# Characterization of a novel *Dictyostelium discoideum* prespore-specific gene, *PspB*, reveals conserved regulatory sequences

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## 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 (Krefft 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 (Jermyn 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 Gα4 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 *Pst*I 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 5′ end of the *PspB* transcript was determined by primer extension as described in Calzone et al. (1987), with the following exceptions: 25 fmole of the oligo 5′-GAACACTTAATAGTCT-CATTC 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 *Sau*3A cloned into the *Bam*HI 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 5′ 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 5′ flanking sequence and 51 bp of coding sequence was excised from the genomic clone as an *Xba*I-*Asp*718 fragment. *Exo*III was used to make 3′ deletions from the *Asp*718 site in the *PspB* coding region. These deletions were *Hin*dIII 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 (*Xba*I-*Hin*dIII) into pSPSpe, a pSP72 vector in which an *Spe*I linker has been cloned into the *Sma*I site. The promoter and 23 bp of the coding region was then cut out with *Bgl*II and *Hin*dIII and cloned into the β-galactosidase and luciferase expression vectors described previously (Haberstroh and Firtel, 1990). The *Hin*dIII site of the luciferase vector was filled-in and a linker adaptor was added to add a *Bam*HI 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.

*Exo*III digestion was used to generate 5′ promoter deletions, which were capped with *Spe*I linkers. Once 5′Δ59 was determined to be the parental promoter, it was cloned (*Bam*HI, *Spe*I) into the *Bam*HI and *Xba*I sites of pSP72. Further 5′ deletions were done in this construct, and *Spe*I linkers were added. The deleted promoters were then cloned into the *Bam*HI and *Spe*I sites of the expression vectors. For 3′ deletions, the Δ59 promoter was cut out of the expression vector as a *Bgl*II, *Bam*HI fragment and cloned into the *Bam*HI and *Bgl*II sites of pSP73. The *Spe*I site was then filled-in with Klenow. 3′ deletions were generated with *Exo*III, and *Spe*I linkers were added. Internally deleted promoters constructs were made by ligating the 3′ deleted promoters into the *Bgl*II and *Spe*I 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 GBF is described in Schnitzler et al. (1994), and the GBF 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′-ACTAGTTCATAAACCCACTCACA-ATTTCACACAAATAAAATCTAGA and 5′-GACTAGTAATAAAAAAC-ACACACATTTACCACACTATAGCT-CTAGA, 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 colinear 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 $43 \times 10^3$, similar to that predicted for the primary products of *Dictyostelium* spore coat proteins (Fosnaugh and Loomis, 1989b).
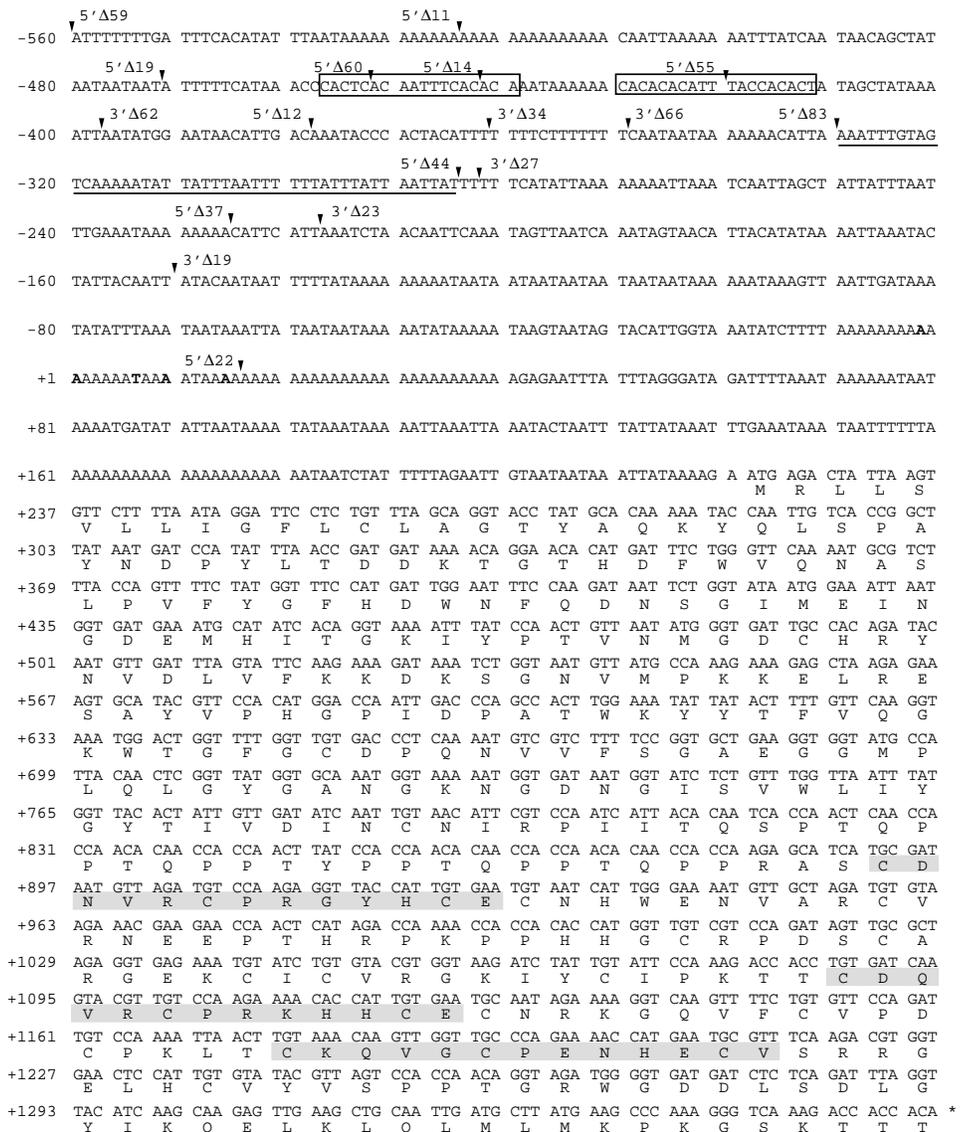


**Fig. 1.** Sequence of the *PspB* genomic sequence comprising the protein coding region, 5′ untranslated region, and 560 bp of sequence 5′ to the transcriptional start. The asterisk represents a TAA stop codon. The primary transcriptional start site is labelled +1, and minor start sites, as determined by primer extension, are in bold. Deletion endpoints used in these studies are also indicated. The promoter sequences similar to the *SP60 (cotC)* CA-rich elements (CAEs) are boxed, and the AT-rich regulatory region is underlined. The CDNVXCPXGYXC in the derived protein sequence are also boxed.

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PSPB        C|D N V R|C P|R G Y H|C E|
            C|D Q V R|C P|R K H H|C E|
            C|K Q V G|C P|E N H E|C V|

SP60/CotC   C|D N V H|C P|K G F N|C K|
            C|S T L Q|C P|P R Y H|C E|
            C|D N T R|C P|P N Y H|C I|

SP70/CotB   C|D K V N|C P|N G Y I|C T|
            C|G N V N|C P|R G Y H|C E|
            C|K T V Q|C P|K G Y K|C K|

SP96/CotA   C|S N T Q|C P|N G F Y|C Q|
            C|D T V Q|C P|Y G Y S|C E|
            C|D N V H|C P|R G Y K|C N|
            C|R N I Q|C P|T G Y R|C E|

DP87        C|D R M R|C P|E G F Y|C E|
            C|G N I Q|C P|W G H Y|C V|
            C|R N Q H|C P|H G Y S|C R|
            C|R G F G|C P|E G S H|C E|
            C|N Q T R|C P|H D Y Q|C E|
            C|K W H R|C P|P G S S|C F|
            C|K V T Q|C P|T D F S|C K|

consensus   C|D N V X|C P|X G Y X|C E|
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**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, 1989a,b; Haberstroh and Firtel, 1990; Ozaki et al., 1993). 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 CPEGHECK 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 *Eco*RV 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 co-regulated 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
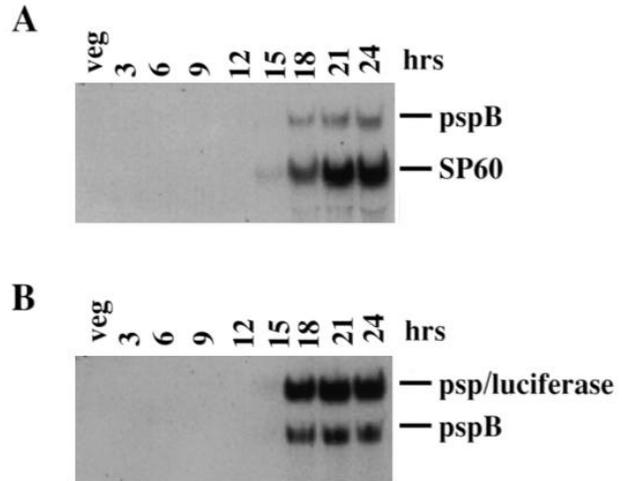


**Fig. 3.** Developmental time course of *PspB* and *SP60 (cotC)* mRNA expression and expression of *PspB/luciferase* fusion gene. RNA was isolated from wild-type Ax-3 cells (A) and from cells transformed with the *PspB/luciferase* fusion gene (including 1.6 kb of 5′ flanking sequence; B) at 3-hourly intervals during development. The RNA blots were probed with the *14E6* and *SP60* cDNAs (A) or the *14E6* cDNA and luciferase coding sequence (B). The development of the cells in this experiment was slightly delayed. In other experiments the prespore genes are often induced after 12 to 15 hours of starvation.

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
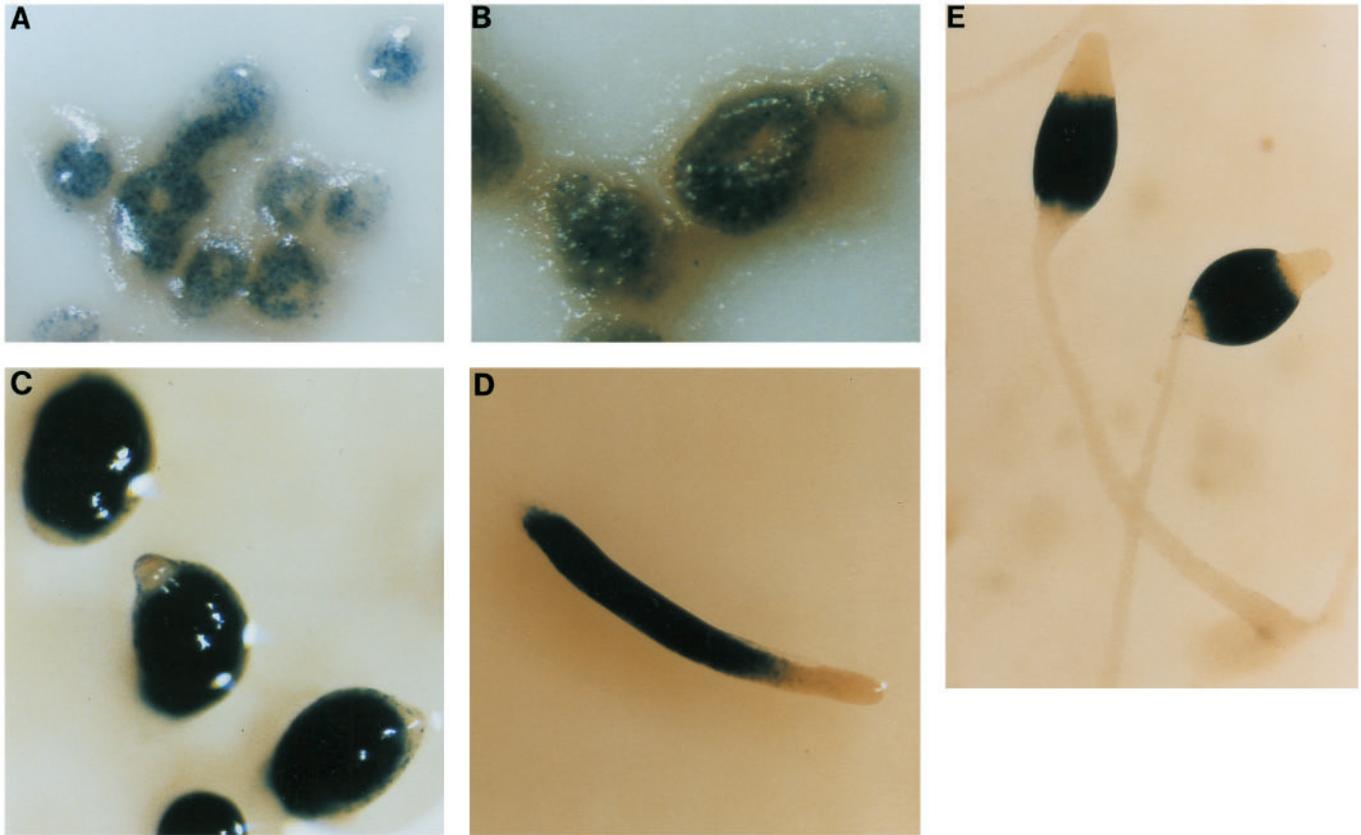
**Fig. 4.** Cytological staining of cells transformed with the 5′Δ59 *PspB/lacZ* construct. Early aggregate (A,B), tipped mound (C), slug (D) and culminant (E) stages are shown. The magnification of B is four-fold greater than that of A.

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 wholemount 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

**A**

### pspB promoter deletions

| | expression level | prespore expression |
|---|---|---|
| 1.4 kb | 100 % | + |
| 5'Δ59 | 25 ± 11 | + |
| 5'Δ11 | 19 ± 6 | + |
| 5'Δ19 | 11 ± 5 | + |
| 5'Δ60 | 0.11 ± 0.03 | + |
| 5'Δ14 | 0.06 ± 0.01 | +/- |
| 5'Δ55 | 0.0011 ± 0.0003 | - |
| 5'Δ12 | 0.005 ± 0.004 | - |
| 5'Δ83 | 0.0003 ± 0.00005 | - |
| 5'Δ44 | 0.0006 ± 0.0004 | - |
| 5'Δ37 | 0.0004 ± 0.00004 | - |
| 5'Δ22 | 0.00009 ± 0.000055 | - |

**B**

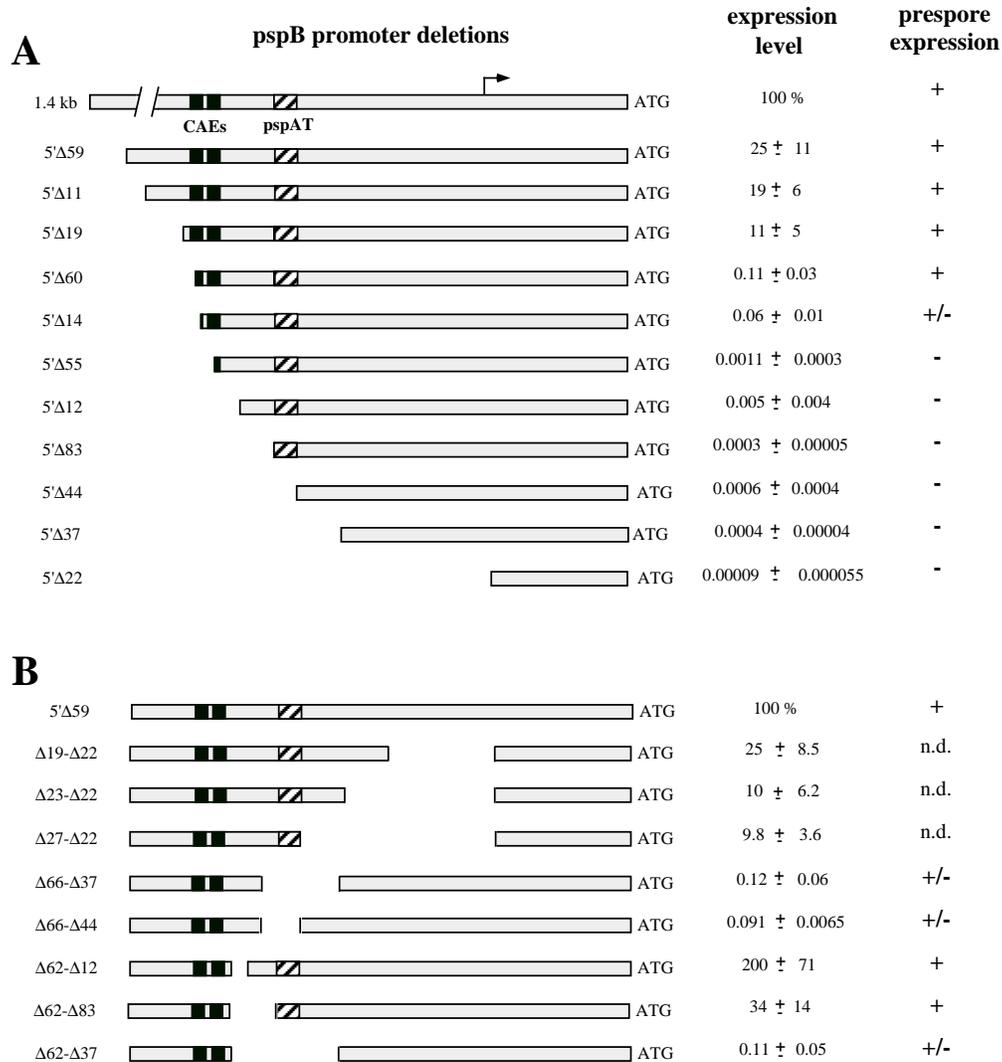| | expression level | prespore expression |
|---|---|---|
| 5'Δ59 | 100 % | + |
| Δ19-Δ22 | 25 ± 8.5 | n.d. |
| Δ23-Δ22 | 10 ± 6.2 | n.d. |
| Δ27-Δ22 | 9.8 ± 3.6 | n.d. |
| Δ66-Δ37 | 0.12 ± 0.06 | +/- |
| Δ66-Δ44 | 0.091 ± 0.0065 | +/- |
| Δ62-Δ12 | 200 ± 71 | + |
| Δ62-Δ83 | 34 ± 14 | + |
| Δ62-Δ37 | 0.11 ± 0.05 | +/- |

**Fig. 5.** Relative levels of developmentally induced expression directed by deleted *PspB* promoters, as assayed by *PspB/luciferase* expression. (A) 5′ deletion analysis. The 1.4 kb full length promoter is listed as (100%). (B) Internal deletions made using the 5′Δ59 promoter (see A) as the parental construct. Expression levels are listed relative to 5′Δ59. The positions of the CA-rich elements (CAEs) and the essential AT-rich region (pspAT) are labeled, and the transcriptional start site is indicated with an arrow. The deletion endpoints are noted in Fig. 1. Standard errors are listed. '+' means expression is prespore-specific; '+/−' means that while expression was detectable in the prespore zone, the levels of β-gal expression were too low to determine whether expression was cell-type-specific (see Results); '−' means there was no detectable expression; n.d., not determined; however, these constructs were tested in the Actin15ΔBgal minimal promoter (see Results).

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,
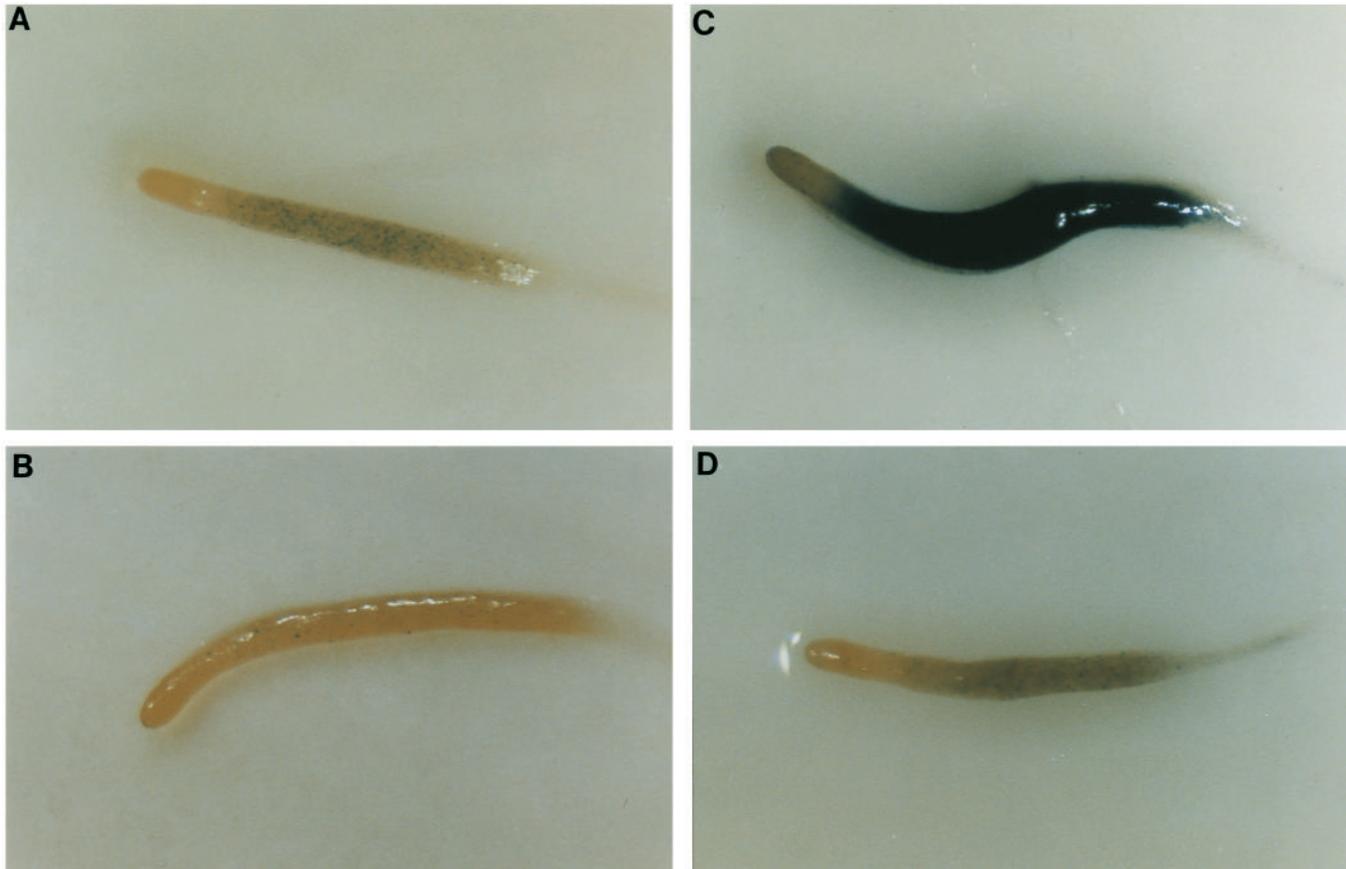
**Fig. 6.** Cytological staining of cells transformed with *PspB/lacZ* promoter deletion constructs. Stable transformants carrying the *PspB/lacZ* constructs 5′Δ60 (A) or Δ66-44 (B) were stained for β-galactosidase activity at the slug stage of development. β-galactosidase expression of Act15-EΔ27 and Act15-EΔ34 transformants is pictured in C and D, respectively. The slugs in C and D were each stained for 3 hours.

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 competed 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) resembles 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; Ceccarelli 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*
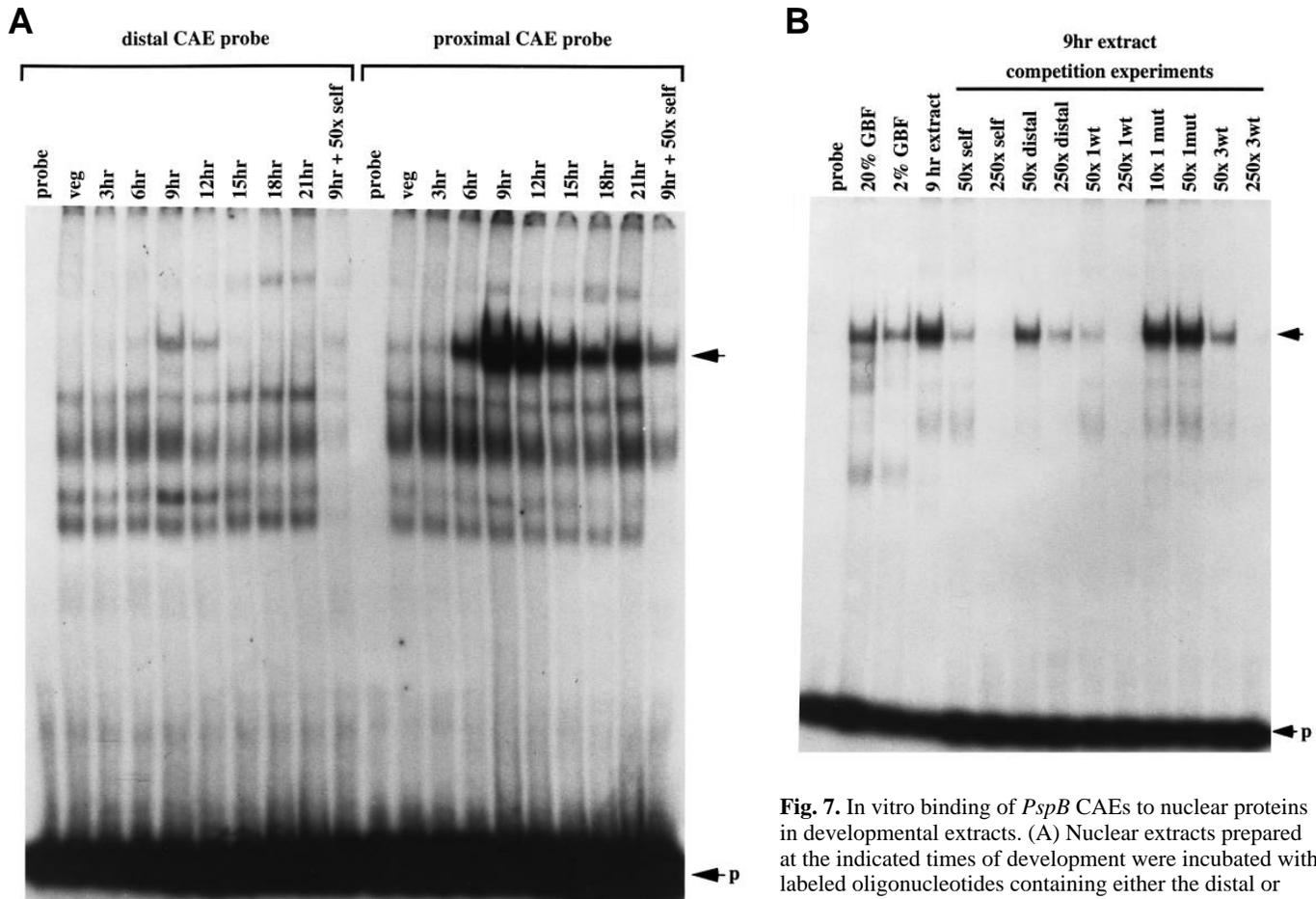
**Fig. 7.** In vitro binding of *PspB* CAEs to nuclear proteins in developmental extracts. (A) Nuclear extracts prepared at the indicated times of development were incubated with labeled oligonucleotides containing either the distal or proximal *PspB* CAEs in the presence of non-specific competitor and electrophoresed in a mobility shift assay (see Materials and Methods). The position of free probe is indicated by 'p'. The complex formed with the developmentally induced activity is indicated by an arrow. (B) The labelled *PspB* proximal CAE-containing oligonucleotide was incubated with either no extract (probe lane), extracts in the final stages of G-box Binding Factor purification (20% or 2% GBF), or nuclear extracts from cells that had been developing for 9 hours. Unlabelled oligonucleotides containing *PspB* proximal CAE (self), distal *PspB* CAE (distal), *SP60 (cotC)* CAE1 (1wt), mutant *SP60 (cotC)* CAE1 (1mut), or *SP60 (cotC)* CAE3 (3wt) were added at the indicated molar excess to compete for binding.

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 CDNVXCPX-GYCE, 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; Hildebrant 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 5′Δ19 and 3′Δ62, and (2) the 46 bp between 5′Δ83 and 5′Δ44. 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 $10^4$-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 3′Δ27) is not required for high levels of prespore-specific expression, as seen in the Δ27-Δ22 internal deletion and the Act15-EΔ27 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).

**A**

```
SP60        C A C A C A C C C A      C A C A C T

            C A C A C A T T A A      C A C A C T

            C A C A C T C C C A A    C A C A C A

pspB        C A C T C A C A A T T T  C A C A C A

            C A C A C A C A T T T A C C A C A C T

consensus   C A C A C A C  (N3-6)    C A C A C A/T
```

**B**

```
pspB     AAA │TTTGTAG│ TCAAAAATATT │ATTTAAT│ TTTTTATTTATTAATTAT

SP60     (A) │TTTGTAA│ AACATAGAACAA│ATTTAAT│ AATTATTTTT


consensus    R TTTGTAR (n=11 or 12) ATTTAAT T's

SP70     G TTTGTAA**     n=11     ATTTAAT TATTTTTATTTTT **

D19      A TTTGTAA**     n=10     ATTTAAT TTTATTGT
```

**Fig. 8.** Comparison of *PspB* regulatory elements with those identified in other prespore genes. (A) The CA-rich regions required for *PspB* promoter expression are similar to the *SP60 (cotC)* CAEs. (B) The essential AT-rich region between 5′Δ83 and 5′Δ44 of the *PspB* promoter are compared to the 37-bp region of the *SP60 (cotC)* promoter required for prespore-specific expression (Powell-Coffman et al., unpublished data), and a consensus is derived. Similar sequences found in the regulatory regions of the *D19 (pspA* and *SP70 (cotB)* promoters are also listed. Asterisks indicate that the sequences are in the reverse orientation.

However, in prior published studies the only prespore promoter element that had been been functionally defined through small deletions and point mutations were the *SP60 (cotC)* CAEs. The strong sequence similarities between the *SP60 (cotC)* and *PspB* CAEs (see Fig. 8A) suggested that they might be functionally homologous. Haberstroh et al. (1991) used point mutations to demonstrate a correlation between the ability of the *SP60 (cotC)* CAEs to increase promoter activity in vivo and the ability of *SP60 (cotC)* CAE-containing oligonucleotides to bind a developmentally regulated nuclear activity in vitro. We have shown here that the *PspB* CAEs also interact with this activity with differential affinities: the proximal CAE binds strongly and the distal CAE binds relatively weakly. The CAE consensus (Fig. 8A) consists of 2 CACACA half sites and resembles a G-box binding factor (GBF) binding site (Hjorth et al., 1990). GBF is induced during development or in response to high levels of extracellular cAMP and has been shown to bind specifically to sites in several developmentally induced promoters, some of which are prestalk-specific (Hjorth et al., 1990, 1991). We have shown here that partially purified GBF forms a complex with the *PspB* proximal CAE in a gel mobility shift assay that migrates at the same position as the complex formed in crude nuclear extracts. GBF has recently been purified, and the gene encoding it has been cloned (Schnitzler et al., 1994). In a concurrent analysis, we have found that the *SP60 (cotC)* CAE binding activity copurifies with GBF and that cloned GBF binds the *SP60 (cotC)* CAEs (Powell-Coffman, Schnitzler, and Firtel, unpublished data).

We, therefore, believe that the *PspB* CAEs are functionally homologous to the *SP60 (cotC)* CAEs.

The AT-rich sequences between 5′Δ83 and 5′Δ44 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 two 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 (TCAATTAAAT) or even-skipped (CAATTAAAT) (Desplan et al., 1988; Hoey and Levine, 1988). Approximately 90% of the nucleotides in *Dictyostelium* noncoding regions are A/T (Kimmel and Firtel, 1982), so specificity of binding to homeobox-like sequences may be problematic. Cooperative binding with factors that recognize neighboring sequences (TTTGTAR 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 (Δ66-44 or the Act15-EΔ34 construct) is extremely low. Consequently, it is difficult to discern the exact specificity of expression directed by these promoters. However, β-galactosidase expression in the Δ66-Δ44 and Act15-EΔ34 transformants is at least prespore-enriched (Fig. 6A,B). This indicates that the factors that interact with the sequences upstream of 3′Δ34 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 complexes 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 regulatory 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.

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