

# The *Dictyostelium* bZIP transcription factor DimB regulates prestalk-specific gene expression

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The *ecmA* gene is specifically expressed in prestalk cells and its transcription is induced by the chlorinated hexaphenone DIF-1. We have purified a novel bZIP transcription factor, DimB, by affinity chromatography on two spatially separated *ecmA* promoter fragments. Mutagenesis of the cap-site proximal DimB-binding site (the –510 site) greatly decreases *ecmA* expression in the pstO cells, which comprise the rear half of the prestalk zone, and also in the Anterior-Like Cells, which lie scattered throughout the prespore region. However, DimB is not essential for normal expression of the *ecmA* gene, instead it spatially limits its expression; *ecmA* is relatively highly expressed in the subset of prestalk cells that coats the prestalk zone, but in slugs of a DimB-null strain, *ecmA* is highly expressed throughout the prestalk zone. Because the –510 site is required for correct *ecmA* expression, we posit a separate activator protein that competes with DimB for binding to the –510 site. DimB rapidly accumulates in the nucleus when cells are exposed to DIF-1, and ChIP analysis shows that, in the presence of extracellular cAMP, DIF-1 causes DimB to associate with the *ecmA* promoter in vivo. Thus, DIF-1 regulates DimB activity to generate a gradient of *ecmA* expression in the prestalk zone of the slug.

**KEY WORDS:** *Dictyostelium*, bZIP, Prestalk, DIF-1

## INTRODUCTION

Primary patterning of the *Dictyostelium* slug creates an anterior prestalk region and a posterior prespore region but, in addition to the coherent mass of prestalk cells that comprises the front one-fifth of the slug, there are Anterior-Like Cells (ALC) scattered throughout the prespore region. Distinct prestalk and ALC subtypes have been identified using *ecmA*, a gene that encodes an extracellular matrix protein.

*ecmA* is more strongly expressed in cells in the front of the prestalk region than in the back and cells in these two locations use spatially separated regions of its promoter (Williams et al., 1989; Early et al., 1993). PstA cells occupy the front half of the prestalk region and employ cap-site proximal promoter elements, while pstO cells occupy the rear half and use cap-site distal promoter elements. Although they were initially identified using the bi-partite promoter of the *ecmA* gene, many other genes are expressed only in one or other region (Maeda et al., 2003). This indicates that the two subtypes are of wide developmental significance and the fact that they differ in their movement patterns within the slug and at culmination supports this notion (Jermyn and Williams, 1991; Abe et al., 1994).

The chlorinated hexaphenone DIF-1 rapidly induces *ecmA* transcription (Williams et al., 1987) and *dmtA*–, a biosynthetic mutant which makes little or no DIF-1, shows a major defect in prestalk cell differentiation (Thompson and Kay, 2000b); pstO-specific gene expression is greatly reduced but pstA differentiation is unaffected. Thus, DIF-1 appears to be essential for efficient pstO cell differentiation but not for pstA cell differentiation. A genetic approach has identified a gene, *dimA*,

that is required for multiple aspects of DIF-1 signalling (Thompson et al., 2004) but its interface with the DIF-1 signalling pathway is unknown.

The protein encoded by *dimA* is a bZIP transcription factor. bZIP proteins are found in all eukaryotes and are characterised by the presence of a basic, DNA-binding region and a closely apposed leucine zipper (Hai and Hartman, 2001; Jakoby et al., 2002; Poels and Broeck, 2004). The leucine zipper mediates the formation of homodimers and heterodimers with other bZIP proteins. We identify a novel bZIP protein that interacts with the *ecmA* promoter and show that it is a DIF-1 responsive regulator of prestalk specific gene expression.

## MATERIALS AND METHODS

### Cell culture, development and transformation

*Dictyostelium* strain Ax2 was grown axenically and transformed by electroporation or, in the case of double transformants, using calcium phosphate precipitation (Nellen et al., 1984). Transformant pools were selected at 50 µg/ml G418 and stained for β-galactosidase activity or double-stained for β-glucuronidase and β-galactosidase (Early et al., 1993).

### DIF induction assays and analysis of *ecmA* gene expression by RT-PCR

Cells were harvested from growth, washed and plated at densities varying between 10<sup>4</sup> and 10<sup>5</sup> cells/cm<sup>2</sup> in stalk medium [10 mM KCl, 2 mM NaCl, 1 mM CaCl<sub>2</sub>, 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 µM and 150 µM, and 5 mM cAMP, and was incubated for a further 6 hours. The medium was then removed and replaced with stalk medium, the same concentration of cerulenin (Kay, 1998) and concentrations of DIF-1 up to 100 nM. 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 are: forward, CCAATTAGCTGTCCA-AAACC; reverse, GCAATCACCTTTACCTCTG. They generate a 480 nucleotide fragment. IG7, a constitutively expressed mRNA, was used as control.

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### Band shift assay, protein purification and mass spectrometry

Nuclear extracts were prepared from slug stage cells (Kawata et al., 1996). For purification, nuclear extract derived from  $3.5 \times 10^{11}$  slug cells was precipitated with 40% (w/v) ammonium sulfate, and subjected to heparin sepharose chromatography. It was then loaded onto a DNA affinity column bearing R1 or R2 oligonucleotides. These were synthesized as a duplicate tandem copy, 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 gel. After staining with Colloidal Blue Staining Kit (Invitrogen), protein bands were excised from the gel, digested with trypsin and analysed by MALDI-TOF mass spectrometry.

### Expression of DimB in *E. coli* and gel retardation assay

The DimB-coding region was cloned in pET28a (Novagene), with the addition of a *Bam*HI site at the N terminus and an *Xho*I site at the C terminus. It was expressed in *E. coli* strain BL21 CodonPlus RIL (Stratagene), as a C terminal HIS tagged fusion protein and purified using a 'Talon' metal affinity column (Clontech). Gel retardation assays were performed, as described above, using approximately 1  $\mu$ g of fusion protein per assay.

### Gene disruption

DimB-coding sequences extending from +1 to +1375 (numbered relative to the ATG) was cloned into a plasmid vector and a *Bam*HI hygromycin resistance cassette was inserted at the unique *Bgl*II site, positioned at nucleotide 600 relative to the initiation codon. Transformants were isolated clonally and screened for gene disruption by PCR and by western blotting.

### Antibody generation

Polyclonal rabbit antisera were generated using the N terminal and C terminal 15 amino acids of DimB as immunogens. The peptides contained a non-coded cysteine residue, respectively at their C and N termini, and these were coupled to an affinity matrix in order to purify the antibody.

### DIF-1 induction of DimB nuclear accumulation

Ax2 cells were developed on non-nutrient water agar to form loose aggregates and DmtA null cells were developed to the tight aggregate stage. Aggregates were mechanically dissociated in KK2 buffer, by trituration through a syringe needle. After dissociation, cells were adjusted to a density of  $2 \times 10^7$  cells in KK2 and treated with 0.01% ethanol or with 100nM DIF-1 in 0.01% ethanol. Cells were fixed on ice in methanol for 10 minutes and stained with affinity purified, anti-DimB antibody. After secondary reaction with Alexa Fluor 488-conjugated goat anti-rabbit antibody (Molecular Probes), samples were analyzed by confocal microscopy.

### ChIP analysis of DIF-1 induced cells

Disaggregated cells, from the loose aggregate stage, were induced by shaking at  $4 \times 10^6$ /ml in phosphate buffer containing 1 mM cAMP and 100 nM DIF-1 for 1.75 hours. They were then fixed with paraformaldehyde (final 1%) in PBS at 4°C for 2 hours. After stopping the reaction with glycine (final 125 mM) and washing, cells were suspended in TNTE buffer [50 mM Tris-Cl (pH 8.0) containing 150 mM NaCl, 1% Triton X100, 0.05% SDS, 2 mM EDTA] containing complete protein inhibitor cocktail (Roche Diagnostics, Germany) and sonicated to yield fragments of 0.3 to 3.0 kb. Immunoprecipitation was carried out from the supernatant in TNTE buffer containing 1% blocking reagent (Roche Diagnostics, Germany): using the anti-DimB antibody or an anti-CudA antibody, and Protein A-Sepharose CL-4B (Amersham Biosciences, Sweden) at 4°C, overnight.

After washing with TNTE buffer and then with 10 mM Tris-Cl (pH 8.0) containing 250 mM LiCl, 1% NP-40, 1% DOC and 1 mM EDTA, DNA was recovered by incubating with 50 mM Tris-Cl (pH 7.5) containing 1% SDS and 1 mM EDTA at 37°C for 15 minutes. After reversing the crosslinking by heating at 65°C overnight and treating with proteinase K (100  $\mu$ g/ml) and RNase (10  $\mu$ g/ml) at 37°C for 1 hour, DNA was purified with a QIA Quick-Spin column (QIAGEN). PCR was performed from immunoprecipitated DNA or from total genomic DNA using the following primers: *ecmA*, AATTATAACCCCATTCGC and AGAGTTTGATGATAACAAGAG (region -1257 to -876); and (as a control) the *gpbA* upstream region, TAAACAAACACACCCAAC and AGGACTTACTAAAATTACAG.

## RESULTS

### The R1 and R2 regions of the *ecmA* promoter bind in vitro to a novel bZIP transcription factor

By combining known expression patterns of *ecmA* promoter deletion constructs (Early et al., 1993) with a limited characterisation of several new deletion constructs (M.F. and J.G.W., unpublished), we designed two oligonucleotides for use in affinity chromatography (Fig. 1).

A 5 to 3' deletion construct of the *ecmA* promoter with a cap-site distal end point at -1041 [construct M (Early et al., 1993)] directs high level expression in *pstA* and *pstO* cells. Further deletion, to -895 [construct N (Early et al., 1993)], reduces expression, equally in *pstA* and *pstO* cells. Hence, oligonucleotide R1 was designed, containing 32 nucleotides of sequence closely bordering the 5' boundary of construct M. Deletion to -531 (construct O) generates the 'standard' *pstA* marker and further deletion to -374 (construct P) greatly reduces expression (Early et al., 1995). Hence, oligonucleotide R2 was designed, containing 32 nucleotides of sequence closely bordering the 5' boundary of construct O.

R1 was used to isolate interacting proteins, by employing its multimerised form in affinity chromatography with slug cell nuclear extracts. After elution the bound proteins were subjected to SDS gel-electrophoresis and the most abundant species identified by mass spectrometry (Fig. 2). Several are RNA-interacting proteins that bind non-specifically to the column. One is a novel bZIP protein that we term DimB (encoded by *dimB*). Another, lower molecular weight species on the gel, DimB', contains a subset of the DimB peptides and is presumably a degradation product of DimB.

When the R2 region was used in affinity chromatography, multiple species were also purified. Again, mass spectrometry showed that several are RNA-binding proteins but one is DimB (Fig. 2). Another of the proteins (asterisked) binds to R2 but not to R1. It is a novel MYB domain protein, that we have named MybE (M.F. and J.G.W., unpublished).

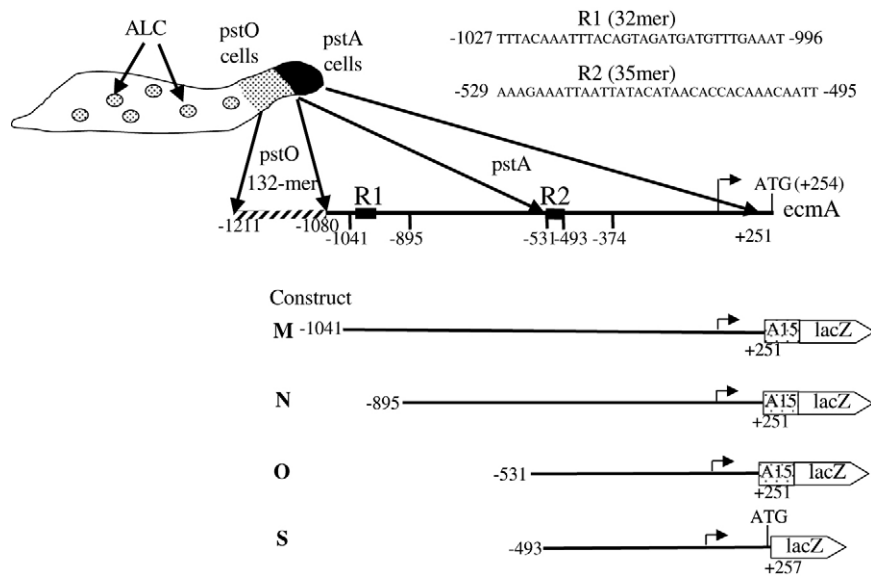
DimB bears significant homology to DimA (Thompson et al., 2004) but is half its size; DimB has a predicted size of 68,447 Da, while DimA is predicted to be 140,259 Da (Fig. 3A). One region of strong homology between the two proteins encompasses the DNA binding/dimerisation domain and an alignment of the basic regions and leucine zippers of DimB, DimA and several other members of the bZIP family is presented in Fig. 3B.

### Determination of the DNA binding properties of DimB

DimB was expressed in *E. coli* as part of a fusion protein, DimB:HIS, in which oligo-histidine is linked to the C terminus. DimB:HIS was employed in band shift assays with either R1 or R2 as labelled probe. DimB:HIS binds to both the R1 and R2 probes, and unlabelled R1 and R2 oligonucleotides are potent inhibitors of the binding: both in self-competition and in cross-competition (Fig. 4; data not shown for R1). Just 10 pmoles of unlabelled R1 or R2 DNA strongly competes for binding of DimB:HIS to a labelled R1 or R2 probe. As negative controls, a discoidin 1 $\gamma$  promoter oligonucleotide, the TTG box (Vauti et al., 1990), and an *ecmB* promoter oligonucleotide, the *ecmB* activator (Ceccarelli et al., 2000), were used as competitors. Both are much weaker competitors than R1 or R2. Thus, recombinant DimB binds with high relative affinity to R1 and R2.

### Identification of the DNA binding sites for DimB

The positions of the DimB-binding sites in R1 and R2 were determined by a mutational scanning technique (Ceccarelli et al., 2000), in which four nucleotide blocks of sequence were replaced



**Fig. 1. The promoter of the *ecmA* gene.** A representation of the *ecmA* promoter showing the positions of the regions known to direct *pstA* and *pstO* specific expression. The minimal region that directs *pstO*-specific expression is a 132 bp subfragment, located just over 1 kb upstream of the *ecmA* cap site (Kawata et al., 1996). The structures of the various mutants mentioned in the text are displayed below (see also Fig. 6B legend). The symbol Act15 indicates the basal promoter elements of the actin 15 gene (Pears et al., 1988). The figure also shows the positions and sequences of R1 and R2, the regulatory elements analysed in the present study.

with the arbitrarily chosen sequence GCGC. The mutant forms were used as competitors in band-shift assays against the respective unmutated probe. In multiple experiments, using varying amounts of competitor, the M6 mutant form of R1 (data not shown) and the M7 mutant form of R2 consistently proved to be relatively ineffective as competitors (Fig. 5A).

Combining the above information with the sequences of several known bZIP binding sites allowed us to deduce a provisional binding consensus (Fig. 5B). The R1 scanning mutation analyses

identified the invert complement of the sequence CATC and this forms part of the sequence CATCA. The R2 scanning analyses identified the related sequence CACA. Together with the cross-species comparison, this suggests that the consensus sequence necessary for binding is A-c/a-A-C-a/c-t/a-C-A (Fig. 5B).

Guided by this consensus, point mutations were inserted into R2. Point mutants R2pM2 and R2pM3, in which one or other of the cap-site distal C residues is changed to A, display reduced binding to DimB (Fig. 6A). This confirms the scanning mutation analysis, in showing that the sequence CACA is necessary for optimal binding of DimB. Point mutations that change all four of the C residues within the proposed consensus region to G, in oligonucleotide R2pM1, eliminate competition activity (Fig. 6A).

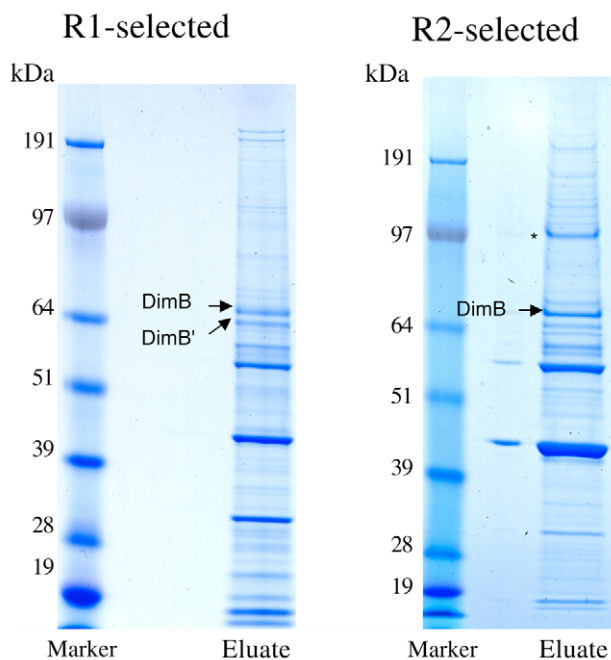
### Point mutations in the proximal DimB binding site greatly decrease expression in *pstO* cells and ALC

R2pM1, the oligonucleotide with the four point mutations that completely abrogate DimB binding, was cloned within a *lacZ* fusion construct to assay its function *in vivo*. This recipient construct, a newly created *lacZ* fusion termed S (Fig. 1, Fig. 6B), has its cap-site distal terminus at nucleotide -493. It differs significantly from published *ecmA* promoter fragment constructs at its cap-site proximal end (legend to Fig. 6B).

Fusion of R2 to S generates a construct, R2S, that is most strongly expressed in the *pstA* region but that is also expressed in cells in the *pstO* region and in large numbers of ALC (Fig. 6B). When this pattern is compared with S and R2pM1S, a striking difference is apparent. With the latter two constructs staining is almost entirely restricted to the *pstA* region, with very few stained cells in the *pstO* region and almost no stained ALC (Fig. 6B). Thus, the cap-site proximal (-510) DimB-binding site is not required for expression in *pstA* cells but is essential for efficient expression in *pstO* cells and ALC.

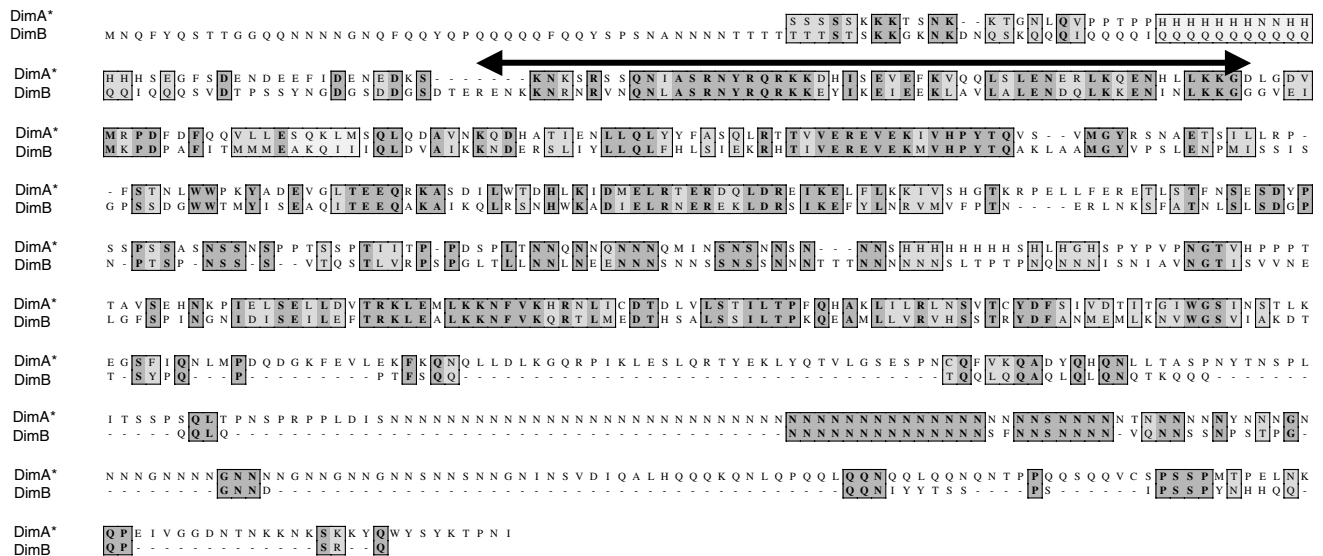
### DimB accumulates during multicellular development

Polyclonal antibodies were raised against an N terminus-proximal and a C terminus-proximal peptide of DimB and purified by affinity chromatography on the respective peptide immunogen. The N

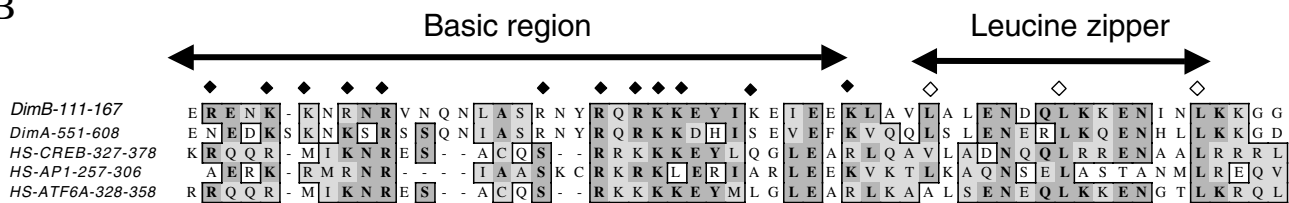


**Fig. 2. Purification of proteins that bind to R1 and R2.** These are gels of the proteins that bind to R1 and R2 affinity columns, stained with Coomassie Blue. The DimB and DimB' proteins were identified by mass spectrometry. Several of the other proteins, selected by both R1 and R2, are RNA-binding proteins. The asterisked species is MybE (M.F. and J.G.W., unpublished).

A



B



**Fig. 3. Amino acid sequence alignments.** (A) Alignment of DimB with DimA. The sequences of the entire DimB protein and a C-terminal proximal fragment of DimA, DimA\*, were aligned using Clustal W. The arrowed line over part of the sequence shows the positions of the proposed DNA-binding and dimerisation domains. (B) Partial alignment of DimB with DimA and other bZip proteins. The indicated segments of the proteins were aligned using Clustal W and the arrowed lines over the sequences show the positions of the DNA-binding and dimerisation domains. Closed diamonds indicate basic residues and open diamonds indicate leucine residues. The other proteins in the alignment are *Dictyostelium* DimA and human CREB, AP1 and ATF6A.

terminus proximal-antibody recognises DimB and a higher molecular weight species (data not shown), and was used only to characterise potential null strains (see below). The C terminus-proximal antibody was used in western transfer to obtain a developmental time course of DimB accumulation (Fig. 7). The only species recognised by this antibody is a protein of 70 kDa, the approximate size of DimB. DimB is present at very low concentration at zero hours, rises in concentration to the tight mound stage and then remains at a relatively constant concentration throughout subsequent development.

### Generation and biological characterisation of a DimB null strain

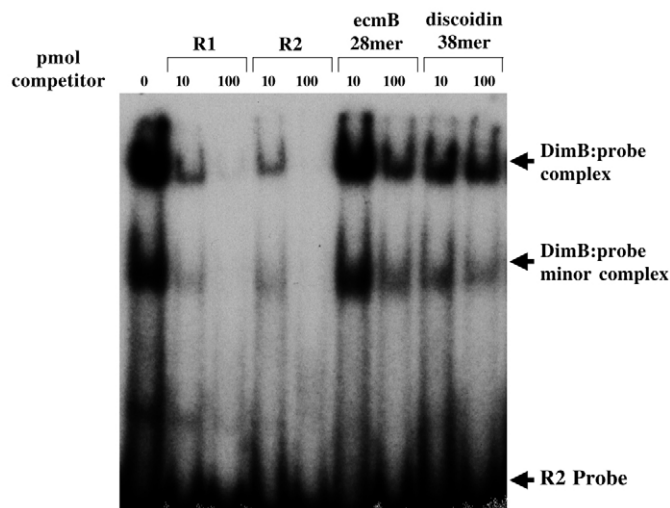
In order to analyse DimB function genetically, a disruption construct, in which a hygromycin resistance cassette interrupts *dimB*, was transformed into cells. Resistant colonies were screened by PCR and clones with an apparent disruption of the *dimB* gene were examined by western transfer, using the N and C terminus-specific antibodies. In wild-type cells both antibodies recognise DimB but in the candidate null cells there is no signal at this position (Fig. 8A and data not shown for the N terminus-derived antibody). Thus, *dimB* is functionally inactivated in the disruptant strains (termed *dimB*-null or *dimB*- strains).

Early development and culmination of the null strains appear normal but at the slug stage several differences from control, random-integrant slugs become apparent. The null strains form elongated slugs (Fig. 8B, upper). When control cells are spotted on agar and exposed to a dim unidirectional source, the slugs that are formed move towards the light source (Fig. 8B, lower). *DimB*- slugs do not leave the point of origin. Examination of the point of origin suggests that this is not caused by a failure to enter the migratory slug stage. It would appear to reflect a defect in the intrinsic ability of the slug to move.

### Analysis of prestalk gene expression in the *dimB*-strain using cell type specific markers reveals no gross defects

In order to analyse total prestalk cell differentiation, a *dimB*- strain and a random integrant strain were transformed with *ecmAO:lacZ*. After development to the standing slug stage, the slugs were stained for  $\beta$ -galactosidase. A control and a mutant field, each containing three slugs, is shown in Fig. 9. These particular control and mutant slugs appear similar in the average relative sizes of their prestalk regions. This conclusion is supported by quantitative analysis of a larger number of slugs, but this revealed a higher level of heterogeneity in the prestalk: prespore ratio in the mutant slug population (explained in the legend to Fig. 9).





**Fig. 4. In vitro binding of DimB to R2.** Full-length DimB:HIS fusion protein was used in band shift assays. The probe is R2 and the unlabelled competitors are R1, R2, a fragment (gatcCTTTAATGTTAGAAATAGGAGATGAAA) from the promoter of the *ecmB* gene and a fragment (gatcAAATCCAAACAAAAAAAAAATTGATTGTTTTT) from the promoter of the discoidin 1 $\gamma$  gene. The *ecmB* oligonucleotide is the activator, that binds to DdSTATa and to an unidentified protein by virtue of repeated GAAA tracts (Ceccarelli et al., 2000). The discoidin 1 $\gamma$  promoter fragment, the TTG element, is an activator of early gene expression but the binding protein is not known (Vauti et al., 1990).

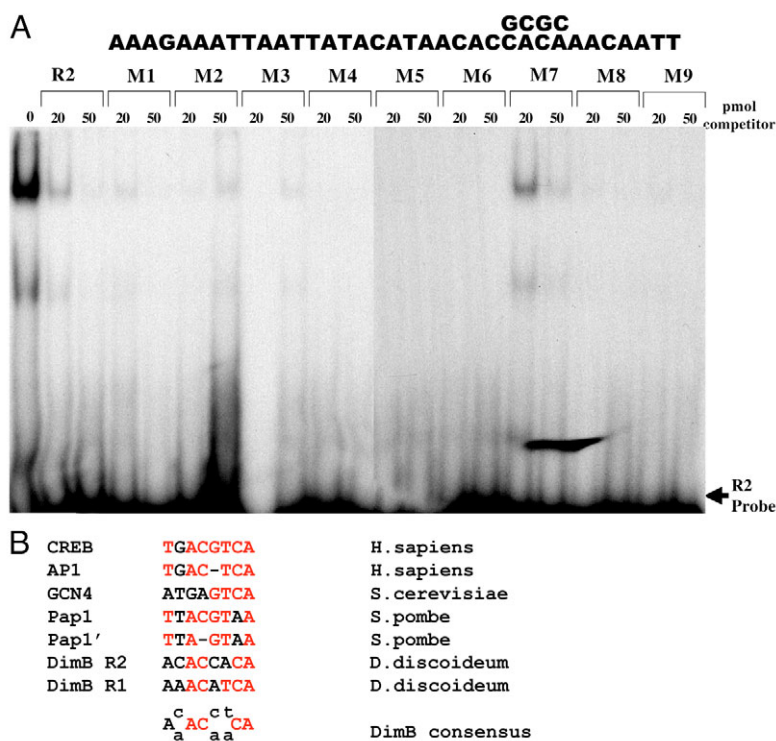
Having failed to detect a consistent difference in the relative size of the total prestalk population in the mutant, the prestalk subtypes were analysed. Cells were transformed with *ecmA:lacZ* [construct O (Early et al., 1993)], *ecmO:lacZ* and *ecmB:lacZ*. Each was a co-transformation with *pspA:gluc*, a construct in which the promoter of the *pspA*, prespore-specific, gene drives expression of  $\beta$ -glucuronidase. Staining was performed at the standing slug stage of development, using a red  $\beta$ -galactosidase chromogen and a blue  $\beta$ -glucuronidase chromogen (Early et al., 1993).

*pspA:lacZ* is, as expected, expressed in the rear of the slug and expression completely abuts the prestalk zone (Fig. 9). As in the control slugs, *ecmA:lacZ* is most highly expressed in the pstA region and *ecmO:lacZ* is expressed selectively in the pstO cells. The presence of a pstO zone is completely reproducible from slug to slug, and is of critical importance because it constitutes a clear difference from the *dimA* and *dmtA* null strains.

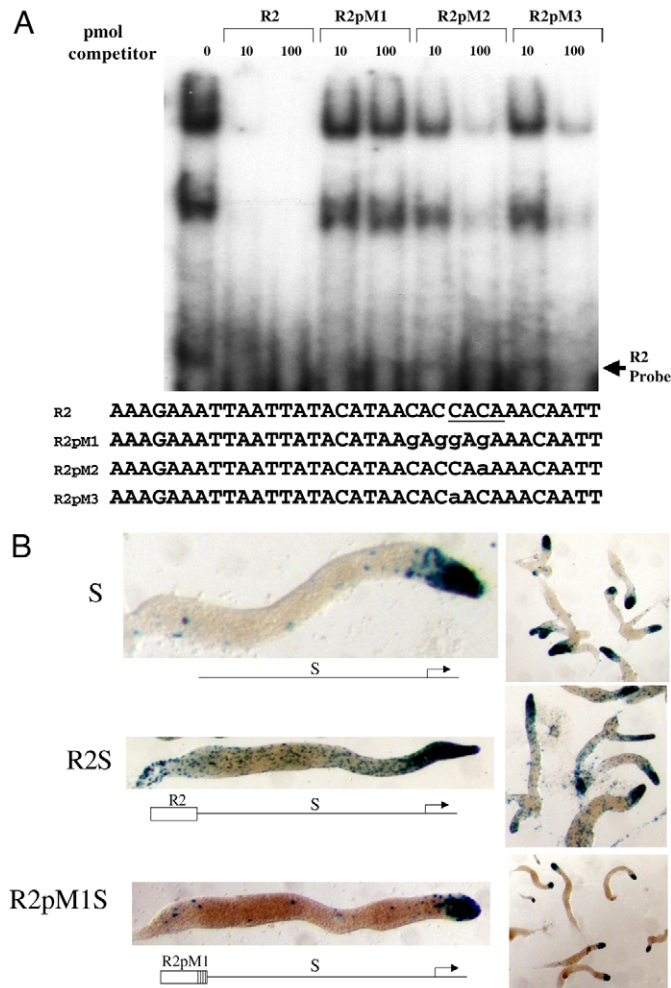
The *ecmB:lacZ* construct is expressed in a cone of pstAB cells within the slug tip and in the group of pstB cells, situated close to the prestalk-prespore boundary (Jermyn and Williams, 1991). Analysis of control and mutant *ecmB:lacZ* transformants at culmination shows the expected pattern, with strong staining in the stalk and the cups that cradle the spore head (data not shown).

#### Prestalk cell differentiation in the *dimB*- strain is subtly aberrant

The above results were obtained using cells developed under overhead light and analysed at the standing slug stage. In addition, the staining times were of 1-2 hours, by which time the enzymatic reactions for the *ecmA*O:lacZ transformants were approaching a plateau. Using *ecmA*O:lacZ marked slugs developed in the presence of a dim uni-directional light source, and with shorter (c. 5-10 minutes) staining times, an

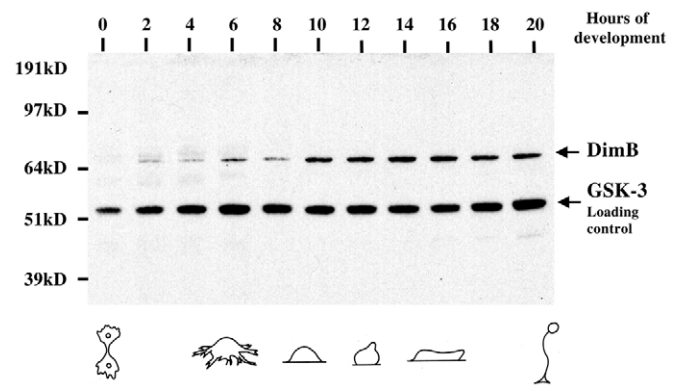


**Fig. 5. Mutational analysis of the binding of DimB to R2.** (A) Binding of DimB to an R2 scanning mutant series. The probe is R2 and the unlabelled competitors are scanning mutants of R2. They are labelled M1 to M9 and each contains a four nucleotide substitution, of GCGC, at the indicated position relative to the unmutated R2 sequence. The position of the mutation that produces a major reduction in competition is shown above the R2 sequence. (B) Proposed consensus sequence for DimB binding and comparison with known bZIP binding sites. This is a manually generated alignment of the R1 and R2 binding sites of DimB with that of several bZIP proteins: human AP1 and CREB, fission yeast GCN4 and the two alternate binding sites of budding yeast Pap1 (Fuji et al., 2000). Residues common to at least four out of the seven sequences are in red.



**Fig. 6. Mutational analysis of R2.** (A) Band shift analysis of the binding of DimB to point mutated forms of R2. The labelled probe is R2 and the unlabelled competitors are R2 and the three R2 mutants illustrated at the base of the figure. The underlined residues in R2 show the position of the scanning mutation that reduced binding; the lower case letters in the mutant sequences show the changes from the wild-type sequence. (B) In vivo analysis of the effect of multiple point mutations in R2. This was determined by creating the indicated fusion constructs and analysing their expression patterns in *Dictyostelium*. All published *ecmA* promoter 5'-3' deletion constructs (Fig. 1) share the same proximal end point at nucleotide +251 (labelled relative to the cap site of *ecmA*), four nucleotides upstream from the translation initiation codon (Early et al., 1993). All these constructs are fused to the vector A15Δbam-gal, which provides the basal transcription signals (Pears and Williams, 1988). By contrast, constructs S, R2S and R2pM1S:lacZ fuse immediately downstream of the ATG initiation codon of *ecmA* to the *lacZ*-coding region, hence using the basal promoter elements of *ecmA* (Fig. 1). The distal end point of S lies at nucleotide -493, just one nucleotide downstream of the cap-site proximal end point of R2. The R2 oligonucleotide has a cap-site distal end point just two nucleotides shorter than construct O (Fig. 1). Hence, construct R2S has a structure that, at its distal end, is very similar to that of construct O. Construct R2pM1S has an identical structure to R2S, except that it contains the four point mutations present in R2pM1.

anteroposterior gradient of staining becomes apparent in the control (Fig. 10). These short staining times also show that staining in control slugs is stronger at the periphery of the prestalk



**Fig. 7. Developmental time course of DimB accumulation.** Ax2 cells were allowed to develop to the indicated stages and harvested: t6=streaming; t10~11=tight aggregate; t12~13=tipped aggregate/first finger; t14-16=slug; t20=mid culminant. Western transfer was performed with the C terminus-specific DimB antibody and with an antibody to GSK3: a gene that is semi-constitutively expressed during development.

region than in the core. The *dimB*<sup>-</sup> slugs display a very different pattern from the control slugs, their staining is uniform throughout the prestalk region.

#### The *dimB*<sup>-</sup> strain is DIF-1 non-responsive

DIF-1 responsiveness in the *dimB*<sup>-</sup> mutant was assayed using a protocol in which cells starved in the presence of cAMP were treated with various concentrations of DIF-1. Expression of the *ecmA* gene was monitored by RT-PCR and, as a quantitation control, parallel RT-PCR was performed for *Ig7*: a constitutively expressed mRNA. In order to minimise the effects of endogenously produced DIF-1, the assays were performed at low cell densities ( $10^4$  or  $10^5$  cells/cm<sup>2</sup>) and the polyketide synthesis inhibitor cerulenin was included at various concentrations (Kay, 1998). Under all conditions tested, there was robust induction of *ecmA* expression in the random integrant cells but no response in the *dimB*<sup>-</sup> cells (Fig. 11).

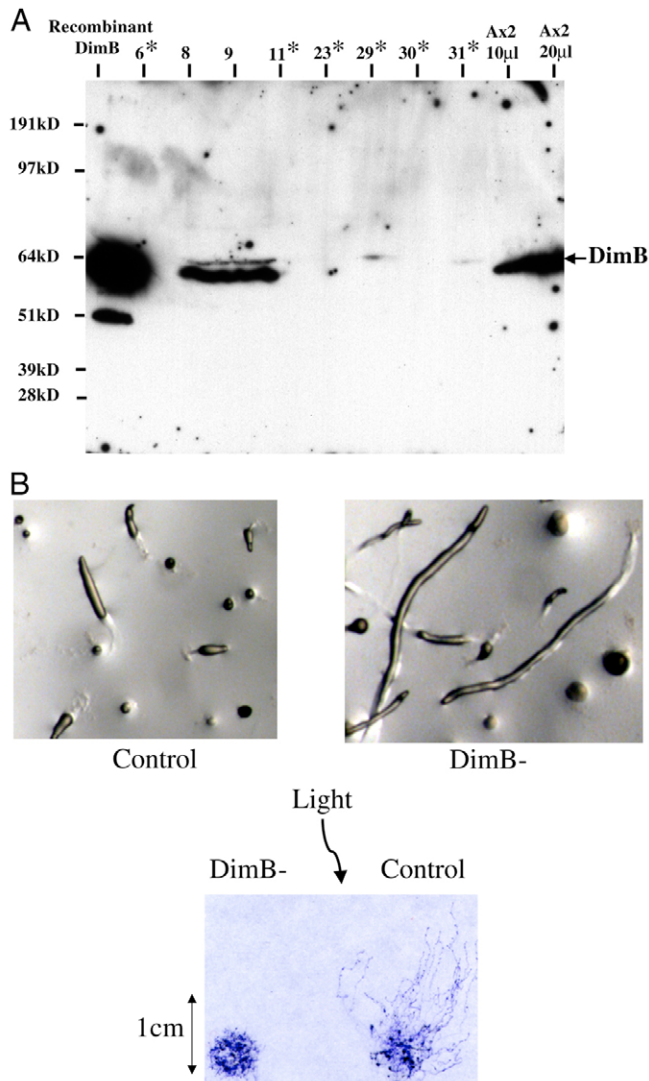
We also analysed cells that were rendered competent to respond to DIF-1 by allowing them to develop normally to the loose aggregate stage. After disaggregation, the cells were shaken in suspension with and without DIF-1 for 2 hours; *ecmA* expression was activated in the control cells but not in the *dimB*<sup>-</sup> cells (data not shown).

#### DIF-1 rapidly induces nuclear accumulation of DimB

In order to determine whether DIF-1 directly regulates DimB loose aggregate, parental cells (data not shown) or tight aggregate stage cells derived from the DIF-1 deficient mutant *dmtA*<sup>-</sup> (Fig. 12) were exposed to DIF-1 and subjected to immunohistochemical staining. Similar results were obtained with both strains. There is a rise in nuclear staining, with a peak three minutes after DIF-1 addition.

#### DimB associates with the *ecmA* promoter when cAMP-treated cells are induced with DIF-1

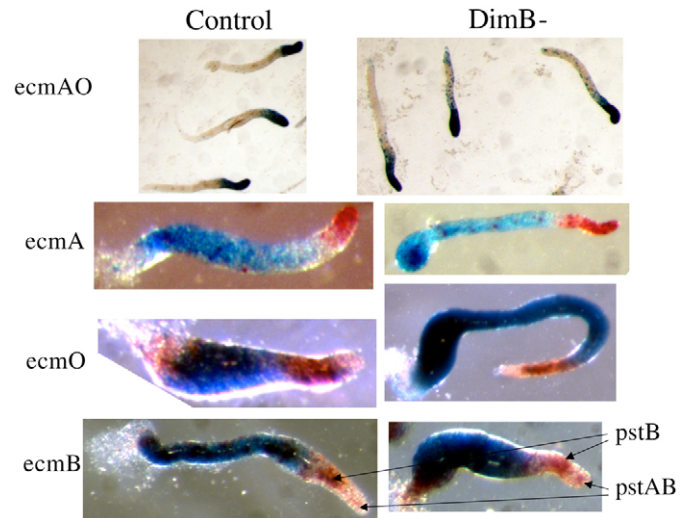
In order to determine whether DIF-1 induces DimB to bind to the *ecmA* promoter in vivo, ChIP analysis was performed. In initial experiments, cells dissociated at the loose aggregate stage were



**Fig. 8 Genetic analysis of DimB function.** (A) Isolation of *dimB*-mutants. A mixture of putative *dimB* disruptants (marked with an asterisk) and random integrants (i.e. clones where the disruption vector integrated at a position other than the *dimB* locus) was analysed by western transfer with the C terminus-specific DimB antibody. The lane on the left contains DimB:HIS fusion protein and those on the right were loaded with Ax2 total protein isolated at the slug stage. (B) Morphological and behavioural phenotypes of the *dimB*-mutant. (Top) A *dimB*- and a control (random integrant) strain were developed on water agar to the slug stage. (Bottom) A *dimB*- and a control (random integrant) strain were developed on water agar in a darkened chamber with a narrow slit for 48 hours. The slugs were then lifted, with their adherent trails, onto a clear plastic sheet which was stained with Coomassie Blue.

incubated for several hours with cAMP, DIF-1 or cAMP and DIF-1. RT-PCR analysis was then used to monitor *ecmA* expression. As was observed previously (Berks and Kay, 1990), a combination of cAMP and DIF proved most effective and these conditions were employed for the ChIP assay.

Chromatin extracted from induced and control cells was precipitated with anti-DimB antibody or with a heterologous antibody. The latter antibody is directed against CudA, a nuclear protein that appears not to be regulated by DIF-1 (Fukuzawa et al.,



**Fig. 9. Whole-mount staining of control and *dimB*- mutant standing slugs.** *dimB*- and control (random integrant) strains were created containing *ecmA*O:lacZ. After development to the standing slug stage, slugs were stained with a blue  $\beta$ -galactosidase chromogen. Quantitative analysis of additional such images was performed to measure the areas occupied by the prestalk and prespore zones. These measurements were taken from photographic images, using the 'magic wand' tool in 'Adobe Photoshop' to select areas with similar signal densities. This revealed more heterogeneity in the apparent fraction of prestalk cells in the mutant but, on average, there was no major difference from the control (control=22 $\pm$ 4.4%,  $n$ =14; *dimB* null=19 $\pm$ 8.9%,  $n$ =15). The greater heterogeneity of the *dimB*-null slugs probably results from their failure to migrate away from their point of origin and from their tendency to break up into fragments. For double staining analysis, *dimB*- and control (random integrant) strains were created containing a *pspA*:glucuronidase reporter and one of the three prestalk lacZ reporter fusions indicated at the left. The strains were developed on water agar to the standing slug stage and the slugs were fixed and stained sequentially for  $\beta$ -glucuronidase (blue) and  $\beta$ -galactosidase (red).

1997). PCR was performed using primers directed against the *ecmA* promoter region. The promoter selected as a control was that of the G protein  $\beta$  subunit gene (*gpbA*), another gene where there is no evidence for DIF-1 regulation. PCR conditions were set such that control genomic DNA gave approximately equal signals with the *ecmA* and *gpbA* PCR primers (Fig. 13).

The apparent abundances of the PCR products generated using the various chromatin samples are expressed relative to the value obtained using the *gpbA* PCR primers with chromatin from cAMP treated and DIF-induced cells, which was immunoprecipitated using the anti-DimB antibody. When the chromatin sample from cAMP and DIF-treated cells was analysed using the *ecmA* promoter region, there was an enrichment of approximately fourfold (Fig. 13): a value that is in good accord with the induction values for *ecmA* itself.

There was no enrichment when cAMP and DIF-1 were omitted (Fig. 13) and a lower, statistically non-significant degree of enrichment when either DIF-1 alone or cAMP alone was used (data not shown). Enrichment depended upon the presence of the DimB antibody; CudA antibody produced no enrichment (Fig. 13). In addition, there was no enrichment when *dimB*- cells were treated with cAMP and DIF-1 (Fig. 13).





**Fig. 10. Single chromogen whole-mount staining of *ecmAO: lacZ* expression in migrating slugs.** *dimB*<sup>-</sup> and a control (random integrant) strain expressing the *ecmAO: lacZ* reporter fusion were developed for 24 hours in a slug migration chamber. The slugs were then fixed and stained to detect  $\beta$ -galactosidase. The staining times were controlled, so as to produce approximately equal intensity staining in the extreme tip of the control and the null mutant. However, the same difference in staining patterns was observed at a variety of staining times up to 30 minutes, at which time the reactions started to plateau.

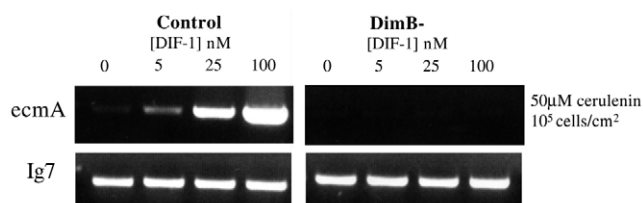
## DISCUSSION

### A novel bZIP protein that binds to two sites in the *ecmA* promoter gene

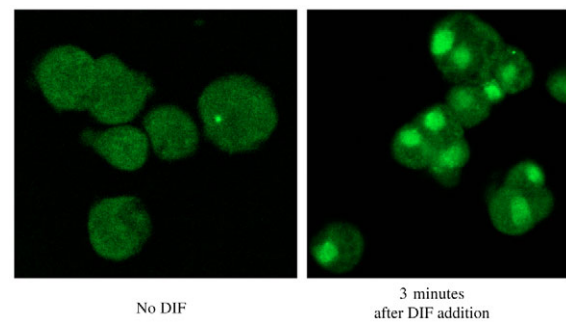
DimB was isolated by virtue of its binding to two spatially separated regions of the promoter of the *ecmA* gene, R1 and R2. Mutational analyses of the DimB-binding sites within R1 and R2 identified a region that is required for binding and this allowed a consensus for the binding sequence to be derived and confirmed (Fig. 5B). It is not a dyad and does not contain the ACGT 'core' sequence that is found in the binding sites of many other bZIP proteins. However, the bZIP family shows a broad range of DNA binding specificities (Fujii et al., 2000), the sites are not always perfect dyads and the binding sites of several well characterised bZIP proteins, e.g. AP1 and GCN4 (Fig. 5B), also lack an ACGT sequence.

### The DimB binding site in R2 is required for expression in *pstO* and ALC

The region within R1 that is essential for DimB binding is contained within the sequence CATCATC. Interestingly, a very similar sequence within the promoter of the *7E* gene, CATCACC, is essential for expression in *pstA* cells and for repressing prestalk



**Fig. 11. Analysis of DIF induction of *ecmA* expression in a control and a *dimB*<sup>-</sup> mutant.** A *dimB*<sup>-</sup> and a control (random integrant) strain were plated at  $10^5$  cells/cm<sup>2</sup>, in the presence of 50  $\mu$ M cerulenin, and treated with the indicated amounts of DIF-1. Total cellular RNA was extracted and the relative abundances of the *ecmA* and Ig7 RNAs (a constitutively expressed gene, used as a loading control) in the two strains determined by semi-quantitative RT-PCR.

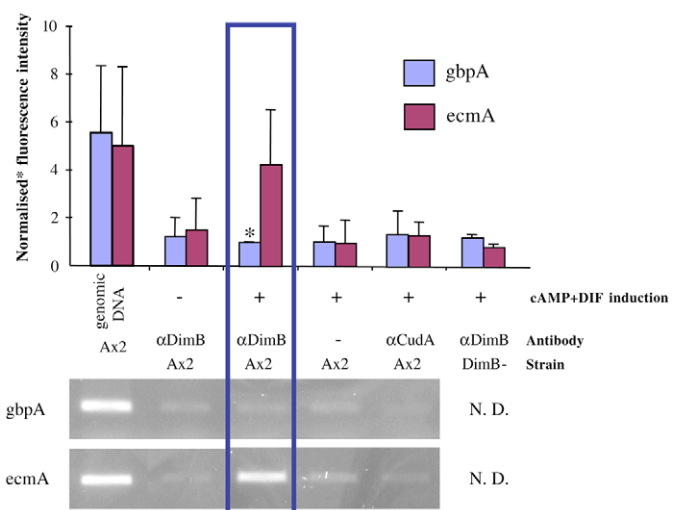


**Fig. 12. DIF-1 induction of DimB nuclear accumulation.** DmtA-null cells at the tight aggregate stage were dissociated, treated with or without 100nM DIF-1 for 3 minutes in shaken suspension and stained using the anti-DimB antibody.

expression in prespore cells (Seager et al., 2001). However, we did not analyse the R1 element functionally. Instead we determined the biological function of the DimB binding site in R2.

As a recipient for cloning R2, and its mutant form R2pM1, a new construct, S, was created and this provided an unexpected result. Construct S contains an *ecmA* promoter fragment that is 38 nucleotides shorter at its distal end than construct O (Early et al., 1993). It also differs significantly from construct O at its cap-site promoter end, in that it employs *ecmA* basal transcription signals (Fig. 1, Fig. 6B). The latter fact probably explains why it is better expressed in *pstA* cells than construct O.

Although it has become the standard *pstA* marker, construct O is weakly expressed and is not totally specific; at extended times of staining it displays a finite level of expression in *pstO* cells and ALC. By contrast, construct S is highly specific to the *pstA* cells. This is



**Fig. 13. ChIP analysis of DimB.** Ax2 cells or *dimB*<sup>-</sup> cells were induced in the manner indicated, and their chromatin was incubated with or without anti-DimB ( $\alpha$ -DimB) or with anti-CudA antibody ( $\alpha$ -cudA). PCR was performed on the immunoprecipitated DNA and the data were analysed as described in the text. The asterisk indicates the sample against which all other fluorescence intensities were normalized. The *ecmA* primers used (see Materials and methods) derive from upstream of R2 but the extent of sonication is such that the R2 region will register in the PCR. In addition, two other primer combinations, which derive from sequences more proximal to R2 were used, in other experiments and they also showed enrichment.



manifest in a highly foreshortened region of prestalk staining and in the almost complete lack of expression in ALC. This expression pattern is, to our knowledge, novel. However, it is in accord with a previous study that suggested that the pstO cells and the ecmO:lacZ expressing ALC may be a unitary population (Abe et al., 1994). The fact that construct S is efficiently and selectively expressed in pstA cells make it a useful marker for future studies.

Construct R2S has, at its cap-site distal end, a very similar structure to construct O but, presumably because of its different cap-site proximal architecture, it is expressed at a much higher level and strong staining is also observed in pstO cells and in ALC. By contrast, R2pM1S has the same, highly pstA-specific, expression pattern as construct S [the construct that lacks R2 (Fig. 6B)]. Thus, the four point mutations that abrogate binding of DimB to R2 in vitro prevent expression in the pstO and ALC. Therefore, within the context of the promoter sequences contained within R2S, the -510 site is essential for pstO and ALC gene expression but not for pstA-specific expression.

There is evidence that DIF-1 selectively induces pstO-specific gene expression (Thompson et al., 2004; Thompson and Kay, 2000b). Hence, the fact that pstO-specific expression is eliminated by the four point mutations present in R2pM1S implies a role for the -510 site in DIF-1 signalling. The corollary, that pstA expression is unaffected by the four point mutations present in R2pM1S, is also in accord with analyses of the DIF-1 deficient mutant *dmtA-*, which suggest that pstA-specific gene expression uses a DIF-1-independent signalling pathway (Thompson and Kay, 2000a).

### DimB establishes a gradient of *ecmA* gene expression in the slug tip

Analysis of the *dimB*-null mutant shows that a prespore marker and the standard prestalk markers are expressed in a similar spatial pattern to the control. The most telling result is the presence of an apparently normal pstO region in the *dimB*-null strain. This is an important difference from both the *dmtA-* and *dimB-* mutants, where there is a defect in pstO cell differentiation. The *dimB* null does, however, show a more subtle difference from the control.

In control slugs, there is both an anteroposterior and a radial gradient of *ecmA* expression, with their respective peaks at the tip and the slug exterior; this creates a thimble shaped cell grouping that ensheathes the prestalk region. These gradients are lost in the *dimB-* strain. Hence, DimB functions as a repressor that reduces *ecmA* expression in the more posteriorly and centrally located prestalk cells.

A similar *ecmA*:lacZ staining pattern has been described previously, again for slugs migrating towards a dim unidirectional light source (Jermyn and Williams, 1991). Interestingly, the pattern was absent when slugs were allowed to migrate under overhead light (Jermyn and Williams, 1991). Perhaps, therefore, exposure to a high light intensity downregulates the repressive activity of DimB and disrupts the *ecmA* expression gradient.

### Functional implications of a gradient of DimB activity

EcmA is a major component of the slime sheath, the extracellular matrix that surrounds the slug and a DimB activity gradient may help ensure that EcmA is selectively synthesised in the region of the slug where it needs to be most highly concentrated: i.e. at the surface. Slug movement is directed by the tip, so if other genes needed for slug migration are modulated in a similar manner to *ecmA*, disruption of their graded expression could account for the lack of slug migration in the *dimB-* mutant.

### Integration of the promoter and genetic analyses: does DimB function as a competitive inhibitor by binding to the site at -510?

It seems paradoxical that: (1) DimB should bind to the -510 site in vitro; (2) mutation of the -510 site should prevent *ecmA* expression in pstO and ALC; yet (3) the *dimB-* mutant should overexpress *ecmA* in parts of the prestalk region. We believe that this can best be explained if the -510 site is bound in vivo by two proteins: an, as yet unidentified, activator; and DimB, functioning as a repressor. The relative occupancy of the -510 site by these two proteins would establish the observed gradients of expression in the prestalk region. One obvious candidate for the activator is the previously mentioned MYB domain protein that binds to R2 but not R1 (M.F. and J.G.W., unpublished).

### DimB, DIF-1 and the regulation of *ecmA* gene expression

Analysis of the *dimB-* mutant provides a further apparent link between DimB and DIF-1 signalling: the mutant is DIF-1 non-responsive in a monolayer assay that measures induction of *ecmA* gene expression. However, *ecmA* and cell-type specific markers derived from its promoter are expressed during multicellular development of the *dimB-* strain.

The monolayer result presumably reflects some limitation of the assay system: deprived of cell-cell interactions and cell-matrix interactions, the signalling inputs to a monolayer cell would be expected to be significantly aberrant. The result can be explained by assuming that under monolayer conditions *dimB* acts alone, either as a direct activator of *ecmA* or as a factor needed to achieve DIF responsiveness. It implies a second protein, which is conditionally redundant with DimB, that receives signalling inputs from other cells and that fulfils these functions in normal development. Nineteen bZIP proteins are encoded within the *Dictyostelium* genome (Eichinger et al., 2005). Hence, there are abundant candidates for such a role. The crucial issue is whether DIF-1 is the normal inducer in the whole organism and here the biochemical evidence is very telling.

### DIF-1 and the activation of DimB

Although the genetic evidence from monolayer cells appears to be misleading, certainly as far as an obligate role for DimB in *ecmA* expression, analysis of the behaviour of DimB establishes a clear link to DIF-1 signalling. DimB rapidly accumulates in the nucleus when cells are treated with DIF-1 and it becomes associated with the *ecmA* promoter after cells are exposed to a combination of cAMP and DIF-1. Many post-aggregative functions require that cells be continuously exposed to a high concentration of cAMP (Kay, 1982; Mehdy et al., 1983; Schaap and van Driel, 1985; Berks and Kay, 1988). Hence, we favour the notion that cAMP is also permissive rather than instructive in this instance.

Thus, we propose that DimB is induced by DIF-1 to accumulate in the nuclei of a subset of the prestalk cells and that, by binding to the *ecmA* promoter at the -510 site, DimB represses *ecmA* gene transcription. How then is DimB activated by DIF-1? Dd-STATc provides a precedent for DIF-1 induced nuclear accumulation of a *Dictyostelium* protein. However, in contrast to the STAT proteins, where there is a paradigm for activation, bZIP proteins display varied activation mechanisms. Some, such as the mammalian CREB protein, are constitutively nuclear and are activated as transcription factors by serine or threonine phosphorylation (Montminy, 1997). Others, such as the fission yeast protein Pap1, are regulated at the level of nuclear export

(Toone et al., 1998). There is, to our knowledge, no precedent for activation of a bZIP by a direct tyrosine phosphorylation event of the kind that initiates the nuclear accumulation of DdSTATc. Presumably, therefore, two wholly or partially separate DIF-1 regulated signalling pathways activate these two different transcription factors.

### Note added in proof

During the course of the above study, we learnt that the same gene was under investigation by Huang et al. (Huang et al., 2005). These authors identified DimB bio-informatically, rather than biochemically, and they showed that it dimerises with DimA. The two studies are in generally good accord and the differences in *ecmA* expression patterns that are observed in the slug are probably explicable by strain differences; they used strain Ax4, whereas we used Ax2.

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