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Changes in the DNA content of amoebae of *Dictyostelium discoideum* during growth and development

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A fluorimetric assay has been used to determine the DNA content of amoebae of *Dictyostelium discoideum* during growth and development. Amoebae grown in axenic culture tended to be multinucleate and had a greater DNA content than amoebae grown with a bacterial substrate, which were mononucleate. During the first 10h of development there was little change in the DNA content of amoebae grown with a bacterial substrate, but the average DNA content per cell in amoebae grown axenically decreased as the amoebae became virtually mononucleate. Amoebae at 10h development that had been harvested during exponential axenic growth were divided into two populations by countercurrent distribution in a polymer two-phase system. DNA content indicated that one population was largely in the G_2-phase of the cell cycle, whereas the other population was largely in the G_1-phase. Similar results were obtained at 10h development with amoebae harvested during the stationary phase of axenic growth, although these amoebae start development all in the G_2-phase of the cell cycle. Spores had a low DNA content, indicating that they were in G_1-phase. It is proposed that all amoebae in G_2-phase after early development differentiate, after mitosis, into spores and that stalk cells are formed from amoebae that remain in G_1-phase after 10h development.

Wild-type strains of *Dictyostelium discoideum* have to be grown with a bacterial substrate, but mutant strains are also available which may be grown either with a bacterial substrate or axenically in a simple, semi-defined medium (Watts & Ashworth, 1970). First attempts to determine the effects of culture conditions on amoebal DNA content indicated that amoebae grown with a bacterial substrate had approximately twice the DNA content of the same number of amoebae grown axenically (Ashworth & Watts, 1970). It seemed probable that the amoebae grown with a bacterial substrate were heavily contaminated with bacterial DNA and therefore had a high DNA content, but later investigations (Leach & Ashworth, 1972; Sussman & Rayner, 1971) showed that such contamination was slight. Leach & Ashworth (1972) then suggested that, if amoebae grown axenically were largely in the G_1-phase of the cell cycle and amoebae grown with a bacterial substrate were mainly in G_2-phase, it would be possible to account for the difference in DNA content between the amoebae in the two culture conditions; however, this explanation also proved to be incorrect (Zada-Hames & Ashworth, 1978*a*). The high DNA content of amoebae grown with a bacterial substrate has remained unexplained and has become even more puzzling with the discovery that these amoebae are mononucleate, whereas amoebae grown axenically, and having a lower DNA content, tend to be multinucleate (Brody & Williams, 1974; Zada-Hames & Ashworth, 1978*a*).

There have been several indications that development and differentiation of *D. discoideum* are dependent on the cell cycle (Katz & Bourguignon, 1974; Zada-Hames & Ashworth, 1978*b*; Durston & Vork, 1978; Woffendin & Griffiths, 1982), and this could be further investigated if it were possible to decide whether amoebae were in G_1-, S- or G_2-phase of the cell cycle from measurements of their DNA content. Amoebal DNA content has therefore been determined by using a fluorimetric assay (Labarca & Paigen, 1980) that is both more specific and more sensitive than the colorimetric assays used previously, and it would seem that this assay gives more reliable estimates of amoebal DNA content. It has also been possible to separate and identify amoebae in different phases of the cell cycle, and it would appear that amoebae in G_2-phase at 10h development differ-

entiate into spores, whereas stalk cells are formed from amoebae in G_1-phase.

Experimental

Materials

'Ultra pure' DNA from *Clostridium perfringens*, 4',6-diamidino-2-phenylindole and glass beads (250–300 μm diameter) were obtained from Sigma. Hoechst 33258 (bisbenzimide) was from Hoechst Pharmaceuticals, Hounslow, Middx., U.K.

The glass beads were washed with 2M-HCl and then distilled water before use.

Dictyostelium discoideum

Amoebae of strain Ax-2 were grown at 22°C with shaking in HL5 medium containing 86mM-glucose (Watts & Ashworth, 1970). Amoebae were harvested during exponential growth (at approx. 2×10^6 amoebae·ml^{-1}) or during the stationary phase of growth (between 1×10^7 and 2×10^7 amoebae·ml^{-1}) when there had been no increase in cell density for 24h. Amoebae of strains NC-4 and Ax-2 were also grown in dual culture with *Aerobacter aerogenes* N.C.T.C. 418 on SM agar plates (Sussman, 1966) and were harvested well before all the bacterial substrate had been consumed. After growth, amoebae were washed in distilled water at 0°C and then in 50mM-NaH_2PO_4/K_2HPO_4 buffer, pH7.4.

Development was on Millipore filters (47mm diameter) at 22°C (Sussman, 1966). Amoebae, 5×10^7 grown axenically or 1×10^8 grown with *A. aerogenes*, were deposited on each filter. Amoebae were washed off filters in 50mM-NaH_2PO_4/K_2HPO_4, pH7.4. Cells were counted in a haemocytometer as soon as possible, and no cell lysis, even of stationary-phase cells, was then detected.

Countercurrent distribution

This was in the dextran/poly(ethylene glycol) two-phase system described by Sharpe *et al.* (1982).

DNA assay

Amoebae were suspended at known density (approx. 10^7 amoebae·ml^{-1}) in buffer A (50mM-NaH_2PO_4 / K_2HPO_4 / 2M - NaCl / 2mM - EDTA, pH7.4). The suspensions were cooled to 0°C and sonicated in a MSE Soniprep 150 instrument at maximum amplitude for 15s. Samples (10 μl) were assayed for DNA by measurement of the increase in fluorescence at 458nm on addition to buffer A containing Hoechst 33258 (0.5 μg·ml^{-1}) at 30°C (Labarca & Paigen, 1980). Fluorescence was detected in a Perkin–Elmer 3000 spectrometer. Excitation was at 356nm.

The increase in fluorescence of Hoechst 33258 on addition of DNA is largely dependent on the AT content of the DNA (Weisblum & Haenssler, 1974), and each assay was calibrated by addition of 10 μl of *Clostridium perfringens* DNA (4.5 μg·ml^{-1}), which has an AT content (73.5%) similar to that of *D. discoideum* DNA (77%). Nevertheless, the assay would still be expected to overestimate slightly the concentration of *D. discoideum* DNA. The concentration of the *Cl. perfringens* DNA was determined on the basis that a solution at 1 mg·ml^{-1} would have an absorbance of 20 at 260nm in a 1cm-light-path cuvette.

Spore breakage

Fruiting bodies at 28–29h development were washed off Millipore filters in distilled water at 0°C and the suspensions were filtered through two layers of nylon mesh (20 μm pore size) to give spore suspensions free of stalks. The spore suspensions were then centrifuged at maximum speed in a MSE Minor centrifuge for 10min and the spores were resuspended at known density (approx. 2×10^8 spores·ml^{-1}) in buffer A at 0°C. The method for breaking the spores was suggested by Dr. D. A. Cotter, University of Windsor, Windsor, Ont., Canada. A 1.0ml portion of spore suspension was added to 2ml of glass beads (250–300 μm diameter) in a 15ml glass centrifuge tube at 0°C. The tube was stoppered and the contents mixed vigorously on a vortex mixer for 6s periods for a total of 2min. In between the 6s periods the tube was cooled in ice for 3s. The contents of the tube were diluted with 4.0ml of buffer A at 0°C and unbroken spores were counted. The number of spores broken could then be calculated. About 70–80% of the spores were broken.

Nuclei

Amoebae were left for 30min in 70% (v/v) ethanol and were then resuspended in 4',6-diamidino-2-phenylindole (0.5 μg·ml^{-1}) (Williamson & Fennell, 1976). Amoebae were viewed, with magnification × 500, under a fluorescence microscope at a wavelength of 300–400nm. The nuclei appeared bright blue. Nuclei were counted in the first 100 amoebae seen in a random field.

Results

Nuclei

Amoebae harvested during exponential growth in axenic culture tended to be multinucleate, so that, on average, each amoeba contained more than one nucleus. Since the proportion of multinucleate cells changed when different batches of yeast extract or peptone were used to prepare the growth medium, as had been found previously by Zada-Hames & Ashworth (1978*a*), amoebae were

Table 1. *DNA content and nuclei in cells of D. discoideum during growth and development*
Amoebae were grown either with a bacterial substrate (*A. aerogenes*) or in axenic culture. Axenic cultures were harvested during exponential growth (at 2×10^6 amoebae·ml^{-1}) or in stationary phase (at 1×10^7–2×10^7 amoebae·ml^{-1}). A fluorimetric assay was used to determine the DNA content of cells. A portion of each sample of amoebae used for the DNA assays was reserved for counting nuclei as described in the Experimental section. Results are given as means ± S.D., with the numbers of observations in parentheses.

Time of development (h)...		0		10		28–29 (spores)
Strain	Growth conditions	Nuclei/cell	DNA (pg/cell)	Nuclei/cell	DNA (pg/cell)	DNA (pg/spore)
Ax-2	Exponential, axenic	1.56±0.081	0.45±0.021 (3)	1.05±0.027	0.23±0.026 (3)	0.16±0.006 (3)
	Stationary-phase, axenic	1.41±0.063	0.59±0.032 (3)	1.06±0.006	0.26±0.031 (3)	
	A. aerogenes	1.00	0.27±0.021 (2)			
NC-4	*A. aerogenes*	1.01	0.25±0.022 (4)	1.01	0.24±0.015 (4)	0.18±0.008 (3)

grown in culture medium made from the same yeast extract and peptone to obtain the results given in Table 1.

It was also found that the average number of nuclei per cell varied slightly during exponential growth, and cultures for DNA assays were harvested when the cell density was 2×10^6 amoebae·ml^{-1} and 40% of the amoebae were multinucleate. When cultures reached the stationary phase of growth, an increasing proportion of the amoebae became mononucleate, but this occurred more rapidly in some cultures than in others. The three cultures examined to obtain the results in Table 1 seemed slow in becoming mononucleate, and 33% of the amoebae were multinucleate. Amoebae grown in axenic culture also tended to become mononucleate during early development and, by 10h development, few multinucleate amoebae (4.3% for exponential-phase cultures, 7.7% for staionary-phase cultures) remained.

Amoebae grown with a bacterial substrate were virtually all mononucleate during growth and development.

Cell DNA content

The DNA content of amoebae is given in Table 1. Amoebae harvested during the stationary phase of axenic growth had a higher DNA content, but contained on average fewer nuclei, than amoebae harvested during exponential growth in axenic culture. This was consistent with previous reports (Soll *et al.*, 1976; Zada-Hames & Ashworth, 1978*a*) that, when amoebae stop growing in axenic cultures, they accumulate in the G_2-phase of the cell cycle. The DNA content of amoebae grown with a bacterial substrate was lower than that of amoebae harvested during exponential axenic growth, and this could be related to the difference between the cultures in the average number of nuclei per cell.

After 10h development, amoebae that had been grown with a bacterial substrate were still essentially mononucleate and their DNA content was little changed. Over the same period of development, the proportion of mononucleate amoebae increased in populations of amoebae that had been grown axenically and harvested either in the stationary phase of growth or during exponential growth, and there was a decrease in amoebal DNA content. Thus, at 10h development, amoebae harvested in the three growth conditions were virtually all mononucleate and had a similar DNA content.

Amoebae harvested during exponential growth in axenic culture can be separated at 10h development into two populations (designated peak I and peak II) by countercurrent distribution in a polymer two-phase system (Sharpe *et al.*, 1982) which separates cells having different surface properties. Amoebae in peak I were found to contain 0.40±0.005 (3)pg of DNA/cell, whereas amoebae in peak II contained only 0.21±0.006 (3)pg of DNA/cell. Similarly, amoebae harvested in the stationary phase of axenic growth were separated into two populations containing 0.39±0 (2)pg of DNA/cell (peak I) or 0.26±0.009 (2)pg of DNA/cell (peak II). It was not possible to count nuclei in the cell samples used for DNA determinations since too few cells could be separated by countercurrent distribution. However, in separate control experiments, it was found that multinucleate cells were evenly distributed between peaks I and II.

Spores derived from amoebae grown axenically and harvested during exponential growth had a low DNA content, similar to that of spores formed from amoebae grown with a bacterial substrate (Table 1).

Discussion

DNA assay procedures

It is difficult to compare results given in Table 1 for the DNA content of amoebae grown axenically with those published previously (Ashworth & Watts, 1970; Leach & Ashworth, 1972; Soll *et al.*, 1976), since cell nuclei were not counted in the previous studies and it is probable that the various

results refer to cultures having different proportions of multinucleate amoebae. Furthermore, the colorimetric assays used in previous studies were calibrated with deoxyribose or calf thymus DNA, whereas the fluorimetric assays were calibrated with DNA from *Cl. perfringens*. Nevertheless, the colorimetric and fluorimetric assays have given qualitatively similar results and have shown that amoebal DNA content increases when axenic cultures pass into the stationary phase of growth, but decreases during development.

Colorimetric DNA assays have suggested that amoebae grown with a bacterial substrate have approximately twice the DNA content of amoebae harvested during exponential growth in axenic culture (Ashworth & Watts, 1970; Leach & Ashworth, 1972), but this has not been confirmed by the fluorimetric assays, which have indicated that it is the amoebae grown axenically that have the greater DNA content. The results obtained with the fluorimetric assay are those that would be expected. In both culture conditions amoebae are largely haploid and grow asynchronously (Sussman & Sussman, 1962; Zada-Hames, 1977; Zada-Hames & Ashworth, 1978*b*), and, since nuclear DNA accounts for at least 70% of the total DNA content of *D. discoideum* amoebae (Leach & Ashworth, 1972; Firtel & Bonner, 1972), multinucleate amoebae from axenic cultures should contain more DNA than the mononucleate amoebae grown with a bacterial substrate. Furthermore, the fluorimetric assay is highly specific for DNA (Labarca & Paigen, 1980), whereas the colorimetric assays are much less specific (Burton, 1956; Ashwell, 1957). It therefore seems reasonable to conclude that the fluorimetric assays have given valid estimates of the DNA content of amoebae grown with a bacterial substrate, whereas the colorimetric assays have overestimated the DNA content of these amoebae.

The cell cycle during D. discoideum development

When amoebae that had been harvested during exponential axenic growth were divided into two populations at 10h development by countercurrent distribution in a polymer two-phase system, the amoebae in peak I had approximately twice the DNA content of amoebae in peak II. It has also been shown that these amoebae are distributed between the G_1- and G_2-phases of the cell cycle and that none are in S-phase or mitosis (Zada-Hames & Ashworth, 1978*b*). It may therefore be concluded that the amoebae in peak I of the countercurrent distribution were largely in the G_2-phase of the cell cycle, whereas the amoebae in peak II were largely in G_1-phase.

Amoebae harvested during the stationary phase of growth in axenic culture were in the G_2-phase of the cell cycle. By 10h development, these amoebae had a DNA content similar to that of amoebae at 10h development that had been harvested during exponential, axenic growth. This would suggest that, during early development, a significant proportion of the stationary-phase amoebae had passed into the G_1-phase of the cell cycle. This was confirmed by the countercurrent distribution, which divided the amoebae into two populations (Sharpe *et al.*, 1982) with markedly different DNA contents. The amoebae in peak I seemed still to be largely in G_2-phase. The amoebae in peak II were largely in G_1-phase, but the peak may also have been contaminated with some amoebae in G_2- and S-phase.

By contrast, Zada-Hames & Ashworth (1978*b*) concluded that stationary-phase amoebae remain in the G_2-phase of the cell cycle during the first 16h of development since, during that time, little increase in cell number could be detected in populations of these amoebae. This could indicate that there is some variability in the behaviour of stationary-phase amoebae during development, but it is also possible that Zada-Hames & Ashworth (1978*b*) underestimated increases in the size of the populations of stationary-phase amoebae during development, and thus the extent to which the amoebae passed through mitosis and into G_1-phase, since stationary-phase amoebae are easily lysed.

Cell fate during development

It has previously been shown (Sharpe *et al.*, 1982) that the two populations of amoebae, obtained by countercurrent distribution at 10h development from amoebae harvested during exponential axenic growth, differ in behaviour during subsequent development and in cohesiveness. Similar differences have been detected between the pre-spore and pre-stalk cells that can be isolated from aggregates at 15–18h development (Raper, 1940; Sampson, 1976; Lam *et al.*, 1981; Ratner & Borth, 1983) and amoebae in peak I of the countercurrent distribution would seem to resemble pre-spore cells, whereas amoebae in peak II seem similar to pre-stalk cells. Furthermore, when genetically marked amoebae from peaks I and II were recombined and allowed to form fruiting bodies, amoebae from peak I all formed spores, whereas stalk cells were formed from amoebae in peak II. Similar results have since been obtained with genetically identical Ax-2 amoebae when it was possible to identify amoebae from peak I and peak II because they had been dyed with different vital stains (P. T. Sharpe & D. J. Watts, unpublished work). Thus it would seem that countercurrent distribution separated amoebae into presumptive spore (peak I) and presumptive stalk (peak II) cells.

Since the amoebae in peak I are also in the G_2-

phase of the cell cycle, it would seem that spores are eventually formed from the amoebae that are in G_2-phase at 10h development. After 12h development, some of the amoebae halted in G_1-phase since 6h development begin to pass through S-phase and into G_2-phase (Zada-Hames & Ashworth, 1978*b*) and all these amoebae also differentiate into spores (Durston & Vork, 1978). Thus it would seem that spores are formed from all amoebae passing into the G_2-phase of the cell cycle during development. However, the low DNA content of spores (Table 1) indicates that these cells are in the G_1-phase of the cell cycle. Pre-spore cells in G_2-phase must therefore pass through mitosis during differentiation into spores, and this would account for the period of mitosis during late development that has been described by Zada-Hames & Ashworth (1978*b*).

Not all the amoebae in G_1-phase at 10h development pass into G_2-phase subsequently (Zada-Hames & Ashworth, 1978*b*), and it must be from among these amoebae remaining in G_1-phase that stalk cells are formed. There is, however, no evidence that all the amoebae staying in G_1-phase after 10h development differentiate into stalk cells, and it is possible that some of these amoebae also differentiate into spores.

Since amoebae in the G_2-phase of the cell cycle during late development differentiate into spores, and stalk cells are formed from amoebae remaining in G_1-phase, there appears to be a relationship between the cell cycle and cell fate during development. It has yet to be shown whether this is a causal relationship, but, nevertheless, there is the possibility that cell fate may be based on the heterogeneity introduced by the cell cycle into a population of otherwise similar amoebae.

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