Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8303–8307, September 1992
Developmental Biology

Chemotactic sorting to cAMP in the multicellular stages of *Dictyostelium* development

(tip formation/directed cell movement/morphogenesis)

DAVID TRAYNOR*†, RICHARD H. KESSIN‡, AND JEFFREY G. WILLIAMS*

*Imperial Cancer Research Fund, Clare Hall Laboratory, Blanche Lane, South Mimms, Potters Bar, Herts., EN6 3LD, United Kingdom; and ‡Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032

Communicated by J. T. Bonner, June 8, 1992

ABSTRACT **_Dictyostelium_ transformants that overproduce the extracellular form of cyclic nucleotide phosphodiesterase and so accumulate a reduced amount of cAMP are blocked in development after aggregation in the form of a tight mound, prior to formation of the apical tip. In such mounds, prespore cell differentiation is repressed, and the apical accumulation of prestalk cells is greatly retarded. When a source of cAMP is placed below the arrested mounds, prestalk cells that would normally migrate in an apical direction instead sort downwards to the substratum. Thus, by acting as the chemoattractant that draws prestalk cells to the apex, cAMP signaling directs the formation of a patterned structure.**

When they are starved, *Dictyostelium* amoebae aggregate into hemispherical mounds that undergo further morphogenetic movements to form fruiting bodies, consisting of a droplet of viable spores supported by a cellular stalk. Aggregation is a chemotactic process, mediated by pulsatile emissions of extracellular cAMP from a signaling center, in which amoebae move up a gradient of cAMP concentration and also relay the cAMP signal (1, 2).

Cellular differentiation occurs from initially uncommitted amoebae within the mound, resulting in an intermingled population of cells, ≈20% of which will go on to become stalk cells, with the remainder forming spores. The prestalk cell population in the migratory slug is heterogeneous. It contains both prestalk A (pstA) cells and prestalk B (pstB) cells, populations that are defined by their pattern of expression of the EcmA and EcmB extracellular matrix proteins (3–7). The slug tip is predominantly comprised of pstA cells but has a central core of pstB cells (3). These lie at the position where the stalk will form during culmination, the stage at which the EcmB protein (the gene product whose expression defines pstB cells) probably plays its major function (4).

During slug formation, pstA cells migrate to the apex of the mound, to yield a structure with a polarized distribution of prestalk and prespore cells that presages the organization of the slug (5, 8, 9). A papilla, or tip, is formed at the apex of the mound, which, during the early stages of its genesis, is primarily composed of pstA cells. Elongation of the tip results in the formation of the first finger, and it is only at this time that the core of pstB cells appears. Depending upon the environmental conditions, the first finger may form a fruiting body *in situ*, or it may flop onto the substratum and migrate for a finite period as a slug. The tip of the aggregate becomes the tip of the migratory slug, where it displays some of the attributes of an embryonic organizer, most notably the ability to direct formation of a secondary axis when transplanted to another slug (10). It is thought that the tip continues to be a source of cAMP signaling during postaggregative stages of development (11–14). Thus an understanding of the formation and properties of the tip is of central importance in understanding *Dictyostelium* morphogenesis.

cAMP plays multiple roles in *Dictyostelium* development. It functions as a chemoattractant during aggregation and regulates the expression of a number of developmentally regulated genes (reviewed in refs. 2, 15, and 16). Among the products of these genes is the secreted form of cyclic nucleotide phosphodiesterase (phosphodiesterase) (17). This enzyme modifies extracellular cAMP gradients and prevents prolonged adaption of the cell surface cAMP receptor (2, 15). Overproduction of the phosphodiesterase blocks development at the tight mound stage, prior to formation of the apical tip (18, 19). The gene has a complex structure, with three different promoter regions: one active during vegetative growth, another active during aggregation, and a third region that directs expression only in prestalk cells (18–20). All three promoters direct the expression of the same protein.

Prespore cell differentiation is induced by cAMP, and high levels of cAMP are thought to be necessary to maintain their differentiated state (21–25). cAMP may also act morphogenetically during slug formation, to direct sorting of prestalk cells to the apex of the aggregate. Prestalk cells, detected by staining with the vital dye neutral red (26), selectively migrate toward cAMP. This occurs in undisturbed submerged agglomerates (27) when stained cells are coaggregated with unstained prespore cells (28) and when a source of cAMP is introduced via a micropipette into developing mounds (28). Overproduction of secreted phosphodiesterase, a condition that would be expected to disrupt cAMP signaling, blocks development at the tight aggregate stage. These observations are consistent with the idea that cAMP directs prestalk cells toward the apical region of the mound, but mutual adhesion of prestalk and prespore cells has been proposed as an alternative mechanism for initial pattern formation (29). Prespore cells appear to be more cohesive than prestalk cells (30, 31). It is therefore possible that prespore cells exclude prestalk cells from the lower regions of the aggregate as a result of their preferential mutual adherence. Consistent with this idea is the finding that manipulation of intercellular adhesion alters both cell fate and cell sorting (32, 33).

The characterization of the phosphodiesterase gene and its promoters (18–20), together with the ability to visualize pstA cells (4), has enabled us to investigate their movement in mounds in which cAMP levels are reduced. Our results suggest that cAMP continues to act chemotactically in the mound to direct the movement of pstA cells to the apex.

MATERIALS AND METHODS

Cell Growth, Transformation, and Development. *Dictyostelium* cells (strain Ax3-L, supplied by Margaret Clarke, Oklahoma Medical Research Foundation, Oklahoma City,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: pstA and pstB, prestalk A and B, respectively.
†To whom reprint requests should be addressed.

OK) were transformed (34) with a mixture of 6 μg of one of two reporter constructs, pEcmA::lacZ (4) or pPspA::lacZ (5), and 8 μg of plasmid pBDE or, in the case of control transformants, with 8 μg of the plasmid pBDEΔ (19). Cotransformants were selected and maintained in the aminoglycoside G418 (Geneticin; GIBCO) at 20–50 μg/ml. For normal development, cells were washed and resuspended in KK_2 (16.5 mM KH_2PO_4/3.9 mM K_2HPO_4, pH 6.1) and then plated for development at a density of 1–3 × 10^6 cells per cm^2 on 2% nonnutrient agar. In experiments where cells were transferred to cAMP, development was performed on washed 325P cellophane (British Cellophane, Bridgewater, U.K.) supported by 2% nonnutrient agar buffered with 20 mM NaH_2PO_4/20 mM K_2HPO_4, pH 6.5. Small pieces of the cellophane were dissected 15–17 hr later, when the cells had formed tight mounds, and transferred to buffered 2% nonnutrient agar containing 0.1–5 mM cAMP or 5′-AMP.

Detection of pstA and Prespore Cells. Aggregates transformed with the pEcmA::lacZ or pPspA::lacZ constructs were fixed for 15 min in Z buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$, pH 7.0) containing 1% glutaraldehyde (35), washed twice (for 15 min each) in fresh Z buffer, and then incubated for 3–8 hr in staining solution [Z buffer containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-galactoside]. In those cases where cells could not be definitively localized in whole mounts, the stained aggregates were frozen in OCT compound (BDH, Poole Dorset, U.K.), and longitudinal (8-μm-thick) sections were collected onto gelatin subbed slides.

To quantitate different cell types, aggregates developing on agar were dissociated in buffer (20 mM Na_2HPO_4/KH_2PO_4/20 mM EDTA, pH 7.0) at a density of 1–2 × 10^6 cells per ml by trituration through a syringe needle. Dissociated cells were washed and fixed as described above before being resuspended at 0.5–1 × 10^7 cells per ml in staining solution. After a 4- to 8-hr incubation at 37°C, 5 μl of the cell suspension was spotted onto poly(L-lysine) subbed (80 μg/ml in 10 mM Tris·HCl, pH 8.0) slides, and the number of stained cells was determined by using bright-field microscopy. As an alternative to β-galactosidase staining, prespore cells were detected by indirect immunofluorescence with a polyclonal antiserum raised against *Dictyostelium mucoroides* spores (36) or with the monoclonal antibody Mud-1 (37).

Phosphodiesterase and cAMP Assays. Cells were developed on nitrocellulose filters (13-mm diameter; Sartorious GmbH) at a density of 1.5 × $10^7/cm^2$. At the times indicated, each filter was vortexed in an ice-cold mixture of 0.2 ml TE (50 mM Tris/4 mM EDTA, pH 7.5) and 0.2 ml of 3.5% trichloroacetic acid. The filter was removed, and the suspension was neutralized by the addition of 0.1 ml of $NaHCO_3$ (saturated solution). After the precipitate was removed by centrifugation, the cAMP concentration in the supernatant was determined by using the isotope dilution method with an assay kit from Amersham.

Extracellular cyclic nucleotide phosphodiesterase was determined by harvesting a filter into 0.2 ml of ice-cold 50 mM Tris (pH 7.5) and then pelleting the cells by centrifugation. The supernatant was removed and combined with a further 0.3 ml of 50 mM Tris (pH 7.5) before being used to wash the filter support pad for 30 min. This solution was then dialyzed for 2 hr in 50 mM Tris (pH 7.5) at 4°C before the cyclic nucleotide phosphodiesterase activity was determined using the isotope dilution method (38).

RESULTS AND DISCUSSION

Two constructs containing differing amounts of sequence upstream of the phosphodiesterase coding region were analyzed; pGP-1 contains both a vegetative and prestalk-specific promoter, whereas pBDE contains only the prestalk-specific promoter (18, 19). Growth in a high concentration of G418 was used to select cotransformants in which the phosphodiesterase gene is present in high copy number and the absolute concentration of extracellular phosphodiesterase is greatly elevated compared to nontransformed strains (refs. 18 and 19; Table 1).

The pGP-1 and pBDE cotransformants differ in that pGP-1 strains commence aggregation somewhat earlier (18, 19). However, the effects of extracellular phosphodiesterase overproduction on pstA cell differentiation and movement are identical in the two strains. Hence, only results obtained with pBDE cotransformants will be described. As a control for any effects that multiple copies of the promoters might have upon development, an analogous construct, pBDEΔ (19), which contains a 1-kilobase internal deletion that renders the phosphodiesterase gene inactive, was also analyzed.

After 5 hr of development, both the extracellular phosphodiesterase activity and cAMP concentration were similar in BDEΔ control and BDE transformants (Table 1). However, at 17 hr of development, a stage at which the prestalk-specific region of the promoter would be expected to have become active, the physiological levels of extracellular phosphodiesterase were 13–19 times higher in BDE transformants than in controls. Surprisingly, cAMP levels were reduced only 2- to 4-fold relative to control cells (Table 1). This discrepancy is unlikely to be the result of the known homeostatic system, whereby synthesis of an extracellular inhibitor protein of the phosphodiesterase is stimulated when cAMP levels fall (40), because the phosphodiesterase assays were performed under conditions where the inhibitor protein is active (39). Perhaps it results from some hitherto unsuspected homeostasis mechanism that operates at the level of cAMP synthesis.

pstA cells (3, 4) were detected by their expression of pEcmA::lacZ, a fusion gene that contains the promoter of the *ecmA* gene (3–5) fused to the *Escherichia coli* β-galactosidase gene as a reporter (35). In untransformed cells (5, 9), and in pBDEΔ transformants (Fig. 1*A*), pstA cells arise at random positions within the aggregate and subsequently accumulate in the tip (Fig. 1*B*), the nipple-shaped structure that forms at

Table 1. Extracellular phosphodiesterase activity and cAMP levels in cotransformants

Clone	Development,* hr	cAMP, pmol per 10^7 cells	Phosphodiesterase,† units/ml
1 BDEΔ	5	9.1 ± 1.1	1.6
	17	23.9 ± 8.2	3.3
2 BDEΔ	5	6.3 ± 2.2	1.0
	17	13.3 ± 2.7	3.2
3 BDEΔ	5	3.3 ± 0.4	1.1
	17	24.8 ± 3.6	3.7
1 BDE	5	8.2 ± 3.0	1.4
	17	5.1 ± 0.9	48.4
2 BDE	5	6.8 ± 3.2	1.2
	17	5.1 ± 1.4	57.7
3 BDE	5	8.8 ± 1.8	1.2
	17	5.4 ± 2.6	61.0

Cells were harvested in the logarithmic phase of growth and plated for development on nitrocellulose filters. The cAMP levels and phosphodiesterase activity were determined as detailed in *Materials and Methods*. Triplicate samples were taken at each time point for the determination of cAMP levels.

*These times of development were chosen because the levels of extracellular phosphodiesterase are known to be normal at 5 hr and overproduced after 17 hr in BDE transformants (19). In addition, cell aggregation had not started at 5 hr of development, whereas the cell sorting process was well underway in the multicellular aggregates of BDE cotransformants and nearly completed in the control BDEΔ cotransformants at 17 hr.

†The extracellular phosphodiesterase was assayed without inactivation of the endogenous inhibitor protein (39).

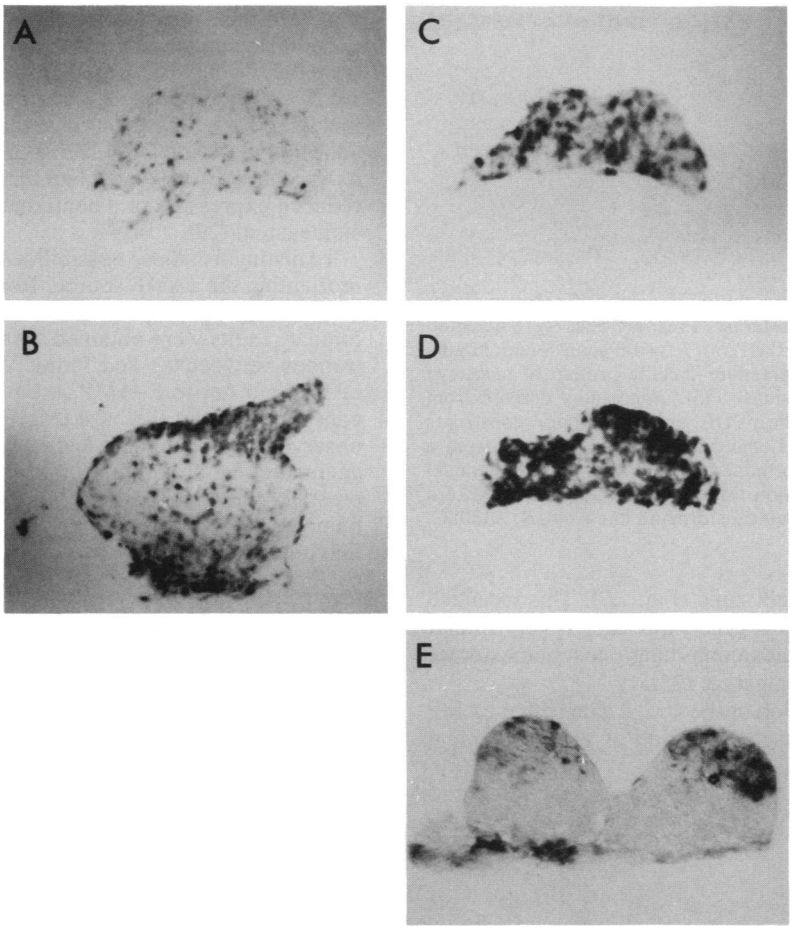

FIG. 1. pstA cell differentiation in aggregates that overproduce the extracellular phosphodiesterase. Aggregates derived from cotransformants containing the pEcmA::lacZ (4) construct together with pBDE or the pBDEΔ (19) construct were analyzed for β-galactosidase activity. Longitudinal sections derived from a control pBDEΔ–pEcmA::lacZ cotransformant analyzed at 10 hr (*A*) and 14 hr (*B*) of development are shown. Similar sections were cut through a pBDE–pEcmA::lacZ cotransformant after 9 hr (*C*), 15 hr (*D*), and 33 hr (*E*) of development. (*A*, ×100; *B*, ×90; *C*, ×125; *D*, ×100; *E*, ×100.)

the top of the aggregate. Once the tip is well extended and most of the pstA cells have moved into it, a relatively smaller number of pstA cells appear in the base of the aggregate (refs. 5 and 9; Fig. 1*B*). In pBDE transformants, pstA cells first appear at the same time and in approximately the same number, as in pBDEΔ aggregates (Fig. 1*C*). However, in pBDE transformants the pstA cells remain widely scattered during the time when sorting to the apex occurs in pBDEΔ aggregates (Fig. 1*D*). Over this time period there is an increase in the number of pstA cells in pBDE aggregates approximately equivalent to that occurring in pBDEΔ aggregates (Fig. 1 *C* and *D* and Table 2). Assuming that it is safe to extrapolate this result to a normal aggregate, this suggests that all pstA cells differentiate by a process that is independent of their position within the aggregate. It therefore helps rule out a possibility that was left open by previous studies (5, 8)—that there is an initial random differentiation of a small number of pstA cells followed by further, positionally specified, pstA cell differentiation in the tip to generate the majority of pstA cells finally present within the slug. It is only after a further 7–9 hr that the pstA cells accumulate in the apex and the base of pBDE transformants (Fig. 1*E*), during which time control transformant and Ax3 cells complete fruiting body formation.

Prespore cells are also scattered when they first appear (5, 43, 44) (Fig. 2*A*) but become concentrated in the basal part of the aggregate (Fig. 2*B*). The fraction of cells that differentiate as prespore cells is reduced in pBDE aggregates (relative to control aggregates; Table 2 and Fig. 2 *B* and *C*), which supports previous evidence that cAMP is necessary for prespore cell differentiation (21–25). The magnitude of this decrease in the fraction of prespore cells seems reasonable, given the 2- to 4-fold reduction in the concentration of cAMP in such aggregates (Table 1). In the pBDE construct, expression of the phosphodiesterase gene is under control of a part of its promoter that directs expression in prestalk cells and this probably explains why there are relatively few prespore

Table 2. Cell type proportions in cotransformant strains

	% cells stained		
	Enzymatic assay	Immunological assay	
Cotransformant	β-galactosidase*	Mud-1†	PSV‡
pBDEΔ–pEcmA::lacZ	16.1 ± 2.8	70.7 ± 6.8	71.0 ± 7.3
pBDE–pEcmA::lacZ	19.0 ± 4.0	47.1 ± 3.6	43.1 ± 3.3

Aggregates were dissociated after 18–21 hr of development, and the cells were fixed and stained for β-galactosidase activity or for prespore-specific antigens. A minimum of 300 cells were counted from duplicate samples. The values given are the means ± SEM from eight experiments.

*The β-galactosidase assay detects cells expressing the prestalk-specific *lacZ* fusion gene.

†The Mud-1 monoclonal antibody recognizes the product of the endogenous *pspA* gene, a prespore-specific marker (41).

‡The PSV polyclonal antiserum was raised against *D. mucoroides* spores and recognizes the products of at least five prespore-specific genes including SP70 and SP96 (42).

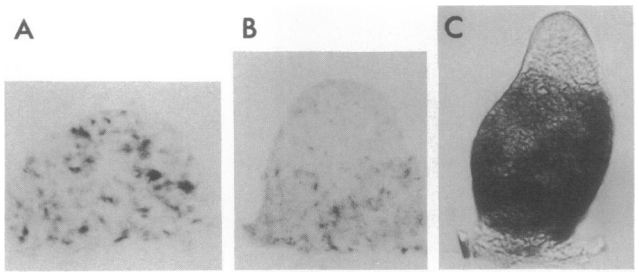

FIG. 2. Prespore cell differentiation in aggregates that overproduce extracellular phosphodiesterase. Prespore cells were detected by their expression of pPspA::lacZ (5), a fusion gene containing the promoter of PsA (5, 41), a prespore-specific protein of unknown function, fused to β-galactosidase (35). Aggregates derived from cotransformants containing the pBDE and pPspA::lacZ constructs were analyzed as in Fig. 1. Longitudinal sections derived from a pBDE–pPspA::lacZ cotransformant analyzed after 10 hr (*A*) and 33 hr (*B*) are shown. (*C*) A section through a late-tipped mound of a pBDEΔ–pPspA::lacZ control cotransformant. (*A*, ×140; *B*, ×130; *C*, ×265.)

cells adjacent to the prestalk zone (Fig. 2*B*). This localized reduction in prespore-specific gene expression is very similar to that observed when beads containing phosphodiesterase are implanted into migrating slugs (25).

The simplest interpretation of the above data is that cAMP signaling continues after the completion of aggregation and that it directs migration of pstA cells to the apex. The eventual accumulation of pstA cells at the apex could result from the residual cAMP signaling in pBDE aggregates (Table 1). However, cAMP acts as an inducer for many proteins, including csA, the best characterized *Dictyostelium* cell adhesion molecule (45). The retardation of cell sorting when extracellular cAMP is depleted might, therefore, result from reduced expression of a component of a selective cell adhesion system (29).

To distinguish these possibilities, we examined the effect of reorienting the cAMP source, from the apex to the base, by transferring pBDE aggregates to agar containing cAMP. Similar results were obtained with a range of cAMP concentrations between 0.1 and 5 mM. Within 90 min of transfer to cAMP, but not to 5′-AMP, the pstA cells accumulated in a concentric ring at the base (Fig. 3 *A–D*). A similar ring was observed by Matsukuma and Durston (28) when they placed aggregates containing a mixture of neutral red-stained prestalk cells and unstained prespore cells onto agar containing cAMP. This behavior of pstA cells may reflect events that occur during normal development, where a ring of basal pstA cells is often observed at the base of tipped aggregates (see figure 3*F* in ref. 5). It could reflect an inhomogeneity in signaling across the base or might be an intermediate phase in the sorting process. After longer incubation, the pstA cells spread throughout the base (Fig. 3*F*).

The striking difference between aggregates exposed to cAMP and control aggregates transferred to AMP is the

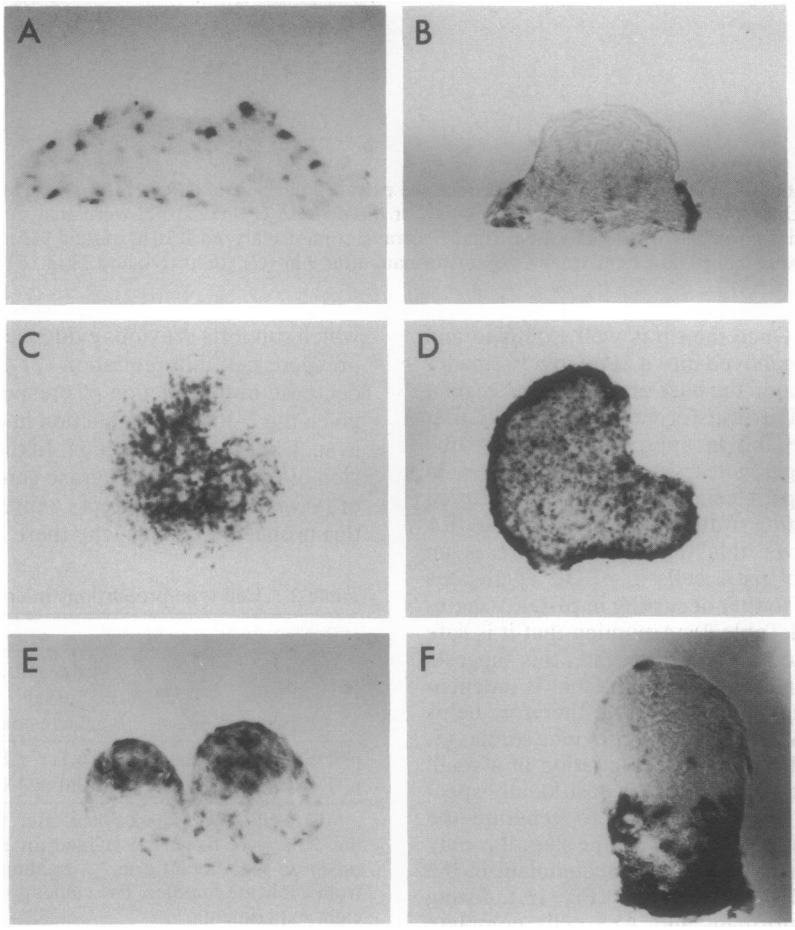

FIG. 3. Directed migration of pstA cells to an exogenous basal cAMP source. Cells cotransformed with pBDE and pEcmA::lacZ were developed to the tight mound stage of development on cellophane and then transferred to agar containing either 5 mM 5′-AMP (*C* and *E*) or 5 mM cAMP (*B*, *D*, and *F*). Longitudinal section through an aggregate stained for β-galactosidase activity immediately before (*A*) and 90 min after (*B*) transfer to cAMP. A whole mount of an aggregate stained for β-galactosidase activity 90 min after transfer to 5′-AMP (*C*) and cAMP (*D*) is shown. Stained longitudinal sections derived from aggregates 5 hr after transfer to 5′-AMP (*E*) or cAMP (*F*). The whole mounts are viewed from their upper surface. (*A*, ×110; *B* and *C*, ×35; *D*, ×160; *E*, ×90; *F*, ×125.)

virtual absence of pstA cells at the apex (Fig. 3 *B* and *F*). This distribution of pstA cells results from reorientation of the direction of sorting, rather than induction by cAMP of a new population of pstA cells that sort downwards. The β-galactosidase marker used to detect pstA cells is stable over the time period of the sorting process (K. A. Jermyn, personal communication), and there is no change in the number of pstA cells during this time (data not shown). We have also observed the sorting process directly. Prestalk cells stain selectively with vital dyes, such as neutral red (26), and we have used this as a marker directly to follow their migration to a basal cAMP source (data not shown). Many such aggregates exposed to a basal cAMP source reestablish the normal apical concentration of pstA cells when transferred back onto buffered agar.

There is considerable previous evidence, albeit indirect, that favors continued cAMP signaling in the multicellular stages (refs. 11–14 and 16; reviewed in ref. 46). The importance of the present results is to show that reduction of the concentration of cAMP within aggregates greatly retards sorting of pstA cells and that their direction of migration can be reversed by reorientating the direction of cAMP signaling. They do not exclude some involvement of selective cell adhesion, but they strongly favor chemotactic sorting of prestalk cells as the primary mechanism directing initial pattern formation. Prestalk cells contain higher amounts of several components of the cAMP detection system than prespore cells, and this may explain their selective migration to cAMP (47–49).

While they explain the chronological delay in the development of phosphodiesterase overproducing cells, these observations do not explain their terminal phenotype and imply a further morphogenetic role for cAMP signaling. During normal development, tip formation occurs at the time prestalk cells start to accumulate in the apex. In pBDE aggregates, pstA cells eventually accumulate in the apex but, despite this fact, a tip never forms. Presumably the impaired cAMP signaling in pBDE aggregates is sufficient to induce cellular differentiation, and eventual polarization of the two cell types, but does not permit the cellular intercalation that is presumably required for tip formation and elongation.

We would like to thank Dr. R. Kay for several helpful suggestions during the course of this work, Dr. J. Franke for his comments on the manuscript, and Dr. M. Faure for making the pBDE and pBDEΔ plasmids.

1. Konijn, T. M., Van de Meene, J. G. C., Bonner, J. T. & Barkley, D. S. (1967) *Proc. Natl. Acad. Sci. USA* **58,** 1152–1154.
2. Devreotes, P. (1989) *Science* **245,** 1054–1058.
3. Jermyn, K. A., Duffy, K. T. I. & Williams, J. G. (1989) *Nature (London)* **340,** 144–146.
4. Jermyn, K. A. & Williams, J. G. (1991) *Development* **111,** 779–787.
5. Williams, J. G., Duffy, K. T., Lane, D. P., McRobbie, S. J., Harwood, A. J., Traynor, D. & Jermyn, K. A. (1989) *Cell* **59,** 1157–1163.
6. McRobbie, S. J., Jermyn, K. A., Duffy, K., Blight, K. & Williams, J. G. (1988) *Development* **104,** 275–284.
7. Wallace, J. S., Morrissey, J. H. & Newell, P. C. (1984) *Cell Differ.* **14,** 205–211.
8. Esch, R. K. & Firtel, R. A. (1991) *Genes Dev.* **5,** 9–21.
9. Williams, J. G., Jermyn, K. A. & Duffy, K. (1989) *Development (Supplement)* **7,** 91–97.
10. Raper, K. B. (1940) *J. Elisha Mitchell Sci. Soc.* **56,** 241–282.
11. Bonner, J. T. (1947) *J. Exp. Zool.* **106,** 1–26.
12. Bonner, J. T. (1949) *J. Exp. Zool.* **110,** 259–271.
13. Rubin, J. & Robertson, A. (1975) *J. Embryol. Exp. Morph.* **33,** 227–241.
14. Maeda, Y. (1977) *Dev. Growth Differ.* **19,** 201–205.
15. Gerisch, G. (1987) *Annu. Rev. Biochem.* **56,** 853–879.
16. Kimmel, A. R. & Firtel, R. A. (1991) *Curr. Op. Genet. Dev.* **1,** 383–390.
17. Klein, C. (1975) *J. Biol. Chem.* **250,** 7134–7138.
18. Faure, M., Podgorski, G. J., Franke, J. & Kessin, R. H. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8076–8080.
19. Faure, M., Franke, J., Hall, A. L., Podgorski, G. J. & Kessin, R. H. (1990) *Mol. Cell. Biol.* **10,** 1921–1930.
20. Podgorski, G. J., Franke, J., Faure, M. & Kessin, R. H. (1989) *Mol. Cell. Biol.* **9,** 3938–3950.
21. Kay, R. R., Garrod, D. & Tilly, R. (1978) *Nature (London)* **271,** 58–60.
22. Barklis, E. & Lodish, H. F. (1983) *Cell* **32,** 1139–1148.
23. Mehdy, M. C., Ratner, D. & Firtel, R. A. (1983) *Cell* **32,** 763–771.
24. Schaap, P., Van Lokeren Campagne, M., Van Driel, R., Spek, W., Van Haastert, P. J. M. & Pinas, J. (1986) *Dev. Biol.* **118,** 52–63.
25. Wang, M., Van Driel, R. & Schaap, P. (1988) *Development* **103,** 611–618.
26. Bonner, J. T. (1952) *Am. Nat.* **86,** 79–89.
27. Sternfeld, J. & David, C. N. (1981) *Differentiation* **20,** 10–20.
28. Matsukuma, S. & Durston, A. J. (1979) *J. Embryol. Exp. Morphol.* **50,** 243–251.
29. Loomis, W. F. (1989) in *Progress in Molecular and Sub-Cellular Biology*, eds. Jeanteur, P., Kuchino, Y., Müller, W. E. G. & Paine, P. L. (Springer, Berlin), pp. 159–183.
30. Geltosky, J. E., Weseman, J., Bakke, A. & Lerner, R. A. (1979) *Cell* **18,** 391–398.
31. Lam, T. Y., Pickering, G., Geltosky, J. & Siu, C. H. (1981) *Differentiation* **20,** 22–28.
32. Siu, C. H., Des Roches, B. & Lam, T. Y. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6596–6600.
33. Houle, J., Balthazar, J. & West, C. M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3679–3683.
34. Nellen, W., Silan, C. & Firtel, R. A. (1984) *Mol. Cell. Biol.* **4,** 2890–2898.
35. Dingermann, T., Reindl, N., Werner, H., Hildebrandt, M., Nellen, W., Harwood, A., Williams, J. & Nerke, K. (1989) *Gene* **85,** 353–362.
36. Takeuchi, I. (1963) *Dev. Biol.* **8,** 1–26.
37. Krefft, M., Voet, L., Mairhofer, H. & Williams, K. L. (1983) *Exp. Cell Res.* **147,** 235.
38. Henderson, E. J. (1975) *J. Biol. Chem.* **250,** 4730–4736.
39. Kessin, R. H., Orlow, S. J., Shapiro, R. I. & Franke, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5450–5454.
40. Rossier, C., Franke, J., Mullens, I. A., Kelley, K. J. & Kessin, R. H. (1983) *Eur. J. Biochem.* **133,** 383–391.
41. Early, A. E., Williams, J. G., Meyer, H. E., Por, S. B., Smith, E., Williams, K. L. & Gooley, A. A. (1988) *Mol. Cell. Biol.* **8,** 3458–3466.
42. Devine, K. M., Bergmann, J. E. & Loomis, W. F. (1983) *Dev. Biol.* **99,** 437–446.
43. Takeuchi, I., Okamoto, K., Tasaka, M. & Takemoto, S. (1978) *Bot. Magazine (Tokyo)* **1,** 47–60.
44. Haberstroh, L. & Firtel, R. A. (1990) *Genes Dev.* **4,** 596–612.
45. Gerisch, G., Fromm, H., Huesgen, A. & Wick, U. (1975) *Nature (London)* **255,** 547–549.
46. Schaap, P. (1986) *Differentiation* **33,** 1–16.
47. Tsang, A. S. & Bradbury, J. M. (1981) *Exp. Cell Res.* **132,** 433–441.
48. Inouye, K. & Takeuchi, I. (1982) *Exp. Cell Res.* **138,** 311–318.
49. Mee, J. D., Tortolo, D. M. & Coukell, M. B. (1986) *Biochem. Cell. Biol.* **64,** 722–732.