

Requirements for the adenylyl cyclases in the development of *Dictyostelium*

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SUMMARY

It has been suggested that all intracellular signaling by cAMP during development of *Dictyostelium* is mediated by the cAMP-dependent protein kinase, PKA, since cells carrying null mutations in the *acaA* gene that encodes adenylyl cyclase can develop so as to form fruiting bodies under some conditions if PKA is made constitutive by overexpressing the catalytic subunit. However, a second adenylyl cyclase encoded by *acrA* has recently been found that functions in a cell autonomous fashion during late development. We have found that expression of a modified *acaA* gene rescues *acrA*⁻ mutant cells indicating that the only role played by ACR is to produce cAMP. To determine

whether cells lacking both adenylyl cyclase genes can develop when PKA is constitutive we disrupted *acrA* in a *acaA*⁻ PKA-C^{over} strain. When developed at high cell densities, *acrA*⁻ *acaA*⁻ PKA-C^{over} cells form mounds, express cell type-specific genes at reduced levels and secrete cellulose coats but do not form fruiting bodies or significant numbers of viable spores. Thus, it appears that synthesis of cAMP is required for spore differentiation in *Dictyostelium* even if PKA activity is high.

Key words: Adenylyl cyclase ACA, Adenylyl cyclase ACR, cAMP-dependent protein kinase PKA, Sporulation, *Dictyostelium*

INTRODUCTION

Genes encoding three distinct adenylyl cyclases have been characterized and shown to be expressed at different stages of development in *Dictyostelium* (Pitt et al., 1992; Soderbom et al., 1999). *acaA* encodes a 12 transmembrane protein that accumulates shortly after the initiation of development and functions in relay of extracellular cAMP pulses. *acgA* encodes a single-pass membrane protein that is expressed only in spores (Pitt et al., 1992; van Es et al., 1996). *acrA* encodes a protein with domains related to histidine kinases and response regulators. The C-terminal portion contains the adenylyl cyclase catalytic domain (Soderbom et al., 1999). ACR is present at low levels in vegetative cells but accumulates dramatically following aggregation (Meima and Schaap, 1999; Soderbom et al., 1999).

Strains in which *acgA* is disrupted develop normally into fruiting bodies but the spores fail to remain dormant when dispersed into an environment of high osmolarity (van Es et al., 1996). Strains in which *acaA* is disrupted do not secrete cAMP and so are unable to chemotactically signal each other. They fail to aggregate unless they are pulsed with extracellular cAMP or they are genetically engineered to overexpress the catalytic subunit of the cAMP-dependent protein kinase, PKA (Pitt et al., 1993; Wang and Kuspa, 1997). Aggregates form under these conditions only when the cells are spread at high densities and result from random collisions rather than from chemotaxis. Strains in which *acrA* is disrupted aggregate and proceed to make fruiting bodies but have a cell-autonomous defect in sporulation such that less than 1% of the cells make

detergent-resistant viable spores (Soderbom et al., 1999). It appears that *acrA* cannot fulfill the role of *acaA* in production of the extracellular cAMP necessary for chemotaxis while *acaA* cannot fulfill the role of *acrA* in production of internal cAMP necessary for terminal differentiation of spores.

Although both *acaA* and *acrA* are expressed during development and their products catalyze the synthesis of cAMP, there are significant differences in the enzymes. ACA is similar to other G-protein coupled membrane-associated adenylyl cyclases while ACR is similar to soluble mammalian cyclases (Buck et al., 1999). ACA is activated when the G-protein coupled surface receptor CAR1 binds extracellular cAMP. In extracts of aggregation stage cells, the activity can be stimulated more than tenfold by adding GTPγS to dissociate the trimeric G protein (Pitt et al., 1992). The activity can also be stimulated by addition of manganese ions which by-pass the need for G-protein-dependent activation (Loomis et al., 1978; Pitt et al., 1992). The activity of ACR in extracts, however, is not stimulated by GTPγS and is maximally active in the presence of magnesium ions (Kim et al., 1998; Meima and Schaap, 1999; Soderbom et al., 1999). During the aggregation stage, between 6 and 12 hours of development, the activity of ACA assayed in the presence of manganese is about fourfold higher than the activity of ACR assayed in the presence of magnesium (Soderbom et al., 1999). However, during culmination, ACR activity is more than tenfold higher than ACA activity. The temporal pattern of accumulation of these activities may partially account for their stage specificity. However, the adenylyl cyclases may have other differences in characteristics that dedicate them to distinct roles. Therefore,

we have analyzed the effects of ectopic expression of *acaA* on the phenotype of cells lacking ACR and the developmental capabilities of strains lacking both of the developmental adenylyl cyclases but overexpressing PKA.

MATERIALS AND METHODS

Chemicals

Triazol Reagent, and Geneticin (G418) were purchased from GibcoBRL, Grand Island, N.Y. Blastidicin S was provided by ICN Pharmaceuticals Inc, Irvine, CA. Biotrak Cyclic AMP [H^3] system was purchased from Amersham Pharmacia Biotech, Arlington Heights, Ill. Nucleopore Track-Etch membrane was a product of the Corning Corp., Corning, NY. Radio-labeled nucleotides were purchased from NEN, Boston, MA, USA.

Cells, growth, transformation and development

Cells of strain AX4 (Knecht et al., 1986), DG1100 (*acrA*⁻) (Soderbom et al., 1999) and AK631 (*acaA*⁻ PKA-C^{over}) (Wang and Kuspa, 1997) were grown in HL5 medium (Sussman, 1987). BlastidicinS was added to the medium at 10 µg/ml to select for cells carrying the Bsr^R gene. Geneticin (G418) was added to the medium at 20 µg/ml to select for cells carrying the NPT II gene. *acrA* was disrupted in the strain AK631 (*acaA*⁻ PKA-C^{over}) as previously described (Soderbom et al., 1999). Strain *acrA*⁻/K was obtained by transformation of DG1100 (*acrA*⁻) with the K-Neo plasmid (*pkaC*::*pkaC*) as previously described (Anjard et al., 1992). Multiple independent transformants were isolated from each transformation and three or four were characterized.

A mutation in *acaA* (L394S) was selected such that its product, ACA, can function effectively in the absence of G-protein-coupled stimulation from the CAR1 receptor (Parent and Devreotes, 1995). The plasmid CP105 carrying this modified gene under control of the actin 15 regulatory region gives high levels of ACA activity even in the absence of cAMP signals. We transformed both wild-type cells and DG1100 strain cells with this construct and selected for G418 resistance using up to 50 µg/ml of G-418. The strains were called AX4 ACA* and *acrA*⁻ ACA* respectively. Both strains aggregate about 2 hours earlier than wild-type cells and form smaller fruiting bodies.

Synchronous development was induced by depositing the cells on nitrocellulose filters supported on pads saturated with buffer (Sussman, 1987). For microscopic examination, structures were deposited on slides in 10-15 µl of 0.1% Calcofluor White ST in 20 mM phosphate buffer pH 6.8 and observed with a Nikon fluorescence microscope.

Quantitation of sporulation

Cells were collected off nitrocellulose filters on which they had developed for 48 hours, dissociated in buffer containing 0.5% Cemusol, incubated for 5 minutes, washed and spread on SM agar in association with *Klebsiella aerogenes* (Shaulsky et al., 1995). The number of plaques formed after 4 days indicated the number of viable spores.

Adenylyl cyclase assay

1×10⁸ cells were collected at each time point, washed in 10 mM phosphate buffer pH 6.5, resuspended in 200 µl of lysis buffer (10 mM Tris pH 8.0, 250 mM sucrose). Cells were lysed through 3 µm pore size nucleopore membranes and the extracts kept on ice. The adenylyl cyclase assay was then performed as described by Soderbom et al. (Soderbom et al., 1999).

PKA assay

PKA activities were determined using the protein kinase A assay system from Gibco BRL (# 13128-012) as previously described

(Anjard et al., 1993). After 20 hours of development on filters, cells were harvested in 20 mM phosphate buffer pH 6.5, centrifuged for 1 minute at 1200 rpm and resuspended in 500 µl extraction buffer containing 1/100 of protease inhibitor cocktail (Sigma P-8215). Cells were lysed by freezing in a dry ice/ethanol bath and rapidly thawing at 20°C. After centrifugation at 12,000 g for one minute, 1-10 µl from each supernatant was assayed at 25°C. The activities are expressed as phosphorylation of Kemptide substrate that can be inhibited by the PKI inhibitor, according to the instructions with the kit. The reaction was linear with extract under the conditions used. Protein concentration was measured using the Bradford assay with BSA as standard.

Northern blot

Northern blot analyses were performed as described by Shaulsky and Loomis (Shaulsky and Loomis, 1993). RNA was isolated using Triazol reagent and 15 µg RNA from each sample was electrophoretically separated on a 0.8% agarose gel and subsequently transferred to nylon membrane (Magna Graph, MSI, Westboro, MA). Probes for *ecmA*, *cotB* and *pkaC* mRNA were generated from appropriate plasmids.

RESULTS

Overexpression of *pkaC* or *acaA* in *acrA*⁻ cells

Overexpression of the gene encoding the catalytic subunit of PKA results in cAMP-independent PKA activity that is able to suppress a block to sporulation resulting from mutations in a variety of genes (Anjard et al., 1992; Wang et al., 1999). We transformed the *acrA*⁻ null mutant DG1100 with a construct in which *pkaC* is expressed under the control of its endogenous promoter. Such transformants were referred to as *acrA*⁻/K. As expected, they showed all the characteristic features of cells overexpressing *pkaC* such as rapid development, small fruiting

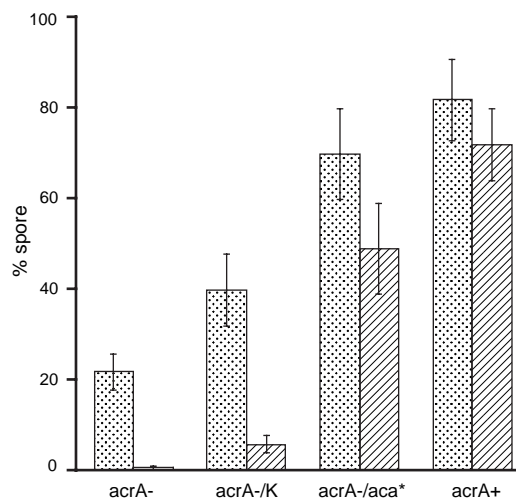


Fig. 1. Proportion of visible and viable spores. Cells were developed on filters for 18 to 24 hours and collected to determine the percentage of visible (stippled bars) and viable (hatched bars) spores compared to the total number of cells. The times at which the maximal proportion of viable spores was found were: 22 hours for *acrA*⁻ cells of strain DG1100; 18 hours for *acrA*⁻/K cells; 20 hours for *acrA*⁻/ACA* cells; 24 hours for *acrA*⁺ cells of strain AX4. Each experiment was repeated at least 3 times.

bodies and the ability of submerged cells in monolayers to form spores (the sporogenous phenotype; Anjard et al., 1992). However, most of the visible spores isolated from sori failed to give rise to progeny when spread on bacterial plates (Fig. 1). At least 80% of the spore-like cells stained with propidium iodide indicating that they were nonviable. Although there was some increase in the number of viable spores, overexpression of *pkaC* was much less effective at rescuing mutants lacking ACR than at rescuing other mutants blocked in sporulation such as those carrying null mutations in *dhkA* (Wang et al., 1999). The number of spores, both visible and viable, in the *acrA*⁻/*K* transformants decreased after 18 hours of development such that after 48 hours sori contained less than 1% spores (data not shown).

To determine whether ACR may function in processes other than synthesis of cAMP, we transformed DG1100 with a construct in which a modified form of *acaA* is expressed under the control of the strong actin 15 promoter. The modified form of *acaA* carries a mutation (L394S) that renders the aggregation stage adenylyl cyclase, ACA, constitutively active even in the absence of G-protein coupled stimulation from the CAR1 receptor (Parent and Devreotes, 1995). After 20–22 hours of development transformants carrying the act15::*acaA** construct formed almost the same number of phase-bright ellipsoid spores as wild-type strains and 70% of these were viable (Fig. 1). However, within the next day most of these appeared to prematurely germinate within the sori such that they took on a glassy appearance and the number of viable spores decreased over 50 fold by 48 hours of development. Spores of the *acrA*⁻; act15::*acaA** strain were even less stable when dissociated from sori after 22 hours of development, treated with detergent and suspended in buffer. Within 3 hours, over 95% of the spores could be seen to germinate and release amoebae. Addition of 200 mM sorbitol, which activates the germination adenylyl cyclase ACG, was ineffective in maintaining dormancy of the spores (data not shown). Wild-type AX4 cells carrying the act15::*acaA** construct form spores slightly earlier than wild-type cells and they remain dormant (data not shown).

These results indicate that expression of ACA* under the control of the actin 15 promoter can substitute for ACR during culmination but is not sufficient for maintaining dormancy. It would also appear that ACR plays no other role essential for sporulation than catalyzing the synthesis of cAMP.

Cells lacking both adenylyl cyclase activities

It was previously shown that a strain lacking ACA but overexpressing PKA (*acaA*⁻ PKA-C^{over}) is able to form aggregates at high cell densities by random collisions and that the aggregates transform into slugs that culminate to form fairly normal fruiting bodies (Wang and Kuspa, 1997). To determine the role of ACR in development of this strain we transformed cells of the *acaA*⁻ PKA-C^{over} strain AK631 with a construct in which *acrA* was disrupted by the blasticidin resistance gene and selected for homologous recombinants. Several independent transformants in which *acrA* was shown to be disrupted were further analyzed. Cells of these strains were found to grow well and, when plated at high cell density, formed tipped mounds and a few small abnormal finger-like structures but no fruiting bodies (Fig. 2). No measurable adenylyl cyclase activity was

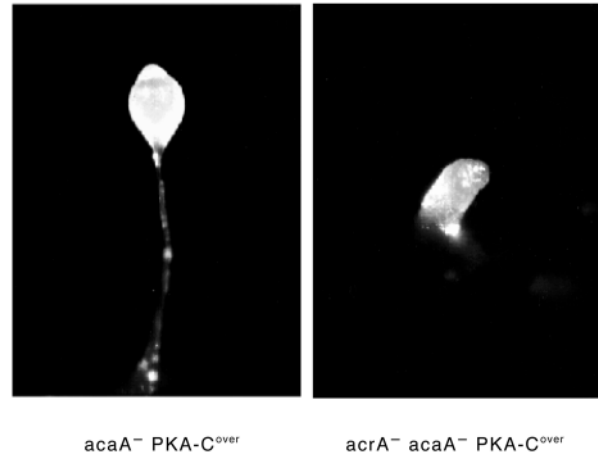


Fig. 2. Terminal structures of strains AK631 and TL130. Cells were deposited on filters at high density and allowed to develop for up to 36 hours before being photographed. As previously described (Wang and Kuspa, 1997) the *acaA*⁻ PKA-C^{over} cells of strain AK631 form fairly normal fruiting bodies. Most of the cells of strain TL130 (*acaA*⁻ *acrA*⁻ PKA-C^{over}) were found in tipped mounds but a few were seen to form small finger-like structures.

found at any stage of development in these strains (data not shown).

One of these *acrA*⁻ *acaA*⁻ PKA-C^{over} strains, TL130, was chosen for further characterization. Although morphological development of strain TL130 appeared abnormal after the mound stage, it is at that stage that the initial cell types diverge and express either prespore- or prestalk-specific genes (Ceccarelli et al., 1991; Shaulsky and Loomis, 1996). Therefore, we analyzed RNA isolated from wild-type and mutant cells, which had developed for 24 hours, with probes from the prestalk gene, *ecmA*, and the prespore gene, *cotB*. Both of these cell-type-specific genes were expressed in cells from strain AK631 (*acaA*⁻ PKA-C^{over}) as well as in cells from strain TL130 (*acrA*⁻ *acaA*⁻ PKA-C^{over}) but the levels of their mRNAs were significantly lower than in wild-type cells (Fig. 3). Thus, constitutive PKA does not seem to be sufficient for normal expression of these post-aggregative genes in the absence of cAMP.

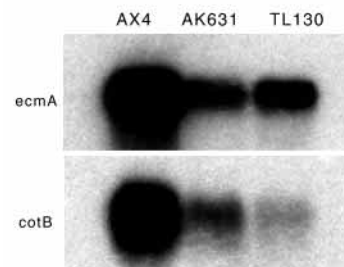


Fig. 3. Expression of the cell-type-specific genes *ecmA* and *cotB*. Total RNA was collected from strains AX4 (wild type), AK631 (*acaA*⁻ PKA-C^{over}) and TL130 (*acaA*⁻ *acrA*⁻ PKA-C^{over}) after 20 hours of development, separated on agarose gels and transferred to nylon membranes. Northern blots were sequentially probed with the prestalk-specific gene *ecmA* and the prespore-specific gene *cotB*.

Staining the finger-like structures after 21 hours of development with calcofluor White ST to make cellulose fluorescent showed that many of the cells were surrounded by extracellular cellulose (Fig. 4B). When wild-type cells were stained at the onset of culmination, similar amoeboid cells were also seen to have cellulose coats (Fig. 4A). About 1% of the wild-type cells at that stage appeared to have differentiated into ellipsoid spores that stained intensely for cellulose (Fig. 4C). A much smaller number of the TL130 mutant cells also had a spore-like appearance (Fig. 4D). While the proportion of wild-type spores increased dramatically over the next few hours of development, the number of visible mutant spores decreased thereafter. After 24 hours of development stalk cells surrounded by cellulose-containing walls could be seen within the stalk tubes of the wild-type strain AX4 (Fig. 4E), while only amorphous clusters of cellulose encased vacuolized cells were seen in strain TL130 (Fig. 4F). Isolated calcofluor-stained mutant cells with the appearance of stalk cells were also seen. Although a few TL130 cells had the appearance of spores at 20 hours of development, the number of detergent-resistant viable spores was less than 10^{-4} . The level of detergent-resistant viable spores decreased as time went on.

Even though cells of strain TL130 are missing both of the developmental adenylyl cyclases, they were expected to have high levels of constitutive PKA activity as a result of expression of *pkaC* driven by the actin 15 regulatory region. PKA activity was measured in extracts of wild-type AX4 cells and cells of strain TL130 (*acrA⁻acaA⁻PKA-C^{over}*) that had developed for 20 hours (Table 1). The specific activity of PKA in strain TL130, assayed in the absence of added cAMP, was as high as the maximal specific activity seen in wild-type cells assayed in the presence of 100 μ M cAMP. It is clear that the *act15::pkaC* construct is sufficiently active late in development to result in high levels of constitutive PKA activity in TL130 cells but that they still do not form significant numbers of viable spores.

Synergy

In those cases where the aberrant development of a mutant strain results from failure to generate the proper intercellular signals, development in chimeric mixtures with wild-type cells can often result in normal morphogenesis. Although sporulation is not rescued in either *acrA⁻* cells or the double mutants lacking both adenylyl cyclases, by developing them together with wild-type cells (Soderbom et al., 1999, unpublished observations), we reasoned that overproduction of PKA in TL130 cells might permit them to respond to signals from wild-type cells. When the mutant cells were mixed with 10% wild-type cells, they coaggregated and formed normal finger structures. Mutant cells labeled with FITC could be seen in both the anterior (prestalk) and posterior (prespore) compartments of these structures. Culmination was initiated after 22 hours of development, resulting in the formation of small fruiting bodies with short stalks. However, when cells were collected from these

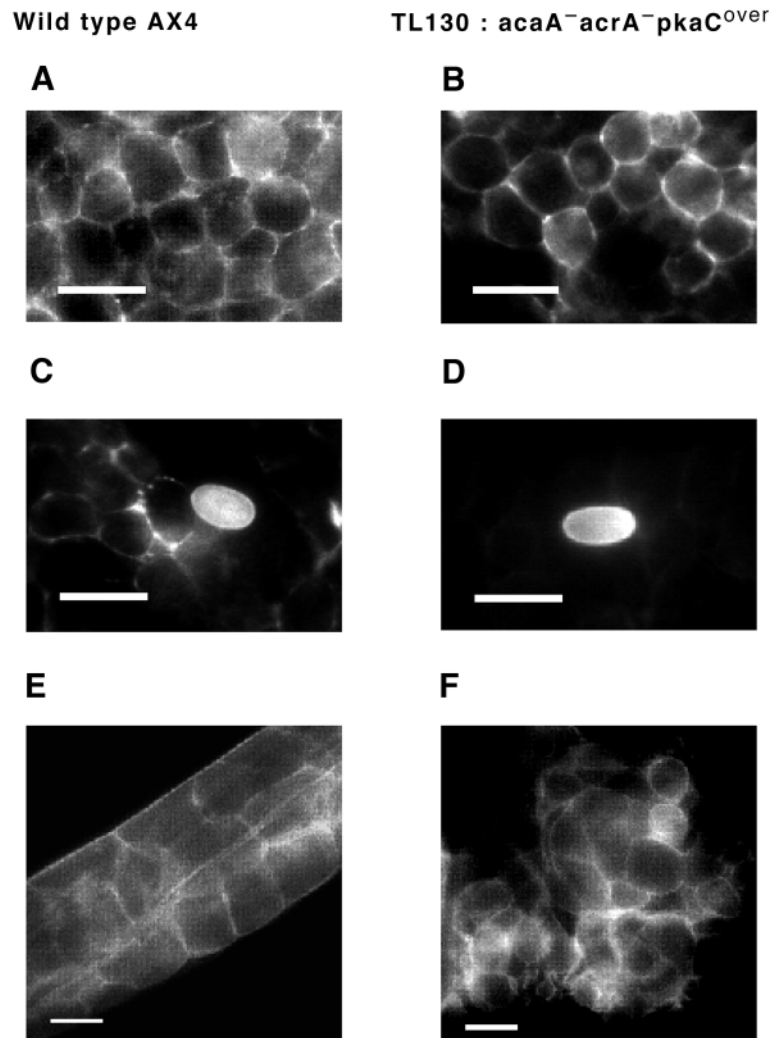


Fig. 4. Calcofluor staining. Cells of strains AX4 and TL130 were developed on filters for 21 hours (A-D,F) or 24 hours (E) before being stained with 0.1% Calcofluor White ST and observed under a fluorescence microscope. Wild-type AX4 cells (A,C,E); *acaA⁻, acrA⁻PKA-C^{over}* cells of strain TL130 (B,D,F). Scale bars, 5 μ m.

fruiting bodies, only 10% were found to be viable after detergent treatment and all of these were genetically wild-type as judged by the phenotype in the resulting plaques. While a few mutant spores may have been made, we would not have observed them if they were less than 10^{-3} of the population. Thus, it appears that the *acrA⁻acaA⁻PKA-C^{over}* cells have a cell autonomous block to sporulation.

Table 1. PKA activity

Strain	PKA activity (pmoles/min/mg protein)	PKA activity in the presence of cAMP (pmoles/min/mg protein)
AX4	34 \pm 7	285 \pm 90
TL130	280 \pm 120	410 \pm 100

Cells were collected after 20 hours of development and assayed for PKA activity with or without addition of 100 μ M cAMP to the reaction buffer. Average of 3 independent experiments.

DISCUSSION

Strains that lack the late developmental adenylyl cyclase due to mutations in *acrA* have a cell autonomous defect in the formation of viable spores but about a quarter of the cells encapsulate into spore-like structures. These non-viable spores fail to exclude propidium iodide and so appear to have permeable spore coats and plasma membranes. The number of spore-like cells decreases rapidly both following dissociation from sori and when left in fruiting bodies (unpublished observations) suggesting that they may undergo premature germination as a result of insufficient PKA activity that is known to be required for maintaining dormancy (Viridy et al., 1999).

Expression of a modified form of the early adenylyl cyclase, ACA, that is independent of G-protein activation in *acrA*⁻ null cells resulted in the formation of detergent-resistant viable spores that were able to exclude propidium iodide. The number of viable spores was highest immediately after culmination and decreased rapidly thereafter as a result of premature germination. It appears that expression of the *act15::acaA** construct in *acrA*⁻ cells resulted in sufficient internal cAMP for terminal differentiation of spores indicating that ACR plays no other role in sporulation than the synthesis of cAMP.

It has been proposed that *Dictyostelium* can develop in the absence of cAMP as long as PKA is constitutively active as a result of overexpression of the catalytic subunit from *pkaC* (Wang and Kuspa, 1997). While *acaA*⁻ PKA^{over} cells that lack the aggregation adenylyl cyclase are unable to chemotactically signal each other, they can form aggregates if plated at high cell density. They then proceed through subsequent stages to form fairly normal fruiting bodies complete with stalks and viable spores (Wang and Kuspa, 1997). No measurable cAMP was found to accumulate prior to culmination in these cells due to rapid turnover (Wang and Kuspa, 1997; Meima and Schaap, 1999). However, we have found that the adenylyl cyclase activity of ACR is essential for morphogenesis beyond the mound stage as well as the differentiation of viable spores. It could be argued that the *act15::pkaC* construct might not be sufficiently active late in development to result in constitutive PKA activity, however direct enzymatic measurements showed that PKA was constitutively active late in development of the strain lacking both developmental adenylyl cyclases but carrying the *pkaC* construct (Table 1). While these mutant cells accumulate mRNA from the prestalk gene *ecmA* to about the same level as seen in their parental strain AK631, the prespore-specific gene *cotB* was expressed at a very low level and almost no spores were formed. When developed in mixtures with wild-type cells, marked TL130 (*acrA*⁻ *acaA*⁻ PKA-C^{over}) cells co-aggregated and were found in both the anterior prestalk region and the posterior prespore region suggesting that they had sorted out normally. Although normal fruiting bodies were subsequently formed, the mutant cells failed to give rise to viable spores further confirming the cell autonomous nature of the *acrA*⁻ defect in sporulation.

While cells of strain TL130 are blocked at the tipped mound stage of morphogenesis when developed as pure populations, they can proceed to form fruiting bodies complete with stalks and sori when developed together with 10% wild-type cells. Although the chimeric fruiting bodies are fairly small, it is unlikely that the stalks are formed only from wild-type cells

since they make up only a minor proportion of the total cells. It is more likely that factors secreted by wild-type cells direct the morphological progression from the finger stage to culmination and induce stalk differentiation among the *acrA*⁻ mutant cells. The signaling factor secreted by wild-type cells is unlikely to be cAMP since neither 5 mM cAMP nor 500 μ M 2' deoxy-cAMP added to 16-hour-developed TL130 cells led to fruiting body formation (unpublished observations).

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