EVIDENCE OF MITOCHONDRIAL SYNCHRONY IN SYNCHRONOUS CELL CULTURES OF YEAST

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Received February 2, 1970

SUMMARY

Diploid cell cultures were grown in liquid media containing 3% glucose or galactose after synchronization by an alternating feeding-and-starving method. There were stepwise increases in rates of nuclear DNA synthesis and malate dehydrogenase and alkaline phosphatase activities, as indications of cell synchrony. Mitochondrial synchrony was demonstrated by stepwise doublings of cytochrome *c* oxidase activity and organelle DNA content, once per cell cycle. In contrast with these data, there were continuous rate increases in culture turbidity, protein content, and oxygen uptake.

From studies of growth and synthesis patterns in synchronously-growing cell cultures (1,2), it has become clear that cellular development occurs as a highly ordered series of chemical events (3). In addition to whole-cell systems, it is apparent that replication of organelle DNA molecules also may occur synchronously (4,5), but there is little specific information concerning synchronous cycles for other biochemical events which are organelle-specific. Our own investigations of mitochondrial activities were undertaken to determine whether or not synchronous organelle behavior occurred in repetitive cell cycles. Such behavior would permit us to examine homogeneous organelle populations using the bulk methods of biochemistry, physiology, and ultrastructure analysis. In effect, we could interpret such data as indicative of changes taking place at the level of the individual organelle and, therefore, exploit the amplification phenomenon to analyze mechanisms and events involved in the formation and regulation of the organelle population of a single cell. We report here some of our findings which indicate that cyclic and sequential changes occur in the mitochondria of synchronous cell cultures.

MATERIALS AND METHODS

Culture system: Cell cultures of the diploid strain iso-N of *Saccharomyces cerevisiae* (6) were synchronized using a modification of the method of

Williamson and Scopes (7), with either 3% glucose or galactose as the carbon source in all appropriate steps. From 3 to 6 liters of culture were grown in a MicroFerm fermentor (New Brunswick Sci. Co.) at 25° ± 0.1, on either carbon source. Samples could be removed when necessary as the culture circulated through a flow system devised to permit the continuous recording of culture turbidity at 515 nm. In all cases, asynchronously-growing cultures served as control populations which otherwise were handled identically to the synchronous cell cultures.

Monitoring cell synchrony: Cell counts and the onset of budding were observed directly with the microscope, according to established criteria (8). In addition, whole-cell DNA (9), whole-cell protein (10), and culture turbidity were assayed throughout the growth period.

Oxygen uptake of whole cells: Three variations of the polarographic method were used for cultures grown on either carbon source. In one method, samples of the culture were removed at 10-min intervals and measured using a Clark-type electrode and a YSI Biological Oxygen Monitor (Yellow Springs, Ohio). Another method permitted continuous recording of oxygen saturation at one electrode inside the fermentor vessel and at another electrode situated near the end of an airtight flow system. The third method involved measurement of oxygen uptake while aeration to the culture was interrupted at regular but brief intervals, similar to the procedure described by Scopes and Williamson (11).

Enzyme activity assays: Samples were removed at 15-min intervals during growth and passed once through a chilled French pressure cell at 18,000 psig. The cell-free supernatant obtained after a 10-min centrifugation at 4,000 X g was stored overnight at -20°, without loss of enzyme activity. Spectrophotometric assays were performed to measure the activities of cytochome *c* oxidase (1.9.3.1, ref. 6), L-malate dehydrogenase (1.1.1.37, 12), and alkaline phosphatase (3.1.3.1, ref. 13). Each assay was performed in duplicate on all samples.

RESULTS

Duration of the cell cycle: Cultures which were grown at 25° on either glucose- or galactose-containing media completed a synchronous cell cycle in 80 to 120 min.

Patterns of increase: Direct microscopic examinations revealed that budding was initiated almost immediately after the completion of the wall which separated the previous set of buds from the parent cells. In one particular synchronous cell culture (Fig. 1) the first round of budding occurred 55 min after the initiation of growth, and a second burst of budding occurred after

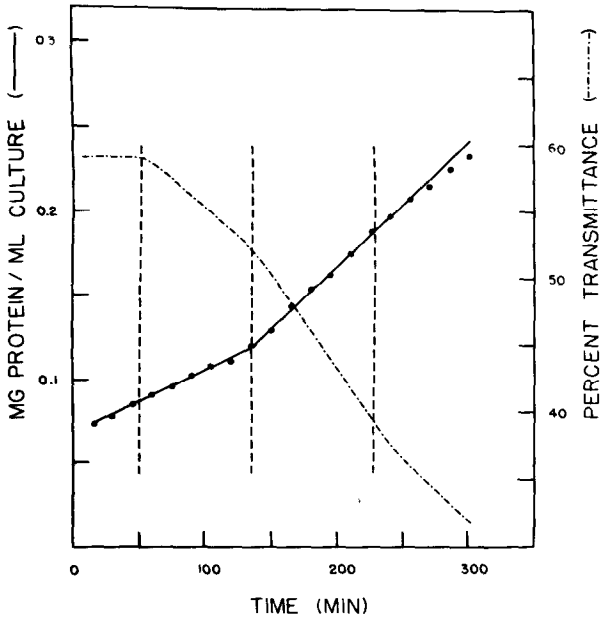

FIGURE 1. Measurements of protein content and turbidity were made during synchronous cell growth of a single culture grown at 25° on 3% galactose medium. Doubling in rate of turbidity increase (shown as decrease in transmittance) occurred at about the same time as the onset of budding cycles (shown by vertical dashed lines). The linear rate of increase in total protein doubled at the same time as the onset of the second budding cycle and the doubling in rate of turbidity increase.

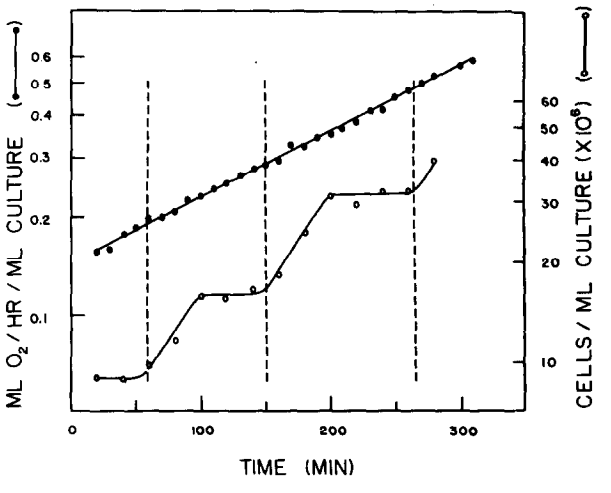

FIGURE 2. Results from synchronous cell cultures showing the exponential increase in rate of oxygen uptake during the entire growth period. Cell synchrony is indicated by the stepwise doublings of cell counts in each cycle. The onset of budding cycles is indicated by vertical dashed lines.

135 min of growth. The first detectable increase in turbidity also appeared after 55 min, with a doubling of this linear rate after 135 min. The linear rate of protein synthesis also doubled at 135 min. From these data (Fig. 1) it can be seen that synchrony begins to decay after the completion of the second cell cycle, which we found to be due primarily to depletion of the carbon source

There were no stepwise increases in oxygen uptake (Fig. 2) regardless of the polarographic method used, the carbon source, or the temperature. Since sampling either was very frequent or continuous, it is unlikely that such increments would have been overlooked if they had occurred at all.

DNA synthesis: Synchronous cultures which were grown at 25° on media containing 3% glucose (= 3% GLU) showed a distinct stepwise increase in DNA content at about the time of bud initiation, as well as an apparent minor increment in each cycle about 20-30 min before budding (Fig. 3). To further enhance the minor-DNA stepwise increase, on the assumption that it represented mitochondrial DNA (= mitDNA), we also monitored cultures in media containing 3% galactose (= 3% GAL). As we had predicted, on the basis of Smith's (14) studies using *Saccharomyces lactis*, the stepwise increases were much sharper in the 3% GAL cultures (Fig. 3).

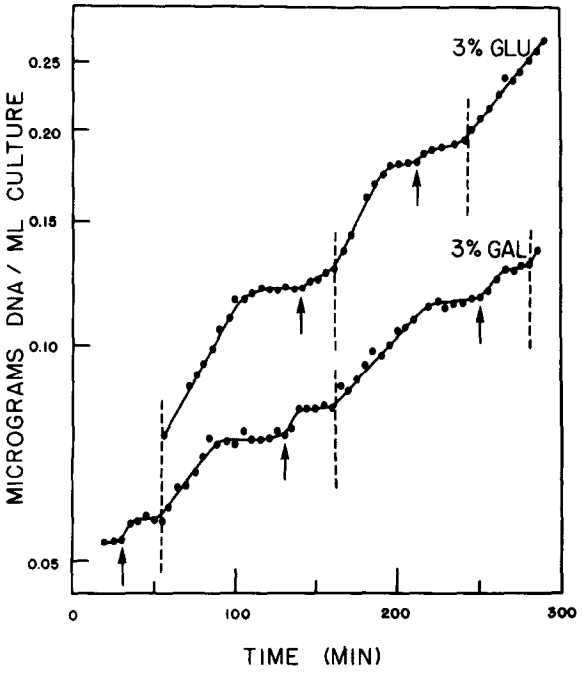

FIGURE 3. DNA synthesis for two single cultures grown at 25° on media containing 3% glucose or galactose. Stepwise increases in the minor-DNA component are shown at the arrows, and onset of budding by vertical dashed lines. Note the enhancement of the minor-DNA increment for cells grown on galactose as compared with glucose.

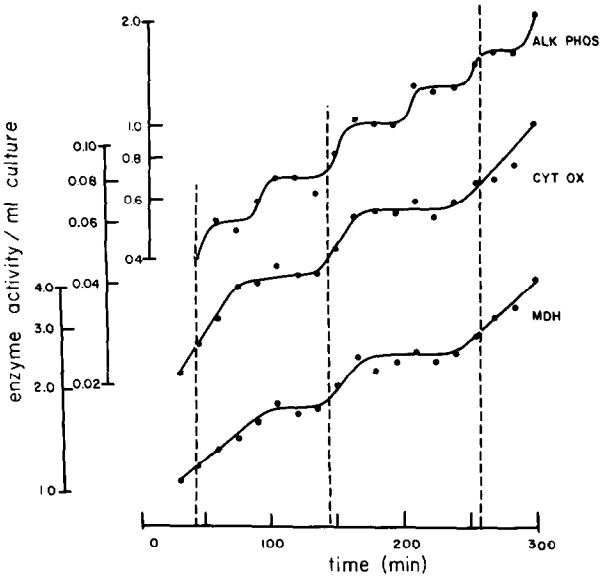

FIGURE 4. Enzyme activities in a single synchronous cell culture grown at 25° on galactose media. Increase in activity of alkaline phosphatase (ALK PHOS) was measured as micromoles nitrophenol produced per min per ml culture; cytochrome *a* oxidase (CYT OX) as the first-order reaction rate constant, $k(sec^{-1})$ per ml culture; and malate dehydrogenase (MDH) activity as nanomoles NADH oxidized per min per ml culture. The onset of budding in each cycle is indicated by a vertical dashed line.

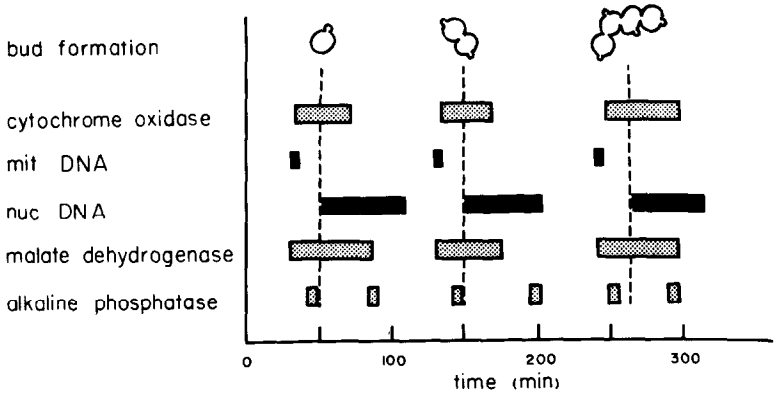

FIGURE 5. The relationships of onset and termination of increase in DNA content and of enzyme activities are shown relative to the time of budding in each cell cycle (vertical dashed line). These values all were derived from a single synchronous cell culture.

Enzyme activities: All three enzyme activities showed stepwise increases (Fig. 4), but while alkaline phosphatase showed two steps of rate increase per

cell cycle, both cytochrome *c* oxidase and malate dehydrogenase exhibited only single stepwise doublings during each cell cycle. The relationships of onset and termination of the several biochemical activity increases which were measured during a single experiment are shown in Fig. 5. Enzyme activity assays of asynchronous control cultures showed essentially exponential rates of increase throughout growth, with no indications of stepwise increments.

DISCUSSION

While complex phenomena often demonstrate continuous change patterns (1), many simple events show discrete stepwise rate increases during the cell cycle (3). It is most likely that individual events also increase in steps in randomly-changing populations but that such patterns are obscured. In our study, asynchronous cell populations always showed continuous rather than stepwise rate increases. On the other hand, in the synchronous cell cultures there were stepwise doubling increments in DNA synthesis and enzyme activities in each cell cycle and continuous increases in rate only for more complex functions.

At present we have only tentatively identified a minor-DNA component as mitochondrial. The minor-DNA content varied according to the physiological state of the culture (15), comprising about 12% of the total DNA for cells grown on 3% glucose but about 20% for those grown on 3% galactose. Such results also were reported for *S. lactis* (14) grown on different carbon sources at different concentrations. Also, yeast mitDNA replicates at a specific time in the cell cycle which is distinct from that for nuclear DNA (5). In one preliminary experiment in which whole-cell DNA was examined after analytical ultracentrifugation in CsCl gradients (16), we found that the mitDNA (ρ = 1.684) had doubled in amount relative to the nuclear DNA (ρ = 1.700) after the step increase in the minor-DNA component but before nuclear DNA synthesis of the succeeding cell cycle. These data also are in agreement with results from a similar study of *S. lactis* (17). Further studies now are in progress to confirm the identity of the presumptive mitDNA.

Malate dehydrogenase and alkaline phosphatase probably are exclusively under nuclear control (18,19) whereas cytochrome oxidase is regulated at least in part by a mitochondrial genetic system (20,21). Since all three enzymes showed stepwise doublings we may conclude that mitochondrial events were synchronized along with the other components in the remainder of the cell. In this connection, if we accept the identity of the minor-DNA component as mitochondrial DNA, it is interesting to note that the abrupt increase in cytochrome oxidase activity occurred at about the time of mitDNA replication. On the other hand, there were substantial time lags between nuclear DNA replication and the onset of stepwise doublings in malate dehydrogenase and alkaline phos-

phatase activities. Although we probably are observing synthesis of new enzyme proteins, we are measuring only activity and thus cannot be certain that a lag is due to a transcriptional or translational control mechanism (22), or perhaps to some other form of regulation.

Our failure to detect stepwise increases in rates of oxygen uptake is in accord with some observations (23), but contradicts others (11,24). It seems unlikely that the various metabolic processes which contribute to oxygen consumption in yeast all would occur sufficiently close together in time to yield a stepwise rate increase. Since we have evidence that mitochondrial synchrony occurred in our cultures, we are inclined to doubt the reality of stepwise increases in oxygen uptake which have been reported by others, unless these are due to species or strain differences.

The present data are sufficiently indicative of mitochondrial synchrony to permit us to explore further questions concerning mitochondrial development and regulation. Such studies now are in progress in this laboratory.

ACKNOWLEDGMENTS

This study was supported in part by U.S. Public Health Service grant AI-07262 and by Contract No. AT(30-1)-3997 from the U.S. Atomic Energy Commission. One of us (S.F.C.) was the recipient of a predoctoral fellowship from the Johnson & Johnson Co., New Brunswick, New Jersey.

REFERENCES

1. Padilla, G.M., Whitson, G.L., and Cameron, I.L. (Eds.), The Cell Cycle, Academic Press, New York (1969).
2. Cameron, I.L., and Padilla, G.M. (Eds.), Cell Synchrony, Academic Press, New York (1966).
3. Mitchison, J.M., Science, 165, 657 (1969).
4. Chiang, K.-S., and Sueoka, N., Proc. Natl. Acad. Sci. U.S., 57, 1506 (1967).
5. Smith, D., Tauro, P., Schweizer, E., and Halvorson, H.O., Proc. Natl. Acad. Sci. U.S., 60, 936 (1968).
6. Avers, C.J., Rancourt, M.W., and Lin, F.H., Proc. Natl. Acad. Sci. U.S., 54, 527 (1965).
7. Williamson, D.H., and Scopes, A.W., Nature, 193, 256 (1962).
8. Williamson, D.H., and Scopes, A.W., Exp. Cell Res., 20, 338 (1960).
9. Burton, K., Biochem. J., 62, 315 (1956).
10. Tustanoff, E.R., and Bartley, W., Canad. J. Biochem., 42, 651 (1964).
11. Scopes, A.W., and Williamson, D.H., Exp. Cell Res., 35, 361 (1964).
12. Ferguson, J.J., Boll, M., and Holzer, H., Europ. J. Biochem., 1, 21 (1967).
13. Torriani, A., Biochim. Biophys. Acta, 38, 460 (1960).
14. Smith, J.D., Ph.D. Thesis, Univ. Wisconsin (1967).
15. Moustacchi, E., and Williamson, D.H., Biochem. Biophys. Res. Commun., 23, 56 (1966).
16. Billheimer, F.E., and Avers, C.J., Proc. Natl. Acad. Sci. U.S., 64, 739 (1969).

17. Tauro, P., Schweizer, E., Epstein, R., and Halvorson, H.O., in The Cell Cycle, Padilla, G.M., Whitson, G.L., and Cameron, I.L. (Eds.), Academic Press, New York, p. 101 (1969).
18. Gorman, J., Tauro, P., LaBerge, M., and Halvorson, H.O., Biochem. Biophys. Res. Commun., 15, 43 (1964).
19. Longo, G.P., and Scandalios, J.G., Proc. Natl. Acad. Sci. U.S., 62, 104 (1969).
20. Sherman, F., Genetics, 48, 375 (1963).
21. Clark-Walker, G.D., and Linnane, A.W., J. Cell Biol., 34, 1 (1967).
22. Halvorson, H.O., Bock, R.M., Tauro, P., Epstein, R., and LaBerge, M., in Cell Synchrony, Cameron, I.L., and Padilla, G.M. (Eds.), Academic Press, New York, p. 102 (1966).
23. Müller, J., and Dawson, P.S.S., Canad. J. Microbiol., 14, 1127 (1968).
24. Osumi, M., and Sando, N., J. Electron Micr., 18, 47 (1969).