Progression of an inductive signal activates sporulation in *Dictyostelium discoideum*

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**SUMMARY**

*spi*A, a marker for sporulation, is expressed during the culmination stage of *Dictyostelium* development, when the mass of prespore cells has moved partly up the newly formed stalk. Strains containing a full-length *spi*A promoter/β-galactosidase gene fusion were stained for β-galactosidase activity at intervals during development. The results indicate that expression of *spi*A initiates in prespore cells at the prestalk/prespore boundary (near the apex) and extends downward into the prespore mass as culmination continues. A spatial gradient of staining expands from the top of the prespore mass and intensifies until the front of activation reaches the bottom, whereupon the entire region stains darkly. The *spi*A promoter can be deleted to within 301 bp of the transcriptional start site with no effect on the relative strength, timing or spatial localization of expression. Further 5′ deletions from −301 to −175 reduce promoter strength incrementally, although timing and spatial expression are not affected. Deletions to −159 and beyond result in inactive promoters. Treatment of early developmental structures with 8-Br-cAMP in situ activates the intracellular cAMP-dependent protein kinase (PKA) and precociously induces *spi*A expression and sporulation. The absence of an apparent gradient of staining in these structures suggests that PKA is equivalently activatable throughout the prespore region and that all prespore cells are competent to express *spi*A. Thus, we postulate that the pattern of expression of *spi*A reveals the progression of an inductive signal for sporulation and suggest that this signal may originate from the prestalk cells at the apex.

Key words: gene expression, *spi*A, 8-Br-cAMP, protein kinase A, culmination, expression gradient

**INTRODUCTION**

Cell differentiation is often regulated during embryogenesis by signals emanating from nearby, but distinct, cell populations (Jessell and Melton, 1992; Slack, 1993). For example, vegetal cells of the *Xenopus* embryo direct mesodermal differentiation of overlying cells by releasing signals which can be mimicked by and may be related to the TGFβ-, FGF- and Wnt-families (Dawid et al., 1990; Slack et al., 1992). Some of these molecules regulate the expression of goosecoid, a homeo-domain protein involved in the activity of the embryonic organizer of the dorsal blastopore lip (Spemann and Mangold, 1924; de Robertis et al., 1992). Likewise, cell-cell interactions dependent upon the evolutionarily conserved hedgehog proteins induce furrow movement in the *Drosophila* retina (Heberlein et al., 1993; Ma et al., 1993) and mediate polarizing activity in the vertebrate limb and central nervous system (Echelard et al., 1993; Riddle et al., 1993). Thus, the position of cells within a developing organism is often critical for determining their ultimate fate.

We are interested in the mechanisms that activate sporulation during culmination of *Dictyostelium discoideum* through the coordination of positional information and morphogenesis. Culmination is a transitional stage of development that is marked by a series of complex cell movements and the terminal differentiations of stalk cells and spores (see Fig. 1B; Raper, 1940; Williams, 1991). Although some of the extracellular and intracellular signals that regulate early development of *Dictyostelium* are well characterized, those that direct terminal differentiation are poorly understood.

At early culmination, an upright structure forms which is composed of an apical tip of prestalk cells supported by a mass of prespore cells. Prestalk cells within the core of the tip differentiate into a stalk and, as cells enter the stalk tube at the top, the tube extends through the prespore cells to the substratum. Upon continued elongation, the stalk extends up and the prespore cells lift off the base. Midway through culmination, when the prespore cells have moved partly up the newly formed stalk, they begin to differentiate into mature spores (see Fig. 1B).

A variety of data indicate that cAMP-dependent protein kinase (PKA) plays an essential role in the regulation of sporulation. As in all eucaryotic organisms, PKA in *Dictyostelium* is inhibited by association of a regulatory subunit with a catalytic subunit (de Gunzburg et al., 1984). When cAMP binds to the regulatory subunit, it dissociates from and thus...
activates the catalytic subunit. rdeC rapid development strains, which lack an active regulatory subunit, form spores precociously in the absence of stalk differentiation (Simon et al., 1992). These strains will also encapsulate spores when developed as monolayers in the presence of cAMP, whereas wild-type strains do not produce spores under these conditions (Abe and Yanagisawa, 1983). Moreover, overexpression of the catalytic subunit or treatment with the membrane-permeable 8-Bromo derivative of cAMP (8-Br-cAMP) to constitutively activate PKA will also induce precocious sporulation (Maeda, 1988; Kay, 1989; Richardson et al., 1991; Anjard et al., 1992; Maeda, 1992; Hopper et al., 1993a; Mann and Firtel, 1993). Neither pkaC-nulls, which do not express a catalytic subunit, nor prespore cells that lack PKA activity due to expression of a mutant regulatory subunit that does not dissociate in the presence of cAMP, will sporulate (Mann and Firtel, 1991; Hopper et al., 1993b).

Although activation of PKA induces sporulation, the developmental signals that mediate this regulation have not been identified. There is some evidence that prestalk cells may play an active role in the terminal differentiation of prespore cells. Laser ablation of the prestalk region at the apical papilla during early culmination prevents sporulation (Klaus and George, 1974) and inhibition of prestalk differentiation, by expression of the dominant-negative regulatory subunit of PKA in prestalk cells, will likewise attenuate spore differentiation (Harwood et al., 1992). Although some rapidly developing mutants produce spores in the absence of normal prestalk and stalk differentiation (Abe and Yanagisawa, 1983), their unregulated PKA, abnormal sensitivity to cAMP and premature sporulation indicate that terminal differentiation in these strains has become dissociated from normal inductive events (Kay, 1989; Simon et al., 1992).

To investigate the extracellular and intracellular signals that regulate sporulation in Dictyostelium, we have initiated a detailed study of the expression of spiA, a marker for sporulation. spiA expression begins in prespore cells during mid-culmination, just prior to spore maturation, and its mRNA continues to accumulate as spores mature (Richardson et al., 1991; Richardson and Loomis, 1992). The spiA gene product, Dd31, is found on the inner surface of the spore coat in mature spores and spiA-null strains produce spores that appear normal but are unstable in the presence of water (Richardson and Loomis, 1992). Mutations that block sporulation also block spiA expression, while both are induced by treatment with exogenous 8-Br-cAMP (Richardson et al., 1991). We have, therefore, used strains containing spiA promoter::lacZ fusions to examine the spatial patterning of sporulation specific gene expression during culmination.

We first observe spiA-driven β-galactosidase expression at the very top of the prespore mass adjacent to the apical prestalk region. A gradient of spiA expression extends downward as culmination proceeds. Analysis of a series of 5′-spiA promoter deletions indicates that the most minimally active promoter retains this spatial and temporal regulation. In contrast, bypassing endogenous spiA induction by treating slugs with 8-Br-cAMP in situ causes precocious expression of β-galactosidase throughout the prespore mass. We suggest that sporulation in Dictyostelium is regulated by an inductive signal that mediates PKA activation within prespore cells and that this signal emanates from the apical tip. This work further establishes an assay to allow identification of signalling molecules that induce sporulation in Dictyostelium.

MATERIALS AND METHODS

Strains

Dictyostelium discoideum strains AX-4 and HL328, and conditions for their growth and development were described previously (Richardson and Loomis, 1992).

spiA::lacZ fusion constructs

The vector pyrBlue2 was derived from DdGal16 (Harwood and Drury, 1990) by replacement of the Nex0 cassette with the 4 kb Spihl-Xbhl fragment of DIV-2 which contains pyr-5-6 (Richardson and Loomis, 1992; Fosbaugh and Loomis, 1993). pyrBlue3 was analogously constructed from DdGal17. The –1090/+261 spiA::lacZ construct was formed by ligating the XbaI-SpeI fragment of pLGL1 (Richardson et al., 1991) into Xbal site of pyrBlue2, fusing the first 24 codons of spiA in frame with lacZ.

Constructs were derived from –1090 by a series of 5′ exonuclease digests or specific cloning of PCR-amplified fragments. For the exonuclease series, the –1090 construct was modified by the insertion of a synthetic polylinker (5′ CTAGAAAAAAAAAGATTGACCTAGGGGCGCCCTTCATAGAGGGCGCCCG3′) into the Xbal site at the 5′ end of the spiA promoter insert. This regenerated the single Xbal site and created additional ApaI, AvrII, Apol and NotI sites. A series of nested, unidirectional deletions from the 5′ end of the promoter was made using the Exo III-Mung Bean Deletion Kit (Stratagene, Inc.). The –1090 plasmid was digested with AvrII, blocked with thiо-dNTPs using Klenow fragment of DNA polymerase I and the plasmid was secondarily digested with NotI. Endpoints of the deleted plasmids selected were determined by DNA sequencing to be +609, +390, +301, −159, −143 and −124.

For the PCR constructs, six oligonucleotides were synthesized, each with an Xbal site at the 5′ end and an additional 13 to 22 nucleotides specific to the desired sequence. The 5′ ends terminated at −241, −225, −211, −193 and −175. Each was used separately in PCR reactions with a second primer which was complementary to the 3′ end of the lacZ gene to amplify specific fragments from the –1090 plasmid. After digestion with Xbal and BamHI, the purified fragments were ligated into Xbal-BamHI cut –1090 plasmid. Constructs were analyzed by DNA sequencing to confirm that no alterations in the sequences were introduced by PCR. Two blunt-end constructs were made by direct subcloning of an EcoRI fragment (−90 to +234) or Xbal-PvuII fragment (−580 to +270) into BamHI of pyrBlue2 and BamHl/BglII of pyrBlue3, respectively.

Transformations

DNA-mediated transformation by electroporation, pyr5-6 selection and clonal isolations were performed as described (Kalpaxis et al., 1991; Richardson and Loomis, 1992). Genomic DNA was isolated from 25 ml of axenically grown cells as described (Richardson and Loomis, 1992). Transformations were confirmed by Southern blot hybridization or PCR amplification using primers specific to spiA promoter and lacZ sequences. Transformants isolated by pyr5-6 selection usually contain random, single copy integrations (Kalpaxis et al., 1991). Several (3-6) transformed strains of each deletion construct were isolated clonally.

β-galactosidase staining

Staining of developmental structures on nitrocellulose filters was by a modification of the agar overlay procedure (P. Morandini and R. H. Insall, unpublished). Nitrocellulose filters were moved to a plastic Petri dish and sprayed gently (with a fine spray) with 1% glutaraldehyde in Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl,
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1 mM MgSO₄, pH 7.0) until just damp. Filters were fixed for 10 minutes, permeabilized with 0.1% NP-40 in Z buffer for an additional 10 minutes, washed twice with Z buffer and incubated with Xgal stain solution (5 mM K₃[Fe(CN)₆], 5 mM K₄[Fe(CN)₆], 1 mM X-gal, 1 mM EGTA, Z buffer) for various times. This procedure gave more consistent staining of culminants than those previously described (Dingermann et al., 1989). In some cases, the structures were counterstained for 30 minutes with 0.02% eosin Y in Z buffer and rinsed with Z buffer.

RNA isolation and primer extensions:
RNA was isolated as described (Kimmel, 1987). Primer extensions were as described (Calzone et al., 1987; Louis et al., 1993).

RESULTS

spiA is expressed in a gradient during culmination:
We have previously shown that 4 kb of genomic DNA containing the entire spiA coding region is sufficient to rescue the phenotype of a spiA-null strain (Richardson and Loomis, 1992). The resultant strains express spiA with the same temporal regulation as that of a wild-type spiA⁺ strain, suggesting that this genomic fragment contains the entire spiA promoter. This 4 kb region includes 1090 bp upstream of the transcription start site (see below). To further characterize spiA expression, a spiA genomic DNA fragment from −1090 to +261 (relative to transcriptional initiation and including the first 24 codons) was fused in frame with the E. coli lacZ gene. This −1090 construct was transformed into Dictyostelium, stable transformants were clonally isolated and its presence was confirmed by Southern blot hybridization (Richardson and Loomis, 1992).

Fig. 1. β-galactosidase staining of spiA/lacZ strains during development. (A) β-galactosidase expression driven by the spiA promoter during culmination. Strains containing the constructs indicated (see Fig. 2A) were plated for development on nitrocellulose filters. Filters were removed for β-galactosidase staining at different developmental stages. Photographs of representative structures are presented. (B) Diagrammatic representation of spiA expression during culmination. spiA expression as indicated by β-galactosidase staining is shown as shaded regions within culminating structures. Labels indicate names of morphological stages and their canonical times of development.
in Fig. 1A and include strains carrying the full-length (−1090) or modified (−580 and −301) promoter constructs (see below). The deduced temporal and spatial pattern of \( \text{spiA} \) expression during development is presented diagrammatically in Fig. 1B. No \( \beta \)-galactosidase activity was observed prior to mid-culmination even after staining for more than 48 hours. \( \beta \)-galactosidase was first detected as the mass of prespore cells lifted off the substratum on the newly forming stalk. Staining initially appeared at the top of the prespore mass and cells below this region did not stain even after prolonged incubation (see Fig. 1A). As culmination continued and the stalk elongated, a front of \( \beta \)-galactosidase expression penetrated down the prespore mass until eventually the entire spore region stained an even intense blue. The protrusion of prestalk cells at the apex remained unstained until it disappeared into the stalk at the end of culmination. Strains that lack the \( \text{spiA} \) promoter construct or contain \( \text{lacZ} \) sequences but lack the \( \text{spiA} \) promoter did not accumulate \( \beta \)-galactosidase that is detected through the end of culmination (Richardson and Loomis, 1992). We show here that the \( \text{spiA} \)-directed \( \beta \)-galactosidase activity appears at the same morphological stage and also accumulates through the end of culmination (see Fig. 1). In addition, strains that contain the −1090 \( \text{spiA} \text{-lacZ} \) fusion construct and overexpress the PKA catalytic subunit in prespore cells, express \( \text{spiA} \) and \( \text{lacZ} \) mRNAs precociously and coordinately (Mann et al., 1994). These data indicate that the sequences from −1090 to +261 contain the entire regulatory region of \( \text{spiA} \) and that the gradient of \( \beta \)-galactosidase that we observe is reflective of the endogenous \( \text{spiA} \) expression pattern.

Sequences mediating developmentally regulated expression of \( \text{spiA} \)

To examine the intracellular mechanisms responsible for the
graded expression of spiA in the prespore cell mass prior to sporulation, we studied a series of 5′ promoter deletions derived from the −1090 spiA/lacZ fusion construct. First, the sequence of the 5′ region of spiA was determined (Fig. 2A) and the transcription start site identified by primer extension of mRNA collected from late culminants. Two reactions using different antisense oligonucleotide primers extending from nucleotide 244 to 217 and from nucleotide 220 to 194 gave identical 5′ termini (Fig. 2B).

5′ deletions were constructed by exonuclease III digestion and by directed PCR amplification. The endpoints of the deletions are indicated (Fig. 2A). Clonally isolated stable, single-copy integrants of each construct were plated for development on nitrocellulose filters and stained for β-galactosidase at intervals during development. Although their relative expression levels varied, constructs −1090 through −175 expressed β-galactosidase at equivalent stages and maximal staining for each was observed in the spore mass of mature fruiting bodies. In constructs −1090 to −193, we saw clear gradients of staining that first appeared in mid-culminants at the top of the spore mass (Figs 1, 3). The −175 construct produced weak, variegated staining that also initiated at the prespore/prestalk boundary (see Fig. 3).

Since there is often variation in synchrony during late culmination and since cells expressing β-galactosidase include spores that are resistant to disruption, we chose not to evaluate the relative levels of β-galactosidase by in vitro quantitation. Rather, we estimated β-galactosidase activity in different strains by observing the relative intensity of staining for similar structures after staining for 1 hour, 5 hours and 25 hours (Fig. 4). These data are summarized in Table 1. Strains carrying the −1090 full-length promoter and deletions −580, −469, −390 and −301 all stain with equivalent intensity. For comparable intensities, deletions −241 and −225 need to be stained approximately ten-times longer, while deletions −211 and −193 decrease promoter activity an additional order of magnitude. The expression of the −175 construct is decreased even further and required more prolonged staining to estimate its extremely low activity (see Fig. 4 and Table 1). Further deletion to −159 and beyond inactivates the promoter below our detection limit.

Exogenous 8-Br-cAMP induces spiA expression and sporulation in situ:

Various models can be constructed to explain the initiation of spiA expression at the prespore/prestalk boundary. Prespore cells near the apex could be activated prior to those at the base as the result of proximity to an inductive signal. Alternatively, prespore cells near the apex may independently express a factor (e.g. PKA) required for sporulation prior to the cells below. The cAMP analog 8-Br-cAMP will induce spiA expression and sporulation in disaggregated prespore cells, while treatment with equivalent amounts of cAMP has no effect (Maeda, 1988; Kay, 1989; Richardson et al., 1991). Unlike cAMP, 8-Br-cAMP will penetrate cells, activate PKA and bypass the natural inductive signal(s). We have investigated spiA expression and sporulation in response to 8-Br-cAMP applied in situ to determine if all cells were equivalent in their inductive competence.

Strain −1090 was developed to various stages on nitrocellulose filters, then filters were cut and portions were transferred to fresh pads saturated with standard developmental buffer containing no additions, cAMP, or 8-Br-cAMP. At various

<table>
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<th>Strain</th>
<th>Culminants</th>
<th>8-Br-cAMP</th>
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<tr>
<td>-1090</td>
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<td>-580</td>
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<td>nd</td>
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<td>-469</td>
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<td>-124</td>
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<td>-90</td>
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<td>HL328</td>
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Culminants and 8-Br-cAMP treated structures were developed and stained for β-galactosidase as described. The relative staining intensities of each of the strains were recorded photographically after staining for 1, 5 or 25 hours. Examples for representative strains are shown in Fig. 4 (for culmination) and Fig. 5 (for 8-Br-cAMP induction). The intensity of blue staining was compared visually and the relative staining levels estimated. Constructs were grouped according to similar staining intensities based on the average of two or more comparisons with each of two or more independently isolated strains. Each ‘+’ reflects a significant (i.e. approximately 10-fold) difference in relative staining intensity. The differences between groups were determined by comparisons with nearest neighbors. It is important to note that the data are somewhat approximate due to non-linearities inherent in β-galactosidase staining and visual quantitation (see Figs 4 and 5).

Abbreviations: nd, not determined; (−), no detectable staining.

Fig. 3. Patterns of spiA expression during mid-culmination. Strains carrying the spiA/lacZ deletion constructs were developed and stained for β-galactosidase. The duration of staining varied, thus the relative intensity of staining is not representative of promoter strength. The red arrow on the −175 construct indicates the position of weak, variegated staining at the prespore/prestalk boundary.
<table>
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<th>Time of $\beta$-Galactosidase Staining</th>
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<tr>
<td>-225</td>
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<td>-193</td>
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<td>-124</td>
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**Fig. 4.** Relative $\beta$-galactosidase staining of the $spiA/lacZ$ deletion constructs at culmination. Development of the indicated strains was terminated during late culmination and stained for the times indicated.
times after transfer, the filters were stained for β-galactosidase. Slug structures that normally do not express spiA/lacZ stained weakly after exposure to 8-Br-cAMP for 2 hours. Treatment for an additional hour resulted in increased stain intensity for slugs and weak staining of earlier structures such as tipped mounds (Fig. 5). Slugs that were not treated with 8-Br-cAMP continued to develop normally; in these experiments they did not culminate prior to staining and did not express spiA (Fig. 5A). Treatment of preculminants in situ with 1 or 40 mM cAMP did not induce spiA expression or the formation of spores (see Fig. 5A). The duration of 8-Br-cAMP treatment required for maximal levels of β-galactosidase activity in situ was only slightly longer than that required for maximal accumulation of spiA mRNA in shaking cultures of disaggregated prespore cells. We also found that, whereas 10-20 mM 8-Br-cAMP was sufficient to induce β-galactosidase expression in situ, 40 mM was required for maximal response.

The most striking result of the 8-Br-cAMP treatment is that staining is present throughout the prespore region (Fig. 5). Thus, not only will 8-Br-cAMP cause induction of spiA/lacZ precociously, all prespore cells appear to be responsive to this induction. 3 hours of exposure to 8-Br-cAMP in situ is also
sufficient to promote the differentiation of prespore cells into spores as monitored by phase-contrast microscopy. These data indicate that all prespore cells, regardless of their position within the developing organism, are competent to express spiA and to sporulate. It would appear that proximity to an inductive signal rather than an inherent difference in inductive competence is responsible for the observed pattern of spiA expression during normal culmination.

To determine if specific sequences within the promoter were differentially sensitive to induction by 8-Br-cAMP, developing strains carrying the complete series of deletion constructs from −1090 through −90 were analyzed. As was observed with the −1090 strain, 8-Br-cAMP treatment resulted in precocious staining of prespore cells in strains carrying constructs −301 through −175, whereas those with larger deletions did not stain detectably (Fig. 5B). Constructs were grouped according to relative staining intensities upon 8-Br-cAMP induction and these grouping are identical to those derived from staining after development (Table 1). These studies indicate that the sites responsible for 8-Br-cAMP-dependent induction of spiA are indistinguishable from those that direct spatial patterning during culmination.

**DISCUSSION**

We had previously shown that induction of spiA is temporally regulated within prespore cells during culmination (Richardson et al., 1991). We now conclude, by examination of β-galactosidase staining of developing organisms carrying the full-length spiA promoter/lacZ fusions, that this induction is also spatially organized. Staining reveals a gradient of β-galactosidase activity that originates in the upper layer of prespore cells, proximal to the prestalk region, and expands distally as culmination proceeds. We postulate that the progression of the spiA expression gradient through the prespore mass illustrates the movement of an inductive signal for sporulation. Based upon an observed change in opacity in the prespore mass during culmination, Bonner (1944) and Raper and Fennel (1952) had also suggested that sporulation initiated at the prespore/prestalk boundary, but microscopic examination of the progression of sporulation during this time has not been conclusive (Watts and Treffry, 1976).

The expression pattern upon in situ treatment of preculminants with 8-Br-cAMP suggests that all prespore cells are equally responsive and competent to express spiA and to sporulate. Thus, it would appear that sporulation is activated by the progression of a regulatory signal, in contrast to a pre-existing gradient of an intracellular regulatory protein. Since laser ablation of the prestalk cells at the apical papilla during early culmination blocks sporulation (Klaus and George, 1974), there also appears to be a required interaction between prespore and apical cells. Thus, an inductive signal for sporulation may emanate from the apical tip and diffuse into or be relayed through the underlying prespore region. Although it is possible that a repressor of sporulation is dispersed through the prespore region but is least concentrated at the prespore/prestalk boundary, disruption of mid-culminants does not induce sporulation. Unless an activator is also diluted during disaggregation this alternative would be unlikely.

The promoter analyses present an intracellular view of spiA regulation. If expression of spiA were under negative control, as exemplified by the Dictyostelium prestalk-specific ecmB promoter (Ceccarelli et al., 1991), the processive truncation of the promoter would result in constitutive activity. Yet temporal and spatial patterning is retained in all spiA promoter deletions that produce detectable β-galactosidase activity. Since β-galactosidase staining was never observed in prestalk or stalk cells or in prespore cells prior to culmination, spiA does not seem to be regulated by release from repression. Rather, expression appears to be an inductive event. Although we cannot yet assess a potential role for sequences −159 to +261, the data suggest that several sequences from −159 to −301 may collectively mediate this positive regulation (see Fig. 2A). Significant incremental reductions in promoter strength are observed with deletions from −301 to −241, from −225 to −211, and from −193 to −175 (see Table 1 and Fig. 2A). The positions of two potential G-Box half sites (Schnitzler et al., 1994), centered at −211 and −183, correlate with incremental reductions in promoter activity. Many developmentally regulated genes in Dictyostelium require G-Box sites for their high level expression but not for determining their specific temporal and spatial expression patterns (Datta and Firtel, 1987; Haberstroh and Firtel, 1990; Hjorth et al., 1990; Ceccarelli et al., 1992; Schnitzler et al., 1994). The −175 deletion lacks potential G-Box sites but still retains spatial and temporal regulation, suggesting that sequences in the region from −175 to −159 (or beyond) may direct the cell type-specific expression observed with spiA. Further analyses of these regions by mobility shift and nuclease protection assay will allow identification of essential DNA sequences and transcription factors to which they bind. The spiA/lacZ fusion constructs also provide a tool to analyze the interplay of such factors.

Consistent with the conclusion that spiA expression and sporulation are regulated by a PKA-mediated intracellular pathway, we have shown that all constructs that express β-galactosidase during culmination (i.e. −1090 to −175) are activated precociously by 8-Br-cAMP and that the relative level of 8-Br-cAMP-inducible β-galactosidase activity for each construct parallels that observed during culmination. This suggests that induction by 8-Br-cAMP or by the apical inducer during culmination is controlled by the same spiA promoter elements. We have also shown that overexpression of the catalytic subunit in prespore cells will precociously induce spiA mRNA in wild-type cells and rescue expression in gat−/null cells, which normally do not exhibit detectable levels of spiA mRNA (Mann et al., 1994). Since PKA activity appears essential for spiA expression and sporulation, a putative apical inducer may stimulate the accumulation of intracellular cAMP, a direct activator of PKA activity, in prespore cells. Of the potential extracellular effectors released by apical prestalk cells during late development, two small molecules have been identified which may regulate cAMP synthesis; these are extracellular cAMP and ammonia.

In addition to the intracellular function of cAMP, secreted cAMP is a primary, extracellular signalling molecule throughout Dictyostelium development (Kimmel and Firtel, 1991; Johnson et al., 1992a; Saxe et al., 1993). Dictyostelium possess cell surface receptors specific for cAMP that are linked through G proteins to adenyl cyclase (Kimmel and Firtel, 1991; Johnson et al., 1992a). Activation of these receptors by extracellular cAMP leads to an increase in intra-
cellular cAMP. The most active site of cAMP synthesis in the developing organism is the prestalk cells of the apical tip (Bonner, 1949; MacWilliams, 1984). Enhanced cAMP synthesis emanating from apical signalling centers at mid-culmination may initiate a gradient cascade of cAMP signal-relay at the prespore/prestalk boundary that passes into the prespore region leading ultimately to graded activation of PKA, induction of spiA and sporulation. Such increases in extracellular and intracellular levels of cAMP have been observed during sporulation (Abe and Yanagisawa, 1983) and prespore and prestalk cells possess multiple cell surface cAMP receptor subtypes with different affinities for secreted cAMP (Saxe et al., 1991; Johnson et al., 1992b; Saxe et al., 1993; Louis et al., 1994). Whereas 20 mM 8-Br-cAMP will induce spiA expression and sporulation, exogenously added cAMP is ineffective (Kay, 1989; Richardson et al., 1991). Although these results may argue against cAMP as the apical signaling pathway, it must be emphasized that during Dictyostelium development application of a constant level of cAMP (Saxe et al., 1991; Johnson et al., 1992b; Saxe et al., 1993) will inhibit certain receptor-linked signalling pathways and that adenyl cyclase activity may only be activated in an appropriate context of receptor stimulation (Kimmel and Firtel, 1991; Johnson et al., 1992a).

Ammonia, another modulator of cAMP synthesis, is formed metabolically during development (Schindler and Sussman, 1977). Ammonia will inhibit entry of the slug into culmination and loss of ammonia by evaporation may control the timing of culmination (Schindler and Sussman, 1977). Ammonia added during early development has a dramatic inhibitory effect on cAMP synthesis emanating from apical signalling centers at mid-culmination (Schindler and Sussman, 1977; Williams et al., 1984). Thus, a decrease in ammonia at late stages of development may result in an increase in cAMP signalling.

Many developmental events that will not occur in pkaC-null cells will proceed in wild-type Dictyostelium in the absence of adenyl cyclase activation if surface receptors for cAMP (but not PKA) are directly activated (Kimmel, 1987). In addition, acaA-null mutants, which lack adenyl cyclase, will form spores at low frequency when developed in the presence of wild-type cells or stimulated with exogenous cAMP (Pitt et al., 1993). Thus, although sporulation will not normally occur in the absence of an active catalytic subunit of PKA, the accumulation of intracellular cAMP is not absolutely required for sporulation, however inefficient the process. This suggests that, in the absence of cAMP, additional factors may influence sporulation. PKA could be activated during culmination through the disproportionate expression of the catalytic subunit relative to the regulatory subunit (Mann et al., 1994) or by an effector other than cAMP. Alternatively, other signal transduction pathways may be able to substitute partially for PKA activation to induce sporulation.

We have suggested that induction of sporulation in Dictyostelium discoideum is non-autonomous, requiring information encoded in the prestalk region rather than by a program incorporated entirely within prespore cells. Although secreted cAMP (or ammonia) is potentially a critical element activating spore differentiation, sporulation may require additional compounds, either singly or in combination. For example, we have suggested elsewhere that expression of pkaC is induced in prespore cells during culmination (Mann et al., 1994). Our ability to analyze potential effectors of spiA expression by their in situ application to developing strains carrying spiA/lacZ fusions may permit identification of these regulators.

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