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Analysis of cell movement during the culmination phase of *Dictyostelium* development

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MPEG Video sequences of some of the described experiments can be viewed via the following Internet address: http://www.zi.biologie.uni-muenchen.de/zoologie/dicty/dicty.html

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SUMMARY

Co-ordinated cell movement of tens of thousands of cells and periodic signals characterise the multicellular development of the cellular slime mould *Dictyostelium discoideum*. We investigated cell movement by analysing time-lapse video recordings made during the slug stage and the culmination phase of *Dictyostelium* development. Slugs viewed from the side showed an even, straight forward movement with the tip slightly raised in the air. Slugs, that had migrated for a prolonged period of time either culminated or showed a behaviour best described as abortive culmination. Culmination is initiated by a local aggregation of anterior-like cells at the base of the slug at the prestalk-prespore boundary, where they form a stationary mass of cells. Prespore cells continue to move forward over this stationary pile and, as a result, are lifted into the air. The stationary group of anterior-like cells thereby end up to the back of the slug. At this point the slug either falls back on the agar surface or continues culmination. If the slug continues to migrate these cells regain motility, move forward to the prespore-prestalk boundary and form a new pile again. In the case of culmination the neutral red stained cells in the pile move to the back of the slug and form a second signalling centre beside the tip. Both centres are characterised by vigorous rotational cell movement. The cells belonging to the basal centre will form the basal disc and the lower cup in the fruiting body. The upper cup will be formed by the prestalk cells rotating most vigorously at the prestalk-prespore boundary. The remaining neutral red stained anterior-like cells in the prespore zone sort either to the upper or lower organising centre in the fruiting body.

Key words: cell movement, morphogenesis, culmination, chemotaxis, digital image processing, *Dictyostelium discoideum*

INTRODUCTION

During the slug and culmination stages the slime mould *Dictyostelium discoideum* shows exceptionally complex co-ordinated cell movements. In the slug, 10^5 single cells behave as a single organism, which shows thermo- and phototactic reactions. The slug moves at a relatively constant speed towards light and initiates culmination at the surface of the forest floor, where conditions for spore dispersal are much more favourable than below the leaf litter (Loomis, 1982). During culmination, the slug forms a fruiting body 200-500 times higher than the diameter of a single cell. A stiff stalk consisting of vacuolated, dead cells surrounded by a stalk sheath forms from prestalk cells which are located in the front of the slug. The major proportion of the former slug cells, the prespore cells in the back of the slug, move along the stalk towards the top.

Morphogenesis in *Dictyostelium* results from differential cell movement. Understanding morphogenesis therefore requires understanding the control of cell movement, especially in the slug and culmination stages (Raper and Fennell, 1952). There is increasing evidence that propagating cAMP waves and chemotaxis towards cAMP organise cell movement and differentiation during the multicellular development (Matsukuma and Durston, 1979; Siegert and Weijer, 1992, 1995; Traynor et al., 1992). Cell tracking experiments of individual labelled cells in aggregation streams and slugs showed that the cells go through periodic velocity and shape changes, which indicates that the cells move in a chemotactic fashion (Condeelis et al., 1990; Siegert and Weijer, 1991). There are several competing theories describing the way a slug moves (Bonner, 1994). According to the most recent model proposed by Siegert and Weijer (1992) based on detailed cell movement studies, the slug is organised by the same principles that control aggregation – chemotaxis and cAMP relay. Observation of cell movement in slugs showed that prestalk cells in the tip rotate perpendicular to the direction of slug migration while the major proportion of prespore cells in the back of the slug move straight forward in a coherent periodic fashion. From this movement pattern it was concluded that the prestalk cell movement is organised by a rotating scroll wave of cAMP, which serves as pacemaker for the formation of planar cAMP waves, which direct periodic forward movement of prespore cells (Steinbock et al., 1993). The prespore cells are responsible for active slug movement. A

still unresolved problem in this model is how the cells in the slug actually move, how they get their traction. Odell and Bonner (1986) proposed a model in which slug movement is described as an 'inverse fountain flow'. According to this model forward movement is achieved only by a ring of cells in the outer layer, which are able to gain traction from the substratum and the surrounding slime sheath. Cells in the middle of the slug cylinder do not participate in slug migration due to a lack of traction. This leads to a net forward flow of the outer cells towards the tip where they become imotile, leading to relative backwards movement in the inner portion of the slug cylinder ('inverse fountain'). A third model presented by Williams et al. (1986) describes slug migration as a 'squeeze-and-pull' mechanism. A group of specialised cells in the anterior part of the slug anchors firmly to the substratum to gain traction. Longitudinal contraction of the posterior cells pulls the back of the slug forward, while radial contraction of the outer cell layer squeezes the inner cell mass forward.

Since these theories are quite contradictory we decided to perform a careful investigation of cell movement in migrating slugs viewed from the side. A certain proportion of cells was labelled with vital dyes in order to observe the movement of single cells in the slug. This should allow identification of a possible backward flow of cells or the formation of adhesion zones. Furthermore we investigated the morphogenetic movements during the initiation of culmination and culmination itself. Little is known about the mechanisms and signals controlling these complicated processes (Raper, 1940; Durston, 1976; Durston and Vork, 1979; Rand and Sussmann, 1983). To investigate the behaviour of many cells simultaneously, we analysed video sequences by digital image processing and a software package described by Siegert et al. (1994), which allows the quantitative investigation of cell flow patterns. This method enabled us to quantitatively measure direction and velocity of tissue movement in all multicellular stages. Our results show that *Dictyostelium* morphogenesis is brought about by periodic signals emitted by the tip and chemotaxis towards these signals (Siegert and Weijer, 1992; Steinbock et al., 1993, Bretschneider et al., 1995) during slug migration. The prestalk region is shown to stay as an organising centre during culmination and will form the stalk as well as the upper cup. Culmination is initiated by a group of anterior-like cells at the base of the slug just behind the prestalk-prespore boundary. They form an immobile mass of cells, which directs the rest of the slug into the air. They contain both ecmA and ecmB expressing cells and are therefore the same as the cells that initiate basal disc formation in the accompanying paper by Jermyn et al. (1996). This group of cells forms by active accumulation and acts as a second signalling centre during culmination. Later these cells will form the basal disc and the lower cup in the fruiting body.

MATERIALS AND METHODS

Strain culture and labelling techniques

Axenic AX-2 cells were grown according to standard culture conditions (Sussmann, 1987). The ecmA and ecmB transformant lines were the same as described by Bichler and Weijer (1994). To initiate development the cells were washed twice in KK2 (20 mM potassium phosphate buffer, pH 6.8). The cells were labelled with the vital dye, neutral red, by incubating them in 0.06% neutral red (in KK2) for 1 minute, followed by a final wash in distilled water. Slugs were obtained by placing drops of cells (10^8/ml) on 1% water agar plates (1% Difco Bacto agar in distilled water). The plates were then incubated in the dark for 24 hours at 18°C. Slugs aged from 24 to 72 hours were used for experiments.

For whole-mount β-gal staining, neutral red stained slugs from the transformant cell lines ecmA- and ecmB-gal were transferred to nitrocellulose membranes. The membranes were fixed by placing them on thick filterpads soaked with Z′-buffer containing 1% glutaraldehyde (Bühl and MacWilliams, 1991). After 10 minutes the filters were fixed for another 10 minutes upside down in the same fixative, washed once in KK2 and photographed using Kodak Ectachrom 320 tungsten diapositive film. Photographs were taken by putting the nitrocellulose membranes on microscope slides and illuminating from below. After photography the filters were stained as recommended by Dingermann (1989) for 12 hours at 37°C and photographed again.

Video microscopy

Video films were made by placing Petri dishes with slugs or culminants on agar on a Zeiss IM 35 inverted microscope equipped with objectives of different magnifications (6.3×, LD20×). Both slugs and culminants were filmed while submerged in mineral oil as described earlier (Siegert and Weijer, 1992). This dramatically enhanced the optical properties of the tissue in light microscopy by removing unwanted light reflection from the slime sheath. Together with a diffuser screen in the light path, vitally stained vacuoles were then clearly identifiable. Furthermore mineral oil prevented slugs from a premature culmination. It was possible to observe slug migration over a time period of several hours. In order to view slugs from the side, a small agar block with a slug on top was cut out of the agar and turned on its side. Around such a tilted agar block a small rubber ring (1 mm high) was placed and the resulting cavity was filled with mineral oil and covered by an oxygen permeable ACLAR film type g3c (Allied Chemical, Morristown, NJ). Video films were made with a Hamamatsu C-2400 Silicon Intensified Target (SIT) camera adjusted to half maximal sensitivity to reduce the illumination intensity of the light. Successive video images were stored in time lapse mode (Δt = 10 seconds) on a Laser Disc recorder (Sony LVR-4000P) for later evaluation.

Digital image processing

Digital image processing was performed with a 486 66Mhz DX2 computer equipped with 1GB hard disc, 20MB RAM and an Imaging Technology AFG board (resolution 768×512 pixel). The video signal was first contrast enhanced to make use of the full dynamic range of the 8 bit digitising unit provided by the image processing board. To reduce noise in the video signal 34 video images were averaged in real time. The calculations of the vector fields were performed with an IBM RISC 6000 computer, Model 350, equipped with 32MB RAM and a 24 bit Grafic Accelerator Board. The gradient method was used to calculate the average movement at every pixel location over a sequence of 16 consecutive digitized video images (512×512 pixel), resulting in a velocity vector field (Siegert et al., 1994). The length of the vector encodes the velocity at that location. Long vectors indicate fast movement and short vectors slow movement. The length of the vectors is plotted in arbitrary units so as to give a good visual impression of both the magnitude and direction of cell movement. The velocity is calculated for every pixel in the image, however in order to reduce noise more than 500 vectors were averaged and displayed as a single vector.

RESULTS

Migration of young slugs

Observation of migrating and culminating slugs from the side

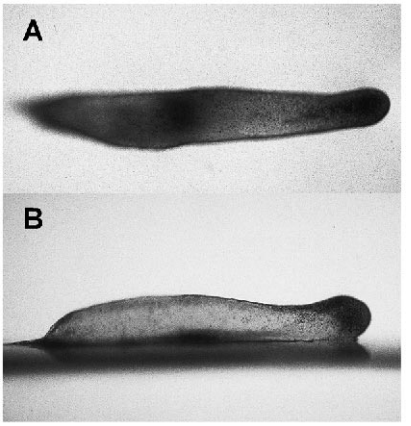

Fig. 1. Top and side view of a neutral red stained slug. (A) View from above. The prestalk zone is visible as a dark stained region to the right. Another dark stained region, showing an accumulation of neutral red stained cells, is seen in the middle of the prespore zone (×63). (B) The same slug viewed from the side. The tip is lifted into the air. The aggregate of neutral red stained cells is located at the base of the prespore zone at the interface between the slug and the substratum.

gave new insight in the mechanisms of slug migration and culmination. However, before these measurements were feasible several problems had to be overcome. In the dark, AX-2 slugs migrate for up to 48 hours and cover a distance of 3-5 cm. If these slugs are placed in the light most of them immediately start to culminate. This effect was avoided by placing the slugs under oxygen permeable mineral oil. From other experiments it is known that this procedure does not impede migration or alter slug behaviour (Siegert and Weijer, 1992). Furthermore, if not placed under oil, the tip of the slug, which is mostly up in the air, dried out too soon. In order to visualise single cell movement the cells had to be labelled by vital dyes.

Fig. 1A shows a typical slug stained with neutral red, a vital dye, which selectively stains prestalk cells in the tip and anterior-like cells in the prespore zone of the slug (Sternfeld and David, 1981). The prestalk-prespore boundary is clearly visible due to a dark staining of the prestalk cells. Furthermore there is a local aggregation of neutral red stained cells, visible in the middle of the prespore zone. A side view of the same slug shows that these neutral red stained cells are located at the base of the slug (Fig. 1B). Observation of young slugs from the side shows that normally the tip is lifted into the air most of the time with a periodic up and downlifting as observed earlier (Vardy et al., 1986). Periodicity was found to be around 10 minutes, in agreement with findings by Breen and Williams (1994). If the tip touched the agar surface slug migration slowed down for a short period and a bulge formed at the prestalk-prespore boundary. When the tip lifted in the air again slug movement speeded up and the bulge disappeared.

Cells in the prestalk zone always showed rotational cell movement perpendicular to the direction of slug migration when the tip of the slug was in the air, while at the same time all cells in the prespore zone moved straight forward in agreement with our previous findings (Siegert and Weijer, 1992). A detailed analysis of single cell movement by manual tracking of labelled vesicles showed that anterior-like cells in the prespore region moved at an average speed of 17.6±9

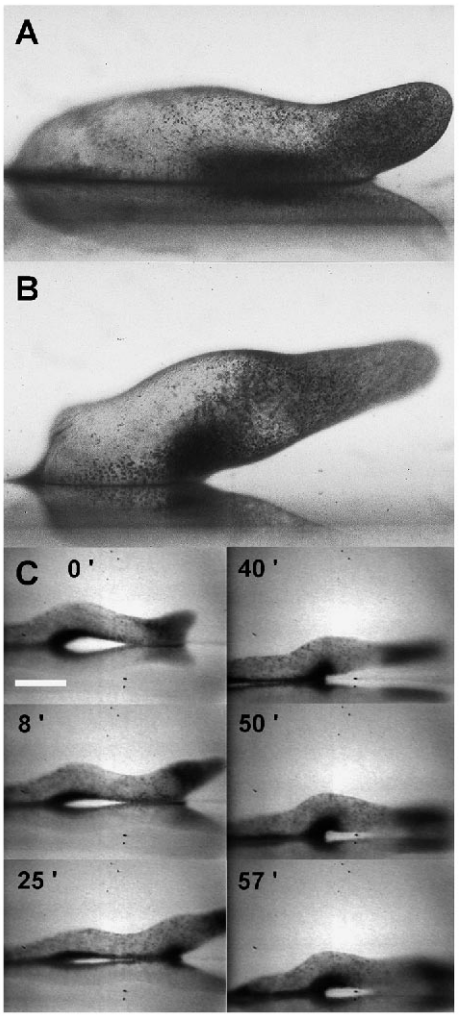

Fig. 2. Time series showing older migrating slugs viewed from the side. (A) Slug shown from the side. The tip is lifted in the air, a pile of anterior-like cells is forming at the prestalk prespore boundary (×200). (B) Same slug 20 minutes later, two thirds of the slug is already lifted in the air. The pile is located at the point of lift off. (C) Series of images of a slug at six successive time intervals. At time 0′ (0 minutes) the slug has just fallen back on the substratum still forming an arch. Note the pile at the last lift off point. At 8′ the tip starts to lift into the air again and the pile at the back is dispersing. At 25′ a new pile is forming at the place of lift off, by recruiting cells from the old pile. At 40′ the observation window was shifted to the right in order to follow slug movement. The tip is out of focus. The new pile is fully developed. At 50′ the tip has fallen down on the substratum again and an arch is formed. At 57′ the pile starts to disappear again. Scale bar: 100 μm.

μm/minute (n = 160). Periodicity of cell movement in the prespore zone was found to be about 3 minutes. Rotational cell movement was most vigorous in the prestalk zone directly adjacent to the prespore zone. We did not observe any backward flow of cells in the interior of the slug or in other parts of the slug. Also we did not observe any clear radial or longitudinal contraction movements as observed by Vardy et al. (1986).

Migration of old slugs

In old slugs (48 hours and more) neutral red staining pattern

intensified as can be seen in Fig. 2A,B. This is due to the fusion of many small neutral red stained autophagic vacuoles to form a few big ones and therefore the staining appears more visible both in prestalk and in anterior-like cells (Durston and Vork, 1979). Apart from a darkly stained tip the number of stained cells in the prespore zone was clearly increased. Most of these cells were found on the ventral side of the slug in a layer close to the agar surface (Fig. 2A). 20 minutes later more than half of the slug was lifted into the air, while the darkly stained basal layer of cells was concentrated at the point where the slug lifted into the air. This aggregation of anterior-like cells was found in 60 out of 84 slugs investigated (70%). The slug can now either culminate or continue to migrate. If the slug continues to migrate it steadily rises into the air until it falls back on the substratum. Fig. 2C shows a sequence of 6 successive video images typical for this type of slug movement. It was found in all AX-2 slugs of this age investigated (n=36). The aggregate of anterior-like cells in the prespore zone plays an important role in this process. These cells form aggregates (piles) of cells on the ventral side of the slug which once formed do not move with respect to the substratum. However at the same time the unstained prespore cells continue to migrate. The non-moving anterior-like cells thereby act like an obstacle in a fluid flow. The prespore cells crawl over this pile and the migrating slug lifts from the substratum into the air until it topples over, back on the substratum. In the course of this process the pile of cells ends up in the back of the slug. As soon as the tip touches the agar surface the pile disappears since the anterior-like cells move towards the prestalk boundary. The cells stay close to the slug substratum interface and move with a higher speed than the surrounding prespore cells. Once they reach the prestalk prespore boundary they become immotile and the whole process can start all over again.

Our results show that there are potentially two different modes of slug migration. Young slugs seem to move forward with the tip up in the air most of the time. The tip may describe a periodic up and down movement. Older slugs show a pronounced lifting in the air which is always accompanied with anterior-like cell pile formation. This latter mode of movement might represent abortive attempts to culminate. We observed many abortive culminations since under our experimental conditions slug migration is favoured. Abortive culminations are also found in old slugs migrating on agar which are not under oil therefore we do not expect them to result from partial anoxia. Supply of excess oxygen does not alter this behaviour either (unpublished observations).

Local accumulation of the anterior-like cell pile is an active process

By analysis of the movement of neutral red stained cells in the prespore zone during pile formation we could show that pile formation is an active aggregation process of the anterior-like cells. Cell movement analysis via calculation of velocity vector fields showed that anterior-like cells located on the dorsal side of the slug move straight towards the accumulation site on the ventral side at the prestalk-prespore boundary (Fig. 3A,B). The vector field clearly indicates the direction of movement of the stained anterior-like cells. The length of the vectors is directly proportional to the velocity. Furthermore it can be seen that the cells in the prestalk zone rotate, as described for normal slug migration.

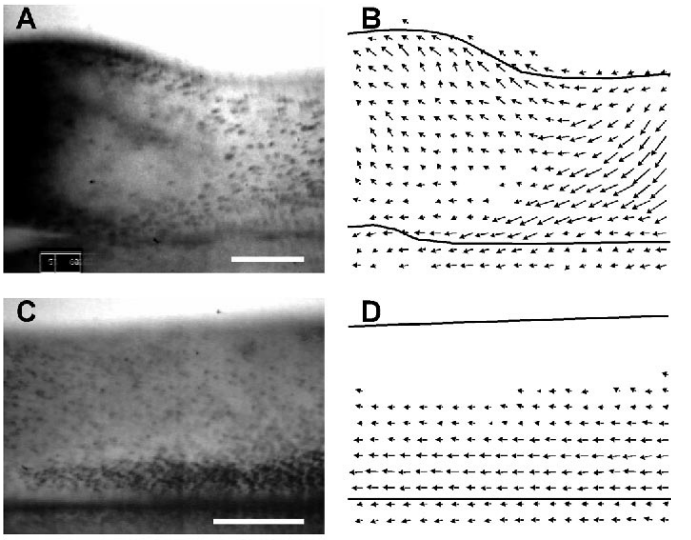

Fig. 3. Formation of the pile of anterior-like cells. (A) Side view of a slug just before the tip (dark stained region on the left) is going to lift off and a pile is starting to form at the base of the prestalk prespore boundary. (B)The vector field shows that pile formation is the result of an active accumulation of a subpopulation of neutral red stained cells at the base of the slug tip. The pile is formed by the directed downward movement of neutral red stained anterior-like cells. The outline of the slug is indicated by black lines. The vectors outside the slug boundary are caused by the reflections of moving cells on the agar surface. (C) Side-view of a slug in the process of pile formation by recruitment of cells from an old pile in the rear of a slug. (D) Vector field showing forward directed movement of a band of neutral red stained cells in the bottom part of the prespore zone. Neutral red stained cells in the upper part of the prespore zone move much slower. Scale bar, (A,B) 50 μm; (C, D)100 μm.

Finally, when as a result of the forward migration of the slug the pile of stationary anterior-like cells ended up in the back of the slug, the slug fell down on the agar again and the pile of cells dispersed. The cells from the pile now started to move forward much faster than the anterior-like cells in other parts of the slug. They started to accumulate again at the prespore-prestalk boundary. Fig. 3C,D show this differential cell movement of anterior-like cells at the dorsal and ventral side of the slug in such a case. According to the vector field there is a gradient of cell movement speeds with the lowest speed on the dorsal and the highest speed on the ventral side. The fast moving anterior-like cells move forward until they reach the prestalk zone, where they stop and form a new stationary pile which causes a lifting of the tip again.

The culmination phase

The culmination phase was investigated in slugs which were exposed to light for more than 15 minutes and had already started culmination at the time when the agar block was cut for observation. In other experiments slugs that had stopped migration under oil and started to culminate were filmed. Immediately preceding the culmination phase we often observed a dramatic change in the movement pattern of the stained anterior-like cells in the prespore zone: the direct forward movement of the cells switched to a path best described as a twisted scroll (not shown). Eventually, a major part of the anterior-like cells collected ventrally at the prestalk-

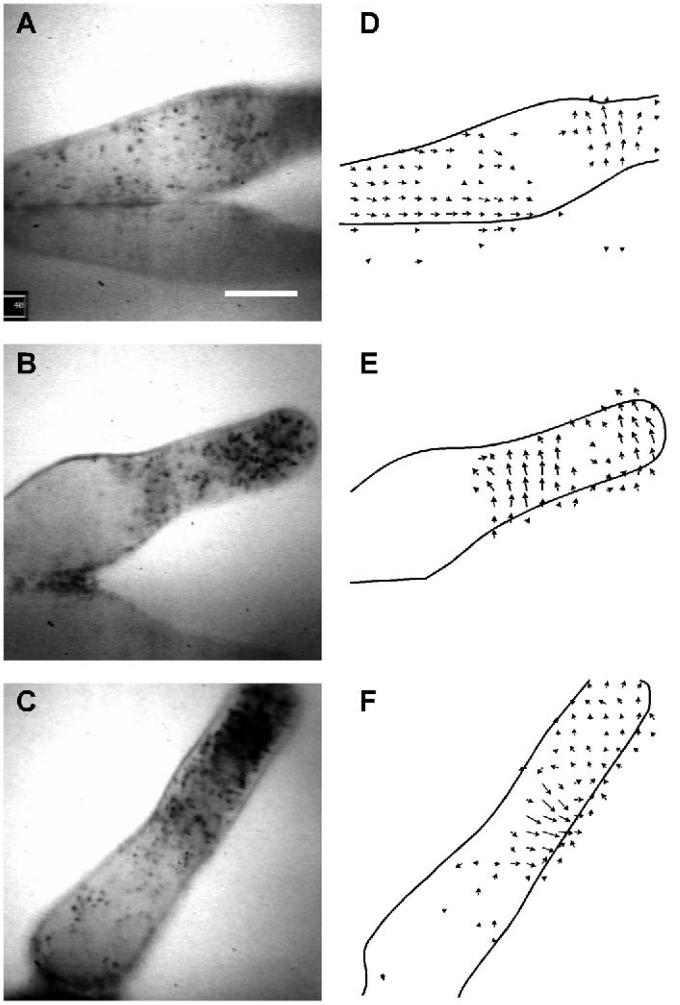

Fig. 4. Formation of a culminant. **(**A-C) Series of successive images of a culminating slug viewed from the side. (D-F) Vector velocity fields corresponding to the images shown in A-C. Rotational cell movement is evident at the prestalk-prespore boundary. During early culmination all the cells in the prestalk zone rotate (D,E). Later during stalk tube formation the prestalk cells rotate most vigorously at the prestalk prespore boundary in the pstO region (C,F). Scale bar, 50 μm.

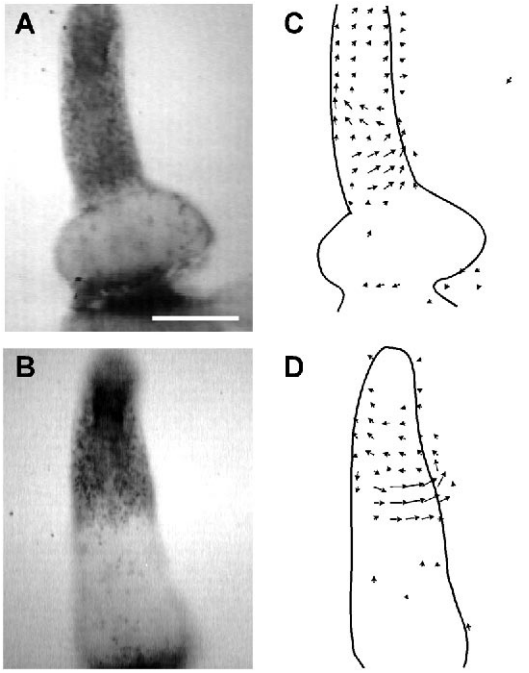

Fig. 5. Cell movement in a late culminant. (A) Mexican hat stage extending up in the air in the process of forming a fruiting body. (B) Same culminant later extending further in the air. The prespore cells have started to move up. (C,D) Velocity vector fields for the images in A and B showing extensive rotational movement in the prospective upper cup regions, while movement in the upper part of the tip slows down in the process of stalk formation. Scale bar, 100 μm.

prespore boundary and stopped movement completely. This led to a process already described above: the prespore cells continued to move forward and lifted the tip high into the air. However, during culmination these structures didn't fall back on the substratum, but the tip shifted backwards towards the centre of the cell mass and a structure resembling a Mexican hat formed (Figs 4,5). This process was always accompanied by a vigorous rotation of the prestalk cells in the elongating tip (Fig. 4B,E). Again rotation was most vigorous at the prestalk-prespore boundary (Fig. 4C,F), while at the very end of the tip cell movement slowed down to almost undetectable. Instead, a stalk formed in this area as can be seen in Fig. 4C and Fig. 5. The whole culminate rests now on a layer of anterior-like cells which is formed by the local aggregation of these cells. On top of this layer is a thick bulge of more or less imotile prespore cells (Fig. 5A) and a vigorous rotating tip (Fig. 5C,D) which extends vertically further into the air. Most of the anterior-like cells in the prespore zone sorted straight to the elongating tip region.

Surprisingly, a second centre of cellular rotation formed at the base of the culminate, which later formed part of the basal disc. This could be clearly observed if the slug was viewed from below as shown in Fig. 6. As can be seen in the time sequence the neutral red stained anterior-like cells in the back of the slug change their movement during the initiation of culmination from forward (Fig. 6D) to S-shaped (Fig. 6E). This S-shaped movement became more and more strongly curved until movement became essentially rotational (Fig. 6F). During this process the front part of the culminate lifted into the air (out of focus). Ultimately the bulge of prespore cells rested on a very thin stalk-like structure made of anterior-like cells. At the base of the bulge we still observed vigorous rotation of anterior-like cells.

This process as viewed from the side is shown in Fig. 7. Observation of cell movement from the side makes clear that the switch in movement pattern during the formation of the basal disc and lower cup is related to an active aggregation of a subclass of anterior-like cells, which derive mainly from the pile cells. During culmination the cells that are going to form the pile are initially moving rapidly forward (Fig. 7A,E), then they stop (Fig. 7B,F) and finally move backward to the region at the base of the culminate (Fig. 7C,G). During this backward movement of the pile cells, the cells in the prestalk zone continue to rotate. Cells in this basal structure destined to form the lower cup start to show a strong rotational movement, which is mostly counter rotational to the direction of

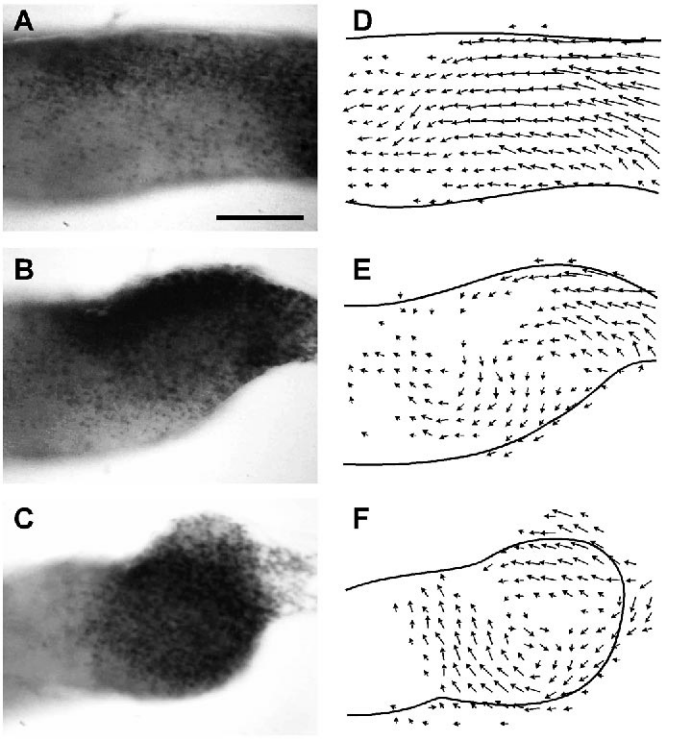

Fig. 6. Sequence of a culminating slug seen from the beneath. (A-C) Successive views of a culminating slug viewed through the agar. (D-F) Vector field corresponding to the images shown in A-C. Initially cell movement is directed forward in the direction of slug migration. When the slug movement stops the cells in the rear start to perform a rotational motion in the plane of focus. Scale bar, 100 μm.

movement of the cells in the prespore zone (Fig. 7D,H) In many culminates we observed at the initial stages rotational movement of nearly all the cells in the culminate. During culmination the anterior-like cells seem to sort out to two different centres, one located at the tip and a second one forming at the base of the culminate.

The pile contains *ecmA*- and *ecmB*-expressing cells

Neutral red stains both prestalk and anterior-like cells. However, it has been shown that anterior-like cells are heterogeneous in the expression of two prestalk specific genes *ecmA* and *ecmB* (Gaskell et al., 1992). To characterize the cell types in the pile of neutral red staining cells further we tested for the expression of the *ecmA* and *ecmB* genes. Colocalisation experiments in slugs (48 hours old) show that the pile of neutral red stained cells contains many cells that express the *ecmB* gene (Fig. 8E-H). Furthermore we found that many cells in these piles also express the *ecmA* gene (Fig. 8A-D).

In a recent study by Jermyn et al. (1996) it was found that basal disc formation was initated by a band of *ecmB*-expressing cells localized at the ventral side of the slug. They concluded from looking at a series of slugs fixed at different stages of culmination that this band of cells moves forward to the pstO zone and finally fuses with rearguard cells to form the basal disc. During this process they also start to express the *ecmA* gene. This behaviour is very reminiscent of the one we have described for the pile of neutral red stained cells that also contains *ecmB* and e*cmA* expressing cells. Therefore we

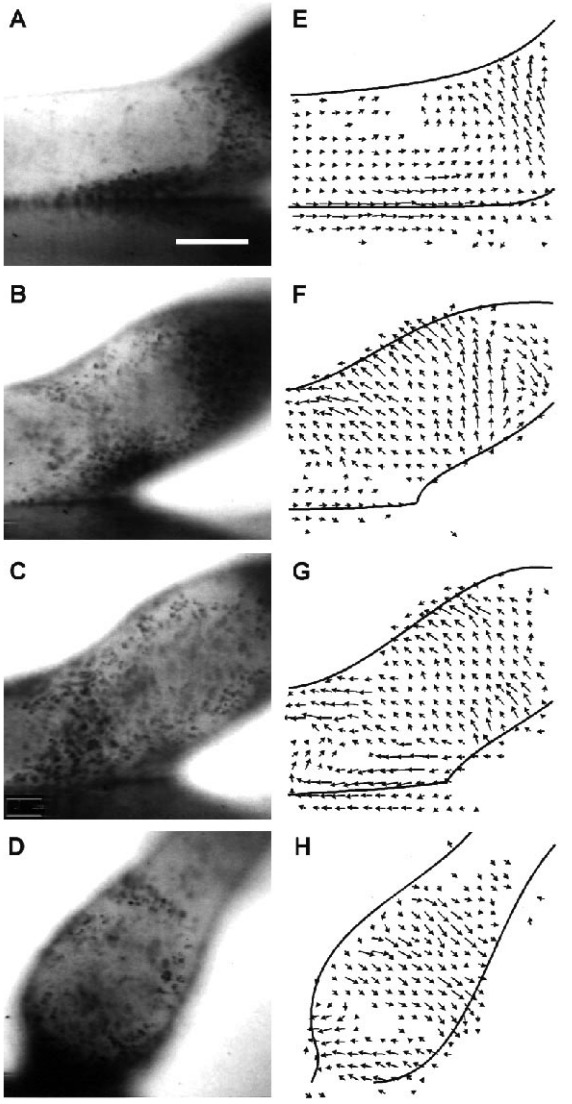

Fig. 7. Culmination as seen from the side. (A-D) Sequence of photographs of successive stages of culmination. The darkly stained prestalk zone is oriented to the right. A and E show the formation of a pile at the prestalk-prespore boundary from a ventral layer of anterior-like cells. B and F show the dissolution of the pile at the place of lift off. (C,G) The pile continues to vanish. The cells from the pile start to move backwards. (D,H) Rotational movement of the cells in the prospective lower cup region. Anterior-like cells in the body of the prespore region show counter rotational movement. Scale bar, 50 μm.

conclude that we are observing the same group of cells as Jermyn et al. (1996).

DISCUSSION

Analysis of slug migration – young slugs

In this study we investigated the mechanism of slug migration and culmination by observation of movement of neutral red stained cells. Cell movement as observed in slugs viewed from the side follows a pattern already known from earlier investi-

gations. Cells in the prespore zone move straight forward in the direction of slug migration (Durston and Vork, 1979; Kakutani and Takeuchi, 1986; Siegert and Weijer, 1992; Breen and Williams, 1994), while cells in the prestalk zone always show rotational cell movement perpendicular to the direction of slug migration (Siegert and Weijer, 1992). This pattern of movement is also represented by the shape of the cell prints on the surface of the slug visualised by staining with Nessler's reagent (Feit, 1994). Cell movement of labelled anterior-like cells in the prespore zone was found to be periodic along the entire dorsal-ventral axis of the slug. Young slugs (24-40 hours) move at a quite constant speed with the tip permanently raised into the air (Fig. 9A). Sometimes we observed a periodic up- and downward movement of the tip, on rare occasions the tip also touched the agar surface. Our measurements of slugs filmed from the side clearly show that rotational cell movement is confined to the tip region that is lifted into the air. Interestingly rotational movement is most vigorous at the boundary between the prestalk and prespore zone.

We believe that the role of the prestalk cells is to deliver coordinating, periodic cAMP signals to the major portion of prespore cells. They do not contribute to slug migration, since the tip is permanently raised at a flat angle above the substratum. Only prespore cells propel the slug forward due to their close contact to the substratum and the surrounding slime sheath. The slime sheath secreted by the tip plays an important role in slug migration in that it provides a rapidly stiffening tube which guides cellular flow in the interior. However, we propose that the slime sheath not only surrounds the slug but is also secreted into the interior of the slug in order to provide traction to all cells in the prespore zone. This is indicated by the fact that all cells along the dorsoventral axis move at the same time a similar distance. If the cells crawled on each other without a supporting matrix there would result a gradient of increasing velocities from ventral to dorsal.

We never observed any backward flow or movement in the central portions of the slug cylinder as proposed by Odell and Bonner (1986), although we analysed more than 100 slugs in 3 different strains (AX-2, NC4, XP55). Time lapse video films and velocity vector analysis clearly demonstrate that all prespore cells move straight forward in the direction of slug migration although at slightly different rates as observed earlier (Kakutani and Takeuchi, 1986). We also never observed any movements in slugs which would resemble the movement pattern proposed by the 'squeeze-and-pull' model of Williams et al. (1986).

Migration of old slugs

Migration in old slugs is characterised by the periodic launching of the slug into the air over a pile of stationary cells at the base of the slug. This pile of cells is mostly made up of anterior-like cells as judged by their intense neutral-red staining. This pile of cells is most likely the same as the band of *ecmB*-expressing cells situated in the front half of the prespore zone that was recently identified by Jermyn et al. (1996). Pile formation may correspond to the formation of 'adhesion zones' as described by Vardy et al. (1986), which have been associated with the deposition of extracellular matrix material, the so-called 'cell prints'. Based on our motion analysis we see no evidence for a role of these adhesion zones in pulling the posterior of the slug forward. Instead our measurements clearly show that the cells continuously flow across the pile, an observation incompatible with a pulling mechanism. After the pile has been formed and the slug lifts into the air it may fall back on the agar again (Fig. 9B) or the slug may stop migrating and initiate culmination (Fig. 9C). Therefore we associate this type of slug movement with abortive attempts to culminate.

Culmination is organised by two signalling centres

From our observations it has become clear that in a culminating slug there are at least two signalling centres. First, there are prestalk cells in the tip that lead all morphogenetic

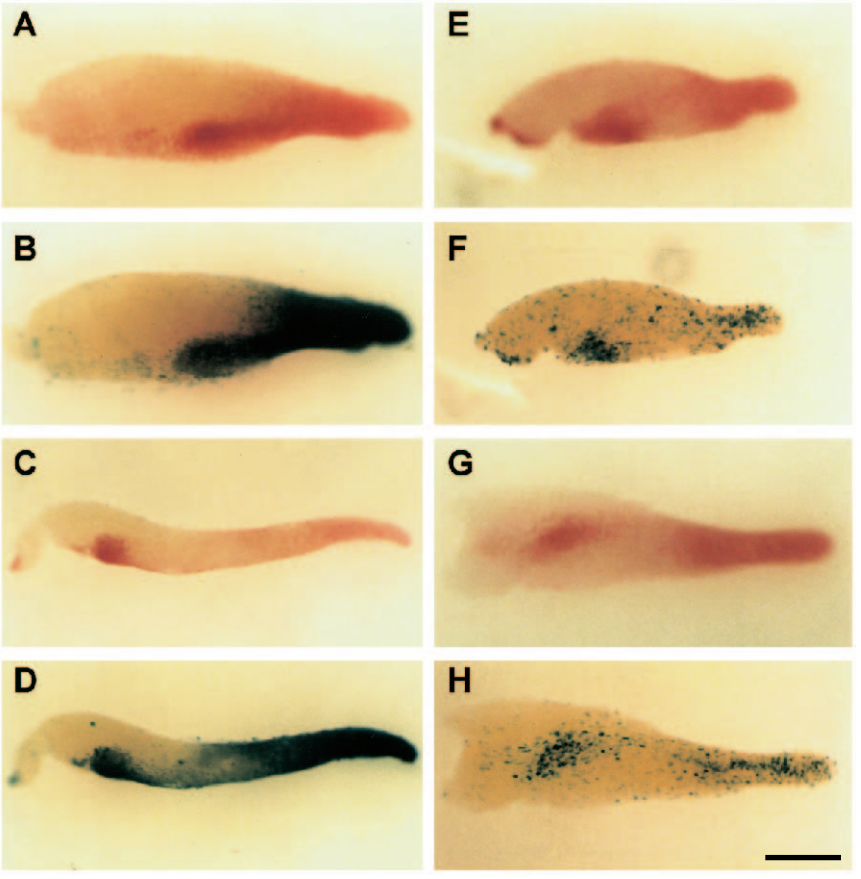

Fig. 8. Expression of *ecmA* and *ecmB* genes in piles of anterior-like cells. (A,C) Slugs of *emcA/lacZ* transformant cell line stained with neutral red. There are piles of anterior-like cells in the front (A) and in the back of the prespore zone (C). (B,D) Same slugs as in A and C but stained for *ecmA* expression (*lacZ* activity). (E,G) Slugs of *emcB/lacZ* transformant cell line stained with neutral red. There are piles of anterior-like cells in the middle of the prespore zone, viewed from the side (E,F) and from below (G,H). (F,H) Same slugs as in E and G but stained for *ecmB* expression (*lacZ* activity). These gene expression patterns were observed in all slugs that showed piles (n=30). Bar=200 μm.

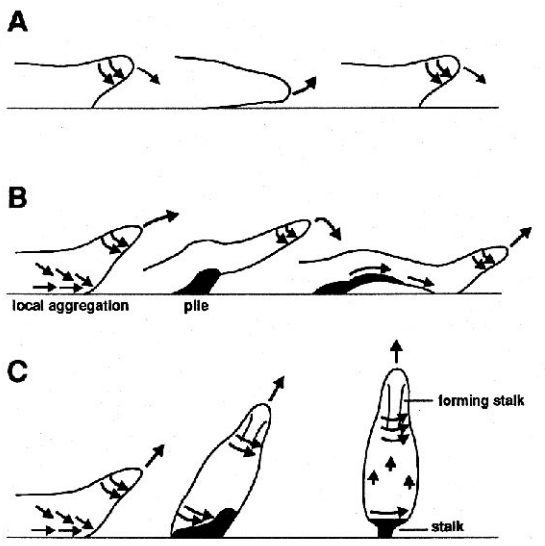

Fig. 9. Scheme of movement of neutral red stained cells during normal migration and culmination. (A) Scheme showing the migration of a young slug. It is characterised by lifting up and down of the slug tip. Cells in the tip rotate as long as the tip is up in the air. (B) Scheme showing migration observed in older slugs. This type of slug movement presumably reflects abortive attempts to culminate. Lifting off is initiated by the active aggregation of anterior-like cells at the prestalk prespore boundary which form a stationary mass of cells. The pile forces the rest of the slug to lift off in the air. The pile of cells is being left behind and gets translocated to the back of the slug. The slug falls back onto the substratum, the pile vanishes and the neutral red stained cells form a new pile at the prestalk-prespore boundary. The cells in the pstO region continue to rotate. (C) Sequence showing full development to culmination. The first image shows the formation of the pile, as well as rotation of the cells in the posterior prestalk (pstO) zone similar to that shown in B. Under influence of the proper environmental signals culmination continues. The whole prespore mass shifts on top of the pile. The cells in the pile start to rotate. These cells will form the basal disc and the lower cup.

movement and are going to form the stalk and upper cup during culmination, and second there are anterior-like cells in the back of the slug (may be part of the rear guard zone) which are going to form the basal disc and the lower cup. Until now it was suspected that the cells in the rearguard zone are a relatively static population. However, our experiments suggest that at least some of these cells are engaged in forming a pile of cells at the prespore-prestalk boundary that is involved in the mechanics of culmination. It appears that this pile is formed by the aggregation of neutral red stained anterior-like cells. This aggregation seems to be an active process since a subpopulation of anterior-like cells moves directly towards this point both from the ventral as well as from the dorsal side of the prespore zone (Fig. 3). Sometimes some of the pile cells actually seem to come from the pstO region, i.e. move actively backward. It seems that the movement of the cells towards the pile is controlled by a signal different from that coming from the tip to which the majority of the cells in the prespore zone react. During culmination the majority of the cells in the prespore zone moves over the pile resulting in the cells of the pile ending up at the back of the slug (Fig. 9C). These cells in the back of the slug start very rapidly to reorganise themselves in a rotational movement pattern, very reminiscent of the rotational patterns seen at the prespore-prestalk boundary.

The nature of the base signalling centre

A major unresolved problem concerns the nature of the signal responsible for the active aggregation of the pile of anterior-like cells. It is possible that the cells, which form the pile, use a similar cAMP signalling system as the cells in the prestalk zone, and that there are really two competing centres. Alternatively the cells in the base might use a signal of different chemical nature. The most attractive possibility is that the cells in the pile use periodic cAMP signals, as suggested by the strong rotational movement observed in the presumptive lower cup region during culmination. It is, however, unclear why the cells that will form the pile seem to react specifically to the signal coming from it while the majority of the cells seem to react to a signal coming from the tip. Possibly these cells use different cAMP receptors leading to different chemotactic activities.

It is interesting to note, that in a recent study of changes in free calcium in strains expressing the calcium-sensitive light-emitting protein, aequorin, there appeared to be two regions in slugs that were characterized by high levels of free calcium (Cubitt et al., 1995). These regions were found in some slugs at the prestalk-prespore boundary and in other slugs in the back. The location of these centers might very well coincide with the pile of cells described in this paper. Furthermore the calcium levels in these regions seemed to oscillate with a period of around 2 minutes. The oscillatory nature of the calcium levels suggests an oscillatory signal. We have tried to determine whether the cells that form the piles move in a periodic fashion. However, we have not yet obtained convincing data either for or against this.

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