Changes in the incorporation of carbon derived from glucose into cellular pools during the cell cycle of *Saccharomyces cerevisiae*

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The rate of incorporation of ^{14}C derived from $[\text{U-}^{14}\text{C}]\text{glucose}$ into cells of *Saccharomyces cerevisiae* X2180(1B) was investigated as a function of the cell cycle. After pulse-labelling of exponentially growing populations, centrifugal elutriation was used to isolate various cell fractions of increasing cell size, representing successive stages of the cell cycle. The total amount of ^{14}C incorporated per cell was found to increase continuously during the cell cycle along with cellular protein content and Coulter counter cell volume. This pattern supports the model of exponential cell growth. In order to evaluate changes in intracellular carbon flow during the cell cycle, chemical extraction procedures were used to obtain four cellular fractions enriched in either low-molecular-mass components, lipid material, polysaccharides or proteins. The distribution of ^{14}C among these cellular fractions varied during successive stages of the cell cycle, indicating cell-cycle-dependent fluctuations in intracellular carbon flow. During the G1 phase the flow of ^{14}C into the low-molecular-mass pool increased markedly; concurrently, the rate of incorporation into the polysaccharide-enriched pool decreased.

Introduction

It has been reported frequently that changes in metabolic activity occur during the cell cycle of *Saccharomyces cerevisiae*. Oxygen consumption, carbon dioxide production, the cellular contents of reserve carbohydrates, as well as the activity of several enzymes involved in, for example, carbohydrate metabolism, seem to vary considerably during the mitotic cycle (Von Meyenburg, 1969; Kuenzi & Fiechter, 1969; Van Doorn *et al.*, 1988*a, b*). It is possible that these metabolic changes are associated with cell-cycle-dependent alterations in the uptake rate of the carbon source and with cell-cycle-dependent changes in intracellular carbon flow. Little is known, however, about these processes during successive stages of the mitotic cycle. In addition to these metabolic considerations, data on the uptake of the carbon source may allow us to discriminate between the linear and the exponential models of *S. cerevisiae* cell growth described in the literature (Mitchison, 1958; Elliot & McLaughlin, 1978, 1979). In fact, the constancy of uptake rates of glucose and glycine during most of the cell cycle of the fission yeast *Schizosaccharomyces pombe* was considered to be experimental evidence for a linear growth pattern

in this micro-organism (Kubitschek & Claymen, 1976).

Hence, the present study was undertaken to investigate the relationship between the rate of glucose uptake and cell cycle stage of *S. cerevisiae*. This report provides data which suggest cell-cycle-related changes in intracellular carbon flow.

Methods

Yeast strain and growth conditions. The yeast used was *Saccharomyces cerevisiae* X2180(1B) (*MAT α SUC2 mal mel gal2 CUP1*; Yeast Genetic Stock Center, Berkeley, Calif., USA). It was grown at 29 °C in a liquid EMM2 medium containing 1% (w/v) glucose (Mitchison, 1970). Cell numbers were determined using a Coulter counter with a 50 μm aperture. The generation time of exponentially growing cells was approximately 170 min.

Kinetics of incorporation of radiolabel. $[\text{U-}^{14}\text{C}]\text{Glucose}$ [specific activity 270 mCi mmol $^{-1}$ (10 GBq mmol $^{-1}$)] was purchased from Amersham. Exponential phase cultures (200 ml) were incubated with $[\text{U-}^{14}\text{C}]\text{glucose}$ [0.15 $\mu\text{Ci ml}^{-1}$ (5.55 kBq ml $^{-1}$); final specific activity 3.3 $\mu\text{Ci mmol}^{-1}$ (122 kBq mmol $^{-1}$)]. At various time intervals, samples (5–15 ml) were withdrawn and immediately filtered through nitrocellulose membrane filters (0.45 μm ; BA85, Schleicher and Schuell). To remove extracellular material, the filters were washed twice at 4 °C with 15 ml 1% glucose. The cells on the filter were resuspended in 1 ml of ice-cold distilled water and washed once by centrifugation. From each suspension 50 μl was used for cell counting and 200 μl was diluted into 5 ml scintillant (299TM, Packard) and used to determine total

Abbreviation: DAPI, 4',6'-diamidino-2-phenylindole.

incorporation of radiolabel. The remainder of the suspension was centrifuged at 4 °C and the pellet was treated as described below.

Chemical cell-extraction procedures. A fixed amount of radiolabelled cells (10^8) was used for the extraction of various cellular components. If the cell pellets contained less than 10^8 labelled cells, they were supplemented with unlabelled cells from an exponentially growing culture. The procedure was a modification of that described for *Candida utilis* by Cowie & Walton (1956).

First, the cell pellets were treated for 1 h in the cold with 250 μ l 10% (w/v) trichloroacetic acid (TCA) with continuous shaking of the suspension. The cells were centrifuged, and the pellets were treated again with 300 μ l TCA for 1 h. After centrifugation, the supernatants of the first and second treatment were combined to give the 'cold-TCA extract'. The pellets were resuspended in 50 μ l of ice-cold distilled water and 200 μ l 96% (v/v) ethanol were added. After 5 min, the cells were spun down and the pellets were resuspended for another 5 min in 300 μ l 96% ethanol, using gentle sonication (MSE sonicator). After centrifugation, the two supernatants were combined to give the 'ethanol extract'. The pellets were resuspended in 75 μ l of water to which 75 μ l 10% TCA were added. After 5 min the cells were centrifuged and suspended again in 250 μ l 5% (w/v) TCA. The suspensions were then incubated at 90 °C for 15 min and the cells were washed in 150 μ l 5% TCA. The three supernatants were pooled to give the 'hot-TCA extract'. Finally, the pellets were suspended in 550 μ l 2% (w/v) NaOH and incubated at 90 °C for 20 min to give clear solutions ('NaOH extract').

Samples (500 μ l) of the various extracts were counted in a liquid scintillation counter with a counting error of less than 3%. Quench curves of [14 C]glucose in water, TCA, ethanol and NaOH were used to calculate d.p.m. from the c.p.m. data. In a separate experiment mixtures of methanol/chloroform (3:1, v/v) and ethanol/ether (3:1, v/v), rather than ethanol alone, were used to extract lipid material. These mixtures gave the same distribution of label among the four extracts as in the experiment with ethanol (data not shown).

The isolated cellular fractions were analysed for their protein, polysaccharide and polynucleotide contents. The 'hot-TCA extract' contained (% by wt): 14% protein (determined by the Lowry method), 69% polysaccharides (anthrone method; Herbert *et al.*, 1971) and 17% polynucleotide (orcinol method; Herbert *et al.*, 1971). The 'NaOH residue' contained (% by wt): 69% protein, 29% polysaccharides and 2% polynucleotide. It was assumed that ethanol extracted all lipids, whereas treatment with cold TCA extracted all compounds of low molecular mass (Cowie & Walton, 1956).

Separation of radiolabelled cells according to size by centrifugal elutriation. Exponential phase cultures (about 400 ml; $1\text{--}2 \times 10^7$ cells ml^{-1}) were incubated with [14 C]glucose [0.25 μ Ci ml^{-1} (9.25 kBq ml^{-1})] for 15 or 30 min. After incubation, the cells were quickly filtered through nitrocellulose filters and the filters were washed with 1 litre of 1% glucose at 4 °C. The cells were resuspended in 20 ml of ice-cold distilled water and washed to give a 12 ml cell suspension. After gentle sonication, 10 ml of this suspension was loaded into the spinning elutriator rotor.

The cells were then fractionated by centrifugal elutriation as described previously (Van Doorn *et al.*, 1988b). Briefly, elutriation in water was done in a Beckman JE6B elutriator rotor at 4 °C at a rotor-speed of 3750 r.p.m. Fractions (100 ml) were collected at flow rates ranging from 13 to 40 ml min^{-1} . In order to evaluate the quality of separation, samples were withdrawn for determination of cell numbers and cell size, for microscopic evaluation and for flow cytometry (Van Doorn *et al.*, 1988b). The remaining cells were collected by filtration, resuspended in 1 ml of ice-cold distilled water and washed once by centrifugation. Samples of this suspension were used for liquid scintillation counting, determination of cell number and protein measurement; the remainder was treated as described above.

Fig. 1. Centrifugal elutriation of exponentially growing populations of *S. cerevisiae* X2180(1B). The inset shows the four different cell types. ●, Unbudded cells; △, budded cells with a single nucleus; ○, cells with migrating nuclei; □, binucleate cells. Cells were stained with DAPI and the proportion of each cell type in a particular elutriated fraction was established microscopically. At least 100 cells were scored per fraction.

Reproducibility of results. All experiments were repeated at least twice with consistent results. Representative results are shown.

Results

Separation of cells according to size by centrifugal elutriation

In previous experiments, centrifugal elutriation was used for cell-size fractionation of *S. cerevisiae* strain DL1 (*MAT α leu2-3 leu2-112 his3-11 his3-15 ura3-251 ura3-372 ura3-328*) grown on a rich medium (Van Doorn *et al.*, 1988b). In this study a different yeast strain and growth medium were used, so the characteristics of cell-size fractionation had to be established.

Fractions from the elutriator were examined microscopically after fluorescent staining of the DNA with DAPI (4',6'-diamidino-2-phenylindole; Williamson & Fennel, 1975). On the basis of nuclear and general morphology the cells were classified into one of the four groups shown schematically in the inset to Fig. 1. Fractions obtained at lower flow rates (13–18 ml min^{-1})

Fig. 2. Cell volume, protein content and DNA content of cells in successive fractions from the elutriator rotor. Cell volume was determined with a Coulter counter equipped with a multichannel analyser and a 50 μm aperture. Relative cell volume ($\bullet$) is given arbitrarily in terms of unit mean cell volume of unbudded cells elutriated at 13 ml min^{-1} . Protein content ($\circ$) is expressed per cell. Mean DNA content of the cells ($\square$) is expressed as the fluorescence (in arbitrary units) of single cells, after staining with Hoechst 33342, as determined by flow cytometry.

consisted almost exclusively of unbudded (G1 phase) cells. Budding cells, in which the nucleus had not migrated into the bud neck, peaked at flow rates of 22 and 24 ml min^{-1} . Most of the budded cells with

migrating nuclei were present in fractions collected at 26 to 32 ml min^{-1} , whereas the fractions at higher flow rates contained predominantly binucleate cells.

DNA content per cell doubled in fractions obtained between flow rates of 19 and 26 ml min^{-1} (Fig. 2), while the beginning of DNA synthesis and the beginning of bud formation appeared to coincide. The protein content per cell as well as the mean Coulter counter volume of the cells showed a sixfold increase between the first and the last fraction (Fig. 2).

To test whether the first fraction contained cells that were dead or non-growing, a suspension of cells in EMM2 medium at 28 $^{\circ}\text{C}$ was elutriated. The first eight fractions were collected in the medium and growth of the cultures was followed. The remainder of the cells was used as a control culture. In all fractions the cell volume increased gradually and without a lag period (data not shown). The duration of the first doubling of cell numbers decreased with increasing flow rates from about 400 min in the first fraction collected to about 250 min in a fraction collected at a flow rate of 21 ml min^{-1} . In all fractions the time required for the second doubling of cell numbers was about the same as that for exponentially growing cells in the control culture (data not shown). In all fractions the percentage of cells unable to exclude the dye fluorescein isothiocyanate (i.e. dead cells) was small (1–2% in most fractions).

These results indicate the effectiveness of centrifugal elutriation in separating exponentially growing populations of *S. cerevisiae* strain X2180(1B) into fractions representing successive stages of the cell cycle.

Fig. 3. (a) Total incorporation of ^{14}C into cells from exponentially growing *S. cerevisiae* X2180(1B) cultures after addition of $[\text{U-}^{14}\text{C}]\text{glucose}$. $\circ$, Cell number (determined with a Coulter counter); $\bullet$, incorporation of label into cells. (b) Distribution of ^{14}C (as a percentage of the total) among the four cellular fractions after addition of $[\text{U-}^{14}\text{C}]\text{glucose}$ to exponentially growing *S. cerevisiae* X2180(1B) cultures. $\square$, Ethanol fraction; $\bullet$, cold-TCA fraction; $\triangle$, hot-TCA fraction; $\circ$, NaOH-soluble residue.

Kinetics of incorporation of radiolabel by an exponentially growing population

Firstly, the kinetics of ^{14}C incorporation from $[^{14}\text{C}]$ -glucose into whole yeast cells and various cellular fractions was investigated. In an exponentially growing population, the rate of incorporation of radiolabel into whole cells initially exceeded the growth rate, whereas after about 2 h incorporation increased in parallel with growth (Fig. 3a).

The distribution of radiolabel among the cellular fractions was different for the various labelling-times (Fig. 3b). Shortly after the addition of $[^{14}\text{C}]$ -glucose there was preferential labelling of low-molecular-mass components (extracted by cold-TCA treatment). After prolonged incubation, a higher percentage of label was found in the protein-enriched NaOH fraction and the polysaccharide-enriched hot-TCA fraction, whereas the percentage of label in the cold-TCA soluble pool had decreased. The sum of label in the four cellular fractions accounted for 95–105% of the label incorporated into whole cells.

On the basis of these results, a $[U-^{14}\text{C}]$ -glucose pulse time of 15–30 min was chosen for subsequent experiments. This allowed sufficient label to be incorporated into all fractions (especially the ethanol-extractable lipid fraction) and was relatively short compared to the generation time of the cells (170 min).

Incorporation of radiolabel during different stages of the cell cycle

The relationship between incorporation of ^{14}C derived from $[U-^{14}\text{C}]$ -glucose and cell cycle phase was investigated. The total rate of incorporation of ^{14}C , expressed per cell, increased continuously by a factor of five in cells from successive cell-size fractions (Fig. 4). When expressed either per μg of protein or per relative mean Coulter counter cell volume, the specific rate of incorporation of label remained virtually constant (Fig. 4). Cell numbers in fractions collected at flow rates of 38 and 40 ml min^{-1} were too low for accurate determination of label incorporation.

The distribution of radiolabel among the various cellular fractions showed cell-cycle-dependent variation (Fig. 5). During the unbudded G1 phase, the relative flow of ^{14}C into the low-molecular-mass (cold TCA) pool increased markedly. Presumably, this increase was compensated by the reduced rate of radiolabel incorporation into the polysaccharide-enriched hot-TCA pool. At later stages of the cell cycle, the percentage of incorporation of ^{14}C into the low-molecular-mass pool increased somewhat further, associated with a simultaneous decrease of incorporation into the protein-enriched

Fig. 4. Incorporation of ^{14}C into cells in the various elutriated fractions. Incorporation is shown per cell (●), per cell with respect to the relative cell volume (○), and per μg of protein (△). Exponentially growing cultures of *S. cerevisiae* X2180(1B) were pulse-labelled with $[U-^{14}\text{C}]$ -glucose and subsequently separated into cell-size fractions by centrifugal elutriation. Dotted lines represent ^{14}C incorporation in cells from the original cell suspension loaded into the elutriator rotor. Cell cycle position was deduced from flow cytometric measurement of DNA content per cell and the (nuclear) morphology of cells after DAPI staining. Abbreviations: ids, initiation of DNA synthesis; be, bud emergence; cds, completion of DNA synthesis; nm, nuclear migration.

NaOH fraction. During all stages of the cell cycle, the protein-enriched fraction accounted for the greater part (39–44%) of ^{14}C incorporation. The incorporation of radiolabel into the lipid-containing fraction was low (2–4%) and nearly constant throughout the cell cycle.

Discussion

Centrifugal elutriation was used successfully to separate exponentially growing populations of *S. cerevisiae* according to the phase of the cell cycle. Advantages and limitations of cell-size fractionation by means of this technique have been evaluated by several authors (Gordon & Elliott, 1977; Ludwig *et al.*, 1982; Barford, 1986; Wheals, 1987; Van Doorn *et al.*, 1988b; Mitchison, 1988). When the various fractions from the elutriator rotor are assigned to particular stages in the cell cycle, the variation in cell-size at any particular cell cycle stage, and the different sizes of mother and daughter cells (Hartwell, 1970; Carter & Jagadish, 1978) must be taken into consideration. Such phenomena may, at least in

Fig. 5. Distribution of ^{14}C (as a percentage of the total) among the four fractions in the various samples from the elutriator rotor. Dotted lines represent distribution in cells from the cell suspension loaded into the rotor. $\square$, Ethanol fraction; $\bullet$, cold-TCA fraction; $\triangle$, hot-TCA fraction; $\circ$, NaOH-soluble residue. Abbreviations, see Fig. 4.

part, explain the observation, also reported previously (Van Doorn *et al.*, 1988b), that volume and protein content per cell increase more than twofold between the first and the last fractions collected from the elutriator rotor. Our observation that the doubling time of cells from the first fraction was longer than the doubling time of the control culture and that this doubling time decreased with increasing cell volume in subsequent fractions, suggests that the first fraction(s) collected contain(s) daughter cells which need to grow to a critical cell size before they are able to divide. The first three or four fractions from our elutriation fractionation may not be considered as representing true stages of the cell cycle. Nevertheless, the conclusion that the uptake of glucose gradually increases during the cell cycle along with the content of protein or with the Coulter counter cell volume is unaffected. The cell-cycle-related changes in distribution of carbon derived from glucose among different cellular pools can still be observed, though less prominently.

The elutriation pattern of *S. cerevisiae* X2180(1B) cells clearly differed from the pattern described earlier for *S. cerevisiae* DL1 (Van Doorn *et al.*, 1988b). Using identical

elutriation conditions, strain X2180(1B) yielded more elutriator fractions (at least eight) enriched in unbudded uninucleate cells than strain DL1 (only three fractions). Furthermore, S phase cells of strain X2180(1B) occurred in fractions obtained at higher flow rates: for DL1 doubling of DNA content was observed between 16 and 21 ml min^{-1} ; for X2180(1B) doubling was between 19 and 26 ml min^{-1} . It is likely that strain-specific differences in cell density, duration of cell-cycle phases and timing of cell separation underlie these differences in elutriation pattern.

The changes in distribution of radiolabel among the four cellular fractions (enriched either in low-molecular-mass components, lipid material, polysaccharides or proteins) indicate cell-cycle-related changes in intracellular carbon flow. This would agree with several reports suggesting changes in metabolic fluxes during the cell cycle (Von Meyenburg, 1969; Kuenzi & Fiechter, 1969, 1972; Van Doorn *et al.*, 1988a, b). Our results should, however, be interpreted as gross metabolic changes, since the chemical extraction procedures used yield extracts that are only relatively enriched in certain major cellular constituents. Hence, further investigations will be necessary to substantiate the carbon flux from carbon source through metabolic pathways to individual cellular components.

Our findings may help to discriminate between the two models of cell growth that have been proposed for *S. cerevisiae*. Mitchison (1958) found linear growth in *S. cerevisiae* cells with a rate of increase of dry mass per cell that changes quite sharply about 20 min after bud formation. Elliot & McLaughlin (1978, 1979) proposed an exponential growth model for *S. cerevisiae*, based on their observation that the rate of synthesis of both total RNA and total protein increases exponentially during the cell cycle. With respect to net uptake rate of all (major) nutrients that enter the cell, a linear growth model would predict constancy in uptake rates per cell with a doubling in rate at a certain stage in the cell cycle, whereas the exponential model would predict continuously increasing uptake rates (Kubitschek & Claymen, 1976; Kubitschek & Edverson, 1977). Constancy of uptake rates (per cell) of adenine and serine during the G1 and G2/M phases of the cell cycle of *S. cerevisiae* and doubling of the uptake rates during the S phase were considered to be in agreement with linear growth (Kubitschek & Edverson, 1977). However, our present finding that the rate of uptake of ^{14}C from [^{14}C]glucose per cell increases continuously during successive stages of the cell cycle (along with cellular protein content and Coulter counter cell volume) supports the model of exponential cell growth.

In similar experiments, Kubitschek & Claymen (1976) found that in *Schizosaccharomyces pombe* the rate of

uptake of [^{14}C]glucose and [^{14}C]glycine per cell remains constant during most of the cell cycle. These data support a linear growth pattern for *Sc. pombe* cells as proposed by Mitchison (1957) and by Wain & Staatz (1973). Thus, the apparent differences between *S. cerevisiae* and *Sc. pombe* with respect to patterns of [$\text{U-}^{14}\text{C}$]glucose uptake during the cell cycle may be explained, at least in part, by differences in the growth pattern of these yeasts.

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