Stepwise Accumulation of an Acid-extractable Protein Fraction in the Budding Yeast, *Kluyveromyces fragilis*, during the Cell Cycle

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SUMMARY

Acid-extractable proteins were obtained from *Kluyveromyces fragilis* by pH titration, and partially characterized. They differ from mammalian histones and from the proteins obtained from other yeasts by similar methods. The largest fraction, extracted at pH 2.2, appeared to be predominantly of cytoplasmic origin. Quantitative changes in this fraction have been followed through the cell cycle and mapped relative to DNA synthesis, nuclear division and cell division. The total amount of such protein/cell doubled about one-third of a cycle after cell division and at a quarter of a cycle before nuclear division. DNA synthesis did not correspond to these doubling times but was almost simultaneous with cell division.

INTRODUCTION

Many organisms including yeasts show marked fluctuations in enzyme activity and in the levels of many cell components through the cell cycle (Mitchison, 1971). What controls these fluctuations is still a matter of considerable speculation but the two main hypotheses both implicate control at the level of transcription, namely that of 'oscillatory repression' (Donachie & Masters, 1969) and of 'linear reading' (Halvorson, Carter & Tauro, 1971). Since this must involve the proteins associated with DNA in the nucleus, it was decided to investigate the most accessible of these, namely the histones, as an initial step. Previous work has indicated that yeasts have proteins which resemble the histones of higher organisms in their extraction properties and occurrence within the nucleus (Tonino & Rozijn, 1966; Duffus, 1971). Duffus (1971) found that these proteins double in amount at a time close to that of DNA synthesis in the fission yeast, *Schizosaccharomyces pombe*. The present paper describes a different sequence of events for the corresponding protein fraction from the budding yeast, *Kluyveromyces fragilis*, formerly *Saccharomyces fragilis* (Van der Walt, 1970).

METHODS

Organism and cultural conditions. *Kluyveromyces fragilis* (NCYC 100) was grown in Oxoid malt extract broth (MEB) at 30 °C with shaking at 160 rev./min in a Gallenkamp orbital incubator. Under these conditions, the cells had a doubling time of 90 min.

Extraction and characterization of acid-extractable proteins. The protein fractions were extracted, as described by Duffus (1971), from asynchronously growing yeast and from a nuclear fraction from such cells (Duffus, 1969), by the method of Murray, Vidali & Neelin (1968). Samples were analysed by acrylamide-gel electrophoresis as described by Johns (1967). Amino acid analyses were performed on a Technicon Auto-analyzer with samples hydrolysed in 5.65 M-hydrochloric acid for 24 h at 105 °C. Immunological analysis

against an antibody for the comparable proteins from *Schizosaccharomyces pombe* was carried out by the double diffusion technique (Ouchterlony, 1964).

Determination of the time of nuclear division. Samples of asynchronous, exponentially growing yeasts were fixed with glacial acetic acid:ethanol (1:3) fixative and stained with Giemsa stain (Ganesan & Swaminathan, 1958). The percentage of binucleate cells was determined microscopically and from this the average time of nuclear division in the cell cycle was calculated (Hoffman, 1949).

Estimation of acid-extractable proteins in samples from synchronous cultures. Synchronous cultures were prepared by the method of Mitchison & Vincent (1965). Samples were taken at 30 min intervals, rapidly frozen in a solid CO₂/ethanol bath and stored at -18 °C until required for analysis. The number of yeasts in each sample was obtained by means of a Thoma haemocytometer. Yeasts were taken to have divided when the bud was two-thirds the size of the mother cell. For analysis of acid-extractable proteins, each sample was thawed rapidly and the yeasts pelleted by centrifuging. The cells were then resuspended in 5 ml acetate buffer, pH 2.8, and passed through an Eaton press (Eaton, 1962). The resultant homogenates were left at 0 °C overnight in order to extract completely all ribosomal and soluble proteins. The homogenates were centrifuged at 200 g for 10 min, the supernatant liquid was removed, and the pellets were resuspended in 2 ml acetate buffer, pH 2.2, and left for 2 h at 0 °C. Thereafter, the other fractions were obtained by serial extraction as previously described (Murray *et al.* 1968) by using 2 ml volumes of the appropriate buffers. The amount of proteins in the extracts was measured by determining the extinction at 260 and 280 nm (Warburg & Christian, 1941).

Accurate determination of the times of doubling of DNA and acid-extractable protein. DNA is replicated at a well-defined point in the cell cycle called the S period (Mitchison, 1971). This makes it possible to calculate the position of the S period by using a technique analogous to that for determining the time of nuclear division. The average DNA content/yeast in an asynchronous, exponentially growing culture at 30 °C was measured as described by Bostock (1970). From this and knowledge of the 2c amount of DNA obtained from synchronous-culture measurements, the fraction of yeasts in the 2c state could be calculated (Hoffman, 1949). The results from synchronous cultures showed that the acid-extractable proteins behaved in a similar fashion to DNA, hence the same method could be applied to determine the average time at which they double during the cell cycle.

Estimation of total cell protein. Samples of asynchronous, exponentially growing cells at 30 °C were homogenized in 1 M-sodium hydroxide solution in an Eaton press. The proteins in the homogenate were extracted by heating the homogenate at 37 °C for 90 min. The supernatant fraction, after centrifuging at 1000 g for 10 min, was used to estimate the protein content by using the biuret method (Gornall, Bardawill & David, 1949).

RESULTS

Characterization of protein fractions

The relative quantities of the protein fractions obtained by the pH-titration technique are shown in Table 1. The fractions obtained at pH 1.80, 1.35 and 0.60 did not separate on acrylamide-gel electrophoresis by the method described by Johns (1967). The electropherograms of the proteins extracted at pH 2.70, 2.20 and 0.97 are shown in Fig. 1. A ribosomal pellet was prepared from the supernatant left after centrifuging a homogenate of yeast from the Eaton press in 1 M-sorbitol:20 % glycerol solution at 30000 g for 30 min. The supernatant was centrifuged at 105000 g for 8 h to obtain this pellet. The protein

Table 1. The relative amounts* of acid-extractable protein obtained by pH titration from *Kluyveromyces fragilis* cells and nuclei in asynchronous culture growing exponentially at 30 °C with full aeration

pH	Protein extracted/cell (g × 10 ¹²)	Protein extracted/nucleus (g × 10 ¹²)
2.20	3.0	0.9
1.80	1.5	1.5
1.35	1.3	1.0
0.97	0.3	0.4
0.60	0.6	0.4

* For comparison, the amount of DNA/cell is 5.8×10^{-14} g.

Fig. 1. Electropherograms of the proteins extracted from *Kluyveromyces fragilis* by the pH titration technique. From left to right, pH 0.97, pH 2.20 and pH 2.70.

present appeared to be totally extractable at pH 2.70 under the conditions described above. However, analysis of a crude nuclear fraction prepared according to Duffus (1969) showed that the ratio of pH 2.20 fraction to DNA was approximately 15:1 as compared to about 50:1 in whole yeasts (Table 1).

The pH 2.20 fractions gave no reaction with the antibody for the same fraction from *Schizosaccharomyces pombe*. The amino acid composition of the pH 2.20 fraction (Table 2)

Table 2. *Amino acid composition of pH 2.20 fraction from Kluyveromyces fragilis compared with the corresponding fraction from Schizosaccharomyces pombe (Duffus, 1971)*

Amino acid	<i>K. fragilis</i>	<i>S. pombe</i>
Lys	9.7	7.6
His	2.4	1.8
Arg	5.2	5.4
Asp	11.1	10.4
Thr	6.1	6.6
Ser	5.2	8.8
Glu	9.5	13.5
Pro	7.0	4.4
Gly	8.5	8.7
Ala	7.0	8.5
Cys	0.0	0.8
Val	6.5	4.7
Met	0.7	1.6
Ileu	4.6	4.9
Leu	8.6	6.2
Tyr	3.1	2.7
Phe	4.5	3.1
Trp	0.1	0.4
Lys/Arg	1.9	1.4

Amounts of amino acids are expressed as moles/100 moles of total recovered amino acids; no corrections are applied for hydrolytic losses of any of the amino acids.

Fig. 2. Bulk change in the pH 2.20 extractable fraction in synchronous cultures of *Kluyveromyces fragilis*.

had a higher lysine to arginine ratio than the corresponding fraction from *S. pombe* or the presumed histone fraction from *Saccharomyces cerevisiae*.

Mapping of the cell cycle

The change in total amount of the pH 2.20 fraction during a normal cell cycle is shown in Fig. 2. The fraction doubles in quantity 20 to 30 min after cell division. The average amount of these proteins/cell in an asynchronous, exponentially growing culture was found to be 3.0×10^{-12} g, which represents 8.7 % of the total protein. Knowing the unduplicated amount of the pH 2.20 fraction from synchronous cultures to be 2.3×10^{-12} g, it was calculated that the exact point of doubling of this fraction was 0.33 of a cycle which, in a cycle of 90 min, corresponds to 30 min after yeast division. The other fractions extracted at pH values below 2.20 appear to behave similarly. On the other hand, the period of DNA synthesis was found to correspond very closely to the time of yeast division. In an asynchronous, exponentially growing culture at 30 °C, 32 % of the cells are binucleate; from this it was calculated that nuclear division must occur about 0.6 of the way through the cell cycle. This means that the nuclei divide 54 min after cell division.

DISCUSSION

In *Kluyveromyces fragilis*, selection synchrony as used here has a useful consequence in producing a uniform population of cells. This yeast tends to form elongated cylindrical cells and other aberrant forms (Van der Walt, 1970). The aberrant forms sediment more rapidly than the normal forms and so are clearly separated from them by the sucrose gradient.

Kluyveromyces fragilis contains none of the classical mammalian histones usually isolated from calf-thymus tissue. The amino acid analysis of the pH 2.20 fraction shows this as does the acrylamide electrophoresis of the other fractions. In addition, there are significant differences between the pH 2.20 extractable proteins from *K. fragilis* and those from *Schizosaccharomyces pombe*, as shown by the failure of the *K. fragilis* fraction to react with the antibody to the *S. pombe* fraction. This is important because it suggests that a study of these proteins might throw considerable light on the evolution and taxonomy of yeasts.

The protein fractions studied constitute about 20 % of the total cell protein if all the fractions obtained at pH 2.20 and below are included. This is about ten times higher than the corresponding figure for *Schizosaccharomyces pombe*, and may correlate with the much faster growth rate of *Kluyveromyces fragilis*. The results show that proteins from a yeast ribosomal fraction are completely removed by pH 2.70 extraction and that the pH 2.20 fraction is mainly contained in the cytoplasm. It appears from their constancy in whole yeasts and in the nuclear fraction in relation to DNA that the proteins obtained at pH 1.80 and below may be nuclear.

Whatever the function of the proteins, the stepwise doubling of the pH 2.20 fraction in quantitative terms represents a considerable change in the nature of the cells. Though it does not occur at the same time as cell division, nuclear division or DNA synthesis, it is likely that it will be found to coincide with other events of fundamental importance.

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