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CO_2 production in cell-free extracts of fission yeast detects cell cycle changes

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SUMMARY

CO_2 production was followed by manometry in starved cell-free extracts of fission yeast stimulated by unstarved cell-free extracts from a synchronous culture. The degree of stimulus, measured by the lag time in CO_2 production, varied markedly during the cell cycle, with a maximum for cells at about mitosis and a minimum for septated cells. Similar differences in lag time were found with unstarved extracts of *cdc13.117* grown at 37°C and 35°C.

Key words: cdc mutants, cell-free extracts, cell cycle, CO_2 production, fission yeast, induction synchrony, *Schizosaccharomyces pombe*

INTRODUCTION

Cell-free extracts, especially of eggs, are powerful tools for following cell cycle changes (e.g. see Murray and Kirschner, 1989; Hutchison et al., 1989), and cell-free extracts of yeast have a long history in metabolic studies (e.g. see Chance et al., 1964; Pye, 1973; Das et al., 1990). We have been interested for some years in cell cycle changes in CO_2 production in intact cells of the fission yeast *Schizosaccharomyces pombe* (Novak and Mitchison, 1986, 1987, 1990a). We show here that CO_2 production in cell-free extracts of fission yeast can also be used to detect cell cycle changes.

MATERIALS AND METHODS

Strains

The wild type (wt) strain 972h$^-$ of *Schizosaccharomyces pombe* was originally obtained from Professor U. Leupold, Bern. The mutants *cdc2.33* and *cdc13.117* are temperature-sensitive conditionals, which form colonies at 25°C but not at 35°C (Nurse et al., 1976). The *cdc2*$^+$ function is required both for mitosis and for an event in G_1 (Nurse and Bissett, 1981; Novak and Mitchison, 1989). The *cdc13*$^+$ function is required for mitosis. It codes for a B-type cyclin that forms an important complex with the cdc2 gene product (Nurse, 1990).

Medium

Cultures were grown in a shaking water bath in EMM3, a minimal medium with phthalate buffer (Creanor and Mitchison, 1982).

Preparation of starved wt extracts

wt cells were grown at 35°C until they were in late exponential phase (10^7 cells/ml). They were collected on a membrane filter, washed with phosphate buffer (0.1 M, pH 6.5), resuspended in the same buffer, and then starved for 4 h at 25°C with shaking. After that time, they were collected on a filter, resuspended in the phosphate buffer and broken by vigorous shaking with an equal volume of 40 mesh glass beads, with intervals in an ice bath. This extract was stored at −70°C without lose of activity when stimulated. Before use, cell debris was removed by spinning for 2 min in a microcentrifuge, although this did not alter the activity.

Preparation of extracts of *cdc2* and *cdc13*

These were prepared in a similar way to that above but with the important difference that the cells were not starved before breakage.

Other methods

CO_2 production was measured by Warburg manometry as described by Novak and Mitchison (1986). Micro-flasks were used in some experiments. CO_2 retention by the buffer was taken into account.

Cell numbers were measured with a Coulter Counter (Industrial model D) with a 100 μm aperture.

Protein was determined by the Pierce BCA protein assay.

RESULTS

General

Our main technique was to add glucose to extracts from unstarved mutant cells and to follow the activation of the

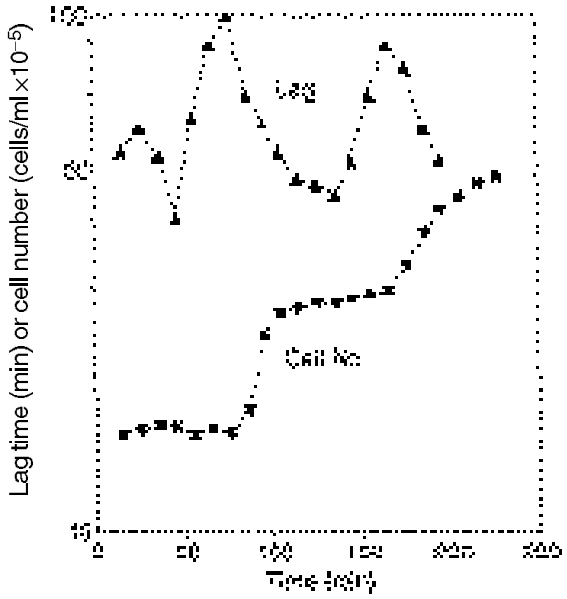

Fig. 1. Induction synchrony with *cdc2.33.* See text for experimental details. Top curve shows the lag time before CO_2 production started in each sample. Bottom curve shows the increase in cell number over time.

glycolytic pathway by measuring the production of CO_2 (the end product of this pathway) by manometry. Typically there was a lag before the rate of CO_2 production started to rise until it reached a maximum value (e.g. see Fig. 2). This does not necessarily mean that the extracts failed to glycolyse during the lag but rather that the glycolytic flux was directed into respiration with no net CO_2 production (see Discussion for further details). The lag was temperature dependent and also increased with dilution of the extract. In order to maintain the concentration of glycolytic enzymes, we increased the lag by diluting the extracts not with buffer but with *starved* wt extracts. On their own, these

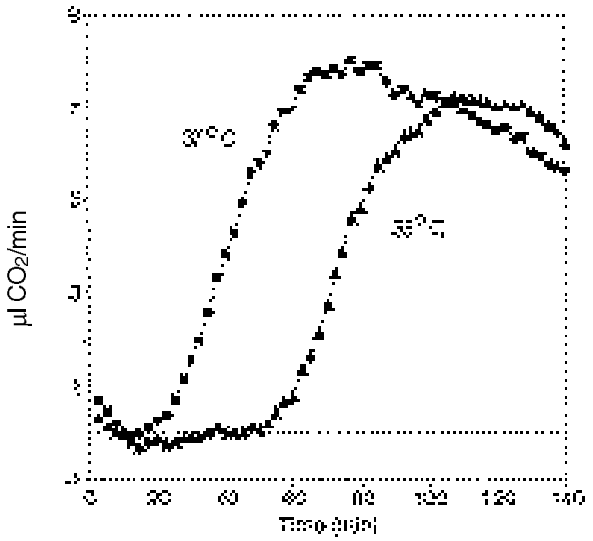

Fig. 2. CO_2 production in extracts from cells grown for 5 h at the temperature indicated. See text for experimental details.

starved extracts were not stimulated into CO_2 production by glucose. Quantitative comparison showed that the CO_2 was produced both by the unstarved and the starved extracts when mixed together. The starved extracts were therefore stimulated into glycolysis by the unstarved extracts and the greater the stimulus the shorter the lag.

Induction synchrony

Asynchronous cultures of *cdc2.33* can be induced to divide synchronously by a block and release experiment (Creanor and Mitchison, 1989; Novak and Mitchison, 1990a). A 2000 ml culture of *cdc2.33* was grown at 29°C to a cell density of 1.6×10^6 cells/ml and then shifted up to 35°C. After 4 h, it was shifted back to 29°C and 100 ml samples taken every 10 min. Extracts from these samples were prepared as described above. Cell numbers were also counted.

Micro-flasks were used for manometry at 29°C, each containing 0.3 ml starved wt extract (10 mg/ml protein) and 50 µl 1 M glucose. To these was added different volumes of the mutant extracts (equal to or less than 0.1 ml) to give 1.5 mg protein, and the volume made up to 0.45 ml with the phosphate buffer.

Fig. 1 shows the cell numbers in the culture and also the lag time before the CO_2 production started rising in each sample. The first small peak is probably a transient effect of the shift down in temperature. Thereafter there is a marked cell cycle pattern of peaks and troughs repeated in two cell cycles. Using the data in Fig. 7A of Novak and Mitchison (1990a), the timing of the peaks (maximum lag) is about the same as the peaks of the septation index. The timing of the troughs (minimum lag) is about at mitosis. Since the shortened cycles in induction synchrony are due to a shortening of G_2, the timing between mitosis and cell division is assumed to be the same as in wt cells at 29°C.

Terminal phenotypes of cdcl3

It was of interest to follow the stimulating effect of extracts of *cdc13.117* grown at different temperatures. We have shown earlier (Novak and Mitchison, 1990a) that when a culture is grown at 37°C, there is a tight pre-mitotic block and only a few cells leak through to septation. But at 35°C, cells leak through the block and accumulate with septa and condensed chromatin.

A 2000 ml culture of *cdc13.117* was grown up at 29°C to a cell density of 2×10^6 cells/ml and then split into two. One was kept at 35°C and the other at 37°C. After 5 h, extracts were made as described above. For manometry, 0.6 ml starved wt extract (10 mg/ml protein) plus 0.1 ml 1 M glucose were put in the main flask compartment, and 0.2 ml mutant extract (10 mg/ml protein) in the side arm. The side arm was mixed in at zero time and CO_2 production followed at 35°C.

The results are shown in Fig. 2. There was a marked difference in the lag time which was 20 min for the 37°C extract and 55 min for the 35°C extract. This is discussed below.

DISCUSSION

It is clear from the synchronous culture results that starved

wt extracts are stimulated into CO_2 production by the addition of unstarved extracts and that the degree of stimulation is cell cycle dependent. The maximum stimulus (minimum lag) comes from cells at about mitosis, and the minimum stimulus (maximum lag) comes from septated cells about 30 min later.

The results from the *cdc13* experiments are analogous. Cells at 37°C, blocked just before mitosis, give a greater stimulus and a shorter lag than the septated cells at 35°C. This is in accord with our earlier results (Novak and Mitchison, 1990a) where these mutant cells at 35°C (but not at 37°C) achieve a constant rate of CO_2 production and we suggested that they have a continuing 'stop CO_2 acceleration' signal. However, there are dangers in using terminal phenotypes. At 35°C, this phenotype is not a normal cell cycle stage and has characteristics of both mitotic and post-mitotic cells (Hagan et al., 1988).

This cell-free system provides a way into metabolic and other studies of the yeast cell cycle. An interesting question is the nature of the stimulus from the mitotic cells. To try and answer this question, we need to understand the reason for the lag. The following explanation of the lag is based on our unpublished experiments with starved wt extracts after addition of glucose and adenine nucleotides (ATP and ADP). Besides measuring CO_2 production, we also followed glucose consumption and ATP concentration.

Since glycolysis starts with ATP-dependent phosphorylation of glucose, the rate of entry of glucose into glycolysis is determined by the availability of ATP. However, the degradation of glucose through glycolysis and respiration produces more ATP than was available initially and this ATP increases the entry of further glucose into the pathway. In other words, glucose utilisation is characterised by autocatalytic production of ATP (Reich and Selkov, 1981). This autocatalysis works efficiently in these extracts which have very low ATPase activity.

The temporal changes in such an autocatalytic system are largely dependent on its initial rate. After mixing a starved and an unstarved extract together with glucose, the initital rate of glycolysis will be determined by the levels of glycolytic intermediates and ATP in the unstarved extract. The glycolytic flux will increase more or less exponentially as a consequence of the ATP autocatalysis, and this can be detected in increasing glucose consumption and ATP level. Addition of ATPase to the extract slows down the process. But the pyruvate produced will be degraded in the citric acid cycle, accompanied by equimolar O_2 consumption and CO_2 production, and there will be no net pressure change.

Fission yeast, however, has a limited respiratory capacity compared to its extensive glycolytic capacity (Hamburger et al., 1977; Novak and Mitchison, 1990b), as in budding yeast. As soon as respiration is saturated, the excess pyruvate will be converted to ethanol and CO_2, which will cause an abrupt pressure increase in the manometer. So the lag is the time period needed for the autocatalytic process to saturate respiration. Thereafter, the glycolytic flux will continue to increase because of the autocatalysis. Finally, the *maximum* rate of CO_2 production is limited by the concentration of adenine nucleotides and can be increased by adding more ATP or ADP to the extracts. Since, however, there were no significant differences in this maximum rate during the cycle (data not shown) or between the *cdc13* extracts (Fig. 2), the total concentration of adenine nucleotides must be very similar in the unstarved extracts. What may vary through the cell cycle are the levels of glycolytic intermediates and the ATP/ADP ratio which could be highest at mitosis. It is still possible that changes in large molecules (e.g. ATPases) could affect the lag period, but further careful studies of the changes in the small molecules will certainly be needed.

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