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DEPENDENCE OF CELL-TYPE PROPORTIONING AND SORTING ON CELL CYCLE PHASE IN *DICTYOSTELIUM DISCOIDEUM*

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SUMMARY

The relationship between the cell cycle phase of vegetative amoebae and prestalk and prespore differentiation in the slug stage were investigated in the slime mould *Dictyostelium discoideum*. Cells were synchronized by release from the stationary phase. Samples were taken at various times during the course of a synchronous cell doubling, fluorescently labelled and mixed with cells of random cell cycle phase from exponentially growing cultures. The fate of the fluorescently labelled cells was recorded at the slug stage. Cells early in the cycle exhibit strong prestalk sorting; cells taken later in the cycle exhibit strong prespore sorting. The period of prestalk sorting occurs immediately following mitosis and lasts about 1 h in a cell cycle of about 7 h duration. Accompanying the altered sorting behaviour is a marked change in the prestalk–prespore proportions in slugs formed from synchronized populations of cells. Cells synchronized early in the cycle form slugs with 55 % prespore cells; cells synchronized late in the cycle form slugs with 90 % prespore. The results are discussed in terms of models for the formation of the prestalk–prespore pattern in slugs.

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* grows as free-living amoebae that multiply by binary fission. Upon starvation the cells collect in multicellular aggregates, which then form slugs and differentiate finally into spores and stalk cells of a fruiting body (Bonner, 1969; Loomis, 1975). Precursors (prespore and prestalk cells) of the final cell types are clearly recognizable at the slug stage. The proportions of prespore and prestalk cells, and spore and stalk cells, are relatively constant over a large range of slug and fruiting-body sizes (Bonner & Slifkin, 1949; Stenhouse & Williams, 1977). Isolated prespore and prestalk pieces can regulate, i.e. regenerate, the missing cell types to restore the normal proportions (Raper, 1940; Sakai, 1973).

Cells grown under different conditions (Leach, Ashworth & Garrod, 1973; Tasaka & Takeuchi, 1981; Forman & Garrod, 1977*a*) and certain mutants (MacWilliams, 1982) have been shown to sort out from each other when mixed at the moment of starvation. It has also been shown that certain density gradient fractions from populations of vegetative stage cells will sort out from each other (Takeuchi, 1969; Weijer, MacDonald & Durston, 1984*b*). This suggests the possibility that cells are already different in respect of certain parameters at the vegetative stage and that during development cells sort out according to this variation.

We had some indication that density gradients separate cells according to cell cycle

phase (Weijer *et al.* 1984*b*) and therefore it seemed likely that the variable property of vegetative cells, according to which the cells sort out during development, would be a cell-cycle-linked property.

In order to test this hypothesis we have mixed fluorescent-labelled, synchronized cells of varying cell cycle phase with exponentially growing cells and determined their location in the slugs that formed. Cells were synchronized by release from stationary phase, a procedure that we have used previously in the characterization of the *D. discoideum* cell cycle (Weijer, Duschl & David, 1984*a*).

Our results indicate that cells that are in *S* phase at the time of starvation show strong prestalk sorting, while late G_2 cells are prespore sorters. Coincident with the change in sorting properties during the cell cycle is a change in the proportions of prespore and prestalk cells in slugs formed solely from cells of a particular cell cycle stage. *S* phase cells form slugs with about 50 % prespore cells while late G_2 phase cells form slugs with about 90 % prespore cells; under these conditions exponentially growing cells formed slugs with about 80 % prespore cells. The existence of subclasses of cells that form slugs with different percentages of prespore cells and show altered sorting behaviour is discussed in the context of altered sensitivity to proportioning signals.

MATERIALS AND METHODS

Strains and growth conditions

All experiments were performed with *Dictyostelium discoideum* strain Ax2, grown axenically according to standard procedures (Watts & Ashworth, 1970) at 23 °C. Under these conditions the cells had a doubling time of $7{\cdot}0 \pm 0{\cdot}5$ h in the concentration range of 2×10^6 to 6×10^6/ml.

Synchronization of cells

To induce synchrony cells were grown to stationary phase and then diluted out into fresh medium. After a lag-phase such cells exhibit a relatively synchronous cell doubling (Yarger, Stults & Soll, 1974; Zada-Hames & Ashworth, 1978*a*). To obtain stationary-phase cells, we diluted exponentially growing cells to 10^6/ml in fresh medium and allowed them to grow for 40 h at 22 °C, at which point the cells had reached a density of $1{\cdot}2\times10^7$/ml. These cells were then diluted into fresh medium at a density of 10^6/ml and the increase in cell number over time was followed. Under these conditions the cells generally showed a cell doubling over a 2- to 3-h period after a lag-phase of 1–2 h.

Cell counting

Cell counts were made with an electronic cell counter (Phywe), using a counting chamber with a 100 μm orifice. Cells were counted in a 1/250 dilution in 0·9 % NaCl. Duplicate cell number determinations never showed more than 2 % variation.

Bulk DNA determinations

Bulk DNA was determined fluorometrically with Hoechst 33258 as the fluorophore (Labarca & Paigen, 1980) and calf thymus DNA as the standard. Frozen pellets of cells or nuclei were resuspended in a solution containing 2 M-NaCl and 2 mM-EDTA in 50 mM-phosphate buffer (pH 7·4); 50 μl of this suspension was then added to 3 ml of the same salt/EDTA solution containing 100 ng/ml Hoechst dye. The fluorescence emission of the sample was measured at 450 nm in a Kontron SFM 19 fluorometer using an excitation wavelength of 350 nm (10 nm slith width). The DNA standard was prepared from a stock solution of calf thymus DNA (Serva no. 18560) dissolved in 5 mM-NaOH

at 1 mg/ml, which was kept refrigerated. A calibration curve was constructed in the range of 0·1 to 3 μg DNA/sample.

Rhodamine staining of cells

Cells were vitally stained with the rhodamine derivative XRITC (Serva no. 34282) essentially according to a method described by Springer & Barondes (1978). Cells were washed three times in 20 mM-potassium phosphate buffer (pH 7·0) and finally pelleted. The pellet containing about 10^7 cells was resuspended in 0·5 ml staining solution (a 1 : 100 dilution of a rhodamine stock solution in 1 ml phosphate buffer).

The rhodamine stock solution contains 50 mg/ml XRITC dissolved in dimethylsulphoxide (DMSO), which was kept frozen in 20-μl portions at −80 °C. To prepare a staining solution one portion of stock solution was dissolved in 2 ml phosphate buffer and sonicated for 2 min at 100 W in a Labsonic 1510 sonicator, and the solution was then centrifuged for 5 min at 1000 ***g*** to precipitate any undissolved stain particles.

Cells were stained for 30 min in this solution while continuously shaken, then washed three times in phosphate buffer and left shaking for another 30 min so that the cells could exocytose any ingested label. The cells were then washed twice with buffer and mixed with unstained cells that had been 'sham-stained' with 1 % DMSO.

Sorting experiments

Cells taken from synchronous cultures at various times were rhodamine-stained and mixed in a 1 : 10 ratio with unstained cells from an exponentially growing population. The cells were pelleted and resuspended in one pellet volume of 20 mM-phosphate buffer (pH 7·0). Drops of this concentrated cell suspension were placed on 1 % water agar plates and incubated in the dark for 24 h at 19 °C after which migrating slugs had formed.

In order to quantitate the sorting of the rhodamine-labelled cells, slugs were divided into three parts, dissociated into single cells, and the percentage of rhodamine-labelled cells in each fraction was counted. Ten to fifteen migrating slugs were collected and dissected into three parts with the aid of a capillary mouth pipette. The slugs were dissected in the following way; tips were collected (~10 % of total cell mass) while the remaining part was divided in half as judged by length. The cells were dissociated by tituration with a micropipette in 40 μl of a Cellulase/salt solution (10 mg/ml Cellulase (Onozuka) in a 20 mM-phosphate buffer containing 0·4 % NaCl and 2 mM-EDTA). The dissociated cells were fixed by addition of 40 μl of 8 % neutralized formaldehyde.

Staining of prespore cells

Whole migrating slugs were collected with a needle and dissociated into single cells as described above. The cells were pelleted, resuspended in 60 % (v/v) methanol, and 5 μl of the suspension was air dried on a glass slide. The cells were post-fixed with 25 μl of 100 % methanol, stained with a rabbit anti-spore serum absorbed with early aggregation-stage cells as described by Takeuchi (1963), washed in KK2 buffer and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit serum. After staining the slides were washed and mounted in phosphate-buffered glycerin (50 %, v/v) (pH 8·0) containing 0·02 % azide. Slides were examined under a Leitz standard microscope fitted with an epifluorescence attachment and scored for prespore cells (cells containing more than two brightly staining vacuoles were scored as positive). For each preparation at least 500 cells were counted.

RESULTS

Cell-cycle-linked sorting of synchronized cells

Stationary-phase axenic cells of appropriate age go through relatively synchronous cell division cycles when diluted out in fresh axenic medium (Yarger *et al.* 1974). Under these conditions the cell cycle consists of a 0·5 h S phase followed by a 6- to 7-h G_2 phase; there is no G_1 phase so that cells enter S phase immediately after mitosis

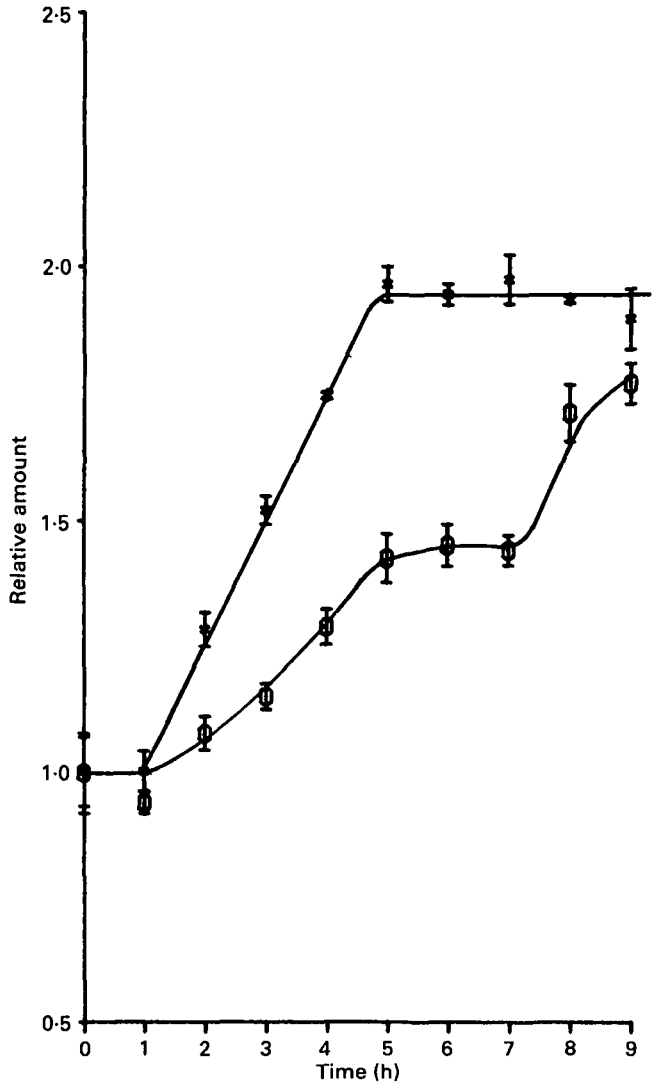

Fig. 1. The relative change in cell number (*) and total DNA content (O) in a synchronized cell culture. The error bars represent the standard deviation of the mean of three determinations.

(Weijer *et al.* 1984*a*). Fig. 1 shows the increase in cell number and in total DNA in a typical population of synchronized cells: cell number doubles between 2 h and 5 h while the total DNA increases about 1·5-fold due to nuclear replication.

To follow the sorting behaviour of cells during the cell cycle, synchronized cells in various cell cycle phases were rhodamine-labelled, mixed with unlabelled exponentially growing cells and allowed to develop into migrating slugs. Fig. 2 shows the distribution of the rhodamine-labelled cells in such slugs. Stationary-phase cells show a strong tendency to sort to the prespore zone of a slug. However, as soon as the cells start to divide, and their nuclear DNA starts to replicate, there is a radical change in

their sorting behaviour from prespore to tip. Later in the cell cycle the sorting tendency changes again to prespore sorting and as soon as the cells enter the second round of division (8 h) the sorting properties of the cells change again to prestalk sorting.

Assuming that the tip (region 1 in Fig. 2) contains 10 % of the cells in the slug, it is possible to calculate the percentage of labelled cells that sort to the tip. The results of two independent experiments are shown in Fig. 3. They indicate that at 2 and 3 h about 50 % of the labelled cells are tip sorters. A second peak of tip sorting occurs at 8 and 9 h in association with the second round of DNA synthesis.

A cell-cycle-correlated shift in prespore/prestalk proportions

To investigate the prestalk–prespore proportions in slugs prepared from synchronized cell populations, we took cells at hourly intervals from a synchronized population and let them develop to the migrating slug stage. Slugs were collected and the proportions of prespore cells determined using prespore antibody. Fig. 4A shows

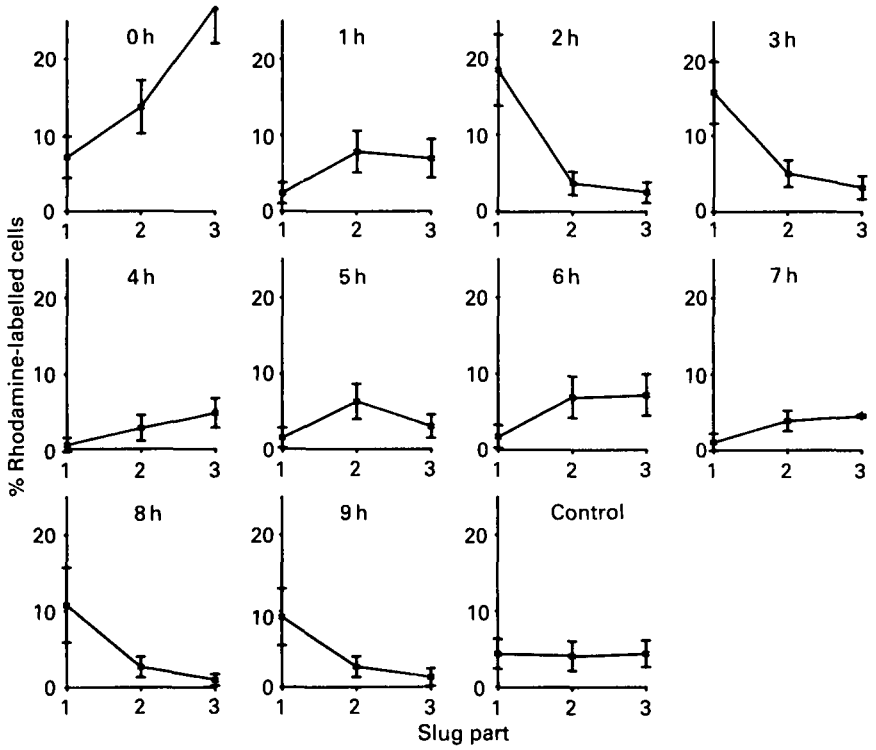

Fig. 2. The distribution of small numbers of synchronized rhodamine-labelled cells in slugs formed from exponentially growing cells. Cells taken at hourly intervals from the synchronized population shown in Fig. 1 were labelled with XRITC, mixed with exponentially growing cells and induced to form slugs. The slugs were dissected into three parts: tip (1), middle (2) and back (3). The percentage of rhodamine-labelled cells in each part was determined. The error bars indicate the 95 % confidence interval for each point.

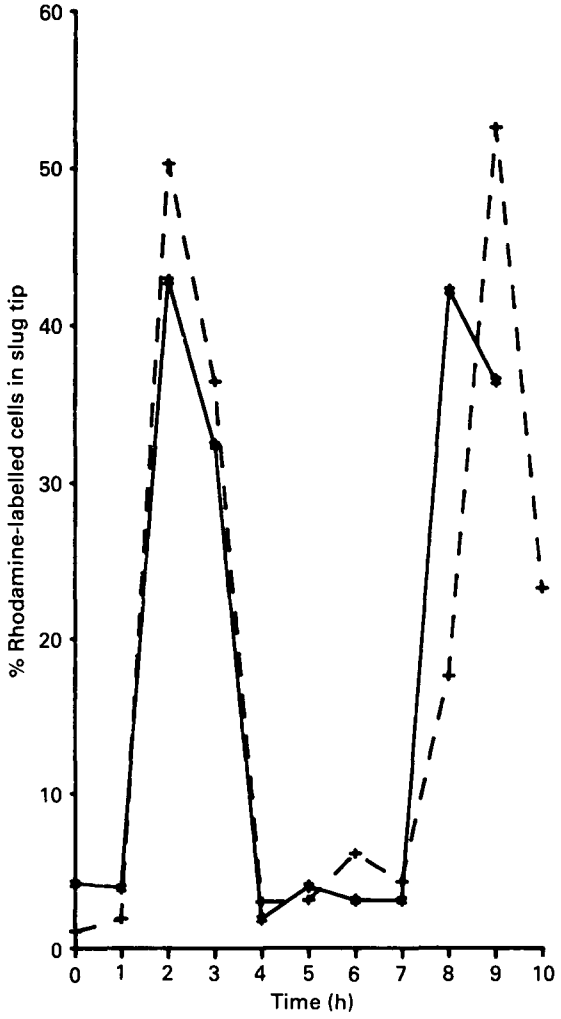

Fig. 3. Percentage of synchronous cells in the anterior 10 % of slugs. The percentage of synchronous cells that have sorted to the tip region was calculated from the distribution of labelled cells in the three parts of the slug (Fig. 2), under the assumption that the tip contains 10 % of all the cells (see Materials and Methods). The results of two independent experiments are shown; (*—*) is the same experiment as shown in Fig. 2.

that there is a drastic change in the proportions of cell types during progression through the cell cycle. Stationary-phase cells, i.e. cells with a strong prespore sorting tendency (Figs 2, 3), form slugs with a high percentage of prespore cells (90 %). Recently divided cells, i.e. those cells with a strong prestalk sorting tendency, form slugs with a very small percentage of prespore cells (55 %). Upon further progression through the cell cycle the proportions change again, with more prespore cells reaching a maximum of sometimes over 90 % in the last phase of the cell cycle. When the cells divide again and enter the second round of DNA synthesis the percentage of prespore cells drops again, as in the first cycle. Control slugs made with cells taken at various

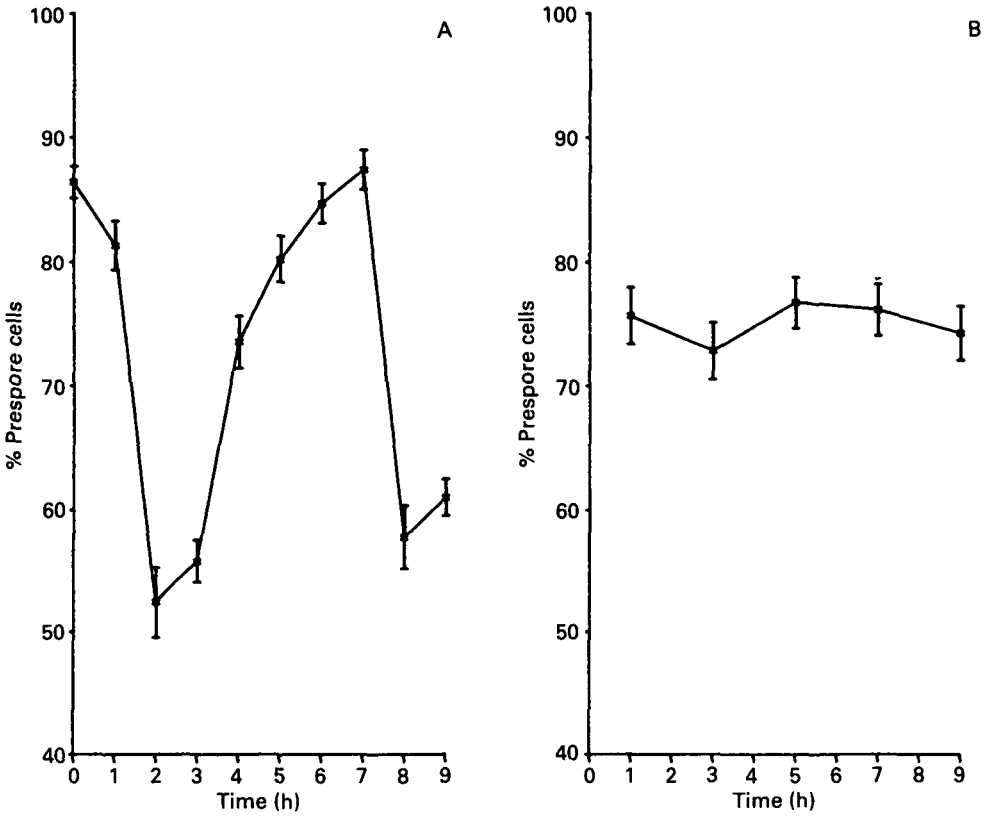

Fig. 4. The variation in the percentage of prespore cells in slugs formed from cells of synchronous (A) and exponentially growing (B) populations. The error bars indicate the 95 % confidence interval for each point.

times from an exponentially growing population show a constant percentage of about 75 % prespore cells, which is typical of slug tissue (Fig. 4B).

DISCUSSION

Our results demonstrate that cell cycle phase at the moment of starvation strongly influences both the sorting behaviour and the prestalk–prespore proportions of differentiating cells. In particular, cells in G_2 phase at the time of starvation are strong prespore sorters and form slugs with a high proportion of prespore cells. By comparison, postmitotic daughter cells, many of which are in *S* phase (Weijer *et al.* 1984*a*), are strong prestalk sorters and form slugs with a high proportion of prestalk cells.

Close examination of the results suggests that the period during which cells are prestalk sorters is relatively short. In Fig. 2, prestalk sorting occurred primarily in the 3 and 4 h samples. At 3 h the cell number has increased by 30 % (Fig. 1); thus about one half of the cells in the sample are postmitotic cells while the other half are still

undivided premitotic cells. The results in Fig. 2 suggest that about half the cells in this sample are prestalk sorters. It is most reasonable to assume that the postmitotic daughter cells are in fact the prestalk sorters, while the premitotic cells are the prespore sorters, since samples enriched in premitotic cells (e.g. 1 and 2 h or 5, 6 and 7 h) are also enriched in prespore sorters. The period during which cells exhibit prestalk sorting appears to be no more than 1 h. Were it any longer one would expect the proportion of prestalk sorters (Fig. 2) and the proportion of prestalk cells (Fig. 4) to increase between 3 and 4 h in parallel with the increase in the proportion of postmitotic daughter cells in the population (Fig. 1). This clearly does not occur.

Under the synchrony conditions used in these experiments the *S* phase lasts about 0·5 h and occurs immediately after mitosis (Weijer *et al.* 1984*a*). Hence many of the cells that exhibit prestalk sorting properties were also in *S* phase at the time of starvation. The significance of this fact is at present not clear (see below).

Stationary-phase cells are prespore sorters

The results in Fig. 2, indicating that stationary-phase cells sort to the prespore region of slugs, appear to contradict earlier findings of Leach *et al.* (1973) indicating that stationary-phase cells are strong prestalk sorters. This apparent discrepancy between our results and those of Leach *et al.* appears to be due to the 'age' of the stationary cells used in the two experiments. In our experiments cells had just reached stationary phase when harvested (see Materials and Methods) and were essentially all viable as judged by their synchronous doubling following dilution into fresh medium (Fig. 1). Such cells are prespore sorters (Fig. 2). By comparison, Leach *et al.* used cultures "in which the cell density had risen by less than 10 % in 24 hours" (p. 649 of Leach *et al.* 1973). In our experience such cultures become strongly alkaline and many of the cells do not divide upon dilution into fresh medium. Since the stationary-phase cells that Leach *et al.* used are rather different from ours, it is perhaps not surprising that they exhibit different sorting behaviour.

Cycling behaviour of prestalk and prespore precursors during development

Because cells continue to cycle slowly during development (Zada-Hames & Ashworth, 1978*b*) it is clear that prestalk and prespore cells do not differentiate in the same phase of the cell cycle in which they were at the onset of starvation. It is, however, possible (from published observations) to estimate qualitatively the cycling behaviour of the precursors to prestalk and prespore cells during development.

There are two periods of cell division during development: one during the pre-aggregation stage and a second during the slug stage. The number of cells from exponentially growing populations that divide during the first phase varies between 20 and 50 % depending on whether cells were grown on bacteria or in axenic medium (Zada-Hames & Ashworth, 1978*b*). When cells synchronized in late G_2 are starved 100 % of the cells divide during the first phase of cell division (Katz & Bourguignon, 1974). During the second period of cell division, another 20–30 % of the cells divide (Zada-Hames & Ashworth, 1978*b*). When exponentially growing cells are pulse-labelled with [^{3}H]thymidine and then immediately starved and allowed to develop it

is found that the labelled cells only begin to proceed through mitosis toward the end of the second period of mitotic activity. Furthermore, the number of labelled cells that actually divide appears to be only a small fraction of the initially labelled cells (Zada-Hames & Ashworth, 1978*b*).

These facts are consistent with the following view of cell cycling during development: most cells in G_2 at the moment of starvation divide in the pre-aggregation period; cells earlier in the cycle (some of which are in *S* phase) at the moment of starvation divide in the slug stage. However, fewer of these cells divide, and *S* phase cells in particular, which were at the beginning of the cycle at the moment of starvation (Weijer *et al.* 1984*a*), appear to divide rarely. The results of Zada-Hames & Ashworth (1978*b*) show, furthermore, that immediately following cell division another round of nuclear replication occurs. Thus, despite cell division, developing cells are formally in the G_2 phase of the cell cycle and have $2n$ nuclear DNA contents (Weijer *et al.* 1984*a*). It is not known to what extent cell growth continues after cell division although it seems unlikely in view of the starvation conditions (see below).

Fig. 5 shows schematically the relationship between cell size and cell cycle position in an exponentially growing cell population: relative cell size (mass) increases from 1 to 2 over the course of the cell cycle (unbroken line). From the above discussion it is clear that cells late in the cycle divide during development while cells early in the cycle in general do not. Assuming that no further growth occurs under starvation

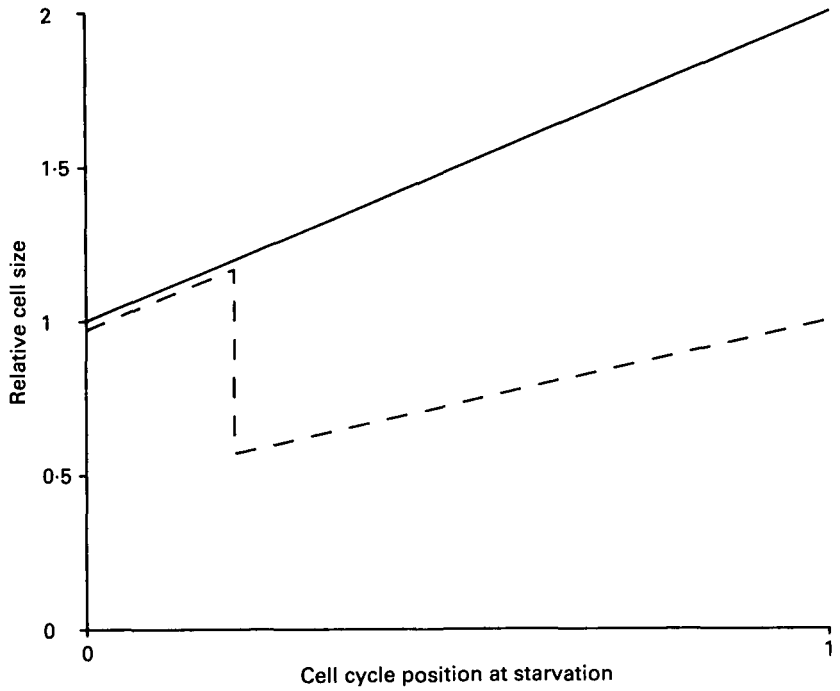

Fig. 5. Schematic representation of the relationship between cell size and cell cycle position at the moment of starvation. The cell cycle is represented on the abscissa from 0 (mitosis) to 1 (mitosis). See the text for explanation.

conditions, cells that divide will have half the size of their parents and thus half the size they had at the moment of starvation. Cells that do not divide during development presumably retain the size they had at the moment of starvation. Under these conditions a new pattern of cell sizes is generated in the slug stage (broken line) in which cells early in the cycle at the moment of starvation are now the largest cells in the population.

In view of the above discussion, one expects prestalk cells to be larger than prespore cells, since cells early in the cycle at the moment of starvation sort to the prestalk region and cells late in the cycle sort to the prespore region (Figs 2, 3). In agreement with this expectation is the observation that prestalk cells have more mitochondrial DNA than prespore cells (unpublished observations). Perhaps this difference in size at the time of prestalk and prespore differentiation is ultimately important in the direction of individual cells into one differentiation pathway or the other.

Comparison with other observations of the cell cycle dependence of sorting behaviour

During the course of these experiments MacDonald & Durston (1984) published an independent series of experiments on cell cycle position and sorting behaviour. These authors concluded that prestalk sorting is favoured by cells in the middle of the cell cycle, which is somewhat later in the cell cycle than the prestalk sorting that we observe. At present it is not possible to resolve this discrepancy. However, it may well be due to differences in the methodology. MacDonald & Durston used the shake-off procedure to collect mitotic cells from exponentially growing populations. Such cells are somewhat smaller than our synchronous cells, which are derived by cell division from stationary cells, since stationary cells are 20% larger than G_2 cells from exponentially growing cultures (Weijer *et al.* 1984*a*).

Since our synchronous cells and those of MacDonald & Durston differ physiologically, it seems likely that they may be capable of more or less cycling during development. Hence, although they start at different cell cycle positions both cells may reach the same cell cycle position at the time of prestalk and prespore differentiation. If cell cycle phase at the time of differentiation controls the decision for prestalk or prespore differentiation, then our results and those of MacDonald & Durston would be in agreement. Alternatively, it seems possible that cell size not cell cycle position controls cell type differentiation (see preceding section). Since our synchronous cells and those of MacDonald & Durston differ in size at the moment of starvation, it seems possible that they reach the same critical size for differentiation at different positions in the cell cycle.

Cells are already different at the vegetative stage and they sort out during later development

The present results with cell cycle fractions as well as those of earlier investigations using density-gradient fractions of cell populations (Takeuchi, 1969; Weijer *et al.* 1984*b*) have shown that it is possible to select certain cells from a vegetative population that will sort out from other vegetative cells when mixed and starved immediately. This implies that populations of vegetative cells are heterogeneous in some parameter

that affects how cells will finally differentiate. Our results indicate that this parameter is related to cell cycle phase.

The present experiments provide evidence that links cells early in the cycle to prestalk differentiation. Since these cells are presumably randomly distributed in the aggregation field and in the early aggregate, the results support the hypothesis that cells differentiate at random and then sort out to form the characteristic slug pattern (Takeuchi, 1969; Durston & Vork, 1979; Tasaka & Takeuchi, 1981; Sternfeld & David, 1981; Meinhardt, 1983). It is, however, still possible that cell cycle phase only affects the *position* to which a cell sorts in a slug while position-dependent events control prestalk and prespore differentiation (MacWilliams & Bonner, 1979; Krefft *et al.* 1984). Hence the present experiments do not resolve the controversy over these two models of differentiation. They do, however, provide a simple explanation for the random distribution of prestalk and prespore precursors that are observed under some conditions during early stages of differentiation (Forman & Garrod, 1977*b*; Tasaka & Takeuchi, 1981).

Correlation between sorting behaviour and shift in prespore/prestalk proportions

We have shown that the type of sorting behaviour correlates with the change in proportions of prespore and prestalk cells in the slug. This correlation has been found in all other cases where it has been investigated. For example: (1) cells grown with (G+) and without (G−) glucose in the medium; G+ slugs contain more prespore cells than G− slugs (Forman & Garrod, 1977*a*), and G+ cells are prespore sorters relative to G− cells (Leach *et al.* 1973; Tasaka & Takeuchi, 1981); (2) the proportioning mutants Hs2 and Hs3, which make more and less prespore cells, respectively, than wild-type cells and also show prespore and prestalk sorting, respectively, relative to wild-type cells (MacWilliams, 1982).

This correlation between sorting behaviour and cell-type proportioning can be qualitatively understood in terms of a simple negative feedback model of proportioning (MacWilliams, 1982; Weijer & Durston, 1984). In such a model the proportions of the two cell types are regulated by inhibitors produced by the cell types. The level of the inhibitors depends on the proportion of the cell types. In our experiments relatively few (10% or less) synchronous cells of a particular cell cycle phase were mixed with exponential cells. In this case it is reasonable to assume that the level of the proportion-regulating signals in the slug is determined by the majority of the cells, i.e. the exponential-phase cells, and therefore they are the same in all slugs. Since synchronous cells of particular phases show shifted proportions, i.e. are prestalk or prespore sorters, it is simplest to imagine that their sensitivity to the proportioning signals has changed. Therefore, it seems plausible that the sensitivity is a cell-cycle-linked parameter.

Thus a population of vegetative cells, which will normally form slugs with about 75% prespore cells, consists of a mixture of cells with variable sensitivities, which, when isolated, form slugs with 55–90% prespore cells.

Many cell-cycle-linked variables have been described in other systems, some of which are cyclic variations in cyclic nucleotide metabolism (Goldberg *et al.* 1974),

changes in ion permeabilities and pump activities (Moolenaar, Mummery, van der Saag & de Laat, 1981; Mummery, Boonstra, van der Saag & de Laat, 1981), which can result in a cyclical variation of internal pH (Gerson & Burton, 1977; Morisawa & Steinhardt, 1982). At present it is not clear whether any of these variables are involved. Nevertheless, it is interesting to note that changes in internal pH (Gross, Bradbury, Kay & Peacy, 1983) as well as cyclic nucleotide metabolism (Weijer & Durston, 1984) have been implicated in proportion regulation in *D. discoideum*.

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