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Development in one dimension: The rapid differentiation of *Dictyostelium discoideum* in glass capillaries

(cellular slime mold/green fluorescence protein/prestalk/prespore/alkaline phosphatase)

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ABSTRACT **When *Dictyostelium discoideum* cells are drawn into a fine glass capillary, they rapidly begin the first steps toward the formation of prestalk and prespore zones. Some of the events occur within a minute or two, whereas others follow later. The cells in the front segment are actively motile and those in the hind segment are passive. The volumes of the segments are proportional for different-sized cell masses, and those proportions are the same as those found in normal slugs. When the cells are stained with the vital dye neutral red, the anterior zone becomes darker simultaneously with the formation of the division line. Green fluorescent protein expressed from a stalk-specific promoter is synthesized mostly in the anterior end. Later, this capillary prestalk zone shows a sharp increase in alkaline phosphatase activity, which is known to be characteristic of prestalk cells.**

Quite by accident it was discovered that if one punctured a migrating slug of *Dictyostelium discoideum* with a small capillary, the cells would rise up into it by capillarity. Here we show that cells in the capillary rapidly differentiate into two zones, an anterior zone whose characteristics are very similar to those found in migrating slugs, and a posterior zone whose size and cell morphology mimic the prespore zone.

Cells in the anterior zone regulate two gene products, both spatially and temporally, in a fashion that mimics the intact slug. Moreover, cells in this region look and behave like prestalk cells: they are motile, contract, and elongate periodically as they move, and many of them follow spiral trajectories. The ratio of anterior to posterior cells in these capillaries measured over a wide range of lengths is very similar to data in the literature for normal slugs.

Our results are of interest from a methodological point of view because the preparation we describe allows one to study cell differentiation and proportioning under circumstances where the object does not move and where virtually all cells can be followed by optical sectioning. Perhaps more interesting, the rapidity with which undifferentiated cells change their morphology and set up clear and well-proportioned zones challenges our current understanding of morphogenesis in the cellular slime molds.

MATERIALS AND METHODS

Strains. The basic experiments were done with *D. discoideum* NC-4 grown on *Escherichia coli* B/r or with transformants of *D. discoideum* Ax3k transformed with vectors carrying the green fluorescent protein (GFP) gene (1) under the control of the *ecmB* (2) or *actin-15* (3) promoters. The transformants were propagated on *E. coli* B/r-1 (4) with G418 (20 μg/ml) under standard conditions (3, 4).

Capillaries. Capillaries were made by drawing out Pasteur pipettes heated over a small flame so that their internal diameters were between 20 and 170 μm. One end of the capillary was pushed gently into a slug or an aggregation center, and a plug of cells soon rose into it by capillarity. The end of the capillary with the cells was then placed in a drop of heavy mineral oil on a slide, with the other end open to the air. In a matter of seconds the cell plug moves up the capillary, pulling the oil behind it. When it was well up the lumen, the air end of the capillary was broken under the oil with a small knife and covered with a coverslip. In this way the capillary was surrounded by oil, and inside there were three compartments: air, cells, and oil.

Fixing and Staining. For these procedures the capillary was not removed from the main portion of the Pasteur pipette. At the end of the experiment the contents of the capillary were blown out into 4% paraformaldehyde in 25 mM Tris·HCl buffer (pH 8.0). To stain the cells with neutral red, a lawn of *E. coli* B/r was just covered with a solution of neutral red (7.5 mg/100 ml) and allowed to dry. Loopfuls of these bacteria were then spotted on 2% agar plates and inoculated with *D. discoideum* NC4 spores. To detect alkaline phosphatase, cell cylinders were blown out into fixative, incubated for 15 min at room temperature, washed twice with AP buffer (100 mM Tris·HCl/100 mM NaCl/5 mM $MgCl_2$, pH 9.5), and stained at room temperature for 1–2 hr in 5-bromo-4-chloro-3-indolyl phosphate (2.5 mg/ml in AP buffer).

Microscopy. Cells and capillaries were observed directly under a microscope with a ×40 objective lens attached to a time-lapse video camera. Capillaries were also imaged with a confocal microscope (Bio-Rad MRC600) using the 488-nm line of an argon/krypton laser for excitation and an LP510 emission filter (3). The center of each capillary was optically sectioned in three sections 5 μm thick and the three images were projected to form one (about 20% of the capillary inside diameter).

RESULTS

General Description of the Capillary Experiment. No matter what the age of the slug, or what part of a slug the cells come from, in less than 1–2 min there will be two distinct bands of cells with the smallest one next to the air bubble. Under the compound microscope it is clear that the bands of cells differ and the cells nearest the air (anterior) adhere closely to one another, while the cells nearest the oil (posterior cells) become rounded and somewhat separate from one another. The division line between the two groups of cells is extraordinarily sharp (Fig. 1*A*).

Cells within the anterior band move dramatically, while cells within the posterior segment are virtually motionless, except for a few cells at the very posterior end, at the cell–oil interface. The motion of the cells in the anterior portion consists of great swirls, which is consistent with the helical movement described by Siegert and Weijer (6). This may be clearly seen in Fig. 1*B*, where a small percentage of the cells

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Abbreviation: GFP, green fluorescent protein.

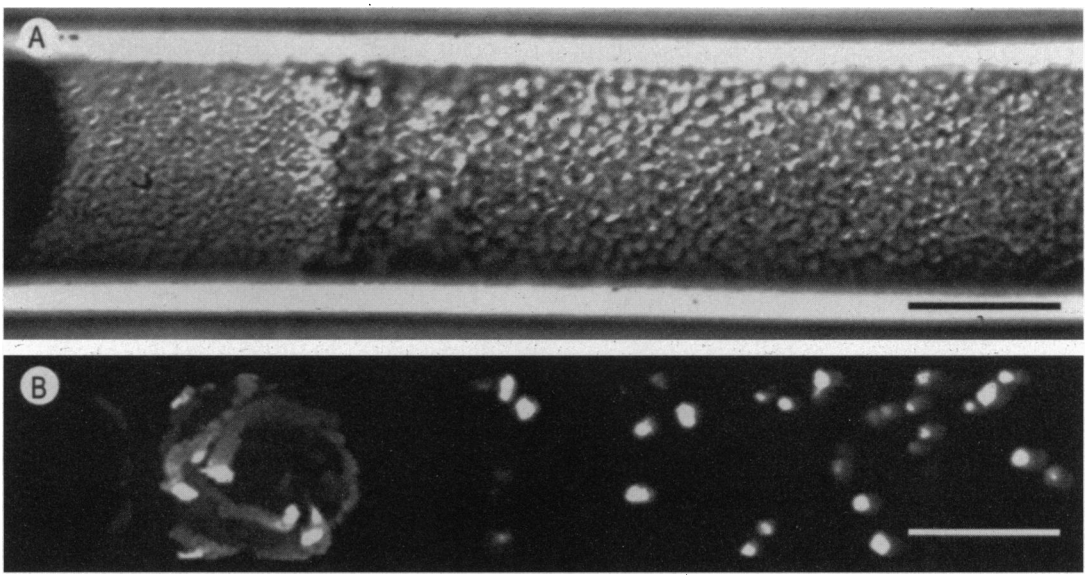

FIG. 1. Anterior ends of capillaries containing cells from migrating slugs of *D. discoideum*. The air bubble is at the extreme left of both capillaries, and the other ends (which cannot be seen) are in contact with mineral oil. (*A*) A view with Nomarski optics showing the cell structure of the two zones and the sharp division line between them. (*B*) Another view showing the difference of the cell movement in the two zones. Approximately 3% of the cells have been labeled with GFP under the control of a constitutive promoter, actin-15, and followed for over 2 hr by confocal microscopy. Successive images have been overlaid, so that each cell leaves a comet-like track of increasing brightness. The fluorescent posterior cells do not move. Frame interval = 2 min; bar = 50 μm.

have been labeled with GFP expressed from a constitutive promoter, *actin-15*, and followed over the course of 2 hr by confocal microscopy. Posterior cells do not move and in appearance are quite distinct from anterior cells (Fig. 2).

It is possible to see clear pulses in a number of instances by the use of time-lapse video microscopy. They are evident at the junction between the anterior and posterior bands as changes in density that move posteriorly. The mean period between pulses is 5.5 min (average of four cases) with a range of 4.7–6.0 min, which is in keeping with what Durston and Vork (7) found for normal slugs.

We emphasize that this two-zone configuration arises very rapidly inside the capillary, and its polarity is invariant regardless of the cell source: the cells can come not only from different regions of a mature slug, but from an immature slug that has not yet migrated or formed two zones when stained with neutral red. This occurs even when one places a band of prespore cells near the air bubble and puts tip cells near the oil. The only difference in this case is that there is initially some motion in the posterior portion, but it soon dies down; the anterior band appears, and the swirling activity starts in the anterior, even though the cells were prespore cells a few minutes before. In addition, if starved pre-aggregation amoebae, or even actively feeding vegetative amoebae, are washed and compacted by centrifugation and drawn up into a capillary, they will also quickly produce two zones.

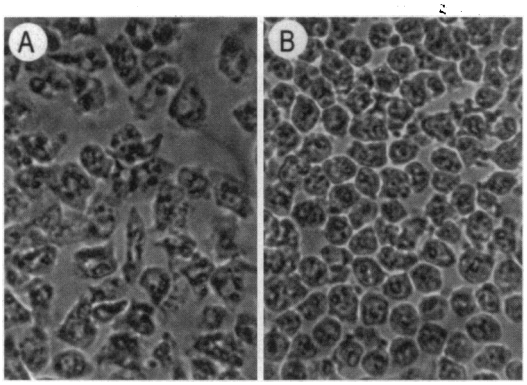

FIG. 2. Cell shape. The contents of a capillary were ejected into a macerating solution [acetic acid/glycerol/water, 1:1:13 (vol/vol)]. (*A*) Anterior cells which are elongate and show many pseudopods. (*B*) Rounded posterior cells. Average diameter of the cells in *B* is ≈4.5 μm.

In another set of experiments air was placed on both sides of the cells in the capillary. In such a case an anterior zone forms immediately at both ends of the cell mass. If the length of the mass is short, one of the zones will slowly decrease in size and within 1–2 hr disappear. If the cell mass is long, both anterior zones remain up to 4 hr, which is the limit before anterior cells cease moving.

If cells are stained with neutral red, it is possible to fill the capillary with bands of red cells and colorless ones, providing a useful method to see whether cell sorting occurs. When the red cells from the anterior tip of a mature slug (and therefore entirely made up of prestalk cells) are placed in the posterior end of a capillary, and colorless cells from the prespore zone of another slug are put in the anterior end, near the air bubble, the division line appears rapidly in the normal fashion at the anterior end. A few of the prestalk, posterior red cells will move anteriorly, but this does not occur until after 2–4 hr. Clearly some cell sorting occurs, but it has no bearing on the rapid formation of zones.

Anterior/Posterior Proportions. Do the two zones described above reflect the beginning of new prestalk/prespore zones, such as is found in normal development? If one measures the volume of both segments of different size cell masses in capillaries of different diameters, one can demonstrate that anterior/posterior volumes are proportional over a large size range (Fig. 3, filled circles). In 35 cases the slope is close to 1, although for each the posterior segment is larger than the anterior one. When this is compared with prestalk/prespore ratios from intact slugs with vital dyes as a marker (Fig. 3, open circles) it is evident that the two sets of data are essentially identical.

Neutral Red Zonation. When cells from the prespore region of young slugs previously stained with neutral red are drawn up into a capillary and quickly placed under a microscope and viewed with Köhler illumination, the division line between the two zones appears simultaneously with the appearance of a darker pink tint in the anterior zone. This transition occurs

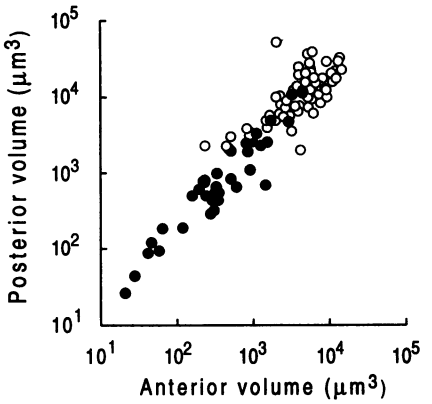

FIG. 3. Full logarithmic graph in which the volumes of the posterior zones from 35 capillaries are plotted against the volumes of their anterior zones (●). These are compared with the volumes of prestalk and prespore regions of 67 intact slugs stained with a vital dye (○). The latter data come from ref. 8. The slope of the regression line for the combined data is 1.038.

within a minute. Since neutral red is known to be a pH indicator, one is led to assume that the cells in the anterior segment have undergone a rapid decrease in pH. This neutral red result will only occur if the cells are from immature, evenly stained slugs; if prespore cell are taken from mature, two-toned slugs, there will be no immediate change in the color of the anterior zone.

All the subsequent experiments reported here were also done with either very young slugs, or aggregation centers. It is obviously important to use cells that have not yet undergone any differentiation if one wants to follow differentiation from the beginning.

Regulation of a Stalk Cell-Specific Promoter. The most telling experiment to support the idea that prestalk–prespore differentiation is occurring in the capillary would be to demonstrate the synthesis of a protein specific for stalk or prestalk cells and show that its synthesis occurs in the anterior zone. This we have done by using a strain of *D. discoideum* transformed with a GFP reporter gene under the control of a promoter specific for stalk and other stalk-related cells, *ecmB*.

As Fig. 4 shows, GFP is expressed in stalk cells and in the cells that will form the upper and lower cups of the mature spore head. This is the known distribution of *ecmB* promoter activity at this developmental stage (9). We have placed cells of this transformant into capillaries and followed them by confocal microscopy. Because there is a certain amount of autofluorescence in the cells (especially in the anterior zone), an untransformed control has also been put in a capillary and laid alongside the experimental one. By 10 min (the minimum time required to set up the preparation), it is clear that in the transformed cells there is more fluorescence in the anterior zone than in the control, and with time there is a steady increase, with no change in the control. In each of four independent experiments, the increase in fluorescence was confined chiefly to the anterior zone, with a few cells in the posterior (Fig. 5). Fluorescent cells in the anterior zone moved actively from one part of the zone to another, while those in the posterior moved no more than one or two cell diameters during the course of these experiments. The data from one experiment, during which cells were followed at 2-min intervals for a period of 120 min, is summarized in Fig. 6. Note that GFP synthesis continues linearly during this time and that there is no change in autofluorescence in the anterior cells of the control.

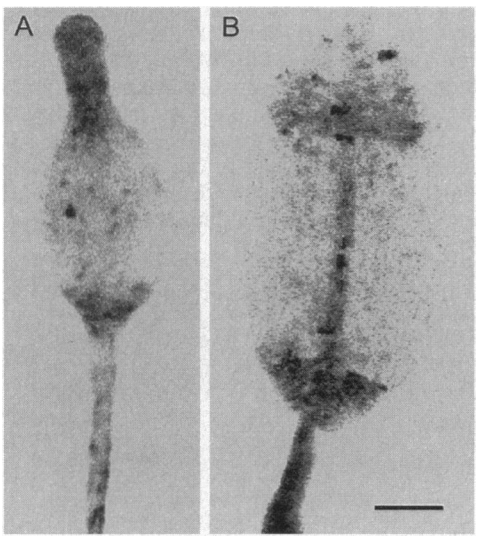

FIG. 4. Two *ecmB*/GFP developing fruiting bodies. Negative images show GFP in stalk cells and upper and lower cups of culminating sorogens in a late (*A*) and mid (*B*) culminant. (Bar = 50 μm.)

Alkaline Phosphatase Distribution. By using capillaries that remain attached to the Pasteur pipette it is possible to hold the cells in the capillary for varying periods of time and then blow them out into fixative and stain them for alkaline phosphatase. Staining increases with time: the longer the cells are held in the capillary before they are ejected into the fixative, the more intense the stain. After 3 min some anterior stain is evident; after 1 hr it is strong (Fig. 7). If the cells are taken from the anterior, prestalk regions of mature slugs the entire cylinder of cells stains for alkaline phosphatase. This is expected, since the cells have already partially differentiated.

DISCUSSION

We have evidence that cells inside a small capillary begin to differentiate very rapidly and therefore might provide a powerful method to study the initial molecular events that lead to spore and stalk cell differentiation.

The first and most striking phenomenon is how fast polarity is set up: the anterior portion is established in under a minute, regardless of the source of the cells. The polarity might have been predicted from the work of Sternfeld (10, 11), who showed that O_2 both attracted amoebae and influenced them in the prestalk direction. It is quite reasonable to assume that the O_2 gradient in the capillary is paramount in establishing the polarity of the cells within the tube.

Once the polarity and the division line have been established in the capillary, there is clear evidence that the anterior cells are on their way to become the prestalk zone—in fact, everything we have done so far fits this interpretation. The most compelling evidence comes from the use of the GFP gene regulated by the *ecmB* promoter. When this promoter is fused to the β-galactosidase gene, the enzyme is expressed primarily in stalk cells, a subset of anterior-like cells with the potential to become stalk cells, and cells in the upper and lower cups of culminating sorogens (Fig. 4) (9). No such massive synthesis occurs on the posterior side of the sharp division line [although there are a few cells that become bright, as one can see in Fig. 5, that might be anterior-like cells (9)].

One puzzling fact is that in cells transformed with an *ecmB*/β-galactosidase fusion gene, the protein is not normally expressed significantly in the prestalk region of normal slugs but appears largely at culmination. That it does appear so rapidly in the capillary might be explained by an old observation of Farnsworth (12), who showed that when amoebae were put into a small cellulose tube they differentiated directly into mature stalk cells. Perhaps confinement hastens the entire differentiation process.

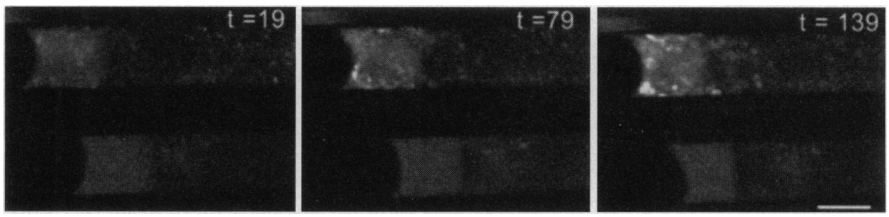

FIG. 5. Time sequence of a capillary containing cells with a GFP reporter gene under the control of the promoter for the *ecmB* gene (whose protein product is found specifically in prestalk and anterior-like cells), as viewed in the confocal microscope. The upper three panels show the experimental capillary; the lower three are controls which were run simultaneously in the same microscopic field. All the cells were taken from aggregation centers (often in the process of forming slugs). Note that there is some fluorescence in the control, but that it remains constant over time, while the tagged cells show a steady increase in fluorescence, as can also be seen in Fig. 6. Time (t) is in minutes. (Bar = 50 μm.)

Additional supporting evidence that the anterior zone in the capillary is prestalk comes from the distribution of alkaline phosphatase. It has been known for a long time that the prestalk region of *D. discoideum* is uniquely high in this enzyme (13, 14). We show the identical result in our capillaries: high levels of the enzyme are found only in the anterior zone.

It is important to point out here that the *de novo* appearance of alkaline phosphatase or GFP in the anterior zone of the capillary can be demonstrated only when the cells come from early stages of development—either from aggregation centers or from beginning slugs. If the cells are from mature slugs that already show prestalk and prespore regions, they will of course show the staining properties characteristic of their source, since both GFP and alkaline phosphatase were present in the transplanted cells.

Despite this fact, and regardless of where the cells come from or their age, the surprisingly rapid formation of the sharp division line between correctly proportioned cell masses invariably occurs. The phenomenon of forming an anterior and a posterior zone, the anterior one with very active, motile cells compared with the posterior cells, is clearly a step that precedes all subsequent differentiation, since it occurs in the first minute. Not only is the division line sharp and the differences in behavior of the cells rapid in their appearance, but it is remarkable that the proportions of their volumes also are established quickly, and those proportions are the same as one finds in normal slugs migrating on an agar surface.

This rapid formation of a division line giving proportionate anterior and posterior segments raises two related problems. First, how can a sharp and correctly proportioned division form so quickly? And second, how can cells respond equally rapidly by changing their shape and motility?

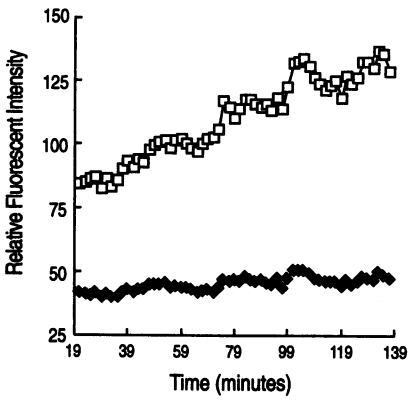

FIG. 6. Graph comparing the change in the intensity of the fluorescence of the anterior zones during the experiment shown in Fig. 5. The open squares come from the experiment with the GFP reporter gene; the filled diamonds are from the control. Fluorescence was measured from confocal images taken at 2-min intervals. Each image was a composite of three focal planes taken through the capillary.

The anterior zones in Fig. 5 are $\approx$50 μm long. Since the prestalk-like zone always forms at the end nearest the air bubble, it is reasonable to assume that the initial polarity is determined by an O_2 gradient. Diffusion coefficients for small molecules, such as O_2 and NH_3, are $\approx 10^{-5}$ cm^2·sec^{-1}, and thus the transit time τ for molecules of this size diffusing in one dimension, $L^2/2D$ (15), is $\approx$1.25 sec. If the diffusion coefficient were restricted to $\approx 10^{-6}$ cm^2·sec^{-1}, the transit time would rise to $\approx$12.5 sec. Both these times are well within the limits we observe, and thus the boundary between anterior and posterior could in principle be set up by O_2 gradients—the distances and times are not too great for reasonable assumptions about diffusion coefficients.

However, if the boundary is determined by a simple gradient, one would expect the length of the anterior zone to be the same in all the capillaries, but this is definitely not the case: the anterior zones vary in length from 25 to 140 μm. Thus we can rule out a simple model involving passive diffusion from a source. It is more plausible to assume that a reaction diffusion mechanism underlies boundary formation, since it is straightforward to model sharp boundaries between two cell masses in terms of reacting and diffusing activators and inhibitors (16). Even so, the synthesis (or release) of activator and inhibitor must be very rapid.

The rapid changes in cell shape and motility associated with boundary formation also require an explanation. It is now clear that GTPase cascades can rapidly trigger changes in cell shape in response to external cues (reviewed in ref. 17). Indeed, platelets, completely lacking a nucleus, change shape in response to phorbol esters in a matter of seconds (18). Perhaps also some of the central initial events are mediated by translational control of prestalk messenger RNA or by the mobilization of proteins required to modify cell response.

There are two cases of rapid change in *Dictyostelium* differentiation that we are aware of, and they might possibly involve the same phenomenon. If young slugs stained with neutral red are submerged under mineral oil, they will (presumably because of the decrease in O_2) within minutes go from a uniformly stained condition to one in which the posterior portion blanches to form a slug with a dark red prestalk region and a faintly stained prespore region (see figure 2 in ref. 19).

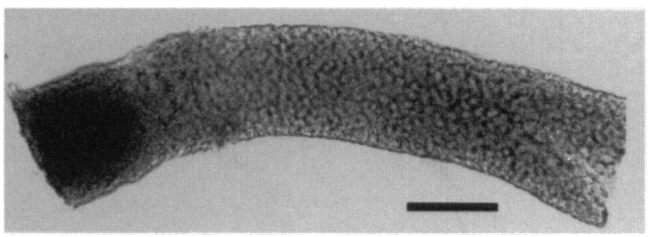

FIG. 7. Distribution of alkaline phosphatase in the cells in a capillary. These cells were in a capillary for 1 hr and then blown out into fixative and stained (see *Materials and Methods*). Note that only the anterior zone stains positively for alkaline phosphatase. (Bar = 50 μm.)

Here, as in a capillary, the sudden reduction in O_2 tension seems to hasten differentiation. The other case is from the work of Gregg and Davis (20), who provided evidence for very rapid effects of position on differentiation in *Dictyostelium mucoroides*. Prespore cells specifically pick up labeled fucose, and when cells move from the prespore to the prestalk zone at the sharp juncture between the two zones, they rapidly, in a matter of 1.5 min, begin to lose the ability to incorporate fucose, as well as losing their prespore vacuoles and other prespore characters. So biochemical changes involved in early differentiation can occur quickly, depending upon the environment of the cells.

There is another conclusion that can be drawn from our experiments. It is generally agreed that differentiation in *D. discoideum* is a composite of two components: (*i*) the sorting-out of cells with stalk or spore tendencies to appropriate regions in the slug and (*ii*) positional information—the anterior region induces prestalk cells, while the posterior region induces prespore differentiation (reviewed in refs. 5 and 21). The positional component is essential to explain the control of proportions between stalk and spore cells and between prestalk and prespore cells. This is confirmed by the results reported here, for regardless of size, the proportions are established very rapidly, and while it is possible to show that there is some sorting-out of the cells in the capillaries, it is not as extensive or as rapid as in normal slugs. Clearly position can overrule sorting-out.

Placing cells in a capillary puts development under a constraint, and surprisingly, the constraint produces a number of advantages. First of all one can readily see the cells begin their differentiation, something that has not been easy to do heretofore. Second, development is in one dimension, which greatly simplifies its analysis. Third, because of the positioning of the cells between air and mineral oil, one can impose polarity that immediately overrides any previous polarity that might have existed. The fourth advantage is that since one gives all the cells an instantaneous start on their road to differentiation, it will be possible to study the timing sequence of early differentiation. For all these reasons the capillary method may be useful to dissect out the molecular causal sequence leading to further development.

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