
Jo Anne Powell-Coffman* and Richard A. Firtel†

Department of Biology, Center for Molecular Genetics, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92039-0634, USA

*Present address: Department of Molecular, Cellular, and Developmental Biology, University of Colorado, Boulder, CO 80309, USA

†Author for correspondence

**SUMMARY**

While *Dictyostelium discoideum* has been studied as a developmental system for decades, and many regulatory proteins have been cloned, the molecular mechanisms of cell-type-specific gene expression are poorly understood. In this paper we characterize a novel prespore gene, *PspB*, and undertake a comparative analysis of the regulatory regions in prespore-specific *D. discoideum* promoters. Sequence alignment of the *PspB* gene product with other prespore-specific proteins identifies a conserved, repeated 12 amino acid cysteine-containing motif that may be involved in spore coat function or assembly. Analysis of the *PspB* promoter identifies two domains essential for developmentally induced promoter activity. The first region includes two CA-rich elements (CAEs) that we show to be functionally homologous to the cAMP-inducible elements previously identified in the *SP60 (cotC)* promoter. The *PspB* CAEs compete with the *SP60 (cotC)* CAEs for binding in vitro to a developmentally regulated nuclear activity. We identify this activity as G-box Binding Factor, a developmentally induced transcription factor. The *PspB* CAEs and adjacent nucleotides direct a very low level of prespore-enriched expression, but high levels of cell-type-specific expression requires a second promoter region: a 46-bp AT-rich sequence that does not resemble the CAEs or any other previously described late gene promoter elements. Comparison of the *PspB* AT element with regulatory regions of the *SP60 (cotC)*, *SP70 (cotB)*, and *D19 (pspA)* promoters reveals an extensive consensus sequence. We suggest that these AT-rich sequences may represent a common regulatory element (or elements) required for prespore gene activation.

Key words: *Dictyostelium discoideum*, prespore genes, DNA binding protein, cis elements

**INTRODUCTION**

During the course of multicellular development, individual cells must sense and integrate extracellular signals in order to implement appropriate changes in gene expression. The cellular slime mold *Dictyostelium discoideum* has proved to be a useful system for studying this complex problem. When food is plentiful, *Dictyostelium* cells exist as free-living amoebae. However, when their food source is depleted, the cells initiate a program of multicellular development. Approximately $10^5$ cells are recruited into an aggregation center via extracellular cAMP-mediated signalling pathways (see Kimmel and Firtel, 1991 for references) to form a multicellular organism. The developmental program culminates in the formation of a fruiting body, in which a stalk elevates a spore mass approx. 2 mm above the substratum (Raper, 1940).

Cellular differentiation into prestalk and prespore cells begins early in development. As cells are still streaming into the aggregate, prestalk cells within the mound sort towards the forming tip (Krefft et al., 1984; Gomer et al., 1986; Williams et al., 1989; Esch and Firtel, 1991). Once the aggregate is formed, 80% of the cells express prespore markers and do not participate in tip formation (Gomer et al., 1986; Williams et al., 1989; Haberstroh and Firtel, 1990; Fosnaugh and Loomis, 1993). The discovery of new cell-type-specific markers and the analysis of prestalk promoters has revealed that there are at least three prestalk cell types (Jermyn et al., 1987; Early et al., 1993). Furthermore, deletions in the *SP60 (cotC)* promoter and cellular transplantation experiments have revealed some heterogeneity within the prespore population (Haberstroh and Firtel, 1990; Haberstroh et al., 1991; Buhl et al., 1993). However, all prespore markers examined to date are expressed uniformly throughout the prespore zone after the tipped mound is formed (Kreft et al., 1984; Gomer et al., 1986; Williams et al., 1989; Haberstroh et al., 1991; Fosnaugh and Loomis, 1993; Ozaki et al., 1993).

Cell-type differentiation in *Dictyostelium* is modulated by both cell-autonomous factors and diffusible morphogens within the aggregate. While there are no classical maternally provided signals to establish the polarity of the multicellular organism, factors such as a cell’s position in the cell cycle at the time of starvation can predispose it to a cell fate (Weijer et al., 1984; Gomer and Firtel, 1987). However, cells are not irreversibly committed to a stalk or spore cell fate until terminal
differentiation (Raper, 1940). Diffusible morphogens within the aggregate function to differentially activate or repress cell-type differentiation. The induction of prestalk- and prespore-specific genes requires high levels of extracellular cAMP, and cAMP is required to maintain prespore expression (Mehdy et al., 1983; Chisholm et al., 1984). Differentiation inducing factor (DIF), a chlorinated alkyl phenone (Town et al., 1976; Morris et al., 1987), has been shown to induce the expression of certain prestalk markers (Jermy et al., 1987; Williams et al., 1987) and to repress the expression of prespore markers (Early and Williams, 1988; Fosnaugh and Loomis, 1991). Adenosine and ammonia have also been identified as diffusible agents that are capable of influencing development (Schaap and Wang, 1986; Feit et al., 1990).

In recent years, cAMP receptors and several intracellular signalling molecules have been cloned and studied through a combination of molecular genetic and biochemical techniques (reviewed by Cubitt et al., 1992; Johnson et al., 1992; Saxe et al., 1993). Some of these molecules, such as the Gα subunit Gt64 and Dictyostelium protein kinase A, have been shown to play essential roles in directing morphogenesis and cellular differentiation (Mann and Firtel, 1991, 1993; Anjard et al., 1992; Hadwiger and Firtel, 1992; Harwood et al., 1992a,b; Mann et al., 1992; Simon et al., 1992; Hopper et al., 1993).

Relatively little is known about how these signals are ultimately integrated at the level of individual cell-type-specific promoters. To understand these events, we must first analyze co-regulated genes and identify the promoter elements and associated nuclear factors that implement specific developmental programs. In this study, we have cloned a novel prespore-specific gene, PspB, and have mapped the cis-acting promoter sequences required for its expression. Our analysis has identified sequences common to regulatory regions in multiple prespore genes that include CA-rich repeats (CAEs) and an AT-rich element essential for PspB promoter function. A fragment containing both the PspB CAEs and the AT element is capable of conferring high levels of prespore-specific expression to a heterologous promoter. Furthermore, we show that the CA-rich elements in the PspB and SP60 (cotC) promoters compete for binding to G-box Binding Factor, a developmentally induced transcription factor, in vitro.

MATERIALS AND METHODS

Growth and development of Dictyostelium

Axenically grown KAx3 cells were used in these studies (Sussman, 1987).

Vegetative Dictyostelium cells were transformed by electroporation, and stable transformants were selected using G418 at a concentration of 15 µg/ml (Howard et al., 1988; Dynes and Firtel, 1989). Once cell lines were established, they were maintained at 25 µg/ml of G418.

To initiate multicellular development, log phase vegetative cells were harvested by centrifugation, washed, and plated on Whatman 50 filters suspended over 12 mM phosphate buffer, as described previously (Haberstroh and Firtel, 1990).

RNA and DNA analysis

To determine the relative copy number of plasmids in stably transformed cell lines, DNA was isolated and analyzed by Southern blot analysis (Nellen et al., 1987).

For developmental RNA blots, RNA was extracted and sized on denaturing gels, blotted to membrane filters, and hybridized to random-primed probes, as described previously (Powell et al., 1992).

The polarity of transcription was determined with strand-specific RNA probes. The 14E6 cDNA was cloned into the PstI site of pSP73, and T7 and SP6 RNA polymerase-derived probes were generated (Haberstroh and Firtel, 1990). These were hybridized separately to blots containing total cellular RNA isolated at different times in development.

The S′ end of the PspB transcript was determined by primer extension as described in Calzone et al. (1987), with the following exceptions: 25 fmol of the oligo S′-GAAACTTATAATGCTT-CATT was incubated with 15 µg total cellular RNA at an annealing temperature of 42°C. The dATP and dTTP concentrations were increased to 250 µM in the extension reaction.

The DNA sequence of the genomic and cDNA subclones was determined by dideoxy DNA sequencing with modified T7 DNA polymerase (United States Biochemical).

Initial sequence analysis computation was performed at the NCBI using the BLAST network service.

Cloning of PspB

Genomic clones that hybridized to the 14E6 cDNA were isolated by screening a library consisting of genomic fragments partially digested with Sau3A cloned into the BamHI site of pAT153L (Haberstroh and Firtel, 1990). One of the genomic clones had a 5.2 kb insert and included the entire coding region and 1.6 kb of S′ flanking sequence. This clone was used for all subsequent subcloning and sequencing. The genomic clone has been named PspB and has been mapped to chromosome I of the Dictyostelium genome (Kuspa et al., 1992).

Expression vectors and deletions

The S′ flanking sequence and 51 bp of coding sequence was excised from the genomic clone as an Xbal-Asp718 fragment. EoxIII was used to make 3′ deletions from the Asp718 site in the PspB coding region. These deletions were HindIII linker-capped, cloned and sequenced. A construct that included 23 bp of coding sequence was used as starting material for the luciferase and β-galactosidase expression vectors. The promoter fragment was then ligated (Xbal-HindIII) into pSPSpe, a pSP72 vector in which an SpeI linker has been cloned into the Smal site. The promoter and 23 bp of the coding region was then cut out with BglII and HindIII and cloned into the β-galactosidase and luciferase expression vectors described previously (Haberstroh and Firtel, 1990). The HindIII site of the luciferase vector was filled-in and a linker adaptor was added to add a BamHI site and facilitate moving promoters between the two constructs. The sequences of the resultant PspB fusion genes are:

ATG AGA CTA TTA AGT GTT CTT TTC AAG CTA GAG GAT

For developmental RNA blots, RNA was extracted and sized on denaturing gels, blotted to membrane filters, and hybridized to random-primed probes, as described previously (Powell et al., 1992).

The polarity of transcription was determined with strand-specific RNA probes. The 14E6 cDNA was cloned into the PstI site of pSP73, and T7 and SP6 RNA polymerase-derived probes were generated (Haberstroh and Firtel, 1990). These were hybridized separately to blots containing total cellular RNA isolated at different times in development.

The S′ end of the PspB transcript was determined by primer extension as described in Calzone et al. (1987), with the following exceptions: 25 fmol of the oligo S′-GAAACTTATAATGCTT-CATT was incubated with 15 µg total cellular RNA at an annealing temperature of 42°C. The dATP and dTTP concentrations were increased to 250 µM in the extension reaction.

The DNA sequence of the genomic and cDNA subclones was determined by dideoxy DNA sequencing with modified T7 DNA polymerase (United States Biochemical).

Initial sequence analysis computation was performed at the NCBI using the BLAST network service.

Cloning of PspB

Genomic clones that hybridized to the 14E6 cDNA were isolated by screening a library consisting of genomic fragments partially digested with Sau3A cloned into the BamHI site of pAT153L (Haberstroh and Firtel, 1990). One of the genomic clones had a 5.2 kb insert and included the entire coding region and 1.6 kb of S′ flanking sequence. This clone was used for all subsequent subcloning and sequencing. The genomic clone has been named PspB and has been mapped to chromosome I of the Dictyostelium genome (Kuspa et al., 1992).

Expression vectors and deletions

The S′ flanking sequence and 51 bp of coding sequence was excised from the genomic clone as an Xbal-Asp718 fragment. EoxIII was used to make 3′ deletions from the Asp718 site in the PspB coding region. These deletions were HindIII linker-capped, cloned and sequenced. A construct that included 23 bp of coding sequence was used as starting material for the luciferase and β-galactosidase expression vectors. The promoter fragment was then ligated (Xbal-HindIII) into pSPSpe, a pSP72 vector in which an SpeI linker has been cloned into the Smal site. The promoter and 23 bp of the coding region was then cut out with BglII and HindIII and cloned into the β-galactosidase and luciferase expression vectors described previously (Haberstroh and Firtel, 1990). The HindIII site of the luciferase vector was filled-in and a linker adaptor was added to add a BamHI site and facilitate moving promoters between the two constructs. The sequences of the resultant PspB fusion genes are:

ATG AGA CTA TTA AGT GTT CTT TTC AAG CTA GAG GAT

CCC GTC GTT-β-GAL

ATG AGA CTA TTA AGT GTT CTT TTC AAG CTA GAG GAT

CCA GGT CTA GCT-Luciferase

EoxIII digestion was used to generate 5′ promoter deletions, which were capped with SpeI linkers. Once 5′Δ59 was determined to be the parental promoter, it was cloned (BamHI, SpeI) into the BamHI and Xbal sites of pSP72. Further 5′ deletions were done in this construct, and SpeI linkers were added. The deleted promoters were then cloned into the BamHI and SpeI sites of the expression vectors. For 3′ deletions, the Δ59 promoter was cut out of the expression vector as a BglII, BamHI fragment and cloned into the BamHI and BglII sites of pSP73. The SpeI site was then filled-in with Klenow. 3′ deletions were generated with EoxIII, and SpeI linkers were added. Internally deleted promoters constructs were made by ligating the 3′ deleted promoters into the BglII and SpeI sites immediately upstream of a 5′ deleted promoter in an expression vector.

β-galactosidase staining

Cells were plated for development on white Millipore filters. At
appropriate times, the organisms were stained for β-galactosidase activity using a modification of previously described procedures (Dingermann et al., 1989; Haberstroh et al., 1990). Filters were allowed to air dry for 1 minute and then placed on Whatman filters saturated with 0.5% glutaraldehyde, 0.05% Triton X-100 in Z buffer. After 5 minutes, additional fixation solution was added to the filters to immerse the organisms for 10 minutes. The cells were washed in Z buffer and stained with X-gal solution (1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, and 0.5 mg/ml X-gal in Z buffer) for up to 3 hours at 37°C.

**Luciferase activity measurement**

Luciferase activity was determined as previously described (Howard et al., 1988; Haberstroh and Firtel, 1990). All relative expression data are mean values from at least two populations of stable transformants, each assayed twice. All values were normalized for the copy number of the vector, as determined by Southern blot hybridization. Copy numbers usually varied by less than two-fold.

**In vitro DNA binding studies**

Nuclear extracts were prepared and gel retardation assays were performed as described previously (Haberstroh et al., 1991). The purification of GFB is described in Schnitzler et al. (1994), and the GFB preparations used in Fig. 7B were prepared by oligonucleotide and DEAE affinity chromatography.

The sequences of the oligos containing the proximal and distal CAEs were: 5′-ACTAGTTCATAAAAAAACACACTACA-ATTTCACAAAAATCTAGTA and 5′-GACTAGTGAATATATATACAAAAACACACTTTACACACTGCTCTAGA, respectively. The sequences of the SP60 CAE-containing oligonucleotides used in competition studies are described in Haberstroh et al. (1991).

**RESULTS**

**Isolation and structural characterization of *PspB***

The 14E6 cDNA was originally identified by Mehdy et al. (1983) in a screen for cDNAs that preferentially hybridized to mRNA from a prespore-enriched cell population. The message complementary to the 14E6 cDNA was shown to be approx. 2 kb and to be induced late in development and in response to high levels of extracellular cAMP. In order to study the regulation of this prespore-specific gene, we used the cDNA to isolate a genomic clone (designated *PspB*) (see Materials and Methods). The polarity of transcription was determined with strand-specific RNA hybridization probes (see Materials and Methods). The 5′ endpoint of the transcript was determined by primer extension and is indicated in Fig. 1.

The entire 14E6 cDNA and approx. 2 kb of the genomic clone (including the protein coding region and 5′ flanking sequence) were sequenced. The cDNA included the entire open reading frame and a short poly(A) tail, and the open reading frame in the cDNA was collinear with that in the genomic clone (see Fig. 1 for sequence information). The difference in size of the mRNA as detected on RNA blots and the cDNA is presumed to be due to the 3′ poly(A) sequences. The predicted relative molecular mass of the *PspB* gene product is 43x10^3, similar to that predicted for the primary products of *Dicystostelium* spore coat proteins (Fosnaugh and Loomis, 1989b).

**Fig. 1.** Sequence of the *PspB* genomic coding region comprising the protein coding region, 5′ untranslated region, and 560 bp of sequence 5′ to the transcriptional start. The asterisk represents a TAA stop codon. The primary transcriptional start site is labelled +1, and minor start sites, as determined by primer extension, are in bold. Deletion endpoints used in these studies are also indicated. The promoter sequences similar to the *SP60* (cauC) CA-rich elements (CAEs) are boxed, and the AT-rich regulatory region is underlined. The CDNXXPCPXGYXGC in the derived protein sequence are also boxed.
**Fig. 2.** Alignment of cysteine repeats in prespore gene products. The amino acids that are absolutely conserved are boxed. The amino acid residues present in at least one third of the repeats are also listed in the consensus sequence and are underlined within each repeat.

PSPB includes a hydrophobic N terminus, which probably targets the protein to the secretion pathway. Otherwise, the protein is relatively hydrophilic. The PspB gene product shares regions of homology with the four prespore proteins SP60 (CotC), SP70 (CotB), SP96 (CotA) and DP87 (Fosnaugh and Loomis, 1989). Fosnaugh and Loomis (1989b) noted that the spore coat proteins SP60 (CotC), SP70 (CotB), and SP96 (CotA) each included variants of the amino acid sequence CPEGCHECK and termed this the ‘prespore motif’. Upon alignment of the PSPB sequence and the DP87 sequence (Ozaki et al., 1993) with those of the three spore coat proteins, we were able to revise and expand this prespore consensus to CDNVXCPXGYXC, with absolute conservation at the cysteine and proline positions (Fig. 2) (see Discussion). This motif is present three times in PSPB, SP60 (CotC), and SP70 (CotB), four times in SP96 (CotA), and seven times in DP87. To investigate further the function of the PspB gene product, the gene was disrupted by inserting a Thy1 marker (Dynes and Firtel, 1989) in the EcoRV site (at 780 bp in Fig. 1), generating a truncated transcript. This cell line developed normally and produced heat-resistant spores (data not shown).

**Temporal and spatial regulation of PspB expression**

As shown in Fig. 3A, PspB expression is temporally coregulated with that of SP60 (cotC), which encodes a spore coat protein and has been shown to be expressed exclusively in prespore cells (Haberstroh and Firtel, 1990). To determine whether PspB expression was also prespore specific, 1.6 kb of sequence 5' to the ATG and 23 bp of protein coding sequence were fused to luciferase and β-galactosidase reporter genes (see Materials and Methods). This promoter region included all the information required to direct proper temporal expression (Fig. 3B), and expression of the reporter gene was prespore-specific (data not shown; see below). Preliminary 5' promoter deletions were constructed and analyzed for luciferase expression. The promoter was deleted to 560 bp 5' of the transcriptional start site (5'Δ59) with only a four-fold reduction in expression levels, representing a 10^5-fold developmental induction in reporter gene expression. Since the region downstream of 5'Δ59 was sufficient for such a high percentage of the total promoter expression, we focussed our studies on these regulatory sequences.

The β-galactosidase expression pattern directed by the 5'Δ59 construct is also prespore-specific, as shown in Fig. 4. Expression is first detected in cells scattered throughout the mound. As PspB is induced and the number of staining cells increases, a central region of non-staining cells can be seen in many aggregates (Fig. 4A,B). SP60/β-gal expression is also initially excluded from the center of many loose mounds (Haberstroh and Firtel, 1990), but the prespore marker SP70(cotB)/β-gal is reported to be expressed uniformly in all early aggregates (Fosnaugh and Loomis, 1993). PspB/β-gal expression levels and staining intensity increase greatly as a ‘tight mound’ forms and a tip emerges (Fig. 4C). The tip elongates upwards to form a ‘first finger’ which, under appropriate conditions, falls over to form a pseudoplasmodium, or slug. The prespore (PspB/β-gal-expressing) cells are restricted to the posterior 80% of the organism (Fig. 4D). The slug migrates to a place suitable for culmination, where it forms a mound, which extends upwards to form a ‘second finger’. The
tip cells then funnel down through the forming spore mass and differentiate into the stalk tube, lifting the spore mass off the substratum. In the slug and in the culminants, fewer than ten PspB/β-gal-expressing cells are ever detected in the tip or in the upper and lower cups of the culminant, while the prespore region stains intensely (Fig. 4D,E). This latter expression pattern is indistinguishable from that of SP60 (cotC) (Haberstroh and Firtel, 1990; data not shown) and is similar to that described for the prespore genes D19 (pspA), SP70 (cotB), and Dp87 (Williams et al., 1989; Fosnaugh and Loomis, 1993; Ozaki et al., 1993).

**Identification of cis-acting regulatory elements**

The only elements previously defined by point mutations or small deletions in a prespore-specific promoter are the three CA-rich elements in the SP60 (cotC) promoter. Deletion or mutation of any of the SP60 CAEs decreases promoter activity 10- to 25-fold (Haberstroh et al., 1991). There are two regions in the PspB 5′ flanking sequence that are very similar to the SP60 (cotC) CAEs (boxed in Fig. 1).

To identify the cis-acting regulatory regions in the PspB promoter downstream of 5′Δ59, a series of 5′ promoter deletions were constructed (see Materials and Methods). For each deletion, PspB/luciferase fusions were assayed to quantitate developmentally induced promoter expression, and PspB/lacZ fusions were used to determine the spatial pattern of expression. The deletion endpoints are indicated in Fig. 1, and the data are summarized in Fig. 5A.

While an 89-bp deletion to 5′Δ19 results in only a further 2-fold reduction in promoter activity, a deletion of another 18 bp to 5′Δ60, which eliminates part of the distal CAE, results in a 100-fold further reduction in promoter activity. The spatial pattern of expression, as determined by β-galactosidase staining, is unchanged, although the intensity of staining is substantially reduced (Fig. 6A). The 10 bp between 5′Δ60 and 5′Δ14 can be deleted with little effect, but a further 5′ deletion to 5′Δ55, which removes half of the proximal CAE, reduces expression levels another 50-fold (Fig. 5A). The activity of the 5′Δ55 promoter is too low to be detected by β-galactosidase staining.

To analyze cis-acting regions downstream of 5′Δ55, we constructed a series of internally deleted promoters. These data are presented in Fig. 5B and the relative levels of expression are compared to 5′Δ59, which is the parental promoter in which the internal deletions were made. In Δ66-Δ44, 65 bp are deleted, and expression levels drop by 1000-fold (Fig. 5A). β-galactosidase expression is almost undetectable by whole-mount X-gal staining, but expression appears to be enriched in the prespore zone (Fig. 6B). The overlapping deletion Δ62-Δ83 only reduces promoter activity 6-fold and does not change the expression pattern (Fig. 5B; data not shown). Taken together, these data indicate that the 46 bp between 5′Δ83 and 5′Δ44 (underlined in Fig. 2; ‘pspAT’ in Fig. 5B) contain sequences essential for high levels of PspB promoter activity.

The 302 bp between 3′Δ27 and 5′Δ22 (Δ27-Δ22) can be deleted with only a 10-fold reduction in promoter activity. This
deletion removes the endogenous start site, and without further studies to compare the efficiencies of the endogenous and alternative start site(s), it is difficult to quantitatively compare the expression levels of these promoters to the parental promoter. However, these data indicated that promoter sequences between 5′Δ27 and 5′Δ59 were sufficient to direct high levels of transcription. To further test this, we cloned this region into the Actin15Δβgal minimal promoter (Early and Williams, 1989; Ceccarelli et al., 1991) to create Act15-EΔ27. As shown in Fig. 6C, cells carrying this construct expressed β-galactosidase specifically in prespore cells. A 3′ deletion to 3′Δ34 (construct Act15-EΔ34) decreased β-galactosidase expression to almost undetectable levels (compare staining intensities in Fig 6C and 6D), but expression was prespore-enriched. The extent to which expression is cell-type specific is difficult to ascertain because of the low level of expression.

**CAE-binding nuclear activity**

The three SP60/cotC CAEs have been shown to specifically bind a developmentally regulated nuclear activity with differential affinities, and point mutations that decrease the binding affinities of SP60 (cotC) CAE1 and CAE2 have been shown to decrease promoter activity in vivo (Haberstroh et al., 1991). To determine whether this activity also interacted with the PspB CAEs in vitro, we incubated oligos containing PspB CAEs with nuclear extracts from a series of developmental time points. As shown in Fig. 7A, both PspB CAEs form a complex with a developmentally induced binding activity,
although the distal CAE binds with much lower affinity than the proximal CAE. The PspB CAE-binding activity and the SP60 (cotC) CAE-binding activity are induced at the same stage of development and give similar relative mobility shifts in gel retardation assays (Haberstroh and Firtel, 1990 and Fig. 7A). Competition studies show that the SP60 (cotC) CAEs compete for binding to the PspB CAE binding activity (Fig. 7B). When 2 µg of 9-hour nuclear extract is incubated with 0.1 ng of labelled PspB proximal CAE probe, approximately 15% of the probe is bound to the developmentally induced activity. The labelled probe is substantially competed out of the complex by the addition of 50-fold molar excess unlabelled PspB proximal CAE or 250-fold molar excess PspB distal CAE. The SP60 (cotC) CAE1 competes for binding more effectively than either of the PspB CAEs. However, 50-fold mutant CAE1, which contains point mutations that decrease CAE1 function in vivo, does not detectably compete for binding. The affinity of SP60 (cotC) CAE3 for the activity is intermediate to that of the two PspB CAEs (Fig. 7B). Thus, the order of binding affinities of the CAEs to the developmentally induced binding activity is SP60 (cotC) > PspB proximal > SP60 (cotC) 2 > SP60 (cotC) 3 > PspB distal.

The SP60 (cotC) and PspB CAE consensus sequences (see Fig. 8A) resemble a G-box Binding Factor (GBF) binding site (Hjorth et al., 1990). GBF binding sites have been described in multiple developmentally regulated promoters, some of which are prestalk-specific and others whose spatial patterns of expression are not known (Hjorth et al., 1989, 1990; Cecarelli et al., 1992). GBF, like the CAE binding activity, is induced during development and in response to extracellular cAMP (Hjorth et al., 1989). Since GBF has recently been purified (Schnitzler et al., 1994), we were able to test whether GBF binds the PspB CAEs. As shown in Figs 3 and 7B, when the proximal CAE is incubated with highly purified GBF, a complex is formed that migrates at the same position as the complex that formed in crude extracts, indicating that the developmentally induced nuclear activity is GBF.

**DISCUSSION**

We have cloned a novel prespore-specific gene, PspB, and have shown its expression pattern to be similar to that described for other prespore markers (Krefft et al., 1984; Gomer et al., 1986; Williams et al., 1989; Haberstroh and Firtel, 1990; Fosnaugh and Loomis, 1993). While Dp87 is expressed in aggregation streams (Ozaki et al., 1993), PspB/β-gal is not detectably expressed until after the cells enter the mound, similar to the SP60/cotC pattern of expression. As the prespore genes D19/pspA, SP60/cotC, SP70/cotB, Dp87, and PspB are expressed in similar spatial patterns in late development, comparative analysis of the essential sequences in PspB
and the promoter regions of other prespore-specific genes should reveal common regulatory elements.

Sequence alignment of the prespore gene products reveals a striking conservation of the cysteine repeat CDNVXCPXGYCE, with absolute conservation at the cysteine and proline positions. This motif is present multiple times in PSPB, SP60 (cotC), SP70 (cotB), SP96 (cotA), and DP87. Interestingly, PsA, a prespore cell surface glycoprotein encoded by D19/pspA does not include any related sequence (Early et al., 1988), and the motif is present only once in the prespore EB4 protein (Hildebrandt et al., 1991a). Neither PsA or EB4 is thought to be involved in spore coat assembly. Although EB4 is packaged in prespore vesicles with the spore coat proteins, the protein is not found in mature spores (Devine et al., 1983; Hildebrandt et al., 1991b). Thus, the CDNVXCPXGYCE repeats may function in the assembly of the inner and outer spore coats.

We have analyzed the PspB promoter regions required to direct proper temporal and spatial expression. Our analysis identifies two regions of the promoter that are essential to direct high levels of cell-type-specific transcription: (1) the 74 bp between 5Δ19 and 3Δ62, and (2) the 46 bp between 5Δ83 and 5Δ44. The first region includes two CA-rich elements (CAEs) very similar to the SP60 (cotC) CAEs. 5′ deletions indicate that both elements are important, and a deletion that removes both CAEs decreases expression 104-fold, such that β-galactosidase expression is not detectable. The second essential promoter region (pspAT) is AT-rich. Deletion of pspAT (as in Δ66-Δ44) is catastrophic to the promoter, reducing promoter activity by >1000-fold. No other regions of the promoter were found to be essential. Removal of the sequences between the CAEs and the pspAT element (in Δ62-Δ83) only reduces promoter activity 6-fold. Furthermore, the region downstream of the pspAT (and 3Δ27) is not required for high levels of prespore-specific expression, as seen in the Δ27-Δ22 internal deletion and the Act15-E Δ27 construct. The expression patterns directed by all of the deleted promoters were assayed in lacZ fusion constructs, and none of the deletions resulted in a novel expression pattern.

CA-rich sequences have been noted in the regulatory regions of several prespore genes (Haberstroh and Firtel, 1990; Tasaka et al., 1992; Fosnaugh and Loomis, 1993; Ozaki et al., 1993).
We, therefore, believe that the \( PspB \) CAEs are functionally homologous to the \( SP60 (cotC) \) CAEs.

The AT-rich sequences between \( 5'\Delta83 \) and \( 5'\Delta44 \) represent a previously undescribed promoter element (or elements). However, given its importance in \( PspB \) promoter activation, we hypothesized that equivalent cis-element(s) might be found in other prespore genes. Concurrent to this study, we defined the region of the \( SP60 (cotC) \) promoter required to confer prespore-specific expression to a minimal promoter and found that both a CAE and a 37-bp AT-rich region are required for this regulatory function (Powell-Coffman et al., unpublished data). Alignment of the \( SP60 (cotC) \) AT element with the \( PspB \) pspAT reveals striking similarities (Fig. 8B). Both regions include TTTGTAR (\( n = 11 \) or 12) ATTTAA, followed by an interrupted T stretch. The promoters of other prespore genes, \( D19 (pspA) \) and \( SP70 (cotB) \), have been characterized by \( 5' \) and \( 3' \) deletions (Early and Williams, 1989; Fosnaugh and Loomis, 1993). We searched regions of the promoters that had been defined as essential by deletion analysis for similar elements and found the pspAT consensus in both promoters (Fig. 8B). A 107-bp deletion of the \( SP70 (cotB) \) promoter that is responsible for a 150-fold decrease in promoter activity includes a pspAT consensus sequence (in the opposite orientation). In the \( D19 (pspA) \) promoter, a 47-bp \( 5' \) deletion that interrupts a pspAT consensus sequence decreases expression below levels detectable by RNA blot analysis. Interestingly, the \( D19 (pspA) \) pspAT upstream sequences (TTTGTAR) are in the opposite orientation of the downstream region, although the spacing between the two is conserved. Fosnaugh and Loomis (1993) have shown that two separable regions of the \( SP70 (cotB) \) promoter are each capable of directing prespore expression. The distal \( SP70 (cotB) \) regulatory domain and the \( SP96 (cotA) \) and \( Dp87 \) promoters each contain variants of the pspAT consensus. However, we have not included these sequences in Fig. 8B because, as of yet, the deletion analysis is not extensive enough to provide evidence for their functional significance. Further analysis of these promoters will determine the regulatory importance of these AT-rich sequences and may help to identify some characteristics of the pspAT consensus which permit variability.

It is intriguing to note that the pspAT downstream sequence shares similarities with the binding sites for the \( Drosophila \) homeobox proteins engrailed (TCAAATTAAAT) or even-skipped (CAATTAAT) (Desplan et al., 1988; Hoey and Levine, 1988). Approximately 90% of the nucleotides in \( Dicyostelium \) noncoding regions are AT (Kimmel and Firtel, 1982), so specificity of binding to homebox-like sequences may be problematic. Cooperative binding with factors that recognize neighboring sequences (TTGTAR or CAEs) may provide that specificity.

As the the pspAT element is absolutely required for physiological levels of transcriptional activation, the levels of expression from promoters lacking this region (\( \Delta66-44 \) or the Act15-E\( \Delta34 \) construct) is extremely low. Consequently, it is difficult to discern the exact specificity of expression directed by these promoters. However, \( \beta \)-galactosidase expression in the \( \Delta66-\Delta44 \) and Act15-E\( \Delta34 \) transformants is at least prespore-enriched (Fig. 6A,B). This indicates that the factors that interact with the sequences upstream of \( 3'\Delta34 \) are sufficient to direct prespore-enriched expression. We have recently demonstrated that GBF mRNA and GBF activity is 2.5-fold
enriched in prespore cells (Schnitzler et al., 1994) and this may contribute to the spatial pattern of expression directed by this CAE-containing domain. Since GBF binding sites are required for the expression on both prespore and prestalk genes (see above), GBF alone presumably does not specify cell-type specific expression. We expect that sequences immediately adjacent to or within the CAEs or in other regions of the promoter promote the binding of prespore-specific complex in the PspB and SP60 (cotC) promoters, while other factors, in combination with GBF, activate promoters in prestalk cells. Consistent with this model, we have shown that CAEs from SP60 (cotC) can replace the G-box (GBF binding site) in the prestalk-enriched gene CP2/pst-cath. This restores developmental and cAMP-induced expression and the expression remains prestalk-enriched, indicating GBF, in itself, does not specify cell-type specific expression (J. Cao, J. A. P.-C., C. Gaskins and R. A. F., unpublished data) Others have noted that the sequences ACACCCAC and CACAC are present in the Gaskins and R. A. F., unpublished data) Others have noted that the sequences ACACCCAC and CACAC are present in the regulation regions of multiple prespore promoters (Fosnaugh and Loomis, 1993; Tasaka et al., 1992). Future studies will examine whether these or other sequences are required to restrict GBF-mediated activation of PspB to prespore cells. Analysis of GBF regulation and the factors that bind the prespore AT elements will enable us to understand better how prespore differentiation is initiated and controlled.

We are grateful to Gavin Schnitzler, who generously provided partially purified GBF, and to Linda Haberstroh, who took half the time points for the nuclear extract developmental time course. We also thank Su Dharmawardhane, Clark Coffman, Gavin Schnitzler, Alexa Clark and Jennifer Roth for critical reading of this manuscript. This work was supported by USPHS grants to R. A. F.

REFERENCES


1610 J. A. Powell-Coffman and R. A. Firtel


(Accepted 25 February 1994)