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Evidence that positional information is used to establish the prestalk-prespore pattern in *Dictyostelium discoideum* aggregates

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Two contrasting mechanisms have been proposed for the establishment of the prestalk-prespore pattern in the multicellular aggregate of the simple eukaryote *Dictyostelium discoideum*. One involves intermingled, non-position-dependent cell differentiation followed by sorting out which produces the pattern of prestalk cells in the anterior region and prespore cells posteriorly. The second mechanism involves patterning according to the position of cells within the aggregate, in which case intermingled cell types are not expected. Here we use a monoclonal antibody (MUD1), recognising a prespore cell surface antigen, to study the initial appearance of prespore cells in aggregates. Quantitative studies were made with a flow cytometer and frozen sections were used to localise the cells expressing the prespore antigen. This antigen first appeared at the onset of tip formation in the centre of aggregates in a position-dependent fashion. The prespore antigen was not detected in the tip region or in streams of cells entering the aggregate. We re-examined the evidence on which the non-position-dependent differentiation model is based. Our results support the positional model for pattern formation.

Key words: Dictyostelium discoideum/monoclonal antibody/proportion regulation/patterning/morphogenesis

Introduction

The generation of patterns is poorly understood in any biological system, although in general there is at least some agreement that positional information (i.e., cells differentiating according to their location) is used in the formation of patterns of different cell types (Wolpert, 1981). The slug stage of the cellular slime mould *Dictyostelium discoideum* is one of the simplest multicellular eukaryotes to exhibit polarity, a definite front and rear, and a pattern. Simplistically this organism is a cylinder of $\sim 10^5$ cells, which is divided into $\sim 20-30\%$ prestalk cells at the tip, an organiser region, and $70-80\%$ prespore cells in the rear (Morrissey, 1982; Williams, 1982). It has been suggested that the prespore/prestalk pattern arises by non-positional (nearest-neighbour) proportion regulation, in which cells differentiate into the correct proportions of prespore and prestalk cells in small groups and subsequently the differentiated cells sort out to their correct positions (Forman and Garrod, 1977; Tasaka and Takeuchi, 1981; Morrissey, 1982). This is an important claim as it provides an alternative explanation for pattern formation to that usually invoked, in which cells are supposed to differentiate according to their position in the organism. Moreover, it has implications concerning how proportion regulation and patterning are interpreted at a molecular level (Meinhardt, 1983). This is particularly true of the types of morphogens expected (Meinhardt, 1983), if indeed proportion regulation is mediated by morphogens and not by other mechanisms (O'Dell *et al.*, 1981). There are several candidate morphogens already identified in *D. discoideum* (Sussman and Schindler, 1978; Gross *et al.*, 1981; Meinhardt, 1983; Williams, 1982; Kay and Jermyn, 1983; Fisher *et al.*, 1984). Before the action of these potential morphogens can be adequately interpreted it is important to clarify whether proportion regulation is positional or non-positional. Here we investigate this question by analysing the appearance of two different prespore markers. One is a cell surface antigen recognised by monoclonal antibody MUD1 (Krefft *et al.*, 1983), the other is the prespore vesicle antigen detected within prespore cells using an anti-spore antiserum (Takeuchi, 1963). Both of these markers suggest that prespore cells differentiate according to their position in the aggregate.

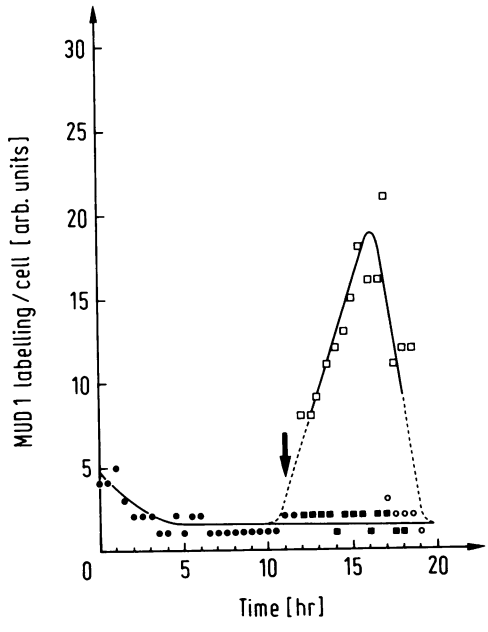


Fig. 1. Complete time course of MUD1 labelling of *D. discoideum* strain NP84 from 0 to 20 h (from vegetative amoebae until development of mature fruiting bodies) measured by flow cytometry. Dissociated cells were incubated with MUD1 and goat anti-mouse IgG $F(ab')_2$–FITC and measured in the flow cytometer. The mean values (arbitrary units) of the level of fluorescence were calculated at each time point for different cell classes. ●——● amoebae (pre-aggregative and aggregative), □——□ prespore cells, ■——■ unlabelled (mostly prestalk) cells, ○——○ mature spores. Mature stalk and basal disk cells are dead and present in a cellulose matrix which prevents their separation to single cells, so they were not measured. Labelling less than five arbitrary units is background. The small amount of labelling in vegetative amoebae is within the levels obtained for background fluorescence. Arrow indicates time of tip formation.

Results

Developmental regulation of the antigen recognised by monoclonal antibody MUD1

We have shown that monoclonal antibody MUD1 recognises a single prespore protein of 30 kd apparent mol. wt. (Krefft *et al.*, 1983). Figure 1 shows that the antigen is present at levels not significantly greater than background labelling on the surface of vegetative amoebae and first appears on the surface of prespore cells in the tight aggregate at tip formation. As development proceeds, the number of molecules of antigen per prespore cell increases until culmination (Figures 1, 2a and 2b), after which the antigen begins to disappear from the cell surface (Figure 1). The antigen recognised by MUD1 is not expressed on the surface of mature spores (Figures 1 and 2c) but it is still found in a membrane-bound state within the spore coat. The presence of the MUD1 antigen inside mature spores is clearly demonstrated by immunoblotting experiments using spores solubilised in SDS and run on SDS gels (Krefft and Grant, unpublished).

The results shown here are for strain NP84, but the same results have been obtained for strain X22 and several other *D. discoideum* isolates.

Immunoblotting experiments have shown that the MUD1 antigen is undetectable in vegetative amoebae (Krefft *et al.*, 1983). The MUD1 antigen appears between 1 and 2 h before it is observed on the cell surface. Figure 3a shows immunoblots and Figure 3b a flow cytometer analysis of the same group of cells. The antigen was very faintly observed in an overloaded immunoblot by 8 h (approximately at tip formation in this experiment) and by 9 h it was clearly present. The flow cytometer data showed that the shift in cell size characteristic of prespore cells commenced at 9 h, when the MUD1 antigen was first clearly apparent inside the cells. One hour later the MUD1 antigen was present on the surface of some cells, and by 12–13 h two distinct populations of cells were apparent (Figure 3b). Hence, during development, the MUD1 antigen is inserted at the cell surface shortly after it can be recognised inside the cells.

Analysis of prestalk/prespore pattern formation using MUD1

Since MUD1 recognises a cell surface antigen specific to prespore cells, we studied its appearance at 15 min intervals in both the flow cytometer and on frozen sections (Figure 4).

In the experiment shown in Figure 4, aggregates with tips were formed after 6.25 h and a small percentage of labelled (prespore) cells were first detectable in the flow cytometer at this time. By ~8.5 h, prespore cells were present as a clearly defined population in the flow cytometer on the basis of labelling with monoclonal antibody and decrease in size. Since strain NP84 develops very quickly, the timing of development varies substantially depending on the stage at which the growth plates are harvested. This is shown in this report by contrasting Figure 1 where MUD1 staining first appeared at 11 h and Figure 4 where labelling was first apparent at 6.25 h. However, we always observed that the appearance of MUD1 staining was closely correlated to the morphological appearance of tips on aggregates.

Analysis of frozen sections prepared in parallel with the flow cytometer study, revealed a striking localisation of cells labelled with MUD1 (Figure 4a). Weakly labelled cells first appeared as a group at the base of the aggregate at 6.25 h. This region of labelled cells extended upwards (7 h) and subsequently filled the prespore area (9 h). It is noteworthy that cells in the streams were not labelled in all 12 sections observed with intact streams (see 7 h aggregate, Figure 4). Nor were labelled cells seen at the tip.

Only with great care was it possible to cut longitudinal sections through these early aggregates which are very fragile. The tip region and cells streaming into the aggregate were particularly fragile and often lost during preparation of sections (e.g., most of the tip is missing from the 7 h section in Figure 4).

Quantitation of prespore cells during pattern formation

Figure 5 shows the percentage of cells in the aggregate which express the MUD1 antigen as determined by curve fitting techniques from the flow cytometer data of Figure 4 (see Voet *et al.*, 1984, for details). The prespore cells, as defined by MUD1 surface label, increased from <5% up to the plateau of ~70% over the period during which the pattern was established (6.25 h to 9 h). These results indicate that prespore cells differentiate at the time the pattern, i.e., spatially separated appearance of prestalk and prespore cells, is established, and not at some earlier time.

Use of a second prespore marker, the anti-spore antiserum

The results presented with MUD1 strongly suggest that the prestalk-prespore differentiation appears in a localised fashion and there is no evidence for a random distribution of

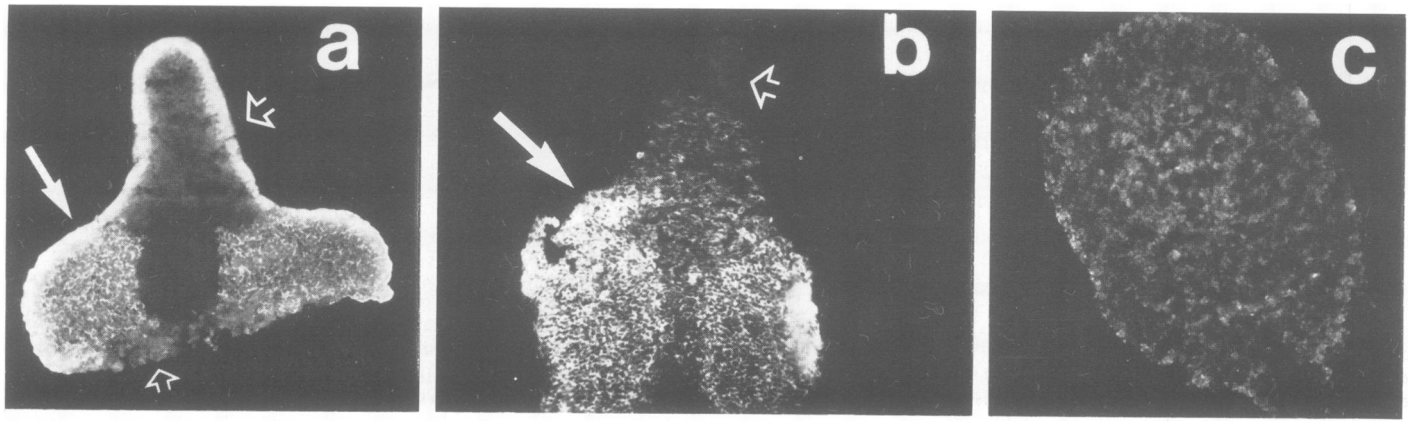


Fig. 2. MUD1 labelling on frozen 5 μm sections of *D. discoideum* late developmental stages: **(a)** early stage of fruiting body formation (culmination), **(b)** late culmination, and **(c)** a spore head. The open arrows in **(a,b)** indicate the region of unlabelled prestalk cells, which later form the stalk, and predisk cells, which form the basal disk region of the fruiting body; the white arrows indicate labelled prespore cells. Individual cells are ~10 μm in diameter.

prespore cells followed by sorting out. The prespore vesicle marker of Takeuchi (1963) has been widely used to monitor prespore cells. We have shown previously that slug cells which are labelled by this antiserum also carry the MUD1 antigen on their surface (Gregg *et al.*, 1982). We have also found that cells labelled by the prespore vesicle antiserum (Takeuchi, 1963) were localised at the base of aggregates shortly after tip formation, and as a column of cells later, in an identical fashion to MUD1 labelling. There was no indication that cells labelled with the prespore vesicle antiserum appeared in an intermingled fashion.

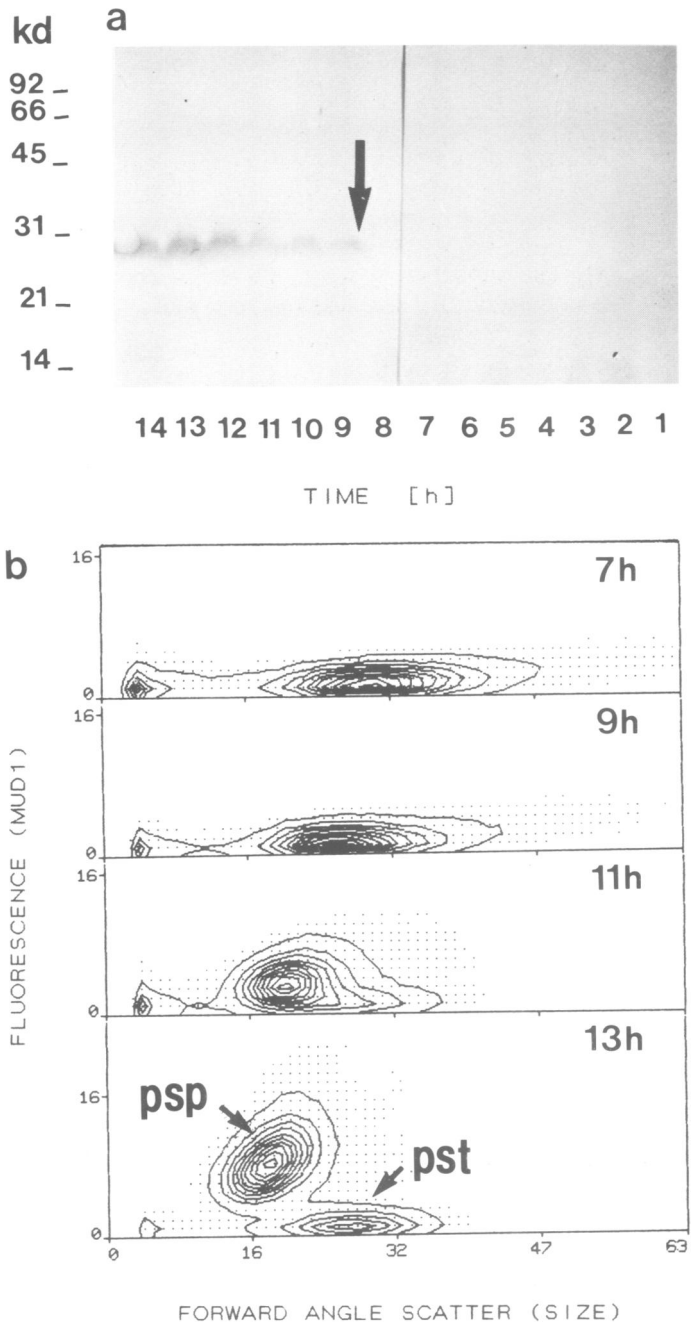


Fig. 3 .(a) Staining with MUD1 of immunoblots from a time course (1 – 14 h) of *D. discoideum* strain NP84 on Millipore filters. Mol. wt. was determined using protein standards shown at left of figure. The arrow indicates the time of tip formation. **(b)** Flow cytometer analysis of the same experiment as that shown in **(a)**. The experiments are visualised as contour plots (dots indicating regions containing 1% or more, and lines at 10% intervals from 5% to 95% with respect to the highest peak) of ~100 000 cells analysed for size (forward angle scatter) and fluorescence due to antibody labelling of MUD1 antigen at the cell surface. psp = prespore cells, pst = prestalk and other unlabelled cells.

Discussion

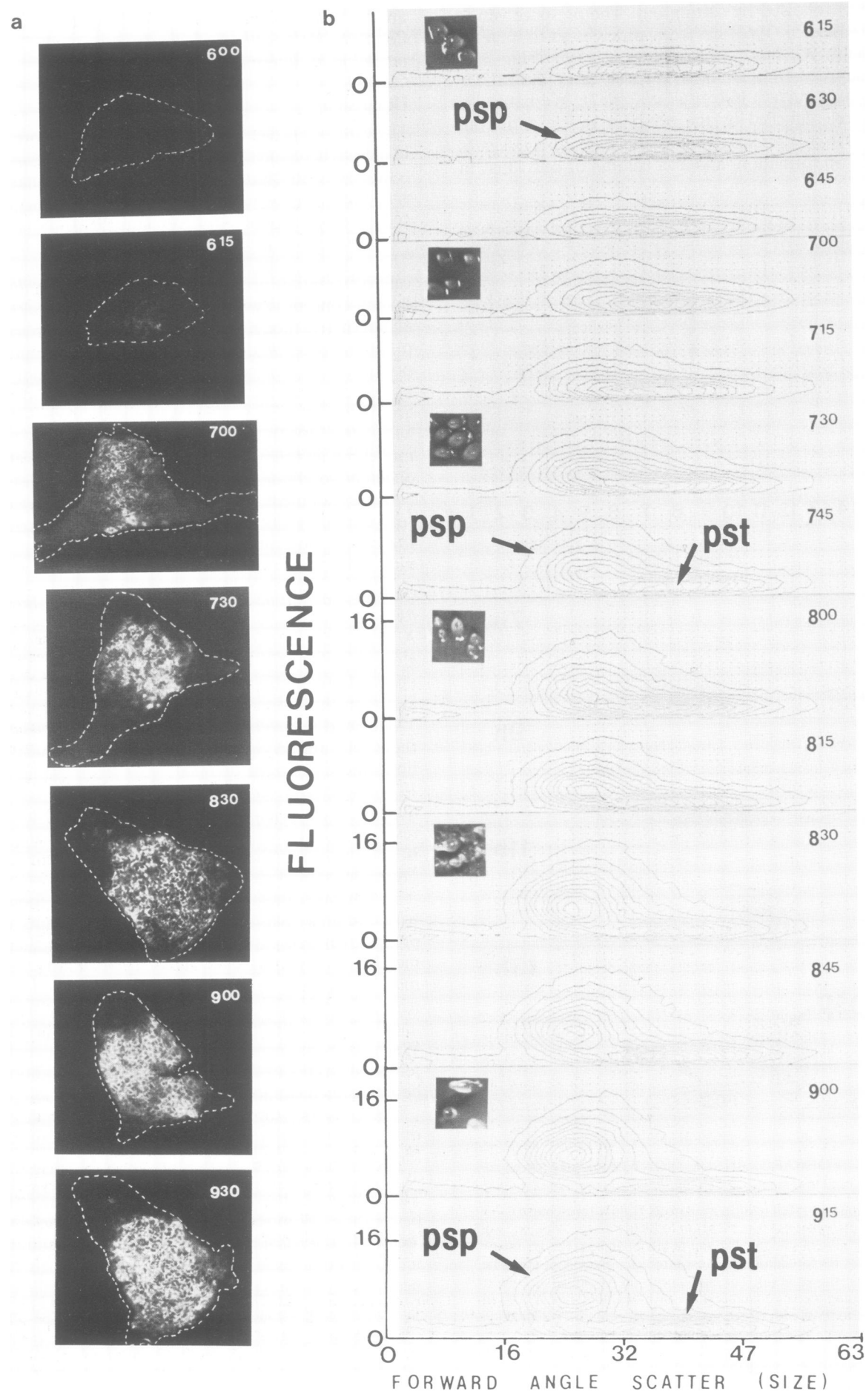
The evidence presented here shows that prespore cells appear in a discrete region of the *D. discoideum* aggregate and not as a random distribution. This is based on two markers of prespore cells: monoclonal antibody MUD1 which recognises a cell surface antigen (Krefft *et al.*, 1983) and an antiserum which recognises prespore vesicle antigen(s) (Takeuchi, 1963). This argues strongly that in the *D. discoideum* aggregate, cells differentiate according to their position in the aggregate. Such a concept is widely held in developmental biology, but an alternative model has been proposed for pattern formation in *D. discoideum* development. This model involves non-positional differentiation followed by sorting out of differentiated cells (Leach *et al.*, 1973; Tasaka and Takeuchi, 1981; Sternfeld and David, 1981; Morrissey, 1982; Schaap, 1983; Meinhardt, 1983). The reasons for accepting the 'sorting out' model are as follows. Firstly, mixtures of cells grown under different conditions or of different genotype sort out during early development. Secondly, disaggregated slug cells clearly sort out to reform the prestalk-prespore pattern. Thirdly, theoretical objections have been raised to the positional model. Here we discuss each of these points.

Sorting out of different types of cells during early development

A key experiment for the sorting out model is one in which glucose grown (G^+) and non-glucose grown (G^-) cells are mixed. Initially the aggregate has a random mixture of cells and then they sort to preferred regions (Leach *et al.*, 1973; Tasaka and Takeuchi, 1981; Meinhardt, 1983). Biochemically these cells are very different; e.g., G^+ cells have at least 10 times more glycogen than G^- cells (Hames and Ashworth, 1974). Moreover, G^- cells make more stalky fruiting bodies than G^+ cells (Garrod and Ashworth, 1972) and are more responsive to cAMP signals (Inouye and Takeuchi, 1982). It is not surprising that cells with such different biochemical properties should sort out.

In principle, these results are analogous to those observed with mutants that sort out, and MacWilliams (1982) has presented evidence that one of his mutants has altered glucose metabolism. A mutation which triggers earlier development may be expected to favour the prestalk region when mixed with wild-type cells. Such preferences are observed (Smith and Williams, 1980; MacWilliams, 1982; Morrissey, 1982). We believe that experiments with mixed cells, either of different genotypes or from different growth conditions, should be interpreted cautiously.

There is no direct evidence, with currently available markers, that differentiation is initially random followed by the sorting out of cells. At the late aggregation stage (Figure 4) the data are clear but have been widely misinterpreted. Takeuchi *et al.* (1982) have stated that prespore cells 'first appear in the cell mound which is about to form a tip and are located in the basal region of the tip'. This claim is in agreement with our results (Figure 4). However, their results have been widely misinterpreted to indicate that cells at first differentiate randomly and then sort out (e.g., Meinhardt, 1983; Morrissey, 1982; Schaap, 1983). To our knowledge the photographs that we present are the first to show prespore differentiation at these very early (and fragile) stages of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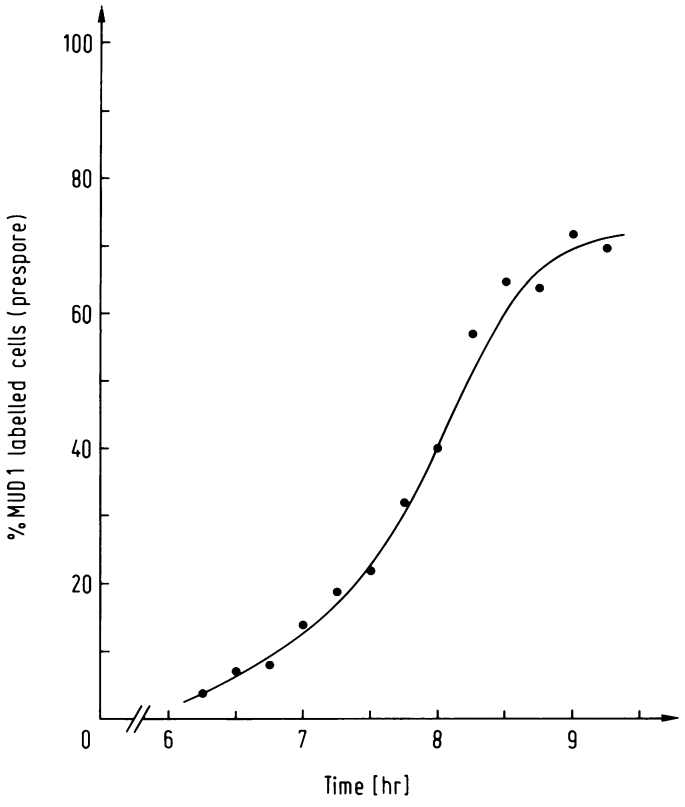

Fig. 5. Percentage of cells labelled by MUD1 (prespore cells) in *D. discoideum* aggregates from 6.25 to 9.25 h. These data show the percentages of prespore cells calculated from the flow cytometer experiment shown in Figure 4.

development.

The only possibility left is that sorting out occurs prior to tip formation, but the presently available markers for distinguishing prespore and prestalk cells are not adequate to test such a hypothesis.

Sorting out of disaggregated slug cells

It is many times documented that slug cells sort out (Durston and Vork, 1979; Sternfeld and David, 1981). This is true of many other disaggregated cell systems involving already heterogeneous cells (Moscona, 1974; Garrod and Nicol, 1981), and is no evidence against a model involving positional information in the formation of differentiated cells. Prestalk cells differ from prespore cells in many ways, including the fact that prestalk and prespore cells are differentially chemotactic to cAMP (Matsukuma and Durston, 1979; Sternfeld and David, 1981). Evidence is accumulating (Krefft *et al.*, 1983) that prestalk and prespore cells differ in cell surface antigens. Sorting out is neither surprising nor unexpected among cells carrying different surface antigens. Sorting out might have a function in sharpening the boundary between the cell types.

Position-dependent change of prespore cells to prestalk cells at the prespore-prestalk boundary has now been observed in most slime mould species (*D. mucoroides*, Gregg and Davis, 1982; *Polysphondylium pallidum*, Hohl *et al.*, 1977; *D. minutum*, Schaap *et al.*, 1983) including *D. discoideum* (Schaap, 1983).

Theoretical objections

It has been pointed out that slugs are too long (1 – 2 mm) to allow time for a positional gradient to be established during the period in which patterning occurs (Morrissey, 1982). However, at the time of pattern formation, the aggregate, which has formed the tip, is a slightly flattened sphere whose diameter is never greater than 0.3 mm (Figure 4). Such dimensions are quite compatible with the establishment of a positional gradient within a few hours (Voet, unpublished).

Time of proportion regulation in D. discoideum

The time at which proportions are established is different for positional and non-positional mechanisms. The non-positional sorting-out mechanism proposes that aggregating cells are already differentiating in streams before the tip, to which the prestalk cells move, is established, whereas in the positional mechanism the proportioning occurs in response to the establishment of a tip. The kind of molecules involved and their ranges are different in the contrasting mechanisms (see Meinhardt, 1983; Kay and Jermyn, 1983).

Materials and methods

Growth and development of D. discoideum

Either V12-derived strain NP84, or NC4-derived strain X22 was grown on SM agar in association with *Klebsiella aerogenes* as described previously (Gregg *et al.*, 1982). Amoebae were collected from agar plates just before all bacteria were consumed (3 x 10^8 amoebae/plate), centrifuged free of bacteria, and 5 x 10^7 amoebae deposited on a 4.7 cm black Millipore filter (HABG 04700) which rested on a 7 cm Whatman No. 17 filter impregnated with LPS buffer (Stenhouse and Williams, 1977). To obtain highly synchronised development, a second Whatman No. 17 filter, soaked in 1 M sodium/potassium phosphate buffer pH 6.0, was fastened to the lid of the Petri dish. The filter in the lid was exchanged every 2 h and the LPS-containing filter was exchanged every 6 h (Newell *et al.*, 1969). A series of such filters were incubated at 21 ± 1°C in dim light. Zero time was taken as the time that the filters were placed at 21°C. Never more than 30 min elapsed between placing cells on the filter and transferring them to 21°C.

Sample preparation for flow cytometry

At intervals of 30 min (or in some cases 15 min) a single plate was removed from 21°C and a 3 x 3 mm square of Millipore filter was excised. The square bearing the slime molds was transferred to a 0.7 ml Eppendorf centrifuge tube containing 0.2 ml 0.15% w/v papain and 5 mM cysteine in 50 mM Tris-HCl pH 7.0. This treatment does not affect cell labelling with MUD1 (Voet *et al.*, 1984). After a 10 min incubation the sample was washed and treated with 0.1 ml of a 1:100 dilution of prespore monoclonal antibody MUD1 ascitic fluid and 0.1 ml of 1:40 diluted goat anti-mouse IgG $F(ab')_2$ (Code 4350 Medac, Hamburg). After 30 min incubation on ice, samples were analysed directly in a flow cytometer (model FACS-IV, Becton Dickinson, Sunnyvale, CA) at ~1000 cells/s (Krefft *et al.*, 1983). The data analysis techniques used to determine the percentage of prespore cells have been described elsewhere (Voet *et al.*, 1984).

Frozen sections

At the same time that the samples were taken for flow cytometer analysis, a photograph of a representative field was taken (Agfa Ortho 25 Professional film) and several sections of Millipore filter were transferred to 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4).

After 4 h fixation at 4°C, the paraformaldehyde was removed and the fixed aggregates were stored in phosphate buffer at 4°C until they were sectioned on the following day. Five micron sections were cut as described previously (Gregg *et al.*, 1982) except that the aggregates were manipulated so that they were lying at right angles to the coverslip before being transferred to the frozen stage. This produced longitudinal sections through aggregates. The

Fig. 4. Detailed time course of the period during which the MUD1 antigen first appears on prespore cells. This sequence is illustrated by **(a)** 5 μm frozen sections of aggregates stained with MUD1 and goat anti-mouse IgG $F(ab')_2$–FITC. The white dotted line shows the outer contour of the section which is not easily visible when cells are unlabelled. Individual cells are ~10 μm in diameter. **(b)** Parallel contour plots of data from the flow cytometer. The small pictures at the left of **(b)** show the morphology of the aggregates taken for flow cytometry. Note that in this experiment tip formation occurred at ~6.25 h; psp = prespore cells, pst = prestalk and other unlabelled cells.

glass slides were dipped at 60°C in a Millipore filtered gelatin-chromate solution containing 0.5 g gelatin in 250 ml of hot distilled H_2O and 0.5 ml of 10% $KCr(SO_4)_2.12H_2O$. The slides were baked at 100°C for 1 h. This treatment was more effective than agarose (Gregg *et al.*, 1982) in promoting adhesion of the sections.

Sections were indirectly stained with monoclonal antibody MUD1 and FITC-coupled (Medac) or rhodamine-coupled (Medac) goat anti-mouse IgG $F(ab')_2$ or with FITC-coupled polyspecific anti-spore serum obtained from Professor I. Takeuchi (Gregg *et al.*, 1982).

Immunoblots

At the same time that the samples were taken for flow cytometer analysis, $\sim 10^7$ cells were removed from a quarter of a Millipore filter, treated for 10 min with 0.15% papain + 5 mM cysteine and centrifuged twice to remove the enzyme. The cell pellet was extracted with 30 μl of 2.5% w/v SDS and 5% w/v mercaptoethanol at 100°C for 5 min. From this sample, 20 μl (containing the extract of $\sim 7 \times 10^6$ cells) from each developmental stage (0 – 14 h) was applied on a discontinuous SDS-PAGE (Laemmli, 1970) employing 15% resolving and 4% stacking gels cast in 11.5 x 13.5 x 0.1 cm slabs. Immunoblotting was as described by Towbin *et al.* (1979), in which proteins were electrophoretically transferred to nitrocellulose sheets (Schleicher and Schüll, BA85), and incubated first with 1:100 dilution of MUD1 ascitic fluid, followed by peroxidase-conjugated staining using 1:1500 dilution of goat anti-mouse IgG peroxidase (Medac, code 6450) and visualised by incubation with 0.05% w/v diaminobenzidine and 0.01% v/v H_2O_2 in 15 mM Na_2/Na-phosphate buffer (pH 6.5).

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