Regulation of Dictyostelium prestalk-specific gene expression by a SHAQKY family MYB transcription factor

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PstA and pstO cells are the two major populations in the prestalk region of the Dictyostelium slug and DIF-1 is a low molecular weight signalling molecule that selectively induces pstO cell-specific gene expression. The two cell types are defined by their differential use of spatially separated regions of the ecmA promoter. Additionally, there are anterior-like cells (ALCs) scattered throughout the rear, prespore region of the slug. They, like the pstO cells, use a cap-site distal ecmA promoter segment termed the ecmO region. When multimerised, a 22-nucleotide subsegment of the ecmO region directs expression in pstA cells, pstO cells and ALCs. It also directs DIF-inducible gene expression. The 22-nucleotide region was used to purify MybE, a protein with a single MYB DNA-binding domain of a type previously found only in a large family of plant transcription factors. Slugs of a mybE-null (mybE-) strain express an ecmAO: lacZ fusion gene (i.e. a reporter construct containing the ecmA and ecmO promoter regions) in pstA cells but there is little or no expression in pstO cells and ALCs. The ecmA gene is not induced by DIF-1 in a mybE- strain. Thus, MybE is necessary for DIF-1 responsiveness and for the correct differentiation of pstO cells and ALCs.

KEY WORDS: DIF-1, Dictyostelium, Prestalk cells, SHAQKY family, MYB transcription factor

INTRODUCTION

The generation of differentiated cell types and positioning them correctly within a developing structure are the two key processes in pattern formation. In the social amoeba Dictyostelium, these two processes are much simplified, relative to higher organisms, but most of the same fundamental principles apply and many of the signalling components are conserved (Kessin, 2001).

Developing cells aggregate together and organise themselves into a fruiting body. Midway through development, approximately one-fifth of the cells in each aggregate differentiate as prestalk cells, while the remainder differentiate as prespore cells. There are two principal sub-classes of prestalk cells, pstA cells and pstO cells (Early et al., 1993; Jermyn et al., 1989). PstA cells occupy the front half of the prestalk region, pstO cells occupy the rear half. The pstO-specific and pstA-specific reporter constructs, normally used to identify these two cell types, derive from the promoter of the ecmA gene (Williams et al., 1987; Early et al., 1993). PstO cells use cap-site distal sequences of the promoter (the ecmO region, Fig. 1), while pstA cells use cap-site proximal sequences (the ecmA region).

The best-characterised inducer of cell-type divergence is the chlorinated hexaphenone DIF-1 (Kay and Jermyn, 1983; Morris et al., 1987). In monolayer assay, DIF-1 induces prestalk and stalk cell differentiation at the expense of prespore differentiation (Early and Williams, 1988; Kopachik et al., 1985; Town et al., 1976; Williams et al., 1987). DIF-1 is produced by the prespore cells as part of a negative-feedback loop that regulates the pstO to prespore cell ratio (Kay and Thompson, 2001).

The ecmA gene is directly induced by DIF-1 (Williams et al., 1987) and in the dmtA– strain, a mutant that is defective in DIF-1 biosynthesis, the pstA-specific marker (ecmA: lacZ) is expressed but the pstO-specific marker (ecmO: lacZ) is not (Thompson and Kay, 2000). These facts suggest that DIF-1 is the physiological inducer of pstO cell differentiation but not of pstA cell differentiation. The situation is, however, complicated by the fact that some pstO markers are expressed in dmtA– slugs (Maeda et al., 2003).

The dimA and dimB genes both encode bZIP transcription factors that are required for DIF-1 responsiveness (Thompson et al., 2004; Huang et al., 2006; Zhukovskaya et al., 2006). DimB binds to the ecmA promoter when DIF-1 is added to cells but the two known DimB-binding sites lie outwith the characterised pstO-specific region (Zhukovskaya et al., 2006). Previous mutational analyses of the ecmA promoter identified a 132-nucleotide subsegment (Fig. 1) that directs pstO-specific gene expression (Early et al., 1993; Kawata et al., 1996). When a G-box, a non-cell-type-specific promoter element (reviewed by Schnitzler et al., 1994), was fused to the multimerised form of a distal sub-region of the 132-mer expression was weakly DIF-inducible. Prestalk-specific gene expression and DIF-inducibility were both abolished when the TTGA repeats, within the distal domain, were subjected to point mutation. The TTGA repeats bind in vitro to the Dictyostelium STAT protein Dd-STATa (Kawata et al., 1997) but Dd-STATa is not the in vivo activator (Mohanty et al., 1999; Araki et al., 1998). There is a DIF-regulated STAT, Dd-STATc, but it also is not required for pstO cell differentiation (Fukuzawa et al., 2001). Thus, identification of the TTGA repeats in the ecmO promoter region led, circuitously, to the identification of the DIF-1:Dd-STATc pathway. However, identifying the inductive pathway that is responsible for pstO cell differentiation remains key to understanding Dictyostelium pattern formation. We have therefore analysed the pstO-specific region of the ecmA promoter further and present evidence that it is regulated by a novel MYB protein.

MATERIALS AND METHODS

Cell growth, transformation and development

Dictyostelium discoideum strain Ax2 was grown axenically and transformed as described (Watts and Ashworth, 1970; Pang et al., 1999). Transformants were selected at G418 concentrations of 10 μg/ml when cells were to be used for DIF-1 induction assays and 50 μg/ml G418 when staining whole mounts for β-galactosidase activity (Dingermann et al., 1989). When first

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isolated, the mybE-null (mybE-) strain grew poorly. Fortunately, the growth problem was overcome by culturing the cells on a plastic substratum in the presence of a heat-killed bacterial food source. After continued passage, the cells spontaneously increased their growth rate. Initially, the mybE- strain also took longer than normal to develop. Again, continued passage alleviated this problem.

DIF induction assay
Transformants, bearing lacZ constructs and selected at a G418 concentration of 10 μg/ml, were harvested from growth, washed and resuspended in stalk medium [10 mM KCl, 2 mM NaCl, 1 mM CaCl2, 10 mM MES (pH 6.2)] containing 5 mM cAMP and 50 μM cefuroxime, with or without 100 mM DIF. The polypeptide synthesis inhibitor, cefuroxime, acts as an inhibitor of endogenous DIF-1 synthesis and use of a low G418 concentration was found to be necessary to prevent a high background level of lacZ expression in the parental control. In some experiments, cells were pre-incubated for 1 hour before addition of cAMP; similar results were obtained with both procedures.

Cell suspension was added into the wells of a 96-well tissue culture plate and incubated overnight at 22°C. Cells were lysed in 100 μl buffer [100 mM HEPES (pH 8.0), 1 mM MgSO4, 5 mM DTT, 2% Triton X-100] containing 1 mM NaCl, 1 mM CaCl2, 10 mM MES (pH 6.2) and incubated at 22°C for 1 hour. The medium was changed to stalk salts, cerulenin, at concentrations between 50 M and 150 M, and 5 mM camp, then incubated for a further 6 hours. Then the medium was removed and replaced with stalk medium, the same concentration of cerulenin (Kay, 1998) and concentrations of DIF-1 up to 100 M. The plates were incubated at 22°C for 16 hours and RNA was extracted using an RNeasy kit (Qiagen) and analysed by RT-PCR using a ‘TITANIUM’ One-Step RT-PCR kit (BD Biosciences). The ecmA primers were: forward, CCAATTTGAACGCTACCCAAACC; reverse, GCAATACCTTTACC-TCTCTG. They generate a 480 nucleotide fragment; IG7, a constitutively expressed mRNA, was used as control.

Nuclear extract preparation and gel retardation assay
Nuclear extracts were prepared from slug stage cells and gel retardation assays performed as previously (Kawata et al., 1996). The probe was made by annealing complementary oligonucleotides with BamHI cohesive ends, derived from the 30-mer sequence (Fig. 2A) (gatccTTATTTTAA-CAGTTACACCCCAATTTTg), followed by labelling with [α-32P]dATP. The CP2 wild-type and mutant oligonucleotides, that were used as G-box/CAE competitors, were: parental, gatccCGGGTGTTTTAAAAAGTTC; reverse, GCAATACCTTTACC-TCTCTG. They generate a 480 nucleotide fragment; IG7, a constitutively expressed mRNA, was used as control.

Protein purification and mass spectrometry
Nuclear extract derived from 3.5×10^11 slug cells was partially purified by precipitation with 40% (w/v) ammonium sulfate, followed by heparin sepharose chromatography. It was then loaded onto a DNA affinity column bearing an oligonucleotide corresponding to the 22-mer. This was synthesized as a duplicate copy, annealed with the complementary strand, multimerized by ligation and coupled to sepharose beads. Bound proteins were eluted with 0.4 M KCl. The eluted proteins were further purified through a second round of binding on the affinity column, concentrated and loaded onto an SDS-polyacrylamide gel. After staining with Colloidal Blue Staining Kit (Invitrogen), protein bands were excised from the gel and digested, in-gel, with trypsin. The resulting peptides were analysed by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionisation) mass spectrometry.

Antibody generation
A rabbit polyclonal antibody was generated and affinity purified, using the C-terminal 15 amino acids of MybE as immunogen. The antibody recognizes MybE but it also detects unrelated proteins. Therefore, it was useful only for western transfer analysis.

Gene disruption
A ClaI fragment of 2.3 kb containing most of the mybE-coding region was cloned into pGEM7 (Promega) and a hygromycin resistance cassette was inserted between two Bal31 sites, located at nt+574 and nt+2079 relative to the ATT initiaion codon. Transformants were isolated clonally and screened for gene disruption by PCR and western blotting.

Expression of a MybE protein fragment in E. coli
The region of mybE-encoding the MYB domain (amino acids 487-818) was cloned in pGEX 5X-1 (Pharmacia). It was expressed in E. coli strain BL21 Codonplus RIL (Stratagene), as a GST-fusion protein, and purified using glutathione-sepharose (Amersham).

RESULTS
Identification of a minimal ecmA promoter fragment
The smallest ecmA promoter sub-fragment known to direct pstO-specific gene expression is 132 nucleotides in length (Kawata et al., 1996). We first showed that a 94-mer, centred within the 132-mer, also directs pstO-specific gene expression when fused to heterologous basal promoter elements (Fig. 1, Fig. 2A). As would be predicted from previous results (Kawata et al., 1996), deletion of distal sequences, to create a 62-mer that lacks the Dd-STATa-binding site, eliminates all detectable pstO-specific gene expression at the slug stage (data not shown). However, this construct is active in culminants, where it directs expression in the upper cup cells (Fig. 2A) – a population that derives directly from the pstO cells (Jermy and Williams, 1991). This observation led us to focus on sequences contained within the 62-mer.

Cap-site proximal elements within the minimal ecmA region are necessary and sufficient for prestalk-specific gene expression
The cap-site distal half of the 62-mer is composed of runs of T residues, interspersed with a few A residues (Fig. 2A). Multimerising a small promoter element sometimes allows it to function in a sequence context where a single copy of the
element is ineffective (Kawata et al., 1996; Powell-Coffman et al., 1994). Therefore, we analysed constructs containing a multimer fused to heterologous basal promoter elements. The multimerised element contains the central 22 nucleotides of the 30-mer (Fig. 3A). The fourfold multimer of the 22-mer (Fig. 3A) directs strong, generic (i.e. pstA, pstO and ALCs) prestalk-specific gene expression (Fig. 3B).

As with most Dictyostelium promoter regions, the 22-mer sequence is extremely AT-rich but two features stood out: a 7-nucleotide imperfect dyad, containing a G and a C residue, and an adjacent C-rich region (Fig. 2A). These two regions were point-mutated within the context of the 94-mer. Mutations 1 and 2, respectively, change the two inner and two outer C residues within the C-rich region into A residues, while mutation 3 changes the C and G residues of the dyad into an A and a T residue (Fig. 4A). In contrast to the unmutated 94-mer, all three mutant forms display scattered, non-cell-type-specific staining (Fig. 4A). Thus, prestalk-specific gene expression requires both the dyad and the adjacent C-rich sequence.

**The 22-mer sequence is both necessary and sufficient for DIF-1 inducibility**

Cells transformed with the lacZ constructs described above were tested for DIF-1 inducibility in a monolayer assay. The 94-mer construct displays a twofold increase in expression in the presence of DIF-1 (Fig. 2B). This is a lower induction ratio than with longer fragments and presumably reflects the absence of elements needed for high expression. Nonetheless, it is reproducible and the 62-mer...
and 30-mer constructs show no significant increase in activity with DIF-1 (Fig. 2B). Point mutations in either the dyad element or the C-rich sequence eliminate DIF-1 inducibility of the 94-mer construct (Fig. 4B). Moreover, when multimerised, the sequences contained within the 22-mer are sufficient to direct strong DIF-1 inducible gene expression (Fig. 3C).

We also analysed dimA–, the DIF-1 non-responsive mutant that contains a disrupted bZIP gene (Thompson et al., 2004), using the 4x22-mer: lacZ fusion as a reporter. The dimA– strain is unresponsive to DIF-1 (Fig. 3D). This provides additional evidence that sequence elements within the 22-mer lie at the end of a DIF-1 response pathway.

**Identification of MybE as the predominant 22-mer binding protein**

When the 30-mer (employed in preference to the 22-mer for technical reasons related to the stability of short AT-rich duplexes) is used as a probe in gel retardation with nuclear extracts prepared from slug cells, one major retarded complex and two minor complexes are observed (respectively marked with an arrow and arrowheads in Fig. 5A). Unlabelled 30-mer, used as a competitor, inhibits formation of the major complex but the minor complexes are relatively unaffected. Variants of the 30-mer, containing the point mutations analysed in the biological experiments described above (Fig. 4A,B), are ineffective as competitors for the major complex (Fig. 5A). Hence, it is a specific complex.

In order to identify interacting protein(s), a slug nuclear extract was twice purified on a multimerised 22-mer affinity column (Fig. 5B). The final eluate contains many protein species and the 10 most abundant of these were identified by mass spectrometry (Fig. 5B; data not shown). Many are predicted to be RNA-binding proteins, presumably sticking non-specifically to the affinity column, but the two highest molecular weight species are MYB family members.

MYB transcription factors contain one to three copies (termed R1 to 3) of a highly characteristic DNA-binding domain (reviewed in Lipsick, 1996). These are typically just over 50 amino acids in length. Each forms a helix-turn-helix in solution and R2 and R3 intercalate into the major groove of DNA (Ogata et al., 1994). There are three previously characterised Dictyostelium MYBs, all containing three MYB domains (Stober-Grasser et al., 1992; Otsuka and van Haastert, 1998; Guo et al., 1999). We have therefore named the two new MYB proteins MybD and MybE, and the genes that encode them *mybD* and *mybE* (annotated as DDB0220512 and DDB0216342 in http://dictybase.org/).

In all of the extracts analysed, MybE was the strongest staining species on the final preparative gel (e.g. Fig. 5B). MybD was present at a lower apparent abundance and in one preparation it was not detectable. It seemed probable, therefore, that the binding of MybD to the 22-mer was in some way artefactual. This led us to concentrate further effort on MybE.

Although most MYB proteins contain two or three MYB domains a large number of plant MYB transcription factors contain only one MYB domain (reviewed by Jin and Martin, 1999). Apart from the presence of one MYB domain, the principal difference between these and orthodox MYB domains is that the amino acids surrounding and including the third of the regularly spaced tryptophan residues, which characterize canonical MYB domains, is replaced by the consensus sequence SH[AL]QKY[RF]. They are therefore sometimes termed the ‘SHAQKY’ family (InterPro Accession Number IPR006447).

An alignment of MybE with several members of the plant family is presented in Fig. 6A. The presence of the sequence SHGQY, at a precisely analogous position to the SHAQKY consensus in the plant MYBs, confirms both MybE and MybD as members of the SHAQKY family. Analysis of the genome sequence reveals five...
Dictyostelium proteins were identified in genome database and aligned using ClustalW. A conserved tryptophan residue and this is replaced by a ‘SHAQKY’-related sequence. (Fig. 2A) Alignment of Dictyostelium SHAQKY proteins. ClustalW was used to align the SHGKY-related sequence. (B) Purification of proteins that bind to a 22-mer DNA affinity column. Slug nuclear extracts were purified and the proteins identified by mass spectrometry.

Characterisation of the MybE DNA-binding site

Having identified MybE as the protein likely to bind the 22-mer in vivo, we analysed the protein-DNA interactions in detail. The C-rich region is essential for biological activity and for competition activity against the major retardation complex in band-shift experiments, using the 30-mer probe (Fig. 4A; Fig. 5A). One possible explanation for this fact is that a second protein binds to the C-rich region and acts synergistically with MybE. G box/CAE elements act by binding the transcription factor GBF (Schnittzler et al., 1994) and are known to synergise with other promoter elements (e.g. Kawata et al., 1996; Powell-Coffman et al., 1994). It was therefore important to determine whether the C-rich sequence in the 30-mer functions via a G box.

This was first analysed using a prototypic G box, from the promoter of the cprB gene (Hjorth et al., 1988; Pears et al., 1985; Pears and Williams, 1987), as competitor in a band shift assay (Fig. 5A). The cprB G box is not an effective competitor against the 30-mer probe.

We also generated a recombinant protein in E. coli, comprising the approximate C terminal half of MybE and encompassing the entire MYB domain, and studied its binding to the 30-mer. The E. coli-derived protein binds to the 30-mer and competitive competition experiments, using the dyad and the C-rich region...
separately, were performed (Fig. 7). Because they are very short elements, each was synthesised in two copies as a direct repeat. A similarly duplicated version of the 30-mer was used as a control. The duplicated 30-mer competes very efficiently but the duplicated C-rich region and the duplicated dyad both fail to compete (Fig. 7). Additionally, all three point mutations within the 30-mer (Fig. 5A), two of which alter the C-rich region, render it ineffective as a competitor (Fig. 7). In combination, these data indicate that the C-rich region is as essential for binding to MybE as the dyad element.

The *mybE* gene is developmentally regulated and cell-type proportioning is highly aberrant in a *mybE* null strain

RT-PCR, with RNA extracted during growth and at various times during development, was used to assay *mybE* expression (Fig. 8A). There is a low but finite level of expression during growth. During development there are two peaks of expression, one early during aggregation the second during early culmination.

A *mybE*-null strain was generated, by replacing most of the coding region sequence with a hygromycin resistance cassette. A large number of randomly selected clones were analysed by PCR and apparent disruptants were subjected to western transfer analysis using a polyclonal antibody directed against a C terminal peptide of MybE. It detects a protein of approximately 100 kDa, the size expected for MybE (Fig. 8B).

One of the strains analysed, the *mybE*- strain, did not detectably express *mybE*. This strain grew and developed poorly when first isolated but after a period in culture it grew and developed well. MybE- and control strains were co-transformed with ecmAO: lacZ, a generic prestalk marker, and also with pspA:gus. The latter construct contains the promoter of *pspA*, a gene that encodes a prespore protein, coupled to β-glucoronidase. Sequential staining with a β-glucoronidase chromogen, X-gluc, and with the β-galactosidase substrate salmon-gal yields a red prestalk region and a blue prespore region (Early et al., 1993).

Migrating *mybE*- slugs are initially much longer and thinner than normal slugs and they subsequently break up into many small fragments. Fig. 9A shows the separated fragments of a *mybE*- slug, with just one complete break point, but many slugs contain multiple incipient break points. With slugs of the control strain, prespore cells occupy the rear 80% of slug length and prestalk cells occupy the front 20%. In the *mybE*- slugs proportioning is extremely variable from slug to slug and presumably depends upon the number of splitting events that had occurred, prior to fixation. The large number of extremely long slugs that predominantly comprise prespore cells, and of fragments that are entirely comprised of prespore cells (Fig. 9A), shows that the prestalk-prespore ratio is greatly distorted in favour of prespore differentiation; however, this was not quantitatively assessed.

Analysis of slugs stained for β-galactosidase alone allows better visualisation of the prestalk sub-populations. MybE- slugs show strong ecmAO: lacZ expression in the very front of the slug, with only a few scattered staining cells in what would normally be the pstO region (Fig. 9B). The heavily stained, anterior region corresponds to the pstA region of a normal slug. In such slugs,
stained for β-galactosidase alone, the ALCs are much more easily visualised and, remarkably, there are almost no stained ALCs in the null strain (Fig. 9B).

Because only one null strain was isolated, it was important to confirm that the phenotypic characteristics are due to MybE inactivation. Therefore, the null strain was transformed with a fusion gene containing the MybE-coding region; linked at its C terminus to GFP and under the control of a semi-constitutive actin promoter. This was a co-transformation with the ecmAO:lacZ marker. The resultant transformants grew and developed well, the slugs were normally proportioned and the ecmAO:lacZ gene displays the normal expression pattern – the hallmark feature being the large number of ALCs that express ecmAO:lacZ (Fig. 9C). Thus, the phenotype observed for the null strain is due to inactivation of mybE.

MybE is essential for normal expression in the major prestalk cell sub-types

Differentiation of the prestalk cell sub-types in the null strain was further investigated using additional reporter constructs.

1. The 4×22-mer: lacZ construct serves as a generic prestalk marker that, like ecmAO:lacZ, is expressed in all anterior prestalk cells and in the ALCs. As would have been predicted from the behaviour of ecmAO:lacZ itself, the 4×22-mer: lacZ construct is expressed in pstA cells but not in pstO cells and ALCs (Fig. 10).

2. EcmA: lacZ is a marker for pstA differentiation. In the control, after very short times of staining, its expression is restricted to the pstA cells but a finite level of staining in pstO and ALCs becomes apparent after longer periods of incubation (Fig. 10; data not shown).

In the null strain, after a short time of staining, expression is almost entirely restricted to the pstA cells (Fig. 10).

3. EcmO: lacZ is expressed in the band of pstO cells that make up the rear half of the prestalk zone and in the ALCs. In the mybE– strain there is no band of pstO cells. This confirms the result obtained using the entire ecmAO:lacZ construct (Fig. 9B). However, there are stained cells in the rear of the slug, in the approximate position normally occupied by the rearguard cells – a subset of the ALCs that is sporadically lost from the back of the migrating slug.

4. ecmB encodes a protein that is closely related to EcmA but in the slug stage it is strongly expressed in a small cone of prestalk cells near the tip: the pstAB cells. In slugs of the mybE– strain, ecmB: lacZ is expressed in scattered cells throughout the slug but there is no cone of pstAB cells (Fig. 10). At culmination, ecmB is activated in prestalk cells as they enter the stalk tube. It is also activated in the upper cup, the lower cup and the basal disc (Jermyn et al., 1996). In the mybE– strain at culmination, ecmB: lacZ is strongly expressed in the stalk and there are stained cells in the positions of the upper cup, lower cup and basal disc (data not shown).

MybE is essential for DIF-1 inducible expression of ecmA

Parental and mybE– mutant cells expressing either the ecmAO: lacZ or the 4×22: lacZ construct were induced with DIF-1 in monolayer assay and lacZ activity was assayed. There is strong induction for both constructs in the parental cells but the two fusion constructs are not induced in the mybE– strain (Fig. 11A). For ecmAO: lacZ, this result was confirmed at the RNA level; by inducing cells with several concentrations of DIF-1 and monitoring lacZ expression using RT-PCR (Fig. 11B). Using the same RNA samples but with ecmA primers, RT-PCR was used to show that the endogenous ecmA gene is, as expected, also non DIF-1 inducible in the mybE– strain (Fig. 11B).
DISCUSSION

The minimal promoter element that directs pre stalk-specific gene expression binds MybE

The minimal ecmAO promoter region that mediates prestalk-specific expression is the fourfold multimerised form of the 22-mer. This is, to our knowledge, the smallest Dictyostelium promoter subregion shown to direct cell-type specific gene expression. Interestingly, although the 22-mer derives from a region that directs expression only within pstO cells, its multimerised form directs expression in pstO cells, pstA cells and ALCs. This implies a close relationship, at the transcriptional level, between the signalling pathways directing differentiation of the two major prestalk cell subtypes.

A dyad within the 22-mer fits the MYB-binding site consensus and is almost identical to a sequence element identified within a subregion of the G6P isomerase promoter that directs prestalk-specific expression. Point mutational analysis showed that this sequence is necessary for prestalk-specific expression but the element was not further characterised (Tabata et al., 2001). As would have been predicted from the sequence of the dyad, the multimerised 22-mer binds in vitro to a MYB protein: MybE.

There is also an essential, C-rich region located downstream of the dyad. There are examples of DNA-binding sites for MYB proteins that extend downstream of the conserved region (e.g. Howe et al., 1991) and the C-rich region in the 22-mer is located very close to the dyad. Hence, it may form part of an extended binding site for MybE.

Similarities between the mybE– strain and other Dictyostelium mutants

The mybE– strain initially grew and developed poorly but, after a period in culture, growth and development improved. It is unclear whether the improvement in development was simply the result of higher cell viability or whether secondary mutations acquired during the adaption process suppressed developmental defects. Therefore, the developmental phenotype we describe must be regarded as a minimal phenotype. However, this phenotype fits a pattern; because there are strong similarities between the mybE– strain and known mutants in DIF-1 signalling.

The morphological phenotype of the mybE– strain is very similar to that of the dimA– and dimB–, DIF response, and dmtA–, DIF biosynthesis, mutants (Thompson et al., 2004; Thompson and Kay, 2000; Huang et al., 2000; Zhukovskaya et al., 2000); all form long thin slugs that frequently split into fragments. The other, striking similarity between the dmtA–, dimA– and mybE– strains is that all three are selectively defective in the expression of ecmAO: lacZ and ecmO: lacZ in pstO cells [the situation with DimB is unclear because it showed such a difference in an Ax3 background (Huang et al., 2006) but not in an AX2 background (Zhukovskaya et al., 2000)]. It will be of interest to determine the signalling relationships between MybE and the DimA and DimB proteins.

Other proportioning mutants also display a change in the extent of the pstO region while retaining a normally sized pstA zone (Han and Firtel, 1998; Chung et al., 1998; Nelson et al., 2000; Ennis et al., 2000). However, the mybE– strain has a more severe defect than any of these strains. It raises the question of whether the mybE- mutant has: (1) a pstO region with no ecmA gene expression but otherwise normal patterning; (2) a pstO region with no expression of ecmA and altered expression patterns of other prestalk-specific genes, or (3) no pstO region. In order to distinguish between these possibilities it will be necessary to analyse additional pstO markers (Maeda, 2003).

MybE is required for the normal differentiation of ALCs

A notable feature of the mybE null, not to our knowledge previously described for any other mutant, is the virtual absence of ALCs that express ecmA promoter-derived markers. The fact that ecmA expression in both pstO and ALCs is greatly reduced is of interest because prior studies suggest a close link between the ALCs and the pstO cells; pstO-specific constructs, derived from the ecmA promoter,
Interestingly, however, there are precedents for SHAQKY proteins mediating induction by a diffusible molecules; the PHR1 protein of Arabidopsis activates gene expression in response to phosphate deprivation (Rubio et al., 2001) and the three OsMYBS proteins of rice mediate gibberelin and sugar regulation of amylase gene expression (Lu et al., 2002). Unusually for Myb proteins, PHR1 functions as a dimer (Rubio et al., 2001) and yeast two-hybrid analysis shows that MybE also forms homodimers (M.F. and J.G.W., unpublished data). Given these similarities, it will be of interest to determine whether DIF-1 and MybE form part of a pathway that resembles a plant signalling pathway.

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