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The cAMP Receptor Subtype cAR2 Is Restricted to a Subset of Prestalk Cells during *Dictyostelium* Development and Displays Unexpected DIF-1 Responsiveness

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Dictyostelium discoidium cells express a family of cell surface cAMP receptors, and these G-protein-coupled receptors are each expressed with unique spatial and temporal patterns. One of these receptors, cAR2, is present during the postaggregative stages of development and our previous work suggests that it is preferentially expressed in prestalk cells. We report here the isolation of the promoter for *carB,* the gene which encodes cAR2. Using this fragment to generate a *carB::lacZ* gene fusion construct, we investigated *carB* expression in detail. Expression is first detected at the tight aggregate stage and subsequently in a pattern reminiscent of the prestalk-specific gene *ecmA*. There are subtle differences, however, with *ecmA* being expressed significantly in the anterior-like cells of the migrating pseudoplasmodium and in the basal disc and lower cup supporting the sorus during terminal development. *carB* is not expressed in any of these places. The presence of these different prestalk cell subtypes was confirmed by double indirect immunofluorescence using anti-cAR2 and anti-**β**-galactosidase antibodies. While virtually all cAR2-expressing cells also express *ecmA::lacZ*, a substantial fraction of *ecmA::lacZ*-positive cells do not express cAR2. We also found the regulation of *carB* gene expression to differ from that of *ecmA*. *carB* expression is induced *in vitro* by extracellular cAMP, but surprisingly, not by DIF-1, a soluble molecule thought to be essential for the initiation of prestalk differentiation. Thus, cAR2 appears to be a cAMP receptor present on a restricted subset of prestalk cells and whose expression does not respond typically to the prestalk inducer DIF-1. DIF-1 sensitivity may, therefore, not be characteristic of all early prestalk differentiation.

INTRODUCTION

Development in the eukaryotic microorganism, *Dictyostelium discoideum*, initially involves the coalescing of up to 10^5 individual cells into a single multicellular organism. This process requires the cessation of cell division, the acquisition of specific chemotactic machinery, and the initiation of a developmental program that includes a progressive series of morphogenetic and cytodifferentiation decisions (Kimmel and Firtel, 1991; Williams, 1991). Development is triggered by starvation (Marin, 1977) and the synthesis and detection of density sensing proteins (Rahti *et al.*, 1991; Gomer *et al.*, 1991; Jain and Gomer, 1994). In response to this trigger, a cell–cell communication system is generated that involves the production and detection of 3′,5′-cyclic AMP (cAMP). Used as a chemoattractant and a signal relay molecule, secreted cAMP mediates the formation of the multicellular organism (Devreotes, 1989). Its detection is also critical for initiating changes in gene expression necessary to progress to the later stages of development (Gomer *et al.*, 1986; Haribabu and Dottin, 1986; Kimmel and Saxe, 1986; Oyama and Blumberg, 1986).

Prior to aggregation, changes in gene expression and concomitant cellular differentiation are equivalent among all the cells (e.g., Gomer *et al.*, 1986). But as cells migrate and form the loose mound, a switch from aggregative to postaggregative gene expression occurs, cell type-specific genes are expressed (Kimmel and Firtel, 1991), and prestalk and prespore cells, the precursors to the terminally differenti-

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ated stalks and spores, can be detected (e.g., Williams *et al.*, 1989; Fosnaugh and Loomis, 1993). The different cell types initially appear randomly distributed in the loose aggregate, but soon collect into coherent groups. The extracellular matrix protein-encoding genes, *ecmA* and *ecmB* have been used to define subclasses of prestalk cells (Jermyn *et al.*, 1989; Williams *et al.*, 1989; Kay, 1994). During tight aggregate formation most *ecmA*-expressing cells move to the top of the aggregate and are present in the tip when it is produced. *ecmB*-expressing cells appear in both the tip and the base of the tight aggregate. As the tip elongates and development proceeds to the migratory, pseudoplasmodium stage, *ecmA* is expressed in the anterior 20% of the organism in two contiguous zones. It is expressed at relatively high levels in the anteriormost region and these have been referred to as prestalk A (pstA) cells. Immediately behind the pstA cells is a region of low, but distinct, *ecmA* expression that defines pstO cells and a portion of the *ecmA* promoter has been identified that regulates pstO-specific expression (Early *et al.*, 1993). There is also a population of *ecmA*expressing cells that is interspersed with the prespore cells, in the rear 80% of the organism, that are referred to as anterior-like cells (ALCs, Sternfeld and David, 1982; Jermyn and Williams, 1991). Normally, pstA cells differentiate into the stalk, pstO cells differentiate into the stalk and a support structure called the upper cup, and the ALCs contribute to the upper cup and two other support structures, the lower cup and the basal disc (Early *et al.*, 1993; Bichler and Weijer, 1994). Stimulation of *ecmA* (and *ecmB*) expression *in vitro* is dependent upon the chlorinated hexaphenone, DIF (Williams, 1991), and they are also under the control of extracellular cAMP, with cAMP stimulating expression of *ecmA* and repressing expression of *ecmB*.

Early in development extracellular cAMP is detected by binding to a high-affinity G-protein-coupled cell surface receptor (cAR1), and much is known about the molecular machinery associated with this system (Devreotes, 1994). Stimulation of several effector systems, including adenylyl and guanylyl cyclases and phospholipase C are all mediated through the activation of cARs (van Haastert and Devreotes, 1993). As development progresses, extracellular cAMP levels rise and the means by which it is detected becomes more complicated. Molecular genetic analysis has revealed the presence of genes for four related, but nonidentical, cARs (cAR1-4) in the *Dictyostelium* genome (Klein *et al.*, 1989; Saxe *et al.*, 1991a,b, 1993; Johnson *et al.*, 1993; Louis *et al.*, 1994). The cARs all appear to be G-protein coupled and are expressed in temporally and spatially unique, but overlapping, patterns (Johnson *et al.*, 1992; Devreotes, 1994). During aggregation cAR3, a second high-affinity receptor, is expressed. It is initially present in most or all cells (Saxe *et al.*, 1991b; Johnson *et al.*, 1993; Yu and Saxe, 1995), but becomes restricted to the posterior region at the time of tip and slug formation (Yu and Saxe, 1995). A lower-affinity receptor, cAR4, is expressed during postaggregative development and is enriched (perhaps 6-fold) in prestalk cells (Louis *et al.*, 1994). Loss of cAR4 function results in abnormal patterns of prespore and prestalk gene expression and aberrant pseudoplasmodium and fruiting body formation, probably as a result of mispositioning of prestalk and prespore cells (Louis *et al.*, 1994). cAR2 is expressed, temporally, between cAR3 and cAR4. We have previously reported that cAR2 expression begins near the time of tip formation and that cAR2-specific RNA is enriched at least 10-fold in prestalk cells (Saxe *et al.*, 1993). Null mutants in cAR2 are defective in forming normal tips, show abnormal regulation of both *ecmB* and a series of prespore genes, and rarely are able to terminally differentiate (Saxe *et al.*, 1993; Yu *et al.*, in preparation). These data are consistent with extracellular cAMP continuing to be critical during the later stages of *Dictyostelium* development and its effects being differentially affected through cAR2 and cAR4.

Our previous work suggested that cAR2 is prestalk-specific, but did not distinguish which of the various prestalk cell types were involved. In the present study we have used two independent approaches to determine cAR2 localization, one measuring promoter/lacZ activity and the other the presence of cAR2 protein. We find that cAR2 is expressed in a pattern that overlaps with, but is not identical to that of ecmA (Williams *et al.*, 1989) and that *carB* and *ecmA* gene expression can be induced in *carB*-null cells. Furthermore, unlike *ecmA*, *carB* expression is not upregulated by the prestalk morphogen, DIF-1. This raises the possibility that DIF-1 is not required for early prestalk differentiation.

MATERIALS AND METHODS

Vectors. The *carB::lacZ* construct was built by blunt-end ligating a *Cla*I fragment of pAB3 (Saxe *et al.*, in preparation) into the *Eco*RI site of pDdgal 16H+ (Harwood and Drury, 1990); the RI site was recreated in the process. The construct includes 1530 bp 5′ of the translation start. Of this, approximately 1 kb is promoter and the rest is 5′ untranslated region (UTR) of *carB* RNA. In addition, sequences representing the first 21 aa of cAR2 are fused in frame to β-gal and the vector multicloning site (MCS). The vector also has restriction sites derived from Bluescript, which was the base vector for pAB3. The *ecmA::lacZ* and pDdgal1 6H$^+$ vectors were the kind gift of J. Williams (MRC, University College, London, UK).

Dictyostelium strains, culturing, and development. All strains described in this study were derived from the thymidine-requiring cell line HPS400 and were grown in axenic, HL-5, liquid medium supplemented (where appropriate) with 100 μg/ml of thymidine. The *carB*-null strain (KO8-1) was constructed by inserting the *thy-1* gene, via electroporation, into the *carB* coding region at a unique *Nde*I site and selecting for thymidine prototropy. KO8-1 cells produced no detectable cAR2 protein and showed the same developmental phenotype as previously reported for *carB*$^-$ cells (Saxe *et al.*, 1993). Strains expressing *carB::lacZ* or *ecmA::lacZ* were transformed by electroporation (Howard *et al.*, 1988) and selected and maintained in axenic medium containing G418 (Gibco/BRL) at 20 μg/ml. For development, cells were washed one time with 12 m*M* PO_4 buffer (pH 6.5) containing 2 m*M* $MgSO_4$ and 0.2 m*M* $CaCl_2$ and placed on filters at a density of 10^7 cells/cm^2.

β*-*Galactosidase staining. For β-galactosidase (β-gal) staining,

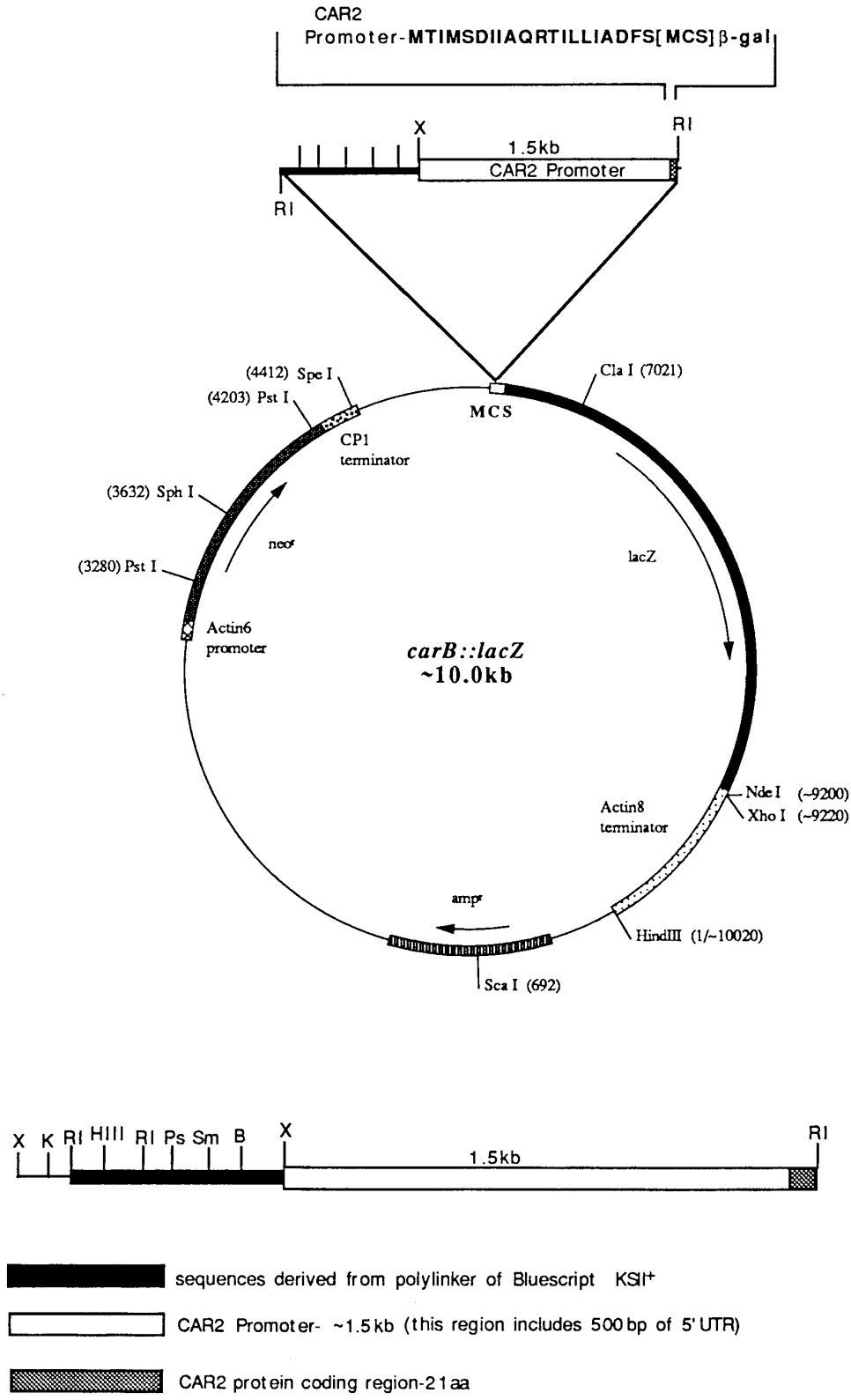


FIG. 1. Structure of the *carB::lacZ* plasmid. (Top) *carB::lacZ* was built as a *Cla*I fragment of pAB3, blunt-end ligated into the *Eco*RI site of pDdgal 16H+ (Harwood and Drury, 1990). RI sites were recreated in the process. The construct includes approximately 1500 bp 5′ of the ATG. Of this, approximately 1 kb is promoter and the rest is 5′-untranslated region of *carB* RNA. (Bottom) The first 21 aa of cAR2 are fused in frame to β-gal and the vector multicloning site (MCS). The construct also has restriction sites derived from Bluescript, which was the base vector for pAB3. See Materials and Methods for details of plasmid construction.

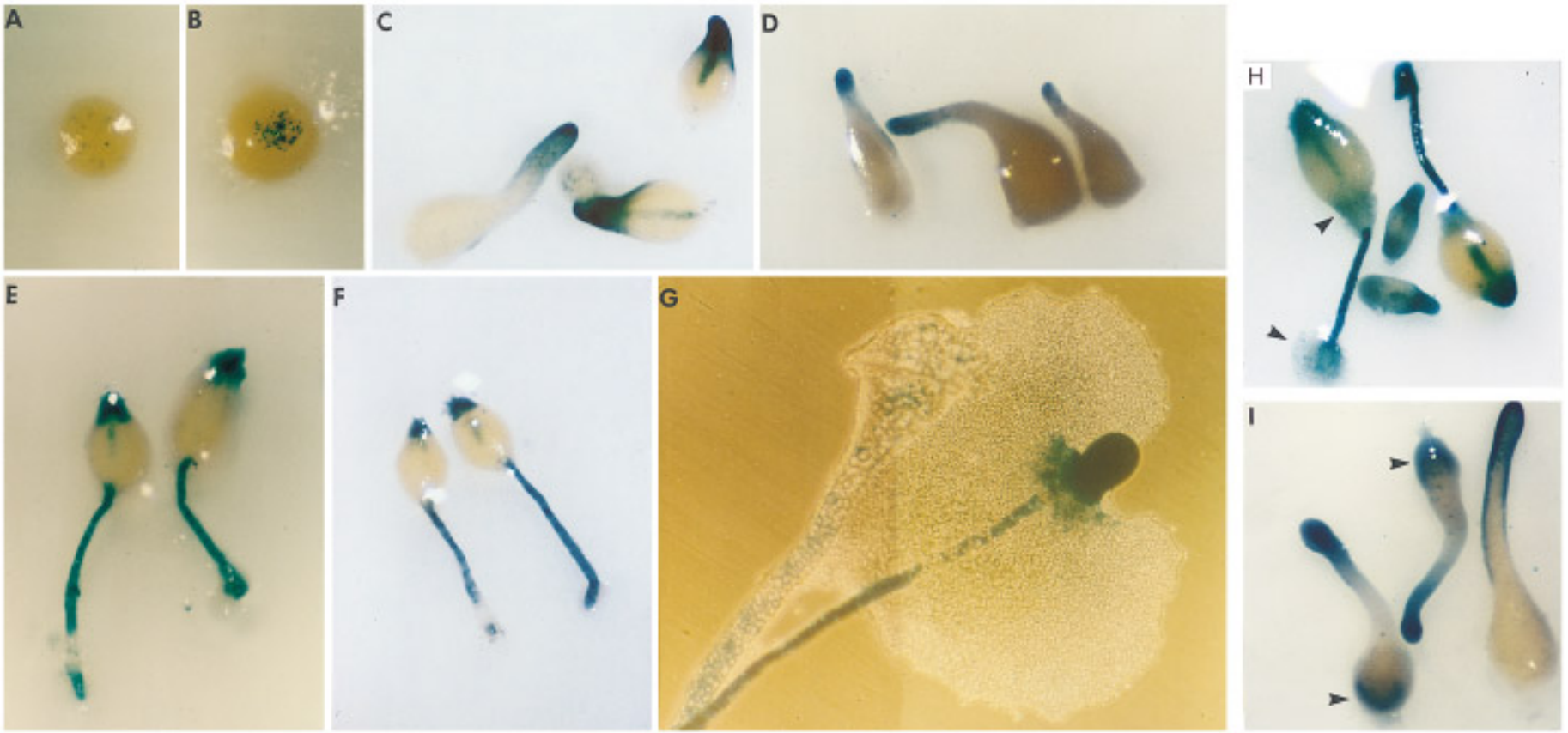


FIG. 2. Expression pattern of *carB::lacZ*. The *carB::lacZ* plasmid was transformed into HPS400 cells and selected with G418^r at 20 μg/ml. Transformed cells were analyzed for *carB::lacZ* expression during development; (A) 10 hr; (B) 12 hr; (C and D) 16–20 hr; (E and F) 24–26 hr; (G) high magnification at 26 hr showing staining in stalk cells; (H) 24- to 26-hr HPS400 cells transformed with *ecmA::lacZ;* (I) 16–20 hr, two slugs on left are HPS400/*ecmA::lacZ* and slug on right is HPS400/*carB::lacZ*. Arrowheads in H and I indicate regions stained by *ecmA* and not *carB*.

structures were gently removed from filters, at various times in development, and permeablized in a small volume of Z-buffer containing 0.1% NP-40 (Dingermann *et al.,* 1989; Fosnaugh and Loomis, 1993) for 20 min, at room temperature. The structures were washed one time in Z-buffer and then fixed with Z-buffer containing 0.5% glutaraldehyde for 10 min. It should be noted that the integrity of terminal fruiting bodies was maintained more completely if the glutaraldehyde step preceded the permeablization. The structures were then washed two times with Z-buffer, stained with 1 m*M* X-gal, and photographed as previously described (Dingermann *et al.,* 1989). Most preparations were stained for 1–2 hr but longer staining periods were also analyzed (see text).

cAR2 antibody production. The peptide NH_2-CNNIETPKENENQNHH-COOH, corresponding to amino acids 333–347 of cAR2, was synthesized. The cysteine at the amino terminus is not from cAR2 but was added for crosslinking purposes. The peptide was coupled to keyhole limpet hemocyanin (KLH) as described (Johnson *et al.,* 1993) and injected subcutaneously into a rabbit. A high titer serum was obtained and used at 1:1000 on Western blots. For immunofluorescence experiments, the cAR2 antibody was affinity purified by binding to preparative amounts of cAR2 size separated on SDS–PAGE and blotted to nitrocellulose. The bound antibodies were released by the low-pH/glycine method (Harlow and Lane, 1988) and diluted fivefold.

Immunofluorescence detection. To detect cAR2 and β-gal expression, cells of the promoter–*lacZ*-containing strains were dissaggregated at various times during development, attached to coverslips, and fixed with 100% methanol. Once dry, the coverslips were placed in PBS containing 0.01% Tween 20 and 5% BSA and incubated at room temperature for 1 hr. They were then removed to a piece of parafilm and 100 μl of primary antibody was added. After incubation for 2 hr at RT, the coverslip was washed three times with PBS/0.01% Tween 20. Secondary antibody was added and incubation and wash procedures were repeated. cAR2 was detected with the rabbit anti-cAR2 antibody and positive cells were visualized with a rhodamine-conjugated goat anti-rabbit secondary antibody. To detect β-gal expression, a mouse anti-β-gal antibody (Sigma Chemicals, St. Louis, MO) and a fluorescein-conjugated goat-antimouse secondary antibody were used. The anti-β-gal and both secondary antibodies were used at 1:1000 dilutions. Fluorescein and rhodamine fluorescence was discriminated using narrow band pass filters on a Zeiss XOphot microscope.

DIF-1 induction of prestalk gene expression. To determine the responsiveness of *carB* gene expression to stimulation by cAMP or DIF-1, cells were washed free of medium, placed on black Millipore filters, and allowed to develop for 13 hr. Cells were harvested into 10 m*M* potassium phosphate buffer, pH 6.4, containing 2 m*M* $MgSO_4$ and 0.2 m*M* $CaCl_2$, disaggregated by pipeting, and resuspended at 2×10^6 cells/ml of the same buffer. Cells were subsequently shaken at 220 rpm and treated with cAMP and/or DIF-1 as described in the figure legends. Samples were collected, RNA isolated and analyzed by Northern blot as previously described (Berks and Kay, 1990; Mehdy *et al.,* 1983). Probes were a 1-kb *Xba*I cDNA fragment of *carB,* a 3-kb *Hin*dIII fragment from plasmid pDdgal 16H+ for *lacZ* detection, and a *ecmA* cDNA whole plasmid. The latter two plasmids were kind gifts from J. Williams (MRC, UCL, London).

RESULTS

Reporter construct for the carB gene. We previously reported the isolation of a gene, now designated *carB,* that encodes cAR2, a member of the multigene family of cAMP receptors in *Dictyostelium*. RNA accumulation data was consistent with *carB* (and by inference cAR2) being expressed largely, if not exclusively, in prestalk cells (Saxe *et al.,* 1993). To further clarify the location of *carB* expression we have produced a *carB* promoter::*lacZ* reporter construct (Fig. 1). A 1.6-kb *Xba–Cla* fragment of genomic DNA was isolated that contains the *carB* promoter, 5′ untranslated material, and sequences that encode the first 21 amino acids of cAR2 (see Materials and Methods for details). Based upon RNase H protection experiments similar to those used to map cAR1 mRNA (Saxe *et al.,* 1991a), the construct contains ~1050 bp upstream of the transcription start site of *carB* (data not shown). This fragment was ligated into the vector pDdgal 16H+ (Harwood and Drury, 1990) producing an in-frame fusion between *carB* and *lacZ*. All subsequent analysis of *carB::lacZ* expression was done with this construct.

Prestalk-specific expression of carB during development. The *carB::lacZ*-containing plasmid was transformed into the axenic strain HPS400 and growing cells were washed free of media, placed on white filters, and allowed to proceed through the developmental cycle. At representative stages, filters were removed and developmental structures analyzed for β-galactosidase activity. As seen in Fig. 2, expression is first detected at the tight aggregate stage in apparently randomly distributed cells (Fig. 2A). Shortly thereafter, as the tip is forming on the aggregate, β-gal staining concentrates on the newly forming tip (Fig. 2B). As development proceeds, *carB*-expressing cells remain localized to the anteriormost region of the slug and the uppermost region of the preculminant (Figs. 2C and 2D). It should be noted that, even with extended staining periods, very few *carB*-expressing cells were detected in the body of the slug or in the posterior regions. This suggests that there is little, if any, *carB* expression in prespore cells or any type of ALC. Consistent with this interpretation is the staining pattern seen at the time of culmination (Figs. 2E–2G). β-Gal-expressing cells are detected in the stalk and in the former tip region, now at the top of the sorus. There is no expression in the lower cup area of the sorus nor in the basal disc. These latter structures are believed to be populated by ALCs and prestalk cells not located at the very tip of the slug (Jermyn and Williams, 1991). The latter cells are clearly distinguished by using *ecmA::lacZ* as a marker (Figs. 2H and 2I) and the differences between *carB* and *ecmA::lacZ* staining are quite reproducible. Because of the potential stability of β-gal in these cells, these experiments only indicate cells that have expressed *carB::lacZ* RNA; they cannot be used, for instance, to establish continued *carB* expression in the stalk.

Immunofluorescent detection of carB/cAR2 expression. The data in Fig. 2 suggest that cAR2 is present in prestalk cells, but only in a subset of the cells expressing ecmA; all cAR2-expressing regions appear to also express *ecmA* but only some of the regions expressing ecmA express cAR2. Because the expression pattern seen for *carB* could reflect

the use of an incomplete promoter, we sought another means of verifying *carB* expression.

As an independent means of analyzing cAR2 expression, we used a rabbit polyclonal antiserum generated against a specific peptide present in the cAR2 carboxyl terminal region (see Materials and Methods for details). The specificity of the antibody was verified by Western blot analysis of axenic cells transformed with a vector that provides for cAR2 protein expression during vegetative growth (Johnson *et al.,* 1992). As previously reported, the antiserum detects a specific protein of apparent molecular mass of 39 kDa (Johnson *et al.,* 1993), and when detected by indirect immunofluorescence, using a rhodamine-conjugated goat anti-rabbit secondary antibody, cAR2 was found to localize to the cell surface (data not shown). Vegetative cells not containing the cAR2 expression vector showed no 39-kDa band on Western blot nor any distinct staining in immunofluorescence studies. The antiserum was then used to compare the expression of bona fide cAR2 with that of β-galactosidase driven by the *carB* promoter.

Cells of the *carB::lacZ*-containing strain were dissaggregated at various times during development and fixed to microscope slides. cAR2-expressing cells were detected with the anti-cAR2 antibody and a rhodamine-conjugated goat anti-rabbit secondary antibody. Expression, in the same cells, of the *carB* promoter was detected using a mouse anti-β-gal antibody and secondary fluorescein-conjugated goat anti-mouse antibody. There appears to be an almost complete concurrence between β-gal antibody and cAR2 antibody staining. In over 400 cAR2 antibody-positive cells, greater than 96% were also positive for the β-gal antibody (data not shown). The converse experiment (i.e., cells first scored for β-gal antibody staining) gave the same results. Based on these data, we suggest the staining pattern presented in Fig. 2 represents the complete distribution of normal cAR2 expression.

Double immunofluorescence experiments also confirmed that cAR2 and *ecmA* are expressed in overlapping but nonidentical patterns. *ecmA::lacZ*-containing cells were analyzed for cAR2 and β-galactosidase activity. As suggested above, cAR2-expressing cells expressed *ecmA,* but a subset of *ecmA*-expressing cells did not express cAR2 (Fig. 3; Table 1). In these experiments approximately 7–9% of the total number of cells in the slug expressed cAR2. Virtually all of these cells also expressed *ecmA* (96%). The small percentage of cAR2-positive cells that do not express *ecmA::lacZ* may merely be the result of staining or detection artifacts, as the percentage varied from 0–5% from experiment to experiment. In the same experiments 12–20% of the cells expressed *ecmA* and approximately 60% of these also expressed cAR2 at the mound stage and approximately 30% colocalized with cAR2 at later stages (Table 1). It is not known, at present, whether the change in percent colocalization of cAR2 and *ecmA* during development is significant. The decrease in coexpression may represent a genuine expansion of the number of *ecmA*-expressing/cAR2-nonexpressing cells as development proceeds. These cells could be ALC/pstO cells that would be expected to express *ecmA* but not cAR2. The same results could be generated if many of the ALC/pstO cells were present at the mound stage but expressing *ecmA* at a level below detection. This is consistent with previous reports (Jermyn and Williams, 1991). Thus, the accumulated data support the idea that cAR2 is expressed in a subset of anterior localized prestalk cells and that its expression pattern overlaps with, but is not identical to, that of *ecmA*.

TABLE 1
Colocalization of cAR2 and *ecmA* during Development

Developmental stage	Percentage of coexpressing cells[a]	
	cAR2 and *ecmA*-positive[b]	*ecmA* and cAR2-positive[c]
Mound	96 (96/100)	62 (31/50)
Slug	96 (96/100)	30 (59/198)

Note. From the time of tip formation until culmination cAR2-expressing cells represent 7–9% of the total cell number, *ecmA/lacZ*-expressing cells represent 12–20% of the total.

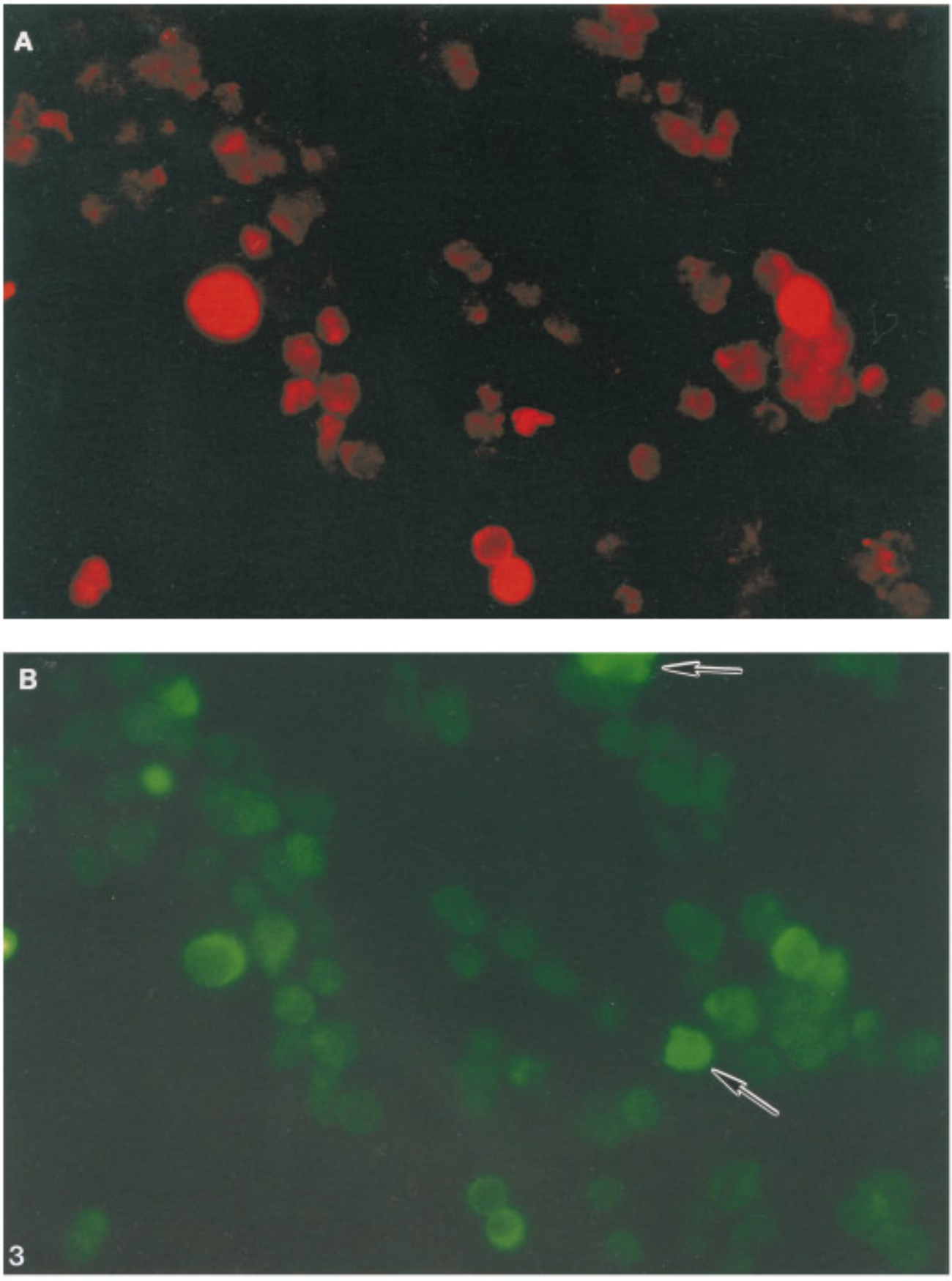
[a] Results given in parentheses are representative of at least three separate experiments.

[b] Based on first detecting cAR2-expressing cells using rhodamine-based indirect immunofluorescence and subsequently detecting *ecmA/lacZ* coexpression using fluorescein-based indirect immunofluorescence (see text for details).

[c] Based on first detecting ecmA/lacZ expression using fluorescein-based indirect immunofluorescence and subsequently detecting cAR2 coexpressing cells using rhodamine-based indirect immunofluorescence.

Initial expression of carB and ecmA is normal in carB- cells. Because *carB*$^-$ cells do not make normal tips and do not complete stalk cell differentiation effectively (Saxe *et al.,* 1993), it was of interest to know whether initial prestalk differentiation was affected in these mutants. Strain KO8-1 (*carB*$^-$) was transformed with *ecmA::lacZ* or *carB::lacZ* plasmids and expression patterns of the resultant strains were observed. Initial expression of both genes seemed to be unaffected by the *carB*$^-$ mutation (Fig. 4). Expression is first detected at ~10 hr of development and expressing cells begin to concentrate by ~14 hr (not shown). Indeed, once differentiation commences, even in the absence of a recognizable tip, many of the *ecmA or carB::lacZ*-expressing cells collect together and are, in some cases, able to become positioned on the top of the mound (e.g., Fig. 4D). Therefore, at least some of the earliest signals regulating prestalk differentiation seem to be shared between *carB* and *ecmA* and are unaffected in a *carB*$^-$ strain.

The carB response to DIF is unlike that of ecmA. The previous experiments suggested that *carB* was expressed in a pattern similar to that of *ecmA* and as such should be inducible by the prestalk-specific morphogen DIF-1. To test this, HPS400/*ecmA::lacZ* cells were allowed to develop on filters to the tight aggregate stage (13 hr, a time when weak


A
B
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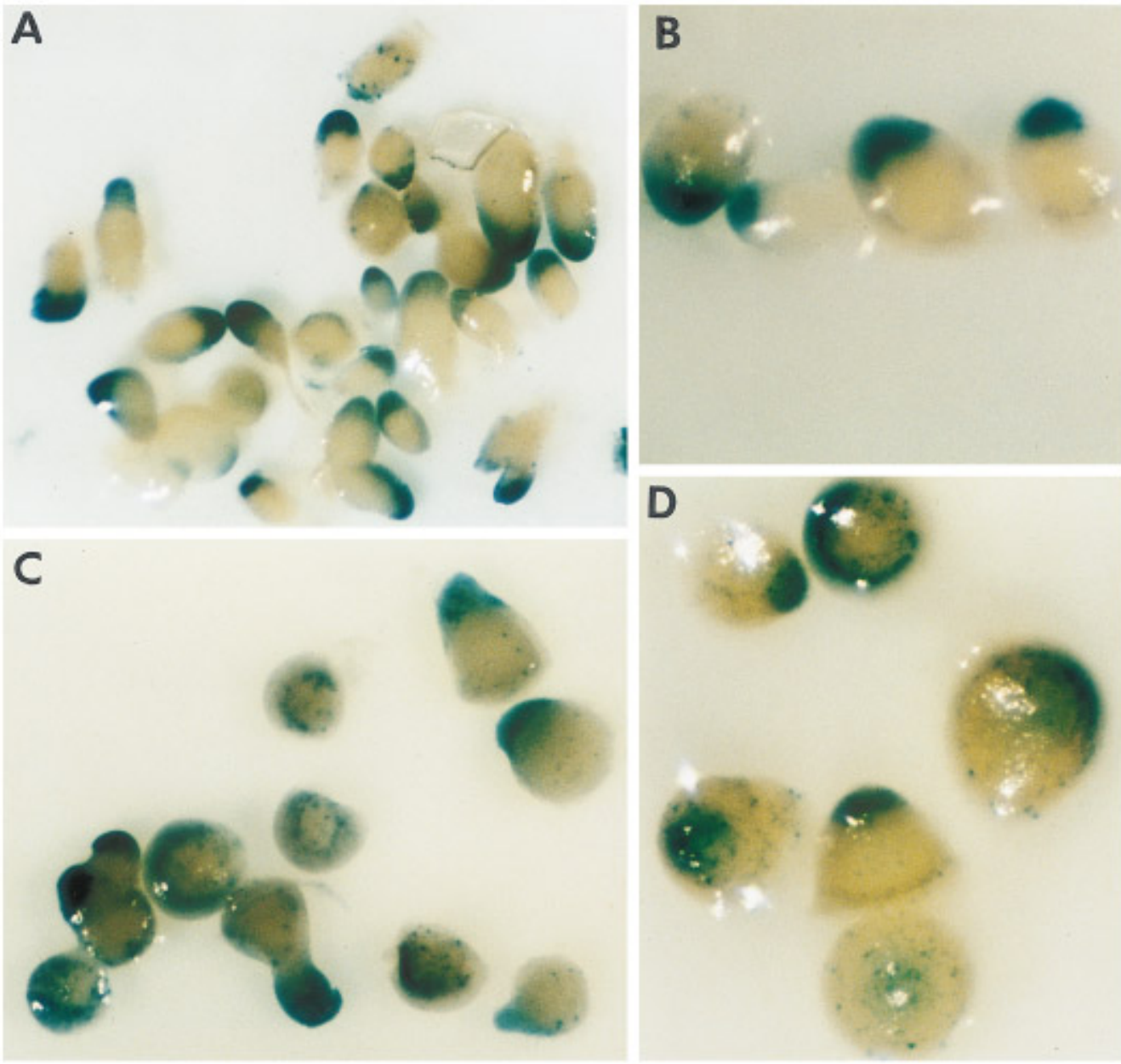

FIG. 4. Expression of *ecmA::lacZ* and *carB::lacZ* in *carB*- cells. The *carB::lacZ* and *ecmA::lacZ* plasmids were transformed into KO8-1 (*carB*-) cells and selected with G418^r at 20 μg/ml. Transformed cells were analyzed for *lacZ* expression during development. (A, B) *carB::lacZ* expression pattern, 18–20 and 30 hr of development, respectively; (C and D) *ecmA::lacZ* expression pattern, 18–20 and 30 hr of development, respectively. (B and D) Evidence that marked prestalk cells can collect together and can become located in their normal position in the aggregate.

FIG. 3. Simultaneous detection of cAR2 and *ecmA* in HPS400/*ecmA::lacZ* cells. Cells from 18-hr structures were disaggregated and analyzed by double immunofluorescence for cAR2 (A) and *ecmA::lacZ* (B) expression. (A) Detection of cAR2 with a rabbit anti-cAR2 antibody followed by a rhodamine-conjugated, goat anti-rabbit antibody; (B) detection of *ecmA::lacZ* with a mouse anti-β-galactosidase antibody followed by a fluorescein-conjugated, goat anti-mouse antibody. Arrows indicate *ecmA::lacZ*-expressing cells that do not express cAR2.

ecmA::lacZ staining was detected), harvested, shaken in liquid suspension, and assayed for the ability of DIF-1 and/or extracellular cAMP to induce *carB* expression. Surprisingly, *carB* expression was not induced by DIF-1, though it was induced by cAMP (Fig. 5). In fact, addition of DIF-1 appeared to block the ability of cAMP to induce *carB* RNA accumulation. Controls showed *ecmA* expression (as detected for convenience by hybridization of a *lacZ* probe to *ecmA::lacZ* RNA) to be inducible by DIF-1, and cAMP as previously reported (Berks and Kay, 1990).

To further analyze the responsiveness of *carB* to DIF-1, a dose/response relationship was established between DIF-1 dose and *carB* RNA expression. HPS400 cells were allowed to form tight aggregates, as above, harvested, and treated with 1 m*M* cAMP plus increasing concentrations of DIF-1. No significant induction of *carB* RNA accumulation occurred in the presence of DIF-1, and even at low DIF-1 concentrations (10 n*M*) the *carB* induction by cAMP was blocked by DIF-1 (Fig. 6). Similar results (data not shown) were obtained with cells harvested at the loose aggregate stage (~2 hr earlier). *ecmA*, in contrast, showed increasing RNA accumulation with increasing DIF-1 to at least 200 n*M* (Fig. 6). The latter data are in complete agreement with previous studies (Berks and Kay, 1990). Thus, it appears that *carB* is a gene, expressed in a subset of prestalk cells, that shows a substantial degree of spatial and temporal overlap with *ecmA*, but whose regulation by the putative prestalk morphogen DIF-1 is substantially different than that of *ecmA*.

DISCUSSION

Our previous work (Saxe *et al.*, 1991, 1993) has led to the suggestion that cAR2 is prestalk localized. The present study extends that effort and establishes that cAR2 is restricted to only a subset of prestalk cells and perhaps the derived stalk structures. We have used two independent methods to verify the localization of cAR2, one using a promoter::*lacZ* construct to visualize *carB* expression *in situ* and the other using a cAR2-specific antibody to identify cAR2 protein-expressing cells. The two methods corroborate one another and together overcome problems of either one alone. Two difficulties are inherent in interpreting promoter::*lacZ* experiments. One involves knowing whether the promoter region used represents the entire regulatory domain of interest. The second concerns the issue of β-galactosidase stability. This includes not only the intrinsic stability of the expressed protein (i.e., very stable vs labile β-gal activity) but whether the stability of the β-galactosidase in anyway mirrors that of the real gene product of interest (here cAR2). We have tried to address the former issue by using a large *carB* promoter-containing fragment with more than 1 kb of sequence upstream of transcription start. The second issue we have addressed by making our β-galactosidase construct a fusion protein that includes the first 21 amino acids of cAR2 at the N-terminus. Whether these efforts have negated the problems or generated different ones (see below) requires further investigation. The high degree of overlap between the β-galactosidase and cAR2 antibody data throughout development suggests, however, that the basic pattern is correct.

It is clear that virtually all cells that express cAR2 also express *ecmA*, but cAR2 is expressed only on a subset of *ecmA*-expressing cells. Taken together the *carB::lacZ* and anti-cAR2 antibody experiments suggest cAR2 accumulation only in the tip, anterior region of the slug and the papillae and stalk of the culminate. These data strongly suggest that cAR2 is expressed only in the anteriormost prestalk cells and not in the majority of ALC/pstOs. But a note of caution should be added. It is conceivable that *carB* is induced in all prestalk and ALC cells, but that the cAR2 protein turns overs rapidly in the ALCs. If this property was incorporated in our cAR2/β-gal fusion proteins, then the distribution of β-gal activity might reflect cAR2 protein distribution more closely than *carB* RNA accumulation. It is also possible that a uniquely rapid turnover of *carB* RNA occurs in ALCs, which could provide the same results. These are more than remote possibilities. β-Gal fusion proteins with different half-lives can show dramatically different distributions even when driven by the same promoter (H. MacWilliams, personal communication). In addition, we have evidence that cAR3 protein distribution may be more restricted than its RNA (Yu and Saxe, 1995). Clearly, cAR2 receptor protein accumulates only in anterior prestalk cells. But until we establish half-lives for both *carB* RNA and cAR2 protein and/or use long half-life β-gal fusion proteins, the precise regulation of *carB* will be incomplete. The distribution of cAR2 is in contrast to the reported *car4::lacZ* pattern that shows *car4* expressed in ALC/pstO as well as pstA cells (Ginsburg *et al.*, 1994; Louis *et al.*, 1994); a pattern very similar to that of *ecmA*. The exact relationship between cAR2 expression and cAR4 and ALC/pstO expression is unknown but is under investigation.

The fact that cAR2 is restricted to only a portion of the prestalk population and only partially overlaps with the other, late, low-affinity cAMP receptor, cAR4, has important implications for both its function and regulation. That *carB* is induced at the time of aggregate formation and cAR2-expressing cells concentrate at the tip are completely consistent with the *carB*$^-$ phenotype. These mutants block at tight aggregate formation and make very unsuccessful attempts at tip formation (Saxe *et al.*, 1993), suggesting that cAR2 expression in the tip is required for its proper morphogenesis. In this context it is interesting to note that *carB::lacZ* (and *ecmA*) expression is induced in the *carB*$^-$ strains and *carB::lacZ*-expressing cells collect together, though not always at the top of the aggregate. The location of cAR2 in the pstA cells is also interesting in the context of terminal stalk differentiation. It has been previously reported that extracellular cAMP induces prespore formation and a drop in extracellular cAMP concentration is needed for stalk cell formation (Hopper *et al.*, 1993). It has been proposed that stalk cell formation requires relieving a re-

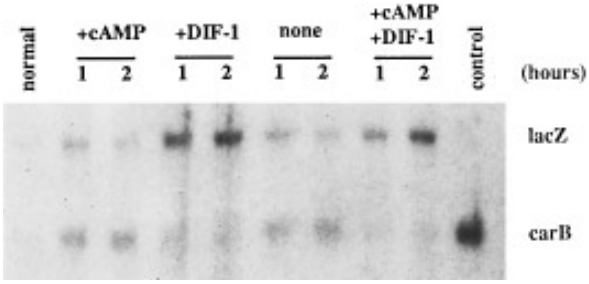

FIG. 5. Regulation of *carB* expression by cAMP and DIF-1. HPS400/*ecmA::lacZ* cells were developed for 13 hr, harvested, disaggregated, and shaken *in vitro* as described under Materials and Methods. Cells were then treated as follows: +cAMP = 1 m*M* cAMP added one time at beginning of shaking; +DIF − 1 = 200 n*M* DIF-1 added one time at beginning of shaking; +cAMP/+DIF − 1 = both cAMP and DIF-1 additions at beginning of shaking. RNA was collected from cells immediately prior to shaking (normal) and at 1 and 2 hr after treatments began (1 and 2 hr points). Control is RNA from 15-hr filter-developed HPS400 cells that express both *ecmA* and *carB*. Blot was probed simultaneously with *carB* cDNA and *lacZ* fragment to detect *ecmA::lacZ* expression. Comparable results were obtained when cAMP and DIF-1 were added as 50 μM and 50 n*M* doses, respectively, every 0.5 hr (not shown).

pression of *ecmB* expression in pstA cells to create pstAB cells. It is conceivable that activation of cAR2 is important for the repression of *ecmB* in pstA cells and when extracellular cAMP levels drop, cAR2 is no longer activated and pstB formation occurs. It has recently been observed (Harwood *et al.*, 1995) that the enzyme glycogen synthetase kinase-3 (GSK-3) is important for repression of *ecmB* expression and null mutant strains in *gskA* are stalkogenous (i.e., form abnormally high numbers of stalk cells at the expense of spore cells). The phenotype of the *gskA*- cells is in many respects the reverse of *carB*- mutants. This has led to the speculation that cAR2, in some manner, regulates GSK-3 activity (Insall, 1995). Presumably, a drop in extracellular cAMP levels would lead to a drop in cAR2 activity. This in turn would result in a decrease in GSK-3 function, relieving the block in *ecmB* expression and promoting pstAB formation. This possibility is presently being investigated. It should be noted that because of similarities in cell type localization (Louis *et al.*, 1994) and null-mutant phenotype, cAR4 could be playing a similar role (Insall, 1995). To understand the importance of cAMP signaling through cAR2 and cAR4, it is going to be critical to establish whether they are providing redundant or unique functions.

Because of the similarity of *ecmA* and *carB* expression, the most surprising data in this study are those showing the effect of DIF-1 on *carB* RNA accumulation. Contrary to expectations, in tight aggregate stage cells, DIF-1 does not induce *in vitro carB* expression and in fact blocks the ability of cAMP to stimulate *carB* RNA accumulation. This is different from *ecmA* regulation, where expression at approximately the same stage is inducible by both cAMP and DIF-1 (Berks and Kay, 1990; Fig. 5). Because *ecmA* is activated by DIF-1 and is expressed only in prestalk and not prespore cells, it has been suggested that DIF is required for prestalk differentiation (Jermyn and Williams, 1995). The regulation of *carB* is inconsistent with that idea in its simplest form. *carB* RNA accumulation is not stimulated by DIF-1 when in parallel experiments *ecmA* expression is clearly upregulated. Because *carB* and *ecmA* were already activated at the time that DIF-1 responsiveness was measured (13 hr), it is possible that earlier *carB* induction is DIF-1 sensitive. We have repeated the experiments for both *carB* and *ecmA* at 10 hr of development, a time when expression of both genes is just detectable. The results were essentially identical (data not shown), but again it could be argued that only an extremely early component of *carB* regulation is positively controlled by DIF-1. A final resolution of this issue may have to await the identification of a completely DIF-negative mutant, but the data presented here (and in Shaulsky and Loomis, this issue) raise the possibility that early prestalk differentiation may not require DIF-1. In some ways the pattern of *carB* expression is similar to that of *Dictyostelium rasD* (Mehdy *et al.*, 1983; Reymond *et al.*, 1984; Jermyn *et al.*, 1987). Jermyn and Williams (1995) have shown that *rasD* is initially expressed not only in prestalk cells but in nearly 50% of the cells of the aggregate. They establish that the prestalk localization of *rasD* is accomplished by differential stability of the *rasD* message in prestalk cells and argue that for those genes which are uniquely expressed in prestalk cells DIF-1 is required for induction. Unlike the *rasD*, however, *carB* is only ever expressed in prestalk cells. From the earliest times we detect *carB*, all cells positive for the cAR2 protein also express *ecmA* and no more than ~10% of the cells ever express *carB*. This suggests to us again that DIF-1 may not be essential for the earliest stage(s) of prestalk differentiation but acts on some, but not all, prestalk genes at a subsequent step. Clearly there is a complex pattern of regulation involved in *Dictyostelium* postaggregative gene expression

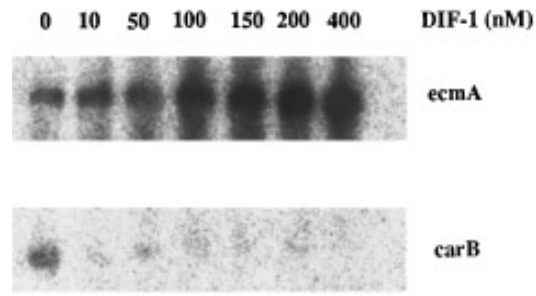

FIG. 6. DIF-1 dose response curve for *carB* expression. HPS400 cells were developed for 13 hr, harvested, and disaggregated as in Fig. 5. Cells were treated with a single dose of 1 m*M* cAMP and single doses of DIF-1 (from 0 to 400 n*M*) as indicated, shaken, and RNA was collected after 1 and 2 hr. RNA was probed with *carB* and *ecmA* cDNAs.

and cell type differentiation, and further studies will be needed to determine the precise role(s) played by DIF.

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