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A repressor controls the timing and spatial localisation of stalk cell-specific gene expression in *Dictyostelium*

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SUMMARY

The *ecmA* and *ecmB* genes of *Dictyostelium* encode related extracellular matrix proteins and both are induced by DIF, the stalk cell-specific morphogen. The *ecmA* gene is expressed throughout the prestalk region of the migrating slug but only later, at culmination, do the prestalk cells express the *ecmB* gene. Expression of the *ecmB* gene is induced at the entrance to the stalk tube and we have identified two, apparently redundant, promoter elements that control this process. They act as repressors, preventing transcription in the tip of the migrating slug and the apical papilla of the culminant. They have a semi-palindromic consensus sequence TTGnCAA, where n is in one case 2 and in the other 4 bp. Either element alone is able to repress *ecmB* promoter activity in prestalk cells. Introduction of a single repressor element into the promoter of the *ecmA* gene changes its expression pattern to resemble that of the *ecmB* gene.

Mutant elements, where n is altered, cause repression during the slug stage but allow premature *ecmB* expression during culmination; suggesting that the effective strength of the inductive signal may increase during culmination. Inhibition of cAMP-dependent protein kinase (PKA) in prestalk cells blocks both stalk cell maturation and *ecmB* gene expression. We show that the block to gene expression correlates precisely with the presence of a functional repressor element and this is consistent with the notion that expression of the *ecmB* gene is controlled by a PKA-dependent release from transcriptional repression.

Key words: *Dictyostelium*, *ecmA* and *ecmB* genes, transcriptional repression, cAMP-dependent protein kinase

INTRODUCTION

The development of multicellular organisms involves the differentiation of cells to form spatially discrete populations which are often separated by very sharp boundaries. A boundary can be generated if specific sets of genes are activated by different concentration thresholds of the same extracellular signal (Wolpert, 1969; Driever and Nusslein-Volhard, 1988; Green and Smith, 1990). With a graded signal, different cell populations will arise at positions where their respective activation thresholds are reached. Additional signals may modulate individual thresholds, to result in more complex morphological patterns (Green et al., 1992), and the boundaries can be sharpened further by overlapping inhibitory signals, which repress gene expression (Small and Levine, 1991; Stanojevic et al., 1991).

In addition to being spatially patterned, populations of cells may pass through different, intermediate states of differentiation and so can be classified by their positions within a temporal sequence. Heterochronic mutations result in the inappropriate timing of gene expression. This disruption to the temporal pattern during development can lead to changes in the final morphology of the entire organism (Sonneborn et al., 1963; Ambros and Horvitz, 1984; Ruvkun and Giusto, 1989; Simon et al., 1992) and may be one of the factors underlying speciation (Gould, 1977; Bonner, 1982). Such heterochronic differences are evident during the process of stalk formation in the Dictyostelids. In contrast to *Dictyostelium discoideum* in which stalk only forms during the final stages of development, many other species, such as *Dictyostelium mucoroides*, continuously produce stalk throughout slug migration (Bonner et al., 1955; Raper, 1984). These differences presumably result, at least in part, from differences in the signalling pathways that regulate stalk cell differentiation. In this paper, we investigate the molecular mechanisms that determine the position and timing of stalk formation in *D. discoideum*.

During *Dictyostelium* development, the precursors of the terminal cell types, the prespore and prestalk cells, are first detectable soon after the cells have aggregated together (Bonner, 1944; Hayashi and Takeuchi, 1976; Takeuchi et al., 1978; Krefft et al., 1983). Prestalk cells appear at apparently random positions within the aggregate and then move upwards to form an apical tip (Williams et al., 1989). The tip seems to act as the coordinating centre for morphogenesis throughout the rest of development. Under environmental conditions that are inappropriate for immediate culmination a migratory slug is formed (Schindler and Sussman,

1977a; Bonner et al, 1982). The anterior fifth of the slug contains the prestalk cells (Sussman, 1951). The majority of cells in the posterior are prespore cells (Raper, 1940; Bonner, 1952; MacWilliams and Bonner, 1979) but there are also cells in the rear which share many of the properties of prestalk cells and which therefore are known as anterior-like cells (ALC; Sternfeld and David, 1981; Devine and Loomis, 1985; Voet et al., 1985).

During culmination, the rear of the slug moves under the tip, repositioning the prestalk cells to form a papilla that sits above the prespore mass. The prestalk cells in the papilla then undergo a set of precisely defined movements. They first travel up the outer region of the papilla and then, just below its apex, they reverse their direction of movement; to pass downwards through the middle of the papilla and to penetrate the underlying prespore cells (Bonner, 1944; Sternfeld, 1992) During this process, the prestalk cells secrete around themselves a cylinder of protein and cellulose known as the stalk tube. Simultaneous with the process of stalk formation, the ALC move to surround the prespore mass; eventually to form the upper and lower cups, structures that cradle the spore head of the mature fruiting body, and also to form the outer part of the basal disc (Sternfeld and David, 1982; Jermyn and Williams, 1991).

The expression patterns of the *ecmA* and *ecmB* genes, and the organisation of their promoters, show that prestalk cells can be subdivided into at least four sub-types. The *ecmA* gene is expressed most strongly in the most anterior ten percent of the length of the slug, in cells termed prestalk A (pstA) cells, and at a much lower level in the cells that are situated between the pstA and prespore regions, the prestalk O (pstO) cells (Jermyn et al., 1989; Jermyn and Williams, 1991). Sequences proximal to the cap site of the *ecmA* promoter are required for expression in pstA cells, whereas more distal sequences direct expression in pstO cells (Early et al., 1993).

The *ecmB* gene is expressed in a population of cells in the migratory slug that occupy a cone shaped region just behind the tip (Jermyn et al., 1989). These derive from the pstA cells, by activation of the *ecmB* gene, and accordingly are termed pstAB cells (Jermyn and Williams, 1991). The cone of pstAB cells in the slug marks the point where the stalk tube will appear at culmination. During culmination, the pstA cells differentiate into pstAB cells as they reach the entrance to the stalk tube and they later mature into vacuolated, dead stalk cells. The proximal part of the *ecmB* promoter is responsible for expression in pstAB cells (Ceccarelli et al., 1991). The gene is, however, also expressed in the ALC, and the distal part of the promoter contains the elements responsible for expression in the upper cup, the subset of ALC that move above the spore head at culmination (Ceccarelli et al, 1991).

In previous studies two complementary approaches have been used to investigate the activation of the *ecmB* gene at the entrance to the stalk tube, the earliest known event in stalk cell differentiation. First, the region of the *ecmB* promoter that directs expression in pstAB cells (−877 to +1, numbered relative to the cap site) has been separated into its component parts. Fragments that lack sequences downstream of position nucleotide −486 direct expression in pstA cells in the migratory slug and in pstA cells within the papilla at culmination (Ceccarelli et al., 1991). This suggests the existence, within the promoter, of a repressor element that prevents premature *ecmB* gene expression in pstA cells.

The second approach has been to analyse the signal transduction processes that control *ecmB* expression. Inactivation of the cAMP dependent protein kinase (PKA) in pstA cells, by selective expression of a dominant inhibitory form of the R subunit of PKA, represses the differentiation of pstA into pstAB cells, stalk tube formation and stalk cell maturation (Harwood et al., 1992a). The block to *ecmB* gene expression at the entrance to the stalk tube caused by the dominant inhibitor, suggests that the putative repressor binding protein may be a target of PKA.

This paper describes the identification and partial characterisation of two, apparently redundant, *ecmB* repressor elements. When these elements are replaced by attenuated repressor elements the temporal and spatial pattern of the promoter is altered, suggesting that the threshold for *ecmB* expression is determined by the strength of the repressor element. In addition the promoter is shown to fall under the regulation of PKA only when such elements are present.

MATERIALS AND METHODS

Cell culture and development

The *D. discoideum* axenic strain AX2 was grown in HL5 medium. Transformation was carried out as described previously (Nellen et al., 1984; Early and Williams, 1987). Clones were selected and grown in the presence of 20 μg/ml G418, except those transformed with pEcmA-Rm, which were further selected on 100 μg/ml G418. For development, harvested cells were washed three times in KK_2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH6.2) and spread onto 0.45 μm nitrocellulose filters (Millipore) or, for slugs, onto 2% non-nutrient agar.

Plasmid construction

PCR products derived from the *ecmB* promoter were digested with *Sau*3A and ligated into the *Bam*HI site of pBluescript™ SK⁻. The integrity of the products was confirmed by sequencing. Each fragment was then excised as a *Bam*HI/*Xba*I fragment and ligated into equivalently digested pA15ΔBam-gal (Ceccarelli et al., 1991), to give plasmids pΔN-gal and pΔG-gal.

Complementary oligonucleotides were synthesised to regions of the *ecmB* promoter. They were designed to anneal to form *Bam*HI compatible cohesive ends. Each set of oligonucleotides was ligated into the unique *Bam*HI site of pΔN-gal. All constructs were subsequently confirmed by DNA cycle sequencing using Circumvent™Exo⁻ (New England Biolabs).

The upper strand sequences of complementary oligonucleotides not shown in the text:

B: GATCTACTATATACATAATCATTGGGAAATCTCATTTTACTTATCA-
CATTGTTTTATTGTTAG
C: GATCCATAGTAAAATTTATTTTGGATATTTCAATTGATTCAAT-
TAAAAAAAACAAAATGCTGTCAAATTATTTATTA
E: GATCCTTATACATATACACCAT

β-galactosidase staining

After development for the appropriate times, culminants or slugs were fixed with 1% glutaraldehyde in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgS0_4$, 2 mM $MgCl_2$) for 15 minutes. After washing twice in Z buffer, they were incubated

in staining solution (0.1% X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in Z buffer) at 37°C (Dingermann et al., 1989).

RESULTS

Localisation of the *ecmB* repressor element

The repressor region of the *ecmB* promoter was previously mapped to a 276 nucleotide segment located between residues −486 and −208 (numbered relative to the cap site). A new promoter deletion was made to localise the repressor more closely. A fragment was generated that spans the region between a *Sau*3A site at nucleotide −1509 and nucleotide −290. As the fragment lacks a TATA box, it was inserted into the *Bam*HI site of the vector pA15ΔBam-gal to create pΔG-gal (Fig.1). This replaces the basal promoter elements of the *ecmB* gene with the cap-site proximal sequences from the *Dictyostelium actin 15* promoter. The 3′ PCR primer was designed to generate a *Bam*HI site at the junction between the *ecmB* and *actin 15* sequences.

A second construct, pΔN-gal, was made that lacks the *ecmB* promoter sequences downstream of position −486. This generates an identical sized 3′ deletion to that previously described by Ceccarelli et al. (1991) but inserts a *Bam*HI site at the deletion junction (Fig.1). Both the pΔG-gal and pΔN-gal constructs retain the distal elements that direct expression in the upper and lower cups. Retaining this region is doubly advantageous, because stalk expression is stimulated by the efficiency elements present within it and the upper and lower cup expression provide internal controls for staining.

These two deletion constructs were introduced into *Dictyostelium* cells. Upon development, cells transformed with pΔG-gal have the same pattern of expression as the intact promoter. There is very little staining in the slug and, during culmination, staining is restricted to the stalk tube and upper and lower cup structures (Fig. 2A). We will refer to this type of staining as the wild-type pattern. As expected, the pΔN-gal transformant stained throughout the entire papilla during culmination (Fig. 2B) and, in the slug, the entire anterior region and the ALC are strongly stained (Fig. 2C). We refer to this type of staining as the unrepressed pattern. In combination, these results map the repressor to the 196 nucleotide region between −486 and −290.

The *ecmB* promoter contains multiple repressor elements

The pΔN-gal construct provides an ideal background in which to test candidate repressor elements. The 196 nucleotide sequence that conferred repression was synthesised as three separate pairs of complementary oligonucleotides: a 43-mer (oligo A), a 64-mer (oligo B) and an 82-mer (oligo C). Each pair anneals to form *Bam*HI-compatible cohesive ends and the double stranded oligonucleotides so generated were separately introduced into the *Bam*HI site of pΔN-gal. Each construct contains a single copy of the oligonucleotide and, because the cloning was not directional, both of the two possible orientations were generated; one in the same orientation as the wild-type promoter and the other in the opposite orientation (Fig. 3).

Constructs containing oligo A have the wild-type staining pattern, indicating that a repressor element is present within it. Both orientations of the oligonucleotide are equally effective. Constructs containing oligo B have the unrepressed staining pattern observed for pΔN-gal, showing that this oligonucleotide does not contain a repressor element. When oligo C is inserted into pΔN-gal, in either orientation, it generally shows a wild-type staining pattern. This suggests the presence of a second repressor element, addi-

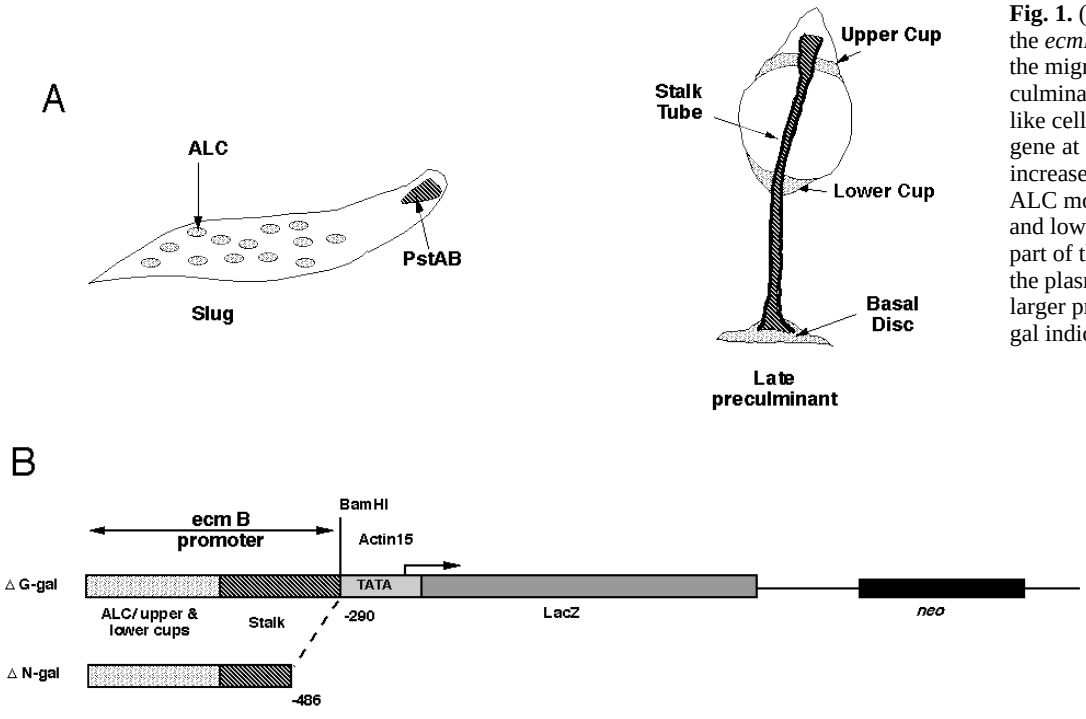

Fig. 1. (A) The regions where the *ecmB* gene is expressed in the migrating slug and maturing culminant. Some of the anterior-like cells (ALC) express the gene at a low level. Thislevel increases at culimination as the ALC move to form the upper and lower cups, and the outer part of the basal disc. (B) Map of the plasmid pΔG-gal, with the larger promoter deletion of pΔN-gal indicated below.

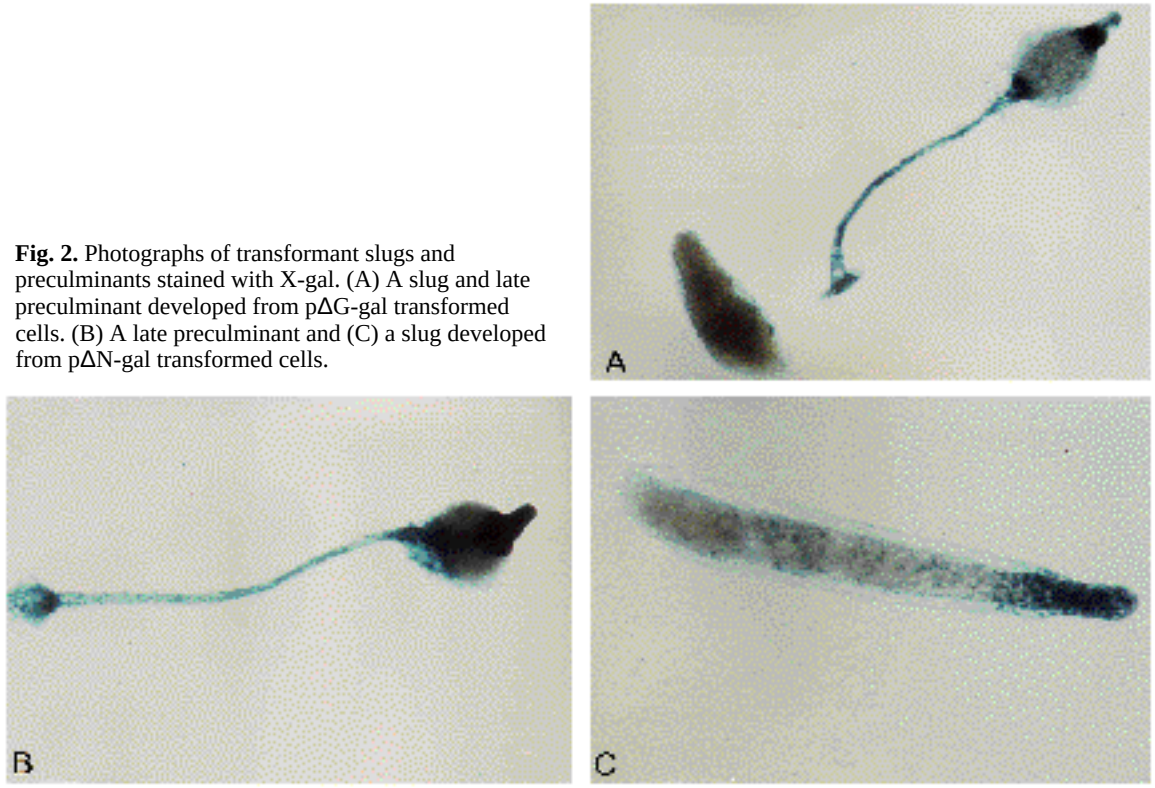

Fig. 2. Photographs of transformant slugs and preculminants stained with X-gal. (A) A slug and late preculminant developed from pΔG-gal transformed cells. (B) A late preculminant and (C) a slug developed from pΔN-gal transformed cells.

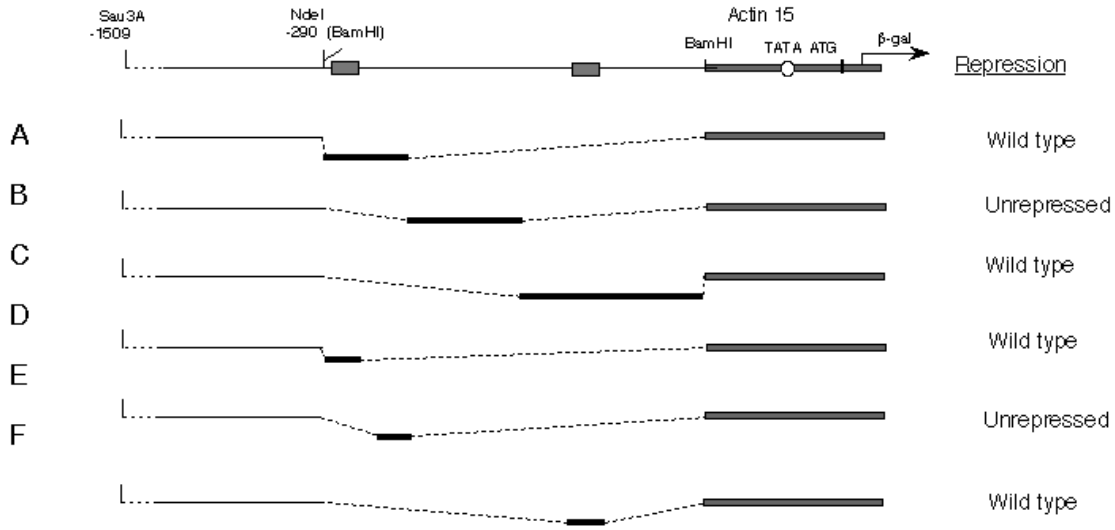

Fig. 3. Diagram showing the location of the *Bam*HI site where oligonucleotides were inserted into pΔN-gal and the regions effectively deleted from the *ecmB* promoter in the resultant constructs, A-F. The positions of the repressor elements are marked by the stippled boxes in the map.

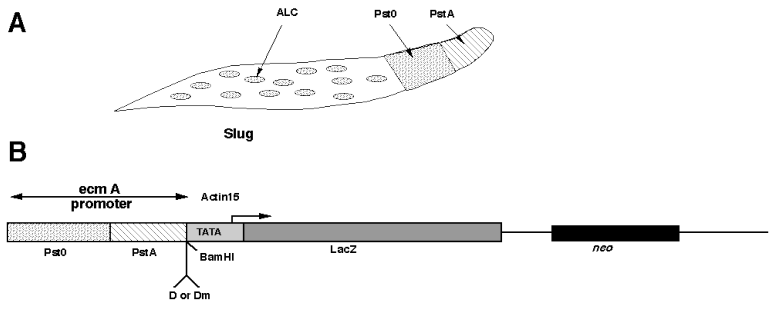

Fig. 4. (A) The pattern of expression of the *ecmA* gene. Pst0 cells express the gene less strongly then pstA cells. (B) Map of the ecmA-oligo D construct which contains the *ecmB* repressor element (oligo D) inserted into the *Bam*HI site at position +40 relative to the *ecmA* cap site. EcmA-oligo Dm has an identical structure, but with oligo Dm replacing oligo D.

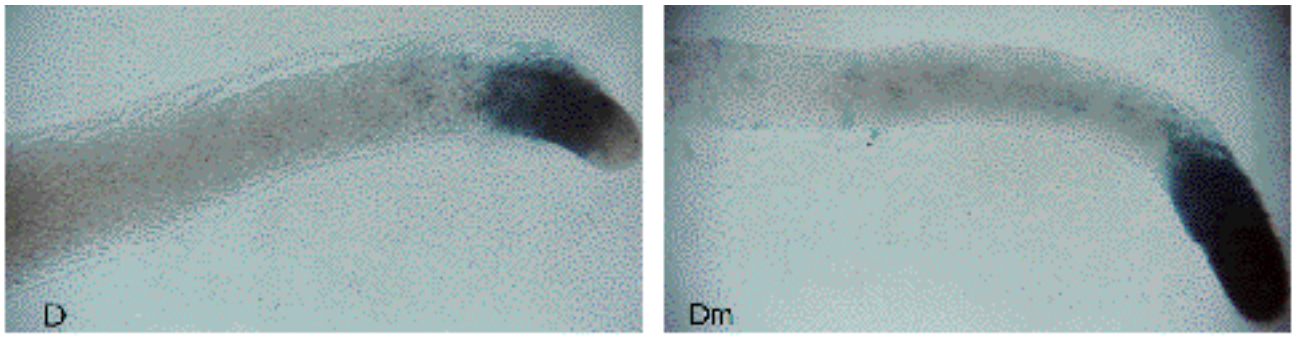

Fig. 5. Photographs of slugs transformed with ecmA/repressor constructs stained for β-galactosidase expression. (D) A slug transformed with ecmA-oligo D construct and (Dm) a slug transformed with ecmA-oligo Dm.

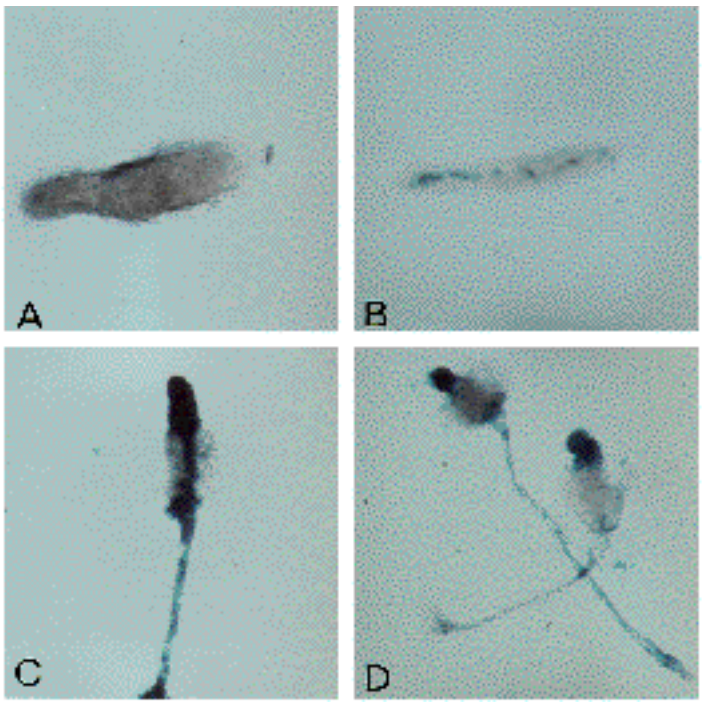

Fig. 6. Photographs of slugs and preculminants developed from cells transformed with oligo D_1 and oligo D_3 constructs. (A,B) Slugs developed from cells transformed with the D_3 construct, and stained for 1 and 5 hours respectively. Preculminants developed from cells transformed with oligo D_3 (C) and oligo D_1 (D) constructs and stained for 1 hour.

tional to that present in oligo A. In some transformant clones that contained oligo C constructs there was no staining in the upper and lower cups. The reasons for this difference from oligo A, and for its clonal variability, are as yet unknown.

Oligo A was further subdivided into a 24-mer (oligo D) and a 23-mer (oligo E), and these were tested by insertion into pΔN-gal. Only oligo D gives the wild-type staining pattern and, again, this is orientation-independent. Oligo D contains the broken palindromic sequence TTGAACAA (see Table 1). A new oligonucleotide was synthesised, equivalent to oligo D (oligo Dm), but with a guanine to adenine substitution to give the sequence TTAAACAA. Constructs containing this oligonucleotide in either orientation give the unrepressed staining pattern, indicating that loss of either the guanine or the cytosine is sufficient to disrupt the element.

One part of the sequence within Oligo C reads TTGATTTCAA. This is the same broken palindrome as oligo D, but it has a larger spacing between the dyad arms. To test whether this element also functions as a repressor, a 23-mer (oligo F) was inserted into pΔN-gal. Constructs containing oligo F have the wild-type staining pattern. Interestingly, they do not exhibit the loss of cup structure staining sometimes seen with oligo C. Presumably, there are sequences within oligo C, but not oligo F, that influence expression from the upper and lower cup promoter elements.

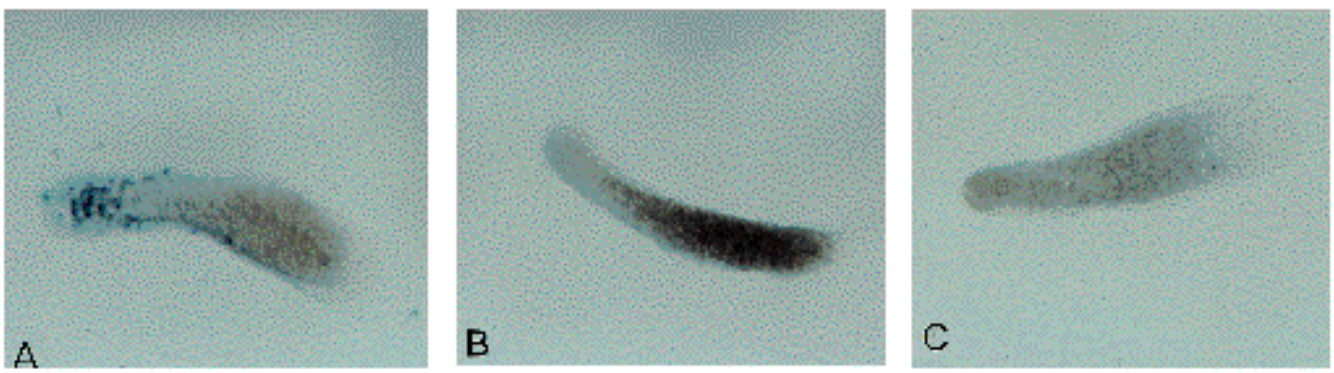

Fig. 7. Photographs of slugs co-transformed with ecmA-Rm and the following oligo constructs: (A) Dm, (B) D and (C) F.

Table 1. Alignment of repressor elements and mutants

Oligo	Sequence*	Repression
D	GATCTGTT**TTG** AA **CAA**CATATATG	wild type
D^{inv}	CATATATG**TTG** TT **CAA**AACAGATC	wild type
Dm	GATCTGTT**TT**A AA **CAA**CATATATG	unrepressed
Dm^{inv}	CATATATG**TTG** TT TA**AA**ACAGATC	unrepressed
F	GATCAAT**TTG**ATTT**CAA**TTAAAG	wild type
F^{inv}	CTTTTAA**TTG**AAAT**CAA**ATTGATC	wild type
consensus	**TTG**(A/T)$_{2/4}$**CAA**	

*Upper strand sequence of oligonucleotide
^{inv}oligonucleotide introduced in opposite orientation

The repressor element inhibits *ecmA* expression in pstA cells

An *ecmA* promoter construct with a conveniently situated *Bam*HI site was used to determine whether the repressor will act upon a heterologous promoter. In this construct (construct A in Early et al.,1993), the *ecmA* promoter region between positions −1694 and +41 is fused, via the *actin15* minimal promoter elements, to the *lacZ* gene (Fig. 4). It gives the same staining pattern as the wild-type promoter. When oligo D is inserted into the *Bam*HI site at +41, there is no expression in pstA cells but pstO expression is unaffected (Fig. 5). The Dm mutant oligonucleotide has no effect on *ecmA* expression.

The effect of changing the central spacing of the element

The fact that the two repressor sequences in the *ecmB* gene differ in separation of the conserved dyad elements prompted us to synthesise and test other spacing variants (Table 2). Oligo 0, which contains no spacer between the dyad elements, does not restore the wild-type staining pattern (data not shown). Oligonucleotides D_1 and D_3 are based upon the sequence of oligo D, but contain one or three adenine nucleotides respectively as their central spacer. Constructs containing either of these oligonucleotides give a wild-type staining pattern at the slug stage (Fig. 6A,B and data not shown for oligo D_1). Remarkably, however, the entire papilla stains during culmination and this occurs at a very early stage, almost immediately after the papilla becomes visible (Fig 6C,D).

The repressor site is required for PKA regulation

Overexpression of Rm, a mutant form of the *Dictyostelium* R subunit that cannot bind cAMP, inhibits PKA activity (Harwood et al.,1992b). One effect of the overexpression of Rm in pstA cells is to block stalk-specific *ecmB* gene expression (Harwood et al., 1992a). We have previously speculated that PKA is required at culmination to inactivate a protein that binds to the repressor elements. This could be either by direct phosphorylation or as part of a kinase cascade. An alternative hypothesis is that PKA is necessary for full activity of other, positively acting regions of the *ecmB* promoter. We have used the *ecmB* promoters that lack the repressor element to investigate this latter possibility.

Co-transformant clones were generated that contain both *ecmA-Rm* and various *ecmB-lacZ* repressor element constructs. Each was examined at the slug stage, when the positively acting sequences are known to be active. Co-transformant clones where the *ecmB* promoter construct lacks a functional repressor element, Dm (Fig. 7A) or pΔN-gal (data not shown), show staining within the anterior prestalk region. Thus inactivation of PKA allows expression in transformant clones where there are no negative elements within the promoter. EcmA-Rm co-transformants with constructs containing either oligo D or F, the functional repressor elements, show no staining in pstA cells (Fig 7B,C). These results are therefore consistent with the notion that PKA regulates the *ecmB* promoter via the repressor element.

DISCUSSION

We have mapped two repressor elements within the *ecmB* promoter. Both are present in the proximal part of the promoter, the region known to direct expression within stalk cells. Each element consists of a dyad of trinucleotide half sites (TTG) with the consensus sequence TTG(A/T)$_{2or4}$CAA, suggesting that the repressor protein probably binds as a dimer. The spacer contains only adenine or thymidine residues, but this may not be too significant as the *Dictyostelium* genome is highly enriched in both these residues (Sussman and Rayner, 1971; Firtel and Bonner, 1972).

One element alone is sufficient to repress expression when inserted into a deletion mutant of the *ecmB* promoter that would otherwise be prematurely and ectopically expressed in pstA cells of the migrating slug and culminant. This apparent functional redundancy raises the possibility of additional repressor elements, located downstream of the most 3′ endpoint of the deletion constructs (nucleotide −290 relative to the cap site). Such an element, with the sequence TTGATCAA, is present at nucleotide −110 and, while we have not tested this sequence for activity, it seems very likely that it would constitute a functional repressor. Hence there may be a threefold redundancy of repressor elements within the *ecmB* promoter.

There is homology between the repressor elements and two elements in the promoter of the rat plasminogen activator inhibitor (PAI-1). These have the consensus

Table 2. Repressor elements with variant central spacing

Oligo	Sequence*	Repression	
		slug	papilla
0	GATCGAAAT**TTGCAA**TTTTAATG	unrepressed	unrepressed
D	GATCTGTTT**TTG**AA**CAA**CATATATG	wild type	wild type
D_1	GATCTGTTT**TTG**A**CAA**CATATATG	wild type	unrepressed
D_3	GATCTGTTT**TTG**AAA**CAA**CATATATG	wild type	unrepressed

*Upper strand sequence of oligonucleotide

TTTGnTCAAT, where n is 1 or 2 nucleotides (Johnson et al., 1992). Similar sequences are also present in the 5′ region of the human fibrinogen β (Huber et al., 1987) and the major histocompatibility complex class II DOβ genes (Servenius et al., 1987). Interestingly, the PAI-1 elements also act as transcriptional repressors.

In *ecmB* elements a central spacing of either two or four nucleotides generates a fully functional repressor sequence whereas the other spacings we have tested do not. Variation between spacing of dyad elements has been previously observed in the binding site for a number of proteins, including the *dorsal* protein in *Drosophila* (Pan and Courey, 1992) and the steriod hormone receptors. In this later case, spacing variation affects hormone specificity (Umesono et al., 1991; Naar et al., 1991) and can even convert an activator element into a repressor (Glass et al., 1988). Insertion of the artificial repressor elements, D_1 and D_3, with variant central spacing, gave a somewhat surprising result. They prevent expression in pstA cells in the migrating slug but allow expression in pstA cells in the papilla during culmination. Thus these 'weakened' repressor elements alter the timing of *ecmB* gene expression.

In combination, these observations give some pointers to the underlying mechanisms that control *ecmB* gene activation. There must be two components, an activation signal and a repression signal. The fact that a single element inserted into the *ecmA* gene prevents expression in pstA cells suggests that the positively acting sequences within the *ecmA* promoter are also susceptible to repressor action. The lack of repression in pstO cells may not necessarily indicate the absense of repressor protein in this cell type or that the pstO-specific activator elements are refractile to repression. Effective repression may require the repressor element to be in a specific position relative to the activator element (Early et al., 1993).

As culmination begins, the strength of the activation signal presumably increases to a sufficient level to override the repressor or, alternatively, the inhibitory signal may decrease to a point where repression is lost. It is also possible that both these processes occur simultaneously. In all three cases, the balance between activation and inhibition will set the threshold for *ecmB* expression. For promoters containing weakened repressor elements, the activation threshold is presumably reduced, so that activation occurs at lower levels of the inductive signal. This would suggest that at culmination the effective inductive signal strength increases throughout the entire papilla, but for an intact *ecmB* promoter the threshold level for activation is only exceded at the entrance to the stalk tube.

In order to understand how the threshold mechanism functions, we need to identify the extracellular signals and trace their intracellular transduction pathways. The activation signal seems likely to be the chlorinated alkyl phenone, DIF (Brookman et al, 1982; Kay et al., 1983; Morris et al., 1987). In an in vitro assay using a *Dictyostelium* mutant that is defective in the production of DIF, induction of both the *ecmA* and *ecmB* genes is dependent upon the addition of DIF (Jermyn et al., 1987; Williams et al., 1987). The extracellular inhibitory signal is, most probably, ammonia. Large amounts of ammonia are produced, by catabolism during development, and experimentally induced, enzymatic depletion of ammonia will cause slugs to undertake culmination (Schindler and Sussman, 1977a).

One of the effects of ammonia treatment is to lower intracellular cAMP levels (Schindler and Sussman, 1977b) and depletion of ammonia is persumably responsible for the elevation of the intracellular cAMP concentration at culmination (Brenner, 1978; Abe and Yanagisawa, 1983). A number of observations suggest that cAMP acts through PKA to induce terminal differentiation along both the spore and stalk cell pathway (Kwong et al., 1988; Kay, 1989; Harwood et al., 1992a; Simon et al., 1992; Anjard et al., 1992; Mann et al., 1992). The results presented here support the idea that PKA controls stalk cell formation by inactivation of the putative repressor protein that regulates *ecmB* gene expression.

Identification of the precise sequence of the repressor elements is an essential step in determining how they function. In other organisms, a number of mechanisms of transcriptional repression have been observed. In its simplest form, repression is caused by direct interaction with activator proteins; either through the formation of transcriptionally inactive heterodimers or by occluding activator proteins that bind to overlapping elements (Levine and Manley, 1989). The *ecmB* repressor elements are located hundreds of nucleotides downstream of the putative activator element, which is located between nucleotides −877 and −757. This suggests that the repressor operates at a distance, as seen in repression by the *Drosphila Kruppel* protein (Licht et al., 1990) and the oncogene *v-erbA* (Damm et al., 1989). Further work will be required to establish whether this is indeed the case.

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