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Controlling Aggregation and Postaggregative Development in *Dictyostelium*

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We have examined the role of cAMP-dependent protein kinase (PKA) in controlling aggregation and postaggregative development in *Dictyostelium.* We previously showed that cells in which the gene encoding the PKA catalytic subunit has been disrupted (*pkacat*⁻ cells) are unable to aggregate [S. K. O. Mann and R. A. Firtel (1991). A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium. Mech. Dev.* 35, 89–102]. We show that *pkacat*⁻ cells are unable to activate adenylyl cyclase in response to cAMP stimulation due to the inability to express the aggregation-stage, G-protein-stimulated adenylyl cyclase (ACA). Constitutive expression of ACA from an actin promoter results in a high level of Mn^{2+}-stimulated adenylyl cyclase activity and restores chemoattractant- and GTPγS-stimulated adenylyl cyclase activity but not the ability to aggregate. Similarly, expression of the constitutively active, non-G protein-coupled adenylyl cyclase ACG in *pkacat*⁻ cells also does not restore the ability to aggregate, although ACG can complement cells in which the *ACA* gene has been disrupted. These results indicate that *pkacat*⁻ cells lack multiple, essential aggregation-stage functions. As the mound forms, high, continuous levels of extracellular cAMP functioning through the cAMP serpentine receptors activate a transcriptional cascade that leads to cell-type differentiation and morphogenesis. The first step is the induction and activation of the transcription factor GBF and downstream postaggregative genes, followed by the induction of prestalk- and prespore-specific genes. We show that *pkacat*⁻ cells induce postaggregative gene expression in response to exogenous cAMP, but the level of induction of some of these genes, including *GBF,* is reduced. *SP60* (a prespore-specific gene) is not induced and *ecmA* (a prestalk-specific gene) is induced to very low levels. Expressing GBF constitutively in *pkacat*⁻ cells restores *ecmA* expression to a moderate level, but *SP60* is not detectably induced. Overexpression of PKAcat from the Actin 15 (*Act15), ecmA* prestalk, and the *PKAcat* promoters in *pkacat*⁻ cells results in significant aberrant spatial patterning of prestalk and prespore cells, as determined by *lac*Z reporter studies. Our studies identify new, essential regulatory roles for PKA in mediating multicellular development. © 1997 Academic Press

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* grows as single-cell amoebae in the presence of a food source. A few hours after this has been depleted, ~10^5 cells stream together to form a tight aggregate that proceeds through a series of morphogenetic steps to produce a fruiting body—a sorus of spores atop a slender stalk with a basal disc. Extracellular cAMP acts through cell surface serpentine/G-protein-coupled receptors (cARs) to regulate numerous essential functions during this developmental cycle (Devreotes, 1994; Firtel, 1995). Extracellular pulses of cAMP at nanomolar concentrations direct the aggregation response in starving cells, including the activation of adenylyl cyclase and the relay of the cAMP chemotactic signal, activation of guanylyl cyclase that is coupled to chemotaxis, and the expression of early genes whose products play essential roles during this and later stages of development [e.g., Gα2, cAR1, and csA (a cell adhesion molecule)] (Devreotes, 1994; Drayer and van Haastert, 1994; Firtel, 1995). Subsequently, as the mound forms, a higher (micromolar), more continuous extracellular cAMP signal produced in the ag-

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gregates (Abe and Yanagisawa, 1983) activates a developmental switch that initiates the multicellular phase of development, including pathways leading to the differentiation and morphogenesis of spatially localized prestalk and prespore cell populations (Firtel, 1995). The high level of cAMP induces and functionally activates the transcription factor GBF, initiating a transcriptional cascade beginning with induction of the postaggregative genes followed by induction of prestalk and prespore cell-type-specific gene expression (Schnitzler *et al.,* 1994, 1995). This process, while cAMP-receptor-dependent, is thought to be G-protein-independent. While much of this pathway has been elucidated, we are only beginning to understand the role of intracellular cAMP in *Dictyostelium* development and, more specifically, the role of its mediator, cAMP-dependent protein kinase (PKA).

Previous studies suggested that intracellular cAMP is not essential for proper development of *Dictyostelium.* Several mutant strains that cannot aggregate, such as the *Synag* strains (Darmon *et al.,* 1975; Theibert and Devreotes, 1986), are defective in the activation of adenylyl cyclase and presumably lack intracellular cAMP. However, some of these mutants develop properly and form spores when mixed with wild-type cells, which can provide the extracellular cAMP signals required for development. Cells in which the adenylyl cyclase gene *ACA* has been deleted by homologous recombination (*aca*$^-$ cells) can be coaxed through development by the addition of extracellular cAMP analogs that do not pass through the cell membrane and cannot activate PKA. These cells express cell-type-specific genes and will produce spores, although at a low frequency (Pitt *et al.,* 1993). These results suggest that intracellular cAMP may not be essential for proper chemotaxis, expression of the aggregation-stage pulse-induced class of genes, or basal multicellular development and morphogenesis.

In contrast to these observations are various studies demonstrating that in *Dictyostelium,* as in *Drosophila* (Perrimon, 1995), cAMP-dependent protein kinase (PKA) plays an essential regulatory role at several stages of development. In *Dictyostelium,* the holoenzyme contains one catalytic subunit and one regulatory subunit. As in other organisms, when cAMP binds to the regulatory subunit, the catalytic subunit is released and active (de Gunzburg and Veron, 1982; Leichtling *et al.,* 1982; Majerfeld *et al.,* 1984; Rutherford *et al.,* 1982). Complementary studies have been carried out using (1) a *Dictyostelium pkacat*$^-$ strain in which the gene for the catalytic subunit of PKA (*PKAcat*) has been disrupted (Mann and Firtel, 1991; Mann *et al.,* 1992), and (2) several strains in which a wild-type or dominant negative mutant form of a PKA regulatory subunit (Rm) is expressed under the control of various promoters (Firtel and Chapman, 1990; Harwood *et al.,* 1992a,b; Hopper *et al.,* 1993b; Simon *et al.,* 1989; Schulkes and Schaap, 1995; Zhukovskaya *et al.,* 1996). In these latter strains, the dominant negative Rm subunit cannot bind cAMP and effectively sequesters the catalytic subunit, thus rendering PKA deficient those cells in which it is expressed.

These studies have demonstrated that PKA activity is required for aggregation (Firtel and Chapman, 1990; Mann and Firtel, 1991; Harwood *et al.,* 1992a; Simon *et al.,* 1989; Schulkes and Schaap, 1995), apparently as part of the signal relay system by which adenylyl cyclase acts to relay the pulsatile aggregation signal. While members of the pulse-induced class of genes are expressed in *pkacat*$^-$ cells (Mann and Firtel, 1991), PKA is required for expression of the phosphodiesterase (PDE) inhibitor (PDI) and for the loss of the vegetative-stage PDE transcript (Wu *et al.,* 1995) and the adenylyl cyclase gene *ACA* is not expressed in cells overexpressing a dominant negative PKA-regulatory subunit (Schulkes and Schaap, 1995). PKA activity is also essential for entry into the morphogenetic stage (Harwood *et al.,* 1992a; Mann and Firtel, 1991), although in which cells and in what capacity is not yet known. *pkacat*$^-$ cells will form coaggregates with wild-type cells, though they do not go on to participate in morphogenesis and fruiting body formation. Rather, they remain as a discrete clump of cells at the base of the fruiting body, which is composed entirely of wild-type cells (Mann and Firtel, 1991). In the multicellular stages, PKA activity is required in prestalk A/O cells for maintained differentiation of prestalk cells, proper slug development, and culmination to commence (Harwood *et al.,* 1992b; Mann *et al.,* 1992; Zhukovskaya *et al.,* 1996), although overexpression of the catalytic subunit in these cells also causes developmental arrest at the tight mound stage (Hopper *et al.,* 1993a; Mann and Firtel, 1993). Adding yet another function to its repertoire, PKA activity is required for prespore gene expression (Harwood *et al.,* 1992a; Mann and Firtel, 1991) and the differentiation of spores (Hopper *et al.,* 1993b). In addition, it has been demonstrated that overexpression of PKAcat in prespore cells, resulting in constitutive PKA activity, is sufficient to cause precocious differentiation of prespore cells into viable spores. Furthermore, expression of the dominant negative Rm subunit in prespore cells, resulting in the inhibition of PKA activation in these cells, inhibits prespore cell differentiation (Hopper *et al.,* 1993a; Mann and Firtel, 1993; Mann *et al.,* 1992, 1994; Simon *et al.,* 1992).

In this paper, we further characterize the *pkacat*$^-$ strain and further elucidate the role that PKA plays in *Dictyostelium* development. We confirm that PKA is required for the expression of adenylyl cyclase, but that the lack of adenylyl cyclase expression in itself is insufficient to account for the inability of *pkacat* null cells to aggregate, indicating that PKA regulates multiple aspects of aggregation. Expression of adenylyl cyclase in *pkacat*$^-$ cells restores some receptor- and GTPγS-stimulated activity, but the kinetics and level of activation are different from those seen in wild-type cells. In addition, we show that PKA is essential for normal postaggregative and cell-type-specific gene expression that is mediated by extracellular cAMP and the transcription factor GBF. *pkacat*$^-$ cells constitutively expressing GBF, however, can induce postaggregative and prestalk gene expression to a moderate level, but not prespore gene expression. To further examine the role of PKA, we overexpressed in *pkacat*$^-$ cells the *PKAcat* gene driven by the Actin 15 (*Act15*), *ecmA* prestalk, and the *PKAcat* promoters. These

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vectors were cotransformed with cell-type-specific *lacZ* reporter constructs to examine the effects of PKAcat overexpression on cell-type differentiation and to further define the functions of PKA in multicellular development. Our results suggest that PKA plays an essential role at various stages of development through the regulation of distinct subsets of genes.

MATERIALS AND METHODS

Cell culture conditions, transformation of Dictyostelium cells, lacZ staining, and general molecular biological methods. All methods have been described previously (Mann and Firtel, 1993; Mann *et al.,* 1992, 1994; Mehdy and Firtel, 1985).

Cyclic AMP assays. Assays were performed as previously published (Devreotes *et al.,* 1987; Mann *et al.,* 1994; Okaichi *et al.,* 1992; Parent and Devreotes, 1995). The cAMP mass assay was performed as described previously (Okaichi *et al.,* 1992). Cells were pulsed with 30 n*M* cAMP for 5 hr, cells were washed, resuspended, and then stimulated with 2′dcAMP, and cAMP accumulation was measured using the Amersham cAMP radioassay kit. Adenylyl cyclase activity in lysed cells was assayed as previously described (Devreotes *et al.,* 1987; Parent and Devreotes, 1995). Briefly, for the adenylyl cyclase activation assays, cells pulsed with 50 n*M* cAMP for 5 hr were washed, lysed, and assayed in the presence of 100 μM ATP, 2 m*M* $MgSO_4$ (basal), 5 m*M* $MnSO_4$, or 40 μM GTPγS and 1 μM cAMP with the addition of exogenous CRAC for 2 min at room temperature. For receptor-mediated activation, cells were pulsed with 50 n*M* cAMP for 5 hr, washed, stimulated with 10 μM cAMP, lysed at specific time points, and assayed for 1 min at room temperature.

Western blot analysis. A polyclonal antibody was raised against a GST fusion protein containing amino acids 306–472 of GBF (Schnitzler *et al.,* 1994). Serum was purified initially by passing the antibody over a column to which GST had been covalently bound (Harlow and Lane, 1988) to remove any GST-specific antibodies. The partially purified serum was then passed over a column to which the GST–GBF protein had been covalently attached and purified GBF antibody was eluted with 100 m*M* glycine (pH 2.5).

Purified GBF antibody was incubated with extracts containing 1.3 mg of protein for 1 hr at 4°C. Forty microliters of a 50% (v/v) solution of Protein A–Sepharose beads in PBS was added for a further 40 min before washing the beads four times with lysis buffer containing 50 m*M* sodium fluoride, 1% NP-40, 2 m*M* EDTA, 0.8 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 m*M* sodium pyrophosphate in PBS. Ten microliters of 2× SDS sample buffer (0.01 *M* sodium phosphate, pH 6.8, 4% SDS, 0.22 *M* DTT, 22% glycerol, 0.02% bromophenol blue, and 0.077 *M* β-mercaptoethanol) was added after the final wash and the beads were boiled for 3 min before loading the supernatant on an 8% SDS polyacrylamide minigel. The proteins were transferred onto Immobilon-P membrane (Millipore) using wet blotting for 1.5 hr at 60 V and the membrane was allowed to dry at room temperature for at least 1 hr. The membrane was incubated for 15 min at room temperature in TBSTG [10 m*M* Tris, pH 7.4, 0.9% NaCl (v/v), 0.05% Tween 20, and 1% goat serum] then for 1 hr in fresh TBSTG containing purified GBF antibody. The membrane was washed in TBSTG as follows: two quick washes, one 15-min wash, and two 5-min washes. The membrane was then incubated for 30 min with TBSTG containing 1:7000 dilution of donkey anti-rabbit IgG linked to horseradish peroxidase and subsequently washed as above. GBF protein was detected by ECl (Amersham).

Gel shift analysis. Mobility gel shift analysis was performed using protocols, probes, and competitors as previously described (Schnitzler *et al.,* 1994). Briefly, cells from each strain were harvested, washed twice, resuspended at a density of 5×10^7/ml in 12 m*M* Na/K phosphate (pH 6.1), and shaken at ~200 rpm. cAMP was added to a final concentration of 500 μM after 5 hr and supplemented to 300 μM after 8 hr of starvation. Cytoplasmic extracts were prepared and mobility shift assays were performed as described previously (Schnitzler *et al.,* 1994).

RESULTS

Regulation of Adenylyl Cyclase in pkacat⁻ Cells

We previously created a *Dictyostelium pkacat⁻* strain in which the PKA catalytic subunit has been disrupted by homologous recombination (Mann and Firtel, 1991). These *pkacat⁻* cells do not aggregate. This strain synergistically aggregates when mixed with wild-type cells, but the *pkacat⁻* cells do not proceed further (Mann and Firtel, 1991). Cells overexpressing the dominant negative PKA regulatory subunit Rm do not show cAMP-stimulated adenylyl cyclase activity (Harwood *et al.,* 1992a). The aggregation-deficient phenotype may therefore be due to an inability to relay the pulsatile cAMP signal, perhaps because of some defect in activation of adenylyl cyclase or the expression of the ACA gene (Schulkes and Schaap, 1995). To investigate this more thoroughly, we performed several assays to quantitate receptor-mediated and GTPγS-stimulated adenylyl cyclase activity. Assays in which adenylyl cyclase activity is measured after stimulation of cells with cAMP show a low basal and little ligand-mediated stimulated activity in *pkacat⁻* cells compared to wild-type cells (Fig. 1A). To determine if this loss of activity in *pkacat⁻* cells is due to low levels of ACA expression, ACA was overexpressed in these cells (*pkacat⁻/ACA* cells) from an Act15 expression vector (Pitt *et al.,* 1992). This resulted in a high basal level of activity and restored ligand-stimulated activity, although the relative increase over the basal level was lower and the kinetics of adaptation of the pathway was lengthened (Fig. 1A). Both the kinetics and the relative level of stimulation were reproducible. $MnSO_4$-stimulated activity (which is a measure of total, functional adenylyl cyclase and stimulates activity in the absence of G proteins) or GTPγS-stimulated activity was also measured. Assays were done in the presence of added exogenous CRAC from wild-type cells, a cytosolic regulator of adenylyl cyclase activity (Lilly and Devreotes, 1994), to eliminate the possibility that it might be rate-limiting in the mutant cells. *pkacat⁻* cells showed little $MnSO_4$-stimulated activity and only a slight GTPγS stimulation of basal activity. Constitutive expression of *ACA* in *pkacat⁻* cells (see below) resulted in higher $MnSO_4$-stimulated activity. These cells also showed GTPγS-stimulated activity; however, the level of stimulation was only approximately twofold in comparison to a sixfold stimulation in wild-type cells. In contrast, previous results showed that

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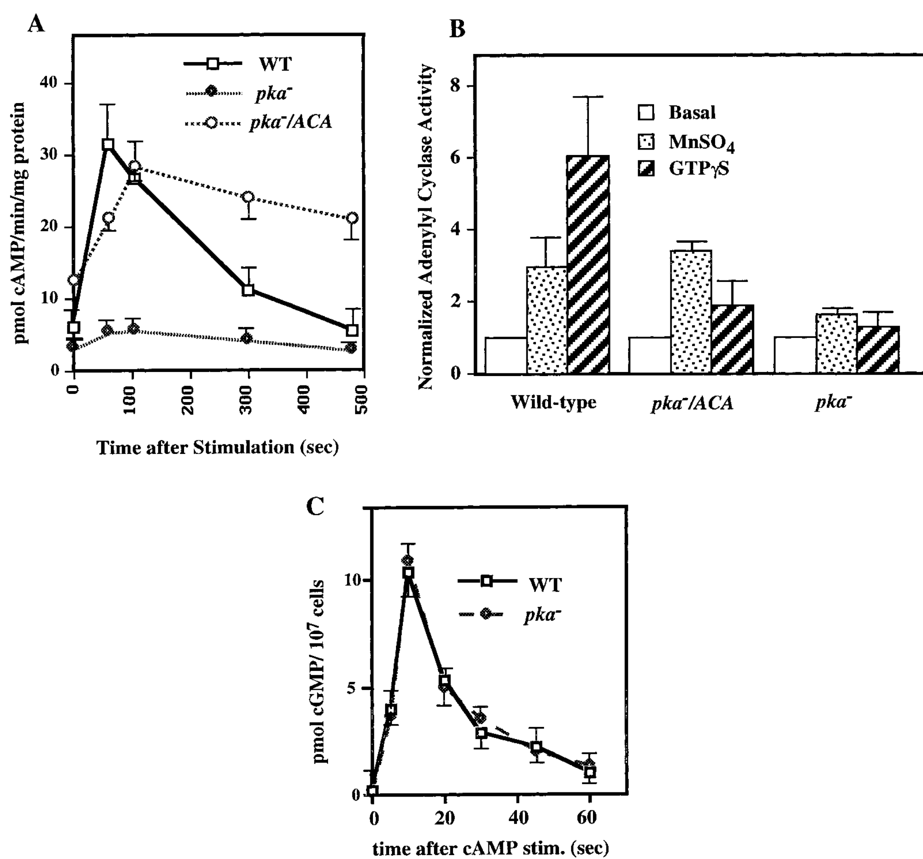

FIG. 1. Activation of adenylyl cyclase and guanylyl cyclase in wild-type, *pkacat*⁻, and complemented *pkacat*⁻ strains. (A) Five-hour-stage cells were stimulated with 10 μM cAMP and lysed at specific time points, and adenylyl cyclase activity was measured as described under Materials and Methods. Results shown represent the means of data taken from at least three independent experiments. Basal activity (pmol cAMP/min/mg protein): KAx-3 wild-type cells, 5.88 ± 1.71; *pkacat*⁻ cells, 3.02 ± 0.30; *pkacat*⁻*/ACA* cells, 12.53 ± 2.03. (B) Cells were prepared as described in A, lysed, and assayed with or without the addition of 5 mM $MnSO_4$ or 40 μM GTPγS and 1 μM cAMP in the presence of exogenous CRAC. The results are normalized to basal levels and represent the means of data taken from three independent experiments. See Materials and Methods for details. (C) Five-hour-stage cells were stimulated with cAMP and the levels of cGMP accumulation were measured as previously described and referenced (Janssens and Van Haastert, 1987; Kumagai *et al.*, 1991).

complemented *aca* null cells overexpressing ACA also showed a higher basal activity, but the kinetics of activation and adaptation and the stimulated increase in basal activity by GTPγS were similar to those of wild-type cells (Parent and Devreotes, 1995). These results indicated that overexpression of ACA restored adenylyl cyclase activity in *pkacat*⁻ cells, but that the activation profile was not identical to that of wild-type cells. In agreement with these results, an *in vivo* cAMP mass measurement assay showed that *pkacat*⁻ cells produce little cAMP in response to stimulation by 2′dcAMP, a cAMP analog that interacts with the cell surface receptor but not the PKA-R subunit and thus does not directly activate PKA or interfere with the assay (data not shown). This defect in the ability of *pkacat*⁻ cells to aggregate is rescued in *pkacat*⁻ cells transformed with the *PKAcat* gene driven by its own promoter [construct PK–PK (Mann *et al.*, 1992; Mann and Firtel, 1993; see below)].

Because little adenylyl cyclase activity is present in *pkacat*⁻ cells, we used RNA blot analysis to determine whether mRNA complementary to the aggregation-stage adenylyl cyclase gene, *ACA,* is present in these cells. Wild-type KAx-3 cells show no detectable signal in vegetative cells. When cells are pulsed with 20 nM cAMP, *ACA* gene expression is rapidly induced (Fig. 2A) (Pitt *et al.*, 1992). If cells are starved without exogenous pulsing, the level of expression is significantly reduced. *pkacat*⁻ cells, however, show no detectable *ACA* transcript (Fig. 2A), indicating that PKAcat activity is essential for the expression of *ACA*. *pkacat*⁻ null cells transformed with the *ACA* expression vector (see above) constitutively expressed ACA transcripts (Fig. 2B) and have receptor-stimulated adenylyl cyclase activity (see above). When these cells were plated for development, they still did not aggregate, suggesting that PKA is also required for a process necessary for aggregation in addition to expression of the *ACA* gene.

aca⁻ cells lack receptor-stimulated adenylyl cyclase ac-

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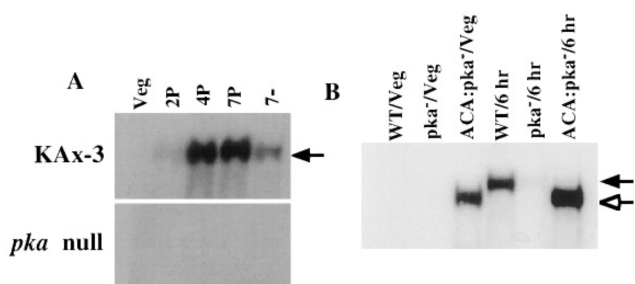

FIG. 2. Expression of ACA in wild-type and mutant strains. (A) RNA blot of RNA isolated from wild-type and *pkacat*$^-$ cells probed for *ACA* expression. Cells were either pulsed with 20 n*M* cAMP every 6 min (samples labeled with a ''P'') or not pulsed (sample labeled with a ''−'') and were taken at the times indicated in hours after starvation. RNA from vegetatively growing cells is labeled ''Veg.'' (B) RNA blot of ACA transcripts from vegetative cells or cells pulsed with cAMP for 6 hr. WT, wild-type KAx-3 cells; *pka*$^-$, *PKAcat* disrupted cells; *ACA*:*pka*$^-$, *PKAcat* disrupted cells expressing ACA from the *Act15* promoter. The solid arrow marks the position of the endogenous transcript. The open arrow marks the position of the *Act15*:*ACA* transcript, which is shorter than the endogenous *ACA* mRNA because of differences in the 5′ and 3′ untranslated regions. See Materials and Methods for details.

tivity and do not aggregate due to the inability to initiate and propagate an extracellular signal of cAMP (Pitt *et al.,* 1992). These cells can be complemented by expression of a new copy of the ACA gene, or the role of ACA can be ''bypassed'' by expression of the constitutively active adenylyl cyclase ACG, which is normally expressed during germination and is not regulated by G-proteins (Pitt *et al.,* 1992). *aca*$^-$/ACG-expressing cells form small aggregates and proceed through development, but do not form aggregation streams due to the nonoscillatory production of cAMP. To examine whether the aggregation-deficient phenotype of *pkacat*$^-$ cells could be complemented by ACG, we expressed *ACG* from the *Act15* promoter (Pitt *et al.,* 1992). Although these cells have constitutive adenylyl cyclase activity (data not shown) as expected from similar results with *aca*$^-$ cells (Pitt *et al.,* 1992), they still do not aggregate, which is consistent with the above results.

Since *pkacat*$^-$ cells coaggregate with wild-type, we expect that they show normal cAMP-stimulated activation of guanylyl cyclase. To confirm this, we assayed the rise in cGMP in response to cAMP and compared that to the response in the parental strain JH10. As shown in Fig. 1C, *pkacat*$^-$ and wild-type cells show a similar activation of guanylyl cyclase in response to chemoattractant.

Regulation of GBF Expression and Binding Activity by PKA

The transcription factor GBF is required for the developmental shift between aggregation and multicellular development (Firtel, 1995; Schnitzler *et al.,* 1994). It binds to GT- and CA-rich sequences known as G-boxes or CAEs found in most postaggregative/cell-type-specific genes that have been examined (Ceccarelli *et al.,* 1992; Firtel, 1995; Fosnaugh and Loomis, 1993; Haberstroh and Firtel, 1990; Hjorth *et al.,* 1990; Schnitzler *et al.,* 1994, 1995). Deletion of the GBF binding sites prevents induction of the gene during multicellular development or in response to extracellular cAMP in suspension assays. Both the expression of GBF and the activation of a receptor-dependent signaling pathway are required for GBF-mediated gene expression (Schnitzler *et al.,* 1995). In initial studies on *pkacat*$^-$ cells (Mann and Firtel, 1991), we showed that the postaggregative gene *rasD,* which is differentially expressed from two promoters during growth and multicellular development, is expressed in *pkacat*$^-$ cells, although the level of expression was reduced, whereas the prespore gene *SP60*/*CotC* is not (Mann and Firtel, 1991; see below). Both of these genes have GBF binding sites and their expression during the multicellular stages is GBF-dependent (Esch *et al.,* 1992; Haberstroh and Firtel, 1990; Schnitzler *et al.,* 1994, 1995). Results in other laboratories showed that expression of the dominant negative Rm subunit from either the prestalk *ecmA* or the prespore *PsA* (*D19*) promoter resulted in a block of the respective cell-type differentiation, suggesting a requirement of PKA for these processes (Harwood *et al.,* 1992b; Hopper *et al.,* 1993b). Furthermore, Hopper *et al.* (1995) showed that expression of Rm from the *PsA* promoter resulted in a significant drop in the rate of *SP60*/*CotC* and *SP70*/*CotB* transcription and in the accumulated level of the respective transcripts. Additionally, they showed that there was a significant decrease in the level of a DNA binding activity that has the properties of GBF as determined by gel shift analysis.

To examine the possible role of PKA in controlling GBF-mediated gene expression, we directly assayed the expression of the endogenous *GBF* gene in *pkacat*$^-$ cells, *pkacat*$^-$ cells constitutively expressing GBF from an *Act15* promoter, and wild-type cells, using a cell suspension assay (Mehdy and Firtel, 1985; Schnitzler *et al.,* 1994, 1995). As previously described, GBF is expressed at a low level in wild-type vegetative cells (Fig. 3A; Schnitzler *et al.,* 1994). Its expression is higher in 5-hr starved cells and is induced to maximal levels in response to cAMP in suspension culture under either conditions in which cell–cell interactions are blocked (fast-shake conditions, Fig. 3A) or conditions in which small agglomerates are formed (slow-shake conditions, Fig. 3A) (Mehdy and Firtel, 1985). In *pkacat*$^-$ cells, there is a similar pattern of GBF expression, although there appears to be a less stringent requirement for cAMP under slow-shake conditions. To distinguish between the endogenous GBF transcript and that expressed from the *Act15*/*GBF* transgene, we created a GBF deletion mutant, GBFΔ1, in which some of the glutamine-rich repeats in the N-terminal region of the protein have been deleted. The GBFΔ1 gene fully complements the *gbf* null mutant as determined by the ability to proceed through development (J.M.B. and R.A.F., unpublished observations), indicating that the deleted sequences are not required for GBF function. The *pkacat*$^-$ *Act15*/*GBFΔ1* cells express a transcript that is shorter than the endogenous GBF transcript. This transcript is ex-

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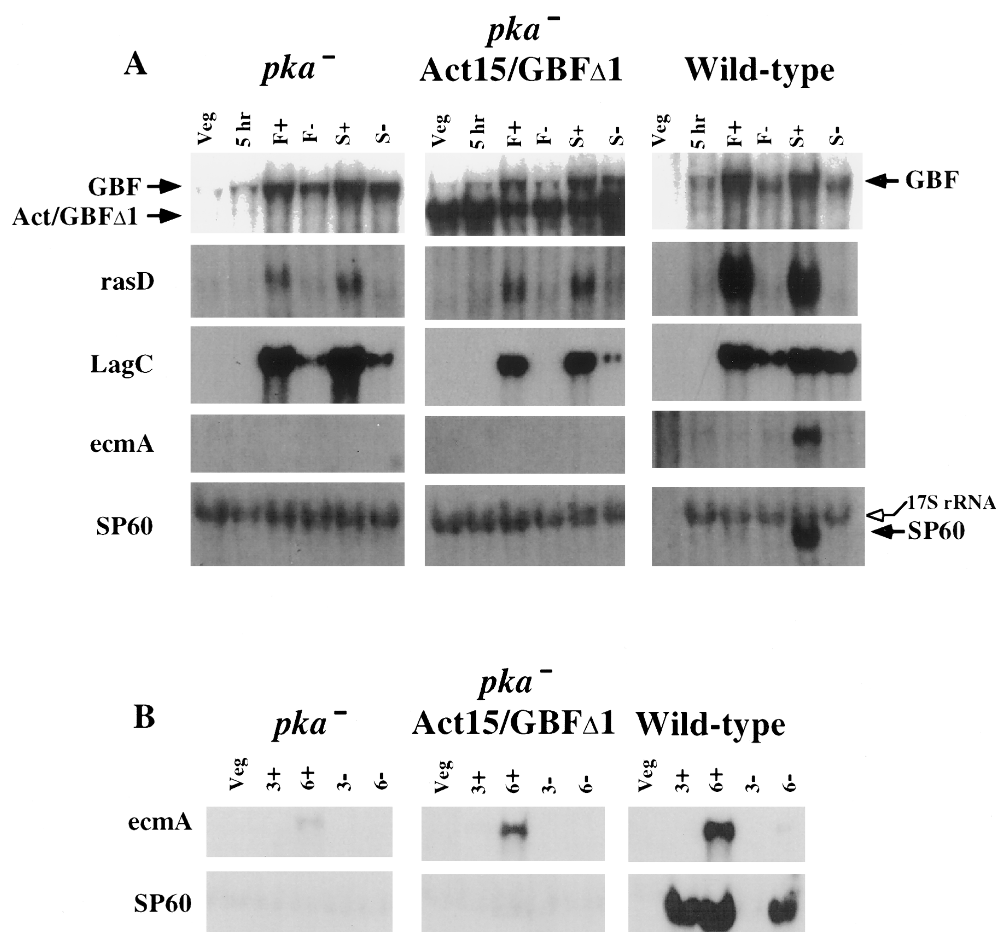

FIG. 3. Regulation of postaggregative and cell-type-specific gene expression in *pkacat*⁻ cells, *pkacat*⁻ cells constitutively expressing GBFΔ1, and wild-type cells. (A) Cells were washed, resuspended in nonnutrient buffer, and shaken for 5 hr. Cultures were split and then assayed under fast-shake (F) or slow-shake (S) conditions with (+) or without (−) 300 μM cAMP. cAMP was supplemented to a level of 100 μM every 2 hr. RNA samples were isolated from cells growing vegetatively (Veg), after 5 hr of shaking suspension (5 hr), and after 6 hr of either fast- or slow-shake conditions. See Mehdy and Firtel (1985) and Dynes *et al.* (1994) for details. Act15/GBFΔ1 is the transcript from the *Act15* promoter expressing GBFΔ1. See text for details. (B) Cells were washed, resuspended in nonnutrient buffer, and shaken for 5 hr with 25 nM pulses of cAMP every 6 min. Cultures were split and assayed under slow-shake conditions with (+) or without (−) 300 μM cAMP. Cyclic AMP was supplemented to a level of 100 μM every 2 hr. RNA samples were taken from cells growing vegetatively (Veg) and from cells shaken for 3 or 6 hr following the initial 5-hr pulsing treatment.

pressed to a high level in vegetative cells and under all conditions of the suspension assays. In these cells, the endogenous *GBF* gene is also expressed, with a pattern similar to wild-type cells, except that the level of induction of the endogenous GBF transcript is lower than in either wild-type cells or *pkacat*⁻ cells (Fig. 3A).

We then examined the accumulation of GBF protein and the ability of GBF to bind to DNA. The experiment presented in Fig. 3A was repeated for all three cell lines. In this case, we also performed GBF gel mobility shift assays and Western blots in addition to the quantitation of GBF RNA levels. Samples were taken from vegetative cells, cells shaken and starved for 5 hr, and cells starved for 5 hr followed by treatment with cAMP for 6 hr. Figure 4A shows that the pattern of GBF transcript expression in this experiment is similar to that observed in the experiment shown in Fig. 3A. Western blot analysis using GBF antibody to quantitate GBF immunoprecipitated from extracts (Fig. 4B) indicates that endogenous GBF protein accumulation in suspension culture is similar to that of the RNA profiles. GBF protein levels are very low in vegetative cells, increase by 5 hr, and are highest in cells treated for 6 hr with cAMP. In accord with RNA blot analysis, endogenous GBF protein levels are reduced in *pkacat*⁻ cells compared to levels in wild-type cells and the level of endogenous GBF in *pkacat*⁻/GBFΔ1 cells is lower than in *pkacat*⁻ cells. GBFΔ1 protein levels increased with development, even though the transcript was slightly higher in vegetative cells than during later development, possibly suggesting a differential stability of GBF protein later in development.

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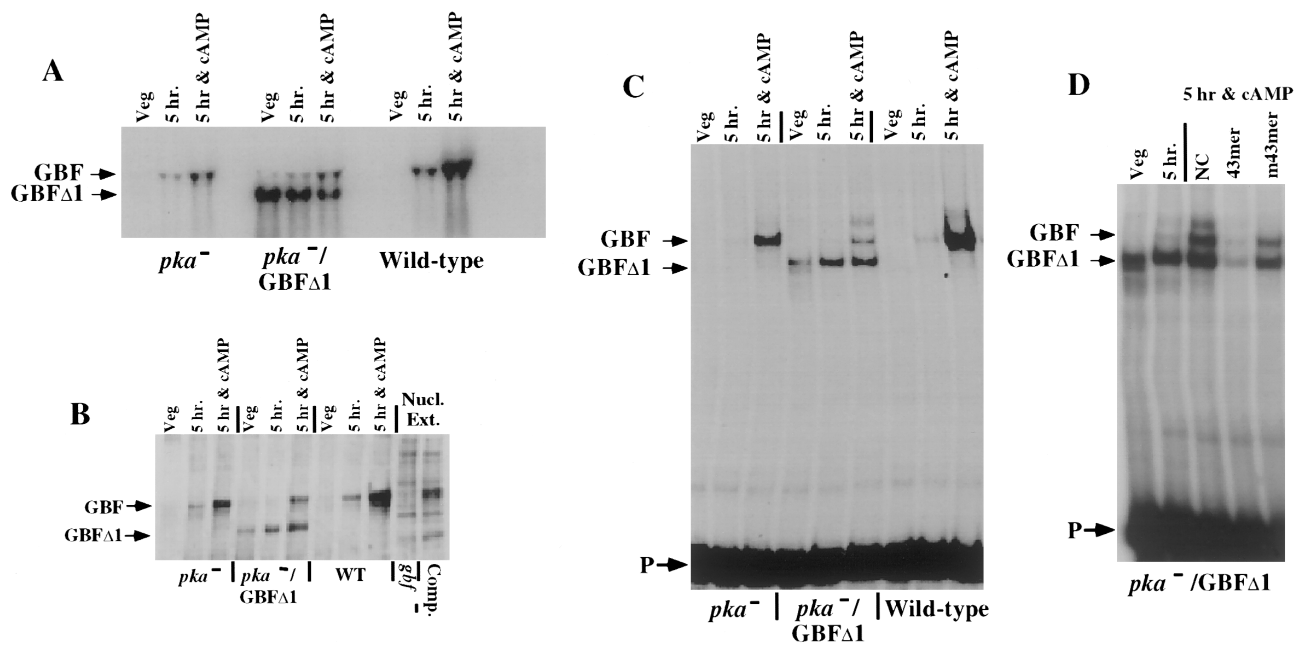

FIG. 4. GBF levels and activity in *pkacat*⁻ cells, *pkacat*⁻ cells constitutively expressing GBFΔ1, and wild-type cells. The strains described in the text were washed and shaken for 5 hr and then for an additional 6 hr in the presence of cAMP as described in the legend to Fig. 3. Time points were taken as shown in the figure. Samples were processed for RNA isolation, GBF immunoprecipitation, and gel shift analysis as described under Materials and Methods. (A) RNA blot analysis. See legend to Fig. 2 for details. (B) Western blot analysis of GBF protein levels. "*gbf*⁻" is a *gbf* null strain and "Comp." is the complemented *gbf* null strain (Schnitzler *et al.,* 1994). (C) Gel shift analysis of GBF in cell extracts as described in Schnitzler *et al.* (1994). (D) Competition analysis of the gel shift of the *pka*⁻/GBFΔ1 strain shown in C with a specific competitor (43-mer) and a specific competitor with mutations that are known to affect GBF binding (m43-mer) as described previously (Hjorth *et al.,* 1990; Schnitzler *et al.,* 1994).

To examine GBF binding activity, we performed a gel shift analysis using a strong GBF binding site (Schnitzler *et al.,* 1994). As shown in Fig. 4C, this directly correlates with the amount of GBF protein as determined by Western blot analysis. Moreover, the results indicate that GBF from *pkacat*⁻ cells binds with the same approximate efficiency as GBF obtained from wild-type cells, indicating that PKA activity is not required for GBF expression *in vivo* or site-specific DNA binding *in vitro.* Gel shifts using mutant and wild-type GBF binding sites as competitors show that the GBFΔ1 protein has the same binding affinity as the endogenous protein (Fig. 4D).

Regulation of Postaggregative and Cell-Type-Specific Gene Expression by PKA

To further examine the function of PKA in regulating genes that are induced during the multicellular stages, we examined the expression of postaggregative and cell-type-specific genes. The postaggregative genes *LagC* and *rasD* and the cell-type-specific genes *ecmA* (prestalk) and *SP60* (prespore) lie downstream from GBF and are induced in response to cAMP in suspension culture (Firtel, 1995; Schnitzler *et al.,* 1994, 1995; Williams *et al.,* 1993a). Postaggregative genes are induced under fast-shake or slow-shake conditions (see above), whereas the cell-type-specific genes are expressed in these assays only under slow-shake conditions (Dynes *et al.,* 1994). Figure 3A shows that the level and pattern of *LagC* expression under these shaking-culture conditions 3 hr after the addition of cAMP were similar in all three strains. In wild-type cells, there was less dependence on exogenous cAMP under slow-shake conditions since agglomerates can produce cAMP. *rasD* was also induced in both strains of *pkacat*⁻ cells under the same conditions as in wild-type cells, although at a significantly lower level (Fig. 3A), as had been observed previously (Mann and Firtel, 1991).

The cell-type-specific genes *ecmA* (prestalk) and *SP60/CotC* (prespore) are expressed in wild-type cells under the above culture conditions only under slow-shake conditions in the presence of cAMP (Fig. 3A). No expression of either of these genes is observed in the *pkacat*⁻ or the *pkacat*⁻ *Act15/GBF* cells under these experimental conditions. However, when the cells are pulsed with 25 n*M* cAMP every 6 min for the first 5 hr after starvation, to mimic cAMP oscillations during aggregation, and then switched to slow-shake conditions, we observe expression of *ecmA* in the *pkacat*⁻ *Act15/GBF* cells and at a barely detectable level in the *pkacat*⁻ cells (Fig. 3B). No *SP60/CotC* expression was seen in any of the strains in which the *PKAcat* gene has been deleted, indicating an absolute requirement for PKA to induce *SP60/CotC* expression (Fig. 4B).

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Partial Rescue of pkacat⁻ Cells with Act15/PKAcat

In previous studies, we rescued the aggregation-minus (agg^-) phenotype of the *pkacat*$^-$ strain by transforming these cells with an extrachromosomal vector carrying the PKAcat coding region driven by the *Act15* promoter. This promoter is active in vegetative cells and at a lower level throughout development in a cell-type nonspecific pattern (Knecht *et al.,* 1986). While the *pkacat*$^-$ *Act15/PKA* cells aggregate, they arrest at the second finger stage of development (Mann *et al.,* 1992). They remain in these finger-like structures even if development is allowed to continue for 48 hr. We repeated these experiments using an integrating vector and obtained similar results (see below).

We cotransformed into *pkacat*$^-$ cells the integrating vector carrying the *Act15/PKAcat* transgene and *lacZ* reporters driven by various cell-type-specific promoters. These cells were plated on filters for β-gal staining, rather than directly on buffered agar, and generally produce slugs as the terminal structure rather than a second finger (Fig. 5). When *pkacat*$^-$ cells expressing *Act15/PKAcat* are developed and stained for cell-type-specific markers (Fig. 5), the staining patterns are similar to those of wild-type slugs. *ecmA/lacZ*-staining cells are present in the anterior ~15% of the slug and comprise two regions, prestalk A cells in the anterior half of this zone and prestalk O cells in the posterior half (Fig. 5C; Early *et al.,* 1993). *ecmB/lacZ* staining also occurs in the tip, as well as in some scattered cells throughout the slug [anterior-like cells (ALCs); Jermyn *et al.,* 1989], as presented in Fig. 5B. In these slugs, there is less intense staining of the anterior prestalk AB region than in wild-type cells (Jermyn *et al.,* 1987), suggesting that this cell type is affected, perhaps rendering the organism unable to initiate culmination. *SP60/lacZ*-staining cells are present in the posterior region of the slug (Fig. 5A), as in wild-type slugs, although there is a greater reduction in the staining observed in the very posterior of the slug (Haberstroh and Firtel, 1990). No staining is observed in *pkacat*$^-$ *Act15/PKAcat* cells carrying *lacZ* driven by the *spiA* promoter, a marker for spore cell differentiation (Richardson *et al.,* 1994; data not shown), indicating that the spore maturation pathway is not induced in these structures. Accordingly, no mature heat- and detergent-resistant spores are formed (data not shown). Earlier studies indicate that *spiA* is prematurely induced in prespore cells when PKAcat is expressed from the *SP60/CotC* prespore-specific promoter (Mann *et al.,* 1994). Very little staining is observed in *pkacat*$^-$ *Act15/PKAcat* cells carrying *lacZ* driven by the *ecmB*Δ*89* promoter, which is induced in prestalk cells as they become stalk cells; however, a very small number of staining cells (<10) are observed scattered throughout many of the slugs (data not shown).

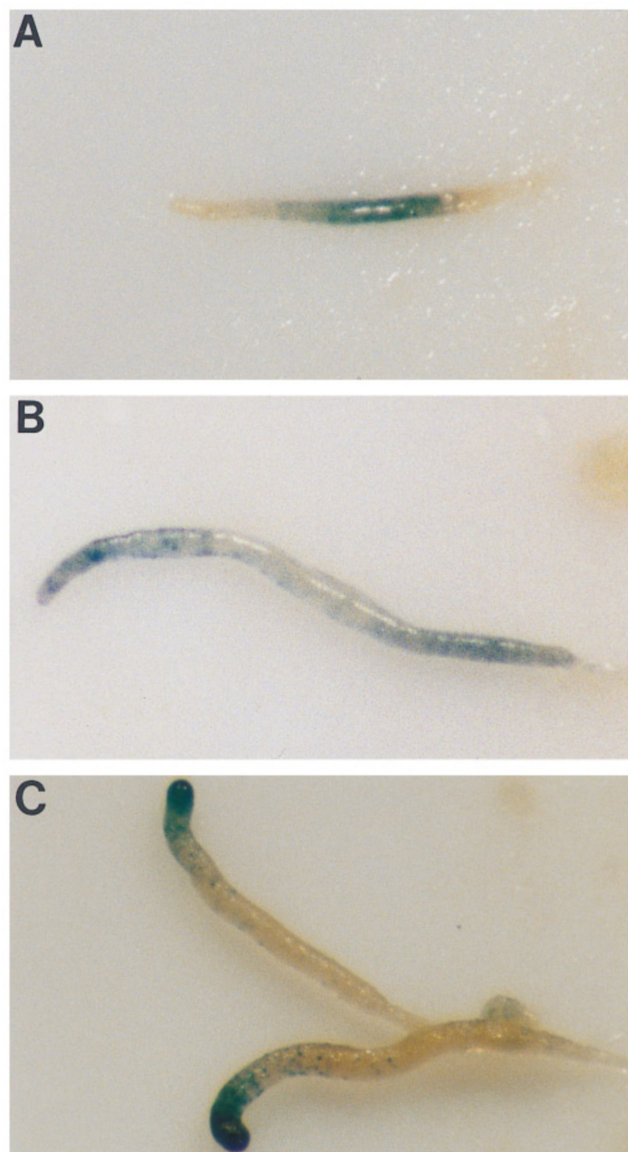

FIG. 5. *lac*Z reporter staining in *pkacat*$^-$ cells expressing the *Act15/PKAcat* transgene and *lacZ* gene fusions driven by various cell-type-specific promoters. (A) *SP60/lacZ.* In wild-type cells, staining is seen throughout the posterior 85% of the slug. In some slugs, the very posterior 5% of the slug (rear-guard cells) shows reduced staining (Haberstroh and Firtel, 1990). (B) *ecmB/lacZ.* In wild-type cells, *ecmB* is expressed in the prestalk AB region, anterior-like cells (ALCs), and the very posterior rear-guard cells (Williams *et al.,* 1993a). (C) *ecmA/lacZ.* In wild-type cells, *ecmA* stains the anterior ~15% of the slug that comprises the more anterior prestalk A and prestalk O regions, an interior cone of cells at the very anterior (prestalk AB cells), and the ALCs, found scattered throughout the slug. Staining in wild-type cells is less intense in the prestalk O region (Early *et al.,* 1993).

Overexpression of PKAcat from Cell-Type-Specific Promoters in pkacat⁻ Cells

We have also expressed the *PKAcat* gene in *pkacat*$^-$ cells from the *ecmA, ecmB,* and *SP60/CotC* promoters to determine whether expression of any of these transgenes could complement the phenotype of *pkacat*$^-$ cells. Neither the *pkacat*$^-$ *ecmB/PKAcat* cells nor the *pkacat*$^-$ *SP60/PKAcat* cells aggregate, presumably since these promoters are not

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expressed until after the aggregate is formed. However, ~20% of the clones of the *ecmA/PKAcat* transformed cells do aggregate and, although some arrest at the tight mound stage (Fig. 6A, panel C), many go on to form small, abnormal fruiting bodies. The morphological phenotype suggests that these strains arrest during culmination. They form final structures that are similar to those observed at early/mid stages of the culmination of wild-type cells. Generally the stalk is short or absent, although some have a stalk of about half the normal length (Fig. 6A, panel E). While expression

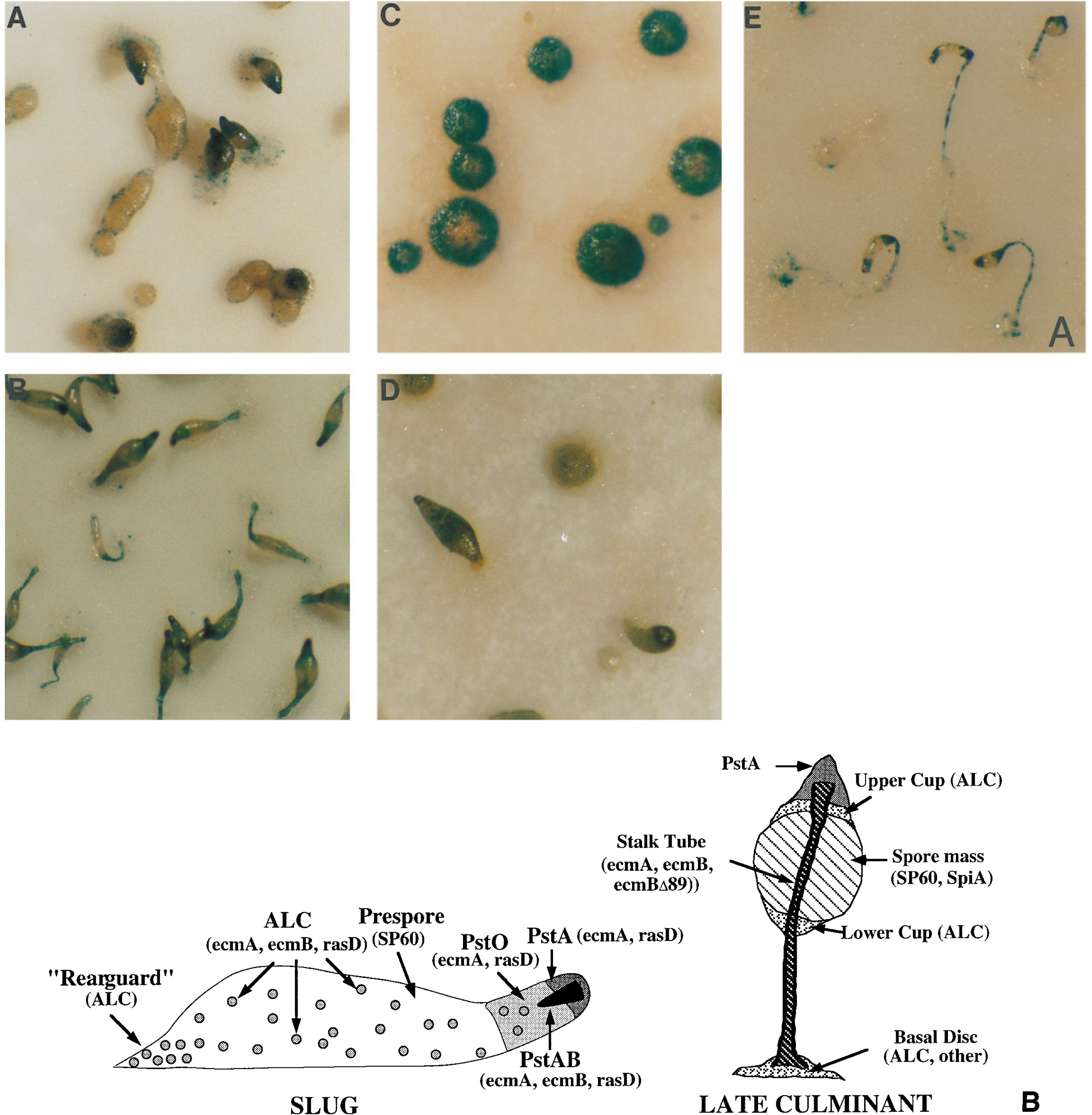

FIG. 6. *lac*Z reporter staining in *pkacat*⁻ cells expressing the *ecmA*/PKAcat transgene and *lacZ* gene fusions driven by various cell-type-specific promoters. (A) Panels A and B, *ecmA/lac*Z; panels C and D, *SP60/lac*Z; panel E, *ecmBΔ89/lac*Z. (B) This illustration depicts the localization in wild-type strains of the various cell types during multicellular development and the pattern of expression of marker genes (Firtel, 1995; Williams *et al.*, 1993a,b).

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of the endogenous ecmA promoter has not been detected in aggregation-stage cells (Jermyn *et al.,* 1987), it is possible that our assay (ability of cells to aggregate) is sufficiently sensitive to very low levels of expression of PKA required to allow observation of this effect. Interestingly, expression of *ecmA/PKAcat* in wild-type cells causes developmental arrest at the mound stage (Hopper *et al.,* 1993a; Mann and Firtel, 1993).

As with the *pkacat*⁻ *Act15/PKAcat* strains described above, we cotransformed cells with *ecmA/PKAcat* and *lac*Z driven by various cell-type-specific promoters. In the terminal stage, the cells expressing the prestalk markers *ecmA* (Fig. 6A, panels A and B) and *ecmB* (data not shown) localize to the tip, neck, and base of the structure. The cells expressing the prespore marker SP60/COTC constitute most of the mass of the structure (Fig. 6A, panels C and D), as they do in a wild-type fruiting body. Cells expressing *lac*Z from the *ecmB*Δ*89* promoter, which is activated in the forming stalk cells, are localized to the stalk as in wild-type cells. In addition, *ecmB*Δ*89* is expressed in cells forming the upper and lower cups where it is not expressed in wild-type cells (Fig. 6A, panel E). For comparison, an illustration showing the staining pattern and cell fate of cells at the slug and mature fruiting body is shown in Fig. 6B.

Overexpression of PKAcat from Its Own Promoter

The most direct method of rescuing a null mutant is to transform into the mutant cells a wild-type copy of the disrupted gene driven by its own promoter. When we transform pkacat⁻ cells with an integrating vector carrying the *PKAcat* coding region and ~1.6 kb of upstream sequences, we observe a variety of phenotypes in the resulting clones, as presented in Fig. 7. These include tight mounds, mounds with protruding stalks and very small, sometimes abnormal sori (Figs. 7E and 7F), mounds from which slugs migrate (see below; Figs. 7D and 7G), short-stalked fruiting bodies (Figs. 7B and 7C), and "kinked" fruiting bodies (Fig. 7A).

The basal mound-and-stalk structures (Figs. 7E and 7F) are reminiscent of the fruiting bodies formed when wild-type cells overexpressing *PKAcat* from the prespore promoter *SP60/CotC* are plated on agar for development (Mann and Firtel, 1993). The phenotypes presented in Figs. 7D and 7G are similar to those observed when *SP60/PKAcat* cells

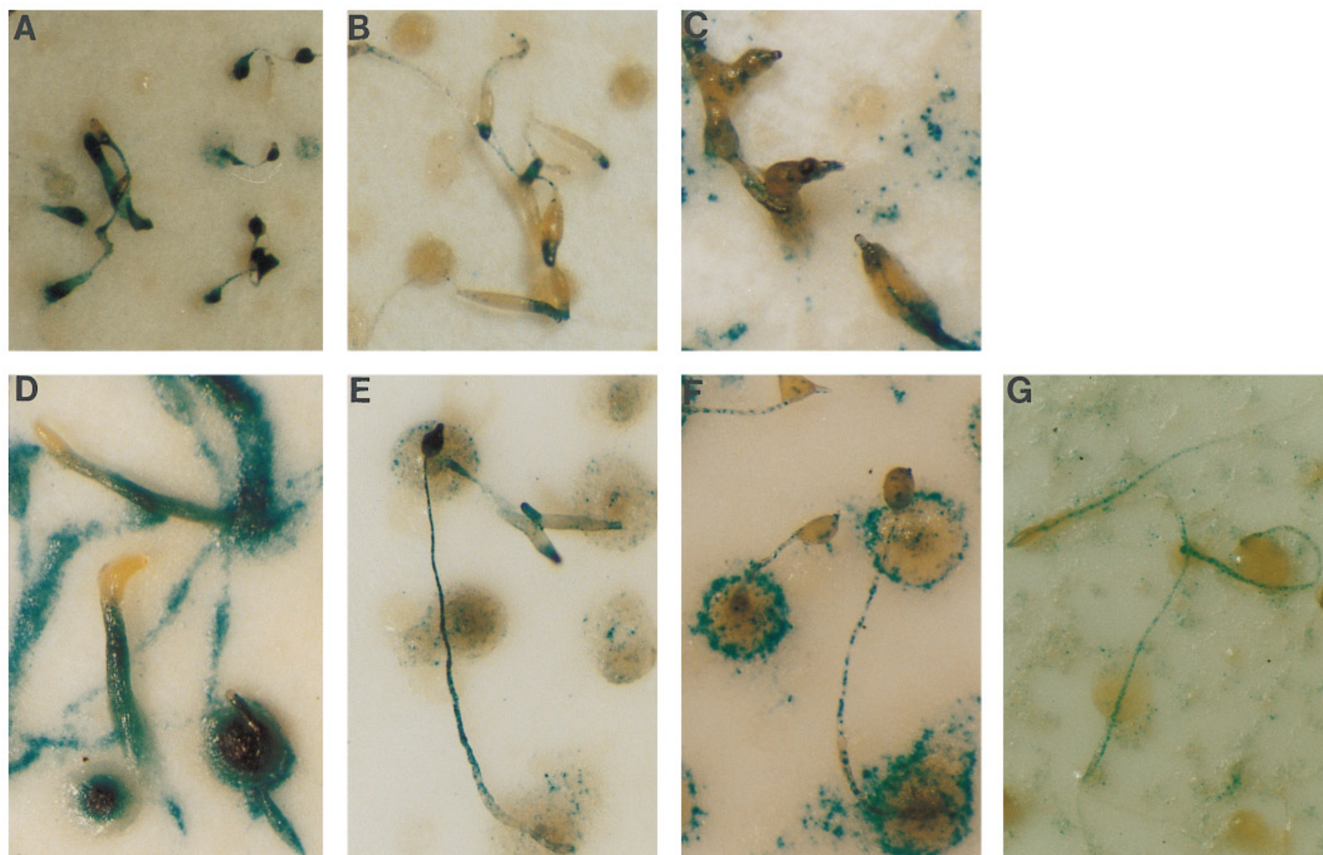

FIG. 7. *lac*Z reporter staining in *pkacat*⁻ cells expressing the *PKAcat*/PKAcat transgene and *lacZ* gene fusions driven by various cell-type-specific promoters. A and B, *ecmA/lac*Z; C and D, *SP60/lac*Z; E, *ecmB*Δ*89/lac*Z. A and D, *SP60/lac*Z; B and E, *ecmA/lac*Z; C and F, *ecmB/lac*Z; G, *ecmB*Δ*89/lac*Z.

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are plated on filters, rather than on agar (Mann and Firtel, 1993). Under these conditions, a basal mound is formed from which a slug-like structure emerges and migrates for a considerable distance, creating a partially cellular tube-like structure that connects the slug to the basal mound. The slug eventually forms a small fruiting body that has a very small spore mass. However, the *pkacat*⁻ *PKAcat/PKAcat*-expressing cells that have this phenotype usually have a smaller basal mound than the *SP60/PKAcat* cells. In both types of structures formed by SP60/*PKAcat*-expressing cells, the basal mound is made up of precociously formed, viable spores. This appears also to be the case for basal mounds formed by *pkacat*⁻ cells expressing *PKAcat* from its own promoter, as determined by microscopic examination.

When *pkacat*⁻ cells were cotransformed with the *PKAcat/PKAcat* construct and a promoter/*lacZ* construct, we were able to determine spatial patterning within the aberrant structures. Cells expressing the prespore promoter *SP60/CotC* (Figs. 7A and 7D) were found in the posterior portion of migrating slugs, basal mounds of terminal structures, and sori. Cells expressing *spiA,* a sporulation marker, show the same pattern as the *SP60*-expressing cells in mature fruiting bodies (data not shown), indicating that these strains form spores prematurely, as do SP60/PKAcat-expressing cells or the RdeC strain that is mutant in the PKA regulatory subunit (Mann and Firtel, 1993; Mann *et al.,* 1994; Simon *et al.,* 1992). Cells expressing *ecmA* (Figs. 7B and 7E) localize to the tips of slugs and sori and to stalks. *ecmB*-expressing cells (Figs. 7C and 7F) are found in the upper and lower cups of sori, in stalks, and at the periphery of basal mounds. These results are similar to those observed in wild-type strains. Cells expressing *lac*Z from the *ecmB*Δ*89* promoter localize to the stalk (Fig. 7G) as they do in wild-type strains. Unlike wild-type strains, no staining is seen in the basal disk.

When PKAcat is overexpressed from its own promoter in a wild-type background, we observe the same variety of phenotypes we obtain when it is expressed in the *pkacat*⁻ background (data not shown). An additional phenotype of these wild-type-background PKAcat-overexpressing cells is that of long, intertwining tube structures, with a small fruiting body but no real basal mound. These tube structures are thicker than those described above and stain for the spore-formation marker *spiA* (data not shown). In contrast, the sorus that eventually forms does not stain for *spiA* expression (data not shown).

DISCUSSION

Role of PKA in Aggregation

Our studies further elucidate the multiple roles that PKA plays in controlling *Dictyostelium* development. We confirm that cAMP-dependent protein kinase is required for normal expression of *ACA,* the gene encoding the aggregation-stage adenylyl cyclase, and show that little cAMP- or GTPγS-stimulated adenylyl cyclase activity is detectable in *pkacat*⁻ cells, in contrast to our previous conclusion (Mann and Firtel, 1991), while cAMP stimulation of guanylyl cyclase is normal. However, this is not the only cause of the aggregation-deficient phenotype, as neither expression of the constitutively active *ACG* gene, which partially rescues *aca*⁻ cells, nor expression of ACA from the *Act15* promoter, which does rescue some cAMP- and GTPγS-stimulated adenylyl cyclase activity in these cells, restores the ability to aggregate. Since *pkacat*⁻ cells can coaggregate with wild-type cells and thus are able to respond chemotactically to exogenous cAMP, we expect that PKA is essential for some other component of the signal relay pathway or for the expression of another gene crucial for aggregation. While no ACA transcripts were detected in *pkacat*⁻ cells, even after extended exposure of the RNA blots, we did observe a low level of Mn^{2+}- and GTPγS-stimulated adenylyl cyclase activity. We expect that there must be a low level of ACA expression, not detectable by RNA blots using whole-cell RNA. As the other *Dictyostelium* adenylyl cyclase (*ACG*), which is specific for germination, is not G-protein-stimulated, and since *aca* null cells show no basal or GTPγS-stimulated activity (Pitt *et al.,* 1992), we expect that the adenylyl cyclase activity that we measure in *pkacat*⁻ cells is ACA.

Previous studies showed that when ACA was overexpressed ~15-fold (compared with wild-type cells), the cells displayed a basal activity which was also ~15-fold greater than that of wild-type cells (Mann *et al.,* 1994a; Parent and Devreotes, 1995). However, the GTPγS-mediated activity was only stimulated 3- to 4-fold compared to 10- to 12-fold in AX3 cells. This phenomenon is presumably due to the limited availability of CRAC or other components in the ACA-overexpressing cells. Indeed, the addition of exogenous CRAC typically potentiates GTPγS activation in these cells (Parent and Devreotes, 1995). However, GTPγS activation of *pkacat*⁻ cells overexpressing ACA only leads to a 2-fold stimulation in adenylyl cyclase activity in the presence of added CRAC, suggesting that, although not essential, PKA activity definitely promotes G-protein-mediated activation of adenylyl cyclase activity in *Dictyostelium* and is thus involved in controlling signal relay and other aspects of aggregation.

PKA Promotes GBF-Mediated Gene Expression

Our results demonstrate that PKA is not required for the induction of *GBF, LagC,* or *rasD,* genes known to require GBF for expression, although the level of expression of *GBF* and *ras*D is significantly reduced in *pkacat*⁻ cells. We investigated the induction of cell-type-specific genes, which are downstream from GBF, in the *pkacat*⁻ cells. We show that under some of our experimental conditions (nanomolar pulses of cAMP during initial starvation), there is a reduced but significant level of *ecmA* expression in a *pkacat*⁻ background that is significantly higher in cells constitutively expressing GBF. The observed induction of *ecmA* in these cells would argue that PKA is not essential for *ecmA* gene expression, but is required for maximal expression. The

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higher *ecmA* expression in *pkacat*⁻ cells constitutively expressing GBF may be due to the higher level of GBF in these cells and/or a higher consequent level of some GBF-dependent gene product required for *ecmA* expression. Since *ecmA* mRNA levels are still below those observed in wild-type cells, it is probable that PKA may regulate a component of the *ecmA* pathway in addition to GBF. Recent results of Zhukovskaya *et al.* (1996) suggest an essential role of PKA in mediating prestalk cell differentiation. They showed that high level of expression of the dominant negative regulatory subunit (Rm) from the prestalk-specific *ecmA* promoter results in an inhibition of prestalk cell differentiation due to the Rm subunit, resulting in a transdifferentiation of prespore cells to prestalk cells. Their experimental procedures are inherently different than ours and directly address a role of PKA in prestalk cell differentiation itself in the multicellular organism, while our data address the ability of cAMP in a slow-shake assay to induce the expression of the prestalk-specific gene *ecmA* in different genetic backgrounds. Our results would indicate that under conditions of high GBF levels, PKA is not essential for a moderate level of *ecmA* expression. Moreover, our results suggest that part of the inhibition of prestalk cell differentiation by the overexpression of Rm is a result of inhibiting either the expression or downstream function of GBF.

PKA Is Essential for Prespore Gene Expression

Our results also show that the prespore gene *SP60/CotC* cannot be induced in the *pkacat*⁻ background under any condition tested, suggesting an absolute requirement of PKA for the prespore pathway. Others (Hopper *et al.*, 1995) have reported that a DNA binding activity with properties of GBF is lost upon expression of the Rm subunit from the *psA* (*D19*) prespore promoter. Our data demonstrate that GBF DNA binding activity is normal in *pkacat*⁻ cells in which GBF expression is induced by cAMP and in *pkacat*⁻ cells in which GBF is expressed constitutively. We have repeated the experiments of Hopper *et al.* (1995) and also observed a decrease in GBF DNA-binding activity after mound formation (unpublished observations). We also examined whether this activity might be reinduced by exogenous cAMP or 8-Br-cAMP since inhibition of PKA activity might impair the ability of cells to stimulate AC and produce cAMP. We observed no increase in GBF levels (data not shown). Our studies indicate that *PKAcat* protein is not essential for GBF function as defined by *in vitro* GBF binding activity and expression of the GBF-dependent gene *LagC in vivo.* Moreover, we have shown that a GBF mutant lacking the only cryptic PKA site (RGIS), which is found between the two zinc fingers, can completely complement the *gbf* null mutation, indicating that direct phosphorylation of GBF by PKA is not essential for its function (J.M.B. and R.A.F., unpublished observations). One possibility is that the reduced level of GBF binding activity observed when Rm was expressed from the *psA* promoter may be due to a reduction of continued GBF expression in this strain resulting from the inhibition of PKA activity. This may be a direct effect on GBF expression (GBF expression is reduced in *pkacat*⁻ cells) or the effect of inhibiting adenylyl cyclase activity resulting in a reduction in cAMP levels (GBF expression requires continuous cAMP stimulation). We propose that if PKAcat is required for the direct activation of a transcription factor required for prespore gene expression, that factor is not GBF but may be a prespore-specific factor or a coactivator that interacts with GBF and is directly regulated by PKA. Currently, the available data do not distinguish between PKA having a direct effect on prespore-specific transcription and PKA being required at some earlier step to make cells competent for the induction of this pathway. For example, we have identified an apparently ubiquitously expressed novel gene product that is required for expression of prespore but not prestalk genes (Yasukawa and Firtel, unpublished observations). PKA may lie in the same pathway as this protein.

Interestly, PKA and the MAP kinase ERK2 have a similar effect on cell-type-specific gene expression. Using a temperature-sensitive ERK2 mutant, we showed that ERK2 is absolutely required for prespore gene expression and *ecmA* expression is reduced in our cell suspension assay, but normal under other conditions (Gaskins *et al.,* 1996). It is possible that both PKA and ERK2 lie on a similar pathway that differentially affects cell-type-specific gene expression.

Results presented in this paper and earlier results (Hopper *et al.,* 1993b; Mann *et al.,* 1994b; Simon *et al.,* 1992) indicate that the correct level of PKA is essential in controlling both the induction and timing of the prespore/spore pathways: too little PKA leads to no prespore cell differentiation, while a high level of PKA activity in prespore cells can lead to precocious induction of sporulation. In contrast, overexpression of PKAcat in *pkacat*⁻ cells from the *Act15* promoter enables development to proceed to the slug stage, in which cells continue to migrate but do not culminate and overexpression of PKAcat in wild-type cells from the *ecmA* promoter results in arrest at the mound stage (Hopper *et al.,* 1993a; Mann and Firtel, 1993). Precocious induction of sporulation by PKA occurs if PKAcat is expressed from the *SP60* prespore promoter or the cloned *PKAcat* promoter, which has a very complex pattern of expression (Mann *et al.,* 1994). The *PKAcat* promoter is expressed in vegetative cells and is further induced during aggregation (Mann *et al.,* 1994b). During the slug stage, its expression is highly enriched in the prestalk A/O region but a lower level of expression is also seen in the prespore zone. During culmination, the pattern of expression switches to one that is significantly more enriched in the differentiating spore cells. This accumulated evidence on the phenotypes of PKAcat overexpression from different promoters suggests that the relative levels of PKAcat activity in prespore and prestalk cells may be critical in controlling tip formation, slug migration, and culmination.

When we transform *Dictyostelium* with a construct carrying the *PKAcat* gene driven by a particular promoter, we assume that PKA is constitutively active in the cells in which that promoter is expressed. The vectors we have used integrate into the chromosome as a tandem array of multi-

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ple copies, a mechanism to confer sufficient resistance to the selection drug G418. As we have shown previously, multiple copies of the *PKAcat* gene driven by a fairly strong promoter lead to significant levels of the PKA catalytic subunit that are sufficient to overload any regulatory subunits present within the cell (Mann and Firtel, 1993; Mann *et al.,* 1992). Although the *PKAcat* promoter is fairly weak, both *pkacat*$^-$ cells and wild-type cells transformed with the *PKAcat/PKAcat* construct display a wide range of aberrant phenotypes. This is most likely due to the integration of multiple copies of *PKAcat,* present in varying numbers, although it may also be due in part to the position of chromosomal insertion. Using RNA blot analysis, we attempted to correlate phenotypes of the *PKAcat/PKAcat*-expressing cells with the levels of *PKAcat* mRNA; our results revealed no apparent correlation. Although the PKAcat promoter region used for these experiments (Mann and Firtel, 1993) is significantly longer than most characterized *Dictyostelium* promoters, we cannot exclude the possibility that additional regulatory sequences are absent. One unexpected phenotype we observed is the induction of the usually stalk-specific promoter *ecmB*Δ*89* in cells that form the upper and lower cups (usually ALCs) when PKAcat is overexpressed from the *PKAcat* promoter. This suggests that PKAcat can activate the *ecmB*Δ*89* promoter in these cells. This is in agreement with previous results suggesting that PKAcat may directly regulate the *ecmB* promoter (Ceccarelli *et al.,* 1991; Williams *et al.,* 1993b).

From studies in several laboratories, it is apparent that cAMP-dependent protein kinase plays numerous important roles in various *Dictyostelium* developmental programs. It is also apparent that PKA must be carefully regulated to ensure normal development. This control is achieved in at least three ways: (1) through spatial and temporal regulation of *PKAcat* transcription, (2) through spatial and temporal regulation of transcription of the gene encoding the PKA regulatory subunit, and (3) through regulation of cAMP.

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