Effects of Different Carbon Fluxes on G_1 Phase Duration, Cyclin Expression, and Reserve Carbohydrate Metabolism in *Saccharomyces cerevisiae*

H. H. W. SILLJÉ,[1]* E. G. TER SCHURE,[1] A. J. M. ROMMENS,[1] P. G. HULS,[2] C. L. WOLDRINGH,[2]
A. J. VERKLEIJ,[1] J. BOONSTRA,[1] AND C. T. VERRIPS[1,3]

*Department of Molecular Cell Biology, Utrecht University, 3584 CH Utrecht,[1] Section of Molecular Cytology, Institute
for Molecular Cell Biology, Biocentrum, University of Amsterdam, 1098 SM Amsterdam,[2] and Unilever Research
Laboratorium Vlaardingen, 3133 AT Vlaardingen,[3] The Netherlands*

By controlled addition of galactose to synchronized galactose-limited *Saccharomyces cerevisiae* cultures, the growth rate could be regulated while external conditions were kept constant. By using this method, the G_1 phase duration was modulated and expression of cell cycle-regulated genes was investigated. The expression of the cyclin genes *CLN1* and *CLN2* was always induced just before bud emergence, indicating that this event marks the decision to pass Start. Thus, G_1 phase elongation was not due to a slower accumulation of the *CLN1* and *CLN2* mRNA levels. Only small differences in *CLN3* expression levels were observed. The maximal *SWI4* expression preceded maximal *CLN1* and *CLN2* expression under all conditions, as expected for a transcriptional activator. But whereas *SWI4* was expressed at about 10 to 20 min, before *CLN1* and *CLN2* expression at high growth rates, this time increased to about 300 min below a particular consumption rate at which the G_1 phase strongly elongated. In the slower-growing cultures, also an increase in *SWI6* expression was observed in the G_1 phase. The increase in G_1 phase duration below a particular consumption rate was accompanied by a strong increase in the reserve carbohydrate levels. These carbohydrates were metabolized again before bud emergence, indicating that below this consumption rate, a transient increase in ATP flux is required for progression through the cell cycle. Since Start occurred at different cell sizes under different growth conditions, it is not just a certain cell size that triggers passage through Start.

Cell cycle progression of eukaryotic cells is primarily controlled at two checkpoints: just before the G_1/S transition and at the G_2/M transition. The main control point in cell cycle progression of the yeast *Saccharomyces cerevisiae* is situated just before the G_1/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 G_1 cyclin-associated Cdc28 kinase activity has been reached (39). The G_1 cyclins Cln1 and Cln2 are 75% homologous and are expressed in late G_1 (39). Cln3 is 20 to 25% homologous to Cln1 and Cln2 and has 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 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 was bound to the *CLN2* promoter in G_1, *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 G_1 cyclins in association with Cdc28 (9). In this way, G_1 cyclin expression is coupled to cell size. The activation of G_1 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, G_2, and M phases is relatively constant, while duration of the G_1 phase may vary widely (5, 15). The effects of growth rate on cell cycle progression have never been investigated in synchronized cultures, and

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* Corresponding author. Mailing address: Utrecht University, Department of Molecular Cell Biology, Padualaan 8, 3584 CH Utrecht, The Netherlands. Phone: 31 30 2532598. Fax: 31 30 2513655. E-mail: Herman.Sillje@molbio.unige.ch.

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 G_1 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 G_1-phase duration, accompanied by an increase in reserve carbohydrates levels. These reserve carbohydrates were metabolized just before bud emergence, thereby transiently 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 Molesay, 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 (calculated 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 6×10^6 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 an 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 by converting galactose to 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) mRNAs, specific gene fragments were labeled with [^{32}P]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 0.1% 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 previously (29). Northern blots were quantified by densitometry on a 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 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. Medium 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 centrifuged for 5 min in an Eppendorf centrifuge at maximal speed, and the supernatant was acidified with 55 μl of 4 M HCl. A 200-μl sample of this suspension was subsequently mixed with 200 μl of a solution of 200 mM sodium acetate (pH 5.5) and 15 mM CaCl₂. Trehalose was degraded to free glucose by incubation of this solution at 37°C with 10 μl of trehalase (Sigma) for at least 12 h. The liberated glucose was measured as described elsewhere (2).

For glycogen 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 addition of 10 μl of 10 M NaOH and spun down for 10 min. Liberated glucose was determined in the supernatant fraction.

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 G_1 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 G_1 phase of 65 min and after about 180 and 540 min in cells with G_1 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 (Fig. 1), due to a 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. Thus, the decrease
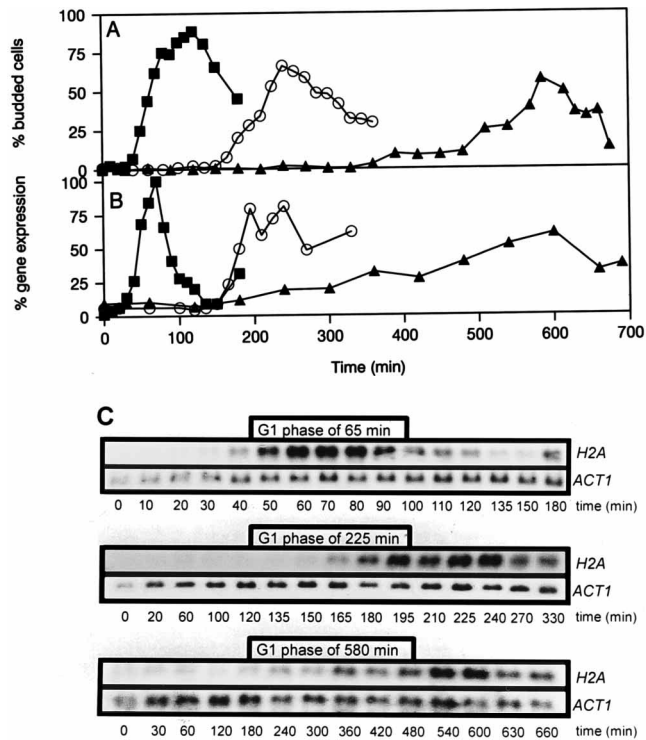
FIG. 1. Cell cycle progression of *S. cerevisiae* under different growth conditions. Synchronous cells, obtained by elutriation, were grown either on 2% galactose or under galactose limitations with different galactose addition rates. In this way, galactose consumption rates, and hence cell growth rate, were regulated. Budding percentages of the different cultures, determined by microscopic counting, are shown in panel A. The expression levels of H2A (B) were determined by densitometric analyses of the Northern blots shown in panel C. The expression values were corrected for the amount of *ACT1* mRNA present in each lane and are relative values, with the highest value set at 100%. Galactose consumption rates: 110 nmol 10^6 cells^{-1} h^{-1} (2% galactose) (■); 41 fmol cell^{-1} h^{-1} (○); 30 fmol cell^{-1} h^{-1} (▲).
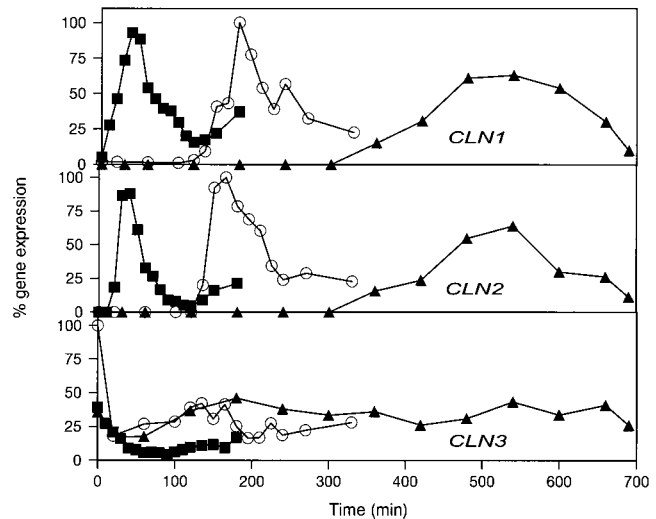

FIG. 2. RNA expression levels of the *CLN* genes under different growth conditions. *CLN1*, *CLN2*, and *CLN3* expression levels were determined by densitometric analyses of Northern blots of the cultures grown at different growth rates as shown in Fig. 1. All expression levels were corrected by the amount of *ACT1* mRNA in each lane. The expression levels of the genes are relative, with the highest value set at 100%. G_1 phase duration: 65 min (2% galactose) (■); 225 min (consumption rate, 41 fmol cell^{-1} h^{-1}) (○); 580 min (consumption rate, 30 fmol cell^{-1} h^{-1}) (▲).

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 G_1 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 G_1 phase durations of 65 and 225 min, a transient increase in *SWI4* levels 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 G_1 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, in this case maximal *SWI4* expression was reached more than 300 min before maximal *CLN1* and *CLN2* expression.

SWI6 expression remained virtually constant during cell cycle progression in the culture with a G_1 phase of 65 min. In the culture with a G_1 phase of 225 min, *SWI6* expression level started to increase transiently after 135 min, reaching a maxi-

mal value at 195 min. In the culture with a G_1 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 increase 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 μm^3 for the cultures with G_1 phases of 65, 225, and 580 min, respectively (Fig. 4). Also, the cell sizes at maximal *CLN1* and *CLN2* expression increased
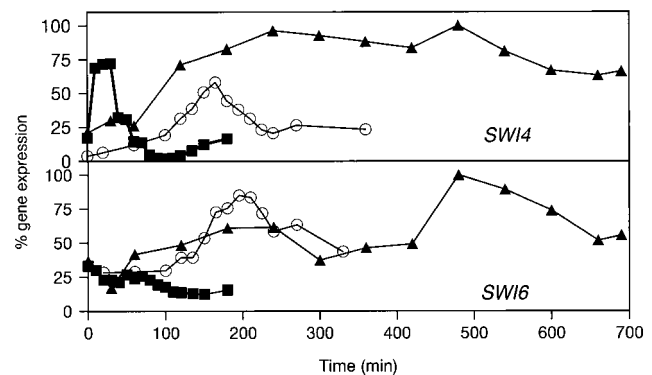

FIG. 3. *SWI4* and *SWI6* RNA expression levels under different growth conditions. *SWI4* and *SWI6* expression levels were determined by densitometric analyses of Northern blots of cultures grown at different growth rates as shown in Fig. 1. The expression levels of the genes are relative, with the highest value set at 100%. G_1 phase duration: 65 min (2% galactose) (■); 225 min (consumption rate, 41 fmol cell^{-1} h^{-1}) (○); 580 min (consumption rate, 30 fmol cell^{-1} h^{-1}) (▲).
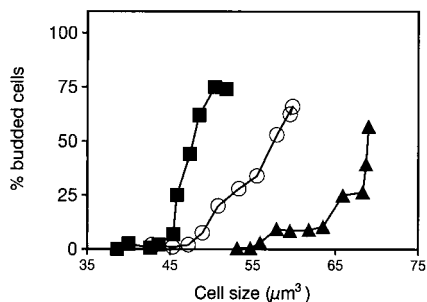
FIG. 4. Correlation between cell size and bud emergence at different growth rates. Cell sizes of the cultures growing at different growth rates were determined at different time points during cell cycle progression, using an electronic particle counter. The percentage budded cells at these different time points is plotted against these cell sizes. G_1 phase duration: 65 min (2% galactose) (■); 225 min (consumption rate, 41 fmol cell^{-1} h^{-1}) (○); 580 min (consumption rate, 30 fmol cell^{-1} h^{-1}) (▲).

FIG. 6. Trehalose and glycogen levels during cell cycle progression in *S. cerevisiae* growing at different growth rates. Synchronized cultures obtained by elutriation were grown in fed-batch cultures having galactose consumption rates of 25 (A) and 36 (B) fmol cell^{-1} h^{-1}. Trehalose and glycogen levels were measured as described in Materials and Methods. ■, percent budded cells; ○, trehalose; □, glycogen.

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,
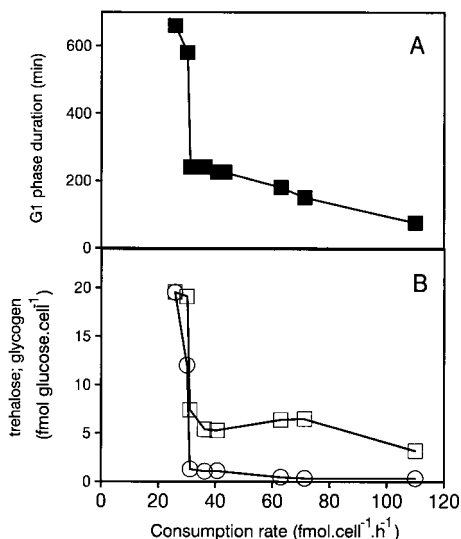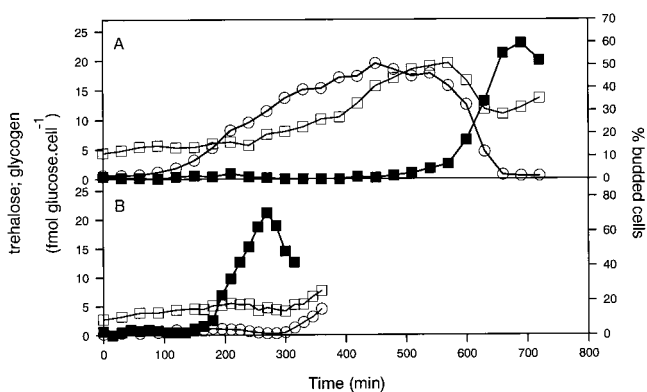
FIG. 5. Correlation between consumption rate, G_1 phase duration, and trehalose and glycogen levels. Synchronous cells, obtained by elutriation, were grown either on 2% galactose or under galactose limitations with different galactose consumption rates. (A) The G_1 phase duration was defined as the time from inoculation until 50% budding was reached. (B) Trehalose and glycogen levels in late-G_1 cells. ■, percent budded cells; ○, trehalose; □, glycogen.

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 of these cultures 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 *CLN1* 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 Futcher (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.

REFERENCES

1. **Baroni, M. D., P. Monti, and L. Alberghina.** 1994. Repression of growth-regulated G1 cyclin expression by cyclic AMP in budding yeast. Nature **371:**339–342.
2. **Bergmeyer, H. U.** 1974. Methods of enzymatic analysis. Verlag Chemie Weinheim, Weinheim, Germany.
3. **Breeden, L. and G. E. Mikesell.** 1994. Three independent forms of regulation affect expression of HO, CLN1 and CLN2 during the cell cycle of *Saccharomyces cerevisiae*. Genetics **138:**1015–1024.
4. **Breeden, L.** 1996. Start-specific transcription in yeast. Curr. Top. Microbiol. Immunol. **208:**95–127.
5. **Carter, B. L. A., and M. N. Jagadish.** 1978. The relationship between cell size and cell division in the yeast *Saccharomyces cerevisiae*. Exp. Cell Res. **112:**15–24.
6. **Dirick, L., T. Böhm, and K. Nasmyth.** 1995. Roles and regulation of Cln-Cdc28 kinases at the Start of the cell cycle of *Saccharomyces cerevisiae*. EMBO J. **14:**4803–4813.
7. **Espinoza, F. H., J. Ogas, I. Herskowitz, and D. O. Morgan.** 1994. Cell cycle control by a complex of the cyclin HCS26 (PCL1) and the kinase PHO85. Science **266:**1388–1391.
8. **Foster, R., G. E. Mikesell, and L. Breeden.** 1993. Multiple *SWI6*-dependent

cis-acting elements control *SWI4* transcription through the cell cycle. Mol. Cell. Biol. **13:**3792–3801.

9. **Futcher, B.** 1996. Cyclins and the wiring of the yeast cell cycle. Yeast **12:** 1635–1646.

10. **Hadwiger, J. A., C. Wittenberg, H. E. Richardson, M. B. Lopes, and S. I. Reed.** 1989. A family of cyclin homologs that control the G1 phase in yeast. Proc. Natl. Acad. Sci. USA **86:**6255–6259.

11. **Harrington, L. A., and B. J. Andrews.** 1996. Binding to the yeast Swi4,6-dependent cell cycle box, CACGAAA, is cell cycle regulated *in vivo*. Nucleic Acids Res. **24:**558–565.

12. **Hartwell, L. H., J. Culotti, J. R. Pringle, and B. J. Reid.** 1974. Genetic control of the cell division cycle in yeast. Science **183:**46–51.

13. **Huang, D., I. Farkas, and P. J. Roach.** 1996. Pho85p, a cyclin-dependent protein kinase, and the Snf1p protein kinase act antagonistically to control glycogen accumulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **16:**4357–4365.

14. **Hubler, L., J. Bradshaw-Rouse, and W. Heideman.** 1993. Connections between the Ras-cyclic AMP pathway and G_1 cyclin expression in the budding yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**6274–6282.

15. **Johnston, G. C., R. A. Singer, S. O. Sharrow, and M. L. Slater.** 1980. Cell division in the yeast *Saccharomyces cerevisiae* growing at different rates. J. Gen. Microbiol. **118:**479–484.

16. **Koch, C., and K. Nasmyth.** 1994. Cell cycle regulated transcription in yeast. Curr. Opin. Cell Biol. **6:**451–459.

17. **Koch, C., A. Schleiffer, G. Ammerer, and K. Nasmyth.** 1996. Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at Start, whereas Clb/Cdc28 kinases displace it from the promoter in G_2. Genes Dev. **10:**129–141.

18. **Küenzi, M. T., and A. Fiechter.** 1969. Changes in carbohydrate composition and trehalase-activity during the budding cycle of Saccharomyces cerevisiae. Arch. Mikrobiol. **64:**396–407.

19. **Lanker, S., M. Henar Valdivieso, and C. Wittenberg.** 1996. Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation. Science **271:**1597–1601.

20. **Lillie, S. H., and J. R. Pringle.** 1980. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J. Bacteriol. **143:** 1384–1394.

21. **Measday, V., L. Moore, J. Ogas, M. Tyers, and B. Andrews.** 1994. The PCL2 (ORFD)-PHO85 cyclin-dependent kinase complex: a cell cycle regulator in yeast. Science **266:**1391–1395.

22. **Nash, R., G. Tokiwa, S. Anand, K. Erickson, and B. Futcher.** 1988. The WHI1$^+$ gene of Saccharomyces cerevisiae tethers cell division to cell size and is a cyclin homolog. EMBO J. **7:**4335–4346.

23. **Nasmyth, K.** 1996. At the heart of the budding yeast cell cycle. Trends Genet. **12:**405–412.

24. **Nasmyth, K.** 1993. Control of the yeast cell cycle by the Cdc28 protein kinase. Curr. Opin. Cell Biol. **5:**166–179.

25. **Nasmyth, K., and L. Dirick.** 1991. The role of SWI4 and SWI6 in the activity of G1 cyclins in yeast. Cell **66:**995–1013.

26. **Neves, J. N., J. A. Jorge, J. M. François, and H. F. Terenzi.** 1991. Effects of heat shock on the level of trehalose and glycogen, and on the induction of thermotolerance in Neurospora crassa. FEBS Lett. **283:**19–22.

27. **Roels, J. A.** 1983. Energetics and kinetics in biotechnology. Elsevier Science Publishers, Amsterdam, The Netherlands.

28. **Salama, S. R., K. B. Hendricks, and J. Thorner.** 1994. G_1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover. Mol. Cell. Biol. **14:**7953–7966.

29. **Sierkstra, L. N., J. M. A. Verbakel, and C. T. Verrips.** 1992. Analysis of transcription and translation of glycolytic enzymes in glucose limited continuous cultures of *S. cerevisiae*. J. Gen. Microbiol. **138:**2559–2566.

30. **Sierkstra, L. N., N. P. Nouwen, J. M. A. Verbakel, and C. T. Verrips.** 1993. Regulation of glycolytic enzymes and the Crabtree effect in galactose-limited continuous cultures of *Saccharomyces cerevisiae*. Yeast **9:**787–795.

31. **Stanbury, P. F., and A. Whitaker.** 1984. Principles of fermentation technology, p. 21–25. Pergamon Press, Oxford, England.

32. **Stuart, D., and C. Wittenberg.** 1995. *CLN3*, not positive feedback, determines the timing of *CLN2* transcription in cycling cells. Genes Dev. **9:**2780–2794.

33. **Ter Schure, E. G., H. H. W. Silljé, A. J. Verkleij, J. Boonstra, and C. T. Verrips.** 1995. The concentration of ammonia regulates nitrogen metabolism in *Saccharomyces cerevisiae*. J. Bacteriol. **177:**6672–6675.

34. **Thevelein, J. M.** 1994. Signal transduction in yeast. Yeast **10:**1753–1790.

35. **Timblin, B. K., K. Tatchell, and L. W. Bergman.** 1996. Deletion of the gene encoding the cyclin-dependent protein kinase Pho85 alters glycogen metabolism in *Saccharomyces cerevisiae*. Genetics **143:**57–66.

36. **Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler.** 1985. In yeast, Ras proteins are controlling elements of adenylate cyclase. Cell **40:**27–36.

37. **Tokiwa, G., M. Tyers, T. Volpe, and B. Futcher.** 1994. Inhibition of G1 cyclin activity by the Ras/cAMP pathway in yeast. Nature **371:**342–345.

38. **Tyers, M., G. Tokiwa, and B. Futcher.** 1993. Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. EMBO J. **12:**1955–1968.

39. **Wittenberg, C., K. Sugimoto, and S. I. Reed.** 1990. G1-specific cyclins of *S. cerevisiae*: cell cycle periodicity, regulation by mating pheromone and association with the p34^{cdc28} protein kinase. Cell **62:**225–237.

40. **Woldringh, C. L., P. G. Huls, and N. O. E. Vischer.** 1993. Volume growth of daughter and parent cells during the cell cycle of *Saccharomyces cerevisiae* **a**/α as determined by image cytometry. J. Bacteriol. **175:**3174–3181.

41. **Yaglom, J., M. H. K. Linskens, S. Sadis, D. M. Rubin, B. Futcher, and D. Finley.** 1995. p34^{Cdc28}-mediated control of Cln3 cyclin degradation. Mol. Cell. Biol. **15:**731–741.