A New Method for Inducing Synchronous Growth of *Dictyostelium discoideum* Cells Using Temperature Shifts

By YASUO MAEDA

Biological Institute, Faculty of Science, Tohoku University, Aoba, Sendai 980, Japan

(Received 24 July 1985; revised 12 December 1985)

A method employing temperature shifts was used to obtain extremely synchronous growth of amoebae of *Dictyostelium discoideum* Ax-2 in axenic medium. When exponentially growing cells at 22.0 °C were shifted to 11.5 °C and shaken for 20 h, they exhibited good synchrony upon further culture at 22.0 °C. A relatively narrow range of low temperature (about 11.5 °C) and of treatment time (20 ± 0.5 h) was necessary for inducing the best synchrony. This temperature-shift method is simple and practical and should be useful for detailed cellular and molecular investigations of growth regulation and of the relationship between the cell cycle and the differentiation of developing cells.

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* normally grows as solitary amoebae by feeding on bacteria and multiplies by binary fission. Cells of the mutant strain Ax-2 can be grown easily in the laboratory either axenically (Watts & Ashworth, 1970) or in association with bacteria (Sussman, 1966). The axenically grown cells show a doubling time of about 7.2 h. All analyses of the *Dictyostelium* cell cycle have been done with axenic cells (Katz & Bourignon, 1974; Zada-Hames & Ashworth, 1978*a*, *b*; Weijer *et al.*, 1984*a*). The results of one study indicate that axenically growing Ax-2 cells have a cell cycle with a G₁ phase of 1.5 h, an S phase of 2.5 h and a G₂ phase of 4.0 h (Zada-Hames & Ashworth, 1978*a*). In contrast, however, Weijer *et al.* (1984*a*) found no G₁ period and an S phase lasting 30 min or less, with most of the cell cycle being in the G₂ phase of 6.5 h.

Despite numerous attempts to obtain synchronous culture systems by a variety of means, many workers have failed to obtain reproducible results; standard methods are either ineffective or result in only partial synchrony. It has been claimed that partial synchrony can be obtained by transferring stationary phase Ax-2 cells into fresh growth medium (Zada-Hames & Ashworth, 1978*a*; Weijer *et al.*, 1984*a*), but this method was found to be unsatisfactory in the present study.

To examine the cellular and molecular basis of the cell cycle and its relationship to the developmental fate of cells, methods must be established for producing more complete synchrony. Such a method is described here which relies on temperature shifts during shaking to induce synchronous growth.

METHODS

Organisms and culture. *Dictyostelium discoideum* Ax-2 was used in all the experiments, and was grown axenically in HL-5 medium containing 1.5% glucose according to Watts & Ashworth (1970). At 22 °C, the cells had a doubling time of 7.2 ± 0.3 h in the density range 2×10^5 to 6×10^6 cells ml⁻¹.

Transfer of stationary-phase cells into fresh medium. To re-examine the observations reported by Weijer *et al.* (1984*a*), cells ($1.2\text{--}1.4 \times 10^7$ ml⁻¹) at the early stationary phase were diluted into fresh medium at a density of $0.5\text{--}1.0 \times 10^6$ cells ml⁻¹ and the increase in cell number was followed. There was generally a considerable number of multinucleate (large) cells, which eventually divided to form several mononucleate cells during shaking. Since the ratio of multinucleate cells to mononucleate cells seemed to increase considerably as the relative volume of the cell

Fig. 1. Growth of stationary-phase *D. discoideum* Ax-2 cells after transfer into fresh growth medium. Cells grown at $22.0 \pm 0.2^\circ\text{C}$ to a density of $1.2 \times 10^7 \text{ ml}^{-1}$ were diluted into fresh medium at a density of $6 \times 10^5 \text{ cells ml}^{-1}$ and shaken at $22.0 \pm 0.2^\circ\text{C}$. Cells doubled with two steps of increase in cell number.

suspension in the shaking flask was increased, 10 ml of cell suspension was shaken in a 100 ml Erlenmeyer flask at 150 r.p.m.

Temperature-shift procedures. Exponentially growing cells ($0.5\text{--}1.0 \times 10^6 \text{ ml}^{-1}$) at $22.0 \pm 0.2^\circ\text{C}$ were transferred to a shaker at $10\text{--}12^\circ\text{C}$. Under these conditions, the cells were cooled to the lower temperature within 20 min. After various periods of shaking, the cultures were warmed to 22.0°C within 1 min by immersion in a water-bath at 25°C , followed by shaking at 22.0°C . Cell counts were taken at 1 h intervals using a haemocytometer. To make the counts accurate, the number of cells (at least 500 cells) contained in the whole measure of the haemocytometer was counted for each sample.

DNA-specific staining of cells. Cells were fixed in methanol and air-dried on cleaned coverslips. The coverslips were incubated for 10 min in 200 mM-KCl and then stained for 1 h with the DNA-specific fluorophore DAPI ($0.5 \mu\text{g ml}^{-1}$ in McIlvain's buffer, pH 7.0), as reported by Leeman & Rush (1982). After a brief wash in the same buffer without DAPI, they were mounted in a 20% (v/v) glycerol solution dissolved in McIlvain's buffer. The fluorescence and number of nuclei in cells were monitored under a fluorescence microscope using UV excitation.

RESULTS AND DISCUSSION

Growth kinetics of stationary-phase cells after transfer into fresh growth medium

When Ax-2 cells ($1.2\text{--}1.4 \times 10^7 \text{ ml}^{-1}$) grown to the early stationary phase were diluted in fresh medium at a density of $0.5\text{--}1.0 \times 10^6 \text{ cells ml}^{-1}$, they exhibited a bimodal increase in cell number during their first doubling (Fig. 1). The first increase in cell number occurred in a period of 1 h after a lag phase of about 1 h. After a further 1 h, the cell number increased gradually over a 4 to 5 h period. In all cases (10 experiments), the cell doubling occurred in at least two steps, though the pattern varied somewhat from experiment to experiment. This variability may depend upon the ratio of multinucleate cells at the time of dilution, since the initial increase in cell numbers could be largely attributed to the break-up of multinucleate cells. Consistent with this view, cell populations with a higher ratio of multinucleate cells seemed to show a more rapid increase in number during the first 1 to 2 h period.

These findings indicated that, apart from the problem of multinucleate cells, stationary-phase cells were arrested in a broad distribution of points in the early G_2 phase. This seems to be somewhat different from the previous observation reported by Weijer *et al.* (1984a), who demonstrated that stationary-phase cells are arrested at a relatively critical point of the late G_2 phase and grow unimodally in fresh medium over a 2 to 3 h period after a lag phase of 1–2 h. Although the reason for this difference is unknown, it may at least partly be due to the difference in methods of counting the cell number. Weijer *et al.* (1984a) made cell counts using an electronic particle counter. Ax-2 cells sometimes form cell clumps during shaking, and also a multinucleate cell often divides into several smaller cells without separating into single cells. Smaller cell fragments without nuclei can also form by pinching off from axenically growing

Fig. 2. Growth of Ax-2 cells following various periods of incubation at low temperature (11.5°C). Cells ($3\text{--}5 \times 10^5 \text{ ml}^{-1}$) growing exponentially at $22.0 \pm 0.2^{\circ}\text{C}$ were shifted to $11.5 \pm 0.2^{\circ}\text{C}$ and shaken for 10 h (a), 14 h (b), 20 h (c), 28 h (d), 36 h (e) or 40 h (f). This was followed by shaking at $22.0 \pm 0.2^{\circ}\text{C}$. The best synchrony was obtained after 20 h at the low temperature.

Ax-2 cells. Such a cell clump or a cell fragment will undoubtedly be recorded by the electronic counter as one cell, thus leading to errors in the cell number. Cell counts with a haematocytometer, as done in the present study, should give better results, because even the cell number in clumps can be correctly determined under a phase-contrast microscope.

Temperature-shifts for growth synchronization

Since the above transfer experiments resulted in partial synchrony, other methods were tested in the hope of obtaining more completely synchronous growth of cells. When Ax-2 cells growing exponentially at $22 \pm 0.2^{\circ}\text{C}$ were shifted to $11.5 \pm 0.2^{\circ}\text{C}$, shaken for 20 h, and then shifted to $22.0 \pm 0.2^{\circ}\text{C}$, they generally expressed good synchrony; cell doubling occurred over about a 2 h period after a lag phase of about 1 h (Fig. 2c). With 19 h or 21 h of the low-temperature treatment there was reduced synchrony. Thus, the growth patterns varied depending upon the period of the low-temperature treatment (Fig. 2). Synchrony indices (F) as described by Blumenthal & Zahler (1962) were plotted against the treatment time at 11.5°C (Fig. 3). The best synchrony was obtained after about 20 h of the treatment, with shorter periods resulting in partial synchrony. Longer low-temperature treatment led to various effects; synchrony was lost after 28 h of treatment, but regained after about 36 h of treatment, and lost again after 40 h of treatment. Essentially the same results were obtained by treatment of the cells at $11.0 \pm 0.2^{\circ}\text{C}$, though the extent of cell synchrony seemed to be slightly inferior to that at $11.5 \pm 0.2^{\circ}\text{C}$. The treatment of the cells at lower temperatures, such as $10.0\text{--}10.5^{\circ}\text{C}$, caused considerable deterioration of synchrony (Fig. 4). The cells which had been treated for 20 h at $10.0 \pm 0.2^{\circ}\text{C}$ exhibited a bimodal growth curve somewhat similar to that shown in Fig. 1. A longer period

Fig. 3. Synchrony indices (F) plotted against treatment time at 11.5 °C. Ax-2 cells growing exponentially at 22.0 °C were shifted to 11.5 °C. After the designated periods of shaking, they were shifted to 22.0 °C, and cell counts taken at 1 h intervals. Synchrony indices (F) were determined according to Blumenthal & Zahler (1962), using the formula $F = N/N_0 - 2^{t/g}$, where the number of cells in a culture increases from N_0 to N in an interval t less than one generation time g .

Fig. 4. Bimodal increase in the number of Ax-2 cells after 20 h of treatment at 10.0 °C. Cells ($5 \times 10^5 \text{ ml}^{-1}$) growing at 22.0 ± 0.2 °C were shifted to 10.0 ± 0.2 °C and shaken for 20 h. This was followed by shaking at 22.0 ± 0.2 °C. The cells exhibit two steps in the doubling of their number, with growth kinetics similar to that shown in Fig. 1.

(24 h) at 10.0 °C gave essentially the same growth kinetics. This was also the case for cells treated at 10.5 ± 0.2 °C. On the other hand, cells treated at 12.0 ± 0.2 °C gave a unimodal growth curve, but the synchrony was less than that shown in Fig. 2(c). Thus, a relatively narrow range of both temperature (about 11.5 °C) and treatment time (20 ± 0.5 h) was of extreme importance for inducing subsequent synchronous growth. There may be a critical point in the late G_2 phase which cells have difficulty passing at 11.5 °C and another point in the early G_2 phase which becomes restrictive at around 10.0 °C, and combined these produce the bimodal growth curve seen at the lower temperature.

In order to examine the temporal change of nuclearity in a synchronized cell population, the cells shaken for 20 h at 11.5 °C were shifted to 22.0 °C, and stained with DAPI after various periods of shaking. As shown in Table 1, there was only a slight change in the ratio of mononucleate cells to binucleate cells over the whole course of the experiment, though the ratio increased slightly in the first 3 h of culture during which almost all cells completed division. The ratio then declined rapidly to the starting level during the next 1 h of culture. There was no significant change in the proportion of cells with three or more nuclei during the experiment.

Table 1. *Change in nuclearity of vegetative cells during synchronized cell-cycle phases in D. discoideum Ax-2*

D. discoideum Ax-2 cells growing exponentially at 22.0 °C were shifted to 11.5 °C and shaken for 20 h to synchronize their cell cycles, followed by a shift to 22.0 °C. After the indicated periods, cells were fixed and stained with DAPI, as described in Methods. Cells with one, two or more nuclei were counted separately. A minimum of 1000 cells were counted for each sample. Values are means $\pm$ SD of three determinations.

Time after shift to 22.0 °C (h)	Percentage of cells		
	No. of nuclei per cell:		
	1	2	≥ 3
-20*	62.3 $\pm$ 3.1	34.3 $\pm$ 2.9	3.4 $\pm$ 1.6
0	65.3 $\pm$ 3.4	32.4 $\pm$ 2.8	2.3 $\pm$ 1.4
1	69.5 $\pm$ 3.6	26.6 $\pm$ 2.4	3.9 $\pm$ 1.7
2	70.7 $\pm$ 3.5	24.5 $\pm$ 2.5	4.8 $\pm$ 1.9
3	76.3 $\pm$ 3.6	21.1 $\pm$ 2.3	2.7 $\pm$ 1.1
4	63.7 $\pm$ 3.3	32.4 $\pm$ 2.4	3.9 $\pm$ 1.4
5	64.1 $\pm$ 3.4	32.2 $\pm$ 2.5	3.6 $\pm$ 1.5
6	62.4 $\pm$ 3.2	32.3 $\pm$ 2.3	5.3 $\pm$ 1.8
7	63.3 $\pm$ 3.7	33.0 $\pm$ 2.6	3.8 $\pm$ 1.6
8	60.9 $\pm$ 4.2	33.1 $\pm$ 3.1	5.8 $\pm$ 2.1

* Cells just before shift-down to 11.5 °C were taken as an unsynchronized sample.

Staining with DAPI revealed that almost all the nuclei contained twice the amount of DNA present in daughter nuclei of anaphase mitotic figures. Weijer *et al.* (1984a) reported that Ax-2 cells lack a G₁ phase and have only a short S phase, and consistent with this, cells with half the interphase nuclear DNA content were rare even in actively dividing cell populations.

In an attempt to further improve cell synchrony, post-mitotic cells, synchronized by growth at 11.5 °C for 20 h and 22 °C for 4 h, were re-shifted to 11.5 $\pm$ 0.2 °C. After various periods of shaking at the low temperature, the growth kinetics of the cells at 22.0 $\pm$ 0.2 °C were analysed. Fairly synchronous populations were repeatedly obtained, for example, after 6 or 13 h of the low-temperature treatment, but cell synchrony was almost completely lost after 8–10 h of the treatment (Fig. 5). Cell synchrony could not be improved by a second treatment at the low temperature, which seemed to cause progressive deterioration of the synchronization. A marked difference in growth kinetics was observed between the first and the second treatments at 11.5 $\pm$ 0.2 °C (Fig. 6). Cell multiplication was almost completely inhibited during the first 20 h period at 11.5 °C, but after 4 h at 22 °C to allow a cell doubling, followed by a return to 11.5 °C, the cell number rose at an increasing rate. These findings suggest that cells may slowly adapt to 11.5 °C and eventually resume growth, thus interfering with synchrony. Possibly, the isolation of clones that are especially sensitive to the low temperature and/or the elimination of multinucleate cells may be required to obtain more completely synchronized cell populations.

It is of interest to note that the cells kept at 11.5 °C for various periods of time go through cycles of synchronous and less synchronous division when shifted to 22.0 °C (Figs 2, 3 and 5). Though there is little division when cells are incubated at 11.5 °C (Fig. 6), it is still possible that the nuclear cycle continues under these conditions, and if this occurred it would result in an increased multinuclearity or ploidy. To test this possibility, the cells shaken at 11.5 °C for various periods were stained with DAPI (Table 2). The results showed that there was scarcely any change in the nuclearity during the periods examined, though the ratio of binucleate cells to mononucleate cells seemed to increase slightly during the first 6 h of culture at 11.5 °C. Such an increase could be related to the small amount of cell division observed during further culture (between 8 h and 16 h shown in Fig. 6). From the observations on fluorescence intensity of nuclei, it was found that almost all the cells had one or more interphase nuclei, each of which contained twice the amount of DNA present in daughter nuclei of anaphase mitotic figures, regardless of the treatment time at 11.5 °C. These findings indicate that neither multinuclearity nor ploidy increases during shaking at 11.5 °C. Thus the cell division cycle does not seem to be

Fig. 5. Effects of a second 11.5°C treatment on growth synchrony of Ax-2 cells. Cell suspensions just after mitosis, obtained by growth first at $11.5 \pm 0.2^{\circ}\text{C}$ for 20 h and then at $22.0 \pm 0.2^{\circ}\text{C}$, for 4 h were re-shifted to $11.5 \pm 0.2^{\circ}\text{C}$. After 6 h (a), 8 h (b), 13 h (c) and 20 h (d) of shaking at the low temperature, the cells were restored to 22°C and their growth followed. Relatively good synchrony was attained after 6 h or 13 h of the second treatment at 11.5°C .

Fig. 6. Growth kinetics of Ax-2 cells during the first and second treatments at 11.5°C . Cells growing exponentially at $22.0 \pm 0.2^{\circ}\text{C}$ were shifted to $11.5 \pm 0.2^{\circ}\text{C}$ and shaken for 20 h. They were then shifted to $22.0 \pm 0.2^{\circ}\text{C}$ and shaken for 4 h, and then returned to $11.5 \pm 0.2^{\circ}\text{C}$ and shaken for 24 h. Note the marked difference in growth kinetics between the first and the second cultures at 11.5°C .

Table 2. *Effect of a low-temperature treatment on the nuclearity of vegetative cells of D. discoideum Ax-2*

D. discoideum Ax-2 cells growing exponentially at 22.0 °C were shifted to 11.5 °C and shaken for the indicated periods. The percentage of cells with one, two or more nuclei was determined, as for Table 1. Values are means $\pm$ SD of three determinations.

Time after shift to 11.5 °C (h)	Percentage of cells		
	No. of nuclei per cell:		
	1	2	≥ 3
0	72.4 $\pm$ 3.5	24.5 $\pm$ 2.3	3.1 $\pm$ 1.4
6	61.6 $\pm$ 3.2	32.9 $\pm$ 2.5	5.5 $\pm$ 1.9
12	73.4 $\pm$ 3.7	22.3 $\pm$ 2.6	4.3 $\pm$ 1.7
16	74.4 $\pm$ 3.5	23.5 $\pm$ 2.5	2.1 $\pm$ 1.1
20	71.0 $\pm$ 3.1	25.9 $\pm$ 2.4	3.1 $\pm$ 1.6
24	71.7 $\pm$ 3.3	25.7 $\pm$ 2.4	2.6 $\pm$ 1.3
28	74.6 $\pm$ 3.9	22.0 $\pm$ 2.1	3.4 $\pm$ 1.6
36	71.6 $\pm$ 3.4	23.4 $\pm$ 2.2	5.0 $\pm$ 1.8
40	76.9 $\pm$ 4.1	19.3 $\pm$ 2.8	3.8 $\pm$ 1.5

tightly coupled to the nuclear cycle timer at the low temperature. In this connection, it is possible that the periodic appearance of synchrony may be due to cyclical changes in the machinery required for cytokinesis. The time intervals between the two synchronous phases seem to become shorter depending on the length of culture at 11.5 °C, as shown in Figs 2, 3 and 5. This could be related to a speeding up of the cytokinesis timer, possibly due to its adaptation to the low temperature.

The temperature-shift method described here for inducing synchronous growth of Ax-2 cells is very practical because of its simplicity and convenience. It should be useful for detailed investigations of growth regulation. Recently, it has been claimed that cells starved at the early G₂ phase of the cell cycle sort out strongly to the anterior prestalk region of the migrating slug (Zada-Hames & Ashworth, 1978*b*; McDonald & Durston, 1984; Weijer *et al.*, 1984*b, c*; Durston *et al.*, 1984). It will be interesting to know if this correlation holds true also for cells synchronized by the temperature shift method. Work is also in progress to determine the exact duration of each cell-cycle phase using the temperature-shift method.

I wish to thank Dr N. Haga of Tohoku University for reading and criticising the manuscript. I also gratefully acknowledge Mr T. Ohmori and Mr K. Takahashi for their invaluable help with the cell counts. This work was in part supported by grants-in-aid (no. 60540442) from the Ministry of Education, Science and Culture of Japan and a grant from the Yamada Science Foundation.

REFERENCES

- BLUMENTHAL, L. K. & ZAHLER, S. A. (1962). Index for measurement of synchronization of cell population. *Science* **134**, 724.
- DURSTON, A. J., WEIJER, C. J., JONGKIND, J. F., VERKERK, A., TIMMERMANS, A. & KULVE, W. T. (1984). A flow fluorimetric analysis of the cell cycle during growth and differentiation in *Dictyostelium discoideum*. *Roux's Archives of Developmental Biology* **194**, 18–24.
- KATZ, E. R. & BOURGIGNON, L. Y. W. (1974). The cell cycle and its relation to aggregation in the cellular slime mold, *Dictyostelium discoideum*. *Developmental Biology* **36**, 82–87.
- LEEMAN, U. & RUSH, F. (1982). Cytofluorometric determination of DNA base content in plant nuclei and chromosomes by the fluorochromes DAPI and Chromomycin A3. *Experimental Cell Research* **140**, 275–282.
- MCDONALD, S. A. & DURSTON, A. J. (1984). The cell cycle and sorting behavior in *Dictyostelium discoideum*. *Journal of Cell Science* **66**, 195–204.
- SUSSMAN, M. (1966). Biochemical and genetic methods in the study of cellular slime mold development. In *Methods in Cell Physiology*, vol. 2, pp. 397–410. Edited by D. M. Prescott. New York & London: Academic Press.
- WATTS, D. J. & ASHWORTH, J. M. (1970). Growth of myxamoebae of the cellular slime mould, *Dictyostelium discoideum* in axenic medium. *Biochemical Journal* **119**, 171–174.
- WEIJER, C. J., DUSCHL, G. & DAVID, C. N. (1984*a*). A revision of the *Dictyostelium discoideum* cell cycle. *Journal of Cell Science* **70**, 111–131.
- WEIJER, C. J., DUSCHL, G. & DAVID, C. N. (1984*b*). Dependence of cell type proportioning and sorting on cell cycle phase in *Dictyostelium discoideum*. *Journal of Cell Science* **70**, 133–145.

- WEIJER, C. J., McDONALD, S. A. & DURSTON, A. J. (1984*c*). A frequency difference in optical-density oscillations of early *Dictyostelium discoideum* density classes and its implications for development. *Differentiation* **28**, 9–12.
- ZADA-HAMES, I. & ASHWORTH, J. M. (1978*a*). The cell cycle during the vegetative stage of *Dictyostelium discoideum* and its response to temperature change. *Journal of Cell Science* **32**, 1–20.
- ZADA-HAMES, I. & ASHWORTH, J. M. (1978*b*). The cell cycle and its relation to development in *Dictyostelium discoideum*. *Developmental Biology* **63**, 307–320.