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Kinetics of Glucose Repression of Yeast Cytochrome c

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The kinetics of glucose repression of cytochrome c synthesis was measured by a radioimmune assay. When 5 or 10% glucose was added to a derepressed culture, the rate of cytochrome c synthesis was reduced to the repressed level with a half-life of 2 min. The addition of 1 or 0.5% glucose repressed the rate of cytochrome c synthesis to the same level as high glucose concentrations but with a longer half-life of 3 min. Glucose repression had no effect on the stability or function of the cytochrome c protein. Cellular levels of active cytochrome c mRNA during glucose repression were measured by translation of total cellular polyadenylic acid-containing RNA and immunoprecipitation of cytochrome c from the translation products. The results of these measurements indicate that glucose represses the rate of cytochrome c synthesis through a reduction in the level of translatable cytochrome c mRNA.

Many cells show a preference for growth on specific carbon sources and manifest this preference by catabolite repression, repressing the synthesis of enzymes used to catabolize one carbon source in the presence of a more favored carbon source. Yeast cells grown on glucose repress the levels of proteins required for respiration and mitochondrial function (reviewed in references 15, 16, 22). We are interested in the molecular mechanisms of catabolite repression of mitochondrial function in yeast for two reasons. First, many of the proteins that function in the mitochondria are encoded in the nucleus (2, 15, 16, 22), and, because yeast is a eucaryote and its DNA is organized like that of other eucaryotes, the mechanisms that regulate proteins in this simple organism may give us some insight into the regulatory mechanisms of more complex organisms. Second, some of the proteins subject to catabolite repression are encoded in the mitochondria, and catabolite repression in yeast involves the coordinate regulation of genes segregated in different organelles (1, 5, 7, 9, 21, 29). Therefore, this system offers the opportunity to learn how organelles might communicate with each other.

Cytochrome c is one of the yeast proteins subject to catabolite repression (12, 18, 23, 27, 30). It is encoded in the nucleus and functions in the mitochondria (25). We hope to use the regulation of the cytochrome c gene expression as a probe for the overall regulation of catabolite-repressed genes. In our initial studies we developed a specific immunological assay for the cytochrome c protein and an in vitro protein synthesis assay for the cytochrome c mRNA (30). We found a fourfold-lower level of active cytochrome c mRNA in cells grown on glucose compared with cells grown on a nonrepressive carbon source. In our present studies, using these same assays, we have determined the kinetics of glucose repression of cytochrome c synthesis.

MATERIALS AND METHODS

Strains. The yeast used for these experiments was a diploid *Saccharomyces cerevisiae* derived from a mating of D311-3A (24) and α_1 131-20 (26).

Growth and labeling of cells. The kinetics of catabolite repression of cytochrome c synthesis were determined by adding glucose to cells growing exponentially on raffinose and then pulse-labeling cells with [^{3}H]lysine at various times to determine the residual level of cytochrome c synthesis. A typical experiment was performed as follows. Cells were grown continuously in log phase for 24 h at 30°C with vigorous shaking on complete medium minus lysine (30), containing 2% raffinose. At a late-log-phase cell density of 2.7×10^7/ml, 2 ml was removed, labeled for 1 min with 100 μCi of [^{3}H]lysine (specific activity, 53.5 Ci/mmol; New England Nuclear Corp., Boston, Mass.), and treated as described below. To 10 ml of the remaining culture, 10 ml of complete medium minus lysine, containing glucose and equilibrated to 30°C, was added to give the desired final glucose concentration. At various times after the addition of glucose, 2 ml of cells was removed and labeled with 100 μCi of [^{3}H]lysine for 1 min. Only about 3% of the [^{3}H]lysine was incorporated into protein during this labeling period. Incorporation was stopped by the addition of 5 ml of a chilled 1 M NaCl-1% lysine solution. The incorporation of [^{3}H]lysine into total protein was assayed as described previously (30). The cells were then washed twice with 1 M NaCl. The cell pellet was combined with 8 ml of chilled unlabeled cells grown to a cell density of 2.7×10^7/ml on complete medium minus lysine, containing 5% glucose. The cell mixture was washed twice more with 1 M

NaCl and then autolyzed overnight at 4°C in a solution containing 0.4 ml of 1 M NaCl and 0.4 ml of ethyl acetate. The amount of [^{3}H]lysine incorporated into cytochrome *c* was determined by specific immunoprecipitation followed by sodium dodecyl sulfate (SDS)-gel electrophoresis of the immunoprecipitate as described previously (30). Routinely, only the small portion of the gel containing and adjacent to the cytochrome *c* marker was sliced and counted. For multiple determinations under a given set of growth conditions, the values of percent incorporated [^{3}H]lysine in cytochrome *c* determined in this fashion agree to within 18%.

In this procedure, the addition of the unlabeled glucose-grown cells was found to increase the efficiency of autolysis and the specificity of the immunoprecipitation.

For each 2 ml of cells labeled, an average of 50 pmol of [^{3}H]lysine was incorporated into protein.

Assay for active cytochrome *c* mRNA. For the preparation of polyadenylic acid [poly(A)]-containing RNA, cells were grown in 2.5 liters of complete medium containing either 5% glucose or 2% raffinose in Fernbach flasks (500 ml/flask) at 30°C with vigorous shaking. At a cell density of 2.7×10^7/ml, cycloheximide was added to a final concentration of 100 μg/ml, and after an additional 1 min at 30°C the cells were quickly chilled.

To follow the kinetics of the disappearance of active cytochrome *c* mRNA, cells were grown under identical conditions in medium containing 2% raffinose. At a cell density of 2.7×10^7/ml, 62.5 ml of 40% glucose was added to each Fernbach flask to give a final glucose concentration of 5%. Growth of the cells was continued for the desired time (5 or 7.5 min in the experiments described below); then cycloheximide was added to a final concentration of 100 μg/ml, and the cells were chilled after an additional 1-min incubation.

The preparation of poly(A)-containing RNA, the in vitro translation in a wheat germ extract, and the immunoassay of cytochrome *c* from among the translation products were all described previously (30).

The preparation of cytochrome *c* and antibody and other methods have been described previously (30).

RESULTS

Reilly and Sherman (20) reported that cells grown on the trisaccharide raffinose are derepressed for mitochondrial functions, including cytochrome *c* levels. We found this observation to be correct, although high concentrations of raffinose did repress cytochrome *c* levels somewhat (Table 1). We decided to study glucose repression of cytochrome *c* in cells grown on raffinose as opposed to a nonfermentable carbon source for two reasons. First, cells grow faster on raffinose than on nonfermentable carbon sources (a 2.5-h generation time on complete medium containing 2% raffinose versus a 4-h generation time on the same medium containing 2% ethanol instead of raffinose). This allows shorter labeling times and better yields of RNA. Second, raffinose supports the growth of mutants deficient in cytochrome *c* or some other mitochondrial function under derepressed conditions (for example, see reference 30).

The experimental protocol for studying catabolite repression involved growing cells for many generations on raffinose until the culture reached a late-log-phase cell density of 2.7×10^7/ml. Glucose was then added, and the cells were pulse-labeled (1 min with [^{3}H]lysine) at various times to follow the kinetics of the repression of cytochrome *c* synthesis. Figure 1 shows

TABLE 1. *Percent cytochrome c[a] in cells grown on different carbon sources*

Carbon source	Cytochrome *c* (%)
5% Glucose	0.050
2% Raffinose	0.287
10% Raffinose	0.145
2% Ethanol	0.305

[a] Percent cytochrome *c* was determined by labeling cells for 1 min (5 min for cells grown on ethanol) with [^{3}H]lysine and determining the percentage of the incorporated label that was incorporated into cytochrome *c* by immunoprecipitation and gel electrophoreis as described in the text.

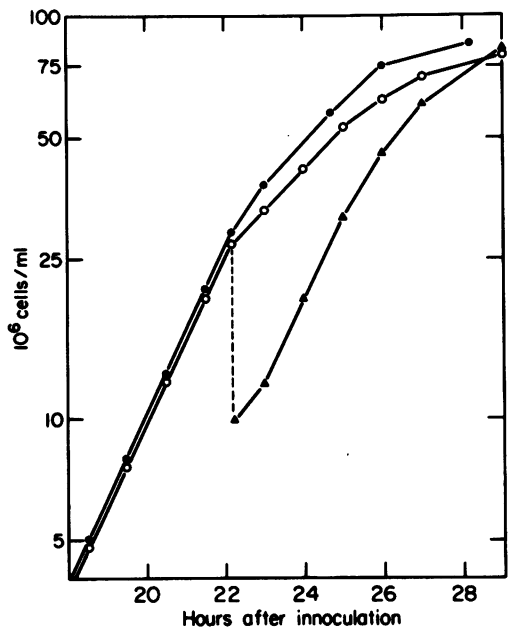

FIG. 1. *Growth curve for cells in medium containing glucose or raffinose. Cells were inoculated at low density and grown for 24 h before each experiment was started. Cell number was determined with the use of a hemocytometer. Cells were grown in medium containing 5% glucose (●), 2% raffinose (○), or 2% raffinose to a cell density of 2.7×10^7/ml and then diluted with medium containing glucose to give a final glucose concentration of 5% (▲).*

the growth curve for a typical experiment. It can be seen that the addition of glucose had no effect on the growth of the cells. The pulse-labeled cells were autolyzed, cytochrome c was immunoprecipitated with specific immunoglobulin, and the immunoprecipitates were subjected to electrophoresis on SDS-polyacrylamide gels. The immunoglobulin is specific for iso-1 and iso-2 cytochromes c as well as for the apoproteins (30); therefore, total cytochrome c protein was measured in these experiments. The results from one such experiment are shown in Fig. 2, in which cells were labeled during growth on raffinose (Fig. 2A) or labeled 2.5 min (Fig. 2B), 5 min (Fig. 2C), or 15 min (Fig. 2D) after the addition of glucose to a final concentration of 5%. The radioactivity under the cytochrome c peak was summed and divided by the total radioactivity incorporated into protein, and the results are expressed as the percent incorporated [^{3}H]lysine in cytochrome c or percent cytochrome c. Figure

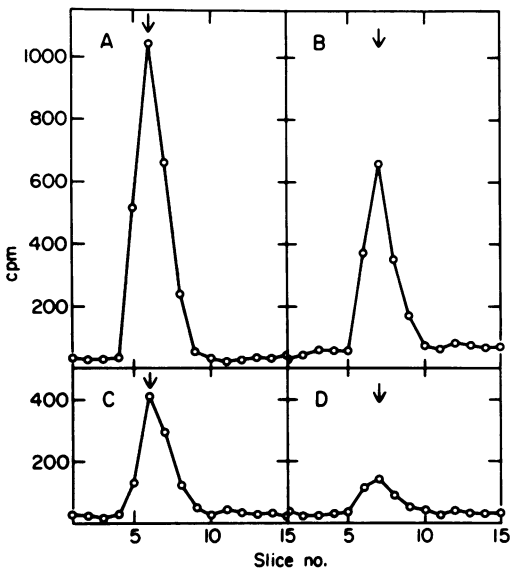

FIG. 2. *SDS-gel profiles of immunoprecipitated cytochrome c from cells labeled during glucose repression. Cells were labeled, and samples were prepared for electrophoresis as described in the text. The arrow designates the position of the unlabeled cytochrome c marker. The direction of electrophoresis was right to left. Only about one-half of each gel containing and adjacent to the cytochrome c marker was routinely sliced and counted. These samples were prepared from cells labeled before glucose addition (A) or labeled 1.5 to 2.5 min (B), 4 to 5 min (C), or 15 to 16 min (D) after glucose addition to a final concentration of 5%. The cells incorporated 61.6, 47.9, 53.2, and 85.8 pmol of [^{3}H]lysine into total protein, respectively. The counts presented here are normalized to an incorporation of 52.6 pmol into total protein to facilitate comparisons.*

3 shows the results of many of these types of experiments for four different glucose concentrations. Glucose concentrations of 5 and 10% gave the same kinetics of cytochrome c repression, whereas 1 and 0.5% glucose gave slower kinetics of repression. Despite the difference in kinetics, the fully repressed values were the same for all four glucose concentrations. It is apparent from Fig. 3 that glucose turned off cytochrome c synthesis extremely fast; within 10 min cytochrome c synthesis was fully repressed by high glucose concentrations. The data for percent cytochrome c in Fig. 3 were replotted as percent derepressed cytochrome c in a semilog plot and are presented in Fig. 4 to show that the rate of cytochrome c synthesis decreased from the derepressed to the repressed rate in a first-order fashion, with a half-time of 2 min for 5 and 10% glucose concentrations and 3 min for 1 and 0.5% glucose.

The effect of glucose is limited to the synthesis of cytochrome c; glucose did not affect the stability or function of the protein. The stability of cytochrome c was determined in a pulse-chase experiment. At 1.5 min after the addition of glucose to a culture growing on raffinose, the cells were labeled for 1 min. Samples were taken immediately after the labeling period and after a 5- and 10-min chase. The amount of [^{3}H]lysine incorporated and remaining in cytochrome c was determined for each sample (Fig. 5). The cytochrome c synthesized during the initial period of repression was still present 10 min later. Therefore, degradation of newly synthesized cytochrome c protein is not an important part of glucose repression. Further evidence that existing cytochrome c is stable in the presence of glucose was obtained by spectrophotometric determinations of the cytochrome c content in whole cells. Cells were grown in medium containing 2% raffinose, and at a cell density of 2.7 $\times$ 10^7/ml, glucose was added to a final concentration of 5%. Samples were then removed at various times, and the cytochrome c content in cells was determined spectrophotometrically. Table 2 shows that the cytochrome c content in cells decreased slowly after glucose addition. The rate of decrease could be accounted for by the repression of cytochrome c synthesis to a low level by glucose, while continuing cell growth diluted the cytochrome c synthesized before glucose addition. No degradation of existing cytochrome c need be involved. The cytochrome c present in the mitochondria was still functional after glucose addition (Table 3). In the same type of experiment as that described above for Table 2, oxygen consumption was measured. Table 3 shows that the cells are capable of respiration long after glucose repression of

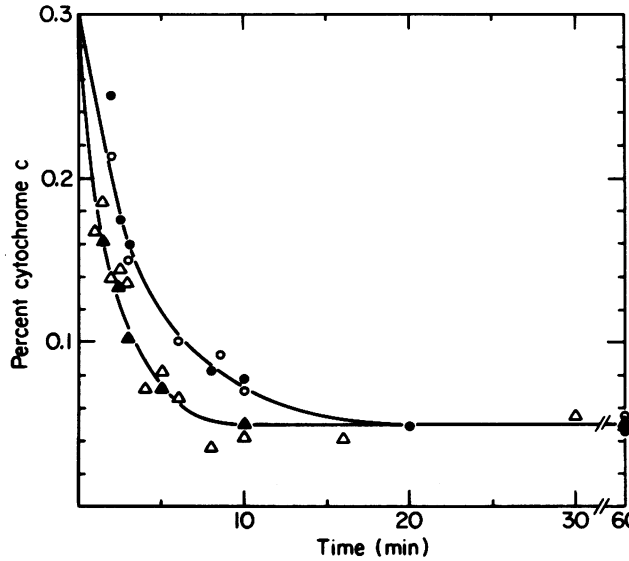

FIG. 3. *Kinetics of glucose repression of cytochrome c synthesis. At zero time, glucose was added to cells grown in medium containing 2% raffinose to a final glucose concentration of 0.5% (○), 1% (●), 5% (△), or 10% (▲). Cells were pulse-labeled for 1 min, and the amount of [^{3}H]lysine incorporated into cytochrome c was measured as described in the text. Percent cytochrome c was calculated by dividing the counts per minute under the cytochrome c peak in an SDS gel (as described in the legend to Fig. 2) by the total counts per minute incorporated into protein. The zero time point was measured as the fully derepressed value of percent cytochrome c determined from cells labeled before glucose addition.*

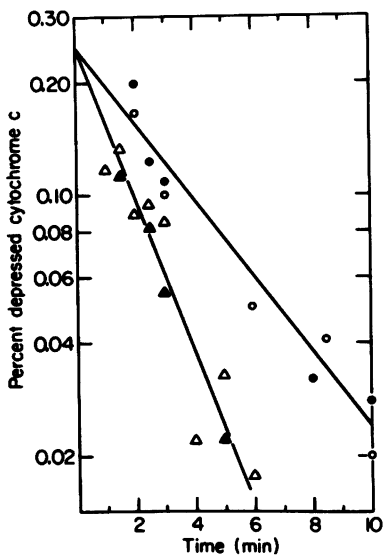

FIG. 4. *Semilog plot of the kinetics of glucose repression of cytochrome c synthesis. The values for percent derepressed cytochrome c were calculated by subtraction of the fully repressed value for percent cytochrome c (0.050%) from each data point in Fig. 3.*

cytochrome *c* synthesis has been accomplished.

The rapid repression of cytochrome *c* synthesis by glucose is paralleled by an equally fast repression of the cellular levels of active cytochrome *c* mRNA. We previously reported an assay for the level of active cytochrome *c* mRNA in cells based upon in vitro translation (30). Poly(A)-containing RNA was prepared from cells and then translated in a wheat germ extract. Nascent cytochrome *c* polypeptide chains were immunoprecipitated from among the translation products and identified by SDS-gel electrophoresis. This assay was used in the present study to determine the level of active cytochrome *c* mRNA during the initial period of glucose repression of cytochrome *c* synthesis. Table 4 shows that the levels of active cytochrome *c* mRNA decreased along with the decrease in cytochrome *c* synthesis. At 5 min after the addition of glucose to a final concentration of 5% to raffinose-grown cells, the level of active cytochrome *c* mRNA was down to 14% of the difference between the derepressed and repressed levels. This number agrees well with the amount of derepressed cytochrome *c* synthesis in cells 5 min after glucose addition (10%, Fig. 4). Similarly, at 7.5 min after the addition of glucose to cells, the level of active cytochrome *c* mRNA had fallen to 7%, the difference between derepressed and repressed levels, whereas the amount of derepressed cytochrome *c* synthesis was 2%. The data strongly suggest that the decrease in active cytochrome *c* mRNA is responsible for the decrease in the amount of cytochrome *c* synthesized upon glucose addition to derepressed cells.

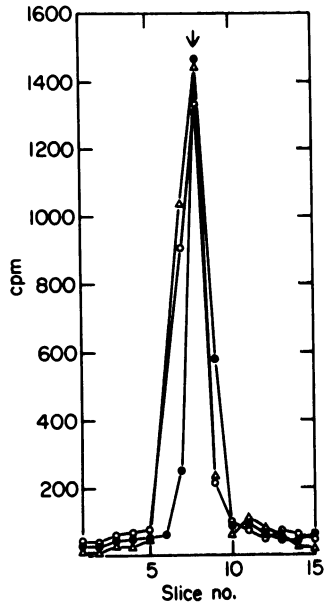

FIG. 5. *SDS-gel profiles of immunoprecipitated cytochrome c from cells labeled and chased during glucose repression. Cells were grown in medium containing 2% raffinose to a cell density of 2.7 × 10⁷/ml, at which time an equal volume of medium containing glucose was added to give a final glucose concentration of 5%. At 1.5 min after glucose addition, 7 ml of cells was labeled for 1 min with 500 μCi of [³H]lysine. After the labeling period, lysine was added to a final concentration of 10 mM, and cell growth was continued. A quantity of 184 pmol of [³H]lysine was incorporated into protein during the labeling period, and very little label was incorporated after addition of unlabeled lysine. Samples (2 ml) of cells were removed and assayed for cytochrome c as described in the text immediately after* (○), *5 min after* (●), *and 10 min after* (△) *the labeling period.*

DISCUSSION

The most striking feature of the results presented here is the rapidity with which glucose repressed cytochrome *c* synthesis. Yet the repression of cytochrome *c* synthesis was not accompanied by repression of cytochrome *c* function. As Table 3 shows, the cells continued to respire long after cytochrome *c* synthesis was fully repressed. The question may be asked, are other proteins that function in the mitochondria and are repressed by glucose repressed with equally fast kinetics? If so, then a new criterion can be applied to putative agents involved in glucose repression. Is their response to glucose addition fast enough to account for a 2-min half-time for repression? If the glucose repression of other mitochondrial proteins has slower kinetics than those for cytochrome *c*, the question becomes, what makes cytochrome *c* so special as

TABLE 2. *Cytochrome c content in cells during glucose repression*

Carbon source	Time after glucose addition[a] (min)	μmol of cytochrome *c* per mg of dry wt[b] (×10⁴)
2% Raffinose		1.89
5% Glucose		0.36
2% Raffinose + 5% glucose	60	1.53
2% Raffinose + 5% glucose	120	1.33
2% Raffinose + 5% glucose	240	0.98

[a] Glucose was added to a final concentration of 5% to cells grown in medium containing 2% raffinose to a cell density of 2.7 × 10⁷/ml.

[b] Cytochrome *c* content was determined in whole cells with a split-beam spectrophotometer as described previously (30).

TABLE 3. *Respiration rates of cells during glucose repression*

Carbon source	Time after glucose addition[a] (min)	Microatoms of O/min per mg (dry wt)[b]
2% Raffinose		0.16
5% Glucose		0.05
2% Raffinose + 5% glucose	30	0.18
2% Raffinose + 5% glucose	60	0.18
2% Raffinose + 5% glucose	120	0.16

[a] Glucose was added to a final concentration of 5% to cells grown in medium containing 2% raffinose to a cell density of 2.7 × 10⁷/ml.

[b] Monitored polarigraphically with a Clarke oxygen electrode (Yellow Springs Instruments model 53).

TABLE 4. *Levels of active cytochrome c mRNA in cells during glucose repression measured by in vitro translation*

Source of poly(A)-containing RNA[a]	10⁶ cpm of [³H]lysine incorporated into total protein	cpm of [³H]-lysine incorporated into cytochrome *c*[b]	Cytochrome *c* (%)
2% Raffinose cells	1.06	1,509	0.142
5% Glucose cells	1.16	448	0.038
2% Raffinose cells, 5 min after glucose addition	1.57	841	0.053
2% Raffinose cells, 7.5 min after glucose addition	0.89	404	0.045

[a] Cells were grown, and poly(A)-containing RNA was prepared and translated in a wheat germ extract as described in the text. A 10-μg amount of RNA was added per 100 μl of translation mix.

[b] Counts incorporated into cytochrome *c* was determined by immunoprecipitation and SDS-gel electrophoresis as described in the text.

to merit such fast repression?

A question of equal interest to us is, how is this repression of cytochrome *c* synthesis accomplished? We believe that our results are suggestive of repression of cytochrome *c* gene expression at the level of transcription or of mRNA processing. The decrease in the rates of cytochrome *c* synthesis that we observed upon glucose addition to raffinose cultures would then be due to the decay of preexisting mRNA. The first-order kinetics of repression (Fig. 5) were consistent with decay of mRNA, as was the parallel decrease in active cytochrome *c* mRNA. The half-life of yeast mRNA has been estimated to be about 20 min by a variety of methods (6, 8, 10, 11, 14, 19, 28). However, Sripata and Warner recently reported that a significant percentage of yeast mRNA's have half-lives of 3 to 5% of the cell doubling time or 3 to 6 min under our growth conditions (Cold Spring Harbor Mol. Biol. Yeast Meet., 1977). In addition, there have been several reports suggesting that the mRNA's for a variety of inducible enzymes have short lifetimes (3, 4, 6, 13, 14, 17). The 2-min half-time we observed for repression of cytochrome *c* falls close to the half-lives of the fastest-decaying mRNA's reported so far. Therefore, our data are consistent with a model of repression involving the decay of an mRNA pool due to a shutoff of mRNA synthesis or processing. Nonetheless, we cannot rule out models of repression involving translational blocks by covalent modification of the mRNA or an increased rate of mRNA degradation.

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