**Int. J. Dev. Biol. 54**: 161-165 (2010)
doi: 10.1387/ijdb.082723hw

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

# Identification of a target for CudA, the transcription factor which directs formation of the *Dictyostelium* tip organiser

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**ABSTRACT The tip of the *Dictyostelium* slug functions much like an embryonic organiser; when grafted onto the flank of a recipient slug, it recruits a mass of prespore cells and leads them away as part of a secondary slug. CudA is a nuclear protein which is expressed in prespore cells where it acts as a specific transcription factor. CudA is also expressed in an anteriorly located group of cells, the tip-organiser, that is believed to constitute the functional tip. We identify an expansin-like gene, *expl7*, that is expressed within the tip-organiser region and which is not expressed in a cudA null strain. The *expl7* promoter contains a region that binds to CudA *in vitro* and this region is necessary for expression in the tip-organiser. These results provide an end-point for a previously defined signal transduction pathway in which regionalised expression of the ACA adenylyl cyclase within the tip-organiser leads to localised cAMP-induced activation of STATa and consequent binding of STATa to the cudA promoter. STATa then induces expression of cudA and cudA directs the transcription of target genes such as *expl7*.**

KEY WORDS: *Dictyostelium, CudA, tip, expansin, gene expression*

Cells within the tip of the *Dictyostelium* slug, that we will term the "tip-organiser" [1], control the slug tissue in a manner analogous to vertebrate embryonic organisers. The tip-organiser also controls the choice between continued slug migration and immediate culmination (Smith and Williams, 1980). It is believed to play these roles by acting as a source of oscillatory cAMP production, signalling to cells behind it in the slug and controlling their behaviour. Consistent with such a notion, the ACA adenylyl cyclase, which is ubiquitously expressed during early development, becomes restricted in its expression to the tip-organiser (Verkerke-van Wijk *et al*., 2001). This triggers a number of signalling events, downstream from ACA, and they constitute a pathway leading to tip-organiser cell differentiation; signalling by the extracellular cAMP, that is produced locally within the tip-organiser, causes tyrosine phosphorylation of STATa so that STATa translocates into tip-organiser cell nuclei (Fukuzawa and Williams, 2000; Dormann *et al*., 2001). Then STATa binds to a dyad site within the *cudA* promoter and this induces *cudA* transcription (Fukuzawa and Williams, 2000).

The *cudA* gene was identified in a random mutagenesis screen as being essential for correct culmination (Fukuzawa *et al*., 1997). CudA is present in the nuclei of a cone of cells at the extreme slug tip and is also present in prespore cell nuclei. The *cudA* null strain is defective in the expression of certain prespore genes, including the *cotC* spore coat protein gene (Fukuzawa *et al*., 1997). The cudA null strain is also defective in control of entry into culmination, a behavioural property that is controlled by the tip region (Smith and Williams, 1980). This defect is corrected if the *cudA* gene is expressed in the cudA null mutant under the control of a promoter fragment that selectively directs expression to the anterior of the prestalk region (Fukuzawa *et al*., 1997). This suggests that CudA has a crucial role in tip cell differentiation and constitutes the evidence that the cudA expressing cells correspond to the functionally defined tip-organiser cells. Because there are no informative homologues in the databases, the molecular function of CudA has until recently been obscure. However, it is now known that CudA directly regulates *cotC* prespore gene expression, by binding to a site in the *cotC* promoter (Yamada *et al*., 2008), and we present evidence that it plays a similar, transcriptional role in tip-organiser cell differentiation.

*Abbreviations used in this paper:* ACA, adenylyl cyclase A; CudA, culmination defective A; ECudA, Entamoeba CudA homologue; expl, expansin-like; STAT, signal transducers and activators of transcription.

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**Note 1:** http://dictybase.org/Dicty_Info/dicty_anatomy_ontology.html

*Accepted:* 6 November 2008. *Final author corrected PDF published online:* 14 August 2009.

ISSN: Online 1696-3547, Print 0214-6282

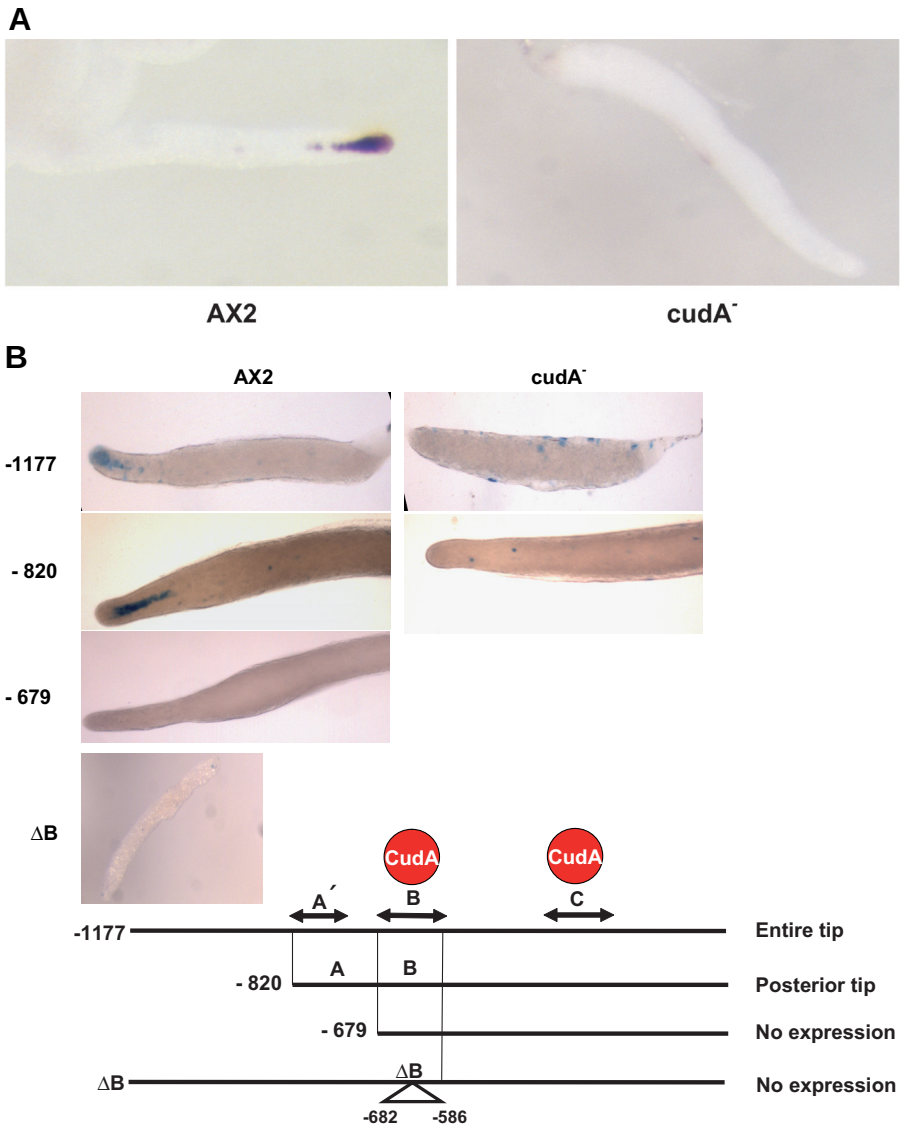


**Fig. 1. Deletion analysis of the expl7 promoter. (A)** In situ *hybridisation of* expl7 *to parental and cudA null slugs. Parental (Ax2) and cudA null (cudA-) slugs were subjected to* in situ *hybridisation using an* expl7 *probe extending from the initiation codon to the stop codon.* **(B)** *Mutational analysis of the* expl7 *promoter. In all four constructs the lacZ gene is fused to* expl7 *at a point 51bp from the initiation codon, retaining the basal transcriptional signals and 5´ non-coding region of* expl7*. Parental (Ax2) and, in some cases, cudA null (cudA-) cells (Yamada* et al.*, 2008) were stably transformed with one of the indicated constructs and slugs were subjected to lacZ staining. As is typical of* Dictyostelium *promoters, the* expl7 *promoter contains long tracts of AT residues interspersed with more GC-rich regions. These GC-rich regions were used as anchor points for PCR and the promoter sequences were scanned for binding of ECudA in a band shift assay, as in Fig 2B. As indicated by the red circles only two regions, B and C, showed binding activity.*

## Results

### Expl7 *mRNA is expressed in the tip and is dependent upon cudA for its expression*

The starting point for this study was a set of prestalk-specific ESTs that were previously categorised by *in situ*-hybridisation at late developmental times (Maeda *et al*., 2003). We selected six (SLF308, SSK861, SLA128, SSL558, SSK348, SSB312) that seemed to most resemble the cudA expression pattern and determined their situ-hybridisation pattern in parental and cudA null slugs. Only SLA128, encoded by the *expl7* gene, has an expression pattern that matches that of the cudA mRNA (Fig 1A). Further consistent with a direct induction of *expl7* by CudA, there is no detectable expression of *expl7* in cudA null slugs (Fig 1A).

### *The* expl7 *promoter can be sub-divided into regions that direct expression in different parts of the tip-organiser*

An *expl7* promoter fragment with a 5' endpoint at -1177 (numbered relative to the ATG initiation codon) was cloned upstream of lacZ and the construct was stably transformed into Ax2 and cudA null cells. In Ax2 cells the construct is expressed with a similar pattern to that observed by *in situ* hybridisation, suggesting that the entire promoter is present. Again, as in the *in situ* hybridisation analysis, there is no expression in the cudA null strain (Fig 1B). In order to narrow the search region for potential CudA binding sites, two 5 to 3´ deletion constructs were generated. Deletion from -1177 to -820 produces a change in the pattern of expression; staining is retained in the rear of the tip-organiser but is absent from the anterior region (Fig 1B). This presumably reflects a difference in spatial patterning between the two regions but we do not know the signalling basis for this difference. Expression within this foreshortened staining region is again dependent upon CudA. When the promoter is shortened further, to -679, all expression is lost (Fig 1B). Thus essential elements for tip-specific expression are located between -820 and -679; we term this region A.

### *A region downstream of the essential region binds to CudA and is essential for tip-specific expression*

A sub-region of A, termed A´ (the remainder of region A is so AT-rich we elected not to analyse it) and region B, derived from promoter sequences downstream of -820 (Fig 1B), were used in affinity chromatography with slug stage nuclear extracts. The eluates were analysed by western transfer using a CudA monoclonal antibody. Region B binds CudA but region A´ does not (Fig 2A). We therefore generated an internal deletion construct, ΔB, that contains the entire promoter except for region B and analysed its expression. It shows no activity (Fig 1B). Therefore, expression in the tip requires sequences located both in regions A and B and region B contains one or more CudA binding sites.

***ECudA binds to multiple dispersed sites within fragment B***

In order to identify potential CudA binding sites within fragment B we performed band shift analysis. This necessitated using a recombinant protein; because there are six *Dictyostelium* CudA homologues that confuse the band shift assay and, for reasons that we do not understand, recombinant CudA is not active in DNA binding(Yamada *et al*., 2008). Hence we used a recombinant form of the Entamoeba CudA homologue, ECudA, that binds to the same site within the cotC promoter as CudA itself (Yamada *et al*., 2008). The probe in the band shift assays is a 66-mer from within the cotC promoter. As expected from the affinity chromatography results (Fig 2A), fragment B is able to compete efficiently with the 66-mer for binding to ECudA while fragment A´ is not active (Fig 2B). This reinforces previous data, established using the cotC promoter, showing that CudA and ECudA have similar DNA binding properties (Yamada *et al*., 2008). ECudA also binds to a region, termed C, that is located between -345 and -221 (Fig 2B) but we did not study this region further. Instead we concentrated our efforts on mapping the site(s) within fragment B.

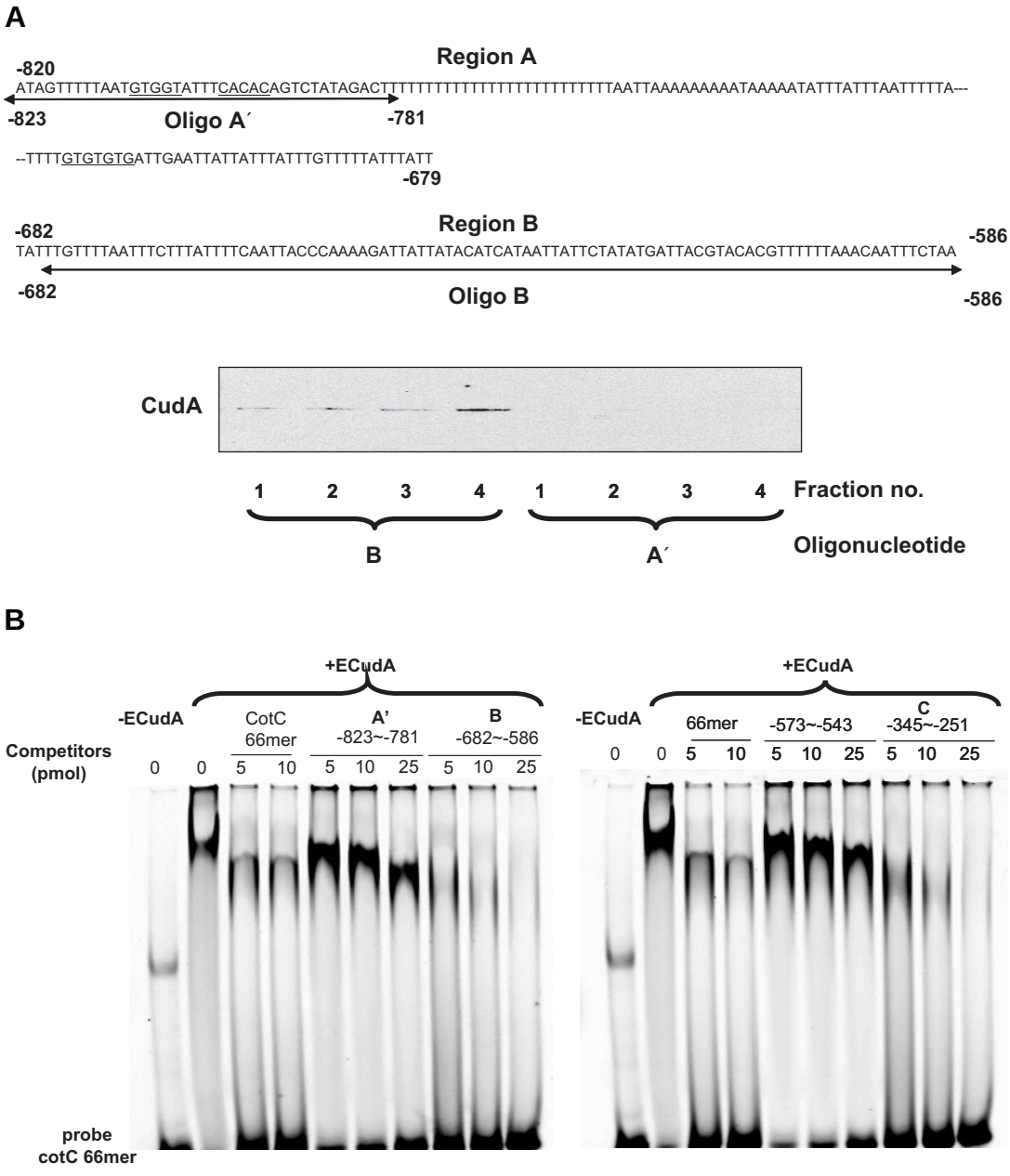


**Fig. 2. Interaction analysis of CudA and ECudA with the expl7 promoter. (A)** *Analysis of the binding of CudA to oligonucleotides derived from the* expl7 *promoter Oligonucleotides A´and B, from regions A and B and with the sequences indicated by the double headed arrows, were used in affinity chromatography with* Dictyostelium *nuclear extracts. The bound protein was eluted in four fractions and analysed by Western transfer for CudA (Yamada* et al.*, 2008).* **(B)** *Band-shift analysis of the binding of ECudA to oligonucleotides derived from the* expl7 *promoter. Recombinant ECudA protein was used in band shift with a 66 nucleotide probe from within the promoter of the cotC gene (Yamada* et al.*, 2008). In the absence of any competitor most of the probe is retarded by binding to ECudA. Oligonucleotide B is at least as effective as the cotC 66-mer as a competitor for binding while oligonucleotide A is almost ineffective at the concentrations employed. The gel at the right analyses a similar experiment using region C and the indicated control, non CudA binding region as probes.*

We first scanned the sequence for close matches to the ECudA binding site previously identified within the *cotC* promoter, GAAttTTC, but there are none (Fig 3). There are, however, four half sites (TTC): two very near the cap-site distal end, a third 41 nucleotides downstream of the first site and a fourth a further 30 nucleotides downstream. When a cap-site distal region containing two half-sites is deleted, to yield BD1, there is a partial reduction in competition efficiency (Fig 3). When a region containing the cap-site proximal half-site is deleted, to yield BD2, there is also a reduction in competition efficiency. Thus deletion from either end of region B reduces competition efficiency. The BD2 construct contains the centrally located half-site and the proximal site and it retains competition activity. When, however, the centrally located half-site in BD2 is

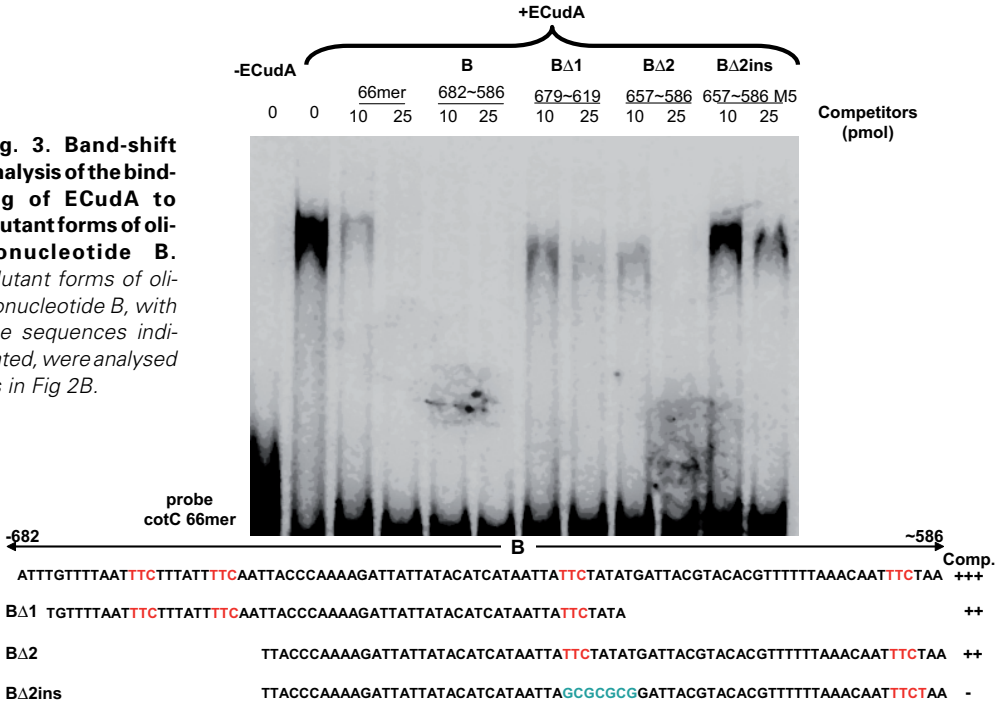


**Fig. 3. Band-shift analysis of the binding of ECudA to mutant forms of oligonucleotide B.** *Mutant forms of oligonucleotide B, with the sequences indicated, were analysed as in Fig 2B.*

replaced with a CG rich sequence, to yield BD2ins, there is a very large reduction in competition efficiency. Thus mutation of three widely separated regions, each containing a TTC sequence, reduce ECudA binding. We therefore believe that there is co-operative binding of ECudA to the half-site sequences. This is in accord with our previous study where we showed that ECudA binds co-operatively to target sequences and presented *in vivo* evidence that a half-site augments the biological activity of CudA as an activator of prespore gene expression (Yamada *et al*., 2008).

## Discussion

*expl7* encodes an expansin. Expansins are found in all groups of land plants, where they interact with cell wall components including cellulose, and they are also found in *Dictyostelium* (reviewed in (Darley *et al*., 2003)). The expression of *expl7* in tip cells presumably reflects the fact that, at culmination, tip cells enter the stalk tube and undergo major changes in cell wall structure that involve the deposition of cellulose. It will be of considerable interest to analyse the function of *expl7* genetically but this may be complicated by functional redundancy between members of the large gene family. The *expl7* gene has a similar pattern of expression to *cudA* and it is not expressed in a CudA null strain. *Expl7* is also not expressed in STATa null slugs (Shimada *et al*., 2004) and this is as expected if CudA is a positive transcriptional regulator of *expl7*; because STATa directs the expression of *cudA* (Fukuzawa and Williams, 2000). ChIP analyses of the cotC prespore promoter indicate that regulation of transcription by CudA is due to its direct binding to the promoter (Yamada *et al*., 2008). We were unable to detect a signal in ChIP analysis of the *expl7* promoter using a CudA antibody (unpublished results) but we believe that this probably reflects a technical limitation; prespore cells comprise 80% of the slug cells while tip cells comprise only a tiny proportion of the slug cell population, making signal detection problematical. Evidence for a direct interaction comes from analysis of the *expl7* promoter.

Two essential regions, A and B, were identified within the promoter and region B displays CudA binding. The binding sites in region B were approximately mapped, using a combination of deletion analysis and sequence replacement, and the regions identified each contain a potential half-site for CudA binding (TTC). The regions are, however, widely spaced and there is a separate CudA binding domain proximal to the cap site, within region C. These facts complicate the biochemical and the functional analysis greatly, hence we have not mapped the sites further. The fact that regions A and B are both essential for expression implies a co-operative interaction between CudA and another transcription factor. Such interactions have been shown to occur with GBF (Ceccarelli *et al.*, 1992; Powell-Coffman *et al.*, 1994, Schnitzler *et al.*, 1994), a zinc finger transcription factor. There are potential GBF binding sites, "G boxes" in region A (underlined in Fig 2A) but heterologous, G boxes, from the CotC promoter (-403 to –386) and from the ecmB promoter (-818 to -802), could not subsume the function of region A (unpublished data).

One related, intriguing issue raised by these results is that of dual specificity; how is CudA able to function as a transcriptional activator in both the prespore and the tip-organiser regions? Presumably CudA interacts with different ancillary transcription factors in the two different cell populations. Given the predominant biological role of the tip-organiser, it will be important to identify such factors.

## Materials and Methods

Cell culture, development, transformation and expression analysis All experiments were performed with the Gerisch isolate of Ax2 and cells were grown, developed and transformed as described previously (Fukuzawa and Williams, 2000). *In situ* hybridisation and lacZ expression analyses were also as described (Fukuzawa and Williams, 2000). Promoter fragments from *expl7* were generated by PCR and cloned into the vector pDd17Gal at the BamHI and BglII sites. The internal deletion mutant was created by sequentially cloning two *expl7* fragments, –1177 to –677 and –573 to +50 into the pDd17Gal vector at its EcoRI and BglII sites, resulting in a 103bp deletion.

### *Analysis of CudA binding*

Slug nuclear extracts were obtained by sonicating nuclei in DB buffer (50mM KPO4, pH7.5, 10% glycerol, 0.5mM EDTA, 0.1mM $ZnCl_2$, 0.1mM $MgCl_2$, 0.01% Brij 35) containing 0.1M NaCl, 2mM benzamidine hydrochloride, complete protein inhibitor cocktail, 10mM sodium fluoride and 1mM sodium pyrophosphate. The oligonucleotides were concatemarised

and coupled to CNBr-sepharose 4B. The slug nuclear extract was pre-cleared with blocked CNBr-sepharose 4B and then incubated with sepharose 4B bearing the oligonucleotide. After washing with DB buffer containing 0.1M NaCl, bound protein was eluted with DB buffer containing 0.4M NaCl. Protein was concentrated by precipitating with 13% TCA and analysed by Western blotting using an anti-CudA antibody.

***ECudA band shift analysis***

The entire ECudA coding region was cloned as A HIS-ECudA fusion construct (Yamada *et al*., 2008) in pET15b (Novagen, Ltd) was expressed and purified over a TALON$^{TM}$ metal affinity resin (BD Biosciences, Ltd). Band shift analysis was performed as described previously (Kawata *et al*., 1996) using oligonucleotides labelled with Cy5-dCTP (Amersham, Ltd). Gels were scanned at 700nm wavelength with the Odyssey Infrared Imaging System (LI-COR, Ltd.).

*Acknowledgements*

*We would like to thank the Wellcome Trust (Project Grant 078971/Z/05/Z) for funding this work.*

## References

CECCARELLI, A., MAHBUBANI, H.J., INSALL, R., SCHNITZLER, G., FIRTEL, R.A. and WILLIAMS, J.G. (1992). A G-rich sequence element common to Dictyostelium genes which differ radically in their patterns of expression. *Dev. Biol.* 152: 188-193.

DARLEY, C.P., LI, Y., SCHAAP, P. and MCQUEEN-MASON, S.J. (2003). Expression of a family of expansin-like proteins during the development of Dictyostelium discoideum. *FEBS Lett.* 546: 416-418.

FUKUZAWA, M. and WILLIAMS, J.G. (2000). Analysis of the promoter of the cudA gene reveals novel mechanisms of Dictyostelium cell type differentiation. *Development* 127: 2705-2713.

MAEDA, M., SAKAMOTO, H., IRANFAR, N., FULLER, D., MARUO, T., OGIHARA, S., MORIO, T., URUSHIHARA, H., TANAKA, Y. and LOOMIS, W.F. (2003). Changing patterns of gene expression in Dictyostelium prestalk cell subtypes recognized by *in situ* hybridization with genes from microarray analyses. *Euk. Cell* 2: 627-637.

POWELL-COFFMAN, J.A., SCHNITZLER, G.R. and FIRTEL, R.A. (1994). A GBF-binding site and a novel AT element define the minimal sequences sufficient to direct prespore-specific expression in Dictyostelium discoideum. *Mol. Cell. Biol.* 14: 5840-5849.

SCHNITZLER, G.R., FISCHER, W.H. and FIRTEL, R.A. (1994). Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in Dictyostelium. *Genes Devel.* 8: 502-514.

SHIMADA, N., MAEDA, M., URUSHIHARA, H. and KAWATA, T. (2004). Identification of new modes of Dd-STATa regulation of gene expression in Dictyostelium by *in situ* hybridisation. *Int. J. Dev. Biol.* 48: 679-682.

SMITH, E. and WILLIAMS, K. (1980). Evidence for tip control of the «slug/fruit» switch in slugs of Dictyostelium discoideum. *J. Embryol. Exp. Morphol.* 57: 233-240.

VERKERKE-VAN WIJK, I., FUKUZAWA, M., DEVREOTES, P.N. and SCHAAP, P. (2001). Adenylyl cyclase A expression is tip-specific in Dictyostelium slugs and directs StatA nuclear translocation and CudA gene expression. *Dev. Biol.* 234: 151-160.

YAMADA, Y., WANG, H. Y., MASASHI, F., BARTON, G. J. AND WILLIAMS, J. G. A new family of transcription factors *Development* 135: 3093-3101.