Int. J. Dev. Biol. 54: 1301-1307 (2010)
doi: 10.1387/ijdb.103141hw

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Synergy between two transcription factors directs gene expression in *Dictyostelium* tip-organiser cells

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ABSTRACT ***cotC*** **requires the transcription factor CudA for its expression in the posterior, prespore cells of the slug, while the** ***expL7*** **gene requires CudA for its expression in the anterior, tip-organiser region. In order to identify additional transcription factors that might mediate tip-organiser specific expression, we performed affinity chromatography on slug nuclear extracts. The affinity matrix bore cap-site distal sequences from region A' of the** ***expL7*** **promoter; an essential region located upstream of the CudA binding domain. One of the proteins purified was G-box binding factor (GBF), a zinc finger transcription factor which binds to G-rich elements, known as G boxes, that are present in the promoters of many developmental genes, including** ***cotC*****. Previous work identified an essential sequence motif within region A' and we show that this element is a G box, that binds recombinant GBF. Moreover, a G box from within the** ***cotC*** **promoter can substitute for region A' of** ***expL7*** **in directing tip-organiser specific expression of** ***expL7*****. Thus the same two transcription factors, CudA and GBF, seem to co-operate to direct both tip-organiser and prespore gene expression. How then is specificity achieved? Replacing a CudA binding region in the** ***cotC*** **promoter with the CudA binding domain from** ***expL7*** **strongly represses** ***cotC*** **promoter activity. Hence we suggest that differences in the topology of the multiple CudA half- sites contained within the two different CudA binding regions, coupled with differences in the signalling environment between tip-organiser cells and prespore cells, ensure correct** ***expL7*** **expression.**

KEY WORDS: *Dictyostelium, cudA, GBF, tip-organiser, prespore, transcription factor*

Dictyostelium develops to form a fruiting body comprised of just two terminally differentiated cell types, stalk and spore cells, but, at the preceding slug stage, prespore cells and several sub-types of prestalk cell can be recognised. The cells of one of the prestalk sub-types constitute the tip-organiser, a tissue that behaves rather like an embryonic organiser and that also directs slug behaviour (Raper, 1940; Poff and Loomis, 1973; Rubin and Robertson, 1975; Smith and Williams, 1980).

CudA is the founder member of a class of transcription factors that are present only in the amoebozoa (Fukuzawa *et al*., 1997). CudA is expressed and nuclear localised in the tip-organiser cells where it is necessary for the transcription of a direct target gene, *expL7*, that encodes an expansin-like protein (Ogasawara *et al*., 2009; Wang and Williams, 2009). CudA acts as a secondary transcription factor in a transcriptional cascade; wherein tip-specific accumulation of the ACA adenylyl cyclase mRNA causes localised cAMP synthesis which triggers STATa activation within the tip (Verkerke-van Wijk *et al*., 2001). STATa then binds to the *cudA* promoter and directs *cudA* transcription. CudA is also expressed and nuclear localised in prespore cells, where it acts as a secondary transcription factor necessary for optimal expression of the *cotC* spore coat protein gene (Yamada, *et al*., 2008).

The *expL7* and *cotC* promoters contain binding sites for CudA, that are essential for optimal expression but that are not sufficient to direct expression when linked to minimal promoter elements (Yamada, *et al*., 2008; Wang and Williams, 2009). Transcription factors generally function co-operatively, with other transcription factors, by binding to synergising promoter elements to achieve the requisite level of gene expression. Often this involves the co-operation of cell-type specific with non cell-type specific transcription factors, such as SP1 (Kadonaga *et al*., 1987). In the case of

Abbreviations used in this paper: GBF, G-box binding factor.

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Accepted: 23 June 2010. *Final author corrected PDF published online:* 15 July 2010.

ISSN: Online 1696-3547, Print 0214-6282

post-aggregative *Dictyostelium* development, a similar role is frequently played by GBF (G-box binding factor).

GBF contains two putative zinc fingers and binds to GT- or, in the opposite strand, CA-rich elements (both relative orientations will be termed G boxes, unless referring to a previously named element) that are frequently located upstream of post-aggregation genes (Hjorth *et al.*, 1988; Hjorth *et al.*, 1990; Pears and Williams, 1987; Schnitzler *et al.*, 1994). G-boxes are essential for optimal gene transcription within the context of their own promoter but are inactive when linked to basal promoter elements. The *cotC* promoter contains three G boxes, termed CA-rich elements or CAEs (Powell-Coffman *et al.*, 1994). They synergise with a downstream TA-rich region to help direct efficient *cotC* expression (Powell-Coffman *et al.*, 1994). Additionally, prespore specificity is endowed by a CudA binding region, termed region B, located between the two cap-site proximal CAEs (Yamada, *et al.*, 2008).

For unknown reasons, recombinant forms of CudA fail to bind at specific promoter sites, hence an Entamoeba orthologue with similar apparent specificity, ECudA, is used for mapping promoters (Yamada, *et al.*, 2008). CudA exists as a dimer and region B of *cotC* contains a complete ECudA binding site, the dyad GAATTTTC, and a more proximal half site, GAA. The *expL7* promoter (Fig. 1A) contains an approximately 100nt ECudA binding region, also called region B, that is essential for tip-specific expression (Wang and Williams, 2009). This region contains several dispersed sub-regions that are necessary for maximal binding by ECudA. There are four potential TTC CudA half-sites within region B but, because of their partial mutual redundancy, they were not precisely delineated by mutation (Wang and Williams, 2009).

CudA is, as stated, present in the nuclei of both the tip-organiser and the prespore cells and is essential for *expL7* and *cotC* expression. What then prevents *expL7* from being expressed in the prespore cells and *cotC* being expressed in the tip-organiser cells; is there an additional transcription factor that confers discrimination between the two promoters? Region A (Fig. 1A and 2A) of the *expL7* promoter is a candidate for the binding site of such a factor. Region A does not bind CudA but is required for *expL7* expression in tip organiser cells (Ogasawara *et al.*, 2009; Wang and Williams, 2009). An essential CA-rich sequence element has been identified in region A by mutational analysis and was proposed to be a site of binding for the DIF-regulated bZIP protein, DimB (Ogasawara *et al.*, 2009). However, no direct evidence was presented for this. In order to determine whether region A might contain ancillary regulatory elements that confer specificity on *expL7*, we undertook an analysis of the proteins that bind to it.

Results

Two known transcription factors are purified by cudA *promoter region A'*

Region A of the *expL7* promoter contains two relatively GC-rich tracts, separated by a 65nt region comprised entirely of A and T residues (Fig. 1A and Fig. 2A). The essential sequences of region A lie in the cap-site distal GC-rich region, region A' (Ogasawara *et al.*, 2009). Hence slug nuclear extracts were purified on an affinity resin bearing the 43nt sequence comprising region A' (Fig. 1A). Multiple proteins were bound (Fig. 1B) but most are either RNA binding proteins, a class of proteins that is often purified in such experiments (Fukuzawa, *et al.*, 2006), or previously un-

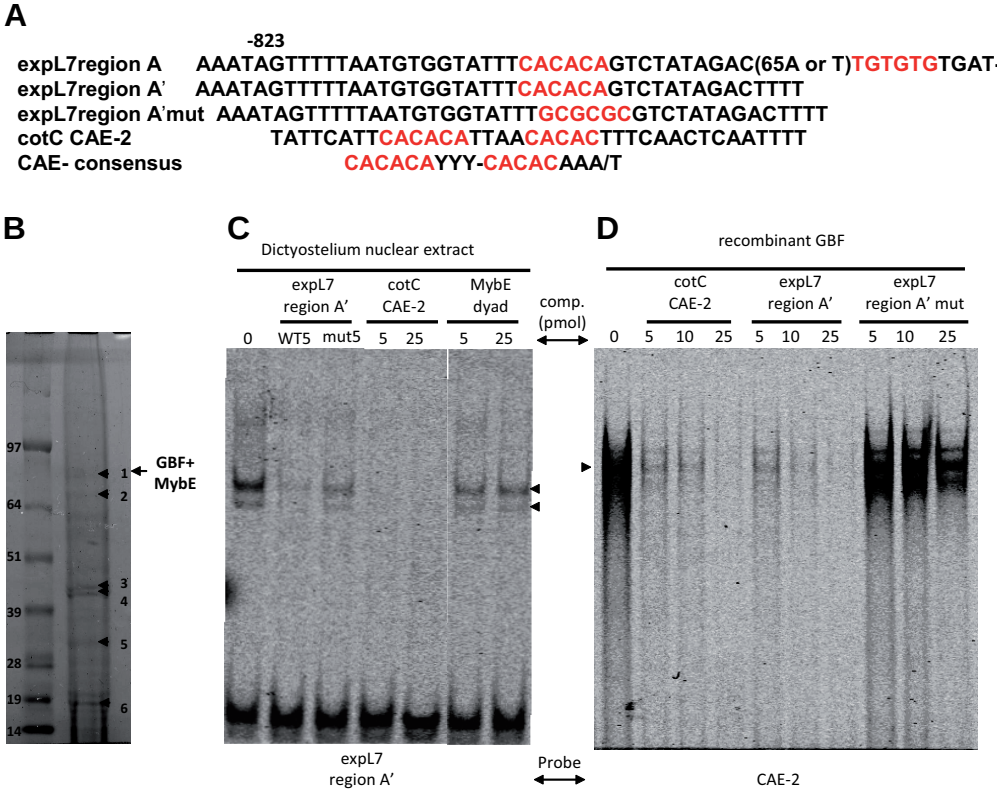

Fig. 1. Affinity purification of nuclear proteins that bind to region A' and band-shift analysis of proteins interacting with region A' . (A) *An alignment, in red, of the CACACA sequence in CAE-2 with the CACACA sequences in region A' of* expL7*. Note that the cap-site proximal GC-containing tract contains a CACACA sequence in its invert complement. Only the distal part of the proximal sequence is shown. It extends another 30nt, with the sequence TGAATTATTATTTATTTGTTTTTATTTATT.* **(B)** *Total nuclear extracts were subjected to affinity chromatography on A' and analysed by mass spectrommetry as described in Methods. Slice 1 contained the only two recognisable transcription factors: MybE (93kDa) and GBF (79kDa). We assume that we are detecting a large degradation product of MybE because slice 1 is tightly centred around a nominal mol wt of 80kD.* **(C)** *Region A' was labeled and used as probe in gel retardation with heparin purified slug nuclear extract and with the indicated competitors.* **(D)** *CAE-2 was labeled and used as probe in gel retardation with GBF produced in E. coli and with the indicated competitors.*

known proteins. There were just two recognisable transcription factors, GBF and MybE. Both were contained within slice 1, the gel slice bearing the largest proteins analysed (Fig. 1B). GBF contains two potential zinc fingers and has a predicted molecular weight of 79kDa (Schnitzler, 1993). MybE is a single Myb domain SHAQKY family protein (Fukuzawa *et al.*, 2006). It has a predicted molecular weight of 93kDa and is required for DIF-inducible gene expression and correct prestalk cell differentiation.

There is a binding site for G-box binding factor (GBF) within region A'

When region A' is used as a probe in a band shift assay with heparin purified nuclear extracts, there is a major retarded band and a minor, faster-migrating band (Fig. 1C). The two retarded complexes apparently contain GBF; because CAE-2, a well-characterised G box located within the *cotC* promoter, is a more potent competitor for binding to the A' probe than is A' itself (Fig. 1C). MybE was also purified in the affinity chromatography but an oligonucleotide sequence from the ecmA promoter containing the MybE dyad does not compete for binding to region A' (Fig. 1C). Also there is no obvious fit to the consensus MybE dyad binding site, AACnGTT, within region A'. MybE could be present as a contaminant in the eluate from the affinity resin or it may bind via an interaction with another protein present in the complex. It was not investigated further.

The consensus site for the binding of GBF to a CAE is CACACAYYYCACACAAA/T (Powell-Coffman *et al.*, 1994) and this region of CAE-2 is indicated in red in Fig. 1A. In region A' there is a sequence with perfect homology to the distal half of the CAE sequence, CACACA, but there is only limited homology to the proximal CACACA element. However, downstream of the long AT tract there is a CACACA sequence in the complementary strand (red in Fig. 1A). GBF functions by binding co-operatively to multiple G box elements, independent of their relative orientation (Pears and Williams, 1988). Hence the downstream sequence could subsume the function of the proximal CACACA element. We mutated the entire distal CACACA element, as indicated in Fig. 1A, and assayed relative competition activity in a band-shift assay using region A' as the probe (Fig. 1C). The mutant form, A' mut, is a significantly poorer competitor than region A' itself (Fig. 1C).

The above band shift assay supports the notion that GBF binds to the *expL7* promoter and maps the binding activity to the CACACA sequence. There are, however, close homologues of GBF in *Dictyostelium* and one of these could, in principle, be responsible for the observed binding. We therefore expressed GBF in E. coli as a HIS fusion protein, purified the protein on metal affinity resin, and performed band shift assays using CAE-2 as probe (Fig. 1D). When used as a competitor, region A' is as effective as CAE-2 itself. Moreover A' mut, the sequence containing mutations that ablate the CACACA element, is much less effective as a competitor (Fig. 1D). Thus recombinant GBF binds to region A' of the *expL7* promoter and it does so by binding to the CACACA element.

The G box is the active element within region A' and a G box derived from cotC *can subsume the function of A'*

The full length expl7 promoter construct, -1117, is expressed throughout the tip-organiser while the fore-shortened, -823 construct is expressed only in the rear part of the tip-organiser (Wang and Williams, 2009 and Fig. 2). The CACA element located in region A' has previously been point-mutated in two of its C residues but, because its parent construct is not expressed at the slug stag, an effect of the mutation was only measurable during culmination (Ogasawara *et al.*, 2009). We therefore generated construct -823M. It is equivalent to construct -823, which we find to be expressed in the posterior of the tip-organiser region at the

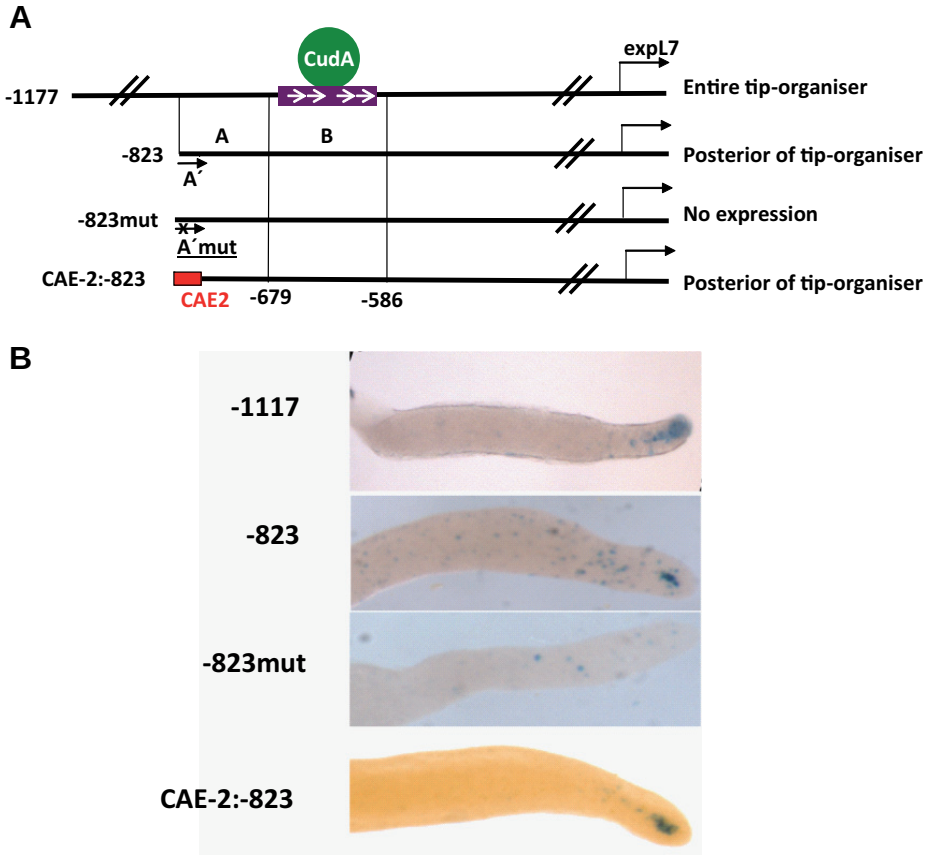

Fig. 2. *expL7* promoters reporter fusions and their expression patterns. (A) *A representation of the explA7 promoter showing the positions of the four proposed CudA half sites, relative to the previously mapped regions A, B and C. Each TTC half site is represented by an arrow. There are also CudA binding sites within region C but they have not been at all mapped to the sequence. In construct -823 mut, region A' was mutated to region A' mut, the sequence shown in Fig. 1A. In construct CAE-2:823, region A' was replaced by the CAE-2 sequence from the* cotC *promoter, again the sequence was as in Fig. 1A.* **(B)** *Cells transformed with the indicated lacZ reporter constructs (Fig. 2A) were allowed to develop to the slug stage, fixed and stained for β-galactosidase.*

slug stage (Wang and Williams, 2009) but contains the same 6 nucleotide substitution (CACACA to GCGCGC) that eliminates binding of region A' to GBF (Figs. 1A and 2A). While -823 is expressed in the expected pattern, -823M is entirely inactive (Fig. 2B). Thus the CACACA element is essential for tip-organiser expression at the slug stage.

If GBF mediates the essential role that region A' plays in tip-organiser gene expression, it should be possible to replace region A' with a generic G box. We tested this using CAE-2 from *cotC*. In construct CAE-2:823 expression is restored in the rear half of the tip-organiser region (Fig. 2B). This mirrors the expression pattern observed for construct -823, where region A' is present (Fig. 2B). Collectively these data imply that GBF synergises with CudA to activate expL7 but we cannot, of course, completely rule out the possibility that another transcription factor with a similar specificity binds region A' *in vivo*.

When transplanted into the cotC *promoter, the CudA binding domain from* expL7 *acts as a transcriptional inhibitor*

The above result shows that region A is required purely because it contains a G-box. Therefore sequences in region A do not dictate that *expL7* should be expressed in the tip-organiser cells but not the prespore cells. That information must be encoded downstream of region A. Region B is essential for tip organiser expression but the only known factor that binds there is CudA and CudA is also necessary for *cotC* expression in prespore cells. There could, however, be a binding site for another transcription factor in region B. Alternatively, differences in the positions and relative orientation of the multiple CudA binding sites between *expL7* and *cotC* might, in some way, determine their different properties.

To help distinguish the above possibilities, we replaced the known CudA binding region of *cotC*, region B in our previous analysis (Yamada, *et al.*, 2008), with region B from *expL7*. The start point was a new construct, cotCΔB; an internal deletion mutant of the full length *cotC* promoter, construct CotC, that lacks region B (Fig. 3A). Expression of this and related constructs, all driving expression of a lacZ reporter, was compared in three separate transformant pools: both by β-galactosidase staining of whole mount slugs and enzymatic assay (Figs. 3 B,C). The latter analysis allows quantitative comparison between the expression levels of the constructs and the use of multiple pools averages out

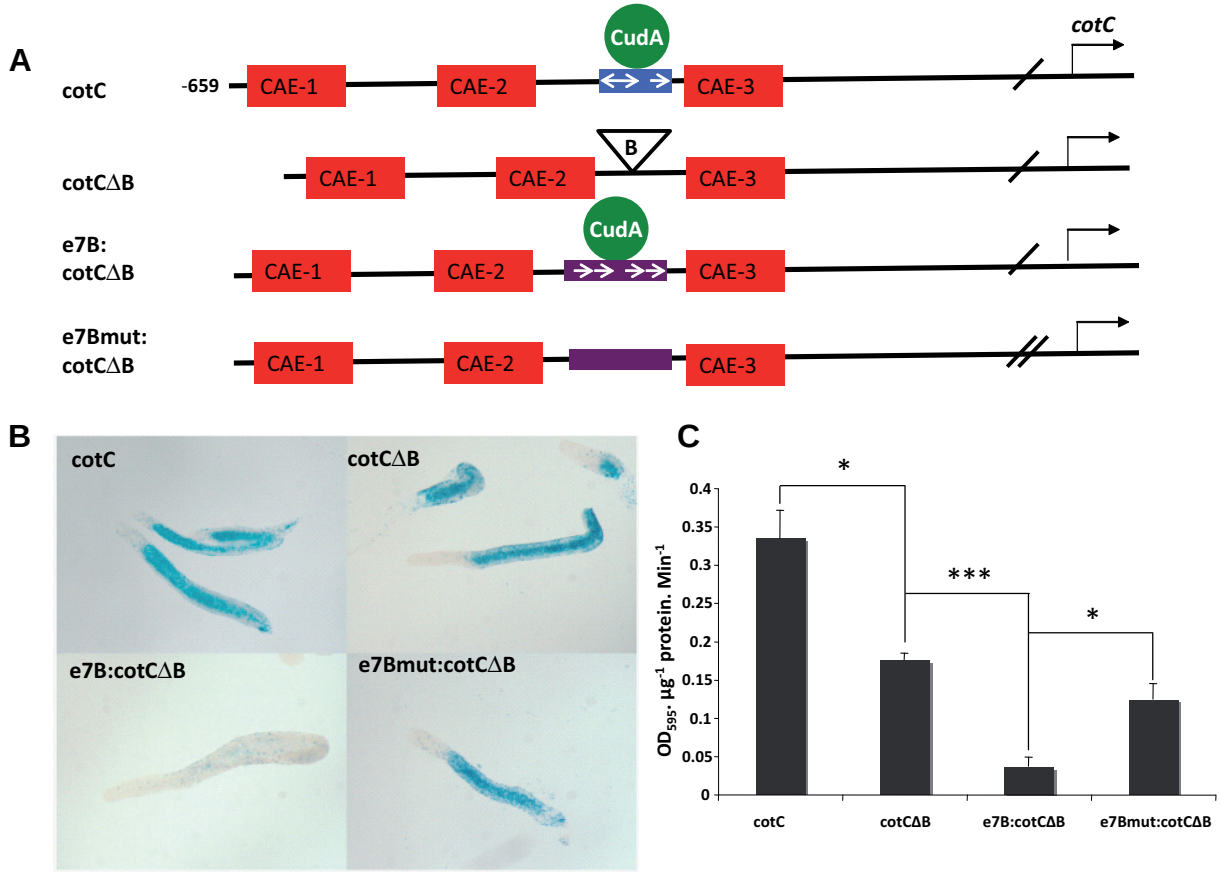

Fig. 3. Insertion of CudA binding region B from *expL7* into the *cotC* promoter. (A) *A schematic representation of the* cotC *promoter and constructs derived from it. The promoter contains three CAE elements, one dyad CudA binding site and one half CudA binding site. Each half site is represented by an arrow. In construct cotCΔB the 66 nucleotide of the CudA binding region is deleted. Constructs e7B:cotCΔB and e7Bmut:cotCΔB were generated from cotCΔB by inserting either wild type or mutant forms of the CudA binding region from the* expL7 *promoter.* **(B)** *Cells transformed with the indicated lacZ reporter constructs (Fig. 3A) were allowed to develop to the slug stage, fixed and stained for β-galactosidase for 30' at 37°C.* **(C)** *Cells transformed with the indicated lacZ reporter constructs (Fig. 3A) were developed to the slug stage, protein was isolated and β-galactosidase enzymatic activity was measured. The average results from three separate transformant pools, assayed in duplicate are shown with S.D. The statistical significance of selected of the results was further assessed using the Student t test and are indicated using asterisks: * p=0.05 and ** p=0.001.*

any effects of copy number.

Despite the deletion, cotCΔB is still expressed in prespore cells, but at a two-fold lower level than cotC (Fig. 3C, significant at p=0.05). The residual level of expression presumably indicates that there are additional CudA binding sites, located elsewhere in the promoter, that are redundant with those in region B. Most likely, these are in the region -659 to -483; because deletion of that region produces a major drop in prespore gene expression and, in a construct with a distal end-point at -457, mutation of the three CudA half-sites in region B greatly attenuates expression (Yamada, *et al.*, 2008).

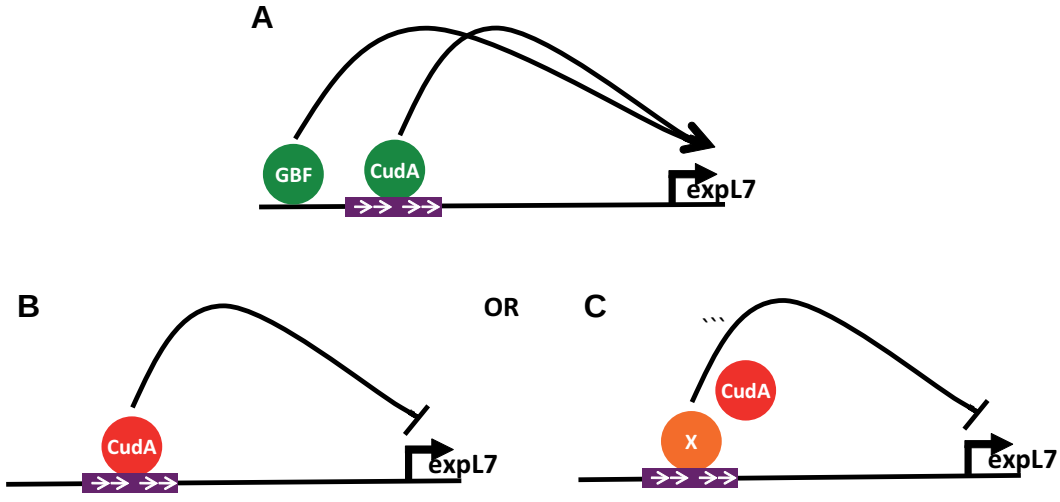

Fig. 4. Models to explain the selective expression of *expL7* in tip organizer cells. (A) *Tip-organiser cell.* **(B,C)** *Prespore cell. The models are explained in the text.*

When region B from *expL7* is inserted into cotCΔB, to generate e7B:cotCΔB, there is almost no expression in the tip-organiser (Figs. 3 B,C, significant at p=0.001). Hence region B from *expL7* does not carry the sequence information necessary to direct tip-organiser expression, at least when within the context of an otherwise prespore-specific promoter. Interestingly however, it exerts a strong inhibitory effect on prespore expression directed by the residual *cotC* promoter elements within cotCΔB (Figs. 3 B,C). Construct e7Bmut:cotCΔB contains block mutations; within the three sub-regions known to be essential for optimal CudA binding and that, between them, ablate the four proposed ECudA half-sites (Wang and Williams, 2009). It does not display the same inhibited expression level as e7B:cotCΔB (Figs. 3 B,C). We are, unfortunately, precluded from performing the symmetrical experiment, to determine whether region B from the cotC gene down-regulates expL7 when it is used to replace region B of expl7. In the case of the expL7 promoter, deletion of region B totally prevents tip-specific expression (Wang and Williams, 2009). Therefore, there is no residual expression to be inhibited by adding in regionB from cotC.

Discussion

The tip-organiser cells are a small sub-set of the prestalk cells that control slug integrity and behaviour. The prespore cells are destined to become spores and differ from the tip-organiser cells in many important ways. Nonetheless, there is a striking similarity in the way two of the markers for these tissues, *cotC* and *expL7*, are regulated; both lie at the end of transcriptional cascades involving CudA and both seem to involve a synergy of CudA with GBF. This congruity is most clearly demonstrated by the fact that a G box from *cotC* can functionally substitute for the G box of *expL7*.

Such interchangeability of promoter elements has been demonstrated previously; using a G box from the DIF regulated ecmB promoter to replace a G box located in a cAMP regulated promoter (Ceccarelli *et al.*, 1992). The present observations, again using two genes with radically different expression patterns, re-inforce the notion of GBF as a general transcription factor which co-operates with regulatory transcription factors to facilitate the expression of most, if not all, classes of post-aggregation genes. A “synthetic” reporter construct, containing multimerised CudA dyad binding sites, fused downstream of CAE-2 to basal promoter elements from the Actin 15 gene, was not expressed in a cell type specific manner (Wang and Williams, unpublished data). Hence we cannot rule out the involvement of other transcription factors in either pathway and, indeed, a cap-site proximal AT-rich region, is known to be important for *cotC* expression (Powell-Coffman *et al.*, 1994).

A negative result from a promoter shuffling experiment is, of course, intrinsically difficult to interpret; the spacing and configuration of the elements may be incorrect or they may require specific basal promoter sequences. We did obtain interesting information when we attempted the less ambitious experiment of using the CudA binding domain of *expL7*, domainB, to replace the characterised CudA binding domain of *cotC*. Rather than the expected stimulation of expression, back up to the level of the undeleted construct, there was a strong inhibition. This observation could explain why *expL7* is expressed in the tip-organiser cells but not in the prespore cells.

We propose that within the signalling environment of tip-organiser cells CudA bound to region B of *expL7* functions, in conjunction with GBF, as an activator of *expL7* transcription (Fig. 4A). Within the signalling environment of prespore cells, either: CudA bound to region B of *expL7* acts as a repressor of *expL7* transcription (Fig. 4B) or CudA is displaced from the *expL7* promoter and replaced by a transcriptional repressor, designated X in Fig. 4C. The nuclear hormone receptor super-family provides precedents where the configuration of half sites determines regulatory polarity (Naar *et al.*, 1991; Carr and Wong, 1994; Kurokawa *et al.*, 1994; Retnakaran *et al.*, 1994; Jacobsen *et al.*, 2009). Similar differences in the topology of CudA half sites could explain why *cotC* shows the opposite beaviour to *expL7* in prespore cells. The inhibitory effect of *expL7* domainB is dependent upon the presence of the four proposed CudA half sites but this observation has to be interpreted cautiously, because the

sites have not been precisely mapped. Transcription factors other than CudA (X in Fig. 4C) could share part or all of the CudA binding specificity and could replace CudA to act as the repressor. There are five CudA orthologues in *Dictyostelium* and one of these may fulfil that function. The fact that deletion of region B from expL7 does not lead to ectopic expression within the prespore region (Wang and Williams, 2009) perhaps indicates that it also contains sequences important for the activation of expression in prespore cells.

Materials and Methods

Cell culture and development

All experiments were performed with the Gerisch isolate of Ax2. Cells were grown, developed, transformed and assayed for lacZ expression as described previously (Fukuzawa and Williams, 2000).

Affinity purification of proteins

Slug nuclear extracts derived from 4 X 10^{11} slug cells were precipitated with 50% ammonium sulfate and subjected to heparin-agarose chromotagraphy and DNA affinity chromatography as described previously (Fukuzawa, *et al.*, 2006). Protein was analysed on 4%-12% Bis-Tris SDS-polyacrylamide gels and selected regions of the gel were analysed by mass spectrommetry (Fukuzawa, *et al.*, 2006).

Band shift analysis

The entire GBF coding region was cloned as a "6XHIS" fusion construct in pET15b (Novagen, Ltd), expressed in E, coli and purified over TALON™ metal affinity resin (BD Biosciences, Ltd). Band shift analysis was performed as described previously (Wang and Williams, 2009) using oligonucleotides labelled with Cy5-dCTP (Amersham, Ltd).

Generation of promoter constructs

The -823 *expL7* promoter construct was described previously (Wang and Williams, 2009). The constructs -823M and CAE-2:-823 were generated by cloning double stranded oligonucleotides respectively containing the sequences A´mut or CAE-2 (sequences as in Fig. 1A) at their distal ends, coupled to the central 65nt AT sequence and the proximal GC-containing sequence (Fig. 1A). They were cloned, with GATC cohesive ends into the BamHI site of *expL7* construct -723; a deletion construct that lacks region A (Wang and Williams, 2009). The *cotC* -659 construct was described previously (Yamada, *et al.*, 2008). CotCΔB was generated by inserting *cotC* promoter fragment -659 to -483 into construct cotC -416 (Yamada, *et al.*, 2008) using XbaI and BamHI. This resulted in a 66nt deletion within the promoter. Using *cotC*ΔB as recipient, *expL7* region B in its unmutated or multiply mutated form (sequences shown below) were inserted into the BamH site with GATC cohesive ends to respectively generate e7B:cotCΔB and e7Bmut:cotCΔB, unmutated domain B: ATTTGTTTTAAT**TTC**TTTATT**TTC**AATTACCCAAAAGATTATTATACATC ATAATTA**TTC**TATATGATTACGTACACGTTTTTTAAACAAT**TTC**TAA and mutated domain B:ATTTGTTTTAGCGCGCGCGCGCGCGCTTACC CAAAAGATTATTATACATCATAATTAGCGCGCGCGATTACGTACACGTTTTTTAAACA GCGCGCGC. The positions of the mutations were based on mutation scanning results (Wang and Williams, 2009), where sequential 8 nucleotide blocks of sequence within domain B were replaced with 8 nucleotide alternating GC tracts. Here the GC tracts are underlined. The suggested CudA half-sites in the unmutated sequence are in bold.

β-galactosidase staining and enzymatic activity analysis

Cells bearing lacZ contructs were developed to the slug stage and either fixed and stained for β-galactosidase (Dingermann *et al.*, 1989) or harvested in lysis buffer (100 mM Hepes, pH8, 1 mM $MgSO_4$, 5 mM DTT, 2%Triton X-100). The protein concentration was determined and 5 ul of protein was added to 100 ul of lysis buffer containing 1 mM CPRG. OD was measured at 595 nm at several time points. LacZ activity is displayed as OD_{595} per ug protein per minute.

Acknowledgements

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

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