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Evidence that a combined activator–repressor protein regulates *Dictyostelium* stalk cell differentiation

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The *ecmA* gene is expressed in *Dictyostelium* prestalk cells and is inducible by differentiation-inducing factor (DIF), a low-molecular-weight lipophilic substance. The *ecmB* gene is expressed in stalk cells and is under negative control by two repressor elements. Each repressor element contains two copies of the sequence TTGA in an inverted relative orientation. There are activator elements in the *ecmA* promoter that also contain two TTGA sequences, but in the same relative orientation. Gel retardation assays suggest that the same protein binds to the *ecmB* repressor and the *ecmA* activator. We propose that DIF induces prestalk cell differentiation by activating this protein and that the protein also binds to the promoters of stalk-specific genes, acting as a repressor that holds cells in the prestalk state until culmination is triggered.

Keywords: activator/*Dictyostelium*/repressor/stalk cells/transcription

Introduction

The phenomenon of developmental regulation, whereby cells within an embryo retain the option of changing their fate for a period of time subsequent to their formation, is not well understood. An essential feature of regulative development is a mode of differentiation that maintains a cell in a flexible, uncommitted state. *Dictyostelium discoideum* utilizes just such a mechanism. The migrating slug contains the precursors of the stalk in its front one-fifth and those of the spores in its rear four-fifths. Both cell types accumulate specific gene products, so they are truly differentiated, but they remain locked in this state until the slug experiences environmental conditions appropriate for fruit formation and culmination is triggered.

Prestalk and prespore cells retain the option of transdifferentiating throughout the period of slug migration, so that if a slug is bisected into a front and a back portion, each part has the potential to form a correctly proportioned fruiting body (Raper, 1940). As they move apically at culmination, the prestalk cells secrete a protein- and cellulose-containing matrix called the stalk tube. They then move downwards, into the stalk tube, where they vacuolate and die. Entry into the stalk tube seems to be the commitment step, leading down a pathway that results in cell death (Raper and Fennell, 1952).

The molecule that induces differentiation into the stalk cell pathway is a chlorinated hexaphenone called differentiation-inducing factor (DIF) (Town *et al.*, 1976; Kay and Jermyn, 1983; Morris *et al.*, 1987). It accumulates at the time the prestalk cells differentiate and, in a monolayer assay, it induces stalk cell formation (Brookman *et al.*, 1982). The *ecmA* gene encodes a component of the slime sheath, the matrix that surrounds the slug, and its transcription is very rapidly induced by DIF (Williams *et al.*, 1987; McRobbie *et al.*, 1988a). The *ecmA* mRNA is selectively localized in prestalk cells, but expression of the gene is complex, in that different parts of the promoter are utilized in different subsets of prestalk cells. pstA cells are located in the front half of the prestalk region and PstO cells are in the back half (Jermyn *et al.*, 1989). Expression in pstA cells is directed by sequences proximal to the cap site of the *ecmA* gene, while expression in pstO cells is directed by more distal sequence elements (Early *et al.*, 1993, 1995).

The *ecmB* gene encodes an extracellular matrix protein that is present in the stalk tube and the basal disc, and also in the upper and lower cups: two ancillary structures that cradle the nascent spore head and possibly help to support it (McRobbie *et al.*, 1988b: Jermyn and Williams, 1991). The EcmB protein is structurally very similar to the EcmA protein (Ceccarelli *et al.*, 1987) and, like the *ecmA* gene, the *ecmB* gene has a complex expression pattern. Sequence elements proximal to the cap site direct expression only in cells that have entered the stalk tube, while distal sequences direct expression in upper cup cells (Ceccarelli *et al.*, 1991). The location of the elements directing expression to the lower cup and basal disc is not known.

The cap site-proximal sequences within the *ecmB* gene, which direct expression in the stalk, can be further subdivided. Two elements located relatively near to the cap site act as repressors, keeping the gene off in prestalk cells until they enter the stalk tube (Ceccarelli *et al.*, 1991). When they are deleted, and the cap site-distal sequences are fused through a heterologous TATA box and cap site to the *lacZ* gene, there is precocious expression in prestalk cells at culmination. If a repressor element is juxtaposed to the cap site of the *ecmA* promoter, it prevents expression in pstA cells (Harwood *et al.*, 1993). Each element contains two copies of the sequence TTGA, in an inverted relative orientation. If the G residues in the two TTGA sequences are mutated, they no longer act as repressors (Harwood *et al.*, 1993).

The trigger for culmination is a reduction in the ambient ammonia concentration as the slug reaches the surface of the leaf litter (Schindler and Sussman, 1977). The intracellular signalling pathway that senses a change in

ammonia concentration seems likely to involve a change in vesicular pH (Davies *et al.*, 1993) and is known to require activation of cAMP-dependent protein kinase (PKA) (Harwood *et al.*, 1992; Simon *et al.*, 1992; Hopper *et al.*, 1993; Mann *et al.*, 1994; Richardson *et al.*, 1994). Indeed, studies with a membrane-permeant cAMP analogue suggest that a rise in intracellular cAMP may be the only requirement for a prestalk cell to turn into a stalk cell (Maeda, 1992; Inouye and Gross, 1993; Kubohara *et al.*, 1993).

Two of the major questions to be resolved in understanding stalk formation are: how does DIF induce prestalk cell differentiation and what is the nature of the repression mechanism that prevents a prestalk cell from turning into a stalk cell before PKA is activated? In the search for the DNA sequence elements that lie at the end of the DIF signal transduction pathway, we have discovered an unexpected link with the stalk cell repression mechanism. It may explain how prestalk cells achieve and maintain an uncommitted, but stable state of cellular differentiation.

Results

Definition of the minimal promoter fragment capable of directing expression in pstO cells

In a previous study, a 164 nucleotide fragment located just over 1 kb upstream of the cap site of the *ecmA* gene was shown to direct expression in pstO cells when fused to a heterologous cap site and TATA box (Early *et al.*, 1993). In initial experiments, we were able to define the minimal region more precisely, by deleting a further 32 nucleotides of sequence distal to the cap site (data not shown), but it proved impossible to reduce the size of the fragment further and also retain expression. We reasoned that the minimal region must be composed of multiple, interacting elements and so we examined its sequence for an indication as to its component parts.

The region can be approximately divided into three domains (Figure 1A): a central region (domain II), that is extremely T rich, flanked at its 5′ and 3′ sides by more GC-rich regions (domain I and domain III). Domain III contains a CA-rich sequence that fits the consensus for G boxes (Figure 1B; Hjorth *et al.*, 1990). These are GT- or CA-rich sequences that are found upstream of very many of the *Dictyostelium* genes expressed after aggregation (reviewed in Schnitzler *et al.*, 1994). G boxes are not, themselves, capable of directing gene transcription, but act synergistically to amplify the effect of other sequence elements within a promoter (Datta and Firtel, 1988; Pears and Williams, 1988; Ceccarelli *et al.*, 1992; Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994). They are binding sites for GBF, a protein with two putative zinc fingers (Schnitzler *et al.*, 1994). For ease of further manipulation, we substituted domain III, which is relatively large, with two very closely spaced GT-rich sequences from the *ecmB* gene (*ecmB* GII in Figure 1B). *EcmB* GII fully substitutes for domain III (data not shown); consistent with the notion that the CA-rich sequence is the active element within domain III and that it operates as a non-specific activator of transcription.

Multiple copies of domain I are sufficient to direct expression in prestalk cells

Domain I seemed the more likely location for the elements that confer prestalk-specific gene expression, because

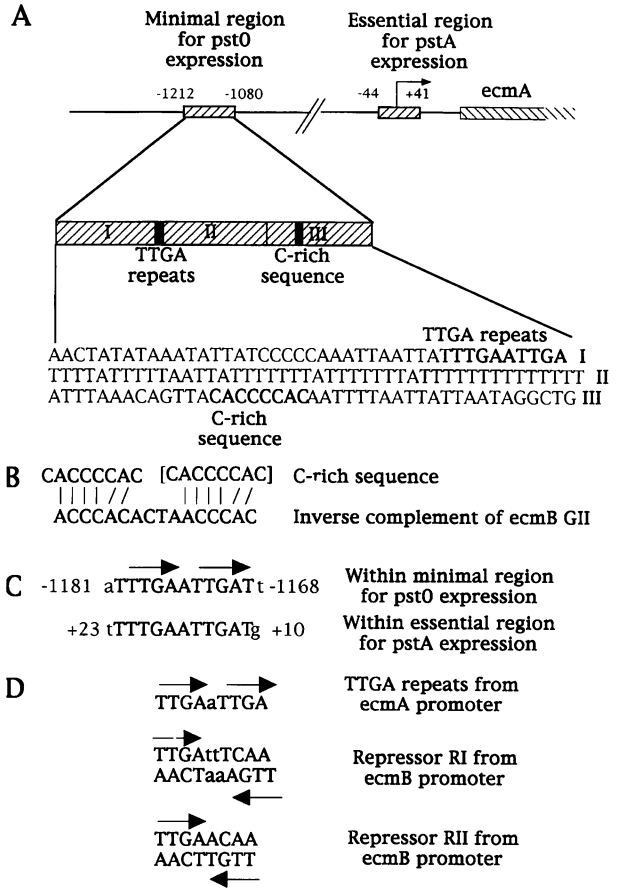

Fig. 1. The organization and DNA sequence of the promoter of the *ecmA* gene. (**A**) The upper part of this panel is a diagram of the *ecmA* promoter showing the regions known to be important in directing prestalk-specific gene expression, with numbering shown relative to the cap site (Early *et al.*, 1993). The lower part depicts in more detail the region directing expression in pstO cells, first schematically and then at the DNA sequence level. The Roman numerals refer to the three sequence domains discussed in the text, and the position of the TTGA repeats, at the end of domain I, and the CA-rich sequence in domain III are indicated by the shaded blocks. (**B**) An alignment of the CA-rich sequence in domain III with *ecmB* GII, a GT-rich region known to be important in obtaining efficient gene expression in the ecmB gene (Kirk, 1994). The sequence in the square brackets is a repeat of the CA-rich sequence and is put for comparison with the second GT (CA-rich as shown here) region in *ecmB* GII. Both the CA-rich sequence and the ecmB GII sequences match the consensus for G boxes (Schnitzler *et al.*, 1994). (**C**) An alignment of TTGA-containing elements from within regions known to be important for expression in pstO and pstA cells showing the 11 bp exact homology. (**D**) A comparison of repressor elements in the *ecmB* promoter with the TTGA repeats in the *ecmA* promoter. RI and RII were formerly called oligo F and oligo D, respectively (Harwood *et al.*, 1993).

domain II consists almost entirely of T residues, so we first investigated its role. A single copy of domain I, which is 53 bp in length, proved inactive when inserted upstream of a basal promoter–*lacZ* fusion gene (data not shown). However, a construct containing four copies of domain I (construct A in Figure 2) directs expression in scattered cells that are mainly confined to the prestalk region (Figure 2). There are expressing cells within the prespore region, but this is expected because large numbers of cells that can utilize the pstO-specific promoter elements are situated there (Abe *et al.*, 1994). They are a subset of the anterior-like cells (ALC), cells that share many of the properties of prestalk cells (Sternfeld and David, 1981,

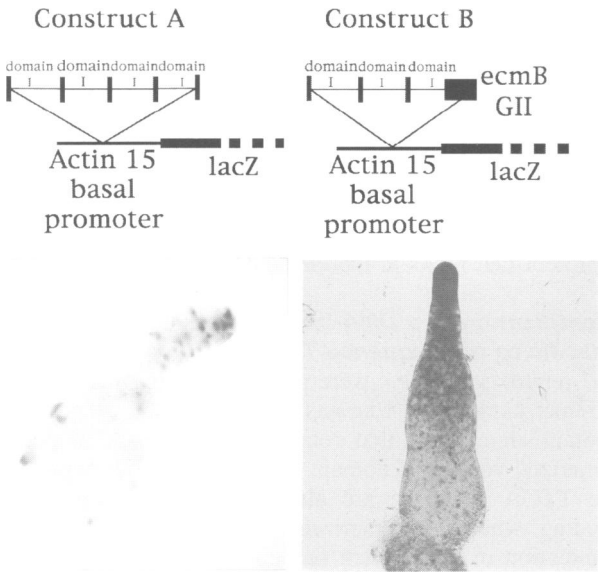

Fig. 2. Structure and expression patterns of constructs A and B. Construct A consists of four copies of *ecmA* domain I fused through an inactive minimal actin 15 promoter to a *lacZ* reporter gene. It was made by the sequential insertion of the domain I 53 bp oligonucleotide, which spans the sequence −1218 to −1165 (Figure 1A), into the *Bam*HI site of the vector *A15ΔBam-gal* (Pears and Williams, 1988; Ceccarelli *et al.*, 1991). After insertion of this oligonucleotide, which has *Bam*HI-compatible ends, a 3′ *Bam*HI site is retained. Construct B has three copies of domain I upstream of the *ecmB* GII oligonucleotide in inverted orientation (sequence shown in Figure 1B), again inserted sequentially into the *Bam*HI site of *A15ΔBam-gal*, leaving a *Bam*HI site at the junction between the two distinct oligonucleotide sequences. The two first fingers/standing slugs, labelled A and B, respectively, derive from cloned cells transformed with constructs A and B, stained for 6 h at room temperature. The structures shown are typical of many examples seen, with clearly prestalk-enriched expression for both constructs.

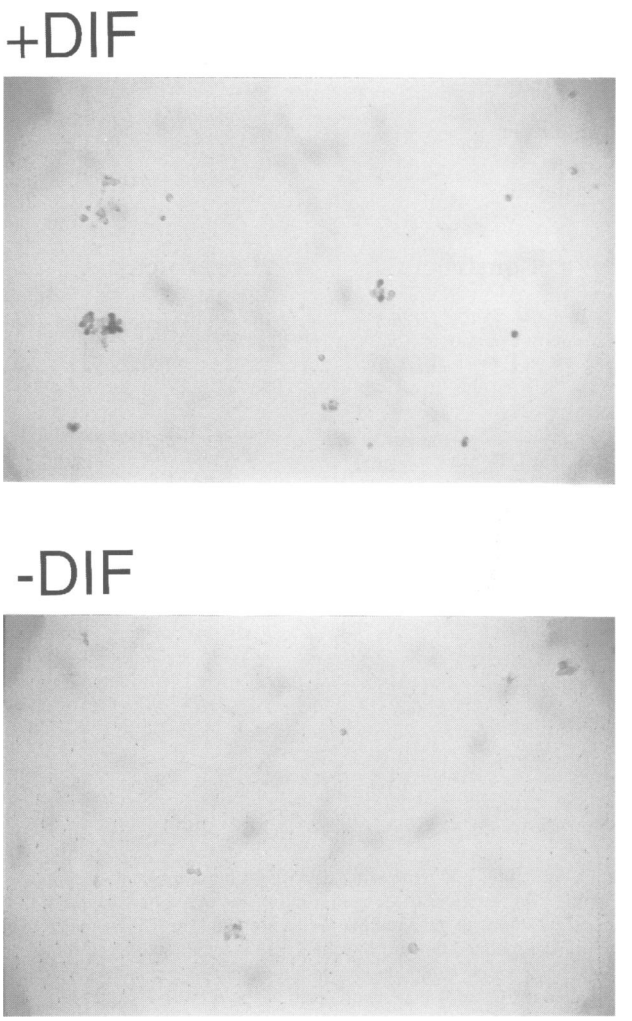

Fig. 3. Induction of gene expression by DIF in cells transformed with a promoter containing domain I fused to *ecmB* GII. HMX44 cells stably transformed with construct B (Figure 2) were plated as monolayer at a density of 2×10^5/ml and induced to differentiate into stalk cells by incubation with DIF (upper panel) or were left untreated (lower panel). This relatively low cell density was used because the pstO element is extremely DIF sensitive (Early *et al.*, 1995), and since HMX44 produces DIF at ~1% of normal levels, pstO expression is seen in the absence of added DIF at higher cell densities. They were then fixed and stained with X-gal *in situ* in the culture dish, overnight at room temperature. Because it is technically difficult to clone HMX44 cells, a pooled population rather than a clone was used. The individual cells expressing at a high level presumably, therefore, have a high copy number of the reporter gene. In scanning many fields, no *lacZ* expression was seen with construct B in the absence of added DIF.

1982). Interestingly, staining is not confined to the pstO region, but extends to the tip of the first finger. Thus, when multimerized, domain I is capable of directing expression in both pstA and pstO cells, albeit weakly.

These conclusions are supported by analysis of a construct (construct B in Figure 2) in which the *ecmB* GII sequence is fused to three copies of domain I. Both the pstA and pstO cells express this construct, and they do so much more strongly than for construct A (Figure 2).

Multiple copies of domain I coupled to ecmB GII constitute a response region for DIF induction

Since multimerizarion of domain I leads to expression in prestalk cells, we next determined whether it contains elements that confer inducibility by DIF. Construct A was transformed into the strain HMX44. This is a mutant defective in the production of DIF, in which differentiation can therefore be made dependent upon exogenously supplied DIF (Kopachik *et al.*, 1983). The transformed cells were exposed to cAMP, to render them competent to respond to DIF, incubated with DIF for 18 h, and then fixed and stained with X-gal. There was no difference between the DIF-treated and the untreated sample (data not shown). We reasoned that the lack of staining over background probably reflects the low level of prestalk-specific gene expression obtained with this construct during normal development (Figure 2). We therefore performed the same experiment with construct B, the construct that also contains *ecmB* GII. Here the result was strongly positive, with 35–40% stained cells being present after DIF treatment and with no detectable expression in the absence of DIF (Figure 3).

Identification of positively acting elements within domain I

The fact that domain I can, when multimerized, direct expression in both pstO and pstA cells suggests that gene expression in the two cell types might be directed by the same DNA sequence element. We therefore searched within the promoter for a region of homology between domain I and the sequences proximal to the cap site that

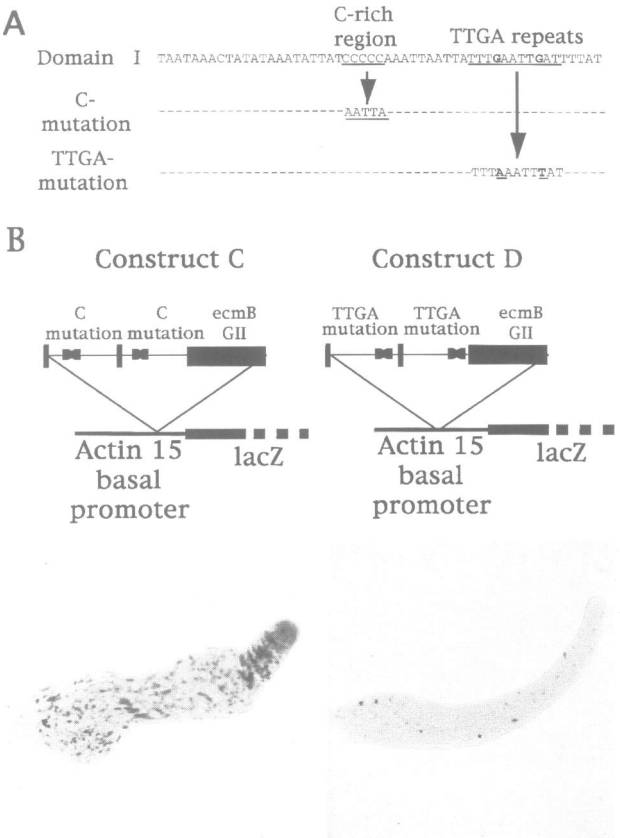

Fig. 4. Structures and expression patterns of point-mutated constructs C and D. (**A**) Structure of domain I and of the mutations that were introduced into it to generate the C mutation and the TTGA mutation. Each of the mutated domain I sequences was synthesized as a dimer of 106 nucleotides, excluding the addition *Bam*HI-compatible ends. (**B**) Construct C consists of two copies of the domain I sequence mutated at the C-rich region, placed upstream of the ecmB GII sequence, which is in the opposite orientation to that in construct B. Construct D has an analogous structure, again with two copies of domain I, this time point mutated within TTGA, and was constructed in an identical manner. The two standing slugs, labelled C and D, respectively, are typical examples derived from cells transformed with constructs C and D. Staining was continued for 6 h at room temperature.

are essential for expression in pstA cells. A construct containing the entire promoter and 41 nucleotides of the 5′ non-coding region is expressed in pstA and pstO cells, but a construct with a 3′ end point 44 nucleotides upstream from the cap site is entirely inactive in pstA cells (Early *et al.*, 1993; Figure 1A). Within the 85 nucleotide region defined by these two constructs, there is an 11 nucleotide sequence that is perfectly complementary to a sequence within domain I (Figure 1C). Each sequence contains two direct repeats of the sequence TTGA (as read in the coding strand for the domain I sequence and in the non-coding strand for the cap site-proximal sequence), separated by a single A residue.

We investigated the role of the TTGA sequences by constructing a mutated form of domain I, in which the G residues in each of the TTGA repeats were altered (Figure 4A). Since there seemed to be one other likely candidate for a control region within domain I, a centrally located C-rich sequence, a separate oligonucleotide with point mutations within it was also made (Figure 4A). The mutated sequences were dimerized and cloned, with ecmB GII, upstream of the minimal promoter (Figure 4B).

Mutations within the TTGA sequences essentially abolish expression, while there is strong expression in the construct containing mutations in the C-rich regions (Figure 4B). This result suggested that the TTGA sequences are at least partially responsible for directing expression in prestalk cells and we therefore searched for a protein that binds to the element.

Identification of a DNA-binding activity that is specific to the sequence TTGA

Gel retardation assays were performed using the domain I 53mer as a probe. Extracts made from slug-stage cells contain an activity that retards the probe. A mutated, dimerized version of domain I, wherein all four copies of the TTGA sequence are altered, fails to compete for binding, whereas a dimerized oligonucleotide containing a mutation in both C-rich sequences competes as effectively as the wild-type form of domain I (Figure 5A). Thus, the retardation activity binds specifically to the TTGA sequences, a conclusion that is supported by competition analysis using a shorter oligonucleotide. Four tandemly repeated copies of the 11 nucleotide sequence that is common to the pstO- and pstA-specific regions of the ecmA promoter (Figure 1C) compete for the binding activity, but somewhat more weakly than the entire domain I sequence (Figure 5C).

The TTGA-binding activity is developmentally regulated. It is weakly detectable at the aggregate stage, reaches a peak at the slug stage and declines thereafter (Figure 5B).

The same protein that binds to the activator sequences in the ecmA promoter binds to the repressor sequences in the ecmB gene

The promoter of the *ecmB* gene also contains TTGA sequences. They form part of the two, mutually redundant repressor elements that keep the gene inactive until cells enter the stalk tube at culmination (Ceccarelli *et al.*, 1991; Harwood *et al.*, 1993). However, the two TTGA sequences that constitute a repressor element are in an inverted relative orientation. In one case (RI; termed oligo F in Harwood *et al.*, 1993), they are separated by two nucleotides and in the other (RII; termed oligo D in Harwood *et al.*, 1993) there are two juxtaposed components, one with the sequence TTGA and the other with the sequence TTGT (Figure 1D). Because of this homology, we next determined whether the TTGA-binding activity identified using *ecmA* promoter sequences as a probe also binds to the *ecmB* repressor elements.

The assay was performed in two ways, using domain I as a probe and RI as a competitor, and vice versa. When domain I is used as a probe, RI competes ~10-fold less efficiently on a molar basis than unlabelled domain I itself. However, the binding is specific, in that a mutant form of RI wherein the two TTGA sequences are mutated (and which is inactive as a repressor element *in vivo*; data not shown) is ineffective as a competitor (Figure 6A). Thus, it would appear that the same TTGA-binding activity that binds to domain I also binds to RI, but that it binds ~10-fold more efficiently to the direct repeat than to the inverted repeat.

The reciprocal experiment, where RI was used as a

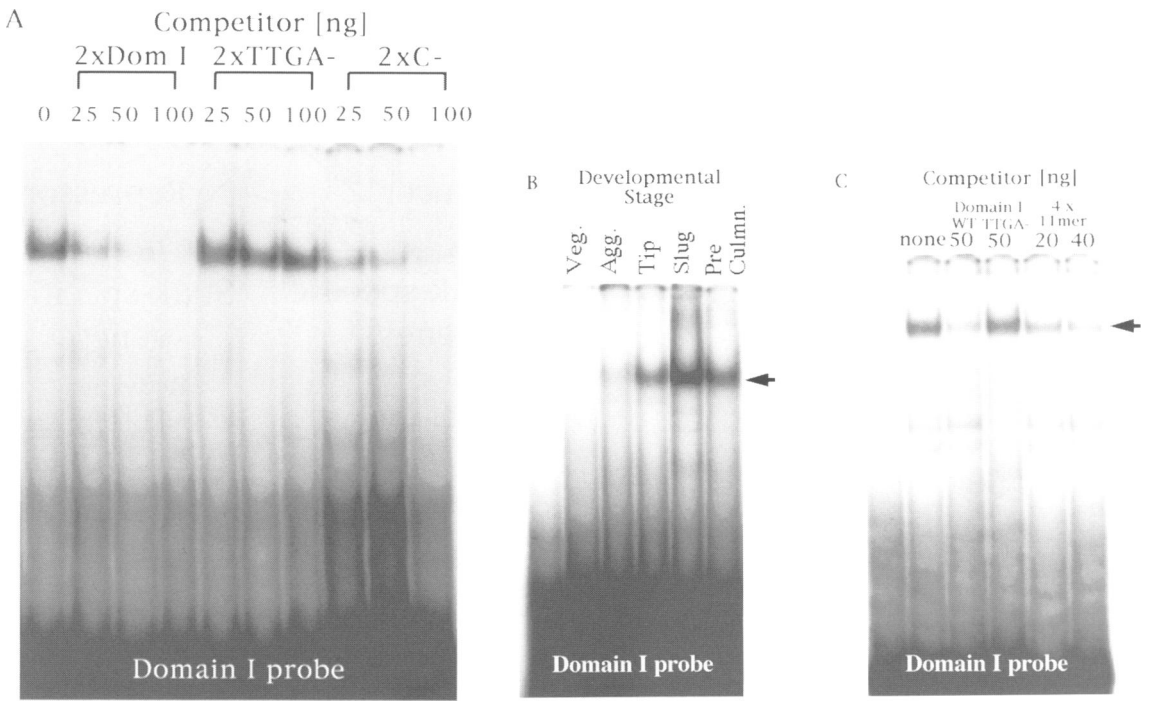

Fig. 5. Identification of a developmentally regulated activity that binds to TTGA elements. (**A**) A nuclear extract from cells at the slug stage of development was partially fractionated as described in Materials and methods, and bound to a labelled oligonucleotide with the DNA sequence of domain I (Figure 1A) in the presence of the indicated competitor. In the tracks labelled 2xDom I, domain I itself was used as a competitor, and in the tracks labelled 2xTTGA- and 2xC-, TTGA-mutated and C-mutated forms of domain I (Figure 4A) were used as competitors, respectively. All competitors contained two tandemly reiterated copies of the indicated sequence. (**B**) Nuclear extracts were prepared and partially fractionated from cells at the indicated stages of development and bound to a labelled domain I oligonucleotide. The same amount of protein (2 μg) was included in each binding mixture. (**C**) Nuclear extract was prepared and partially fractionated from cells at the slug stages of development, and bound to a labelled domain I oligonucleotide in the presence of the indicated competitor. In the track labelled WT, the domain I sequence itself was used as a competitor (Figure 1A), and in the track labelled TTGA-, the TTGA-mutated form of domain I (Figure 4A) was used as a competitor. Unlike (A), these competitors contained a single unit of the indicated sequence. In the tracks labelled 4×11mer, a 76 bp oligonucleotide was used as a competitor containing four tandemly reiterated copies of the 11 nucleotide consensus sequence (Figure 1C). This sequence was interspersed with three randomly chosen distinct spacer sequences, such that each copy of the 11mer has a 20 bp periodicity. The sequence of 4×11mer, which also has *Bam*HI-compatible ends, is 5′-GATCTTTGAATTGATAATCTAGAATTTGAATTGATTACAAGTTATTTGAATTGATGTAATTCTAT-TTGAATTGATG-3′.

probe, confirms the above result. Domain I is, again, a much more effective competitor than RI (despite the fact that this is an RI probe), but the biologically inactive form of domain I, wherein the TTGA sequence is mutated, is ineffective as a competitor (Figure 6B). We also performed gel retardation with the RI probe using RII as a competitor. RII competes with an ~10-fold lower affinity than RI but, again, studies using a mutant oligonucleotide show that the binding is specific (data not shown).

Discussion

Gene expression in prespore cells requires a combinatorial interaction between a GT-rich sequence, a G box and a neighbouring AT-rich sequence (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al.*, 1994). The results presented here indicate that a similar situation exists on the prestalk pathway of cellular differentiation. When four copies of the 53 nucleotide region that we term domain I are multimerized together, there is specific expression in pstA and pstO cells, but expression is weak and is only detectable in a small fraction of the prestalk population. When domain I is multimerized and fused to *ecmB* GII, a GT-rich sequence which cannot itself drive expression, there is a high level of expression in most or all of the prestalk cells. Therefore, although we have not shown directly that it binds to GBF, the *ecmB* GII sequence behaves like a G box in that it is unable to activate expression in isolation, but will amplify the effect of another DNA sequence element.

Two pieces of evidence indicate that at least one of the active elements in domain I is the 11 bp, AT-rich region that contains two, directly repeated copies of the sequence TTGA. There is the indirect evidence of the identity with an 11 bp sequence that lies within a region known to be essential for expression in pstA cells, and there is the direct evidence that point mutations introduced into the two TTGA sequences render domain I inactive. We cannot rule out the existence of other interacting elements within domain I nor of sequence context effects. Indeed, although the 11 nucleotide region of homology competes for TTGA-binding activity, using domain I as a probe, we were unable to obtain gene expression by multimerizing the 11mer and inserting it upstream of a heterologous minimal promoter (data not shown). It is, however, difficult to interpret a negative result when small pieces of DNA are artificially recombined together, so it is necessary to reserve judgement on this point.

Given that it derives from a region that is only capable of directing expression in pstO cells, it is interesting that multimerizarion of domain I leads to expression in both pstA and pstO cells. There are, however, several potential

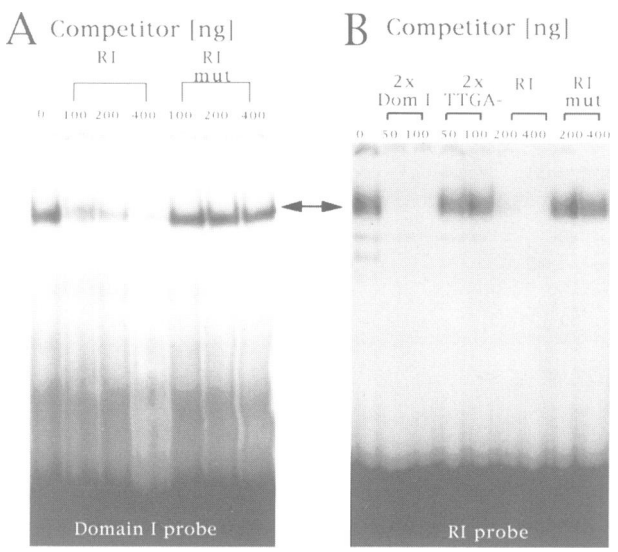

Fig. 6. Evidence that the same activity binds to TTGA elements within the *ecmA* activator and the *ecmB* repressor sequences. (**A**) A nuclear extract from cells at the slug stage of development was partially fractionated as described in Materials and methods, and bound to a labelled oligonucleotide with the DNA sequence of domain I (Figure 1A) in the presence of the indicated competitor. In the tracks labelled RI, an oligonucleotide (5′-GATCAATTTGATTTCAA-TTAAAG-3′) with the DNA sequence of the RI repressor element from within the *ecmB* promoter was used as a competitor, and in the tracks labelled RI mut, a mutated form of RI used as a competitor (DNA sequence, 5′-GATCAATTTAATTTTAATTAAAG-3′). (**B**) A nuclear extract from cells at the slug stage of development was partially fractionated as described in Materials and methods, and bound to a labelled oligonucleotide with the DNA sequence of RI [see (A) above] in the presence of the indicated competitor. In the tracks labelled 2xDom I, domain I was used as a competitor, and in the tracks labelled 2xTTGA-, a mutated forms of domain I was used as a competitor. These competitors contained two tandem copies of the single sequence shown in Figure 4A. In the tracks labelled RI, an oligonucleotide with the DNA sequence of the RI repressor element was used as a competitor, and in the tracks labelled RI mut, a mutated form of RI was used as a competitor [see (A) above].

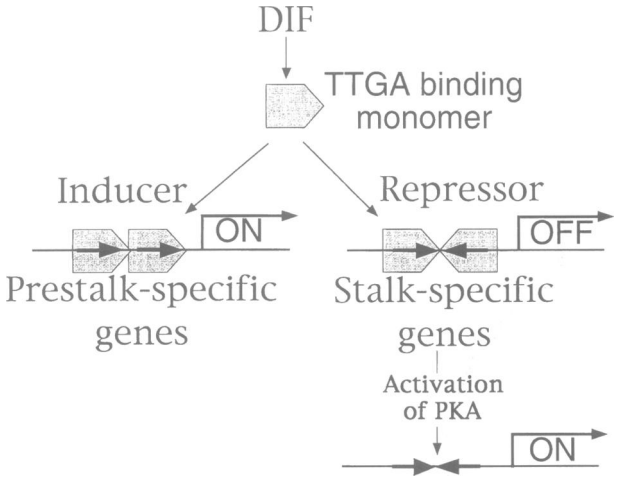

Fig. 7. A model for the regulation of differentiation along the stalk cell pathway. DIF is proposed to activate a TTGA-binding protein which then binds to the promoters of the genes that are specifically transcribed in prestalk cells. It binds as a head-to-tail dimer and activates gene transcription. It also binds to the promoters of genes that are only expressed when a prestalk cell enters terminal differentiation to become a stalk cell. Here it binds in a head-to-head orientation and acts to prevent transcription during slug migration. Within the part of the *ecmB* promoter that directs expression specifically in the stalk tube, there is an activator region of 130 nucleotides (Ceccarelli *et al.*, 1991). When placed upstream of a heterologous cap site and TATA box this region, which lacks a repressor element, directs expression in prestalk cells prior to their entry into the stalk tube. We assume that cells acquire the ability to activate *ecmB* transcription via this activator region as a consequence of becoming prestalk cells, while simultaneously being kept switched off by the repressor. Once a cell has become a prestalk cell, its further differentiation into a stalk cell becomes independent of DIF. Instead, when culmination is triggered, cAMP levels within the prestalk cell rise, PKA is activated, the repressor protein dissociates from the stalk-specific genes and they are transcribed. By making the reasonable assumption that there is a high intracellular cAMP concentration in cells incubated in the presence of a high concentration of exogenous cAMP, such a dominant effect of PKA would explain how the addition of DIF is able to induce the entire process of stalk cell differentiation in monolayer assays where cells are normally exposed to a millimolar concentration of cAMP (Kay and Jermyn, 1983).

explanations for this result and we do not yet have enough information to discriminate between them. It could be that sequences within domain II or domain III contain a repressor element that prevents expression in pstA cells, or it could be a consequence of the multimerization of sequences present within domain I itself.

Domain I, when multimerized and fused to *ecmB* GII, is able to direct DIF-inducible gene expression. Again, because *ecmB* GII is inactive in isolation and because point mutations show that the TTGA repeats in domain I are essential for gene expression, we favour the notion that the repeats are the end point of the DIF signal transduction pathway. If so, the binding protein that interacts with the TTGA repeats must be activated by DIF in some way. It is possible that it is activated directly by DIF, as there is a DIF-binding protein that has a similar developmental time course of appearance to the TTGA-binding activity (Insall and Kay, 1990). The DIF-binding protein is, however, more abundant in the cytoplasm than the nucleus (Insall and Kay, 1990), while the TTGA-binding activity is predominantly nuclear. Addition of DIF to an extract containing the TTGA-binding protein has no detectable effect on its gel retardation activity (data not shown), but this could simply reflect the fact that all the protein within the extract is already in a stable complex with DIF.

Further evidence that the TTGA-binding protein is a central regulator on the prestalk to stalk cell pathway of differentiation comes from the demonstration that it, or a protein with very similar binding specificity, also binds to elements that regulate stalk cell differentiation. The two repressor elements in the *ecmB* gene contain two TTGA sequences, but they are in an inverted relative orientation. The TTGA-binding activity interacts with them specifically, but with a lower affinity than to the direct repeats in the *ecmA* promoter. The fact that the direct repeats act as a better competitor than the inverted repeats, even when the inverted repeats are used as a probe, argues in favour of the same, or very similar, proteins being involved.

One simple way to interpret these results is to posit a multi-step process for stalk cell formation (Figure 7).

(i) An uncommitted cell is exposed to a sufficiently high concentration of DIF to trigger the DIF intracellular signalling pathway and hence activate the TTGA-binding activity. This first binds to the direct repeats, present in the promoters of prestalk-specific genes such as *ecmA*, because these have a relatively high affinity for the activity.

We assume that it is a dimeric protein, that the monomeric subunits bind to the direct repeats in a head-to-tail relative orientation and that in this configuration they function as activators of gene transcription. (It could, of course, be a heterodimer, composed of two different TTGA-binding proteins, but because this would not alter the essential nature of the model we will assume a homodimer.)

(ii) As the concentration of the TTGA-binding protein increases, it interacts with the low-affinity, inverted repeats present in the promoters of stalk-specific genes. Here, the monomeric subunits bind in a head-to-head fashion and they act as repressors of gene transcription. The thyroid hormone (T3) receptor provides a close precedent for such behaviour. Direct repeats of a T3 response element half-site direct constitutive transcription which is repressible by T3, while inverted repeats of the same motif direct T3-inducible gene expression (Naar *et al.*, 1991).

(iii) Abundant evidence shows that the intracellular signal that triggers terminal stalk cell differentiation is a rise in intracellular cAMP. This acts, via PKA, to negate the effect of the repressor elements present within the promoter of the *ecmB* gene and presumably of other stalk-specific genes (Harwood *et al.*, 1992; Maeda, 1992; Simon *et al.*, 1992; Hopper *et al.*, 1993; Inouye and Gross, 1993; Kubohara *et al.*, 1993; Mann *et al.*, 1994). We assume that even though the DIF signalling pathway may still be functioning at culmination, PKA acts dominantly to override its effects and so cause removal of the repressors.

We term this the 'induce and hold' model, because the activated TTGA-binding protein induces cells to differentiate as prestalk cells, but then holds them in this state until culmination is triggered. If the model is correct, then there is an elegant economy of resources on this pathway, with a single regulatory protein performing two opposing functions.

Materials and methods

Cell culture, transformation and development

Cells of the AX-2 strain were grown and transformed as described previously (Watts and Ashworth, 1970; Early and Williams, 1987). Transformant clones were selected for a high level of gene expression by plating amoebae in association with *Klebsiella aerogenes* and assaying for β-galactosidase activity using an *in situ* detection method (Buhl *et al.*, 1993). Cells of the strain HMX44 (Morrison and Harwood, 1992), an axenic derivative of HM44 (Kopachik *et al.*, 1983), were transformed as for AX-2 cells, with the exception that selection was initially at 80 μg/ml G418 and then increased to 200 μg/ml to maximize expression levels. The analysis of gene expression of the HMX44 transformants was performed on pooled populations. Development of AX-2 cells to the standing slug stage was performed by washing exponentially growing cells in KK2 (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH 6.2) and plating them at a density of 5×10^7/ml on millipore filters resting on buffer-soaked prefilters placed in Petri dishes within a humid chamber.

Analysis of gene expression

Filters bearing the developing structures were transferred to 1% glutaraldehyde in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 2 mM $MgCl_2$) for 10 min, and washed twice in Z buffer without fixative (Dingermann *et al.*, 1989). They were then incubated in Z buffer containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K[Fe(CN)_6]$ and 1 mM X-gal at 22°C until the appropriate degree of staining was obtained. The reactions were stopped by the addition of 1 mM phenylethyl-β-D-thiogalactoside, and the samples were mounted in gelvatol before photography.

Induction of marker gene expression by DIF

Subconfluent, transformant HMX44 cells were washed twice with KK2 and plated in submerged monolayer culture in stalk salts buffer (Kopachik *et al.*, 1985), 10 mM 4-morpholine ethane sulfonic acid (MES), 2 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 5 mM cAMP, 200 μg/ml streptomycin sulfate and 15 μg/ml tetracycline (pH 6.2). Incubation, at a density of 2×10^5/ml, was in the presence of 5 mM cAMP for the first 8 h. The cells were then washed three times with stalk salts buffer before addition of buffer containing 5 mM cAMP or 5 mM cAMP additionally supplemented with DIF (a kind gift of Dr R.R.Kay) at a concentration of 50 nM. After a further 18 h, the cells were fixed *in situ* and stained overnight at 37°C using X-gal as described above.

Preparation of nuclear extract and gel retardation assay

Axenically growing AX-2 cells (1×10^9 cells) were harvested and washed with KK2 as above, and allowed to develop on 2% water agar plates (Nunc Bio-Assay plates, 24.5×24.5 cm). Cells were harvested at the slug stage, unless otherwise stated, and a crude nuclear extract was prepared, exactly as described by Insall and Kay (1990), except that the concentration of NP-40 was 1%. Saturated $(NH_4)_2SO_4$ solution was added to the crude extract to a final concentration of 20% and the tube was incubated in an ice bath for 60 min and centrifuged at 13 000 *g* for 15 min in a microcentrifuge. Additional saturated $(NH_4)_2SO_4$ solution was added to the supernatant to bring the final concentration of $(NH_4)_2SO_4$ to 60%, and the tube was incubated and centrifuged as above. The pellet was suspended in 200–400 μl of dialysis buffer [10% glycerol, 50 mM potassium phosphate (pH 7.5), 2 mM $MgCl_2$, 400 mM KCl, 200 μM TLCK, 500 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT)] and dialysed against dialysis buffer. The dialysate was centrifuged to remove insoluble material, dispensed into small portions and stored in liquid N_2.

For the gel retardation assay, 2–4 μg of fractionated nuclear extract were used. Binding reactions contained 5 μl of the extract diluted with dialysis buffer, 2 μl of binding buffer [20 mM HEPES–KOH (pH 7.9), 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 40% glycerol, 2 mM DTT, 1 mM PMSF, 0.02% NP-40], 0.5 μg of poly(dA-dT)·poly(dA-dT) (Pharmacia), 25–400 ng of the indicated competitor and 2 ng of probe in a 10 μl total volume. Pre-binding was performed in an ice bath in the absence of the probe for 60 min, and then the probe was added and the tube incubated at room temperature for 30 min. The binding mixture was then electrophoresed on a 5% native polyacrylamide gel.

The probes were made by annealing complementary oligonucleotides either for the domain I region of the *ecmA* promoter or for the RI repressor element of the *ecmB* promoter (Figure 1). These were both made with *Bgl*II cohesive ends, and were labelled with [α-^{32}P]dATP (6000 Ci/mmol, Amersham) using the Klenow fragment of DNA polymerase I. Unincorporated nucleotides were removed by two sequential passages through a 'Push Column' (Stratagene).

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Note added in proof

We have recently shown that the archetypal G box derived from the non-cell type specific gene *cprB* (Datta and Firtel, 1988; Pears and Williams, 1988) resembles *ecmB* GII in that it is able to amplify prestalk-specific expression directed by three tandemly arrayed copies of domain I.