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Transcriptional Regulatory Elements of the Phosphodiesterase-1 Gene of *Dictyostelium discoideum*

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Abstract: The *Dictyostelium discoideum* cyclic nucleotide phosphodiesterase 1 (PDE1; PdsA) is synthesized during different stages of the developmental cycle and is controlled by three stage-specific promoters. The aggregation-specific (*PDE1-A*) promoter is induced by extracellular 3', 5'-cyclic adenosine monophosphate (cAMP) and is developmentally regulated. Regulatory elements of the *PDE1*-A promoter were analyzed by serial deletions and by subcloning promoter segments. The full-length promoter was found to consist of disperse and redundant developmental regulatory elements. A region -556 to -450 relative to the transcription start site is critical for extracellular cAMP-mediated transcription. Nuclear proteins that are synthesized *de novo* during development bind to this 106 bp fragment in gel shift assays. The level of binding activity is enhanced significantly by cAMP treatment. Treatment of nuclear extracts with phosphatase indicated that protein phosphorylation is essential for DNA binding activity. A GT-rich motif within this 106-bp region was identified, which is similar to a consensus sequence for cAMP responsive elements characterized in other *Dictyostelium* genes.

Key words: Phosphodiesterase, cAMP, *cis*-acting element, *trans*-acting factor

INTRODUCTION

The development of an organism depends on an array of processes by which genes are spatially and temporally regulated. Transcription factors can serve as switches turning specific sets of genes on or off at different times during development. Progress in characterizing transcription factors has led to the realization that single genes are governed by combinations of positive and negative factors acting on *cis*-acting regulatory elements. Transcription factor activity is regulated not only by controlling transcription of the cognate genes, but often by posttranscriptional modulation. The goal of the studies described here was to examine the *cis*-acting elements that control the transcription of the cyclic nucleotide phosphodiesterase gene (*PDE1*; *PdsA*) in response to cAMP in *Dictyostelium discoideum*.

Dictyostelium discoideum is a eukaryotic microorganism which undergoes a relatively simple process of development that is fundamentally similar to that of development in more complex organisms (Aubry and Firtel, 1999; Kimmel and Firtel, 2004; Mahadeo and Parent, 2006; Williams, 2006). Extracellular cAMP is a key regulator of *Dictyostelium* development. Initially, cAMP is the chemoattractant that gathers cells into an aggregate during the early stage of development (Firtel, 1991; Nebl *et al*., 2002; Saran *et al*., 2002; Manahan *et al*., 2004; Strmecki *et al*., 2005). Throughout development, cAMP regulates gene expression by regulating transcription (Gerisch, 1987). Extracellular cAMP acts analogously to peptide hormones of animals and its effects are mediated through G-protein-linked receptors (Newell *et al*., 1990; Firtel, 1991; Cubitt *et al*., 1992). Different cAMP receptors and different intracellular signaling pathways modulate aggregation-stage and later

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cell-type specific gene expression (Kimmel, 1987; Wu *et al*., 1995; Weening *et al*., 2003). Ultimately, these signaling pathways impinge on *cis*-acting regulatory sequences of target genes. Many *cis*-acting elements have been identified in *Dictyostelium,* including cAMP responsive elements (CRE) and elements that control cell type specific gene expression (Hjorth *et al*., 1990; Vauti *et al*., 1990; Ceccarelli *et al*., 1991; Desbarats *et al*., 1992; Harwood *et al*., 1993; Hori, 1994; Richardson *et al*., 1994; Yoder and Blumberg, 1994; Ramalingam *et al*., 1995; Miller *et al*., 1996; Gollop and Kimmel, 1997; Favis *et al*., 1998; Fukuzawa and Williams, 2000; Warner and Rutherford, 2000; Weening *et al*., 2003; Hirose *et al*., 2005; Shimada *et al*., 2005; Wiles *et al*., 2006).

PDE1 was the first of six *Dictyostelium* phosphodiesterase genes to be identified (Barra *et al*., 1980; Bader *et al*., 2006). The gene product, PDE1, is dual specificity cAMP and cGMP phosphodiesterase. It is a member of the class II phosphodiesterases, a group confined primarily to lower eukaryotes (Bader *et al*., 2006). PDE1 is both secreted and bound to the cell surface (Orlow *et al*., 1981) and provides the primary activity for the degradation of extracellular cAMP that is used to coordinate aggregation, morphogenesis and gene expression (Bader *et al*., 2006). The importance of *PDE1* is demonstrated by the developmental defects of mutants that are either deficient in PDE1 (Barra *et al*., 1980) or that overproduce it (Faure *et al*., 1988; Hall *et al*., 1993).

PDE1 is a single-copy gene that exhibits a complex pattern of regulation involving alternative splicing and the use of three independent, stage-specific promoters active during vegetative growth, cellular aggregation and later multicellular development (Podgorski *et al*., 1989). An analysis of the *PDE1* late development promoter revealed that critical *cis*-acting transcriptional regulatory elements are found in a transcribed region that is copied into the 5' untranslated region of the late development-specific transcript (Weening *et al*., 2003).

Transcriptional regulation mediated by the aggregation-stage *PDE1* (*PDE1*-A) promoter is the focus of the studies reported here. The aggregation-stage promoter becomes active shortly after cells enter development (Franke *et al*., 1987), which in *Dictyostelium*, is triggered by nutrient deprivation and modulated by autocrine factors that provide information on cell density (Clarke and Gomer, 1995). Pre-starvation factor (PSF), a protein that induces the expression of many early developmental genes (Clarke and Gomer, 1995) and extracellular cAMP (Franke *et al*., 1987) upregulate transcription from the *PDE1*-A promoter. Sequences necessary *PDE1*-A promoter activity reside in 1.5 kb of DNA upstream of the transcription start site for the aggregation-specific PDE1 transcript (Faure *et al*., 1990).

The *PDE1*-A promoter was analyzed in this study to learn if its activation upon nutrient depletion and by extracellular cAMP was controlled by independent regulatory elements and, if so, to identify the regulatory sequences. We define as temporal control elements (TCEs) those sequences required to activate transcription as cells enter development. TCEs were found to be dispersed over broad regions of the *PDE1*-A promoter with no single distinct region responsible for temporal control. In contrast, a discrete region important for cAMP induction (a cAMP response element or CRE) was identified. In addition to the identification of *cis*-acting elements, gel mobility shift assays revealed the existence of at least one sequence-specific DNA-binding protein that recognized the CRE. The DNA-protein interactions were developmentally regulated, cAMP inducible and dependent on protein phosphorylation.

MATERIALS AND METHODS

Enzymes and Chemicals

Most reagents and enzymes used in this study were purchased from New England BioLabs, Inc. (Beverly, MA, USA). [α-^{32}P]dATP (3,000 Ci mmol^{-1}) was obtained from Dupont NEN Research Products (Boston, MA, USA). Other chemicals were from Sigma (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Dictyostelium Discoideum Culture Conditions

Cells of the axenic strain AX3 were grown in HL5 medium [1% glucose, 0.5% yeast extract, 0.5% thiotone peptone, 0.5% proteose peptone, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4 and 0.005% dihydrostreptomycin sulfate, pH 6.4 to 6.6] at 22°C (Franke and Kessin, 1977). Transformants were cultured in the same medium with 20 μg mL^{-1} G418. For development in liquid culture, cells at a density of 2 to 5×10^6 mL^{-1} were collected by centrifugation and then resuspended at 2×10^7 cells mL^{-1} in Sorensen's buffer [14.7 mM KH_2PO_4 and 2.0 mM Na_2HPO_4, pH 6.0) and shaken at 120 rpm. Cells were treated with cAMP by adding either an initial concentration of 1 mM cAMP or by adding cAMP droplets at 6 min intervals to give a 20 nM cAMP concentration with each drop.

Transformation of Dictyostelium Cells

AX3 cells were transformed either by electroporation (Dynes and Firtel, 1989; Rizzuto *et al*., 1993) or by the method of calcium phosphate DNA precipitation (Nellen and Saur, 1988). Stably transformed cells were selected in HL5 medium supplemented with 20 μg mL^{-1} G418 (geneticin), 40 and 0.5 μg mL^{-1} amphotericin B. Transformants were picked and spread on plates with *Klebsiella aerogenes*. Transformants showing wild type development were then transferred back to HL5 medium containing G418.

Construction of Serial Deletion Clones

A previous study indicated that an upstream1.5 kb fragment of *PDE1* contained all sequences necessary for aggregation-stage promoter activity (Fig. 1; Faure *et al*., 1990). This fragment was excised

Fig. 1: Sequence of the *PDE1* aggregation-specific promoter. The arrows denote the 5' to 3' and 3' to 5' deletion endpoints. Numbering is relative to the start site of transcription

by BclI digestion from the plasmid p2B2-3 (Podgorski *et al*., 1989) and was cloned into the BglII site of the shuttle vector pAV-CAT II (May *et al*., 1989). For the 5' to 3' deletions, the recombinant plasmid was cut with KpnI and BamHI before initiating ExoIII deletions. Nested deletions were performed using ExoIII and mung bean nuclease to create unidirectional deletions. Five microgram of restriction enzyme-digested DNA in 54 μL of ExoIII buffer [66 mM Tris-HCl, pH 8.0, 0.66 mM $MgCl_2$ and 200 units of ExoIII exonuclease] were incubated at 37°C. At 1 min time intervals (about 100 bp DNA deleted per minute), 2.5 μL aliquot of the DNA mixture was added to 7.5 μL Mung bean nuclease solution [30 mM NaOAc, pH 4.5, 50 mM NaCl, 1 mM $Zn(OAc)_2$, 5% glycerol and 0.8 unit of mung bean exonuclease]. Samples were incubated at room temperature for 30 min before stopping the reaction by adding 1 μL stop solution [300 mM Tris-HCl, pH 8.0 and 50 mM EDTA] and heating at 65°C for 10 min. After cooling to room temperature, 2 μL of Klenow mixture [20 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$ and 0.3 unit of Klenow DNA polymerase] and 1 μL of 125 μM of each dNTP were added and incubated at 37°C for 5 min. Plasmids were recircularized by adding 20 μL of blunt-end ligation buffer [250 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 0.25 mM ATP, 5 mM DTT, 25% PEG-8000 and 6 units of T4 DNA ligase] at room temperature overnight. Deletion end points were confirmed by DNA sequencing. A clone with an endpoint at -121 (construct H, Fig. 3) was identified as a suitable vector that contained only the basal promoter. The 3' to 5' nested deletions and truncated fragments were generated by PCR amplification using primers that created SacI sites at both ends. PCR fragments were cloned into the construct D or H (Fig. 2). The orientations of the inserts were determined by restriction digestion and DNA sequencing.

RNA Isolation and Analyses

Total RNA was extracted as described (Chomczynski and Sacchi, 1987; Franke *et al*., 1987). Northern blots were performed, according to Podgorski *et al*. (1989). Probes were labeled with ^{32}P by the random-primer method (Feinberg and Vogelstein, 1983). The 1.1 kb fragment of the *PDE1* coding region was obtained from pGP1 (Faure *et al*., 1990) and labeled for use as a probe of endogenous *PDE1* expression. The 0.7 kb fragment of the CAT gene from pAV-CAT II was labeled as a probe of reporter gene activity. Quantification of RNA was performed by analyses of digitized images of autoradiograms using the NIH Image Program (Masters *et al*., 1992).

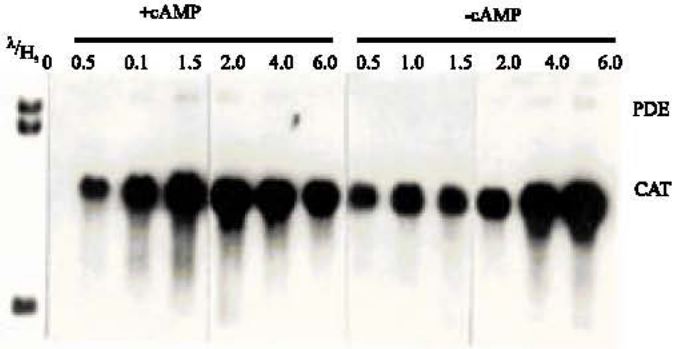

Fig. 2: Time course of *PDE1* promoter activity in shaken suspension culture with or without cAMP. λ/H_3 shows the 2.3, 2.0 and 0.6 kb DNA size markers. Development was initiated by transferring cells to non-nutrient buffer at t_0. Times are shown in hours. CAT shows CAT reporter mRNA expression and PDE shows endogenous *PDE1* mRNA. The average copy of the reporter gene is about 100-200 versus a single copy for the endogenous *PDE1* gene

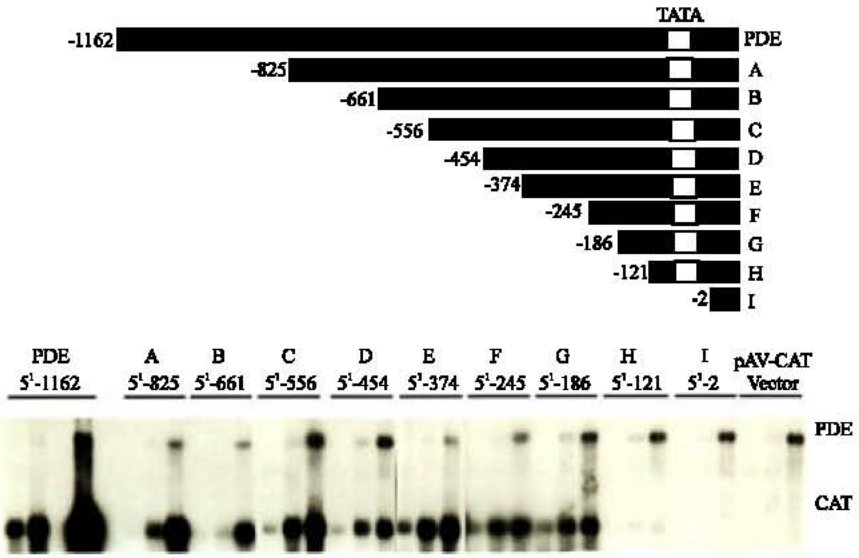

Fig. 3: The 5' to 3' nested deletions of *PDE1* promoter and their transcriptional activity. Each set of three lanes shows northern blots prepared using RNA extracted from cells shaken for 0 and 1.5 h without cAMP and 1.5 h with 1 mM cAMP, respectively

Preparation of Nuclear Extracts

Nuclear extracts were prepared as described by Haberstroh *et al.* (1991). cAMP was added either at a 1 mM initial concentration or as drops added to give a 20 nM concentration every 6 min for 3 or 6 h. After 0, 1.5, 3, or 6 h in shaker culture, cells were harvested and resuspended in 1 mL of ice-cold nuclei preparation buffer [25 mM Tris-HCl, pH 7.5, 5 mM $Mg(OAc)_2$, 0.5 mM EDTA and 5% sucrose]. Two hundred microliters of a 20% Nonidet P-40 solution was added and the cells were incubated on ice for 5 min. After centrifugation in a microfuge at 4°C for 5 min at 10,000 rpm, the nuclear pellet was resuspended in cold nuclei preparation buffer and treated again using the same procedure. Pelleted nuclei were resuspended in 150 μL ice-cold nuclear protein extraction buffer [20 mM Tris-HCl, pH 7.5, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 1 mg mL^{-1} benzamide, 2 μg mL^{-1} chymostatin, 1 μg mL^{-1} leupeptin, 0.1 μg mL^{-1} mg mL^{-1} TPCK (N-tosyl-L-phenylalanine chloromethyl ketone), 0.1 mg mL^{-1} TAME (N-α-p-tosyl-L-arginine methyl ester), 5 μg mL^{-1} antipain, 0.1 mg mL^{-1} TLCK (N-α-p-tosyl-L-lysine chloromethyl ketone), 0.1 mg mL^{-1} PMSF (phenylmethylsulfonyl fluoride) and 0.2 mg mL^{-1} phenanthroline]. Nuclear proteins were extracted on ice for 30 min with occasional flicking of the tube. Extracts were centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was dialyzed at 4°C for 2 h, against two changes of 1000x volume of dialysis buffer [20 mM Tris-HCl, pH 75, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.1 mg mL^{-1} PMSF]. Protein concentrations were determined by the Coomassie blue G250 using a kit from Pierce Chemical Co. (Rockford, IL, USA). Nuclear extracts were aliquoted and stored at -80°C.

Gel Mobility Shift Assays

The protocol for gel mobility shift assays was modified from Chodosh (1988), Haberstroh *et al.* (1991), Hennighausen and Lubon (1987) and May *et al.* (1989). Assay conditions were optimized in pilot experiments that tested the effects of different compositions of binding buffer, the percentage of polyacrylamide, different running buffers and the concentration of non-specific and specific competitors. The 106 bp cAMP responsive element (CRE) of the *PDE1*-A promoter was used as a

probe for gel shift assays. The probe was prepared by PCR amplification performed with 10 μCi (3000 Ci mmole^{-1}) of ^{32}P-dATP. The labeled DNA was purified by gel electrophoresis. Typically, probes were labeled to a specific activity of ~2×10^{7} cpm μg^{-1} and were at a concentration of ~2 ng μL^{-1}. Standard binding reactions were performed in a 20 μL volume containing 2 ng of labeled DNA, 500-fold weight excess (1 μg) of pBR322 DNA, 5 μg nuclear extract, 1x binding buffer [20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 10 mM DTT, 1 mM EDTA and 5% glycerol] and other competitor DNAs as indicated in the figures. For dephosphorylation of nuclear proteins, 5 μg of nuclear extract was incubated with 2 units of CIAP at 37°C for 45 min. DNA-protein binding reactions were carried out at room temperature for 20 min. The binding reaction was loaded onto a 4% native polyacrylamide gel with 37.5:1 ratio of acrylamide to bisacrylamide. The gel was eletrophoresed in 1x TBE buffer at 200 V for 3 to 4 h in the cold room. Gels were dried onto 3-MM paper under vacuum and prepared for autoradiography.

RESULTS

Establishing Conditions for Dissecting Temporal and cAMP-Mediated Transcriptional Activity

PDE1 gene expression is under the regulation of three distinct promoters specific for vegetative growth, cell aggregation and late development (Faure *et al*., 1990; Hall *et al*., 1993). The aggregation-specific promoter resides within 1.5 kb of 5' upstream noncoding sequences (Faure *et al*., 1990). In the present study, we investigated the *PDE1*-A promoter to identity DNA regulatory sequences that confer temporal and cAMP responsiveness. Temporal responsiveness is defined as the induction of transcription as cells enter development following nutrient depletion. cAMP responsiveness is defined as an increase in expression levels in cAMP-treated relative to untreated developing cells. Transcription directed by the *PDE1*-A promoter begins very early (within 30 min) in response to nutrient depletion, well before cells begin to secrete cAMP. Therefore, it is possible to distinguish effects on transcription mediated by cAMP from effects on transcription triggered by nutrient depletion. To determine conditions that accentuate the difference between temporally- and cAMP-induced promoter activity, transformants containing the reporter vector with the full-length *PDE1*-A promoter were starved in shaken suspension in the presence or absence of 1 mM cAMP and harvested for RNA extraction at intervals throughout the first 6 h of starvation. Total RNA was analyzed for *CAT* reporter gene and endogenous *PDE1* transcripts (Fig. 2).

CAT mRNA was undetectable at the start of starvation (t_0) but was present 30 min later in both cAMP-treated and cAMP-untreated cells. *CAT* mRNA levels increased more rapidly in the cAMP-treated cells, reaching a maximum at ~1.5 h. Without cAMP, *CAT* and *PDE1* mRNA levels increased gradually throughout 6 h of starvation. Longer exposure of this blot revealed that endogenous *PDE1* mRNA levels paralleled those of the *CAT* mRNA, although *PDE1* mRNA was not visible at all time points in the exposure shown in Fig. 2. Based on this analysis, 1.5 h of starvation was chosen as the time point that provided the greatest difference between cAMP-treated and cAMP-untreated cells. In subsequent experiments, the increase in the level of *CAT* mRNA between 0 and 1.5 h of starvation in cultures not treated with cAMP was taken as an indication of temporal induction and the difference between cAMP-treated and cAMP-untreated cells after 1.5 h of starvation was taken as a measure of cAMP-responsiveness.

Promoter Motifs Defined by 5' to 3' Serial Deletions

A set of ten 5' to 3' serial deletion clones was selected for promoter activity analysis (Fig. 3). To eliminate the effects of variable integration position and DNA copy number, populations of transformants were used for the analyses of each construct. RNA was extracted from cells immediately after suspension in buffer and after 1.5 h of starvation in the presence or absence of 1 mM cAMP.

Representative results of a Northern blot for *CAT* and *PDE1* mRNA are shown in Fig. 3. Quantitative analyses of the autoradiograms revealed two deletions that resulted in major declines in temporally-induced promoter activity (Fig. 3 and Fig. 7A). There was a 2-fold reduction associated with removal of the -1162 to -825 region and a 10-fold reduction associated with excision of the -186 to -121 region. Although construct B shown in Fig. 3 appears to significantly reduce promoter activity, normalization to levels of endogenous *PDE1* transcript reveals that its activity is similar to that of construct C to G, or roughly 50% of the full-length control.

cAMP responsiveness dropped by half when the -1162 to -825 region was deleted (Fig. 3 and 7A). Removal of the region between -556 to -454 essentially eliminated induction by cAMP. Deletion to -121 (construct H) resulted in very low and unregulated promoter activity. Construct H contains little more than the putative TATA box. Transcriptional activity was undetectable using construct I that removed the TATA box (Fig. 3 and 7A).

Constructs of 3' to 5' Serial Deletions

The series of 5' deletions could only map the upstream limits of the regulatory elements. To more precisely delineate critical regulatory regions, a set of 3' to 5' deletions was prepared. Deletion between -209 and -121 (construct J, Fig. 4) decreased temporal induction, but had little effect on induction by cAMP (Fig. 7A). Deletion to -412 (construct K) had virtually no additional effect on promoter activity. Further deletion to -548 (construct L) removed the CRE defined in the 5' to 3' series. This reduced but did not eliminate cAMP inducibility (Fig. 4 and 7A). This result suggested that the remaining 5' upstream sequences contain another CRE. The final construct in the 3' to 5' deletion series (M; Δ -745 to -121) severely reduced temporal induction but still retained CRE activity. These results indicated that: (1) there are at least two CREs, one between -556 and -450 and a second within the -1170 to -746 region; (2) the region between -209 and -121 is important for temporal induction and (3) sequences between -746 and -548 augment promoter activity.

Internal Truncations Confirm the Existence of the Proximal CRE

To confirm the existence of the proximal CRE in the -661 to -454 region, two internal truncation constructs were created to eliminate this putative CRE. In construct N (Δ -746 to -454), the promoter activity was only slightly inducible by cAMP. When a smaller region encompassing the CRE was excised (construct O; Δ -548 to -454) cAMP inducibility was eliminated (Fig. 4). These two internal truncations confirm the existence of the CRE within the -547 to -455 region. Unexpectedly, the putative upstream CRE (-1170 to -746) identified by the 3' to 5' deletions did not function in the context of internal truncation clones.

Refining the Limits of the CRE

The results described above indicated the existence of at least one CRE in the -661to -454 region. The ability of this region to function as a CRE out of its normal context was investigated by linking fragments containing all or portions of this region in both normal and inverted orientation to the basal promoter (construct H). Inverting these sequences reduced the effectiveness of the CRE. Fragments -661 to -361 (P), -661 to -450 (Q) and -556 to -450 (S) conferred at least partial cAMP inducibility. In contrast, the region -661 to -519 (R) did not function as a CRE (Fig. 5 and Fig. 7A). These results suggest that the 106-bp (-556/-450) region functions as a core CRE and that the sequences between -661 and -519 augment CRE activity.

The CRE Activates Transcription

Two models can be proposed for the mechanism of CRE activity. One is that the CRE functions as a positive element that activates the *PDE1* gene only when bound by cAMP-activated transcription

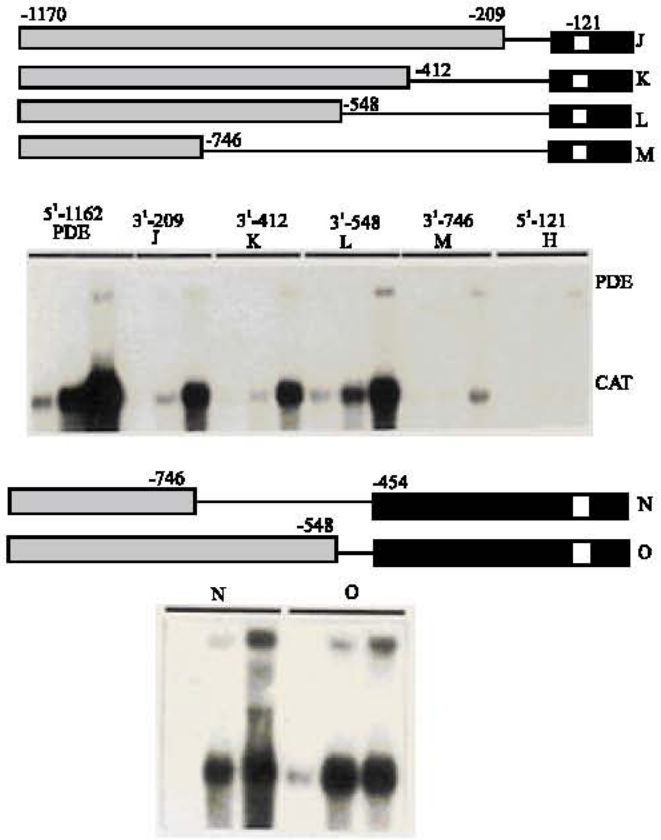

Fig. 4: The 3' to 5' serial deletions of the *PDE1* promoter and their transcriptional activity. Constructs J to M were fused to the basal promoter (construct H). Constructs N and O were fused to construct D, in which the major CRE was deleted. RNAs in each set of three lanes are as described in Fig. 3

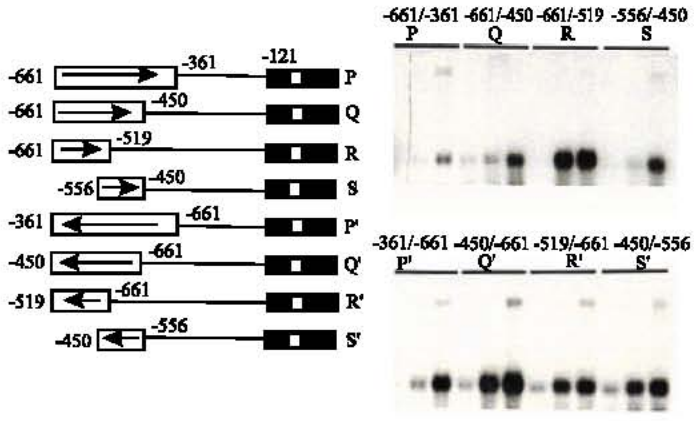

Fig. 5: Transcriptional activities of PCR-amplified *PDE1* upstream region fragments. RNAs in each set of three lanes are as described in Fig. 3

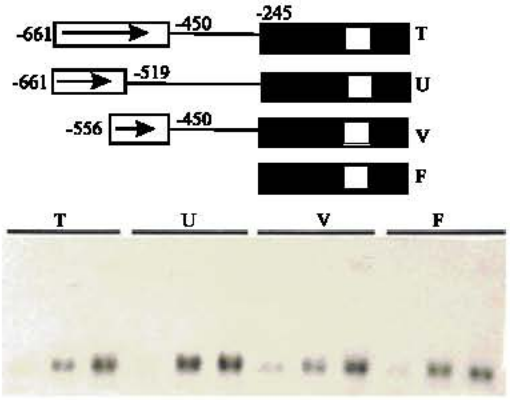

Fig. 6: Test for negative or positive regulation by the CRE. Different fragments containing the CRE were linked to a promoter fragment that allowed temporal control but lacked a CRE (construct F). Each set of three lanes shown is as described in Fig. 3

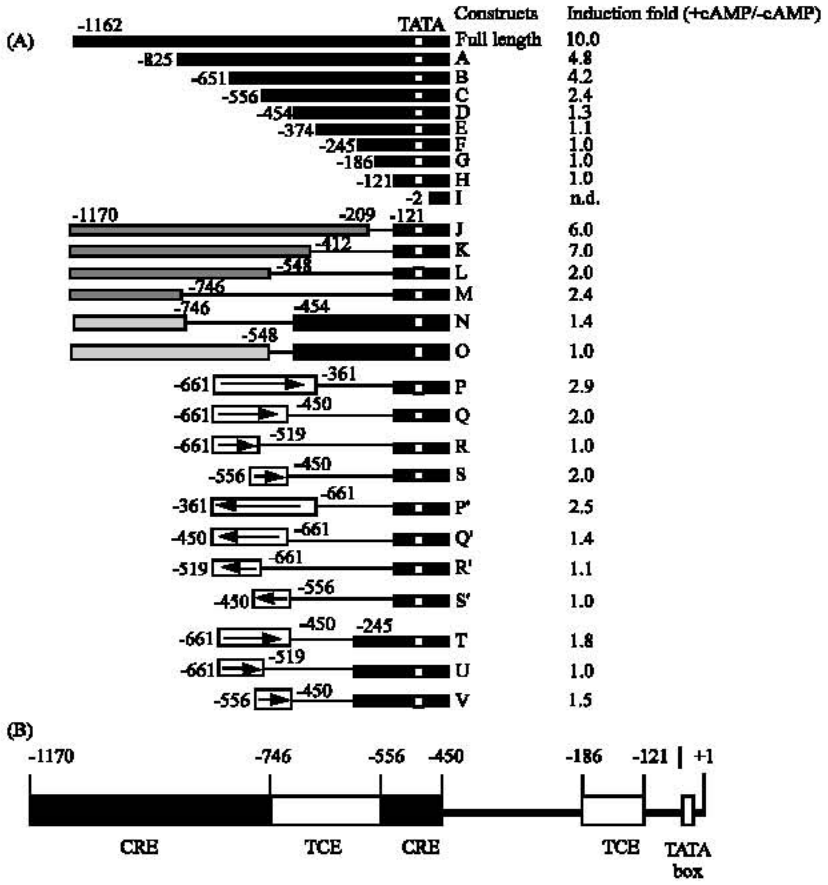

Fig. 7: Summary of the cAMP-inducibility of each construct and the major regulatory regions of the aggregation-specific promoter. (A) Summary of the cAMP-inducibility of each construct. (B) A diagram of the critical regulatory regions within the *PDE1* promoter. The downstream temporal control TCE and CRE elements were more precisely defined than were the upstream regulatory elements

factors. Another model is that cAMP relieves repression mediated by an inhibitory transcription factor. To distinguish between these models, fragments containing the CRE were linked to construct F, which is regulated temporally but not by cAMP (Fig. 3). If the CRE acted as a positive regulator, linking it to construct F should show that (1) in the absence of cAMP, there is no effect on the promoter activity of construct F and (2) in the presence of cAMP, promoter activity is induced. Fusion of fragments included the CRE (-661/-450 construct T and -556/-450 construct V), but not the fragment without the CRE (-662/-519 construct U), yielded results consistent with the model of positive regulation (Fig. 6, 7A and B).

Trans-Acting Factors Interact with the Proximal CRE

Gel mobility shift assays were employed to investigate DNA-binding proteins that interact with the proximal *PDE1* CRE (-556/-450). The -556 to -450 DNA fragment was amplified by PCR and incubated with crude nuclear extracts isolated from vegetative AX3 cells and AX3 cells developing in the presence or absence of cAMP. Two types of cAMP treatment were used in these studies: the continuous high level cAMP exposure used in the deletion analysis and more physiologically realistic

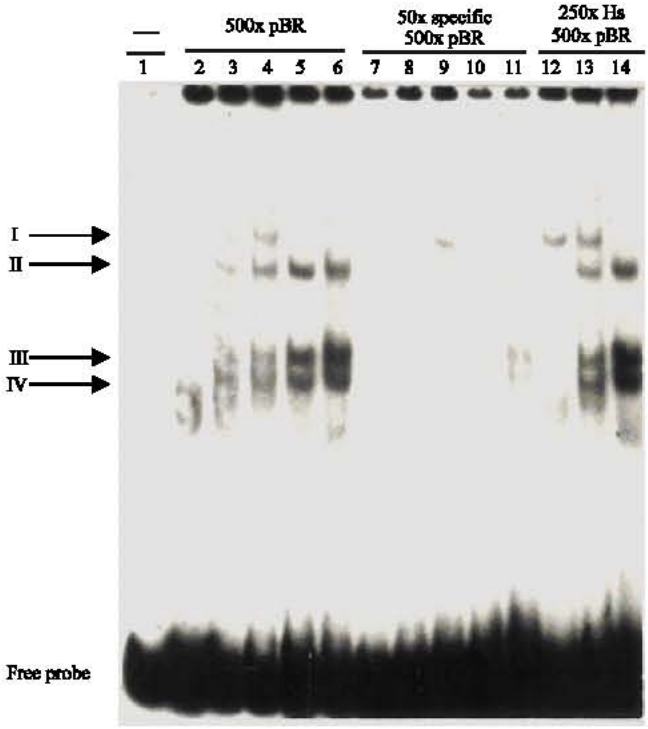

Fig. 8: DNA-protein complex formation as a function of developmental stage and cAMP treatment. Three sets of lanes show gel mobility shift results using extracts from cells starved for 0 h (lanes 2, 7 and 12), 1.5 h (lanes 3 and 8), 1.5 h with 1 mM cAMP (lanes 4, 9, 13), 3 h with cAMP pulses (lanes 5 and 10) and 6 h with cAMP pulses (lanes 6, 11 and 14). The first set of binding assays was performed using a 500-fold weight excess of pBR322 as competitor; the second and third sets were performed with the same amount of pBR322 plus either a 50-fold excess of unlabeled (cold) probe DNA or a 250-fold excess of herring sperm (HS) genomic DNA, respectively

conditions of low concentrations of cAMP delivered in pulses. Under conditions of cAMP pulses, the aggregation-specific promoter is maximally active at 6 h of development (Franke *et al*., 1991). Therefore, the developmental induction of potential DNA-binding factors was examined in cAMP-pulsed cells developed for 0, 3 and 6 h.

Four shifted complexes were detected in extracts from developing cells (Fig. 8). Three of these, marked bands II, III and IV, were effectively competed by a 50-fold excess of cold probe. These three complexes disappeared completely when the excess of specific competitor was increased to 100-fold (data not shown). Competition for binding was sequence-specific, as adding an additional non-specific competitor (herring sperm DNA) with the standard non-specific competitor DNA (pBR322) had no effect on binding. Shifted complex I was detected primarily in extracts of cells starved for 0 and 1.5 h and was not competed by unlabeled specific competitor. It is likely that complex I is due to nonspecific binding. The binding complexes II, III and IV showed that the interaction between the CRE and binding proteins was developmentally regulated as the binding was detected only when the extracts from developing cells were used. The intensity of each band increased during the 6 h course of the experiment. All three bands were detectable by 1.5 h of starvation. Significantly, the intensity of each band at 1.5 h of starvation was increased if cells were exposed to 1 mM cAMP, suggesting that a very rapid synthesis or activation of proteins occurred upon starvation. The appearance of the DNA-protein complexes and CRE activity are tightly correlated.

The Role of Protein Synthesis in the Induction of DNA Binding Activities

The aggregation-stage *PDE1* transcript does not accumulate if starving cells are treated with cycloheximide, an inhibitor of protein synthesis (Franke *et al*., 1987). We found that cycloheximide

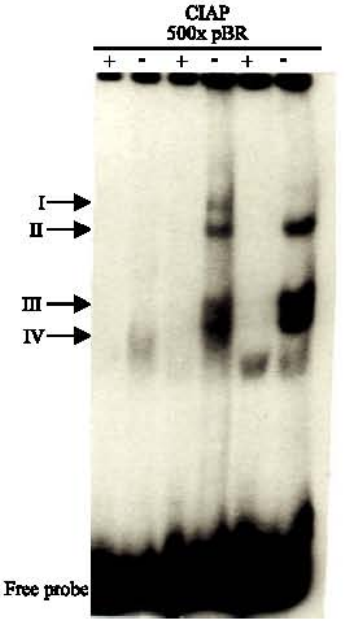

Fig. 9: The effect of phosphatase treatment on DNA-protein interactions. Nuclear extracts prepared from cells starved for 0 and 1.5 h with 1 mM cAMP, or 6 h with pulses of cAMP were treated with 2 units of calf intestine alkaline phosphatase (CIAP) for 45 min at 37°C. + and -denote the presence or absence of CIAP. Binding assays were performed under conditions as described in the text

treatment prevents formation of the DNA-protein complexes (data not shown). These findings indicate that the protein or proteins binding to the *PDE1* CRE must be synthesized as cells enter development.

The Effect of Protein Phosphorylation on DNA Binding

The activity of many transcription factors is regulated by post-translational modifications such as protein phosphorylation. The effect of protein phosphorylation on the interaction of proteins with the *PDE1* CRE was tested by treating nuclear extracts with calf intestine alkaline phosphatase (CIAP). In all cases in which DNA-protein binding was detected (1.5 h starvation with 1 mM cAMP and 6 h starvation with pulses of cAMP), CIAP treatment eliminated the formation of DNA-protein complexes (Fig. 9). This effect is not due to proteolytic degradation of nuclear proteins during incubation, as mock-treated extracts demonstrated strong binding activity. These results suggest an important role for protein phosphorylation in the interaction between the CRE and sequence-specific binding proteins.

DISCUSSION

The full-length *PDE1*-A promoter contains multiple *cis*-acting elements. Two regions between -746 to -556 and -186 to -121 were important for temporal induction as cells enter development. These regions appeared to have overlapping activity as the presence of one or the other allowed temporal induction to occur. The -186 to -121 sequence had an especially strong effect on developmental induction. We did not note any previously reported *Dictyostelium* regulatory motifs within this region. The -746 to -548 region contains CA-rich motifs in the area centered around -620 that are similar to those important for some *Dictyostelium* prespore promoters. If these CA-rich motifs are important in early developmental activation of the *PDE1*-A promoter, they are unlikely to be bound by the same transcription factors that function in later multicellular stages of development when prespore genes become active.

Two regions of the *PDE1*-A promoter functioned as CREs. One CRE was only broadly localized within the -1170 to -746 region. The second CRE was more tightly delimited as lying within 106 nucleotides between -556 and -450. Linking either of these sequences to a basal promoter conferred cAMP inducibility. Both CRE-containing regions also allowed weak temporal induction in the absence of cAMP. A similar organizational scheme involving disperse and redundant regulatory elements that interact to provide full promoter activity occurs in the *SP70* gene (Fosnaugh and Loomis, 1993).

Within the -556 to -450 region that allowed temporal and cAMP-induced transcriptional activation is a sequence that forms one half of an inverted repeat in the regulatory region of the divergently transcribed *impA* and *dia1* genes (Hirose *et al*., 2005). This sequence is TCAAAAAATTC in the *PDE1*-A promoter and TTCAAAAAGTTC (and its adjacent inverted repeat GAATTTTTGAA) in the intragenic region separating *impA* and *diaA*. *impA* is expressed in vegetatively-growing cells and is rapidly downregulated at the onset of development. *diaA* exhibits mirror-image kinetics and is inactive in growing cells and rapidly induced upon the growth-to-development transition. Deletion of a 112 bp region that contains the inverted repeat prevented the transcription of *impA* and *dia1* in both growth and development (Hirose *et al*., 2005). Notably, Hirose *et al*. (2005) reported closely related sequences in the regulatory regions of *cAR1* and *gdt1* (Hirose *et al*., 2005). Similar to *impA* and *dia1*, at the growth-to-development transition, *cAR1* transcription is activated and *gdt1* transcription is downregulated.

In addition to the *impA/dia1*-related motif, a single perfectly symmetric GT-rich motif (TTGTGTGTGTT) occurs within the -556 to -450 region (Fig. 1). A GT-rich motif (GTGTG) is important for cAMP-responsiveness of the *gp80* gene (Desbarats *et al*., 1992) and complementary CA-rich sequences mediate cAMP-responsiveness of many other *Dictyostelium* genes (Esch *et al*., 1992; Fosnaugh and Loomis, 1993; Hjorth *et al*., 1990; Vauti *et al*., 1990).

Three developmentally-induced DNA-protein complexes were detected using the -556/-450 CRE fragment as a probe in gel shift assays. These three different complexes may arise by the binding of three different proteins to three separate DNA binding sites or by binding of a single DNA-binding protein to one site followed by the assembly of additional proteins into a complex.

The control of DNA-protein interactions through protein phosphorylation is a major eukaryotic regulatory mechanism (Yamamoto *et al*., 1988; Jackson *et al*., 1990). We found that protein phosphorylation regulates protein association with the major *PDE*-A CRE. Possible kinases involved in phosphorylating the CRE-binding protein or proteins include protein kinase A (PKA) and ERK2. A PKA knockout mutant reduces the level of *PDE1* mRNA and weakens the gene's response to cAMP (Wu *et al*., 1995) and PKA and ERK2 collaborate in the establishment and control of the cAMP oscillatory circuit (Maeda *et al*., 2004).

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