered through a 0.22-µm-pore-size filter. The supernatant fractions were stored at −20°C until galactose determination. Galactose concentrations were measured as galactonic acid with β-galactose-dehydrogenase at the expense of one molecule of NAD per molecule of galactose. Reactions were performed in 50 mM imidazole buffer (pH 7.0) with 5 mM MgCl2, 100 mM NAD (Boehringer GmbH, Mannheim, Germany), and 10 µl of β-galactose-dehydrogenase (Boehringer). The conversion of NAD to NADH was measured spectrophotometrically with an Ultraspec 3000 spectrophotometer (Pharmacia Biotech BV, Woerden, The Netherlands). Galactose consumption was calculated by subtracting the amount of residual galactose from the amount of galactose added per time unit divided by cell number.

Gene expression. For detection of the CLN1, CLN2, CLN3, SWI4, SWI6, and H2A (histone 2a) mRNA's, specific gene fragments were labeled with [-32P]dCTP, using a T7 quick prime labeling kit as specified by the manufacturer (Pharmacia Biotech). Procedures for the separation of 5 µg of total RNA, blotting of the gel, and hybridization, as well as and washing conditions, were as described previously (29), with the following modifications. Hybridization of fragments was performed at 65°C with 0.1% sodium dodecyl sulfate (SDS) in the hybridization mixture instead of 0.5%. Washing conditions for fragment hybridization were as follows: 2 min with 2 × SSC (1 SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, followed by two incubations for 20 min at 65°C with 2 × SSC-0.1% SDS.

Blots were stripped several times by boiling in 1% SDS for reprobing with different probes. As an internal control for the amount of RNA loaded on the gel, an ACT1 (actin) oligonucleotide was used as the probe, essentially as described (28). Northern blots were hybridized with DNA probes as described in Methods. Personal Densitometer SI. Different exposure times were used in order to obtain reliable densitometry data. All scanning data were corrected for the ACT1 level measured in each lane.

To compare the expression levels of different cultures with each other, the 40-min, 195-min, and 540-min RNA samples of the cultures with G1 durations of 65, 225, and 585 min, respectively, were separated on one gel, blotted, and subsequently hybridized with the different probes. The quantified data were corrected for the amount of ACT1 in each lane. In this way, the expression levels could be compared directly without having differences in probing efficiencies or hybridization conditions. These time points were used because all genes examined were expressed at those time points. The scan data of the different cultures were subsequently corrected for these values.

Determination of trehalose and glycogen levels. Samples of 25 ml were centrifuged at 4,000 rpm for 3 min at 4°C. Maltose was discarded, and cells were suspended in ice-cold water and centrifuged again. The obtained cell pellets were quickly frozen into liquid nitrogen and stored at −80°C until further processing.

Trehalose and glycogen were extracted essentially as described before (26). One ml of a 0.25 M Na2CO3 solution of 60°C was added to the frozen cell pellets, which were subsequently placed in boiling water. After 20 min, a 450-µl sample was taken from each sample for trehalose determination; after 2 h of boiling, 450 µl was taken for glycogen determination.

For trehalose determination, the 450-µl sample was directly acidified with 67.5 µl of 6 M acetic acid; 200 µl of this solution was mixed with 800 µl of acetate buffer (pH 4.8) and incubated at 37°C with 2 µl of amyloglucosidase (Sigma). After at least 12 h of incubation, the solution was neutralized by adding 10 µl of 10 M NaOH and spun down for 10 min. Liberated glucose was measured as described elsewhere (26).

RESULTS

Synchronous fed-batch system. To investigate the effect of carbon flux on cell cycle progression, we used synchronous cultures in which the growth rate could be modulated. This was performed by inoculation of daughter cells, obtained by elutriation, in minimal medium with 0.4 mM galactose, in which subsequently galactose solutions of different concentrations were pumped. Under these conditions, the residual galactose concentration in the medium and hence the galactose consumption rate, remained virtually constant (data not shown). The effects of three different galactose consumption rates, 110 (2% galactose), 41, and 30 fmol cell
-1 h
-1, on bud emergence are shown in Fig. 1. The mean G1 phase durations, defined as the time from inoculation to 50% budding, were reached after 65, 225, and 580 min, respectively, for these three consumption rates. In all cases, S phase was initiated around bud emergence, as determined by the relative H2A expression levels (Fig. 1B and C). The decrease in the maximal percentage of budded cells in the cultures with decreasing consumption rate was due to the fact that the cells passed Start less simultaneously at lower growth rates (Fig. 1).

Cell cycle-regulated gene expression. Since cell cycle progression is mainly regulated by changes in cyclin gene expression, we next investigated the expression of the genes under these different growth conditions (Fig. 2). Maximal CLN1 and CLN2 expression levels were reached after about 40 min in cells with a G1 phase of 65 min and after about 180 and 540 min in cells with G1 phase durations of 225 and 580 min, respectively (Fig. 2). In the latter culture, the maximal CLN1 and CLN2 expression levels were about twice as low as in the other cultures. This corresponds to the lower maximal percentage of budded cells in this culture compared to the other cultures. Thus, the decrease in the number of cells which simultaneously pass Start. However, the total amounts of CLN1 and CLN2 expression seem to be the same, as can be deduced from the area beneath each graph, indicating that maximal levels of CLN1 and CLN2 expression per cell are the same under all conditions.
in growth rate did not result in a decreased rate of CLN1 and CLN2 expression but resulted in a delayed onset of CLN1 and CLN2 expression. Except for the high expression at time zero in the culture with a G1 phase of 225 min, almost no changes in CLN3 expression were observed (Fig. 2). However, CLN3 expression seems to be about four times higher in the slower-growing cultures than in those grown on 2% galactose.

Expression of the transcriptional activator genes SWI4 and SWI6. Expression of the cyclin genes CLN1 and CLN2 is mainly regulated by the transcriptional activators Swi4 and Swi6 (4). The relative expression levels of SWI4 and SWI6 are shown in Fig. 3 for the three different growth conditions. In all cultures, SWI4 expression increased before CLN1 and CLN2 expression. In the cultures with G1 phase durations of 65 and 225 min, a transient increase in SWI4 expression was observed, with maximal expression levels after 30 and 165 min, respectively, which is about 10 to 20 min before maximal CLN1 and CLN2 expression. In the culture with a G1 phase duration of 580 min, SWI4 expression reached a maximal expression level after about 180 to 240 min and remained high till about 540 min.

Thus, SWI4 expression remained virtually constant during cell cycle progression in the culture with a G1 phase of 65 min. In the culture with a G1 phase of 225 min, SWI6 expression level started to increase transiently after 135 min, reaching a maximal value at 195 min. In the culture with a G1 phase of 580 min, a transient increase in SWI6 expression was observed around 420 min, reaching a maximal level around 480 min. Thus, CLN1 and CLN2 expression and SWI6 expression increased around the same time in the galactose-limited cultures, indicating that regulated SWI6 expression may have a regulatory role at decreased growth rates.

Cell size at Start increases with decreasing growth rates. Because cell size is generally regarded as an important parameter for cell cycle progression (6), the cell sizes at bud emergence were determined. The mean cell volumes at 50% budding were about 48, 57, and 69 μm³ for the cultures with G1 phases of 65, 225, and 580 min, respectively (Fig. 4). Also, the cell sizes at maximal CLN1 and CLN2 expression increased...
with decreasing growth rates. These observations clearly demonstrate that cell cycle progression does not occur at a particular cell size.

**Cell cycle progression at different galactose fluxes.** Since the method of galactose-limited synchronized fed-batch cultures appeared useful to regulate the G_1_ phase duration, the relationship between consumption rate and G_1_ phase duration was further investigated. By using different rates of galactose addition, the galactose consumption rates were modulated between 110 (2% galactose) and 25 fmol cell\(^{-1}\) h\(^{-1}\). As expected, the mean G_1_ phase duration increased with decreasing galactose consumption rate (Fig. 5A). Surprisingly, a biphasic relationship was observed between G_1_ phase duration and galactose consumption rate. Between galactose consumption rates of 110 and 31 fmol cell\(^{-1}\) h\(^{-1}\), G_1_ phase duration increased linearly from about 65 to about 240 min. However, at a consumption rate lower than 31 fmol cell\(^{-1}\) h\(^{-1}\), G_1_ phase duration increased markedly from 240 to 580 min and more. Thus, a switch point was located around a consumption rate of 31 fmol cell\(^{-1}\) h\(^{-1}\). This switch was not due to a change in metabolism from respiratory-fermentative to fully respiratory, since ethanol was produced only in the cultures with consumption rates of 110, 71, and 63 fmol cell\(^{-1}\) h\(^{-1}\) (data not shown).

**Correlation between G_1_ phase duration and trehalose and glycogen levels.** Since at low growth rates reserve carbohydrates are synthesized in *S. cerevisiae* (21), the strong increase in G_1_ phase duration could be due to the onset of reserve carbohydrate synthesis. Therefore, the levels of the reserve carbohydrates trehalose and glycogen were measured in the different cultures just before bud emergence occurred (late G_1_ phase). A striking correlation between these levels and the G_1_ phase duration was observed (Fig. 5B). At consumption rates higher than 31 fmol cell\(^{-1}\) h\(^{-1}\), the levels of trehalose and glycogen were low and varied between 0.4 and 1.3 and between 3.2 and 7.4 fmol (glucose) cell\(^{-1}\), respectively. At a consumption rate of 25 fmol cell\(^{-1}\) h\(^{-1}\), below the switch point in G_1_ phase duration, the levels of trehalose and glycogen increased markedly to 19.6 and 19.5 fmol (glucose) cell\(^{-1}\), respectively. Thus, at a consumption rate of 25 fmol cell\(^{-1}\) h\(^{-1}\), about 14% of the total amount of galactose consumed is converted to reserve carbohydrates. Therefore, the formation of reserve carbohydrates explains at least partially the relative elongation of the G_1_ phase as shown in Fig. 5A.

**Changes in reserve carbohydrate levels during cell cycle progression.** To investigate the function of these reserve carbohydrates, the levels were monitored during cell cycle progression in cultures grown at different growth rates. Figure 6A shows trehalose and glycogen levels and percentage of budded cells for a culture grown at a consumption rate of 25 fmol cell\(^{-1}\) h\(^{-1}\). Cells started budding after about 600 min, and 50% budding was reached after 660 min. Trehalose levels increased from 0.5 to 19.6 fmol (glucose) cell\(^{-1}\) during the first 450 min (Fig. 5A) and remained constant until about 540 min, followed by a rapid decrease to 0.5 fmol (glucose) cell\(^{-1}\) at 690 min. Glycogen levels increased under this condition from 4.4 to 19.5 fmol (glucose) cell\(^{-1}\) at 600 min, followed by a rapid decrease to 10.9 fmol (glucose) cell\(^{-1}\) at 660 min. Thus, both glycogen and trehalose levels increased during G_1_ and were metabolized again at bud emergence.

In Fig. 6B, the same parameters are shown for a culture grown at a consumption rate of 36 fmol cell\(^{-1}\) h\(^{-1}\). Cells
started budding after about 190 min, and 50% budding was reached after 240 min. Only minor changes in trehalose and glycogen levels were observed in this culture compared to the culture with a consumption rate of 25 fmol cell\(^{-1}\) h\(^{-1}\). Interestingly, glycogen and trehalose levels markedly increased in this culture when the first cells entered G\(_1\) again, probably because at this point the galactose flux per cell decreases due to the increase in cell numbers.

Thus, below a particular galactose consumption rate, a concomitant increase in G\(_1\) phase duration and levels of reserve carbohydrates occurs; the latter are metabolized just before bud emergence, thereby transiently increasing the energy flux. From the culture with a consumption rate of 25 fmol cell\(^{-1}\) h\(^{-1}\), it was calculated that the ATP flux increased transiently from 0.4 to 0.9 pmol cell\(^{-1}\) h\(^{-1}\) around the time of bud emergence.

**DISCUSSION**

Using a combination of two well-known methods in yeast biology, elutriation and fed-batch culturing, we investigated the relationship between nutrient flux, G\(_1\) phase duration, and cell cycle-regulated gene expression. With this new approach, we observed several interesting phenomena.

In all cultures tested, expression of the cyclin genes CLN1 and CLN2 always occurred just before bud emergence. Thus, the increase in G\(_1\) phase duration at low growth rates was not due to a slower accumulation of CLN1 and CLN2 mRNA levels. This finding indicates that the decision to pass Start is made at the onset of CLN1 and CLN2 expression. The maximal expression levels of CLN1 and CLN2 per cell were about the same under all growth conditions examined. No clear cell cycle-dependent changes were observed in CLN3 expression, which is in accordance with observations of others (39). In all cultures, SWI4 expression was activated before CLN1 and CLN2 expression, which is consistent with its role as a transcriptional activator of CLN1 and CLN2 (4). However, whereas SWI4 expression increased just before CLN1 and CLN2 expression in cultures with a G\(_1\) phase of between 65 and 240 min, SWI4 was expressed much earlier than CLN1 and CLN2 in slower-growing cultures with an elongated G\(_1\) phase. Since it has recently been shown that SBF can be bound to SCB without activating transcription, an additional activation step at SBF has to occur (11, 17). This activation of SBF in late G\(_1\) might therefore represent an important step in transcriptional regulation of HO and CLN2 expression at low growth rates, and hence the transcriptional levels of SWI4 and SWI6 do not have to reflect SBF activity.

We observed a strong increase in G\(_1\) phase duration below a particular consumption rate which coincided with a strong increase in intracellular reserve carbohydrate levels. These stored carbohydrates were metabolized before bud emergence, resulting in a transient increase in the glycolytic flux and hence in the ATP flux. Also in glucose-limited continuous cultures, simultaneous changes in budding and reserve carbohydrate levels were observed when cells were grown at low growth rates (18). Apparently the reserve carbohydrates may need to be metabolized at low consumption rates before bud emergence in order for the specific ATP flux required for cell cycle progression to be reached.

Protein kinase A (PKA) is an important regulator of trehalose and glycogen metabolism and represses the synthesis of these carbohydrates at high growth rates. Thus, the accumulation of reserve carbohydrates below a particular flux is likely to be due to a decreased PKA activity (34, 36). Also CLN1 and CLN2 expression is influenced by PKA activity; both very low and very high PKA activities have been shown to repress the expression of CLN1 and CLN2 (1, 14, 37). A low PKA activity at low growth rates might thus relieve reserve carbohydrate metabolism and at the same time delay CLN1 and CLN2 expression, resulting in a concomitant increase in G\(_1\) phase duration. Thus, modulation of PKA activity may play an important role in regulating cell cycle progression in response to environmental conditions.

Under the conditions tested, an increase in cell size was observed with decreasing growth rate. This finding is in contrast with other studies (5, 15), in which an increase in cell size at very high growth rates was found. This difference is probably a result of the different methods used to study cell size at bud emergence in relation to the growth rate. In those experiments, cell sizes were measured either in cultures growing exponentially at different growth media or in continuous cultures at steady-state conditions. In our conditions, however, daughter cells were harvested from cultures growing on 2% galactose and were subsequently incubated under different limitation conditions. The fact that cells can pass Start at different cell sizes indicates that the proposed model of Futercher (9), in which a particular nuclear Cln3 concentration determines passage through Start, is probably more complicated, and changes in metabolism might affect the onset of CLN1 and CLN2 expression either via changes in Cln3 levels or via changes in PKA activity.

Under the conditions tested, the extracellular conditions remained the same, indicating that no particular extracellular condition determines cell cycle progression, but that under these conditions cell cycle progression is dependent only on the galactose consumption rate. It will therefore be important to understand how carbon metabolism and cell cycle progression are linked to each other. As discussed above, one good candidate for integrating cell cycle progression and metabolism is PKA. Another interesting candidate is the Pho85 protein kinase, which is activated in G\(_1\) upon binding of the G\(_1\) cyclins Pcl1 and Pcl2 (7, 21). Pho85 has recently been shown to be a regulator of glycogen metabolism and to downregulate glycogen synthase 2 at the protein and transcriptional levels (13, 35). It will therefore be interesting to see if at low growth rates Pho85 is involved in regulating glycogen and trehalose metabolism in a cell cycle-dependent way.

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