Developmental and spatial regulation of a Dictyostelium prespore gene: cis-acting elements and a cAMP-induced, developmentally regulated DNA binding activity

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Summary

Previously, 5' deletion analysis revealed three important upstream regions within the regulatory region of the cAMP-induced, prespore gene SP60 of D. discoideum, each of which contains a CA-rich sequence element (CAE: consensus CACACAYYYCACAAAA/T). In this study, we have made site-directed mutations within these CAEs and examined their effect on reporter gene activity (luciferase or lacL). Point mutations within or deletion of the distal CAE (CAE-1), middle CAE (CAE-2) or proximal CAE (CAE-3) result in substantial decreases in promoter activity at 18 h of development or in response to cAMP. lacZ fusions made with the CAE mutant promoters produced novel β-gal staining patterns that suggest the presence of one or more morphogen gradients within the prespore zone of the slug and indicate that the CAEs are also important in regulating the spatial patterning of SP60 expression in the multicellular aggregate.

Gel mobility shift assays were used to identify activities from crude nuclear extracts that bind oligonucleotides containing the CAEs. One of the binding activities is not observed in extracts from vegetative cells or cells in early development and is induced during multicellular development with kinetics similar to those of SP60 gene expression. This activity is also induced in response to cAMP and specifically binds the wild-type CAE-1- and CAE-2-containing oligonucleotides. CAE-1 and CAE-2 oligonucleotides containing point mutations within the CAE core sequence neither bind to nor compete for the cAMP-induced, developmentally regulated factor(s) and result in substantial reductions in expression levels when substituted for the wild-type CAEs in vivo. The correlation between in vitro binding and in vivo function suggests that the CAE-1/CAE-2 binding activity may be involved in regulating cAMP and developmentally induced expression of SP60. A second, constitutive in vitro binding activity with high affinity to CAE-3 is also described. Models are proposed to relate the binding activities with the effects of the mutations on the spatial patterning of SP60-lacZ expression.

Key words: Dictyostelium, gene regulation, cAMP, cis-elements, trans-factors, DNA binding.

Introduction

Upon starvation, Dictyostelium vegetative amoebae initiate a 24 h multicellular developmental program that culminates in the formation of a fruiting body containing spores and stalk cells. Approximately 6–7 h after starvation, cells start to aggregate in response to pulsatile signals of cAMP and form a loose mound by ~10 h, a tipped aggregate by ~12 h, and a migrating slug by ~16 h. The appearance of prespore and prestalk cells, the precursors to the spores and stalk cells, can be first detected at the late aggregate stage as determined by the expression of cell-type-specific markers (see Williams, 1988; Firtel et al. 1989; Devreotes, 1989 for review). Prespore-specific genes (e.g. genes encoding spore coat proteins) and one class of prestalk-specific genes (e.g. pst-cath/CP2 or Dd-ras) are induced by cAMP. As with aggregation and early, cAMP-pulse-induced gene expression, the induction of both classes of cAMP-induced, cell-type-specific genes is activated through G-protein-linked receptors. Physiological studies, however, suggest that different cAMP receptors and/or intracellular signalling pathways may mediate aggregation-stage and cell-type-specific gene expression (Mehdy and Firtel, 1985; Reymond et al. 1984; Schaap and van Driel, 1985; Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Gomer et al. 1986a,b; Mann and Firtel, 1987; Schaap and Wang, 1986; Kimmel, 1987; Blumberg et al. 1988; Spek et al. 1988; Kumagai et al. 1989; Ginsberg and Kimmel, 1989;
Peters et al. 1991). A second class of prestalk-specific genes is induced by the morphogen DIF at approximately the same time in development as the cAMP-induced prestalk genes (Williams et al. 1987). [DIF, Differentiating Inducing Factor, has been identified as a chorinated hexaphenone (Morris et al. 1987).]

Promoter–lacZ, or other promoter–reporter gene fusions and antibodies raised against cell-type-specific proteins have allowed the examination of the spatial patterning of prestalk and prespore cells and the morphogenetic processes required for the formation of spatially distinct cell types within the migrating slug and culminant (Takeuchi, 1963; Kreft et al. 1984; Gomer et al. 1986; Datta et al. 1986; Williams et al. 1989; Haberstroh and Firtel, 1990; Esch and Firtel, 1991). Analyses have shown that cells expressing cAMP-induced prestalk-specific genes and the DIF-inducible gene ecmA first appear as ‘randomly’ scattered cells within the aggregate and constitute approximately ~10–15% of the total cells (Williams et al. 1989; Esch and Firtel, 1991). These cells then appear to sort, possibly in response to cAMP signals, to form the tip. In contrast, prespore cells first appear as a spatially distinct population of cells within the central region of the developing mound with no observed staining within the skirt of cells moving toward the aggregation center. A spiral or ring pattern then develops and staining cells are excluded from the center of the developing aggregate (Kreft et al. 1984; Gomer et al. 1986; Gomer and Firtel, 1987; Williams et al. 1989; Haberstroh and Firtel, 1990; Powell and Firtel, unpublished observations). By the slug stage, the prespore gene-expressing cells constitute the majority of the posterior 80–85% of the migrating slug, while the prestalk cells constitute the anterior 10–15% and the basal cells, which are identified by the expression of a subpopulation of prestalk-specific markers, constitute the posterior 2–5%.

We previously described the cloning of the gene encoding the SP60 spore coat protein and, using SP60–lacZ fusion studies, showed the expression of the gene to be spatially localized and prestalk-specific (Mehdy et al. 1983; Haberstroh and Firtel, 1990). Using SP60–luciferase gene fusions, we showed that luciferase levels are induced ~10^2 to 10^6-fold during development or in response to high levels of cAMP in the developing culture. 5' deletion studies identified three ~100 bp upstream domains lying between 650 and 300 bp 5' to the CAP site, each containing a common sequence motif, CACACA(Y)YCACA/T (CAE). 5' deletions sequentially removing each ~100 bp domain resulted in 10- to 50-fold drops in the level of expression assayed during multicellular development or in response to cAMP in shaking culture. Contrasts lacking all three domains showed no developmental or cAMP-mediated induction. Internal deletions have also defined a cis-acting element within the 300 bp proximal to the third CAE (CAE-3) that is required for SP60 expression. Moreover, in transformants expressing an SP60–lacZ construct carrying a 5' deletion that removes the 5' region containing the first CAE (CAE-1), the β-galactosidase (β-gal) activity is restricted to the anterior 1/3 of the prespore domain with the expression levels decreasing toward the posterior of the slug. A larger 5' deletion that removes the region containing the two 5'-most CAEs (CAE-1 and CAE-2) results in β-gal expression that is further restricted to the very anterior of the prespore zone at the boundary between the prespore and prestalk regions. From these studies, we proposed that the CAEs may be essential cis-acting elements responsive to cAMP-mediated regulation of SP60 expression. We also suggested the presence of a gradient of cAMP-responsiveness within the slug to account for the observed expression patterns seen in the mutants.

In this manuscript, we directly examine the function of the CAEs and show that mutations that only affect these regions have the expected reduction in SP60 promoter function. An internal deletion of the third (3'-most) CAE results in a gradient of expression opposite to that seen with 5' deletions removing the distal CAEs. In addition, using DNA mobility shifts in native gels, we identify a developmentally regulated, cAMP-inducible putative trans-acting factor that specifically interacts with the two 5'-most CAEs but not with CAEs carrying mutations that are inactive in promoting expression in vivo. We also identify a second factor that is present constitutively throughout development and has a high affinity for the third CAE (CAE-3).

Materials and methods

Site-directed mutagenesis of CAEs

The BamHI, SpeI fragment of 5’Δ20 contains the 663 bp upstream of the transcriptional start and the first 49 bp of 5' untranslatable region of the SP60 gene (Haberstroh and Firtel, 1990). This fragment, which contains the three CAEs, was subcloned into BamHI and SpeI-digested BSKII bluescript (Stratagene) vector to create BSKIIΔ20. BSKIIΔ20 was then digested with BamHI and XbaI to liberate the promoter-containing fragment which was subcloned into the BamHI and XbaI sites of M13mp19 creating M13mp19-5’Δ20. M13mp19-5’Δ20 single-stranded DNA was prepared as previously described (Maniatis et al. 1982) to serve as the antisense (relative to the coding) template for site-directed mutagenesis using the sense mutant CAE primers shown in Fig. 1. Site-directed mutagenesis was performed according to the manufacturer’s instructions using the Amersham Oligonucleotide-directed in vitro Mutagenesis Kit Version 2.

Clones potentially carrying mutant SP60 promoter fragments were screened for the desired mutation by dideoxy-chain termination sequencing using Sequenase (USB) with single-strand phage templates. Replicating form (RF) DNA was prepared from clones containing the appropriate mutation and was digested with BamHI and SpeI. The ~700 bp mutant promoter fragments were then subcloned as BamHI–SpeI fusions into BamHI–SpeI digested SP60–luciferase or SP60–lacZ vector backbones to create the appropriate expression vectors (see Haberstroh and Firtel, 1990 for maps).
Dictyostelium transformation, development, and cAMP assays

For β-gal or luciferase expression studies, vectors were transformed by electroporation into Dictyostelium vegetative cells and stable, G418^R transformants were selected (Howard et al. 1988; Dynes and Firtel, 1989). Cell lines were maintained under selection (10 µg/ml^-1 of G418). All methods have been described previously (Haberstroh and Firtel, 1990).

Vegetative cells, axenically grown to log-phase in HL-5 nutrient media with G418 at a concentration of 10 µg/ml^-1, were harvested by centrifugation, washed and resuspended in 12 mM sodium phosphate buffer (pH 6.1). The cells were then plated for multicellular development on Whatman 50 filters as has been described (Haberstroh and Firtel, 1990). Cells were harvested from the filters at various stages during development as determined by visual examination with the aid of a dissecting microscope. Loose aggregates generally occurred at ~9 h into development, while early culminants were observed at ~18 h. For luciferase assays, developing cells were isolated in 100 mM glycyl-glycine buffer with protease inhibitors and quick-frozen in a dry ice/isopropanol bath as previously described (Howard et al. 1988).

For nuclear extracts, untransformed KAx-3 cells were grown as described above, except in the absence of G418. These cells were plated for development on 12 mM phosphate-containing agar plates. Cells were followed visually throughout development under the dissecting microscope and harvested at various stages during development. Nuclear extracts were prepared by a modified protocol of Lee et al. (1988) (see Preparation of nuclear extracts).

Slow shake/cAMP induction experiments were done as described previously (Mehdý et al. 1983; Mehdý and Firtel, 1985; Haberstroh and Firtel, 1990). Briefly, vegetative cells were harvested by centrifugation and resuspended at 5×10^6 cells/ml in 12 mM phosphate buffer. Cells were then shaken at 150 revs min^-1 (10 ml/125 ml flask) for 6.5 h. The speed of the shaker was reduced to 120 revs min^-1 and cAMP was then added to one culture (+cAMP) to a final concentration of 300 µM. Cyclic AMP was again added to the +cAMP culture to a concentration of 100 µM every 2 h. Cells were harvested at 16 h and processed according to either the luciferase assay or the nuclear extract protocol.

Preparation of nuclear extracts

Nuclear extracts were prepared by a modification of a previously described protocol (Lee et al. 1988; Radler-Pohl et al. 1990). Approximately 1×10^6 cells were harvested as described above. These cells were resuspended in 300 µl of ice-cold 10 mM Tris 7.9, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM DTT, plus 1× protease inhibitors (1 mg/ml^-1 benzamidine, 2 µg/ml^-1 chymostatin, 1 mg/ml^-1 leupeptin, 0.1 mg/ml^-1 TPCK, 0.1 mg/ml^-1 TAME, 5 µg/ml^-1 antipain, 10 µg/ml^-1 aprotinin, 0.1 mg/ml^-1 TLCK, 0.1 mg/ml^-1 PMSF, 0.2 mg/ml^-1 phenanthrolines) and incubated on ice for 15 min. Cells were then lysed by two passages through a 3/an pore poly (dl-dC) (non-specific competitor or with [32P]dATP (ICN) and unlabeled dCTP, dTTP and dGTP for oligonucleotides used as 'cold' competitor or with [32P]dATP (ICN) and unlabeled dCTP, dTTP and dGTP for oligonucleotides used as 'probes').

Labelled double-stranded oligonucleotides (probe) were incubated in the presence of either 1 µg of poly (da-dt) or 0.75 µg poly (da-dt) or 0.25 µg poly (dl-dc) (non-specific competitors, Sigma) either in the presence or absence of 0.5 µl-2 µl (0.5 µg total protein) of the appropriate nuclear extract in 20 µl total sample volume. Binding incubation was carried out in 1× binding buffer (20 mM Tris 7.9, 100 mM KCl, 12.5 mM MgCl_2, 0.1 mM EDTA, 1 mM DTT) for 15 min on ice followed by 2 min at 25°C. Where competition is indicated, the specified molar excess (relative to probe) of unlabelled specific oligonucleotide was added to the sample at the onset of binding incubation. Samples were loaded onto a 4 % native polyacrylamide (30 acrylamide: 0.8 bis-acrylamide) in 0.5× Tris–borate buffer and electrophoresed for ~400 volt-hours at 4°C. The gels were then dried and exposed to X-ray film for autoradiography. Gel retardation binding studies and competitions were repeated at least three times.

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Results

Analysis of CAE function

The SP60 upstream regulatory region contains three related CA-rich sequences (CAEs) (see Fig. 1) that 5' deletion analysis has implicated in the cAMP induction and developmental regulation of this gene (Haberstroh and Firtel, 1990). To determine directly the regulatory importance of the CAEs, we specifically altered the CAEs using site-directed mutagenesis (see Materials and methods). The 663 bp upstream to the SP60 transcriptional start (construct 5' Δ20) was previously shown to be sufficient in conferring full cAMP responsiveness and proper developmental regulation to reporter genes. To examine the function of the CAEs within this promoter background, small deletions or individual nucleotide changes were introduced in the CAEs (Fig. 1), and these mutant promoters were then fused to either the firefly luciferase or the E. coli lacZ reporter genes and assayed for developmental induction by cAMP (Table 1) and spatial expression (Fig. 2).

Dictyostelium cells were transformed with either the full-length promoter construct 5' Δ20-luciferase (three intact CAEs) or a luciferase gene fusion carrying a mutation in CAE-1 (CAE1M-luciferase), CAE-2 (CAE2D-luciferase) or CAE-3 (CAE3A2-luciferase) (see Fig. 1 for sequence end-point and Fig. 5A for maps). To assay developmentally induced expression, stable transformants were plated for multicellular development as previously described (see Materials and methods; Haberstroh and Firtel, 1990). Luciferase activity was measured in extracts from early culminants (18 h of development), the developmental stage of maximal SP60 mRNA expression, and the data are shown in Table 1. The level of 5' Δ20-luciferase mRNA has been shown to parallel that of luciferase activity, consistent with luciferase activity being directly proportional to the expression of the reporter gene (Haberstroh and Firtel, 1990). All fusions show extremely low levels of expression in vegetative cells (data not shown), consistent with previous findings (Haberstroh and Firtel, 1990). Expression of 5' Δ20-luciferase at 18 h is used as an internal standard in each experiment and given a relative value of 100%.

The importance of the CAE-1 and CAE-2 elements

Table 1. Relative luciferase expression levels

<table>
<thead>
<tr>
<th>Construct</th>
<th>Mutation</th>
<th>CAEs affected</th>
<th>% of 5' Δ20 at 18 h</th>
<th>% of 5' Δ20 in + cAMP slow shake</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' Δ20</td>
<td>Parental</td>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5' Δ23</td>
<td>5' deletion</td>
<td>CAE-1</td>
<td>8.3±1.3</td>
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</tr>
<tr>
<td>CAE-1M</td>
<td>Point</td>
<td>CAE-1</td>
<td>4.4±1.2</td>
<td>nd</td>
</tr>
<tr>
<td>CAE-2D</td>
<td>Point</td>
<td>CAE-2</td>
<td>9.8±2.2</td>
<td>1.8</td>
</tr>
<tr>
<td>CAE-3A2</td>
<td>Internal</td>
<td>CAE-3</td>
<td>6.3±1.8</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Comparison of 18 h developmentally induced or cAMP-induced expression in slow-shake culture luciferase levels from Dictyostelium transformed with 5' deletions or site directed CAE disruptions/luciferase gene fusions. Data were calculated and expressed as a percentage of 5' Δ20/luciferase transformants developed and assayed as internal controls with each experiment. Value of 5' Δ20 was taken as 100% activity as described in the text and previously (Haberstroh and Firtel, 1990). Error is given as ± S.E.M. Each developmental experiment was run at least 3 times. Percentages were then averaged over many trials resulting in the values shown above. Mean expression level of 5' Δ20 was 2.9×10⁵ LU µg⁻¹ protein±1.0×10⁵, nd, not determined.
Fig. 2. Spatial expression of CAE mutants. Slugs were fixed and stained for β-galactosidase activity as described before (Haberstroh and Firtel, 1990; Materials and methods). Four site-directed mutant CAE1M-, CAE2D-, CAE2Δ1- and CAE3Δ2-lacZ transformed slug staining patterns are shown along with the parental 5’Δ20-lacZ. [Note that in Dicyostelium, spatial patterning is independent of the size of the aggregate. Thus, although this culminant has substantially fewer cells than the slug shown, the spatial pattern is the same as seen for SP60-lacZ culminants (Haberstroh and Firtel, 1990)]. Arrows indicate the anterior of the slug (prestalk region). Note the solid dark 5’Δ20-lacZ staining pattern compared to that of CAE1M-lacZ, which exhibits a gradient pattern which decreases anterior to posterior within the prespore zone. The CAE1M slug in panel a of CAE1M was stained for approximately the same length of time as the slugs in the other panels. The slug in CAE1M panel B was stained for ~10 times longer. CAE2D/ and CAE2Δ1-lacZ transformants show no such gradient and stain lightly and fairly homogeneously across the entire prespore region (see Results). CAE3Δ2-lacZ transformants have a unique staining pattern which is characterized by a prespore gradient which is inverse to that of CAE1M-lacZ transformants. When 5’Δ42-lacZ or untransformed slugs are fixed and stained, no β-galactosidase activity is detected (data not shown; Haberstroh and Firtel, 1990; Eash and Firtel, 1991). The staining patterns seen were reproducible in stable transformants from several independent transformations. The observed results were the same with whole transformed populations and clonal isolates from independent transformations. The results shown are representative photographs.
mutated to A residues in CAE-1, whereas the CAE2D-luciferase carries a double point mutation of CAE-2 in which two of the C residues were mutated to G residues. In each case, the remainder of the upstream region is identical to that of 5'Δ20-luciferase (see Fig. 2). Each of these site-directed mutations results in an ~10- to 20-fold reduction in the 18 h expression level relative to that of 5'Δ20. A small internal deletion within CAE-3 (CAE3Δ2-luciferase) results in an ~20-fold reduction, strongly suggesting that the CAEs themselves are cis-acting regulatory regions essential for full developmental induction of the SP60 gene. Data are summarized in Table 1.

Analysis of spatial patterning of CAE-1, CAE-2 and CAE-3 mutations
To examine the effect of CAE mutations on the spatial pattern of expression, site-directed mutant promoters CAEI M, CAE2A1 (which carries an internal deletion of CAE-2, see Figs 1 and 5A), CAE2D and CAE3Δ2 were fused to the lacZ reporter gene and transformed into Dictyostelium. Stable, G418-resistant transformants were plated for development and at the slug stage fixed and stained in situ for β-galactosidase activity (see Materials and methods). Results are pictured in Fig. 2 and summarized in Fig. 5A. The point mutation of CAE-1 results in a reduced level of staining expressed in a prominent anterior–posterior gradient within the prespore zone that is not observably different from that seen with 5'Δ23, a 5' deletion that removes ~100 bp including CAE-1 (Fig. 5A; Haberstroh and Firtel, 1990). A deletion or point mutation of CAE-2 (CAE2Δ1 or CAE2D) also results in a reduced intensity of staining compared to that of 5'Δ20-lacZ transformants. For both of these constructs, however, the staining pattern is predominantly uniform within and restricted to the prespore zone (no staining is seen in the anterior prestalk domain) (Fig. 2). In both cases, the very posterior of the slug does not stain. Deletion of CAE-3 (CAE3Δ2) also results in a reduced level of staining, consistent with the low level of expression observed with the CAE-3Δ2-luciferase construct. Interestingly, the CAE3Δ2-lacZ expression was also graded but with a substantially higher level of expression in the posterior of the slug relative to the level in the anterior of the prespore zone. The expression pattern of CAE3Δ2-lacZ thus appears to be a 'reverse' gradient when compared to the CAE1M-lacZ staining pattern, which decreases posteriorly within the prespore zone (Fig. 2). Moreover, the sharp boundary seen between the prestalk and prespore zones for 5'Δ20 or 5' deletions of CAE-1 and CAE-2-containing regions was absent. However, a few β-gal-positive cells are sometimes seen for full promoter constructs, although at a lower frequency (Haberstroh and Firtel, 1990). At present the significance of these anterior staining cells is not known. In transformants carrying constructs in which all three CAEs are deleted (5'Δ42-lacZ) or in control cells lacking a lacZ expression vector (strain KAx-3), no β-galactosidase staining is observed (data not shown; Haberstroh and Firtel, 1990).

Regulation by exogenous cAMP
Previous results have shown that both the endogenous SP60 gene and the SP60 promoter–lacZ fusions are induced in slow-shaking culture in response to exogenous cAMP (see Material and methods for details). Under these conditions, the endogenous SP60 gene and other prespore-specific genes are induced to a high level. Some prespore gene expression is observed in the absence of added cAMP due to endogenous cAMP produced in the small agglomerates of cells formed under slow shaking conditions (Mehdy and Firtel, 1985; Gomer et al. 1986; Haberstroh and Firtel, 1990). To examine the effects of the CAE-2 and CAE-3 mutations on the ability of the cells to be induced by cAMP, slow shake assays were performed. The results (see Table 1) show that the level of cAMP-induced expression in the slow-shaking assay was similar to that at 18 h of development for each construct and that promoters carrying CAE-2 or CAE-3 mutations show a 10- to 20-fold lower level of expression relative to the parental construct. The level of expression in the ‘plus-cAMP’ cultures was 50- to 100-fold higher than in the ‘no-cAMP’ cultures (data not shown). These data indicate that the CAEs are important in the ability of the SP60 promoter to respond to cAMP and may be directly involved in mediating the cAMP induction pathway.

Analysis of CAE binding proteins
Our cis-analysis of the CAEs indicates that all three are essential for maximal developmental and cAMP-induced as well as proper spatial expression. Considering the importance of the CAEs in controlling SP60 expression in vivo, we have used gel mobility shifts to assay for CAE binding proteins extracted from cell nuclei at different times throughout development. Fig. 3 shows the complexes formed in the presence of non-specific competitor DNA using oligonucleotides containing CAE-1, CAE-2, or CAE-3 as 32P-labeled probes (see Fig. 1 for sequences). Incubation of extracts with the CAE-1 oligonucleotide yields two predominant complexes when electrophoresed on a native polyacrylamide gel (see Materials and methods). A more rapidly migrating complex (band A) is observed at moderate intensities using extracts from vegetative cells and cells from 3 h in development. The intensity of this band then increases 2- to 3-fold with extracts from 6 h cells (early aggregation), a time when a few SP60-expressing cells are seen in the forming aggregate (Haberstroh and Firtel, 1990) and then remains constant through 21 h (early culmination). A more slowly-migrating complex (band B) is first detectable with the 6 h extract. The binding activity increases in the 9 h extract and is present in extracts through 21 h of development. Reproducibly, we observe the highest band 'B' activity with extracts from 9 h developing cells (mound stage). The specificity of the bands is shown by competition experiments using the 9
Fig. 3. Developmental time course of CAE-binding proteins. (A) Nuclear extracts from a developmental time course (vegetative through 21 hr) were incubated with labeled CAE-1 oligonucleotide (1wt) in the presence of non-specific competitor and electrophoresed in a mobility shift assay (see Materials and methods). Complex(es) are labeled A and B. For comparison, CAE-1 probe (1wt) was incubated with nuclear extract from cAMP-induced single-cell shaking culture (see Materials and methods) to show that complexes formed have a similar, if not identical, relative mobility to those formed with extracts from developing cells. Specific competition by cold oligonucleotides containing the indicated CAE (wild-type oligonucleotides: CAE1wt, CAE2wt, CAE3wt or mutant oligonucleotides: CAE2D, CAE1M) for specific complexes present in the 9 hr or 18 hr extract is shown (see Fig. 1 for sequences of the probes/competitors). All specific competitors were in 50-fold molar excess relative to probe. An oligonucleotide containing CAE-3 (3wt) was also used as labeled probe for nuclear factors in cAMP induced single-cell shaking culture and is included in this assay for comparison (last lane; complex labeled A'). (B) Same as Fig. 3A except oligonucleotide containing CAE-2 (2wt) was used as probe. Again, major specific complex(es) are labeled A and B. Labeled oligonucleotides containing CAE-3 (3wt) or CAE-1 (1wt) were incubated with 18 hr extract and assayed on the same gel for comparison of relative mobilities and relative affinities. As in Fig. 3A, specific competition is shown for 9 hr extract. CAE2wt and CAE1wt probes were also incubated with nuclear extract from single-cell shaking culture (slow shake) which had been starved either with or without exogeneous cAMP (+cAMP/-cAMP; see Materials and methods). The complexes are labelled A and B. (C) Oligonucleotide containing CAE-3 (3wt) was used as probe for nuclear factors present in developmental extracts. Complexes are labeled A' and B'. Oligonucleotide 3wt probes incubated with extracts from shaking culture (+cAMP) are also shown, along with specific competition for the complex(es) formed (cold 3wt 200-fold molar excess relative to probe).
When a CAE-2-containing oligonucleotide is used as a probe, two complexes with the same mobility and similar initial developmental pattern of expression as the complexes formed with the CAE-1 probe are observed. As can be seen in Fig. 3B, the relative band intensity compared to using labelled CAE-1 on the same gel shows that CAE-2 forms complexes less efficiently than CAE-1. Considering that the mobilities and developmental induction of the activity are the same as that with the CAE-1 probe, we believe that the proteins forming these complexes may be the same or closely related in the extracts from 9h cells (see Discussion). The lower intensities of the complexes are consistent with the competition data showing that CAE-1 oligonucleotides compete better than CAE-2 oligonucleotides for the CAE-1 complexes. This is confirmed by the observation (see Fig. 3B) that CAE-1 also competes better than CAE-2 for the CAE-2 complexes. We also observe that CAE-3 is a poorer competitor than CAE-2 for complex labelled B (Fig. 3B; other data not shown), while the mutant CAE-2 oligonucleotide (CAE-2D) does not compete for the band B activity but does compete for the band A. CAE-1M competes to a moderate degree for the band A activity and competes for the upper band activity to a similar extent as CAE-3. Whether the CAE-1 or CAE-2 oligonucleotide is used as probe, the relative intensities of the two complexes (B to A) are similar for both at 9h. A comparison of the developmental pattern of the more slowly migrating complexes with CAE-1 versus CAE-2 probes (labelled 'B' in both cases) shows that with CAE-2, the band is substantially stronger within the 9h extract lane and decreases in intensity at later stages, whereas the intensity of band A remains approximately constant. The 9h extract exhibits the most intense band B with the CAE-1 probe, but the relative intensities of the band formed with the CAE-2 probe with extracts from later stages are significantly weaker than those with the CAE-1 probe [e.g. compare the 21h extract which shows a relatively strong complex (band B) with the CAE-1 probe but not with the CAE-2 probe]. Competition experiments show that CAE-2 oligonucleotide is an efficient competitor for the slowly migrating complex(es) formed with the CAE-2 probe, while mutant CAE-2D (ineffectual as a cis-element in vivo) does not compete. CAE-1 is an even stronger competitor than CAE-2, whereas the mutant CAE-1 competes very poorly. CAE-3 competes at approximately the same efficiency as CAE-2. We have also used CAE-1M and CAE-2D as probes and no detectable complexes running at the position of the slower-migrating band (band B) were formed, but weak complexes were seen to migrate near the position of band A (data not shown).

When the CAE-3 oligonucleotide is used as a probe, we see a constitutive binding activity, labelled A', which produces a much broader and more intense band than the rapidly migrating band A seen with the CAE-1 and CAE-2 probes (see Fig. 3C and comparative data in Fig. 3B). Band A' appears to be composed of several complexes, one of which may or may not be the same as that formed with the CAE-1 and CAE-2 probes. CAE-1 and CAE-2 show little or no competition for the band A' complexes (data not shown). Since the CAE-3 does not compete well for the CAE-1 band 'A' complex and CAE-1 does not compete well for the CAE-3 band A' complex, at least some of the complexes running in this area of the gel formed by CAE-1/CAE-2 probes and CAE-3 are probably formed by different activities. CAE-3 and CAE-1M show the best competition for these complexes, but the level of competition is still relatively low (data not shown). It is possible that one or more of the complexes constituting the CAE-3 band A' compete differentially with various CAE-containing oligonucleotides. A small amount of a complex, labelled B', is observed using CAE-3 as a probe that has similar mobility and developmental kinetics to complex B seen with CAE-1 and CAE-2 (Fig. 3C). This band is competed with CAE-1 and CAE-2 oligonucleotides (data not shown), suggesting that the band B and B' complexes may be due to the same binding activity. We only see the B' complex with some extracts having a high level of B complex activity as measured using the CAE-1 probe, presumably because of the low affinity of the factor for the CAE-3 sequence.

To examine the effect of treatment of cells with cAMP on the formation of DNA-binding complexes in vitro, we made nuclear extracts from cells from slow-shaking cultures starved in the presence or absence of exogenous cAMP (see Materials and methods). As can be observed in Figs 3B and 4A, the complexes formed with CAE-1 and CAE-2 probes with extracts made from cells treated with exogenous cAMP had mobilities and competition properties similar to the complexes formed with 18h extracts, except that the faster-migrating complex(es) in the case of CAE-2 form a broader band A that is stronger than band B. The extract made from cells not given exogenous cAMP showed the fastermigrating complex and only a very low level of complex B. Several additional complexes are seen in both the plus (+) and minus (−) cAMP extracts. These are not seen with extracts from the developmental time points and are not competed by unlabelled probe and therefore appear to be non-specific (see Fig. 4A and below).

When incubated with extracts from slow-shaking cells, the CAE-3 probe shows a pattern of complexes similar to that seen with either vegetative cell or 18h cell extracts (Fig. 4B). In this experiment, in which the autoradiogram is exposed for a longer time than that shown in Fig. 3C, a more complex pattern of bands is observed; however, none of the band A' complexes is cAMP regulated (data not shown). The presence of the slower complex, band B', is variable with plus-cAMP extracts and is not seen in the experiments shown in Fig. 3C but is observed in others (Fig. 4B). No complexes running at the position of the CAE-1/CAE-2 B complexes are detected.

Competition experiments performed with CAE-1
and CAE-2 probes and the plus-cAMP cell extracts using the CAE-1, CAE-2, CAE-3, CAE-1M, and CAE-2D oligonucleotides give similar results to those seen with the 9 or 18h nuclear extracts (see Fig. 4A). Competition using the CAE-3 probe and CAE-3 oligonucleotide is shown in Fig. 3C (other competition data with CAE-3 probe not shown). As can be seen in Fig. 4A, the slower, cAMP-inducible complex, band B, is competed well with the CAE-1 oligonucleotide and less well with the CAE-2 and CAE-3 oligonucleotides. When CAE-1M or CAE-2D probes are incubated with nuclear extract from cAMP-induced cells, only very weak, faster-migrating complexes are observed (see Fig. 4B).

Discussion

In this manuscript, we use internal deletions and point mutations to examine the functional roles of the CAEs in controlling the expression of the cAMP-induced, developmentally regulated prespore-specific gene \(SP60\). Point mutations in CAE-1 or CAE-2 result in a significant drop in the level of luciferase expression, consistent with both sequences being necessary for a high level of developmental and cAMP-mediated expression. The quantitative effects are similar to those seen with relatively large (80–100 bp) internal deletions that remove either CAE and surrounding sequences (5'A23 and construct 2lirs; Haberstroh and Firtel, 1990) and suggests that it is the CAEs that are essential in regulating \(SP60\) expression. \(\beta\)-gal expression studies show that the expression pattern is fairly uniform across the prespore region with mutations in the \(SP60\) promoter that alter CAE-2. This is in contrast to the gradient seen when CAE-1 is mutated (CAE1M–\(lacZ\)). The lack of a significant observed change (see Results for description) in the spatial patterning with mutations affecting only CAE-2 could be due to differences in the role of CAE-1 and CAE-2 (see discussion below). Unexpectedly, an internal deletion of CAE-3, which also results in a reduction in the level of luciferase expression, yields a graded pattern of \(\beta\)-gal activity that is strongest in the posterior of the slug. Since deletion of CAE-1 or CAE-3 results in opposite gradients, it is possible that the two elements are responsive to different trans-acting factors and/or signalling pathways that are not uniformly active in the prespore domain of the slug. This is consistent with differences in the types of complexes formed in vitro with CAE-1 and CAE-3 probes. Since the two types of graded patterns of expression appear to be ‘opposing,’ it is possible that proper spatial patterning of \(SP60\) expression results from an interaction of these two components to yield
uniform expression over the entire prespore domain. It is of interest that deletions of domains containing CAE-1 still maintain a very sharp prespore–prestalk boundary, which is less evident when an internal deletion of CAE-3 is examined. The two cis-acting domains (CAE-1 and CAE-3) may therefore be responsible for defining expression of SP60 in distinct regions of the slug. We cannot exclude the possibility that other sequences within the upstream region may also be involved in controlling this differential graded pattern of expression within the prespore domain seen with the 5' deletion mutants. Our studies indicate that small changes within these sequences result in major changes in the quantitative and qualitative expression patterns.

Our analysis of potential trans-acting factors using gel mobility shifts has identified at least two activities that interact with CAE-1- and CAE-2-containing oligonucleotides. A similar, faster-migrating complex formed with CAE-1- and CAE-2-containing probes is seen at low/moderate levels with extracts from growth and early development and then, in the case of CAE-1 band A, increases at about the time of aggregation when SP60 expression is first detected within a subset of cells. A second slower-migrating complex (band B) first appears at very low levels with extracts made from early aggregating cells and is present at high levels starting at the late aggregation/early mound stage, consistent with the rapid increase in SP60 expression at this time in development (Mehdy et al. 1983; Saxe and Firtel, 1986; Fosnaugh and Loomis, 1989; Haberstroh and Firtel, 1990). The mobilities, developmental pattern and the relative level of competition with different oligonucleotides strongly suggest that the same or closely related activities are interacting with both CAE-1 and CAE-2. Some differences are seen between the B complexes formed with these two CAEs using 9 and 15 or 18 h extracts which could result from changes in the affinity of the factor for CAE-2, possibly the result of a modification of the factor. Sequential 5' deletions of both of these elements result in a very strong anterior–posterior gradient of expression which is not seen when CAE-2 internal mutations are examined, even though CAE-2 is required for full promoter activity. Interestingly, CAE-2 appears to have a lower affinity for the protein(s) present in the B complexes formed in vitro, suggesting an SP60 promoter lacking only CAE-2 may not be as sensitive to the physiological factors responsible for this gradient in vivo as a deletion in CAE-1. This apparent reduced interaction of CAE-2 with the factor forming complex band B, with no apparent change in the affinity of the factor forming complex band A as measured in gel shift assays, may indicate discrete differences in the roles for the CAE-1 and CAE-2 in regulating the anterior–posterior pattern of SP60 expression.

Our results are consistent with the model in which the protein(s) that form the more slowly migrating complex (band B) are directly involved in regulating both developmental and cAMP-inducible expression. The appearance and increase in binding activity correlates with the developmental expression of SP60. In shaking culture, little if any band B complex is seen using extracts from cells shaken in the absence of cAMP, while the activity is induced in response to exogenous cAMP to a level above that seen in developing aggregates. A biological role for this binding activity is also supported by a direct correlation of the ability of CAE-1 and CAE-2 probes to form complexes and the requirement of these sequences for full expression in vivo. Mutations that alter CAE-1 and CAE-2 at only three and two base pairs, respectively, within the entire 5'A20 upstream region result in a 10- to 20-fold drop in expression and a loss in binding activity in vitro. Since activity is not seen in cells shaken in the absence of cAMP, we expect that the factor(s) are responsive to cAMP signalling. It is possible that the activity(ies) involved in this slower-migrating complex (band B) are themselves induced by cAMP and/or in response to other developmental signals or may be a constitutively expressed protein whose binding activity requires a post-translational modification, possibly in response to cAMP receptor-mediated signalling pathways. While the factor(s) responsible for the faster-migrating complexes (band A) formed with CAE-1/CAE-2 probes increase somewhat during development, the binding activity is not detectably enhanced upon cAMP stimulation of the cells. Moreover, it is competed for by the CAE-1M oligonucleotide. We cannot correlate factors associated with CAE-1/CAE-2 complex band A and cAMP-induced SP60 expression.

We also observe that, while the sequences of CAE-1/CAE-2 and CAE-3 are quite similar, there is a spacing difference of 1 base pair separating the two repeated CACACA motifs of CAE-1 and CAE-2 when compared to those of CAE-3. However, when one of the A residues within this spacer region was removed, this deleted CAE-3 oligonucleotide still had the same binding properties as the unmodified CAE-3 but with a 5- to 10-fold lower affinity (unpublished observations), suggesting that other differences within the CA-rich core or flanking sequences are essential for binding specificity. In vivo experiments show that the CAE-3 is important for both the level of expression and the pattern seen. We would therefore expect that one or more of the faster-migrating complexes contain a factor that is essential for regulating SP60 spatial patterning. Although we observe no change in the complexes formed between the plus- and minus-cAMP extracts, we previously showed that 5'A21, which lacks both CAE-1 and CAE-2 and contains CAE-3, is responsive to cAMP. This suggests that either CAE-3 is responsive to cAMP signalling or a more proximal sequence also responds to cAMP.

The combined results imply that full expression over the entire slug prespore region requires the interaction of factors with the CAE-1/CAE-2 and CAE-3 sequences. We also note that the 5' deletion removing both CAE-1 and CAE-2 has a substantially greater effect on expression than the sum of the effects of mutations affecting either CAE-1 or CAE-2. This is consistent with a cooperative interaction of the factors that may be interacting with these sequences, with
Fig. 5. (A) Schematic representation cis-analysis. Constructs 5'Δ20, 5'Δ23, and 5'Δ21 are 5' deletion mutants used as controls in this paper and described in detail previously (Haberstroh and Firtel, 1990) and are included as a comparison for the data described in this manuscript. Constructs CAE1M, CAE2D, CAE2Δ1, and CAE3Δ2 are the site-directed mutants described in this manuscript.

Lightly shaded boxes within the distal part of the promoters represent CAEs, while more proximal darkly shaded boxes represent as yet unidentified cis-elements. (B) Cartoon model of effects of CAEs in regulating spatial expression of SP60. In the simplest case, factor B interacts predominantly with CAE-1 and CAE-2, while CAE-3 interacts with A'. Results suggest that CAE-3 and its factor A' are important for the anterior expression and factor B is important for the expression seen in the posterior. Since factor B activity is responsive to cAMP, the proposed gradient could be the result of a gradient of either B, cAMP, or some part of the signal transduction pathway (e.g. receptor, G protein, effector). In the figure, the gradient manifests itself in the relative level at which the factor binds to its site in that region of the slug. Data suggest that CAE-1 and CAE-2 respond to a positive factor (possibly cAMP-responsive based on DNA-binding experiments) present in a gradient decreasing anteriorly within the prespore zone. CAE-3, on the other hand, would mediate the positive response to a second factor present in an opposing gradient decreasing posteriorly within the prespore zone. We suggest that CAE-2, which has a lower affinity for the cAMP-responsive element, is not essential for the spatial patterning and that CAE-1 and CAE-3 can respond, although less efficiently, to both gradients to obtain a fairly uniform pattern of expression. In this simplest model, binding activity A is not used, but it may function as a modulator of B binding, giving the cell more control over the level of expression by cAMP signalling. With this model, the site would not be unoccupied and it would require a higher concentration of active B to displace A. Under these conditions, the gene would not be expressed until a threshold response was received. The number of +s represents the relative occupancy of the site. Note that the CAEs function synergistically and thus the response is not linear. ‘Prox.’ represents the proximal regulatory element (Haberstroh and Firtel, 1990). An alternate model would consist of a posterior-to-anterior gradient having a negative effect on prespore differentiation and prespore-specific gene expression, possibly DIF (Brookman et al. 1987), and a positive anterior-to-posterior gradient inducing the prespore gene. CAE-3 may mediate both the negative response to DIF and the positive response to cAMP via two different transcription factors. One could imagine a situation like that described for the competitive interaction of glucocorticoid receptor (GR) and c-Jun (Yang-Yen et al. 1990; Schule et al. 1990).
described in Figs. 5A and 5B, respectively. For the simplest model, two gradients, one anterior-to-posterior and the other posterior-to-anterior, are shown. The model is proposed in terms of responsiveness of the CAE-1/CAE-2 elements to factor B and CAE-3 to factor A'. This could be due to a spatial gradient in the factor itself, a morphogen such as cAMP, or intracellular signaling responses. Other more complex models employing inhibitory (e.g. DIF) as well as activating (e.g. cAMP) gradients are possible (see legend to Fig. 5B). Based on studies using phosphodiesterase to reduce cAMP levels and adenosine, an antagonist to prespore gene expression, Wang et al. (1988) proposed an anterior-to-posterior increasing gradient of cAMP. This is consistent with our data that suggest the B factor complex is important to posterior expression. Our model, however, does not describe how the gradient is set up or maintained. We believe that the initial patterning is established by the sorting of cells developing down either the prespore or prestalk pathway (Esch and Firtel, 1991). It is possible that an anterior-to-posterior gradient of an inhibitor of spore differentiation or the presence of an activator in the posterior could be used to maintain this pattern. Since the cells are totipotent until fairly late in development, just prior to culmination, some mechanism is needed to maintain the established border between the prestalk and prespore regions.

We have previously identified a putative cAMP-induced, developmentally regulated trans-acting factor (GBF) that interacts with other GT/CA-rich sequences (designated G boxes) that were first identified in a cAMP-inducible gene (e.g. pst-cath/CP2) preferentially expressed in prestalk cells (Datta and Firtel, 1987, 1988; Pears and Williams, 1987, 1988; Hjorth et al. 1989, 1990). In addition, this factor binds a CA-rich (coding strand) sequence, which is very similar to the CAE of SP60, from another cAMP-inducible gene, DG17, whose spatial pattern of expression is not known. Other biochemical studies (Schnitzler, Haberstroh, and Firtel, unpublished observations) indicate that GBF and the developmentally regulated binding activity identified here share similar binding characteristics to several oligonucleotide probes but have some differences in their binding-condition requirements. These differences may indicate differences in the binding factors themselves, other components required for the formation of the specific complexes, or differences in the biochemical conditions used. Both GBF and the factor that forms CAE-1 complex B are presently being purified, and analysis of the properties of the purified proteins should allow us to examine any biochemical differences in the factors in detail (Schnitzler and Firtel, unpublished observations). Since extracts are made from whole organisms containing a mixture of cell types, it is not known whether GBF or the factors described in this manuscript are found in all cells or are cell-type-specific. When the genes encoding these proteins are cloned, it will be possible to examine the spatial pattern of expression of these proteins, in order to understand better their function in cell-type-specific gene expression. At the present time, it is possible that specific components of these activities required for prespore versus prestalk may share non-overlapping spatial patterns of expression that may correlate with cAMP-induced prestalk or prespore gene expression. It is also possible that all factors may be found in the same cells and give the cell ability to respond under different developmental or environmental conditions by the formation of specific complexes for different CA or G-boxes.

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References


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