A spatial gradient of expression of a cAMP-regulated prespore cell-type-specific gene in *Dictyostelium*

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Previously, we identified a class of genes in *Dictyostelium* that are prespore cell-type specific in their expression in the multicellular aggregate and are inducible by cAMP acting through cell-surface cAMP receptors. In this paper, we report the cloning and analysis of the regulatory regions controlling the expression of one such gene that encodes a spore coat protein, SP60. By use of a fusion of the firefly luciferase gene and *Escherichia coli* lacZ (expresses β-galactosidase [β-gal]), we have identified cis-acting regions required for proper spatial and temporal expression in multicellular aggregates and for cAMP induction in shaking cell culture. Deletion analysis suggests that a CA-rich element (CAE) and surrounding sequences present three times within the 5'-flanking sequence are required for proper regulation. SP60–lacZ fusions that include all three of these regions express lacZ only in the posterior ~85% of migrating slugs (prespore zone). Studies show that SP60 is expressed during mid to late aggregation, and SP60–lacZ-positive cells are spatially localized as a doughnut-shaped ring within the forming aggregate. Cells within the skirt that surrounds the aggregate and that are still migrating into the aggregate do not stain. Sequential 5' deletions of CAEs and surrounding regions affect the expression level of SP60–luciferase in response to developmental signals and cAMP, as well as the spatial pattern of SP60–lacZ. Deletion of the first (most 5') of these regions restricts the spatial expression of SP60–lacZ fusions to the anterior of the prespore zone. When both the first and second regions are removed, the expression level drops, and the staining is restricted to the prespore/prestalk boundary. Furthermore, the staining pattern that is seen with these two deletions is present as a gradient from anterior to posterior within the prespore zone. Deletion of all three regions results in a loss of both cAMP and developmentally induced expression. These results suggest the presence of a gradient within the prespore zone that differentially affects the activity of promoters containing different numbers of response elements.

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*Dictyostelium discoideum* grows as a single-cell vegetative amoeba. Cells remain in this vegetative state until food is depleted. On starvation, cells initiate a multicellular developmental program in which ~10^6 cells form a multicellular aggregate. During this time, the cells do not feed or divide; thus, cell division and multicellular differentiation are separated in this organism. The aggregation process is mediated chemotactically by extracellular cAMP that interacts with cell-surface receptors to activate two intracellular signal transduction pathways: one involving a cAMP relay pathway and one controlling cell motility and induction of genes whose products are required for aggregation [Janssens and van Haastert 1987; Firtel et al. 1989]. A loose aggregate is formed by 10 hr. By 15–16 hr, a migrating slug or pseudoplasmodium develops that culminates to form a mature fruiting body containing ~85% spores and ~15% stalk cells. The slug contains predominantly two cell types that are spatially localized: The anterior 15% contains prestalk cells, whereas the posterior 85% contains predominantly prespore cells [Loomis 1982]. The mechanisms regulating whether a vegetative cell eventually differentiates into a prespore/stalk cell or a prespore/spore cell are partially understood. Cells starved early in the cell cycle (S and early G2; there is no detectable G1) preferentially differentiate into prestalk cells, whereas those starved in mid and late G2 differentiate into prespore cells [Weijer et al. 1984; Gomer and Firtel 1987b]. After the pseudoplasmodium has formed, cAMP, adenosine, NH4^+, and the morphogen DIF all play roles in regulating gene expression, patterning, and subsequent differentiation [see Mehdy et al. 1983; Chisholm et al. 1984; Williams et al. 1984; Mehdy and Firtel 1985; Schaap and van Driel 1985; Gomer et al. 1986b; Oyama and Blumberg 1986; Schaap and Wang 1986; Williams et al. 1987, 1988; Wang et al. 1988].

We and others have isolated cDNA and genomic clones for a number of genes preferentially expressed in...
either prespore or pseudoplasmodium stages, whereas prespore RNA is first detected as the aggregate is forming and is maximal at the tight aggregate/pseudoplasmodium stages, whereas prespore RNA is first detected at the loose/tight aggregate stage and is maximal in the pseudoplasmodium/early culminant stages (Mehdy et al. 1983; Saxe and Firtel 1986). The gene product for one prespore gene, that for the prespore coat protein SP70 [see below], has been specifically localized in the posterior prespore cells in migrating slugs and in maturing spore cells (Gomer et al. 1986a). Both prespore and pseudoplasmodium stages can be induced in single-cell culture by cAMP (Barklis and Lodish 1983; Mehdy et al. 1983; Mehdy and Firtel 1985; Saxe and Firtel 1986). Analyses using pharmacological agents, cAMP analogs, and mutants that affect signal transduction have indicated that induction of both prestalk and prespore gene expression by cAMP is mediated through cell-surface receptors that are coupled to G proteins (Schaap and van Driel 1985; Gomer et al. 1986b, Haribabu and Dottin 1986; Oyama and Blumberg 1986; Kimmel 1987; Firtel et al. 1989). Recent studies suggest that diacylglycerol [DAG] and 1,4,5-inositol triphosphate (1,4,5-IP₃) are the second messengers involved in this induction (Ginsburg and Kimmel 1990). Although some of the extracellular (cAMP) and intracellular [DAG and 1,4,5-IP₃] messengers have similar effects in the regulation of the expression of these genes, other studies indicate that the intracellular pathways regulating the expression of these two classes of genes are different (Gomer et al. 1985; Mehdy and Firtel 1985; Blumberg et al. 1988; Speck et al. 1988).

Molecular analysis has identified the cis-acting elements controlling cAMP induction and developmental expression, as well as a developmentally regulated trans-acting factor controlling their expression of the cAMP-inducible early prestalk genes [Datta and Firtel 1987, 1988; Pears and Williams 1987, 1988; Hjorth et al. 1989]. However, no information is available on the molecular requirements for prespore gene expression, nor is the spatial expression of these genes within the multicellular aggregate understood. To understand better the regulatory pathways controlling prespore cell-type-specific gene expression, we have now examined the cis-acting regulatory region of the spore coat SP60, which is one of a set of three proteins (SP60, SP70, and SP96) that make up the majority of the proteinaceous component of the spore coat (Devine et al. 1983; Erdos and West 1989). SP60 and SP70 gene expression is coordinately regulated at the level of transcription during multicellular development in wild-type cells and in a number of mutant strains affected in their temporal pattern of differentiation, and in response to cAMP and cAMP analogs in shaking culture (Mehdy et al. 1983; Gomer et al. 1985, 1986; Mehdy and Firtel 1985; Saxe and Firtel 1986; Fosnaugh and Loomis 1989). Within the pseudoplasmodium and early culminant, these proteins are localized in vacuoles in prespore cells as a layer covering the internal side of the vacuole membrane. During the maturation of spores during culmination, these vacuoles fuse with the plasma membrane (Devine et al. 1983; Erdos and West 1989). As a result, the inside of the prespore vacuole becomes the spore coat extracellular matrix. By use of lacZ and firefly luciferase gene fusions, we describe the spatial patterning of the prespore zone and show that 650 bp of the 5' flanking region contains all of the necessary signals for proper cAMP regulation and spatially and temporally correct cell-type-specific expression. This region contains a short CA-rich element (CAE) that is repeated three times. Sequential 5' deletions of each repeat and surrounding sequences, as well as some internal deletions that remove these regions, result in substantial reductions in cell-type-specific expression. In addition, sequential deletion of these regions causes an anterior-to-posterior decreasing gradient of expression within the prespore zone, suggesting the presence of some type of gradient within this region of the aggregate. Possible models for this differential expression of SP60 are presented.

Results

Cloning and structure of the SP60 prespore gene

The cDNAs 3-E2 and 9-C3 were originally identified as being complementary to 1.8-kb mRNAs preferentially expressed in prespore cells, and they were shown to be inducible by cAMP in shaking culture (Mehdy et al. 1983; S. Datta and R.A. Firtel, unpubl.). Although the two cDNAs do not cross-hybridize, they both hybridize to the same genomic fragments [see below]. Because both cDNAs are substantially shorter than the mRNA, they were presumed to be nonoverlapping, partial cDNA clones. The two cDNAs were used to isolate the prespore gene from two genomic libraries, and two overlapping genomic clones were obtained [see Materials and methods]. The cDNAs within the genomic clones were localized by restriction mapping and DNA blot hybridization. The polarity of transcription was determined through the use of SP6- and T₇-derived strand-specific RNA hybridization probes [see Materials and methods]. The possibility of other transcription units within the genomic fragments was ruled out by use of different regions of the genomic clones as hybridization probes against RNA isolated from various times in Dictyostelium development. A single transcription unit was identified that mapped to the region complementary to the two cDNAs [data not shown; see Fig. 3]. A map of the genomic clone indicating the location of the prespore gene and positions of the two cDNA clones is shown in Figure 1.

Approximately 2 kb of the genomic clone and a portion of the cDNA clone 3-E2 that maps toward the 5' end of the prespore transcription unit were sequenced [see Fig. 2]. A single open reading frame in the cDNA was identified that was colinear with the sequence of

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Figure 1. Genomic map of the SP60 gene. The structure and partial restriction maps of two overlapping genomic clones (see text) containing the SP60 gene are presented. The location of the coding region, the start of transcription, and a mapped exon in the amino-terminal region of the SP60-coding sequence are indicated. The location of the 9-C3 and 3-E2 cDNAs are shown. The genomic clones overlap from the BglII site to the PstI site in the center of the map (the region between the two cDNAs). One genomic clone extended from the XbaI site to the PstI site. The second clone was the BglII fragment from the BglII site immediately 5' of the PstI site. For details of the cloning procedures, see Materials and methods. The positions of homology to the two cDNA clones (c3E2 and c9C3) are shown. Also presented is an expansion of the region between the BglII site at position –1335 (relative to the cap site) and the PstI site within the coding region. The sequence of this fragment is presented in Fig. 2. The position of several unique restriction sites that were used for deletion constructs are shown. The region between the BglII and SapI sites shown in boldface type is a reference point for the deletion map shown in Fig. 5.

the genomic clone except for the presence of a 120-bp intron within this region of the genomic clone. The intron, as with other noncoding regions, is very AT-rich and has 5' donor and 3' acceptor sequences that agree with the consensus splice sequence for Dictyostelium genes (Kimmel and Firtel 1983). The 5' end of the mRNA was determined by S1 nuclease mapping (see Fig. 3A) and is marked on the sequence (see Fig. 2). A putative TATA box lies at –36. Analysis of the extremely AT-rich 5'-upstream regions shows three homologous CA-rich sequences (which we designate CAE) (CACA-CAACG-CAACG) that have been labeled box 1, box 2, and box 3. These CACA boxes are followed by an A+CT-CAAN+ sequence –15–30 bp downstream.

Analysis of the derived protein sequence for the region sequenced indicates the presence of four repeats of six amino acids (GDWNNN) in the amino-terminal region that is preceded by a putative transmembrane leader sequence. This is followed by a region of ~70 amino acids that is rich in proline and cysteine. A search of the literature indicated that the 6-amino-acid repeat is identical

Figure 2. Sequence of 5'-flanking region and amino-terminal-coding sequence of SP60. The ATG translation initiation codon was determined from the open reading frame that is contiguous through the 3'-E2 cDNA. The A-rich sequence 5' to the ATG initiation codon is similar to that present in other Dictyostelium genes (Kimmel and Firtel 1983). The intron, determined from the discontinuity of the genomic and cDNA sequence, is shown. The three CACA-rich sequences (CAE boxes) are labeled and numbered 1–3, 5' – 3'. The TATA box at nucleotide –36 is underlined, and the 5' start of transcription as determined from S1 mapping is shown (+1). The derived open reading frame is indicated. The position of the 5' deletion end points and the unique XbaI and NdeI restriction sites used for mapping of the cis-regulatory regions are shown, as is the fusion point (3'Δ2) for all luciferase and lacZ constructs.
to that obtained by Loomis and collaborators from a partial protein sequence and derived amino acid sequence of a short cDNA encoding the amino-terminal region of SP60 (Dowds and Loomis 1986; Hong and Loomis 1988). Both the identity of the derived amino acid sequence with the protein amino acid sequence and the prespore-

**Figure 3.** S1 nuclease mapping of the 5' ends of the SP60 endogenous and SP60–luciferase transcripts. [A and B, top] Probe B is not protected by the endogenous SP60 mRNA because the homology is limited to the very AT-rich, 5'-untranslated region, which shows little RNA specificity without protein coding sequences. S1 nuclease analysis was performed using single-stranded probes uniformly labeled with [32P]dCTP, as described in Materials and methods. The probe for the endogenous transcript contains 37 nucleotides of the SP60 protein-coding region and 774 nucleotides of 5' sequences. The probe for the SP60–luciferase construct contains 99 nucleotides from the luciferase gene and 265 nucleotides from the SP60 gene. Note that the 5' ends of each probe contain the 17-nucleotide primer used for making the probe and either 49 nucleotides (endogenous RNA probe) or 30 nucleotides (fusion RNA probe) of the M13 polylinker. These are digested by the S1 nuclease. The sequencing ladder is made from the same template and primer. Because the sequencing ladder still has the primer and polylinker sequences attached, the positions of the size of the S1 nuclease products are corrected for the length of the primer to identify the 5' position of the mRNA. [Top] [A] A set of major S1 nuclease-protected bands are seen [large arrowhead]; [B] a set of major [large arrowhead] and minor [small arrowhead] bands are seen. [A and B, bottom] Gel analysis. The sequencing ladder is shown in lanes ATGC in both A (endogenous transcript) and B (SP60–luciferase transcript). [A] Lanes 17hr indicate the probe hybridized to RNA from KAx-3 cells developed for 17 hr and then digested with S1 nuclease. Lane con is the probe hybridized to E. coli tRNA and then digested with S1 nuclease. [B] Lanes veg and 18hr indicate the probe hybridized to RNA from vegetative and 18-hr developed cells, respectively, and digested with S1 nuclease. Lane KAx-3 is the probe hybridized to RNA from 17-hr untransformed cells (strain KAx-3) and then digested with S1 nuclease. Lane con is as in A.
specific expression of 3-E2/9-C3 complementary mRNA indicates that the isolated gene encodes the spore coat protein SP60. The amino acid sequence of purified SP60 begins with the glycine corresponding to amino acid 23 in our derived amino acid sequence [Dowds and Loomis 1986], suggesting that the leader is removed during processing. The four hexa-amino acid repeats and the proline- and cysteine-rich region that follows may be important motifs required for building a complex spore coat structure. The hydrophobic leader sequence is presumably involved in membrane insertion and subsequent localization of the SP60 coat protein in the prespore vesicles. The SP60 gene has also been cloned, and the coding region has been sequenced by Fosnaugh and Loomis (1989).

Figure 4 shows the developmental kinetics of SP60 mRNA and that of another major prespore protein, SP70. SP60 mRNA is not expressed in vegetative or early developing cells and can first be detected in cells at 10 hr of development, shortly after the aggregate is formed. It is maximally expressed at the slug and culminant stages. SP70 mRNA shows a slightly different pattern. It is first detectable at 12-15 hr, peaks at 22.5 hr, and decreases slightly by 25 hr. Because both the SP60 and SP70 probes are being hybridized to the same blot, we do not believe that these differences are due to experimental variations. This is consistent with previous data from our laboratory [Mehdy et al. 1983; Saxe and Firtel 1986] on SP60 and SP70 expression and similar to that of Loomis and co-workers [Hong and Loomis 1988; Fosnaugh and Loomis 1989], although they observe a greater coordination of SP60 and SP70 expression.

Identification of cis-acting regulatory regions

To identify the cis-acting regulatory regions of SP60, sequences between the upstream BglII site, 1335 bp 5' from the cap site, and a linker inserted after codon 7 were fused in-frame to the firefly luciferase reporter gene in a Dictyostelium transformation vector (see Fig. 9). This plasmid was used as a basis for constructing a series of ExoIII 5' deletions that were capped at the 5' end with a HindIII linker. The structure of the vector with the positions of the deletions used in subsequent studies is shown in Figure 5. The complete structure and a partial restriction map of the vector are shown in Figure 5A. The SP60–luciferase fusions were transformed into the wild-type axenic strain KAx-3, and stable transformants were isolated.

To examine the temporal pattern of expression, transformants were plated for development on filters. A partial developmental time course of luciferase specific activity in cells carrying the parent SP60–luciferase gene construct [2IN] is shown in Table 1. The expression of the promoterless 2R10 vector is also presented. Expression of an Actin15–luciferase fusion gene [VII Luc (Howard et al. 1988)] was included as an internal control for the assay. The expression of the parent [2IN] fusion gene containing 1.3 kb of SP60 upstream is highly developmentally regulated, like that of the endogenous gene. There is a very low, but detectable, level of luciferase activity in vegetative cells [see Discussion]. By 9 hr, as the aggregate is forming, luciferase specific activity starts to increase, is very high at 18 hr, and increases another two- to fivefold by 23 hr late in culmination [data for 23 hr not shown]. As shown in the RNA blot in Figure 4, SP60 RNA levels are maximal between 20 and 23 hr. The increase in luciferase specific activity from the level in vegetative cells is $10^4$ to $10^5$-fold, and the maximal specific activity of the parental construct is similar to that of VII Luc, which uses the Actin 15 promoter, one of the strongest Dictyostelium promoters. This is consistent with the high level of accumulation of the SP60 mRNA and is not unexpected for a major spore structural protein.

The 5' end of the gene fusion mRNA was determined by 51 nuclease experiments (Berk and Sharp 1977) with a 409-nucleotide probe from the XbaI site at nucleotide +99 of the luciferase-coding sequence through nucleotide −154 (construct 5'Δ28) of the SP60 5' flanking sequence isolated from the SP60–luciferase fusion gene and includes some polylinker sequences. The cap site mapped within 5 nucleotides of the cap site of the endogenous SP60 mRNA [see Figs. 2 and 3B].

We then examined the expression of the different SP60–luciferase constructs in vegetative cells and in cells at 18 hr of development. In some experiments, expression at 10 hr of development, a time when SP60 mRNA can first be detected on RNA blots, was also assayed. The data from several experiments are presented as luciferase specific activity and as a percentage of the level at 18 hr of construct 5'Δ20 (see Table 1). 2R10 and VII Luc were used as internal controls in all transformations and in all developmental studies. In examining the results, we note that there is experiment-to-experiment variability in the levels of luciferase activity when cells transformed with a specific construct are assayed. This is due to variability in synchrony of the developing cells.

**Figure 4.** Developmental time course of expression of SP60 and SP70 mRNAs. A developmental time course was performed as described in Materials and methods. Cells were plated for morphological development and harvested at the times indicated. RNA was extracted and analyzed by RNA blot hybridization. RNA blots were hybridized with 9-C3 (SP60 probe) and 2-H3 (SP70 probe) cDNA inserts (for details, see Materials and methods). Aggregation occurred between 7 and 10 hr, the tight aggregate stage was ~12 hr, and early culminants were harvested at ~18 hr.
in different experiments and to the very rapid rate of increase in luciferase activity over time in the developmental periods that were assayed. The overall qualitative and quantitative effects of the deletions on SP60-luciferase expression are consistent. The general pattern of expression with the deletion constructs suggests four major classes of deletion, A–D, defined by the level of expression (Table 1; Fig. 5). Deletions removing ~670 bp from the 5′-flanking sequence of the parent construct (2IN, -1335) to -663 (5′Δ20) result in a three- to fivefold reduction in the level of expression at 18 hr and a decrease in vegetative expression and have been grouped as class A. The next two deletions (5′Δ38 and 5′Δ23, class B) remove another ~80 bp of 5′ sequence and a major portion or all of the first CAE (box 1), as well as intervening sequences, and show an ~20-fold drop in the level of expression. Deletions 5′Δ21 and 5′Δ25 (class C) remove ~100 bp more, including box 2, and result in a further ~50-fold drop in activity. Although the luciferase specific activity is <0.1% that of

Table 1. Effect of 5′ deletions on luciferase specific activity

| Construct* | Activity at specified developmental times | Maximum sp. act. (%) |
|------------|------------------------------------------|---------------------|
|            | [LU/mg protein]                           |                     |
|            | t = 0 hr t = 10 hr t = 18 hr              |                     |
| parent (-1335) | a 6 254 4.8 x 10^6 480 |                     |
|            | b 215 n.d. 1.3 x 10^6 542 |                     |
|            | c 0.2 74 1.25 x 10^6 230 |                     |
| Class A    |                                         |                     |
| 5′Δ9 (-1033)* | b 22 3.8 x 10^3 1.0 x 10^6 100 |                     |
| 5′Δ12 (-866)* | b 46 n.d. 2.4 x 10^6 100 |                     |
| 5′Δ20 (-663) | c 0.5 120 5.4 x 10^6 100 |                     |
| Class B    |                                         |                     |
| 5′Δ38 (-595) | b 13 n.d. 1.1 x 10^4 4.6 |                     |
| 5′Δ23 (-570) | b 24 130 9.6 x 10^3 1.0 |                     |
| Class C    |                                         |                     |
| 5′Δ46 (-516) | a 26 n.d. 4.8 x 10^3 5 x 10^-1 |                     |
| 5′Δ21 (-464) | a 6 20 99 1.0 x 10^-2 |                     |
| 5′Δ42 (-357) | b 4.8 n.d. 168 7 x 10^-2 |                     |
| 5′Δ53 (-203) | c 0.14 27 (3 x 10^3) 5 x 10^-1 |                     |
| 5′Δ28 (-154) | b 3.8 n.d. 58 2.4 x 10^-2 |                     |
| Class D    |                                         |                     |
| 5′Δ53 (-90) | a 8 1.6 3.6 x 10^-3 |                     |
| 5′Δ36 (-20) | a 4.5 1.6 3.6 x 10^-3 |                     |
| 5′Δ35 (-10) | a 24 n.d. 5.6 6 x 10^-4 |                     |
| 5′Δ42 (-203) | b 2.5 n.d. 2.0 8 x 10^-4 |                     |
| 5′Δ28 (-154) | a 4.5 1.6 3.6 x 10^-3 |                     |
| 5′Δ36 (-20) | a 24 n.d. 5.6 6 x 10^-4 |                     |
| 5′Δ35 (-10) | a 1.0 2.2 1.3 1 x 10^-4 |                     |
| 2R10 (no promoter) | a 3 2.4 0.3 3 x 10^-5 |                     |
| VIILuc | b 3 8.8 x 10^6 9.5 x 10^6 9.8 x 10^6 980 |                     |

Luciferase activity in light units (LU) per microgram of total protein is given for stable transformants during multicellular development. Three developmental times were assayed: At 0 hr, the cells are vegetative and growing logarithmically; the 10-hr time point corresponds to a loose aggregate; the 18-hr time point represents early to mid-culmination. At 18 hr, the cells develop very rapidly and there is some asynchrony in the population. In each experiment, cells were harvested at approximately the same point in development, as determined visually. Data from three different experiments (a, b, c) are presented. For each time point, the absolute specific activity of luciferase (LU/mg protein) is given. However, because of experiment-to-experiment variability due to the biology of the system, data were not averaged. Construct 5′Δ20 and the promoterless construct 2R10 were used as internal controls in every experiment. To allow comparisons between experiments and to quantify the effect of each deletion on reporter gene activity, the level of expression for each construct is also presented as a percentage of the level of 5′Δ20 luciferase specific activity for that particular experiment. Cells transformed with an Actin 15–luciferase fusion gene [VIILuc (Howard et al. 1988)] were used in all assays as an internal control for the luminometer; these results are given for experiment a only. The parent [2IN], 5′Δ23, 5′Δ21, 5′Δ42, 5′Δ28, 5′Δ38, 5′Δ46, and 5′Δ53 were also all assayed relative to 5′Δ20 and other constructs in several other experiments that are not shown, and these data were consistent with that shown. Data for 2R10 (no promoter) is shown only for experiment a. The deletions have been grouped into classes (A–D) as described in Results according to the relative level of maximum luciferase activity.

*a Constructs 5′Δ9 and 5′Δ12 were each assayed relative to 5′Δ20 in a separate set of experiments. All constructs were assayed multiple times.

*b For this experiment, the level of 5′Δ21 expression was >10-fold higher than in the other two experiments shown and in two additional experiments not shown.
5′Δ20 in these deletions, the activity is developmentally regulated and increases 15- to 25-fold between starvation and 18 hr. Deletion 5′Δ46, which removes the first CAE of CAE box 2, as well as 56 bp between 5′Δ23 and 5′Δ46, results in a 5- to 10-fold reduction in expression at 18 hr relative to the class B deletion 5′Δ23.

Subsequent deletions that remove box 3 and surrounding sequence (class D) result in a basal expression that does not show significant change during development. The expression level of these constructs in vegetative cells is approximately the same as that of 2R10 (promoterless construct). The specific activity of 2R10 is approximately constant through development.

RNA isolated from stable transformants expressing the 2IN, 5′Δ20, and 5′Δ23-luciferase constructs was used for a developmental RNA blot. The relative levels of SP60-luciferase RNA are consistent with the levels of luciferase activity in cells carrying the different deletion mutants [see Fig. 6A]. Figure 6B shows that the luciferase probe does not hybridize to untransformed cells.

A series of internal deletions were also made using the unique Rsal and Ndel sites in the 5′-flanking sequence (see Materials and methods). An internal deletion of 76 bp between -538 [Rsal] and -466 [5′Δ21]—construct 21irsa—which removes the second CAE [box 2] and surrounding sequences, results in a level of expression similar to that of deletion 5′Δ23 [deletion of the first CAE [box 1] and surrounding sequences], as assayed by luciferase activity [1.2 × 10^6 for 21irsa; 1.5 × 10^6 for 5′Δ23, and 3.0 × 10^6 for 5′Δ21]. These results suggest that the two regions between -664 and -571 and between -566 and -465 perform a common function. When a fragment lying between -664 and the Ndel site at -383 bp, which contains all three CAEs, was placed on deletions at -154 [5′Δ28] or -90 [5′Δ53], no developmental induction of luciferase activity was observed [data not shown], indicating that sequences lying 3′ to the CAE-containing domains are also essential for expression.

**Regulation by cAMP**

Previously, we have shown that the endogenous SP60 mRNA is inducible by cAMP in slow-shake culture, using the 3-E2 cDNA probe [Mehdy et al. 1983]. To determine whether the SP60-luciferase construct was so regulated, cells transformed with the 2IN-, 5′Δ20-, 5′Δ23-, 5′Δ21-, and 5′Δ42-luciferase fusion genes were washed free of nutrient medium and placed in slow-shake culture in buffered salts. At 6.5 hr, one-half of the culture was given high, continuous levels of cAMP, whereas the other half was not. Cells were harvested at 16 hr, and luciferase activity was measured in extracts. As shown in Table 2, the +cAMP extracts had substantially higher luciferase activity (~40-fold) than the -cAMP cultures. As the regions containing CAEs and surrounding sequences are sequentially deleted, the levels of cAMP-inducible expression drop in parallel with the level of expression seen in multicellular development, suggesting that these regions are needed for both cAMP and developmental induction. Deletion of all three regions (5′Δ42) results in a loss of cAMP induction. There is some induction in the -cAMP cultures that is variable from culture to culture. This has also been seen when endogenous gene expression is assayed by RNA blots and is believed to be due to low levels of...
Prespore cis-acting regulatory regions

Spatial expression of fusion gene products

The firefly luciferase reporter gene provides an extremely sensitive and quantitative expression assay, and the above data indicate that the SP60–luciferase fusion gene shows the proper temporal pattern of expression and is inducible by cAMP. However, cytological localization of the fusion gene product in aggregates is not possible when using available antibodies and fixing procedures that work well for Dictyostelium [Gomer et al. 1986a; Gomer 1987a, b]. To determine whether the fusion gene products are preferentially expressed in prespore cells, we have made SP60–lacZ gene fusions (SP60–lacZ). The parental construct (5′Δ20-gal) contains the 5′Δ20 upstream region, which is sufficient for complete temporal and cAMP regulation (see above), and the SP60-coding sequence to codon 7 fused in-frame to the Escherichia coli lacZ gene at codon 9 in the same vector background as the SP60–luciferase gene fusions (see Fig. 9). The position within the SP60 sequence used in the fusion is identical to that used for the luciferase gene fusion. Stable 5′Δ20–lacZ transformants were plated for development and stained for β-gal activity [see Materials and methods]. Figure 7 (I and II) shows the staining pattern of slugs and early culminants of these transformants. Control, untransformed cells at the same developmental stages are shown in Figure 7I and do not stain. As can be seen, the posterior 85% of migrating slugs and culminants [i.e., the entire prespore zone] expressing the 5′Δ20–lacZ fusion construct stains uniformly and very strongly, whereas the anterior remains unstained except for several random cells (see Discussion). Fruiting bodies show preferential staining in the sorocarp containing the spore mass. The pattern of staining seen with this construct is indistinguishable from that seen by immunofluorescence with an antibody that recognizes a common post-translational modification of the spore coat proteins SP60, SP70, and SP96 and with an anti-SP70 antibody made against a fusion protein expressed in E. coli (Takeuchi 1963; Gomer et al. 1986a). This is also consistent with earlier two-dimensional gel results examining the expression of SP60 in dissected anterior and posterior portions of migrating slugs and mature spores [Devine et al. 1983; Morrissey et al. 1984]. A similar pattern is seen for the prespore gene D19, which encodes a protein found in the slime sheath surrounding the migrating slug, through the use of monoclonal antibodies and lacZ fusion constructs [Kreft et al. 1984; Early and Williams 1988; Early et al. 1988; Dingermann et al. 1990]. Untransformed cells [see

Table 2. Induction by cAMP

| Luciferase activity [LU/μg protein] | −cAMP | +cAMP |
|-----------------------------------|-------|-------|
| Parent                            | 1.9 × 10⁴ | 3.1 × 10⁴ |
| 5′Δ20                             | 2.0 × 10⁴ | 4.8 × 10⁴ |
| 5′Δ23                             | 3.6 × 10⁴ | 1.4 × 10⁴ |
| 5′Δ21                             | 18     | 3.6 × 10³ |
| 5′Δ42                             | 3.3    | 5.6    |

Vegetative cells were harvested, washed, and resuspended in MES-PDF and shaken at 130 rpm for 6.5 hr. cAMP was added to 300 μM in one-half of the suspensions and readed every 3–4 hr to 100 μM. Cells were harvested at 16 hr and assayed.

Figure 6. RNA blot hybridization of SP60–luciferase expression. (A) RNA was isolated from 2IN (parental), 5′Δ20–, and 5′Δ23–luciferase constructs at the times shown. RNA was size-fractionated, blotted, and hybridized with SP60 cDNA (9-C3) or luciferase [lucif.] probes. The RNA was from experiment c, shown in Table 1. The 18-hr lanes for Δ20 and Δ23 contain only one-half the RNA in the other lanes. (B) RNA was isolated from vegetative cells (veg) and 10 and 18 hr of multicellular development from 5′Δ20 luc transfectants and also from control, untransformed cells at 18 hr (con-18hr). These were blotted and probed with either a luciferase-coding region [lucif] or SP60 cDNA (9-C3).
Figure 7. (Continued on facing page.)
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Figure 7. Cytological staining of SP60–lacZ constructs. Individual panels are labeled. Staining was performed as described in Materials and methods. (I) Photographs were taken through a dissecting microscope. All aggregates are stained for approximately the same amount of time (5 hr). (A) S′Δ20 (complete promoter). Migrating slugs. The unstained anterior prestalk zone is visible. (B) S′Δ20. Early culminants and fruiting bodies. (C) S′Δ20, 9- to 10-hr early aggregates. A ring of stained cells is visible. The very center and skirt of cells around ring are unstained (see text). (D) Control aggregates, slugs, early culminants (untransformed cells). No staining. (E) S′Δ23 (promoter lacking first CAE and adjacent sequence), 9- to 10-hr early aggregates. Stained cells (small arrow) create a circular pattern around the unstained center. (F) S′Δ23. Slugs and early culminants. The tip (small arrow) and posterior prespore zone (larger arrow) are unstained. There is a gradient of stain in the anterior of the prespore zone. (G) S′Δ21 (lacking first two CAEs). Standing slugs. Staining is restricted to the anterior of the prespore zone (small arrow). (II) Photographs were taken through a compound microscope of slug and aggregates dissected off a Millipore filter and placed on a glass slide. (A) A migrating slug. (B) A view of an aggregation center at the early to mid-aggregation stage showing a ring of stained cells. The majority of the aggregation streams still entering the aggregate were lost in the dissection; however, a skirt of unstained cells is seen at the periphery. The very center of the aggregate shows no staining. (C) A side view of a slightly later stage (tipped aggregate). Note that some cells stain more intensely.

Fig. 7I) and cells transformed with a lacZ construct lacking a promoter (not shown) show no detectable staining.

We have also examined the level and spatial pattern of expression using lacZ fusions with S′Δ23, S′Δ21, and S′Δ42 promoter regions. S′Δ23 and S′Δ21, which lack the first (5′-most) and second regulatory domains, respectively, showed sequentially reduced expression relative to that observed with the S′Δ20 construct, as might be expected from results with the luciferase assay [Fig. 7I; cells were all stained for ≈5 hr]. Significant staining can be seen with S′Δ20–lacZ in 5–10 min. Little staining can be seen with S′Δ23–lacZ in the first hour. This suggests that even within the cells that express β-gal activity, the promoter activity of the S′Δ23 construct is significantly reduced from that of S′Δ20. Moreover, S′Δ21–lacZ stains more slowly than S′Δ23. In addition, with the subsequent deletions, a decreasing gradient of expression from the anterior to the posterior of the prespore zone is observed, as the cells that stain became progressively restricted to the anterior of this region. In slugs and early culminants from the S′Δ23–lacZ-expressing cells, staining is observed in the anterior one-third to one-half of the prespore zone, with a gradient of decreasing staining toward the posterior. In S′Δ21–lacZ transformants, staining was restricted to a collar of prespore cells at the interface with the prestalk zone. Transformants carrying S′Δ42–, S′Δ28–, and S′Δ53–lacZ gene fusions showed no detectable staining [data not shown]. These data are in agreement with the results from similar constructs with luciferase as the reporter gene [see Table 1]. It is also worth noting that with both S′Δ20–lacZ and S′Δ23–lacZ constructs, some cells stain substantially stronger than others during the tipped aggregate stage [Fig. 7II] in and early culminants [data not shown].

The sensitivity of the luciferase assay has shown that prespore gene expression begins to be induced at the time of aggregate formation, earlier than previously observed from less sensitive RNA blot data with total RNA. Therefore, we examined the spatial distribution of expression at the early and mid to late stages of aggregation. Figure 7, I and II, includes photographs of the lacZ pattern of expression at these earlier stages. When viewing the aggregate from above, one sees staining in the body of the aggregate, except in the very center. At
this point in development, an estimated 20% of the cells are staining. This nonstaining central region eventually forms the anterior tip region of the migrating slug and the prestalk zone, which does not express SP60-lacZ fusions (Fig. 7I). The skirt around the aggregate contains the streams of cells entering the aggregate. As can be seen, the cells within the skirt show no detectable β-gal staining. Therefore, even in early aggregation, only the cells that will become localized in the prespore zone are detectably expressing the fusion gene product. Within an hour of the stage shown here, the formation of the multicellular aggregate will be complete and a pronounced tip will have formed. Eventually 80–85% will express the 5’Δ20-lacZ fusion protein. Aggregates of 5’Δ23-lacZ transformants also show slight staining of a ring of cells within the same region as is observed for the stronger 5’Δ20 construct. The 5’Δ21 construct shows no detectable staining at this point (not shown), presumably because of the weakness of the promoter.

Figure 7II shows a side view of aggregates at mid [Fig. 7IIB] and late [Fig. 7ICC] aggregation that have been dissected off the Millipore filters. As can be seen, staining is preferentially localized to a ring of cells within the aggregate mass, suggesting that expression of this gene may be controlled, in part, by spatial determinants. Some of the cells do show more intense staining. This pattern demonstrates that many of the cells that stain positively are already localized at this earlier stage.

Discussion

We cloned the gene encoding the spore coat protein SP60 and analyzed the sequences required for expression of the gene. Using two different SP60-reporter gene fusions, we show that the 5′-flanking sequences contain all of the necessary sequence for proper temporal, cAMP, and spatial expression of the fusion gene within the developing aggregate as compared to the endogenous gene. These results are consistent with the cytological localization of the SP70 gene product from immunofluorescence studies by using an antibody made against an SP70 fusion protein expressed in E. coli. They are also consistent with the results of Takeuchi and co-workers, who used antibodies against a carbohydrate moiety common to prespore proteins [Takeuchi 1963; Tasaka et al. 1983; Takemoto et al. 1985; Gomer et al. 1986a]. The localized developmental pattern of SP60 expression reveals the initial patterning of prespore cells and is consistent with the involvement of spatial determinants in this process and with the results of Krefft et al. [1984] for the D19 gene product.

When a series of 5′-deletion mutations were analyzed, two general patterns were observed. First, major decreases in the level of expression were observed that correlated with sequential deletion of the three CAE boxes and surrounding sequences. The level of expression, as assayed by luciferase activity, drops precipitously with each CAE and surrounding sequence deletion. However, as the β-gal staining indicates, the decrease is not equal across all cells within the prespore zone. Thus, comparison of the absolute levels of luciferase specific activity for the different deletion classes does not give an accurate picture of relative promoter strength within a given cell but, instead, gives an indication of relative expression levels as averaged across the entire prespore zone. However, the varying β-gal staining intensity of expressing cells between deletion classes is consistent with the quantitative analysis of the deletion series by using the luciferase assay. Deletion of all three boxes correlates with the loss of all expression, except a basal level that is ~10-fold below that normally observed during culmination. Analysis of the internal deletions suggests that the two 5′-most CAEs and surrounding sequences are equivalent in controlling the level of developmentally induced expression. Although the decreases in expression correlate with the sequential deletion of each CAE, we have not formally established that these sequences or the AT-rich sequences surrounding the CAEs are specifically required for high levels of expression.

Because the deletions are 5′ to the cap site, we infer that the differences in luciferase expression reflect differences in the rate of transcriptional activity. This is in agreement with developmental changes in the endogenous and fusion gene mRNA levels and nuclear run-on studies of the endogenous SP60 gene [R.A. Firtel, unpubl.]. The sequence homology between the CAEs is evident and suggests that they may bind a transcription factor required for expression of this and possibly other coordinately regulated genes. Another prespore-specific protein is encoded by a gene containing homologous CAEs in its upstream regulatory region [J.A. Powell and R.A. Firtel, unpubl.]. It is possible that the CAE boxes represent a prespore cell-type-specific element or an element required for cAMP regulation. Moreover, this sequence is homologous to one from DG17, which has been shown to confer cAMP and temporal regulation to a heterologous gene [Hjorth et al. 1990]. Although the three CAEs are quite homologous, there are nucleotide sequence differences, the significance of which is not presently known. Analysis of the regions required for expression of the DIF-inducible, prestalk-specific gene (st63) shows a number of GC-rich sequences, none of which looks similar to the CAEs of SP60 [J. Williams, pers. comm.]. The 5′-flanking region of the cAMP-inducible cyclic nucleotide phosphodiesterase gene contains several CA/GT-rich regions, but their structures are different from those in SP60, and the function of these sequences or surrounding sequences in regulating the expression of this gene is not known [Podgorski et al. 1989].

Clearly, sequences more proximal to the cap site than the CAE-containing domains are also required for proper expression because the region from −663 to the Ndel site at −383, which contains all three CAE-containing domains, does not rescue function when fused to 5′Δ53 (−90) or 5′Δ28 (−154). Therefore, the region downstream of the Ndel site also contains essential cis-acting elements. Construct 5′Δ42 is missing all three CAEs and their surrounding sequences, but very little of the proximal domains. When the 80-bp region containing essen-
tial cis-acting elements from the cAMP-induced prestalk gene \textit{pst-cath} (Datta and Firtel 1988; Hjorth et al. 1989) is added to this deletion, no developmental regulation is observed (L. Haberstroh and R.A. Firtel, unpubl.). Thus, it is possible that many elements specifying patterns of developmental regulation in \textit{Dictyostelium} are promoter-specific.

Deletions between the upstream BglII site and the 5'Δ20 deletion result in a gradual three- to fivefold decline in the level of expression, both in vegetative cells and at 18 hr of development. The expression in vegetative cells is extremely low (>10^4-fold lower) relative to that observed at 18 hr. Luciferase appears to be very stable in \textit{Dictyostelium} cells (when the enzyme is expressed from an actin promoter that is turned off early in development, it is present at 18 hr), and the low level of activity is that present in growing cells accumulates over the entire vegetative cell cycle, which is ~10 hr. In contrast, developing cells show an ~10^4-fold increase between 10 and 18 hr of development. Because no SP60–luciferase RNA can be detected in vegetative cells by any assay we have tried, we cannot tell whether the luciferase is being expressed from the same or a different promoter used for developmental expression. The sensitivity of the luciferase assay is sufficiently high that random, nonspecific initiations from within the upstream region could be detected using this assay. The level of vegetative expression correlates with the length of the 5' flanking region rather than any specific sequence.

\textbf{Evidence for a gradient regulating SP60 expression within the prespore zone}

The SP60–lacZ fusion protein is almost exclusively expressed in the prespore cells. In some aggregates, several cells are seen to stain in the anterior region or, in some cases, in the maturing stalk when mid-stage culminants are examined. There is no obvious pattern to these cells, and they represent ~1% of the cells in this region, most slugs and culminants show staining only within the posterior prespore zone. Staining of early aggregates, when cells are still moving into the aggregate, shows a novel pattern, indicating that the cells expressing SP60–lacZ are localized at this early stage. Because Sp60–β-gal-staining cells represent ~80% of the cells several hours later at the slug stage, many of the cells that are still moving into the aggregate must still be recruited into the prespore zone, possibly as they enter the ring of cells described in Figure 7. This suggests that spatial information may be essential in forming the prespore zone. Dingermann et al. (1990) have shown that lacZ expression driven by an actin promoter takes place in all cells during growth and early development, and slugs show uniform staining from the anterior to the posterior. Moreover, a lacZ fusion with a prestalk gene (\textit{Dd-tas}) shows strong preferential staining in the anterior tip of the prestalk zone in migrating slugs (R.K. Esch and R.A. Firtel, in prep.), indicating that our observations are not the result of an artifact of the staining procedure. We also note that for 5'Δ20SP60–lacZ transformants, the entire posterior 85% of migrating slugs stains, including the tail ends. One may have expected that the very posterior, which is believed to produce basal cells that express anterior markers, might not stain. However, recent results show that basal cells of the tipped aggregate that express the DIF-inducible genes st56 and st63 are sloughed off when slugs form and then begin to migrate (Jermyn et al. 1989; J. Williams, pers. comm.). This is also seen for the spatial expression of \textit{Dd-ras}–lacZ (R.K. Esch and R.A. Firtel, in prep.) and thus accounts for a lack of non-SP60–lacZ-expressing cells in the posterior of migrating slugs.

A novel finding is that sequential deletions result not only in a reduced maximal level of expression but also in restricted spatial expression. Although a gradient of β-gal staining is not observed with the full SP60 promoter–lacZ construct (5'Δ20–lacZ), even in slugs stained for a short period of time, sequential deletions within the distal regulatory region do result in such a gradient. A promoter deletion with only one CAE-containing region shows detectable expression only at the very anterior of the prespore zone. A promoter carrying two boxes and surrounding sequences shows expression in this collar region and in a posteriorly decreasing gradient in the anterior ~50% of the prespore domain. Deletion analysis suggested that at least two of the three CAE-containing 5' domains are functionally equivalent elements. The β-gal staining pattern of the 5' deletion constructs therefore appears to have masked the presence of a spatial gradient within the prespore zone that becomes evident with lacZ expression from sequentially weaker promoters (see Fig. 8). This could be a gradient of an essential transcription factor, possibly one recognizing the CAE. Moreover, the effects of the CAE and surrounding sequences are multiplicative, which is in agreement with the effect of sequential deletions on luciferase levels. This would be similar to the proposed mechanism by which bicoid-responsive elements in the \textit{hunchback} regulatory region have been suggested to recognize a bicoid gradient to establish a discrete zone of \textit{hunchback} transcription (Driever et al. 1989; Struhl et al. 1989). The observation that a decreasing number of distal elements results in a decrease in the proportion of the prespore zone that stains suggests that the CAEs and surrounding regions contain sequences that may be recognized by transcription factors that work cooperatively for the expression of SP60. In such a scenario, active transcription factors would be present across the prespore zone of the slug in a gradient from the anterior (higher concentration) to posterior (lower concentration) within the cells. In the \textit{bicoid}/\textit{hunchback} example, the syncytium allows an internal gradient of bicoid to rise across the entire organism. In contrast, \textit{Dictyostelium} is composed of individual cells and would require transmission of some external signal across cells membranes into an internal, active transcription factor gradient across the organism. Such an active transcription factor gradient could arise in response to an anterior-to-posterior gradient of a positive morphogen (e.g., cAMP),

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cAMP receptors, or intracellular effectors or to an inverse gradient of an inhibiting morphogen such as DIF. DIF, known to inhibit the transcription of the prespore gene \(D19\) [Early and Williams 1988] and induce a specific class of prestalk genes [Williams et al. 1987], is paradoxically localized primarily in the posterior of the slug [Brookman et al. 1987]. Whether DIF is present in a gradient within the posterior region is not known.

We are still left with the question of how the two zones are established and what determines the very sharp division between the two. Because \(SP60-lacZ\) expression can be detected first in a marginal zone within the early aggregate, the information necessary to activate expression in specific cells must be established by this point. Previously, several laboratories demonstrated that the propensity of single cells to differentiate into prespore or prestalk is based on the position of the cell in the cell cycle at the time of initiation of development (starvation) [MacDonald and Durston 1984; Weijer et al. 1984; MacWilliams et al. 1985; Gomer and Firtel 1987].

Perhaps we are dealing with a two-factor differentiation scheme: (1) cell cycle position at starvation (some “organizing” cells), and (2) position within the forming aggregate in which cells that are uncommitted at the time of starvation are induced by their position within the aggregate and the relative concentrations of various morphogens.

Materials and methods

General methods

Axenically grown KAx-3 cells were used in these studies [Mehdy et al. 1983]. Vegetative Dictyostelium cells were transformed by electroporation, and stable transformants were selected at a concentration of 10 \(\mu\mathrm{g}\)/ml, as described previously [Howard et al. 1988; Dynes and Firtel 1990]. Whole transformed populations were utilized for the analyses. Transformed populations were maintained as logarithmically dividing cells.

RNA and DNA analysis

RNA was isolated from cells and size-fractionated on denaturing gels, blotted, and hybridized with the appropriate clones, as described previously [Mann and Firtel 1987]. DNA was isolated from transformants and from untransformed large cultures of Dictyostelium, as described previously [Datta and Firtel 1987]. S1 mapping of the 5' ends was performed with single-stranded DNA probes labeled with \(\Delta^{32}\)PdCTP and derived from M13 phage DNA, by using the standard sequence primer and the Klenow fragment of DNA polymerase I [Berk and Sharp 1977, Maniatis et al. 1982, J.A. Powell and R.A. Firtel, in prep.]. After polymerization, the product was digested with HindIII. This linearizes the M13 vector and produces a full-length probe fragment. In addition, partial-length fragments from the probe region plus fragments complementary to the M13 backbone are also produced. The products are denatured, and the full-length single-stranded probe is isolated on urea-containing partial denaturing sequencing gels.

The probe for mapping the endogenous \(SP60\) transcript extended from the \(Hpal\) site at +146 (5' end of probe) to the 5' \(\Delta20\) HindIII site (−663) (see Fig. 3A, top and bottom). RNA from 18-hr untransformed cells was used. To map the \(SP60-luciferase\) transcript, a probe from the \(XhoI\) site (+99) of luciferase (5' end) to the HindIII site of 5' \(\Delta28\) at −154 and RNA from 18-hr 5' \(\Delta20/SP60-luciferase\) transformed cells were used [see Fig. 3B, top and bottom]. Because the \(SP60-lacZ\) and \(SP60-luciferase\) fusion vectors are identical upstream from the \(SpeI\) site, which lies within the 5'-untranslated sequence and thus downstream from the mapped endogenous cap site, the cap site for the \(SP60-luciferase\) fusions was not mapped.

The polarity of transcription was determined with strand-specific RNA probes. The 3-E2 cDNA was cloned into the \(PstI\) site of pSP73 [Promega] and linearized at either the \(BglII\) site [for a T7 RNA polymerase-derived probe] or the \(XhoI\) site [for an SP6 RNA polymerase-derived probe]. The linearized DNAs were transcribed by using T7 and SP6 polymerase [Promega], respectively, with a \(\Delta^{32}\)P[CTP], according to the manufacturer's directions. These were hybridized separately to blots containing RNA from 18-hr cells. The hybridization pattern was then related to the DNA sequence of the cDNA in SP73.

DNA sequencing

Dideoxy DNA sequencing with modified T7 DNA polymerase

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Model for prespore zone gradient. [Top] Cartoon of the \(SP60\) promoter–\(lacZ\) staining response (intensity and localization of expression) and cAMP and developmental response for the various promoter constructs. [Bottom] Model showing two possible gradients within the prespore zone to explain \(SP60\) promoter–\(lacZ\) responses. Positive gradient could be transcription factors or parts of the cAMP receptor/intracellular signaling pathway. Negative gradient could be DIF, adenosine, or some other metabolite/morphogen.
was performed on subclones, on all 5' deletions, and on the 3'-E2 cDNA.

Cloning of SP60

SP60 genomic clones were isolated by screening two genomic libraries constructed by use of mapping data for Dictyostelium genomic DNA digested with various restriction enzymes and hybridized with 3-E2 and 9-C3 cDNA inserts. One library contained a BgIII limit digest of Dictyostelium DNA cloned into the BamHI site of pAT153. The second library contained Dictyostelium genomic DNA, double-digested with PstI and XbaI and inserted into an XbaI limit/PstI partial digest of a modified pAT153 plasmid vector. This modified pAT153, designated pAT153L, contained the polylinker from pSP73 (Promega Biotech) inserted between the EcoRI site and the SalI site of pAT153. The insertion of the polylinker was accomplished by ligating a blunt-ended BgIII site on one end of the polylinker into a blunt-ended EcoRI site from pAT153 and the Xhol site at the other end of the polylinker into the SalI site of vector sequences. The cloning deleted the unique EcoRI, HindIII, Clal, BamHI, EcoRV, Sphl, and SalI sites and ~675 bp of the vector, including the sequences required for tetracycline resistance. These sites, however, as well as other unique restriction sites, are present in the polylinker. Because of the cloning protocol, the BgIII and Xhol sites in the polylinker were lost in the cloning. The vector has two PstI sites, one in the AmpR gene and one in the polylinker. The unmodified pAT153 vector had previously been used successfully to construct Dictyostelium genomic libraries (J. Williams, pers. commun.). In our hands, both pAT153 and pAT153L work substantially better for cloning Dictyostelium genomic sequences than pUC-based vectors. Unusually AT-rich sequences. The cloning deleted the unique HindIII linker. The full-length and individual SP60 promoter constructs were then cloned into the vector Cath-Luc13 (Howard et al. 1988) [see Fig. 8] in which the pst-cath promoter had been deleted. Cath-Luc13 was originally derived from VIILuc (Howard et al. 1988). Deletion end points were determined by DNA sequencing.

The lacZ construct was made by isolating the lacZ gene from pC4lacZ and pC4AUGlacZ (a gift from C. Zuker), filling in the 5' overhang of a BamHI site, adding a HindIII linker (10-mer), and linking this in-frame to the SP60 3' Δ2 deletion used to construct the luciferase fusions. This was then cloned into the same vector backbone as that used for the luciferase gene [see Fig. 9].

Luciferase and lacZ fusions carrying different promoter deletions were made by exchanging the restriction fragment containing the promoter from the 5' unique BgIII and SpeI sites in the 5'-untranslated sequence of SP60.

Development and cAMP assays

For multicellular development, axenically grown log-phase vegetative cells were harvested by centrifugation, washed, and plated on Whatman No. 50 filters in MES-PDF buffered salts, as described previously. Development was followed visually under a dissecting microscope. Cells were harvested at the loose aggregate (9–10 hr) and early culmination stages (~18 hr).

For developmental RNA blots, RNA was extracted and sized on denaturing gels, blotted to membrane filters, and probed with nick-translated or random-primed probe, as described previously (Mann and Firtel 1987). For the luciferase assays, cells were harvested in glycyl-glycine buffer with protease inhibitors and quick-frozen in a dry ice–ethanol bath, as described previously (Howard et al. 1988).

Slow-shake culture was done as described previously (Mehey et al. 1983, Mehey and Firtel 1985). Briefly, vegetative cells were harvested and washed as described above and resuspended in MES-PDF at 5 x 10⁶ cells/ml. Cells were shaken at 130 rpm [10 ml/125-ml Erlenmeyer screw-cap flask] for 6.5 hr. cAMP was added to one-half of the cultures to 300 μM. CAMP was readded every 3–4 hr to a concentration of 100 μM. Cells were harvested at 16 hr.

Cytological staining

Dictyostelium cells were plated for development as described above, except that they were plated on white Millipore filters. At appropriate times, aggregates were stained for β-gal activity using a modification of the procedure of Dingermann et al. [1990]. Cells were fixed for 15–45 min in 0.5% glutaraldehyde in Z’ buffer, washed in Z’ buffer, and stained with X-gal [BRL] in the presence of potassium ferrocyanide and potassium ferricyanide at 37°C (Dingermann et al. 1990). Extended staining [24 hr at 37°C] showed a very slight blue tinge in untransformed aggregates, presumably due to an endogenous Dictyostelium β-gal activity. This activity is minimal at pH 6.9, used in the staining in these studies, and was not reduced by the presence of 2 mM EGTA. The dark blue color is seen in the transformants of 5'Δ20SP60–lacZ gene fusion constructs after 2–4 hr. Note that more than one independent, stably transformed population was used for each construct. In addition, the staining pattern remained unchanged in experiments performed over a period of several months.

Luciferase activity measurement

Luciferase activity was assayed as described previously. The Monolight 2001 luminometer, used for these studies, gives a
linear response up to $10^6$ light units (LU). Machine background with either buffer or extracts from untransformed cells is 80–100 LU and was subtracted from the value obtained in each assay. Extracts were diluted, and an aliquot equivalent to between 0.01 and 50 μl of the original extract in 300X total was used to obtain light units that ranged between 2,000 and 500,000 per assay. The assay was linear over all dilutions, and luminometer readings were used and performed as described previously [Howard et al. 1988]. Total protein amounts were determined using the Bio-Rad protein reagent.

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