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 cooperation of cell-type specific with non-cell-type specific transcription factors, such as SP1 (Kadonaga et al., 1987). In the case of...

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

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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 GAATTTC, 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 binding sites 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 unrecognised transcription factors. Region A of the *expL7* promoter contains two relatively GC-rich tracts, separated by a 65nt region comprised entirely of A and T, termed region A' (Ogasawara *et al*., 2009). G-boxes are essential for CAT-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 unrecognised transcription factors.

**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 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 unrecognised transcription factors.
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 potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (Fig. 1C). MybE was also purified in the affinity chromatography but an potent competitor for binding to the A’ probe than is A’ itself (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 CACACA YYYCACACA AA 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 Dictostelium 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 stage, 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...
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=0.001). 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).

When region B from expL7 is inserted into 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 region B 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 GFB. 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 GFB as a general transcription factor which cooperates 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 GFB, 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 behaviour to expL7 in prespore cells. The inhibitory effect of expL7-domains is dependent upon the presence of the four proposed CudA half sites but this observation has to be interpreted cautiously, because the

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.
sites have not been precisely mapped. Translation 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 fulfill 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 chromatography 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 spectrometry (Fukuzawa, et al., 2006).

Band shift analysis
The entire GBF coding region was cloned as a "6XHIS" fusion construct in pET15b (Novagen, 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, inserted into the BamH site with GATC cohesive ends to respectively in its unmutated or multiply mutated form (sequences shown below) were inserted into the BamH site with GATC cohesive ends to respectively (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 spectrometry (Fukuzawa, et al., 2006).

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