Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10561–10565, October 1994
Developmental Biology

Expression of cAMP-dependent protein kinase in prespore cells is sufficient to induce spore cell differentiation in *Dictyostelium*

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Communicated by Eric H. Davidson, June 23, 1994

ABSTRACT **The activity of cAMP-dependent protein kinase (PKA) is required for proper development at several stages during the *Dictyostelium* life cycle. We present evidence that activation of PKA is rate-limiting for the differentiation of prespore cells to spores and that PKA activation may be the developmental trigger for sporulation. Strains that overexpress the gene encoding the catalytic subunit of PKA (*PKAcat*) or lack a functional regulatory subunit (*rdeC* strains) undergo rapid, heterochronic development. We show that overexpression of *PKAcat* in prespore cells is sufficient to directly induce expression of the spore maturation marker *spiA* and differentiation to spores, in a cell-autonomous manner. Moreover, overexpression of *PKAcat* in prespore cells can bypass a mutation that blocks an earlier developmental step to induce *spiA* expression. Our results suggest that the regulatory pathway in prespore cells between the activation of PKA and *spiA* induction/spore maturation is quite short and that *PKAcat* expression in prespore cells may mediate spore differentiation at the level of transcription. This induction of sporulation requires the prior activation of the prespore cell pathway. In addition, we show that β-galactosidase activity expressed from a *PKAcat* promoter/*lacZ* reporter construct is highly enriched in the anterior prestalk A region during the tipped aggregate, slug, and early culminant stages and that this pattern switches abruptly to a prespore pattern at the time of spore maturation, supporting the proposed role of PKA in this process.**

Dictyostelium discoideum grows as vegetative amoebae in the presence of a food source. When this is depleted, ≈10^5 cells aggregate and proceed through a morphogenetic program, culminating in the formation of a fruiting body containing a spore mass borne aloft by a slender stalk. Aggregation to form the multicellular organism and subsequent morphogenetic movements and gene regulation, directed by extracellular signals such as cAMP acting through cell surface receptors, have been fairly well defined (1–7). However, the role of *intracellular* cAMP in development is not well understood. In eukaryotes, cAMP functions as a second messenger to activate cAMP-dependent protein kinase (PKA), which is involved in a diversity of regulatory pathways (8). In *Dictyostelium*, as in other systems, intracellular cAMP binds to the regulatory subunit of PKA, causing dissociation and activation of the catalytic subunit, PKAcat (9–11).

Recent evidence indicates that proper regulation of PKA is required for normal development. Examination of *pkacat* null mutants and strains overexpressing dominant negative mutants of the regulatory subunit indicates that PKA is required for aggregation, entry into the morphogenetic stage, prespore cell differentiation, and culmination (12–20). Moreover, *rdeC* mutants, which show rapid development, abnormal morphogenesis, and precocious spore formation, lack a functional regulatory subunit (21, 22) and are presumed to have active PKA in all cells that express the catalytic subunit. However, studies on these mutants could not address where and when in the developmental program PKAcat functions. Overexpression of the *PKAcat* coding region from its own and various cell-type-specific promoters renders the enzyme constitutively active in only particular cells and leads to aberrant morphologies (17, 18, 20, 23). The phenotype of cells expressing *PKAcat* from the prespore-specific promoters *SP60* (*cotC*) and *D19* (*pspA*) provides evidence that PKA may play a direct role in spore differentiation.

When *SP60/PKAcat* cells are plated for development on phosphate-buffered agar, a culminant forms rapidly that consists of a mound of spores with a protruding stalk and a very small sorus (18, 20). When plated on Millipore filters over buffered agar, the morphology is different. A basal mound of spores (containing ≈75% of the cells) again rapidly forms, but a finger-like structure protrudes and falls onto the substratum (18) (see Fig. 1). This becomes a slug-like structure that migrates for a relatively great distance, remaining attached to the basal mound by a partially cellular tube. The "slug," which becomes smaller as it migrates and sloughs off cells into the elongating tube, eventually culminates to form a very small "fruiting body." By using cell-type-specific *lacZ* reporter constructs, it was shown that the distribution of cell types within the slug is significantly altered. In wild-type slugs, the posterior 80% is almost entirely prespore cells, and the anterior 20% is prestalk cells, with the apical 5–10% prestalk A cells and prestalk AB cells present as a small core within this prestalk A-cell region (the remaining ≈10% are prestalk O cells). In *SP60/PKAcat* strains, the anterior 50% of the small slug is a mixture of prestalk A and prestalk AB cells. Prespore cells make up almost the entire basal mound and are also found in the tube and at the very posterior of the slug. The pattern in the slug remains stable, even as the slug continues to migrate and lose cells into the extending tube. This suggests that the cells toward the posterior of the slug are redifferentiating into prespore cells as slug migration occurs.

In this paper, we further examine the developmental roles of PKA and show that expression of *PKAcat* at the onset of prespore cell differentiation is sufficient to very rapidly induce these cells to express the sporulation marker *spiA* (24, 25) and to mature into spores. In addition, expression of *PKAcat* in prespore cells of a *gα4* null mutant, blocked after formation of prespore cells, allows some steps in spore maturation to proceed. Further, we analyze the wild-type spatial expression pattern of the *PKAcat* promoter to show that *PKAcat* is

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Abbreviations: PKA, cAMP-dependent protein kinase; PKAcat, PKA catalytic subunit; *PKAcat*, gene for PKAcat.

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expressed throughout the mound, and then in prestalk A cells during the finger and slug stages. As the prespore mass rises above the substratum, expression of *PKAcat* abruptly shifts to a prespore pattern. Our results suggest models by which differential regulation and expression of PKA controls maturation of prespore cells into spores.

MATERIALS AND METHODS

Dictyostelium strains were grown, transformed with expression constructs, clonally isolated, and developed as described (13, 18). Cells were stained for β-galactosidase expression from *lacZ* reporter constructs as described (26, 27). All cell lines were selected and maintained in medium with G418 at 20 μg/ml, except those carrying the *PKA/lacZ* construct, which were maintained with G418 at 60 μg/ml. Mature, viable spores were assayed as described (28).

For Northern analysis, cells were developed on filters and harvested at various time points, and RNA was extracted (29). The RNA was electrophoresed in formaldehyde/agarose gels, with 6 μg of total RNA per lane. The size-separated RNAs were then blotted and hybridized as described (29), with probes labeled by random priming.

The *SP60/PKAcat* expression vector has been described (17), as have the vectors for *ecmA/PKAcat*, *ecmB/PKAcat*, and *SP60/lacZ* (18). The *PKAcat/lacZ* vector was created by replacing the *SP60* (*cotC*) promoter in *SP60/lacZ* (26) with upstream sequences of *PKAcat*. A genomic subclone of *PKAcat* that includes 1.6 kb of upstream sequence was digested with exonuclease III to remove most of the coding sequences and capped with *Hin*dIII linkers. The 3′ endpoint was found to include sequences for the first 27 amino acids of the protein. The fragment was cloned between the *Bgl* II and *Hin*dIII sites of the *SP60/lacZ* vector from which the *SP60* promoter fragment had been removed, then the *Hin*dIII

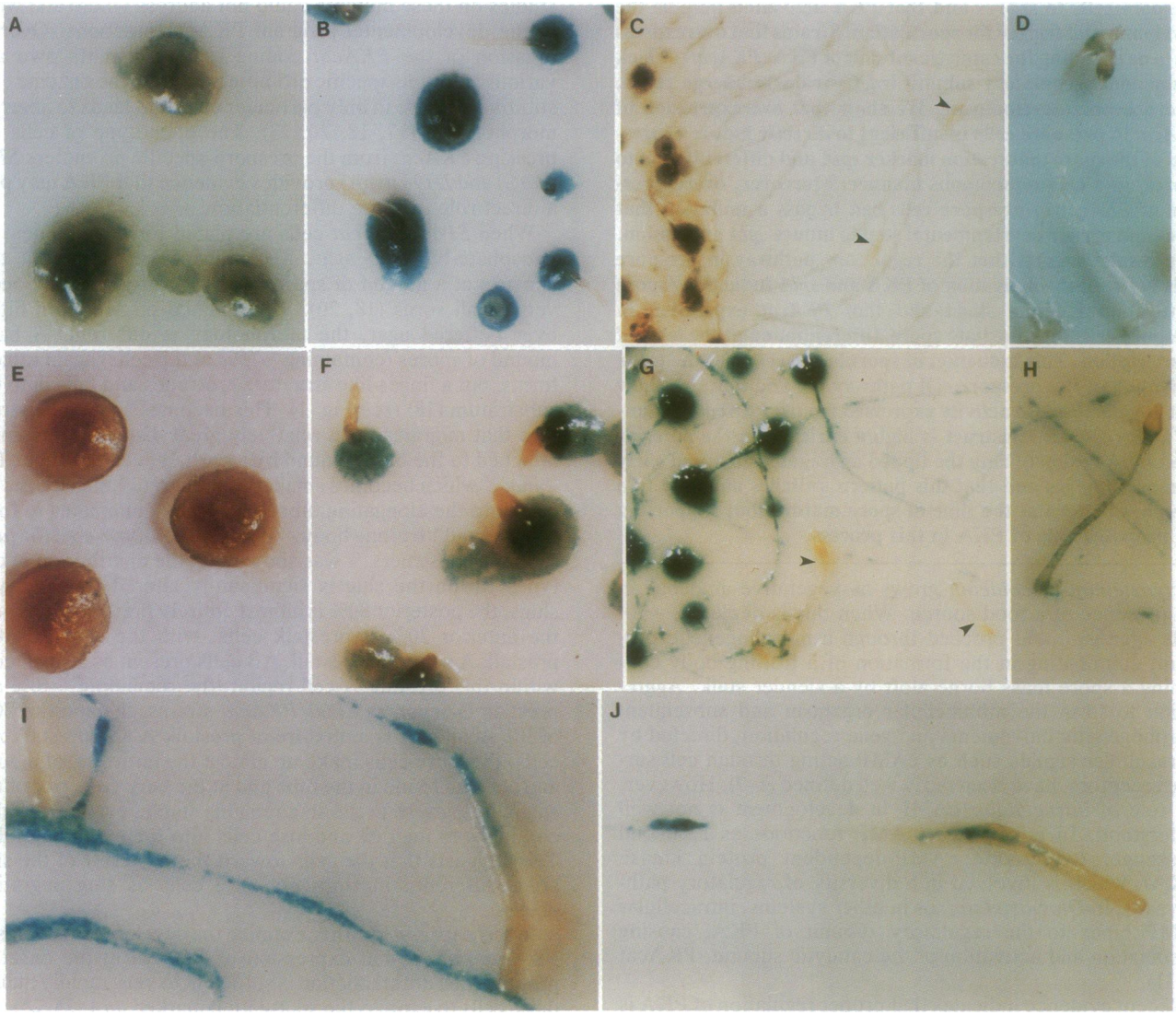

FIG. 1. *SP60* and *spiA* expression in KAx-3 *SP60/PKAcat* cells. Presented are the patterns of β-galactosidase activity staining of *SP60/PKAcat* cells expressing either *SP60/lacZ* (*A–D* and *I*) or *spiA/lacZ* (*E–H* and *J*). *A* and *E* show 10.5-hr developed aggregates; in *E*, *spiA/lacZ*-staining cells are visible, though <1% of the cells stain at this stage. *B* and *F* show basal mounds as the finger-like structures protrude and begin to fall over. *C* and *G* present a lower-magnification view of basal mounds (stained dark blue) and migrating slug-like structures. Some of these slugs, which in general are difficult to see at this magnification, are indicated by small arrowheads in *C*. The tubes can be seen as long stringlike structures. In *G* the arrowheads indicate mature fruiting-body-like structures rather than slugs. Also, the patches of staining cells in the tubes are more clearly seen. *D* and *H* show a higher-magnification view of the fruiting-body-like structures. *I* and *J* provide a higher-magnification view of migrating slugs; in both strains, staining cells are present at the posterior of the slug and as patches of cells within the tube.

site was opened, filled in by Klenow DNA polymerase, and religated to make the fusion in-frame. The filled-in *Hin*dIII site created an *Nhe* I site.

RESULTS

Induction of a Spore Differentiation Marker by PKA. Differentiation of prespore cells to spores can be induced by very high levels of the membrane-permeant cAMP analog 8-Br-cAMP, presumably by activating PKA (30, 31). The rapid development and sporogenous phenotype of *rdeC* mutants and cell lines overexpressing *PKAcat* from a prespore-specific promoter support this theory (18, 20, 22). To determine where in the developmental program PKA functions to control sporulation, we have studied the sporulation marker *spiA* in strains overexpressing *PKAcat* in only specific cells. *spiA* encodes a spore coat protein that is synthesized as spores mature and can be induced by high concentrations of 8-Br-cAMP but not cAMP (24, 25). It is tightly linked to spore formation and thus serves as an excellent marker for sporulation.

Wild-type cells were cotransformed with the *SP60/PKA* vector (see Introduction) and either an *spiA/lacZ* or an *SP60/lacZ* construct. In *SP60/lacZ* transformants staining for β-galactosidase activity can be clearly seen at ≈10 hr into development, at the time of tight aggregate formation and before tip formation (Fig. 1). In *spiA/lacZ* transformants, staining is first observed 1–1.5 hr later, and maturing ellipsoid spore cells form by 13–14 hr (data not shown). Although *spiA/lacZ* staining in the basal mound is slightly delayed relative to *SP60/lacZ* staining, the *spiA/lacZ* staining pattern within the small migrating slug (see Introduction) closely parallels that of *SP60/lacZ*. It has been previously shown (8) that as the slug migrates, *SP60/lacZ* expression is continuously induced in the most posterior cells as the older cells are "sloughed off" into the tube. As seen in the close-up of the slugs (Fig. 1 *I* and *J*), the *spiA/lacZ* staining pattern is indistinguishable from that of *SP60/lacZ*, indicating that, in these structures, *spiA* is being induced almost immediately after and presumably in the same cells as *SP60* (*cotC*).

We examined the developmental expression of *SP60*, *PKAcat*, and *spiA* in *SP60/PKAcat:spiA/lacZ* and KAx-3 strains by Northern analysis (Fig. 2). *SP60* is induced by 11–12 hr in both cell lines. *PKAcat* expression, driven by the *SP60* (*cotC*) promoter, is strongly induced between 12 and 16 hr in the experimental strain, in contrast to its low-level expression in wild-type cells. In *SP60/PKAcat* cells, the endogenous *SP60* (*cotC*) gene and *SP60/PKAcat* are expressed for a shorter period than in wild-type cells. This correlates with and is probably due to the rapid procession of development and spore formation in this strain. Expression of the endogenous *spiA* gene is detectable very early in the *PKAcat*-overexpressing cells, by 14 hr of development, ≈8 hr earlier than in wild-type cells. We observe *spiA/lacZ* staining in a small number of cells by 11 hr of development, due to the greater sensitivity of staining relative to RNA blots. The endogenous *spiA* gene and the *spiA/lacZ* transgene have similar kinetics and are induced shortly after *SP60* (*cotC*).

ecmA/PKAcat and *ecmB/PKAcat* strains, which overexpress *PKAcat* in prestalk A/O and prestalk AB cells, respectively, arrest as aberrant tipped aggregates (18). Although prespore markers are induced, mature spores are not produced and *spiA* expression is not detected (data not shown). In addition, we observe no staining in *spiA/lacZ* cotransformants of these strains (data not shown).

Induction of Sporulation Markers in *gα4*-Null Strains. Cells lacking the G-protein subunit α4 (*gα4*-null cells) arrest at the first finger stage to form an organism with aberrant morphology and essentially no spores (28). These cells express the endogenous *SP60* (*cotC*) gene and the *SP60/lacZ* transgene (Fig. 3*A*) but do not express *spiA/lacZ* at any stage in development up to 26 hr (Fig. 3*C*). To examine whether PKAcat overexpression in prespore cells can bypass the developmental block and induce *spiA* expression, we cotransformed *gα4*-null cells with *SP60/PKAcat* and either *SP60/lacZ* or *spiA/lacZ* and examined β-galactosidase expression. In *gα4:SP60/PKAcat* cells, the spatial patterns of β-galactosidase expression from the *SP60* (*cotC*) and *spiA* promoters are similar at 16 hr of development (Fig. 3 *B* and *D*) and at 22 and 26 hr (data not shown). *spiA/lacZ* and *SP60/lacZ* staining can also be seen at 13 hr; however, at

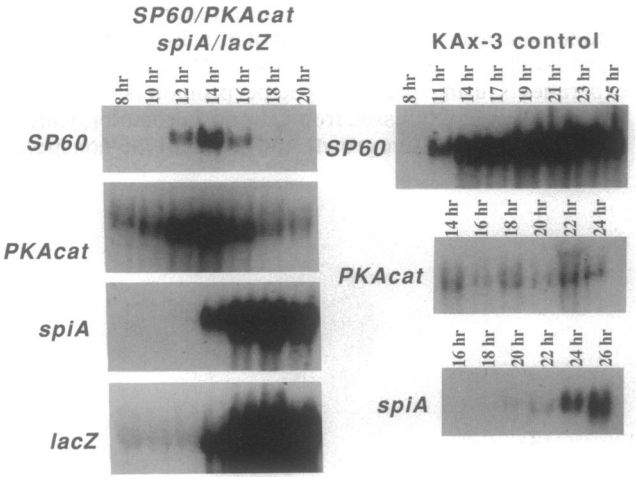

FIG. 2. Northern blot analysis of the developmental time course of *SP60/PKAcat:spiA/lacZ*-expressing cells and KAx-3 control cells. RNA was isolated from these two strains at the time points indicated and then probed to determine the temporal expression pattern of *SP60*, *PKAcat*, *spiA*, and *lacZ*. In the experimental strain, *PKAcat* RNA derives from both endogenous expression and that induced from the *SP60* promoter. Different time points were used for the *SP60/PKAcat* and the wild-type strain because of the difference in developmental timing in the two strains.

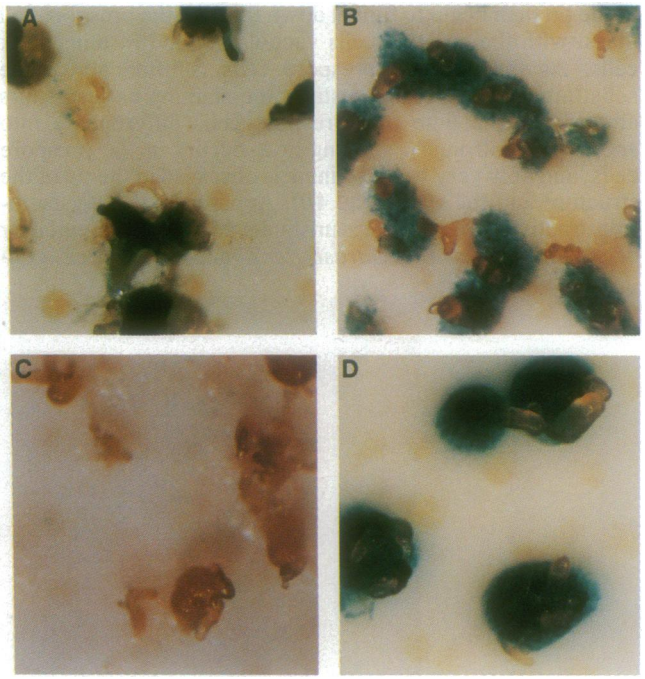

FIG. 3. Development of *gα4*-null cells and *gα4:SP60/PKA* cells. (*A* and *B*) *SP60/lacZ* staining pattern in *gα4*-null cells at 22 hr (*A*), and in *gα4 SP60/PKAcat* cells at 16 hr (*B*). (*C* and *D*) *spiA/lacZ* staining pattern in *gα4* null cells at 22 hr (*C*) and in *gα4:SP60/PKAcat* cells at 16 hr (*D*).

11–12 hr, we observe staining for *SP60/lacZ* cells but not for *spiA/lacZ* cells (data not shown). In assays for mature spores, no difference is noted between *gα4* null cells and the *gα4:SP60/PKAcat* strain (see *Discussion*).

The terminal stage of the *gα4*-null strains remains very light in color, whereas the *gα4:SP60/PKAcat* strain turns yellow-tan (data not shown). The spore mass of wild-type cells also becomes yellow-tan, due to the production of a pigment during sporulation. This suggests that genes involved in spore pigment production are induced in *gα4:SP60/PKAcat* cells and that the PKA effect on the spore cell differentiation pathway is not restricted to *spiA* induction. The morphology of the *gα4:SP60/PKAcat* strain is somewhat different from that of the *gα4*-null strain, indicating that overexpression of *PKAcat* in the prespore cells of this strain also leads to morphological changes.

Wild-Type Expression Pattern of *PKAcat*. To better understand the mechanisms by which PKA functions during various stages of *Dictyostelium* development, we examined the spatial expression pattern of the *PKAcat* promoter, using a *PKAcat/lacZ* reporter containing 1.6 kb of 5′ upstream sequence. *PKAcat* is developmentally regulated and expresses a transcript during vegetative growth that disappears upon starvation (14, 32); a shorter transcript is induced during aggregation. When *PKAcat/lacZ*-expressing cells are examined histochemically in whole mount preparations, the early aggregate shows a low level of staining throughout, with a small percentage of intensely staining cells randomly distributed (data not shown). As the aggregate forms a tip and then a finger, which falls over to become a slug, the tip or anterior region stains more intensely, indicating a prestalk A-enriched pattern of expression (Fig. 4*A*). Strong staining is not seen in prestalk O cells, but scattered staining is observed throughout the organism. Identical results were obtained for several independently transformed populations and clonal isolates.

The prestalk A-enriched pattern of *PKAcat/lacZ* staining continues through early culmination when the migrating slug forms a second finger structure (Fig. 4*A*). The prestalk cells at the tip then begin to funnel down through the prespore cell mass, with the prestalk cells vacuolating and forming stalk cells. As the mass of prespore cells is lifted above the substratum by the forming stalk, the staining pattern shifts abruptly at the time that prespore cells differentiate into spores. The strongest staining now occurs in the prespore cells, and only very weak staining is observed in the tip of the structure and the stalk (Fig. 4*B*). Apparently the β-galactosidase activity we observe is unstable, as has been previously demonstrated for the *DdrasD/lacZ* and *PTP1/lacZ* constructs (27, 33), since staining is lost in the prestalk/stalk cells as culmination progresses.

spiA/lacZ staining in wild-type cells occurs in a gradient as culmination progresses, with cells at the top of the prespore mass staining first (34). No such gradient is observed in *PKAcat/lacZ* cells stained for β-galactosidase activity (data not shown), although staining in these cells is far less intense than in *spiA/lacZ*-expressing cells, and it is possible that such a gradient would go undetected.

DISCUSSION

In this paper, we directly demonstrate that overexpression of *PKAcat* in prespore cells at the time of prespore gene induction leads to rapid activation of the *spiA* promoter in these cells and to the rapid onset of spore maturation. Our results suggest that activation of PKA by some means is the direct molecular trigger to induce sporulation. When *PKAcat* is overexpressed from the prespore promoter *SP60* (*cotC*), we presume that PKA becomes constitutively active in these cells. Most likely the ratio of catalytic subunit to regulatory subunit increases to the point that the level of free catalytic subunit rises significantly, regardless of intracellular cAMP concentration. Even if some feedback mechanism exists to provide for increased regulatory subunit synthesis, equilibrium kinetics would dictate that the level of free catalytic subunit is greater in *SP60/PKAcat* cells than in wild-type cells.

The relatively rapid (1–1.5 hr) induction of the *spiA* promoter following overexpression of *PKAcat* in the prespore cells of the basal mound suggests that PKA activation in prespore cells at culmination is an essential step in a rapid signal transduction pathway that induces *spiA* and, perhaps, other spore maturation genes. We suggest that PKA is mediating its effect directly at the level of gene expression. Consistent with this, identical *spiA* promoter elements are required for induction by PKAcat or 8-Br-cAMP, or during sporulation (unpublished observation and ref. 34). Expression of *spiA* occurs almost immediately after the *SP60*-driven induction of *PKAcat* in the cells at the posterior of the small slug; this suggests that at least some elements of this inductive pathway are already present in these redifferentiating cells. Overexpression of *PKAcat* in prespore cells is sufficient to rapidly induce spore differentiation in a cell-autonomous manner, but this cannot be achieved in other cell types simply by the overexpression of *PKAcat*, as shown by experiments using prestalk-specific promoters. Moreover, when *PKAcat* is expressed from an *Act15* promoter, which is active during vegetative growth and in a cell-type-nonspecific

FIG. 4. Wild-type expression pattern of endogenous *PKAcat*. (*A*) Finger and slug stages have a prestalk-enriched pattern of expression, with staining primarily observed in the anterior of the structures. A small culminant also is seen. (*B*) In the culminant the spore mass stains, indicating a switch in *PKAcat* expression to a prespore/spore pattern.

manner during development, *spiA* expression is not observed until prespore genes are induced and occurs only in the prespore population (unpublished observation). It appears, then, that some level of "prespore competence" must first be achieved before PKA can induce spore differentiation.

Our studies with *gα4*-null cells suggest that *PKAcat* expression is sufficient to induce some spore differentiation steps and to bypass aspects of the developmental program that are arrested in these cells, although there is no increase in the number of viable spores. It is possible that Gα4 signaling may be essential for other, independent aspects of spore or prespore differentiation that may intersect with the pathways regulated by PKA.

In *rdeC* cells, the level of PKA activity is a function of the expression level of the endogenous *PKAcat* gene and the stability of the subunit. When *PKAcat* is expressed in wild-type prespore cells from the *SP60* (*cotC*) promoter, spore differentiation is even more rapid than in *rdeC* strains and the morphology is distinct (this paper and ref. 18). The difference in phenotype between the two strains is most likely due to differences in the absolute level of PKA activity in the various cell types. In particular, the endogenous pattern of *PKAcat* expression indicates that PKA activity would be low in *rdeC* prespore cells during the early stages of multicellular development and high in prestalk A cells. By directly expressing *PKAcat* in prespore cells, we have shown that induction of *spiA* and spore differentiation is mediated by PKAcat activity; the kinetics of the response suggest that PKA has a direct effect on the process.

The timing of PKA activation in prespore cells is critical for normal morphogenesis and optimal propagation of the species. Constitutively active PKA causes premature spore differentiation; the spores remain at the base of the organism rather than atop a stalk. This is prevented in wild-type cells by the precise regulation of PKA activity, which might occur in any of several ways. It is possible that intracellular cAMP levels are carefully controlled and rise in prespore cells at the time of spore maturation, perhaps in a graded fashion that follows the observed gradient of *spiA* expression in the forming sorus (34). This is supported by the early observation that intracellular cAMP increases at culmination (21); however, new studies show that intracellular cAMP is not absolutely required for spore cell differentiation (see below).

A second possible scenario is that PKA activity in prespore cells is regulated at the level of transcription of either or both of the subunits. We show that expression of the catalytic subunit is induced in prespore cells at the time of spore maturation; perhaps this occurs without the concomitant induction of regulatory subunit expression, in effect rendering the enzyme independent of cAMP at this time in these cells. However, the gradient observed for *spiA* expression is not detected in *PKAcat/lacZ*-expressing cells, though our ability to see such a gradient may be limited by the low level of expression of the *PKAcat* promoter. Small increases in PKA activity may be sufficient to drive this maturation process. The gradient of *spiA* expression may be due to an undetected gradient of *PKAcat* transcription or perhaps to the graded presence of some other element of the pathway that leads from PKA activity to *spiA* induction.

Evidence that, in some situations, PKA activity can be regulated independently of intracellular cAMP levels is provided by the analysis of strains in which the adenylyl cyclase gene *ACA* has been deleted so that they cannot produce intracellular cAMP. Normally, *aca*-null cells do not aggregate or produce spores, but they can be induced to form agglomerates by shaking slowly in buffer with pulses of cAMP. When these large agglomerates are plated on filters and supplied with high levels of cAMP analogs that do not bind the PKA regulatory subunit, a small percentage of the cells (≈5%) are able to form very small fruiting bodies with viable spores (35). To solve the paradox this presents with our conclusions that PKA activity is required for spore differentiation, one must invoke a mechanism whereby the cells that develop and form spores are able to induce PKA activity in the absence of intracellular cAMP.

We thank Jo Anne Powell-Coffman for helpful discussions. This work was supported, in part, by Public Health Service grants to R.A.F.

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