Two distinct populations of prestalk cells within the tip of the migratory *Dictyostelium* slug with differing fates at culmination

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SUMMARY

The *ecmA* gene of *Dictyostelium* encodes an extracellular matrix protein and is selectively expressed in prestalk cells. We show that its promoter contains discrete elements that direct expression in different subpopulations of prestalk cells. Prestalk(pst)A cells occupy the front half of the prestalk region. Expression in pstA cells requires DNA sequences close to the cap site of the gene and a separate, upstream region that acts in combination with the gene proximal sequences. PstO cells are situated in the rear half of the prestalk region and at least two separate and redundant promoter regions direct expression within them. All constructs that are expressed in pstO cells are also expressed in anterior-like cells (ALCs); cells that resemble prestalk cells but which are scattered throughout the prespore region.

This observation suggests that pstO cells and ALCs may be very similar in their properties. If development occurs under conditions in which a migratory slug is not formed, there is an ordered movement of cells into the stalk tube. PstA cells enter the stalk tube first, followed by a proportion of the pstO cells. The remainder of the pstO cells contribute to the upper cup, an ALC-derived subpopulation of prestalk cells which is located at the apex of the spore head. After prolonged slug migration, a discrete pstO zone appears not to be maintained and, at culmination, pstO cells are found scattered throughout the stalk.

Key words: *Dictyostelium*, promoter analysis, double enzymatic staining, multiple prestalk cell types, anterior-like cells

INTRODUCTION

One important approach to the study of development is to isolate genes specifically expressed in a particular cell lineage. This serves a dual function. It provides markers that can be used to follow cellular differentiation and morphogenesis and, by examining the regulation of the specific genes, it is possible to trace the inductive pathways controlling cellular differentiation. In *Dictyostelium*, the developmental programme yields two differentiated cell types, stalk cells and spores; the former providing a supporting structure which aids dispersal of the latter. Stalk cells and spores are derived from prestalk and prespore cells, respectively. Prestalk cells make up the anterior one-fifth of the length of the aggregate at the migratory slug stage (Bonner, 1952; Sternfeld and David, 1982). An equivalent number of anterior-like cells (ALCs), which cytologically and biochemically resemble prestalk cells, are scattered throughout the rear (Sternfeld and David, 1981; Devine and Loomis, 1985). Prespore cells constitute the remainder of the posterior region.

It has recently become apparent that both the prestalk and prespore cell populations are heterogeneous and that cellular differentiation is, therefore, a more complex process than originally believed (Jermyn et al., 1989; Haberstroh and Firtel, 1990). Successive 5 deletions of the prespore-specific cotC (SP60) promoter, that remove CA-rich elements, restrict expression to increasingly anterior parts of the prespore region (Haberstroh and Firtel, 1990). This suggests a gradient of induction within the prespore zone, which could result in the differential expression of prespore genes. Prestalk cell heterogeneity was discovered by studying the ecmA and ecmB genes. They encode extracellular matrix proteins, which form part of the slime sheath and the stalk tube (McRobbie et al., 1988a,b), and their expression is dependent upon, and inducible by, the stalk cell morphogen DIF (Jermyn et al., 1987; Williams et al., 1987).

By linking the promoter sequences of the *ecmA* and *ecmB* genes to reporter genes, and introducing the constructs into *Dictyostelium*, it has proven to be possible to mark prestalk cells specifically (Jermyn et al., 1989; Williams et al., 1989; Jermyn and Williams, 1991). Expression of the two genes, visualised in this way, defines several different subclasses of prestalk cells. In the first experiments, an immunologically detectable marker was used (Jermyn et al., 1989). Expression of the *ecmA* gene was detected in cells occupying the front 10% of the length of the slug but was apparently absent in the rear half of the prestalk zone. Cells expressing the *ecmA* gene were designated prestalk(pst)A cells, while the cells in the posterior half of the prestalk zone, which appeared not to express the gene, were termed

pstO cells. Subsequently, a much more sensitive, enzymatic marker, obtained by fusion of the *ecmA* promoter to a *lacZ* reporter, showed that the *ecmA* promoter is active throughout the prestalk region but at a lower level in the pstO zone (Jermyn and Williams, 1991).

The tip, or papilla, of the culminating structure is composed of pstA cells and, as cells enter the stalk tube entrance, they activate expression of the *ecmB* gene (Jermyn and Williams, 1991). The small fraction of prestalk cells within the slug tip that express the *ecmB* gene form a forward facing cone of cells. They were originally designated pstB cells but, because they have subsequently been shown to express both the *ecmA* and *ecmB* genes, they have been renamed pstAB cells (Gaskell et al., 1992). The core of pstAB cells seems to constitute a type of stalk tube primordium that may result from periodic, abortive attempts by the slug to enter culmination (Sternfeld, 1992).

Prestalk cells and ALCs are selectively stained by vital dyes such as neutral red. The ALCs form a population of cells distinct from those within the anterior prestalk region, with differences in their pattern of gene expression, detected using 2-D gels, and in their fate at culmination (Sternfeld and David, 1981; Devine and Loomis, 1985). At culmination the ALCs move to surround the emerging spore head to form the upper and lower cups, structures that cradle the spore head (Sternfeld and David, 1982; Jermyn and Williams, 1991). They also form the outer part of the basal disc, a conical-shaped structure that supports the culminant (Jermyn and Williams, 1991; Sternfeld, 1992). In the migratory slug, the *ecmA* and *ecmB* genes are expressed at a low level in a subset of the ALCs, with some ALCs expressing both genes, some expressing one or the other gene and some ALCs apparently expressing neither gene (Gaskell et al., 1992). During culmination, expression of the *ecmB* gene becomes elevated in the ALCs as they sort to surround the spore head (Jermyn and Williams, 1991).

An insight into the regulation of the differentiation of pstA into pstAB cells has been obtained by analysis of the *ecmB* promoter. It contains distinct regulatory regions: a distal region that directs expression in the upper cup and a proximal region that directs expression within the stalk tube, and which may also contain elements that direct expression in the lower cup and basal disc (Ceccarelli et al., 1991). Here we undertake a similar analysis of the *ecmA* promoter and show that pstO cells are a discrete class of prestalk cells. They can be distinguished from pstA cells by their ability to direct expression from two separate subregions of the promoter, a property that they share with ALCs. We have also exploited the ability to mark a distinct prestalk cell subpopulation to study the process of culmination.

MATERIALS AND METHODS

Plasmid constructs and sequencing

The *ecmA* promoter fusion construct pDd63CAT (Jermyn et al., 1989) was used as a template for dideoxy sequencing and polymerase chain reaction (PCR). Primers used for sequencing (20-25 nucleotides long) were completely complementary, while *Bam*HI or *Bgl*II restriction enzyme recognition sites were added to the

ends of the oligonucleotides used for PCR (30-40 nucleotides long). PCR was performed over 20 cycles using Taq DNA polymerase (Boehringer Mannheim) in 100 µl reaction volumes containing 10 mM Tris-HCl, 4.5 mM MgCl$_2$, 50 mM KCl, 0.1 mg/ml gelatine, pH 8.3. 10 ng of template and a final concentration of each dNTP of 200 µ were used. DNA fragments generated by PCR were phenol extracted, restricted, gel purified, ligated into the *Bam*HI site of actin15 Bam-gal (Pears and Williams, 1988; Ceccarelli et al., 1991) and transformed into *Escherichia coli*. Two isolates of each construct were purified as large-scale plasmid preparations and used for independent *Dictyostelium* transformations.

Dictyostelium transformation, culture and development

Transformants of the axenic strain AX-2 were isolated as described previously (Nellen et al., 1984; Early and Williams, 1987) and grown in axenic medium containing G418 at 20 µg/ml. In our experience, the safest method of analysis is to study pooled populations derived from a large number of individual clones. This averages out any problems with integration site and/or copy number. Hence, total populations were analysed except for a few instances (listed in the legend to Fig. 4) where multiple clonal isolates were also studied. For development, cells were washed in KK2 (16.5 mM KH$_2$PO$_4$, 3.8 mM K$_2$HPO$_4$, pH 6.2) and plated on nitrocellulose filters or 2% Bacto Agar (Difco) plates. Incubation was at 22°C in humid chambers. Slugs were encouraged to migrate by exposure to low level unidirectional light, and then to culminate by temporary removal of the Petri dish lid and exposure to overhead light.

Histology

For -galactosidase (-gal) staining, aggregates were fixed in 1% glutaraldehyde in Z buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 2 mM MgCl$_2$) for 15 minutes, and washed twice in Z buffer without fixative (Dingermann et al., 1989). Samples were then incubated in staining buffer (Z buffer containing 5 mM K$_3$(Fe(CN)$_6$), 5 mM K(Fe(CN)$_6$) and 1 mM X-gal) for various times before mounting in Gelvatol and photography. For double staining with X-gluc and Magenta-gal, aggregates were fixed in 50 mM NaHPO$_4$ (pH 7.0) containing 0.5% (w/v) glutaraldehyde for 5 minutes and washed twice in 50 mM NaHPO$_4$ (pH7.0) for 15 minutes each. They were then stained for -glucuronidase activity at 37°C in 50 mM NaHPO$_4$ (pH 7.0), 5 mM Fe$_2$(CN)$_4$/Fe$_3$(CN)$_6$, 1 mM X-gluc (5-bromo-4-chloro-3-indolyl glucuronide, Molecular Probes, USA). When they were adjudged to be sufficiently stained, the aggregates were transferred to -gal staining buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 2 mM MgCl$_2$, 5 mM Fe$_2$(CN)$_4$, 5 mM Fe$_3$(CN)$_6$) containing 1 mM Magenta-gal (x-bromo-y-chloro-3-indolyl galactoside, BioSynth AG, Germany). After the required time of staining, the reaction was stopped with phenylethyl- -D-thiogalactoside [Sigma, USA] and the samples mounted and photographed as above.

RESULTS

A distal region of the *ecmA* promoter is active in pstO cells but not in pstA cells

The sequence of the 1950 nucleotides immediately upstream of the *ecmA* coding region, extending to −1694 with respect to the cap site, is shown in Fig. 1. When the entire promoter is joined to the *lacZ* gene, with a fusion point just downstream of the initiation codon of the *ecmA*

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TTATTATTATTTTTTTATTTTTTTATTTTTTTTAACTTTACAAAGCATATAAACTGAAAATAAATAAATTATTTAAACATATTTAACTCATTTAACTTATTTTTATTTTT

TTATTTTACGATAAATTTTGGATAGCAATAAAAAAAAAAAAAAATTTGTTATTAAAAATTTGAGGTTAAATTGTTATTAATTTCATTAAAAAAAAATTAATTCAAAAAAAAA
                    -1344◄┐                                                                    -1269┌►
AAAATATGAGTTGTTCAATTTCATGGTGTCAAAATATTAAAAAAACATACATTAACAATATTTTAATTAACCCACAAAAACAAATTTCAAACACAATATTAATATTCA
                                -1218◄┐      ┌► -1212
AACTCACTTAATTATAACCCCCATTCGCTATTTTATCAAATTCACTATTGTAATAAACTATATAAATATTATCCCCCAAATTAATTATTTGAATTGATTTTATTTTTA
                              ┌► -1111
ATTATTTTTTTATTTTTTTATTTTTTTTTTTTTTATTTAAACAGTTACACCCCACAATTTTAATTATTAATAGGCTGATTTTGTGGGTAAAAAGAGTGGTATTTTTTT
◄┐-1048 ┌►-1041
GGGTGATTGTTTTAATAATCAAATTTACAAATTTACAGTAGATGATGTTTGAAATTAAATAAATTAAAAGTGTATGTTTTATTTAATTTTTTTTTTTTTTTTTTTATAT
                                                      ┌► -895
TGATTTTTATATTGATTTTTTATTATTGTTAATTGTACTTTTTAATCTCTTGTTATCATCAAACTCTACACAAATAGGTTATTTACAAAATCTATTAAAATTATTCAA
                            -797◄┐
ATATAGGTGTGGGTTTGTTGTGTGTGAATATATTAATATAAATTTGTGATTTTGTTATTGTTTTTAAACATTACTGTTGTTTTTTTTTTTTATTTATGTGGTTTAGAG

AGTTTGAGATAGTGAGGTTTTTTAATTATGATTTCATATACAATTGAAAAAATAAAAAAATATTTAATAATATGATTATGCATTATTTGATGTTATGGTTGTAAACTCG
                  -599◄┐                                                      ┌► -531
TGAGGGGGTTGGTGATTTTATAGTTGTAAAAAATTTTGCAATAAAATATATTATGTAAACTGATACTTTATACTATTGGAAAAGAAAAAAAAGAAATTAATTATACAT
                              -451◄┐
AACACCACAAACAATTTCTATTTTCAAACACACACATGTGATATTTTTAATTTTTCATAAAAATACCTTGTGTACATATAAACAGATAGTACAATAATGAAAAAAAAG
                        ┌► -374
TTATCTCACTCTCTATTTAAAATAATTAACACACTTTCACCAAAATTATACACCACCACCATAAATAATAAAAATAAAAAATAGAATAAAAAAAATAACAATAATGAT
          -282 ◄┐                                                                  ┌► -201
AATAAATAATATAATGAAACATTGGGGTTGTATAAAAAAACAATTTAAAAAAATACATACATACACATATTATTATTGTTAAAAAAATGTATATTTTATATTGCGTAA
                                                  ┌► -131 ◄┐ -120
TGGTTTTGCGGTCTTTTTAAGAATATCACTAATCAAATATGATCGACAATCCAATTATTTTTAATTTACCATACTTTATTTTTTTTTAAAATTAATTTTAATTTTATTT
                                                                +1
TATTTTTATTTTTATTTTTTTGTTTATATTTTTTTTTTTTTTGTGTAAATATTTTTTGTATAAATATTTGATTATTTTAATTTTATTTTCATCAATTCAAAAATTAAC
          +41◄┐
TTTTAAATTGGAATATATTTAAATAATTTAATATTTCAATATATTTATTTATTTTAAATAAATAAAT[AAAAAAAAAAAAAAAAAAAAAAAAAA]TAATATTTATTT

ATTAAAAAAAAAAAAAAAATCATTTAAAAAAAAAAAAAAAAAAAAAAAACAAAAAAAAAAAAAAGTAGGAAAAAGGAATTAGTAAAAACTAAAATCATTAGTTTAAAAATTA
          +250◄┐
TAACGTTGAAAATG
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Fig. 1. The nucleotide sequence of the region upstream of the *ecmA* gene. The sequence was determined using synthetic oligonucleotides complementary to relatively GC-rich regions of the sequence. The length of the run of A residues shown in brackets could not be precisely determined. The position of the cap site derived from primer extension analysis (data not shown) is marked (+1). The end points of the 5′ and 3′ deletion constructs analysed are also shown.

gene, β-gal accumulates in pstA cells and pstO cells (Jermyn and Williams, 1991). In Fig. 2 we show the staining pattern for the intact promoter using a novel method of double enzymatic staining, where prespore cells are stained by virtue of their expression of the the β-glucuronidase gene (Jefferson et al., 1986) via a prespore-specific promoter (*psA:gus*), and pstA cells, expressing the *ecmA:lacZ* fusion gene, are stained with a red substrate. As expected (Jermyn and Williams, 1991), the level of expression is higher in pstA cells than in pstO cells, at this time of staining there being no detectable expression within the pstO cells.

In order to investigate the activity of the different parts of the promoter, we investigated the activity of subfragments inserted into the actin15 Δ Bam-gal vector (Pears and Williams, 1988; Ceccarelli et al., 1991). In this vector, an internally deleted version of the actin15 promoter is fused to the *lacZ* gene. Although it is inactivated by the removal of essential regulatory elements, it still provides a TATA box and cap site. Defined fragments of the *ecmA* promoter were generated by PCR and inserted into actin15 Δ Bam-gal (Fig. 3).

The first 3′ deletion (Fig. 3, construct A), which removes sequences to a point 41 nucleotides downstream of the cap site (i.e. to nucleotide +41) but which retains its 5′ boundary at nucleotide −1694, confers a different pattern of expression from that of the intact promoter (Fig. 4A). There is expression in pstA cells and pstO cells but at approximately equal levels in both cell types. One possible expla-

nation for this result is that there is an element located in the 5′ non-coding region of the *ecmA* gene, downstream of nucleotide +41, which is required for high level expression in pstA cells. Alternatively, the lowered expression in pstA cells may result from the presence within construct A of TATA boxes and cap sites from both the *ecmA* and *actin 15* genes.

Upon removal of an additional 161 nucleotides (Fig. 3, construct B) there is a further, and much more dramatic, change in the pattern of expression (Fig. 4B). Staining is now absent from the tip of the slug and is confined to a band of cells in the posterior half of the prestalk zone and to ALCs. The loss of staining in tip cells that accompanies deletion of sequences between nucleotides −120 and +41 is not the pattern expected for a non-specific reduction in *ecmA* expression, because with the intact promoter there is stronger staining of the β-gal reporter gene in the tip than in the posterior part of the prestalk region (Jermyn and Williams, 1991 and Fig. 2). It indicates the existence of at least two, distinct *ecmA*-expressing cell populations and, using previous nomenclature (Jermyn et al., 1989), we term the anterior cells, pstA cells, and the posterior cells, pstO cells.

Identification of a distal promoter fragment that directs expression in pstO cells

Sequences between nucleotides −120 and +41 are required for expression in pstA cells, whereas sequences located

more distally with respect to the cap site direct expression in pstO cells and in ALCs. When 3′ sequences from the *ecmA* promoter are further deleted, to an end point at nucleotide −1048 (Fig. 3, constructs C-G), the pattern and intensity of pstO staining remains unchanged from that of construct B (Fig. 4C). With a fragment that has its 3′ end point at −1218 (construct H) there is no band of pstO cells, although some weakly staining cells are present, scattered throughout the slug (data not shown). In a construct with a 3′ boundary at −1344 (construct I) all staining is lost (data not shown). Based upon these deletion endpoints, an internal fragment spanning the region between −1212 and −1048 was generated and again inserted into the actin15′ Bam-gal vector (construct J). This fragment was weakly but clearly active in both pstO cells and ALCs (Fig. 4D). Thus, a region of 164 nucleotides of the *ecmA* promoter, distal to the cap site, directs expression in pstO cells when transferred to a heterologous promoter.

At least two separate regions within the promoter are capable of directing expression in pstO cells

A set of 5′ deletion constructs was made following the same procedure used for the 3′ deletion constructs, i.e. the insertion of PCR-generated fragments into actin15 Bam-gal. This analysis identified a second element capable of directing expression in pstO cells.

Fig. 2. Analysis of the domains of expression of the *ecmA* and *psA* genes by double enzymatic staining. The *psA* gene encodes a cell surface protein of unknown function and is expressed only in prespore cells (Early et al., 1988). The *psA:gus* construct contains the promoter of the psA gene coupled to the β-glucuronidase gene of *E. coli* (Traynor and Williams, manuscript in preparation). Transformant cells containing the *ecmA:lacZ* (Jermyn and Williams, 1991) and *psA:gus* genes were allowed to develop to the slug stage and were then fixed and stained overnight with X-gluc, a β-glucuronidase substrate that gives a blue colour when hydrolysed. The slugs were then stained for 30 minutes at room temperature with Magenta-gal, a β-gal substrate that gives a red colour when hydrolysed. If slugs are incubated with Magenta-gal for longer than a few hours the prestalk region becomes stained throughout its length (data not shown).
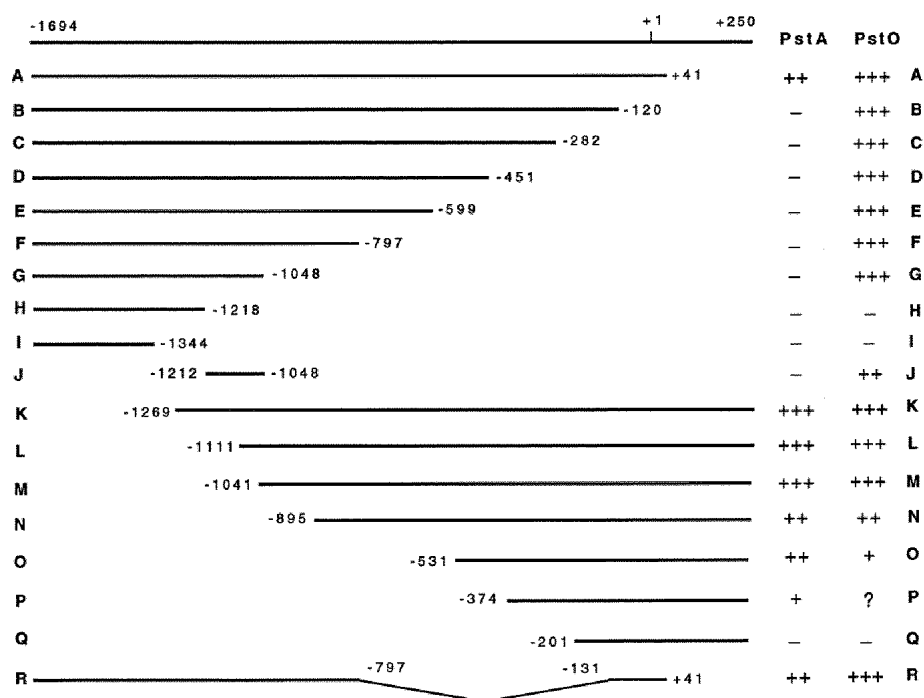

Fig. 3. Structure of the constructs used in the analysis of the *ecmA* promoter. The extent of *ecmA* promoter sequence retained in each deletion construct is shown in relation to the full-length non-coding sequence. Promoter fragments were generated by PCR using oligonucleotides designed to give *Bam*HI-compatible ends. For 3′ deletions, the *Bam*HI site is restored at the 3′ end, whereas for 5′ deletions, the 5′ end was chosen. This allowed the construction of internal deletions, by combining 5′ and 3′ deletions. Fragments were inserted into the *Bam*HI site of the A15′ Bam-gal vector (Pears and Williams, 1988; Ceccarelli et al., 1991). The cloning site lies within the internally deleted, and hence inactive, actin 15 promoter which is in turn fused to the *lacZ* gene of *E. coli*. The actin 15 promoter retains sequences necessary for the initiation of transcription. On the right, the strength (+++ = wild-type) and pattern of expression seen for each construct is indicated. In order to guard against PCR artefacts, two independent DNA isolates of each construct were analysed by transformation into *Dictyostelium*.

In all these constructs (Fig. 3, constructs K-Q), the 3′ boundary of the *ecmA* gene sequences was located at nucleotide +251, 4 nucleotides upstream of the start of the coding region. Construct K, which has a 5′ boundary at

−1269, is expressed in both pstA and pstO cells. As the 5′ boundary is moved to nucleotide −1041 (constructs L and M), there is very little reduction in expression in pstO cells (Fig. 4E). Because there is no overlap between construct M
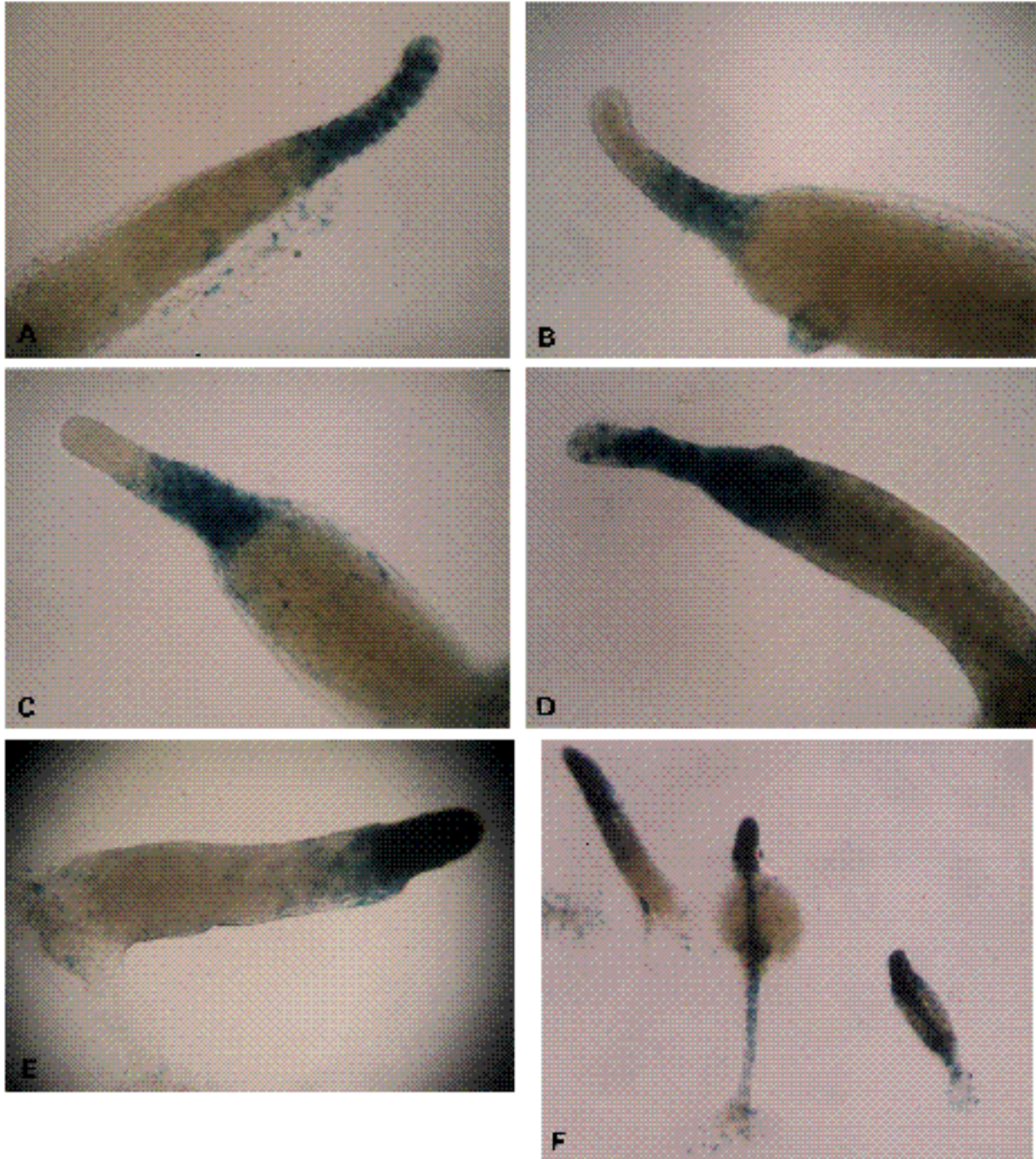

Fig. 4. Analysis of the expression of *ecmA* promoter fragments coupled to the β-gal gene. Whole mounts of developing structures are shown, stained for varying periods of time with X-gal. For A-D, cloned transformant strains were analysed, but the structures shown in E and F are derived from pooled populations. No differences in expression patterns between pools and clones were ever seen, but slight variation in expression levels among clones was observed. (A) First finger stage, transformed with construct A and stained for 4 hours. (B) Same stage, transformed with construct B and stained for 4 hours. (C) Same stage, construct G, 4 hours staining. (D) Same stage, construct J, stained for 10 hours. (E) Early slug, construct M, stained for 4 hours. (F) Construct R, stained for 4 hours, showing (left-right) slug, late preculminant and early preculminant.

and the distal promoter fragment shown to be highly active in pstO cells (construct J), there must be at least two, non-overlapping pstO elements.

As sequences 5′ to the gene are further deleted, there is a gradual reduction in the level of gene expression in both cell types. Construct O, with a 5′ boundary at −531, shows a low level of expression in pstO cells, indicating there to be a second pstO element downstream of this point (data not shown). With only 374 nucleotides upstream of the cap site remaining (construct P), the signal strength is so reduced that it is impossible to judge whether pstO cells are stained, although there is clearly still a low level of expression in pstA cells. When only 201 nucleotides remain (construct Q), all expression is lost.

Combinatorial action of elements from different parts of the promoter

The behaviour of constructs A and B shows that sequences required for expression in pstA cells are located within the region −131 to +41. This region is not, however, sufficient for expression in pstA cells because construct Q, with a 5′ end point at nucleotide −201, is totally inactive. The gradual reduction in gene expression that is observed as sequences between −1041 and −201 are removed suggests the existence of ancillary elements which are required for efficient expression in pstA cells. Construct R strongly supports this interpretation, because it shows that these ancillary elements can be provided by a fragment containing one of the two pstO-specific regions. It contains the region −120 to +41, coupled to a distal region of the promoter (nucleotides −1694 to −797). In isolation (Fig. 3, construct F), this distal region is expressed in pstO cells but not in pstA cells, while the proximal fragment is inactive in both cell types (data not shown). In construct R where the two are coupled together there is expression in both pstA and pstO cells (Fig. 4F).

The behaviour of pstO cells during culmination shows there to be an ordered movement of prestalk cells into the stalk tube

Cells transformed with either construct B or construct G, both of which express the β-gal marker at high levels in pstO cells, were used to follow the behaviour of pstO cells during culmination. Cells were allowed to develop on nitrocellulose filters, a condition in which most aggregates culminate in situ without first forming a migratory slug. A band of pstO cells is initially detectable at the first finger stage (Fig. 4B,C), although some scattered staining cells can be detected in tipped mounds (data not shown). The unstained tip of pstA cells and the band of pstO cells remain visible until the mexican hat stage.

As the stalk begins to form, the pstA cells move into the stalk tube, and the size of the unstained portion of the tip is progressively reduced (Fig. 5A,B). In preculminants, there is expression within the lower cup and outer basal disc. We assume that this derives from ALCs, which
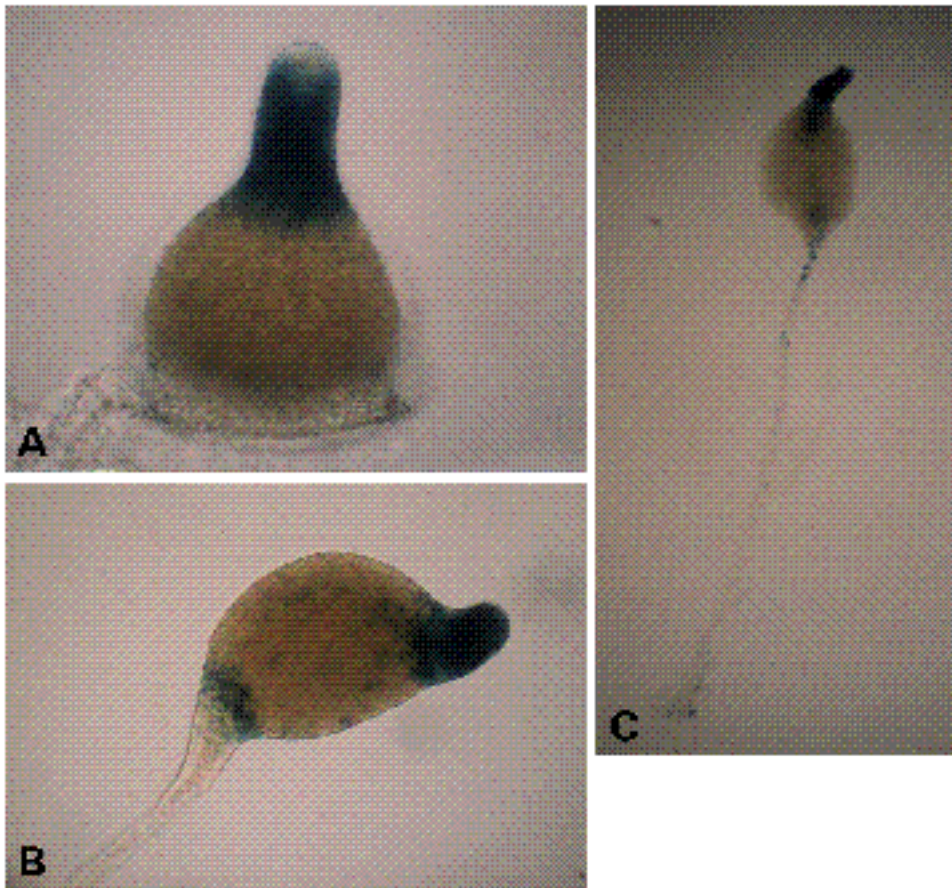

Fig. 5. Analysis of pstO expression patterns during culmination. Aggregates were taken from filter development and stained for 4 hours with X-gal. (A) A mexican hat structure, formed by a clone transformed with the pstO marker construct B. (B) A preculminant, derived from a clone transformed with the pstO marker construct G. (C) A culminant, from the same development as B.

express the pstO marker, because it is known that during culmination some ALCs move downward to the base, where they either remain to form the outer part of the basal disc or move up with the spore head to form the lower cup (Sternfeld and David 1982; Jermyn and Williams 1991). The remaining ALCs move upward, and so the band of expressing cells at the prestalk-prespore boundary presumably derives in part from ALCs that moved there during

culmination and in part from pstO cells that were located there prior to culmination (Fig. 5B).

During the final stages of culmination, as the stalk elongates, the unstained tip disappears and a proportion of the pstO cells enter the stalk tube, the rest contributing to the upper cup (Fig. 5C). The pstO cells extend no further down than the top third of the length of the stalk tube, the bottom two-thirds of the stalk being totally unstained. Thus, pstO
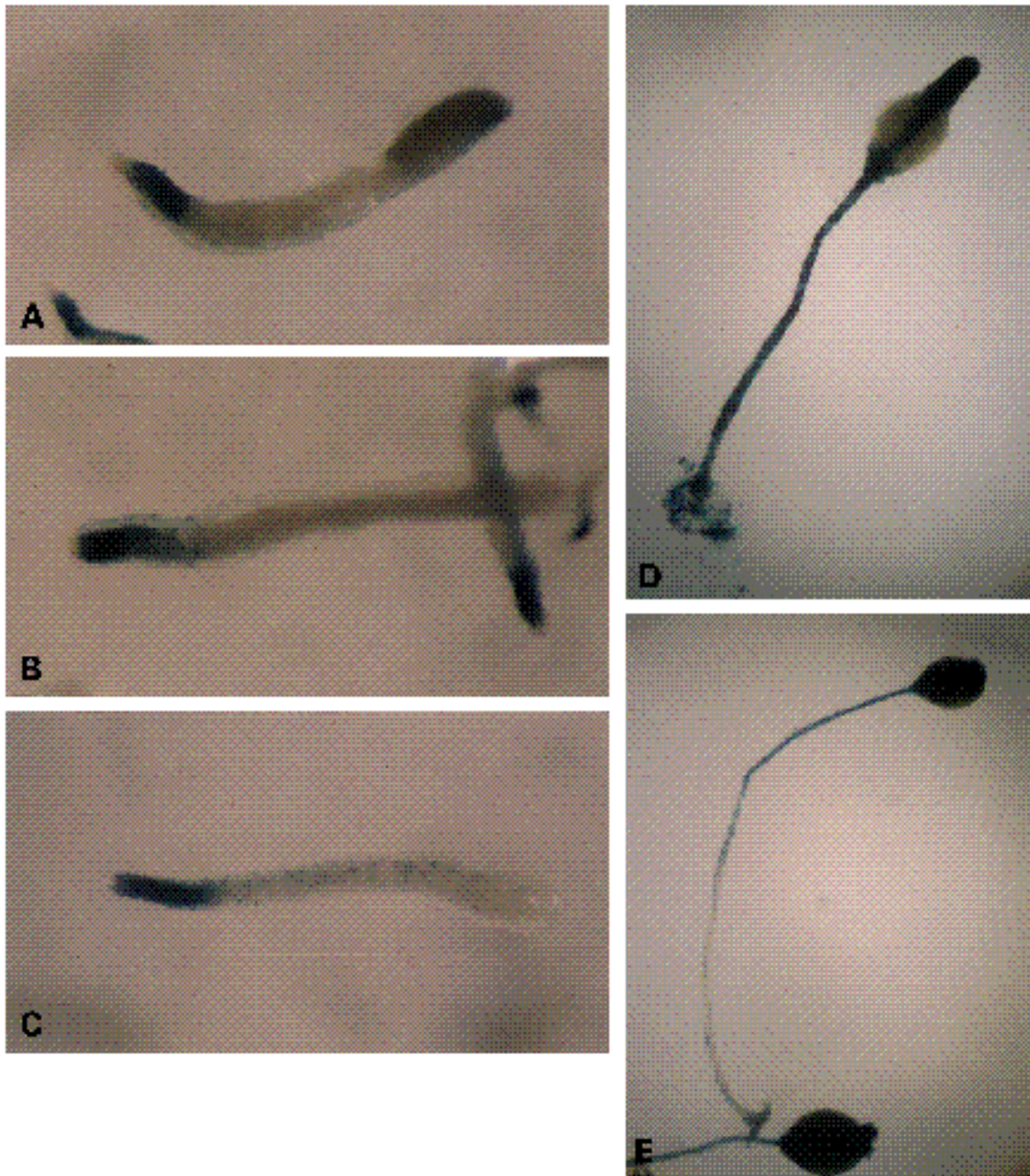

Fig. 6. The effect of slug migration on pstO expression patterns. Slugs, formed from a *Dictyostelium* clone transformed with the pstO marker construct G, were stained after varying times of migration: (A) 3 hours, (B) 5 hours, and (C) 7 hours. (D) A culminant derived from a slug migrated for approximately 8-10 hours. (E) A culminant from the same development as in D taken from the original cell streak, so that minimal migration would have taken place before culmination. Staining was for 4-6 hours in all cases.

cells can undergo one of two different fates: they can become either a stalk cell or an upper cup cell.

Slug migration results in the appearance of pstO cells in the slug tip

The sharp demarcation between stained, pstO cells and unstained, pstA cells is not maintained if aggregates are developed on agar, a condition that encourages slug migration. As the slug begins to move, the pstO band widens and extends foward into the pstA zone, so reducing the size of the unstained region (Fig. 6A-C). After 6-10 hours of migration, the tip is entirely stained. There is a gradual shift in the boundary between staining and unstained zones, leading us to believe that pstO cells cells move forward to mix with the pstA cell population. However, the possibility that a change in cell identity of pstA into pstO cells has occurred during slug migration cannot be excluded.

We next analysed the culmination of slugs that had migrated and in which discrete pstO and pstA zones had disappeared. Slugs that had been allowed to migrate for 10 hours were induced to culminate, by a combination of exposure to light and removal of the Petri dish lid. They were compared with culminants on the original cell streak, which would have migrated for a much shorter distance, if at all. After 10 hours of migration, the stalks of the resulting culminants were entirely stained (Fig. 6D), whereas in the culminants from the cell streak, about half of each stalk was stained (Fig. 6E).

DISCUSSION

We have shown that pstO and pstA cells are separate populations of prestalk cells by demonstrating a qualitative difference in their patterns of gene expression; namely that the expression of the *ecmA* gene in pstO cells, known to be at a lower level than in pstA cells (Jermyn et al., 1987), is a result of the use of different elements within the *ecmA* promoter (Fig. 7). We have analysed the these elements and used a construct specifically expressed in pstO cells to study the processes of slug formation and culmination.
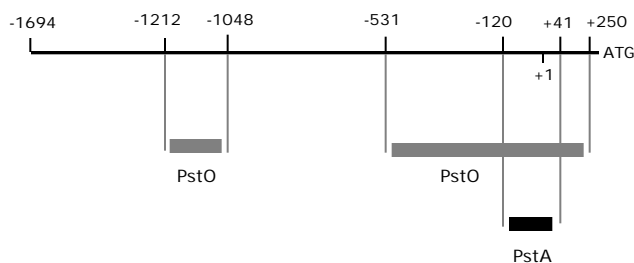

Fig. 7. The structure of the *ecmA* promoter. The region between −1212 and −1048 is independently active in pstO cells. Sequences upstream of +250 and downstream of −531 are also active in pstO cells, albeit weakly, and the presence of a third pstO element between −1048 and −531 has not been ruled out. The region between −120 and +41 is essential for expression in pstA cells, but is inactive in the absence of upstream sequences (see text for details).

Analysis of the *ecmA* promoter

At the time of slug formation, pstA cells are found in the tip (Jermyn et al., 1989), while pstO cells reside in the posterior, prestalk region. This could reflect a difference in the ability of these different cell types to respond to chemotactic signals produced by the tip (Schaap, 1986) or it may reflect a differential response to, or distribution of, inductive conditions within the tip. Discrimination between these possibilities will require determination of the signals that direct pstA and pstO cell differentiation. As a step towards this, a 164-nucleotide region that directs expression in pstO cells and ALCs has been defined. Within this 164-nucleotide minimal region there are several G-rich sequence motifs. They resemble previously identified elements that are known to be important in obtaining efficient expression of developmentally regulated genes (Datta and Firtel, 1987; Pears and Williams, 1987; Ceccarelli et al., 1992) but further analysis will be required before we can identify the element or elements that actually specify expression.

Because a fragment that contains sequences downstream of nucleotide −1041 and that does not, therefore, overlap with the 164 nucleotide fragment is active in pstO cells, there must be one or more additional pstO-specific elements within the *ecmA* promoter (Fig. 7). The 5 deletion analysis shows there to be a pstO-specific element located downstream of −531, but the presence of a third pstO-specific element located between nucleotides −1041 and −531 certainly cannot be ruled out. While multiple, apparently redundant, elements direct expression in pstO cells, the sequences that direct expression in pstA cells seem to be organised somewhat differently. The region between −120 and +41 is necessary for expression in pstA cells but is not sufficient, because a fragment with a 5 end point at −201 (construct Q) is inactive. However, it is possible to restore expression in pstA cells by fusing sequences between −1694 and −797 to a fragment derived from the region −131 to +41. Thus, a combinatorial interaction of discrete, separated sequence elements appears to be required for expression in pstA cells.

These results show that the *ecmA* promoter has a modular structure, as do the promoters of the *ecmB* gene and the cAMP phosphodiesterase gene (Podgorski et al., 1989; Faure et al., 1990; Ceccarelli et al., 1991). This form of promoter organisation may be common to genes expressed in development, perhaps because it allows the fine tuning of expression patterns in response to inductive signals. Interestingly, the organisation of the *ecmA* promoter bears some resemblance to that of the *ecmB* gene. In the *ecmA* gene, the pstO/ALC region lies upstream of a region that is capable of directing expression in pstA cells. In the *ecmB* gene, an element directing gene expression in upper cup cells (which are derived from ALCs) is positioned upstream of nucleotide −858, while a region capable of directing expression in anterior prestalk cells lies between nucleotides −877 and −757 (Ceccarelli et al., 1991). This latter, positively acting region is under the control of a repressor region that prevents gene activation until cells pass into the stalk tube at culmination (Ceccarelli et al., 1991).

The origin and fate of pstO cells

Cells expressing the *pstO:lacZ* gene first appear as scattered cells within the aggregate. Because of the dispersed nature of the pstA-specific elements, we have not as yet been able to generate a pstA-specific construct. Hence, we have not been able to determine whether pstA cells are also scattered in the aggregate when they first arise. The fact that pstA cells in the slug tip show no staining with a pstO-specific construct shows that it is not obligatory to be a pstO cell before becoming a pstA cell. In fact, the two cell types seem to be quite distinct, because if slug migration does not occur the pstA and pstO zones remain unmixed during culmination. There is an ordered movement of cells into the stalk tube, the non-stained pstA cells being followed by a proportion of the stained pstO cells. A similar approach has been used to infer an ordered movement into the stalk in the related Dictyostelid, *Polysphondylium pallidum* (Vocke and Cox, 1992).

The presence of staining in the stalk of transformants containing pstO-specific constructs merely indicates that the pstO promoter element has been active at some earlier time not that it is actively transcribed in the stalk. Also, we cannot rule out the possibility that some, or all, pstO cells activate gene expression via the pstA-specific element during culmination. As a consequence, it is not possible to distinguish between two alternative stalk cell differentiation pathways, pstO to stalk cell <u>or</u> pstO to pstA to stalk cell. The pstO cells that do not enter the stalk tube contribute to the upper cup so that, unlike the pstA cells, pstO cells have more than one possible fate.

The effect of slug migration on pstA and pstO cells

A more complex situation arises during slug migration, because cells expressing the *ecmA* gene via the pstO-specific elements then appear in the tip. There is no re-assortment after the cessation of migration, so that the unstained tip does not reappear and during culmination the entire stalk is stained. This means either that the pstA cells no longer enter the stalk tube preferentially or that the original cellular identities have not been maintained, i.e. that pstA cells start to express the *ecmA* gene via the pstO-specific elements during prolonged slug migration. Transdifferentiation during slug migration is a well characterised phenomenon in *Dictyostelium*. Previous studies of prespore cell diffentiation show that clear prestalk-prespore cell compartmentalisation is lost during slug migration (Tasaka and Takeuchi, 1983; Harwood et al., 1991) and transplantation experiments between the anterior and posterior prestalk regions of slugs provide some support for respecification during migration (Bühl and McWilliams, 1991).

Possible functional significance of the discrete pstA and pstO regions

The function of pstO cells is at present unclear. Both the pstO and pstA cells express the *ecmA* gene but at different levels. It may be important to slug structure that there be a uniform amount of *ecmA* protein in the slime sheath. In this case, more *ecmA* protein would need to be produced per cell in the front of the slug, because the tip contains fewer cells per cross-sectional area than does the back of the prestalk zone. We assume that this is not the only significance of the distinction between pstA and pstO cells. *EcmA* expression via distinct promoter elements presumably reflects cell type differences that result in differential expression of other genes important in morphogenesis.

There is evidence for functional differences between the prestalk cell types. The studies of Bühl and MacWilliams (1991) show that pstO and pstA cells return to their original location when misplaced by transplantation, and isolated cells from the back half of the prestalk region differ from cells within the tip in that they are less dependent upon exogenous cAMP for their induction into stalk cells by DIF (Kwong et al., 1988). The product of at least one gene, the 5′-AMP nucleotidase enzyme, is selectively localised at the prestalk-prespore boundary during culmination (Armant and Rutherford, 1979). It will be of interest to determine whether the 5′-AMP nucleotidase gene, and genes involved in cell movement and cAMP signalling, are regulated by the same mechansims that control expression of the pstO-specific region of the *ecmA* promoter.

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