Pattern formation in *Dictyostelium discoideum*

I. Development of prespore cells and its relationship to the pattern of the fruiting body

By D. FORMAN¹ AND D. R. GARROD²

*From the Department of Biology, University of Southampton*

**SUMMARY**

Immunofluorescent staining of the prespore cells of the cellular slime mould *Dictyostelium discoideum* was carried out using a heterologous spore antibody. The highly specific staining of the prespore vesicles (PSVs) within the prespore cells enabled quantitative determinations to be made of the rate and extent of development of these cells throughout the life cycle.

The results showed that PSVs first appeared in a large proportion of the cells shortly after the cells had chemotactically aggregated into multicellular masses. During the later phases of the life cycle, the proportion of cells containing PSV increased, as did the fluorescent intensity of their PSVs, until the early culmination stage of development when 85-90% of the total cell population contained PSVs. Lowering the temperature of development delayed the onset of vesicle formation and decreased the proportion of prespore cells in the total cell population. Changing the growth conditions of the cells prior to multicellular development also had a significant effect on the proportions of prespore cells, as did the use of a mutant known to give rise to fruiting bodies with a reduced number of spores.

The comparability between these estimates of prespore cell proportions at culmination and previously reported spore:stalk ratios within fruiting bodies confirms the view that PSVs are reliable indicators of prespore cells. The finding that temperature and growth conditions and the use of mutants all of which are known to affect spore:stalk ratios, also all affected prespore proportions in the expected direction, adds further weight to this argument.

The fact that prespore cells are beginning to differentiate early in the multicellular phase of the life cycle and the related finding that such differentiation always precedes formation of the grex tip are results of considerable importance to the development of a model for pattern formation in *D. discoideum*.

**INTRODUCTION**

The fruiting body of the cellular slime mould, *Dictyostelium discoideum*, consists of a mass of spores on top of a cellular stalk and thus represents a simple linear pattern of differentiation. The pattern is size-invariant, the ratio of spore to stalk being roughly constant irrespective of the size of the fruiting body. The fruiting body is formed from a polarized mass of cells, the grex,

¹ Author's address: Department of Bacteriology and Immunology, University of Glasgow, Western Infirmary, Glasgow, G11 6NT, U.K.
² Author's address (for reprints): Department of Biology, University of Southampton, Medical and Biological Sciences Building, Southampton, SO9 3TU, U.K.
there being correspondence between a cell’s position within the grex and whether it forms spore or stalk. Cells at the anterior end of the grex form the stalk, while cells from the posterior end form the spores. These precursors of spore and stalk have been called ‘prespore’ and ‘prestalk’ cells, respectively (Raper, 1940; Bonner, 1944).

The terms ‘prespore’ and ‘prestalk’ can be justified by more than cell position within the grex since a number of differences between the two cell types have been discovered (listed by Garrod & Ashworth, 1973). The major distinguishing feature is the exclusive possession by the prespore cells of vesicles containing material which is thought to be extruded from the cells at culmination to form the coat of the spores (Hohl & Hamamoto, 1969; Takeuchi, 1972). These prespore vesicles (PSV) have been identified by electron microscopy (Maeda & Takeuchi, 1969; Gregg & Badman, 1970), and by the powerful technique of immunofluorescent staining with an antibody against the spore coat (Takeuchi, 1963).

Even in the absence of additional knowledge, the immunological evidence would seem to many to argue most persuasively that the prespore cells are the precursors of the spores of the fruiting body. This in turn would imply that the anterior–posterior prestalk–prespore pattern which has been shown to exist in the grex (Takeuchi, 1963; Gregg, 1965) is the precursor of the stalk-spore pattern of the fruiting body. However, it has been suggested recently that the fruiting body pattern may be formed only during the actual process of fruiting body construction and the presence of prespore cells in the grex bears no relation to the final pattern (Farnsworth, 1973; Farnsworth & Loomis, 1976).

The reason why such a controversy can exist is that the mechanism of pattern formation in *D. discoideum* is not understood. Although numerous hypotheses have been put forward (reviewed by Loomis, 1975) we strongly felt that insufficient experimental work had been done on the descriptive basis of pattern formation to provide a firm basis for any of these hypotheses. Our first intention in this study has been, therefore, to provide more descriptive information on the development of prespore cells in *D. discoideum*. Secondly, we have sought evidence on whether the prespore and prestalk cells in the grex are related to the final pattern of the fruiting body.

**MATERIALS AND METHODS**

**Growth and development of cells**

*Dictyostelium discoideum* cells (strain Ax-2) were grown vegetatively in axenic conditions (Watts & Ashworth, 1970) on a rotary shaker at 22 °C and 140 rev./min (radius of rotation 2-75 cm). For most experiments the growth medium was supplemented with 86 mM glucose. Cells were harvested in the exponential phase of growth when they were at a density of $2-4 \times 10^6$ per ml. Cells were harvested by centrifugation at 350 g for 5 min at 4 °C. They were
Pattern formation in *D. discoideum* 217

then washed twice in cold distilled water and resuspended in distilled water at a density of $1 \times 10^8$ per ml.

Ax-2 cells supplied with bacterial nutrients, *D. discoideum* cells of the mutant strain P-4 (kindly provided by Dr J. Gross), and *Dictyostelium mucoroides* cells were grown by inoculating spores with *E. coli* B/r on a standard agar medium (Sussman, 1966) and incubating at 22 °C. Vegetative cells were harvested in cold distilled water after 40 h of growth and centrifuged at 350 g for 5 min at 4 °C to remove excess bacteria. The cells were washed three times in cold distilled water and resuspended at $2 \times 10^8$ per ml.

Vegetative cells were allowed to develop by placing 0.5 ml of the cell suspension over the surface of washed 47 mm Millipore filters (AABPO4700) resting on absorbent pads saturated with 1.6 ml of a buffered salt solution at pH 6.5 (1 litre 50 mM phosphate buffer with 1.5 g KCl, 0.5 g MgCl$_2$.6H$_2$O, 0.5 g streptomycin). The Millipore filters in Petri dishes were incubated in dark humid conditions at 22 °C or other experimental temperatures. In such conditions cells develop with a high degree of synchrony (Sussman, 1966).

**Preparation of single cell suspensions**

In most experiments single cell preparations were required from the multicellular cell masses formed after aggregation. Such cell masses were dissociated by the method of Takeuchi & Yabuno (1970). Cells were washed off a Millipore filter at the requisite time in 50 mM Tris-HCl buffer pH 7.0 and centrifuged at 700 g for 10 min at 4 °C. They were then resuspended in Tris-HCl containing 25 mM dimercaptopropanol (BAL) and 0.1 % pronase. Cell masses were maintained in this solution for 15 min during which time they were triturated with a Pasteur pipette. This treatment reliably dissociated aggregates and grexes into single cell suspensions. The cells were centrifuged at 700 g for 10 min and resuspended in standard salt solution (Bonner, 1947) at $2 \times 10^6$ per ml. This suspension was poured on top of glass coverslips contained in a Petri dish and the cells were allowed to settle out and adhere to the coverslips for 30 min at room temperature. The salt solution was decanted and the cells fixed for 3 min in ice-cold methanol. The cells were then washed in phosphate buffered physiological saline (PBS) at pH 7.0 and kept in PBS until required for staining.

**Preparation of spore specific antibody**

As specificity is known to be enhanced by utilizing spore antigens from a different species of *Dictyostelium* (Takeuchi, 1972), it was decided to produce antibody from spores of *D. mucoroides*. Such spores were collected from fruiting bodies of *D. mucoroides*, formed after 2–3 days development on agar at 22 °C, using a wire loop and suspended in PBS. The saline was lightly centrifuged (100 g for 1 min) in order to pellet out any contaminating stalk. The spores were then spun out of the supernatant at 700 g for 10 min and resuspended in a small amount of PBS. They were then frozen and thawed in order to lyse any
remaining vegetative cells, and washed in PBS. Finally the spores were sus-
pended in distilled water, lyophilized and stored at 4 °C until required.

Spores were inoculated into New Zealand White rabbits, from which a pre-
injection sample of blood had been obtained from the marginal ear vein. Ten mg
of freeze-dried spores suspended in 1 ml of physiological saline were injected
intravenously into the marginal ear vein. This was followed by a series of five
subcutaneous injections each containing 5 mg of spores into the neck and back
on days 5, 23, 30, 40 and 52. In total 35 mg of spore material was injected which
contained 2.5 mg of soluble protein (Lowry, Rosebrough, Farr & Randall, 1951).
Two days after each of the four booster injections, trial bleedings were made
from the ear vein and the serum antibody level was assessed using precipitin
and Ouchterlony double diffusion tests (Ouchterlony & Nilsson, 1973). Seven
days after the final injection, the rabbits were bled out by heart puncture.
Blood was left to clot for 1 h at room temperature and, after ringing, overnight
at 4 °C. The serum was then taken off and centrifuged to remove any remaining
cells. It was stored in aliquots at −27 °C.

Testing the serum antibody

For the precipitation and Ouchterlony tests, an antigen solution was prepared
by sonicating 10 mg lyophilized D. mucoroides spores in 1 ml PBS (0.7 mg
protein per ml) containing 0.5% sodium deoxycholate. Fragments were dis-
carded by centrifugation at 29000 g for 30 min. After the final booster injec-
tion, dilutions of the antigen to 0.62 μg protein/ml and to 175 μg protein/ml
gave positive reactions in the precipitin and Ouchterlony tests respectively.

Using undiluted antigen solution on the Ouchterlony double diffusion plates
with the antiserum caused the formation of three precipitation lines. After
absorption of the serum with vegetative cells from axenically grown D. dis-
coideum (Takeuchi, 1963) a single specific precipitation line was produced.

For immunofluorescent use it was found appropriate to dilute such absorbed
antiserum by 1 in 100.

Staining of single cell preparations

The dissociated cells, methanol fixed on coverslips, were stained by adding one
drop of the diluted D. mucoroides absorbed antispore serum. This was allowed to
react for 20 min at room temperature in moist conditions before being washed
off in PBS for 10 min. One drop of anti-rabbit immunoglobulin (sheep) conju-
gated with fluorescein isothiocyanate (Wellcome Reagents Ltd) was then applied
to the cells for 10 min at room temperature. A further 20 min wash in PBS was
then given and the coverslips mounted in 90% glycerol:10% PBS.

Control staining was carried out using non-immune pre-injection serum
followed by the commercial conjugate. Controls were also made using the
conjugate alone. At no time was any specific staining observed with such
controls.
Microscopy and counting

Cell preparations were observed on a Zeiss Universal microscope using either phase contrast or illumination with an HBO 200 mercury arc lamp, BG 38 and BG 12 exciter filters, and Zeiss barrier filters 44 and 50.

When it was necessary to assess the proportion of specifically stained cells, a field of cells on the coverslip was selected at random and the total number of cells counted under phase contrast illumination. The same field was then observed under u.v. light and the number of cells containing the specifically stained fluorescent vesicles was counted. Where possible counting was carried out using a ‘blind’ procedure.

RESULTS

Development of PSV during the life cycle

The time course of appearance of PSV in log phase cells allowed to develop on Millipore filters is shown in Fig 1. The first distinctive appearance of the fluorescent vesicles was evident in a large number of cells (65 ± 1-4 % s.d.) after 12 h of development. This was 3-4 h after the chemotactic aggregation stage and corresponded approximately to the time of first appearance of the grex tip. At an even earlier time, from about 10 h of development onwards, a diffuse specific staining could be detected but fluorescence was not localized in distinct vesicles (Fig. 2) making it difficult to estimate in what proportion of the cells it appeared. Specific staining was never observed in cells prior to aggregation. As development proceeded through the standing, migrating and culminating grex stages there was a gradual increase in the proportion of cells with PSV. The rate of increase in prespore cell numbers varied between experiments but by 18-19 h of development (early culmination) there was stabilization of the proportion of prespore cells at 85-90 % of the total cell population. After the early culmination stage, prespore determinations were of little relevance without an additional estimate of the number of mature spores and stalk cells, neither of which could be readily assessed.

Besides these quantitative changes in the number of cells containing prespore vesicles there was also a marked increase in the fluorescent intensity of vesicles during development from aggregation to early culmination (Figs. 2 and 3). This was accompanied by a decline in background cytoplasmic staining so that by 14 h of development it was relatively easy to distinguish between prespore cells and unstained cells, presumably prestalk cells (Fig. 4).

Effect of temperature on PSV development

The temperature at which cells develop is known to affect the spore:stalk ratio in fruiting bodies of D. discoideum. Lowering the temperature increases the proportion of stalk cells (Bonner & Slifkin, 1949; Farnsworth, 1975). We felt that if the prespore:prestalk ratio in the grex is relevant to the pattern of
the fruiting body, this ratio should be affected by temperature in the same way as the proportions of the fruiting body.

Figure 5 shows the results of experiments in which Ax-2 cells were allowed to develop at different temperatures between 15 and 22 °C and the proportion of prespore cells in the population counted. Since the speed of development varied with temperature prespore cells were counted at two-hourly intervals from the time of their first appearance until early culmination at each temperature. Mature fruiting bodies were formed 2–3 h after the final reading at each temperature.

Between 15 and 22 °C there was an almost linear relationship between temperature and the number of prespore cells in the population at the early culmination stage. Above 22 °C we found no further increase in the proportion of prespore cells.

Low temperature delayed the onset of vesicle formation by slightly less than 1 h for every 1 °C drop in temperature below 22 °C. At all temperatures the
first appearance of vesicles coincided approximately with the onset of grex tip formation. Thereafter the proportion of prespore cells increased for 6–8 h when culmination began at all temperatures. Thus the time between tip formation and the onset of culmination was roughly constant at different temperatures. This was because the period of migration was shortened at low temperature. At 15 °C for example there was little or no migration.

**Effect of growth conditions on PSV development**

It has been shown that cells of *D. discoideum* Ax-2 grown under different conditions form fruiting bodies with different spore:stalk ratios. Thus fruiting bodies of log-phase cells grown in axenic medium supplemented with 86 mM glucose (glucose cells) had a spore:stalk ratio of 3:95:1, whereas fruiting bodies of log-phase cells grown in unsupplemented medium (N.S. cells) had a spore:stalk ratio of 2:70:1 (Garrod & Ashworth, 1972). (These spore:stalk ratios were determined by volume measurements on fixed fruiting bodies formed after development on Millipore filters.) If the prespore:prestalk ratio in the grex is relevant to the spore:stalk pattern of the fruiting body the proportion of prespore cells in glucose grexes should be greater than that in N.S. grexes.

Figure 6 shows the prespore:prestalk ratios in early culminating grexes of cells grown under these different conditions. The proportions of prespore cells in the populations was 89 % for log phase glucose cells and 79 % for N.S. cells. Clearly the prespore:prestalk ratios of the populations differ in the same way as the spore:stalk ratios of their fruiting bodies.
Fig. 3. Cells dissociated from early culminants, after 18 h of development on Millipore filters, stained and observed as in Fig. 2. Note the large number of vesicles with bright fluorescence. × 760.

Fig. 4. Cells dissociated from grexes, after 14 h of development on Millipore filters, stained and observed as in Fig. 3. Note the distinction between stained and unstained cells. × 760.
Development of PSV in mutant P-4

*D. discoideum* P-4 is a mutant which has a reduced proportion of spores in the fruiting body (Hohl & Raper, 1964) and an increased susceptibility to cyclic-AMP induction of stalk cells (Chia, 1975). If the prespore:prestalk ratio in the grex is relevant to the pattern of the fruiting body, the proportion of prespore cells in P-4 grexes should be similarly reduced.

Figure 7 shows P-4 cells which were allowed to develop on Millipore filters at 22 °C exhibited a marked reduction in the proportion of prespore cells at the early culmination stage compared with Ax-2 cells. A reduction in the proportion of prespore cells in P-4 was found at all stages of the life cycle subsequent to the first appearance of PSV. The development of P-4 was delayed by 3–4 h compared with Ax-2 glucose cells. On Millipore filters, P-4 formed a large number of fruiting bodies with thickened stalks. We did not obtain a substantial number of normal fruiting bodies as have been found with P-4 developing on cellophane membranes (Chia, 1975; Hamilton & Chia, 1975).
DISCUSSION

Relationship to pattern formation

These results enable us to make two main points in relation to pattern formation. Firstly, all of the conditions affecting the spore:stalk ratios of fruiting bodies which we have used, affect the proportion of prespore cells during the grex stage of the life cycle in the same direction. It seems unlikely that the material in the prespore vesicles which our antibody binds is a non-specific sorus binding material as has recently been suggested (Farnsworth & Loomis, 1976). This is because thorough washing of the spore with saline before inoculation completely disrupted spore heads into single spores and should have removed any such material. Rather, we feel that the antibody is against the spore coat and the antigen in the PSV therefore represents spore coat material. Even if the suggestion of Farnsworth & Loomis were correct, staining with antibody would still indicate which cells were involved in forming spores, for the sorus is that part of the fruiting body which consists of spores. We conclude that the prespore proportion in the grex is directly related to the spore:stalk ratio of the fruiting body and that there is a direct casual relationship between possession of PSV and differentiation into spores. We therefore
disagree with the model of Farnsworth (1973) which proposes that pattern formation occurs only during the process of culmination.

Secondly, prespore cells can first be identified by means of fluorescent PSV at the stage of the late aggregate, approximately at the time when the grex tip first begins to form. Both Müller & Hohl (1973) and Hayashi & Takeuchi (1976) report identification of PSV at about this time but in a smaller proportion of cells. Since we could detect specific cytoplasmic fluorescence which was not collected into vesicles up to 2 h before the first positive identification of PSV, it seems likely that differentiation begins even earlier than the late aggregate stage. This view is reinforced by the finding that two cell populations which, if mixed, sort out to the prestalk and prespore regions of the grex respectively
can be separated by isopycnic centrifugation from populations of preaggregation cells (Takeuchi, 1969; Bonner, Sieja & Hall, 1971; Maeda & Maeda, 1974). Therefore, much evidence now suggests that the first stage in pattern formation, the diversification of prespore and prestalk cell types, probably begins early in the life cycle, possible before the multicellular stage is reached and certainly before the grex develops overt polarity by forming a tip. This point is of major importance in relation to the model for pattern formation which we develop in the following paper (Forman & Garrod, 1977).

Relationship to other work

Our time course of accumulation of prespore cells is intermediate between those of Müller & Hohl (1973) and Hayashi & Takeuchi (1976). The former found a gradual linear increase in numbers of prespore cells from late aggregation to early culmination, while the latter found a sudden increase at late aggregation with no further increase in the later stages of the life cycle. Factors which may have contributed to discrepancies between the three studies are firstly, the use of different strains of *D. discoideum*, secondly the use of different vegetation growth conditions and thirdly the use of different conditions for development. Both Müller & Hohl and ourselves allowed cells to develop on Millipore filters, a condition which shortens the time of grex migration compared with development on non-nutrient agar which was used by Hayashi & Takeuchi. It will be very important to establish whether there is a gradually increasing proportion of prespore cells or whether the full complement of prespore cells appears soon after aggregation.

It is useful to comment on the merits and disadvantages of our techniques for detecting prespore cells compared with the techniques of other workers. The main advantage of our procedure of staining cells which have been dissociated from grexes is that it enables us to deal with very large numbers of cells. Techniques involving the detection of PSV by electron microscopy (Müller & Hohl, 1973; Farnsworth & Loomis, 1976) are necessarily more laborious and therefore deal with smaller numbers of cells. A disadvantage of our technique is that it does not enable us to count prespore cell numbers in individual grexes. We therefore cannot yet comment on the curious result obtained by Farnsworth & Loomis (1976) by counting PSV with the electron microscope that the number of prespore cells is constant irrespective of the size of the grex.

Our figures for the number of prespore cells in the population at early culmination (between 75% and 89% for all Ax-2 cell populations developed at 22 °C) agree well with spore:stalk ratios in fruiting bodies obtained by other workers (Bonner, 1967; Farnsworth, 1975). It should be pointed out that spore:stalk ratios obtained by making volume measurements on fruiting bodies (Bonner & Slifkin, 1949; Garrod & Ashworth, 1972) invariably give lower spore:stalk ratios than counting prespore cells or spores. Volume measurements make no pretence at being able to estimate cell number. The
discrepancy with ratios obtained by cell counting techniques arises because the formation of spores from prespore cells involves a decrease in cell volume while the formation of stalk cells from prestalk cells involves an increase in volume. There are thus many more spore cells per unit volume of the spore head than there are stalk cells per unit volume of stalk.

We would like to thank Lynette Banham, Alistair Nicol and Sherilee Taylor for excellent technical assistance. This work was supported by the Science Research Council. D. Forman was in receipt of a S.R.C. Studentship.

REFERENCES


(Received 4 January 1977)