Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8076–8080, November 1988
Developmental Biology

Disruption of *Dictyostelium discoideum* morphogenesis by overproduction of cAMP phosphodiesterase

(gene expression/differentiation/pattern formation/chemotaxis)

MICHEL FAURE, GREGORY J. PODGORSKI, JAKOB FRANKE, AND RICHARD H. KESSIN

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, July 25, 1988 (received for review April 8, 1988)

ABSTRACT **The development and cellular differentiation of *Dictyostelium discoideum* are disrupted in transformants secreting high levels of the cyclic nucleotide phosphodiesterase. The aggregation of these cells in the early stage of development proceeds rapidly and without the formation of organized streams. The later stages of development, in which differentiation into stalk and spore cells normally takes place, are completely blocked so that the transformants remain in spherical clusters of undifferentiated cells that do not elaborate the tip structure that regulates morphogenesis. These effects are due to overproduction of extracellular phosphodiesterase and demonstrate the role of cAMP during the aggregation phase of development as well as in the control of differentiation and pattern formation.**

When *Dictyostelium discoideum* amoebae are starved, they initiate a program of development that results in the formation of a multicellular organism composed of differentiated cell types. Individual cells aggregate by chemotactic movement toward signaling cells that are sources of cAMP. During the first hours of starvation the cells elaborate the biochemical apparatus necessary to synthesize, secrete, detect, and destroy cAMP (see refs. 1 and 2 for reviews). The molecules that are required for aggregation include an adenylate cyclase, a cAMP receptor, a secreted cyclic nucleotide phosphodiesterase (phosphodiesterase), and a phosphodiesterase inhibitor. The phosphodiesterase reduces extracellular cAMP levels between cAMP pulses, playing a role analogous to acetylcholinesterase in neuronal transmission. The essential role of the phosphodiesterase during aggregation has been demonstrated by studies of mutants that lack the enzyme and fail to aggregate unless it is supplied (3, 4). The phosphodiesterase is postulated to play a role in later morphogenesis when the cells further differentiate and undertake a complex series of movements to construct a fruiting body composed of spore and stalk cells (see ref. 5 for a review). During the elaboration of the fruiting body the phosphodiesterase transcript is restricted to prestalk cells (6).

A single phosphodiesterase gene encodes an enzyme that can be localized to the extracellular face of the plasma membrane or secreted from the cell (7). The phosphodiesterase mRNA is induced during development to levels ≈40-fold higher than those of growing cells (8).

We have introduced multiple copies of the cloned gene into cells, which then secrete large amounts of the enzyme. Overproduction of secreted phosphodiesterase does not impede growth but alters aggregation and blocks postaggregation morphogenesis. We find that transformants overexpressing the phosphodiesterase aggregate precociously and without the normal inward migration of streams of cells. The cells have the capacity to control the activity of the extracellular phosphodiesterase by secreting an inhibitor glycoprotein (9), but transformants carrying multiple copies of the phosphodiesterase gene have overwhelmed this homeostatic mechanism.

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.

EXPERIMENTAL PROCEDURES

Conditions for the growth and development of the *D. discoideum* strain AX3 have been described (6). A mixture of 12 μg of the vector pB10TP-1 (10), which carries the marker for resistance to G418, and 12 μg of the plasmid pGP-1 or pGP1ΔBst was prepared for the transformation of cells. pGP-1 carries the complete phosphodiesterase gene and pGP1ΔBst contains a phosphodiesterase gene that lacks 1 kilobase (kb) of the coding region. The construction of these plasmids is described in *Results*. The procedure for transformation was that of Nellen *et al.* (11) with the following modifications. The glycerol shock was for 5 min. Transformants were selected with 20 μg of G418 per ml for 4 days and with 5 μg/ml for an additional 4 days. Surviving cells were harvested and allowed to grow under nonselective conditions on SM/5 plates with *Klebsiella aerogenes* as a food source (12). Single colonies were picked and transferred to HL/5 medium containing 10 μg of G418 per ml. The transformants were maintained in this medium or on nonnutrient agar plates containing G418 and spread with autoclaved bacteria as a food source. Transformants gradually lost copies of the phosphodiesterase gene unless continuously exposed to G418.

The spectrophotometric assay and the radiometric assay of phosphodiesterase activity have been described (13). Procedures for RNA extractions and transfers (Northern blots) were according to Franke *et al.* (6). Southern blots were prepared as described by Lacombe *et al.* (8). The probe used for Northern and Southern blots was a 0.9-kb *Asu* II–*Eco*RI fragment of the phosphodiesterase cDNA (8) labeled with [α-^{32}P]dATP (111 TBq/mmol) by the random priming method of Feinberg and Vogelstein (14).

RESULTS

Expression of the Introduced Phosphodiesterase Gene. We used a phosphodiesterase cDNA as a probe to recover the genomic clone pGP-1 that contains a 4.7-kb *Bcl* I fragment of *D. discoideum* DNA inserted into the *Bam*HI site of pBR322 (Fig. 1*A*). Sequences within pGP-1 encompass the translated portion of the gene, 2.3 kb of 5′ flanking DNA, and 0.6 kb of 3′ flanking DNA. pGP1ΔBst is a derivative of pGP-1 that was prepared by excising the internal *Bst*XI fragment of pGP-1 that contains 1 kb of the protein coding region. pGP1ΔBst is shown in Fig. 1*A* and was used as a control in experiments testing the biological effects of phosphodiesterase overproduction. pGP-1 and pGP1ΔBst were introduced into *D. discoideum* AX3 cells by cotransformation with the vector pB10TP-1 (10), which carries the selectable marker for resistance to G418. Transformants selected by G418 were

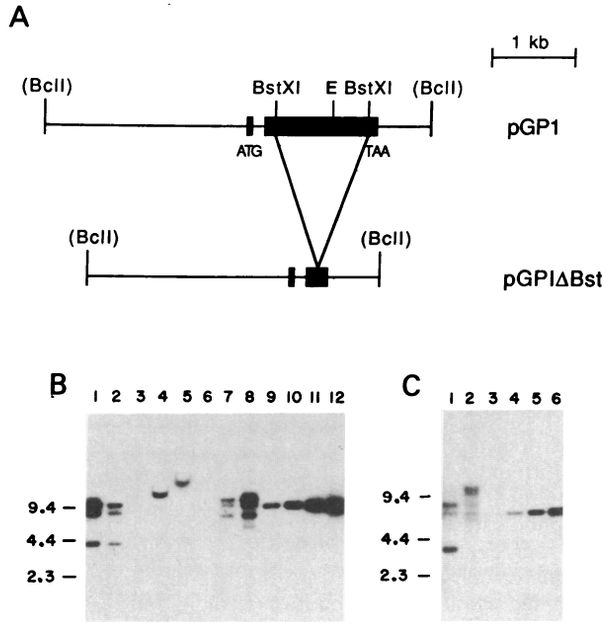

FIG. 1. Plasmids used for transformation and Southern blot analysis of transformant DNA. (*A*) Schematic representation of the plasmids used in the cotransformation experiments. Only the inserts are shown; for pGP-1 and pGP1ΔBst the *Bcl* I genomic fragments are inserted in the *Bam*HI site of pBR322 (15); *Bst*XI and *Eco*RI (E) sites are shown. Coding sequences are shown as thick lines and noncoding regions are shown as thin lines. pGP-1 carries the complete phosphodiesterase coding sequence. pGP1ΔBst has been generated from pGP-1 by cutting with *Bst*XI, digesting with mung bean nuclease to produce blunt ends, and religating the plasmid. (*B*) Southern blot of DNA from cells cotransformed with pGP-1 and pB10TP-1. DNA was extracted by the method of Reymond (16). Following restriction enzyme digestion, agarose gel electrophoresis, and Southern blotting (17), membranes were hybridized with a probe derived from the phosphodiesterase coding region. Numbers on the side indicate the size in kb and the positions of the *Hin*dIII digestion products of phage λ DNA. Lanes 1–4, DNA has been digested with *Eco*RI; lanes 5–12, DNA has been digested with *Hin*dIII. Lanes 1 and 8, 75 ng of AX3-PDE$_2$ DNA; lanes 2 and 7, 75 ng of AX3-PDE$_1$ DNA; lanes 3 and 6, 75 ng of AX3 DNA; lanes 4 and 5, 2 μg of AX3 DNA; lanes 9–12, calibration standards containing 0.34 ng, 0.68 ng, 1.36 ng, and 2.40 ng, respectively, of pGP-1 DNA, which corresponds to 25, 50, 100, and 200 gene copies per cell for 75 ng of genomic DNA. (*C*) Southern blot analysis, performed as described in *B*, of DNA from cells cotransformed with pGP1ΔBst and pB10TP-1. Lanes 1 and 2, 75 ng of AX3-PDEΔ DNA digested with *Eco*RI and *Hin*dIII, respectively; lanes 3–6, calibration standards containing 0.3 ng, 0.6 ng, 1.2 ng, and 2.4 ng, respectively, of pGP1ΔBst DNA, corresponding to 25, 50, 100, and 200 copies per cell for 75 ng of genomic DNA.

found to have integrated these plasmid sequences into chromosomal DNA in numbers that ranged from 25 to 200 copies per cell. Southern blots prepared with DNA from cotransformants carrying pGP-1 or pGP1ΔBst and probed for the phosphodiesterase gene are shown in Fig. 1 *B* and *C*. Lanes 3 and 6 in Fig. 1*B* contain 75 ng of genomic DNA from nontransformed AX3 cells, which is too little to detect a signal at the exposure time used. Comparison of the hybridizing fragments in transformant DNA with that of linearized pGP-1 plasmid DNA, included as a calibration standard for gene copy number, shows that the integrated sequences are not inserted in a simple head-to-tail array. Bands larger and smaller than pGP-1 were detected, which indicates that sequence rearrangement of the transforming DNA has occurred. This result is common in the cotransformation of *D. discoideum* cells and is believed to be due to recombination between the two plasmid sequences before insertion into the chromosomal DNA (18). By comparing the intensity of the bands from the transformants with the signal observed in nontransformed cells and with calibration standards prepared with pGP-1 or pGP1ΔBst, we estimate that AX3-PDE$_1$ carries 25–50 copies of the phosphodiesterase gene, AX3-PDE$_2$ contains 100–150 copies of the phosphodiesterase gene, and AX3-PDEΔ harbors 100 copies of the pGP1ΔBst sequence.

When Northern blots of mRNA from AX3-PDE$_1$ and AX3-PDE$_2$ were analyzed, we found that the amount of mRNA produced by growing cells of these transformants is proportional to gene copy number (Fig. 2*B*). A comparison of the signal intensities indicates at least a 100-fold overproduction of phosphodiesterase mRNA by AX3-PDE$_2$ during growth. Two sizes of phosphodiesterase mRNA are detected in nontransformed cells; a 1.8-kb transcript is found in growing and developing cells, and a 2.2-kb transcript is observed shortly after the initiation of starvation (Fig. 2*A* and ref. 6). Comparison of the mRNAs from transformed and nontransformed cells shows that only the 1.8-kb transcript is overproduced by the transformants (Fig. 2*B*), indicating that elements required for the production of the 2.2-kb developmental transcript are absent in pGP-1. AX3-PDEΔ produces a 0.8-kb mRNA (data not shown), demonstrating that the deleted phosphodiesterase gene is functional and can be transcribed following transformation.

Extracellular phosphodiesterase is secreted by the transformants in amounts proportional to gene copy number (Table 1). Growing cells of the transformant AX3-PDE$_2$ produce 160-fold more phosphodiesterase than AX3 cells. The generation time of cells transformed with pGP-1 is similar to that of nontransformed cells. The relative overproduction of phosphodiesterase in the transformants falls during development; AX3-PDE$_2$ produced only 30-fold more phosphodiesterase than AX3 after 3 hr of starvation. This decrease relative to growing cells appears to be due to a combination of two factors; the levels of 1.8-kb phosphodiesterase mRNA decline during starvation of the transformants (Fig. 2*B*), whereas in the developing nontransformed cells significant amounts of the 2.2-kb phosphodiesterase mRNA are produced (Fig. 2*A*). The control transformant

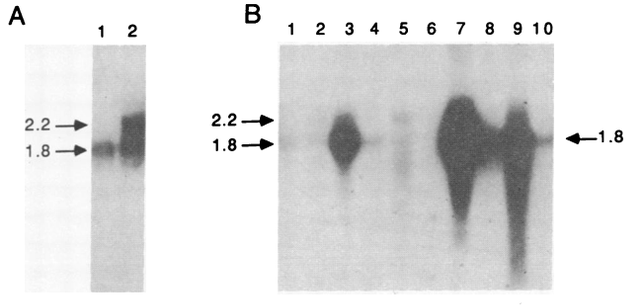

FIG. 2. Levels of phosphodiesterase mRNA in transformed and nontransformed cells. Total RNA was extracted and processed for Northern blotting according to Franke *et al.* (6). Blots were hybridized with the phosphodiesterase coding region probe. (*A*) RNA from AX3. Lane 1, 5 μg from cells growing in HL/5 medium; lane 2, 5 μg from cells shaken for 3 hr in 17 mM Na_2/K-phosphate buffer, pH 6.0/50 μM $CaCl_2$ (starvation buffer). (*B*) RNA from AX3, AX3-PDE$_1$, and AX3-PDE$_2$ cells. Lane 1, 5 μg from growing AX3; lane 2, 5 μg from AX3 cells after 3 hr of shaking in starvation buffer; lanes 3–6, RNA from a transformant (AX3-PDE$_1$) containing 25 copies of the phosphodiesterase gene: 5 μg (lane 3) and 0.5 μg (lane 4) of RNA from growing cells and 5 μg (lane 5) and 0.5 μg (lane 6) of RNA from cells shaken for 3 hr in starvation buffer; lanes 7–10, RNA from a transformant (AX3-PDE$_2$) containing 200 copies of the phosphodiesterase gene: 5 μg (lane 7) and 0.5 μg (lane 8) of RNA from growing cells and 5 μg (lane 9) and 0.5 μg (lane 10) of RNA from cells shaken 3 hr in starvation buffer. Arrows indicate the positions of the 1.8-kb and 2.2-kb RNAs.

Table 1. Extracellular phosphodiesterase activity in control and transformed cells

Strain	Approximate PDE gene copy number	Growth, units/ml	3 hr of starvation, units/ml
AX3	1	11	14
AX3-PDE$_1$	25	62	30
AX3-PDE$_2$	200	1750	450

Cells were grown, harvested, and washed as described in the legend of Fig. 2. Starvation took place in flasks shaken at 22°C. Cellular supernatants were recovered and dialyzed overnight against 50 mM Tris (pH 8.0) at 4°C. Samples were assayed after treatment by dithiothreitol to destroy the specific phosphodiesterase inhibitor (9). PDE, phosphodiesterase.

AX3-PDEΔ produces phosphodiesterase at levels comparable to nontransformed cells.

The extracellular phosphodiesterase secreted by cells of AX3-PDE$_2$ and AX3-PDEΔ developing on filters was assayed (Fig. 3). If extracellular phosphodiesterase is assayed in the presence of the inhibitor, a condition that provides a physiologically relevant measure of the extracellular phosphodiesterase levels, the difference in phosphodiesterase activity between these strains approaches 100-fold. The phosphodiesterase inhibitor is produced by developing cells and functions to inactivate the extracellular phosphodiesterase (9, 19). When the cysteine-rich inhibitor glycoprotein is inactivated by reduction with dithiothreitol (9), AX3-PDE$_2$ cells are found to produce 10-fold more enzyme after 22 hr of development than AX3-PDEΔ cells (Fig. 3).

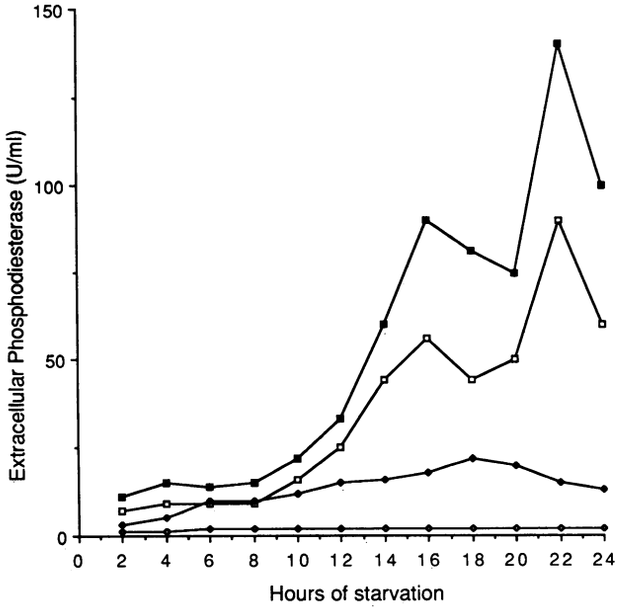

FIG. 3. Time course of extracellular phosphodiesterase accumulation of cells developing on filters. The extracellular phosphodiesterase was collected from 9×10^7 cells on two 42.5-mm Whatman 50 filters by washing the cells and filters with 4 ml of starvation buffer. Cells were pelleted and the supernatant was used to extract the enzyme from the pads by shaking for 15 min before collecting the liquid. Phosphodiesterase activity is expressed in units/ml of the extraction solution. One unit is defined as 1 nmol of cAMP hydrolyzed in 1 min. The phosphodiesterase inhibitor was inactivated by incubation at 35°C for 1 hr in the presence of 2 mM dithiothreitol. Corrections were made for the slight inhibition of the phosphodiesterase by dithiothreitol. Open symbols represent values before inhibitor inactivation and solid symbols represent values after inactivation of the inhibitor. ◇ and ◆, Extracellular phosphodiesterase from AX3-PDEΔ; □ and ■, extracellular phosphodiesterase from AX3-PDE$_2$.

In normal cells a variable fraction of phosphodiesterase activity is localized to the membrane (20). By using a radiometric assay, we measured membrane-bound phosphodiesterase activity residing on the extracellular face of the plasma membranes of living cells (21). After 14 hr of development on filters, we detected 0.13 unit of membrane-bound phosphodiesterase per 10^7 AX3-PDEΔ cells and 0.16 unit per 10^7 AX3-PDE$_2$ cells. Assays of membrane-bound phosphodiesterase at 17 hr of development in these transformants also failed to detect any significant differences. The excess enzyme activity in the transformants results from overproduction of the extracellular form of the phosphodiesterase.

Effect of Phosphodiesterase Overproduction on Development. *D. discoideum* transformants that overexpress the phosphodiesterase gene exhibit profound alterations in their patterns of development. Fig. 4 shows the aggregation of the overexpressing transformant AX3-PDE$_2$ and the control transformant AX3-PDEΔ. A comparison of AX3-PDE$_2$ (Fig. 4, column a) and AX3-PDEΔ (Fig. 4, column c) indicates that phosphodiesterase overproduction in AX3-PDE$_2$ leads to rapid aggregation without the characteristic formation of streams of cells migrating into the aggregation center. The rapid aggregation is seen after 6 hr of starvation when AX3-PDE$_2$ cells have already formed sizable clusters, whereas AX3-PDEΔ remains as single cells. The aggregation and later development of the AX3-PDEΔ control cells is identical to nontransformed AX3 cells. By 9 hr of starvation small clusters of 10–20 AX3-PDEΔ cells begin to form whereas AX3-PDE$_2$ amoebae are found in large masses containing 1000 or more cells. After 15 hr of starvation another major difference is observed between AX3-PDE$_2$ and AX3-PDEΔ. AX3-PDEΔ is organized into large streams of cells migrating into a central region (Fig. 4, column c), whereas AX3-PDE$_2$ shows no streaming behavior at any stage of aggregation (Fig. 4, column a). The normal aggregation of AX3-PDEΔ indicates that the effects we observe in phosphodiesterase overproducing strains such as AX3-PDE$_2$ are caused by the phosphodiesterase and not by factors related to the high copy number of phosphodiesterase regulatory or flanking sequences present in these cells.

When the phosphodiesterase inhibitor glycoprotein was added to the developing transformed cells the timing of aggregation was restored to that of control cells. Transformed cells treated with inhibitor form inwardly migrating streams (Fig. 4, column b). In the experiment shown in Fig. 4, column b, 80 units of purified inhibitor per well were added. Addition of 8 units per well was not sufficient to restore aggregation to its normal time course. The inhibitor functions solely in the extracellular medium and therefore the abnormal aggregation of the transformant is due to effects of phosphodiesterase acting outside the cells.

Cells expressing multiple copies of the phosphodiesterase gene that are allowed to develop on a solid substratum are unable to form fruiting bodies. Fig. 5 shows the terminal structures of AX3-PDEΔ and AX3-PDE$_2$. The introduction of multiple copies of the deleted phosphodiesterase clone pGPΔBst has no effect on development in AX3-PDEΔ (Fig. 5*a*), whereas overproduction of phosphodiesterase in AX3-PDE$_2$ severely alters morphogenesis (Fig. 5*b*). The terminal structures formed by AX3-PDE$_2$ cells are rounded masses that do not exhibit the distinct tip that organizes cell movement and is an essential feature of postaggregation development. Phase-contrast microscopic observation of disaggregated cells of AX3-PDE$_2$ obtained from the terminal structures revealed no differentiated spore or stalk cells. The aberrant postaggregation development of cells overexpressing the phosphodiesterase cannot be explained by the confinement within the aggregate of phosphodiesterase produced early in development, because when AX3-PDE$_2$ aggregates are dissociated, washed to remove extracellular phospho-

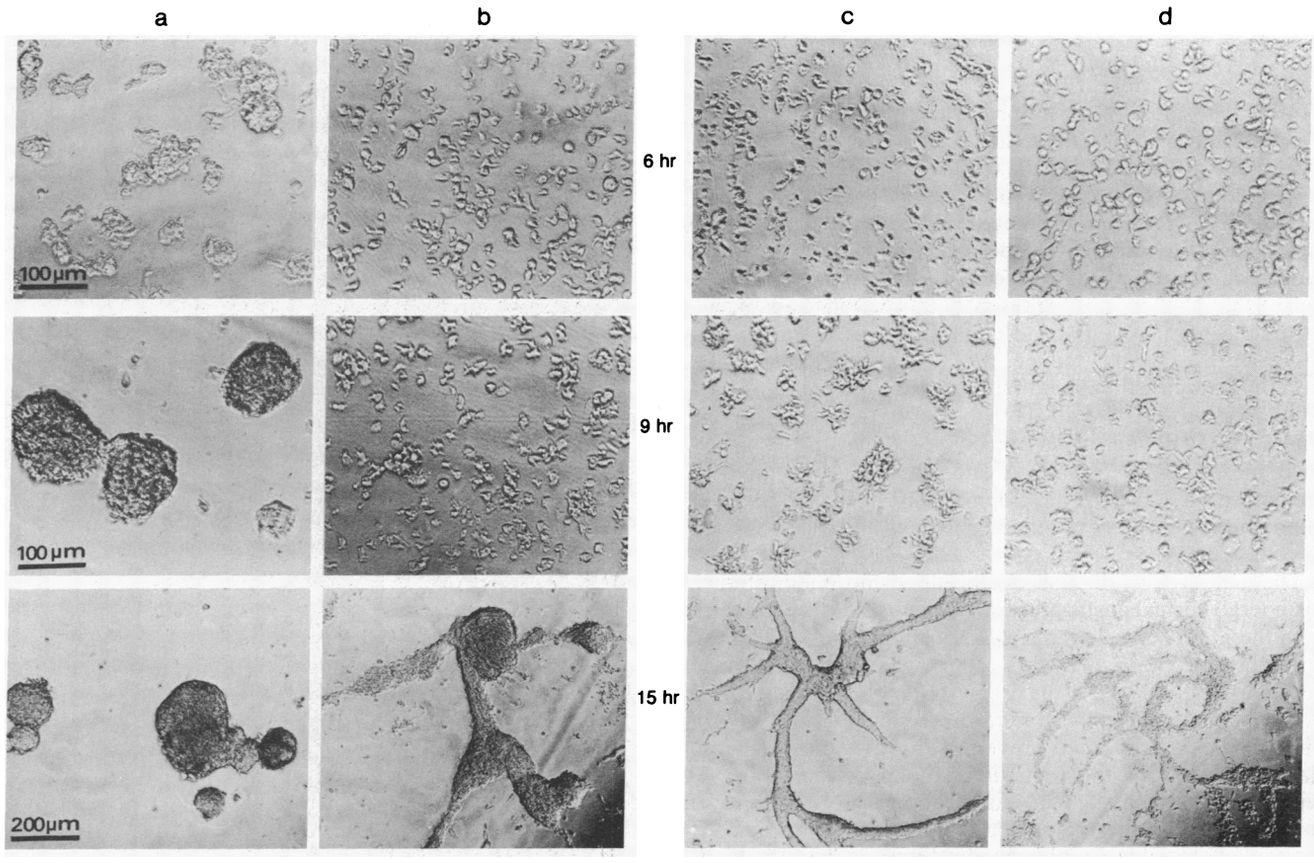

FIG. 4. Aggregation of *D. discoideum* amoebae transformed with the phosphodiesterase gene. Growing amoebae were harvested and washed, and 2×10^5 cells in 0.25 ml of starvation buffer were incubated in 15-mm microwell chambers at 22°C. Purified phosphodiesterase inhibitor was added to 320 units/ml (9) to cells shown in columns b and d. AX3-PDE_2 cells are shown in columns a and b. AX3-PDEΔ cells are shown in columns c and d. Observation times in hours are indicated.

diesterase, and then allowed to reaggregate rapidly, the same abnormal structures are formed. We have not succeeded in restoring normal fruiting bodies by addition of inhibitor to the transformant. When equal numbers of AX3-PDE_2 and AX3 cells are mixed and allowed to develop, the final structures remain defective. The degree to which aggregation and later development are altered in transformants overproducing the phosphodiesterase is related to the phosphodiesterase gene copy number. AX3-PDE_1 cells harboring 25–50 copies of the phosphodiesterase gene are able to form small aggregates that develop with a morphologically distinct tip. A small portion of the aggregates completes development and forms mature fruiting bodies.

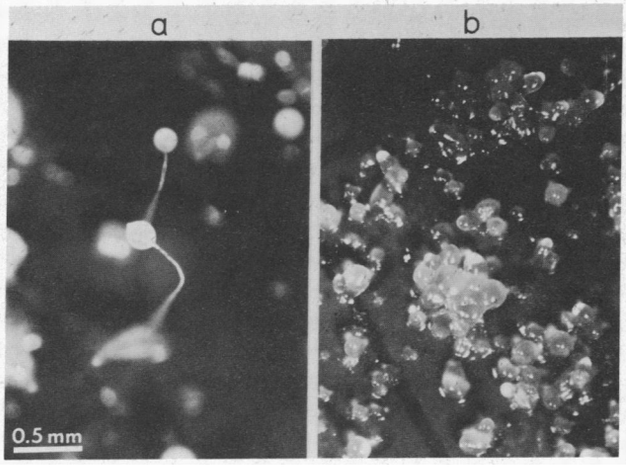

FIG. 5. Development on Millipore filters of AX3-PDEΔ (*a*) and AX3-PDE_2 (*b*). Cells were prepared for development as described by Sussman (12) except that starvation buffer (17 mM Na_2/K-phosphate buffer, pH 6.0/50 μM $CaCl_2$) was used. Photographs were taken after 30 hr.

DISCUSSION

The overproduction of extracellular phosphodiesterase leads to profound effects on the development of *D. discoideum*. Our prediction was that high levels of phosphodiesterase would prevent cells from aggregating, since cell aggregation in *D. discoideum* is mediated by cAMP. However, the transformants overproducing phosphodiesterase do aggregate, although more rapidly and without formation of streams of migrating cells. The collection of the cells into aggregates indicates either that the cAMP signaling that normally mediates chemotaxis is not eliminated by these levels of enzyme or that the amoebae utilize a cAMP-independent mechanism for aggregation under these unusual conditions of development. Aggregation directed by levels of cAMP far lower than normal may be possible. It has been shown that 1 nM pulses of cAMP are as effective as 100 nM pulses for the induction of cAMP binding sites and the cell adhesion molecule contact site A (22). Preliminary examination of time lapse video tapes indicates that the high-copy number transformants migrate directionally into aggregates by alternating between periods of movement and periods when amoebae are stationary (D. Wessells, D. Soll, M.F., and R.H.K., unpublished experiments). This form of migration is observed during chemotaxis that is controlled by cAMP signaling. The precocious aggre-

gation of the transformants that occurs without cell streaming was shown to result from the depletion of extracellular cAMP due to elevated phosphodiesterase activity because normal aggregation is restored if the secreted enzyme is inhibited with the phosphodiesterase inhibitor glycoprotein (Fig. 4). Wier (20) has also observed that adding phosphodiesterase significantly reduces the period required for aggregation. One explanation for these observations is that the cAMP that is secreted during the earliest stages of development normally accumulates to some extent before significant amounts of phosphodiesterase have been secreted. Accumulation would cause any cAMP receptors present at this period to adapt and become less sensitive to cAMP. Introduction of more phosphodiesterase would reduce the background of cAMP, thereby removing the inhibition resulting from partial receptor adaptation. This would allow the cell to detect early weak pulses of cAMP and would precociously trigger the appearance of the cAMP signaling and response system. The premature induction of developmentally regulated genes is often observed when exogenous pulses of cAMP are applied to cells (22–24).

The inability of cells that overproduce phosphodiesterase to undergo normal postaggregation development is consistent with the proposed role for cAMP signaling in differentiation and morphogenesis. cAMP is required for the early differentiation of all cells up to the branch point of the prespore and prestalk cell lineages (25). The presence of high levels of phosphodiesterase would prevent postaggregation differentiation because of the reduction or elimination of cAMP within the aggregates. Our examination by phase-contrast microscopy of cells in the terminal structures formed by strains overproducing phosphodiesterase failed to detect differentiated spore or stalk cells. The transformants that secreted high levels of phosphodiesterase never progressed to the slug stage of development, in which the organism exhibits a polar organization of prestalk and prespore cells along an anterior–posterior axis. cAMP, which is known to be produced by the tip of the slug (26, 27), has been proposed to play a role in the development and maintenance of polarity within the aggregate (28). The failure of the transformants that overproduce phosphodiesterase to form an organizing tip or slug is consistent with this hypothesis.

The segment of DNA containing the phosphodiesterase gene used for the transformations directs the synthesis of the 1.8-kb form of the phosphodiesterase mRNA. We have shown that elements residing in regions upstream of the DNA segments present in pGP-1 are required for production of the 2.2-kb transcript (unpublished data). The overproduction of extracellular but not membrane-bound enzyme by cells transformed by pGP-1 suggests that sequences found only in the 2.2-kb mRNA play a role in directing membrane attachment.

We thank Bernd Wurster and Michiel M. van Lookeren Campagne for helpful discussions and Jeffrey Williams and Anne Early for providing the plasmid pB10TP-1. This work was supported by National Institutes of Health Grants GM331306 to R.H.K. and GM10174 to G.J.P.

1. Gerisch, G. (1987) *Annu. Rev. Biochem.* **56,** 853–879.
2. Kessin, R. H. (1988) *Microbiol. Rev.* **52,** 29–49.
3. Darmon, M., Barra, J. & Brachet, P. (1978) *J. Cell. Sci.* **31,** 233–243.
4. Barra, J., Barrand, P., Blondelet, M.-H. & Brachet, P. (1980) *Mol. Gen. Genet.* **177,** 607–613.
5. Schaap, P. (1986) *Differentiation* **33,** 1–16.
6. Franke, J., Podgorski, G. J. & Kessin, R. H. (1987) *Dev. Biol.* **124,** 504–511.
7. Podgorski, G. J., Franke, J. & Kessin, R. H. (1986) *J. Gen. Microbiol.* **132,** 1043–1050.
8. Lacombe, M.-L., Podgorski, G. J., Franke, J. & Kessin, R. H. (1986) *J. Biol. Chem.* **261,** 16811–16817.
9. Franke, J. & Kessin, R. H. (1981) *J. Biol. Chem.* **256,** 7628–7637.
10. Early, A. E. & Williams, J. (1987) *Gene* **59,** 99–106.
11. Nellen, W., Datta, S., Reymond, C., Sivertsen, A., Mann, S., Crowley, T. & Firtel, R. A. (1987) *Methods Cell Biol.* **28,** 67–1000.
12. Sussman, M. (1987) *Methods Cell Biol.* **28,** 9–29.
13. Orlow, S. J., Shapiro, R. I., Franke, J. & Kessin, R. H. (1981) *J. Biol. Chem.* **256,** 7620–7627.
14. Feinberg, A. P. & Vogelstein, B. (1984) *Anal. Biochem.* **137,** 266–267.
15. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, W. W., Crosa, J. H. & Falkow, S. (1977) *Gene* **2,** 95–113.
16. Reymond, C. D. (1987) *Nucleic Acids Res.* **15,** 8118.
17. Southern, E. (1975) *J. Mol. Biol.* **98,** 503–517.
18. Nellen, W. & Firtel, R. A. (1985) *Gene* **39,** 155–163.
19. Malchow, D., Nagele, B., Schwarz, H. & Gerisch, G. (1972) *Eur. J. Biochem.* **28,** 136–142.
20. Wier, P. (1977) *Differentiation* **9,** 183-191.
21. Shapiro, R. I., Franke, J., Luna, E. J. & Kessin, R. H. (1983) *Biochim. Biophys. Acta* **758,** 49–57.
22. Yeh, R. P., Chan, F. K. & Coukell, M. B. (1978) *Dev. Biol.* **66,** 361–374.
23. Mehdy, M., Ratner, D. & Firtel, R. A. (1983) *Cell* **32,** 763–771.
24. Barklis, E. & Lodish, H. F. (1983) *Cell* **32,** 1139–1148.
25. Williams, J. G., Pears, C. J., Jermyn, K. A., Driscoll, D. M., Mahbubani, H. & Kay, R. R. (1986) in *Regulation of Gene Expression*, eds. Booth, I. & Higgins, C. (Cambridge Univ. Press, Cambridge, U.K.), pp. 277–298.
26. Bonner, J. T. (1949) *Exp. Zool.* **110,** 259–271.
27. Maeda, Y. (1977) *Dev. Growth Differ.* **19,** 201–205.
28. Schaap, P. & Wang, M. (1986) *Cell* **45,** 137–144.