Development 125, 3689-3698 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV3870

Sequence-specific protein interaction with a transcriptional enhancer involved in the autoregulated expression of cAMP receptor 1 in *Dictyostelium*

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Accepted 24 June; published on WWW 25 August 1998

SUMMARY

Major stages of *Dictyostelium* development are regulated by secreted, extracellular cAMP through activation of a serpentine receptor family. During early development, oscillations of extracellular cAMP mobilize cells for aggregation; later, continuous exposure to higher extracellular cAMP concentrations downregulates early gene expression and promotes cytodifferentiation and cell-specific gene expression. The cAMP receptor 1 gene *CAR1* has two promoters that are differentially responsive to these extracellular cAMP stimuli. The early *CAR1* promoter is induced by nM pulses of cAMP, which in turn are generated by CAR1-dependent activation of adenylyl cyclase (AC). Higher, non-fluctuating concentrations of cAMP will adapt this AC stimulus-response, repress the activated early promoter and induce the dormant late promoter. We now identify a critical element of the pulse-induced *CAR1* promoter and a nuclear factor with sequence-specific interaction. Mutation of four nucleotides within the element prevents both in vitro protein binding and in vivo expression of an otherwise fully active early *CAR1* promoter and multimerization of the wild-type, but not mutant, sequence will confer cAMP regulation to a quiescent heterologous promoter. These *cis* and *trans* elements, thus, constitute a part of the molecular response to the cAMP transmembrane signal cascade that regulates early development of *Dictyostelium*.

Key words: DNA-binding protein, cAMP regulation, Promoter, Receptor, *Dictyostelium*, Transcriptional enhancer

INTRODUCTION

The non-sexual phases of the *Dictyostelium discoideum* life cycle are composed of unicellular growth and multicellular development (see Firtel, 1995; Kimmel and Firtel, 1991; Loomis, 1996; Rogers, 1997; Williams, 1995). The transition from growth to development is triggered by an increase in cell density and a depletion of nutrients. Under these conditions, cells secrete and respond to extracellular cAMP. Early during development, oscillations in extracellular cAMP concentrations control cell migration and the formation of multicellular aggregates. The cAMP oscillations are generated through balanced activities of a sensitized/desensitized receptor-mediated pathway for G protein-dependent adenylyl cyclase stimulation and of a secreted cAMP phosphodiesterase. As development proceeds, extracellular cAMP concentrations increase and differentiated prestalk and prespore cells appear. These cells organize into a specific pattern within the aggregate that can be followed through slug formation and the terminal phases of development (Early et al., 1993; Williams, 1995).

Four 7-span, cell surface receptors for extracellular cAMP, encoded by separate genes (*CAR1*, *CAR2*, *CAR3* and *CAR4*), control cell movement, aggregation, cytodifferentiation, pattern formation and developmentally regulated gene expression (Johnson et al., 1992, 1993; Ginsburg and Kimmel, 1997; Klein et al., 1988; Louis et al., 1994; Saxe et al., 1991a,b, 1993). The receptor subtypes are ~50% identical in amino acid sequence, and have unique binding affinities for cAMP and distinct temporal and cell-specific expression patterns.

CAR1 is expressed at the earliest stages of development and is under multiple modes of regulation. During rapid growth at low cell densities, CAR1 receptors are not detected (Klein et al., 1988; Saxe et al., 1991a,b), but as cell densities increase, low level expression of *CAR1* is initiated (Louis et al., 1993; Rathi et al., 1991; Saxe et al., 1991a,b). The other receptor subtypes do not accumulate to significant levels during this period (Johnson et al., 1993; Louis et al., 1994; Saxe et al., 1993). cAMP binding to CAR1 is required to establish the early events of cAMP signal-relay. Response of CAR1 protein to secreted, oscillating waves of cAMP promotes a 20- to 50-fold induction of CAR1 and other components of the signal-relay machinery (Firtel, 1995; Johnson et al., 1993; Kimmel, 1987; Klein et al., 1987, 1988; Loomis, 1996; Louis et al., 1993; Saxe et al., 1991a,b). Following aggregation and a rise in extracellular cAMP concentrations, *CAR1* mRNA levels decline, although expression continues at reduced levels through culmination

(Johnson et al., 1993; Kimmel, 1987; Klein et al., 1988; Louis et al., 1993; Saxe et al., 1991a,b). There are ~75,000 CAR1 cAMP-binding sites per cell during early development; by culmination, these sites have declined by a factor of 20 (Johnson et al., 1993; Kimmel, 1987; Klein et al., 1987).

The sequences required for expression of CAR1 involve two functionally distinct promoters that initiate transcription at separate sites but encode identical proteins (Louis et al., 1993; Saxe et al., 1991a,b). The early *CAR1* promoter is expressed at low levels during dense growth and is induced to high levels during early development on solid substrata or during differentiation in shaking cultures with nM pulses of cAMP. The early promoter is repressed during aggregation or by continuous exposure to high (>300 μm) concentrations of cAMP. These latter conditions promote CAR1 adaptation, effectively uncoupling the receptor from adenylyl cyclase, and activate the late *CAR1* promoter. The late promoter is expressed at low levels through culmination. Both the early and late *CAR1* mRNAs are preferentially represented in prestalk cells relative to prespore cells,

A transcription factor has been identified that mediates gene activation by a non-fluctuating cAMP stimulus after aggregation (Schnitzler et al., 1994, 1995), but molecular events required for cAMP pulse-induced gene expression during early development have not been well characterized. We have begun an analysis of nuclear elements essential for control of early *CAR1* expression and identified a specific DNA-protein interaction that may underlie part of a mechanism for the autoregulation of *CAR1* gene expression and for initiating and repressing early developmental events through CAR1-mediated transmembrane signalling.

MATERIALS AND METHODS

Growth, development and DNA-mediated transformation of *Dictyostelium*

Ax-3 and G418-resistant cell lines were grown and developed (Louis et al., 1993; Williams et al., 1989) or were differentiated in suspension culture in 10 mM sodium phosphate, pH 6.4, 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ at 2×10^6 cells/ml at 200 revs/minute, conditions that do not support endogenous cAMP signalling (Kimmel, 1987). Cultures received cAMP in ~30 nM pulses at 6 minute intervals or were maintained at >300 μM cAMP (Kimmel, 1987).

Ax-3 cells were transformed and selected for G418-resistance using *lac*Z expression vectors (Louis et al., 1993; Williams et al., 1989). Transformations were confirmed by Southern and/or PCR analyses. Multiple, independent transformants with the same construct were used to confirm consistency of results.

Isolation and hybridization of RNA and DNA

Total RNA was prepared, size separated on formaldehyde/agarose gels and transferred to nitrocellulose (Kimmel, 1987). Probes were radiolabeled by the random primer method using [α-^{32}P]dCTP and hybridized to RNA blots at 37°C in 0.8 M Na^+, 50% formamide (Wahl et al., 1987).

5′-deletion, internal mutant and concatamer constructs

The early *CAR1* promoter/*lac*Z fusion (Louis et al., 1993) was cleaved at the 5′-end of the promoter with *Bam*HI and at the *Kpn*I site, 29 nt further upstream. Digestion with exonuclease III, followed by repair with mung bean nuclease and recircularization, generated a family of 5′-deletions from −1010 that were linked to an identical upstream sequence (Barnes, 1987). Deletion junctions were sequenced by dideoxy chain termination using double-stranded DNA (Mierendorf and Pfeffer, 1987; Sanger et al., 1977).

For Box A and Box B mutants (see Fig. 4), G and C nucleotides were substituted with T and A, respectively. A mutated Box A oligonucleotide primer and a *lac*Z 5′ oligonucleotide were used to construct the −746A promoter, which was ligated into a *lac*Z vector. Sequencing confirmed that −746A only differed from −746 at the 4 nt Box A sites.

Wild-type and mutated Box A GAC-DR oligonucleotides (−743 to −712) with *Bam*HI and *Bgl*II cohesive ends were used for concatamer construction. Individual oligonucleotides were phosphorylated, self-ligated, digested with *Bam*HI and *Bgl*II, resolved with 4% Nusieve agarose gels, and 6-mer DNA fragments purified and cloned into the *Bam*HI site of *Actin15ΔBam*/*lac*Z. The orientation and sequence of the constructs were confirmed by DNA sequencing.

β-galactosidase staining

Nitrocellulose filters with developed structures were fixed with a gentle spray of 1% glutaraldehyde in Z buffer and stained as described (Richardson et al., 1994).

Analysis of proteins by immunoblotting

5×10^7 cells were washed twice in phosphate buffer (14.7 mM KH_2PO_4 and 2 mM Na_2HPO_4) and suspended in 500 μl of the same buffer. After one freeze/thaw cycle and 1 minute vortexing, lysates were centrifuged. Equal amounts of supernatant proteins were mixed with 4× Laemmli buffer, heated (90°C, 4-5 minutes) and analyzed using 10-20% gradient Tricine polyacrylamide gels (Novex Experimental Technology). Immunoblotting was performed using rabbit β-galactosidase polyclonal antiserum (Burnette, 1981).

Preparation of nuclear extracts

10^8 cells were suspended in 1 ml of 25 mM Tris pH 6.0, 5 mM magnesium acetate, 0.5 mM EDTA and 5% sucrose. 200 μl of 20% Nonidet-40 (NP-40) was added and the tube gently mixed. After 5 minutes at 4°C, nuclei were pelleted at 2000 ***g*** for 5 minutes. The pellet was washed in the same buffer containing 4% NP-40. Nuclear extracts were prepared (Gollop and Kimmel, 1997). Protein concentrations were measured (Bio-Rad and Integrated Separation Systems) and extracts adjusted to 3 mg/ml in 50 mM Tris pH 7.9, 50 mM NaCl, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 0.25 mM Zn_2Cl and 0.1 mg/ml PMSF.

DNA-electrophoretic mobility shift assays

Double-stranded wild-type (WT) and mutated (Box A and Box B) oligonucleotides spanning positions −743 to −712 were synthesized (see Figs 1, 4), gel-purified and radiolabeled by fill-in. Assays contained 1 μl of labeled probe (2 fM), 1.6 μl of 6x-binding buffer (100 mM Tris at pH 7.5, 0.25 M NaCl, 50 mM DTT, 5 mM EDTA and 25% glycerol), 1 μl (500 ng) of $(dAT)_n/(dAT)_n$ as non-specific competitor, 2 μl of nuclear extract in a total volume of 10 μl. The reactions were incubated at 4°C, 15 minutes. Competitor DNAs were also filled-in under identical conditions. In competition experiments, nuclear extracts were preincubated for 10 minutes with unlabelled competitor; subsequently, radiolabeled DNA was added and incubated for an additional 10 minutes. The protein-DNA complexes were analyzed by 8% or 10% nondenaturing polyacrylamide (acrylamide/methylbisacrylamide ratio of 29:1) gel electrophoresis with 45 mM Tris borate/1 mM EDTA as running buffer. Gels were prerun for 1 hour at 180 V at 4°C and electrophoresis was allowed to proceed for 3.5 hours at 200 V at 4°C. The gels were dried and autoradiographed. Mobilities of the complexes were compared to controls incubated in the absence of nuclear extracts.

DNase I footprinting

Mobility shifts were performed with a 269 bp *CAR1* fragment, end-

labeled on the 3′ strand at position −756 and extending to −487. The *CAR1*-specific DNA-protein complex and uncomplexed free DNA were located by autoradiography and excised from the gel. The free DNA served as the native DNA control. The gel slices were incubated with various concentrations of DNase I without Ca^{2+} or Mg^{2+} at room temperature for 10 minutes. $MgCl_2$ was added to 2 mM, $CaCl_2$ was added to 5 mM and the reactions were continued for another 2 minutes and then terminated (Papavassilou, 1993). The DNA was eluted, resuspended in DNA sequencing loading buffer and electrophoresed on a 6% denaturing gel. The probe was also subjected to chemical sequencing for G+A sites (Maxam and Gilbert, 1977).

In gel UV-crosslinking

DNA-protein interactions were performed in 50 μl reactions with 4 fM DNA and 0.36 μg/μl protein. Reactions were separated on 2% low melting point agarose gel for 3 hours at 80 volts at 4°C. The gel was irradiated with 300 nm UV light for 30 minutes (Wu et al., 1987). The specific DNA-protein complex was identified by autoradiography, excised, incubated for 5 minutes at 95°C in protein loading buffer and then electrophoresed in a 4-20% SDS-glycine gradient polyacrylamide gel. After electrophoresis, the gel was dried and the labeled protein was visualized by autoradiography.

RESULTS

Sequences required for *CAR1* expression during early development

Early *CAR1* promoter sequences −1010 through +283 directs developmental expression of a fused reporter gene (Louis et al., 1993) and regulates gene induction by 20 nM pulses of cAMP during differentiation in shaking culture and repression by continuous exposure to >300 μM cAMP. We created a series of 5′-deletions from nucleotide −1010 to define upstream elements that regulate *CAR1* expression during early development. Stable *Dictyostelium* transformants containing early *CAR1* promoter/*lac*Z fusions were developed on solid substrata and expression analyzed with northern blots. All the deletions retain the endogenous transcription initiation site; active constructs produce an identical *CAR1*/*lac*Z fusion mRNA and functional β-galactosidase.

5′-promoter deletions from −1010 through −746 show comparable levels of *lac*Z mRNA induction during early development (Fig. 1). Differences observed relate to vector copy number variation within the individual transformants, slight developmental asynchronies among them and/or position effects of the inserts. Deletions beyond −741 had wild-type levels of *lac*Z mRNA during vegetative growth but did not exhibit developmentally induced expression. The sequences within this region and a summary of the data for all the 5′-deletions examined are shown in Fig. 1.

The −746 5′-deletion is the minimal construct regulated as the endogenous gene (Louis et al., 1993; Saxe et al., 1991a,b). Expression (Fig. 2A) during development is induced >20-fold during cAMP signal-relay and aggregation (5 hours) and declines during mound formation (10 hours). Although the early *CAR1*/*lac*Z fusion mRNA is relatively unstable, the resultant β-galactosidase protein (Fig. 2B) and enzymatic activity (data not shown) are more stable and detected during later developmental stages.

Differentiation of *Dictyostelium* in shaking culture with cAMP accelerates early development, with more rapid induction and repression of *CAR1* than in cells developed on solid substrata (Kimmel, 1987; Louis et al., 1993; Mann and Firtel, 1987; Saxe et al., 1991a,b). −746 cells incubated under conditions that do not promote endogenous cAMP signalling (−) did not express *lac*Z (or *CAR1*) mRNA (Fig. 2C) significantly above that during growth (V). Addition of cAMP to 20 nM at 6 minutes intervals (P) promoted high-level expression of *lac*Z (and *CAR1*). Enhanced repression of *lac*Z (and *CAR1*) was observed in cultures adjusted to and maintained at 300 μM cAMP (Fig. 2C).

Spatial expression of the early *CAR1* promoter

Density-fractionated prestalk cells (Ratner and Borth, 1983) exhibited a 3- to 4-fold enrichment of *CAR1* mRNA sequences (Saxe et al., 1991a,b) and, by inference, CAR1 cAMP-binding sites (see Schaap and Spek, 1984) relative to prespore cells. However, these data were inadequate to ascertain the distribution of *CAR1* mRNA among the various subpopulations of prestalk cells (Ratner and Borth, 1983; Williams, 1995). Since β-galactosidase is fully accumulated by ~5 hours of development and is relatively stable (see Fig. 2B), cytological staining for β-galactosidase activity within multicellular aggregates was used to examine the fate of cells that initially expressed *CAR1*/*lac*Z.

Dictyostelium transformants carrying the −1010 and −746 early *CAR1* promoter/*lac*Z fusions were developed on filters and stained in situ for β-galactosidase activity at various developmental stages (Fig. 3). Both constructs exhibited indistinguishable distributions of staining. As aggregation mounds form, prestalk and prespore patterns become apparent. Prestalk A cells differentiate at the periphery of the mound and subsequently spiral inward toward the aggregation center (Ceccarelli et al., 1991; Early et al., 1993, 1995; Williams, 1995). Although all cell types expressed β-galactosidase, there

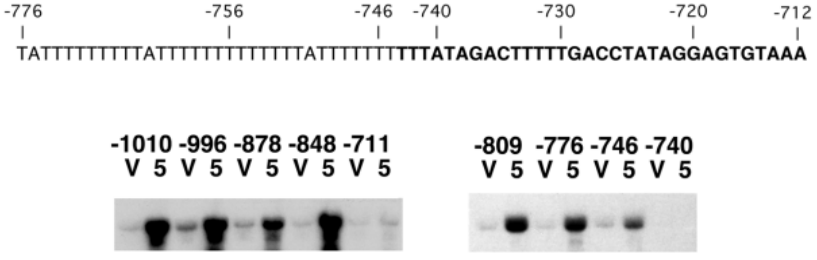

Fig. 1. Expression of *CAR1*/*lac*Z early promoter deletions. The sequence of the *CAR1* early promoter (GenBank accession number L09637) from −776 to −712 is shown. The bold sequence −743 to −712 was used in mobility shift assays to examine sequence-specific protein binding (see Figs 4, 5). Cells carrying promoter-deletion fusions of *CAR1*/*lac*Z were grown in axenic media (V) and synchronously developed on solid substrata for 5 hours. Total RNA was size separated on denaturing gels and blotted for hybridization to *lac*Z. The left panel was deliberately overexposed to show *lac*Z mRNA expression in growing cells. Deletions −1010, −996, −878, −848, −821, −809, −776, −775, −756 and −746 showed identical patterns of *lac*Z induction during development or during differentiation in suspension. Deletions −740, −739, −711, −540, −222, and −101 were developmentally inactive.

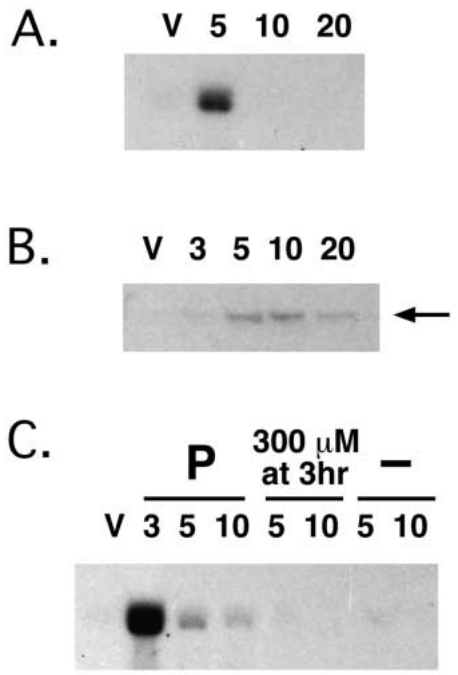

Fig. 2. Expression of –746 *CAR1*/*lac*Z fusion. Cells carrying the –746 promoter-deletion fusion of *CAR1*/*lac*Z were grown in axenic media (V), harvested and washed, and either developed on solid substrata or differentiated in shaking cultures. (A) Developmental expression of *lac*Z mRNA. At times indicated (in hours) cells were collected and total RNA was isolated, size separated on denaturing gels, and blotted for hybridization to *lac*Z. (B) Developmental expression of β-galactosidase protein. At times indicated (in hours) cells were collected and protein extracts were prepared, size separated on denaturing SDS-gels and blotted for reaction to anti-β-galactosidase serum, as indicated with the arrow. (C) cAMP regulated expression of *lac*Z mRNA. Cells were differentiated in suspension culture with pulses of cAMP to 20 nM at 6 minute intervals (P), 300 μM cAMP from 3 hours after pulsing, or no exogenous cAMP (–). At indicated times (in hours) cells were collected and total RNA isolated, size separated on denaturing gels and blotted for hybridization to *lac*Z.

was proportionally more staining at the mound periphery and at the tips of the aggregate and extended (first) fingers (Fig. 3). These data are consistent with enriched expression of *CAR1* in prestalk A cells. As development proceeded this pattern was retained. The anterior prestalk region of the slug (Fig. 3) routinely showed greater β-galactosidase staining than did the posterior region with prespore and anterior-like cells. At culmination β-galactosidase staining can be detected in both stalks and spores (data not shown).

Collectively, the data (see Figs 1-3) indicate that the –746 construct is the most minimal that retains all the elements required for temporally, spatially and cAMP regulated expression of *CAR1* during early development and suggest that a critical regulatory element is located near position –740 of the *CAR1* early promoter.

Specific protein interaction at a GAC direct repeat segment of the early *CAR1* promoter

Sequences –776 through –712 of the early *CAR1* promoter are shown in Fig. 1; *CAR1* sequences –821 to –738 are ~98% A + T (GenBank #L09637; 26). The extended homopolymeric $(dT)_n$ stretches, which are commonly observed in non-protein coding regions of the *Dictyostelium* genome (Kimmel and Firtel, 1983), are largely dispensable, but sequences near –740 may be required to interact with a specific nuclear factor essential for regulated *CAR1* expression during early development.

Nuclear extracts were prepared from *Dictyostelium* during growth (V) and at various developmental stages and were incubated with a radiolabeled, synthetic double-stranded oligonucleotide spanning nucleotides –743 to –712 (see Figs 1, 4). DNA-protein complexes were separated by native polyacrylamide gel electrophoresis and several nucleoprotein complexes were formed with the various nuclear extracts (Fig. 4). Identical mobility patterns were obtained with oligonucleotides that were extended to –746 or truncated to –740 but which also included an additional 5 bp of plasmid sequence that are linked to the promoters in the in vivo expression constructs (data not shown). To identify complexes that exhibited *CAR1*-specific interactions, we examined oligonucleotides carrying mutations at several G+C nucleotide clusters (see Fig. 4; Materials and Methods). The oligonucleotide, with Box A mutations at the GAC direct repeat (positions –737, –735, –729 and –727), is unable to form the predominant, high mobility complex, which we define as *CAR1*-specific. Conversely, mutations in Box B, at downstream G clusters (positions –721, –720, –718 and –716), behave as wild type (Fig. 4). Although multiple *CAR1*-specific complexes are often apparent (see also Figs 5,10), detection of the lower forms

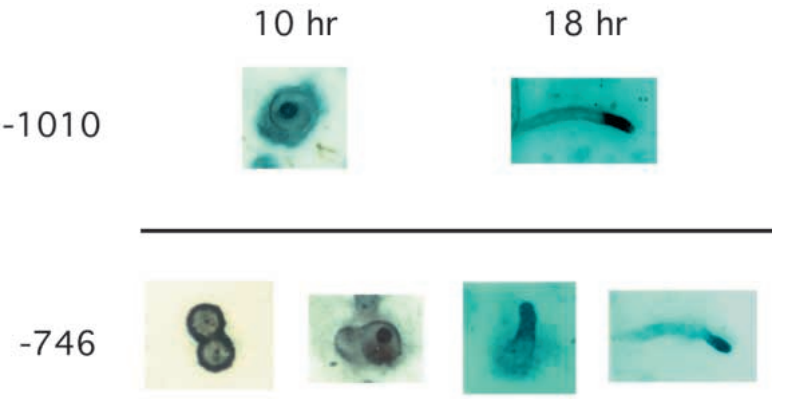

Fig. 3. Spatial expression of full-length (–1010) and –746 *CAR1*/*lac*Z fusions. Cells carrying –1010 or –746 promoter fusions of *CAR1*/*lac*Z were developed, fixed, and stained in situ for β-galactosidase activity. Structures in the lower panel are, from the left, aggregates, tipped mounds, first fingers and slugs (with anteriors to the right).

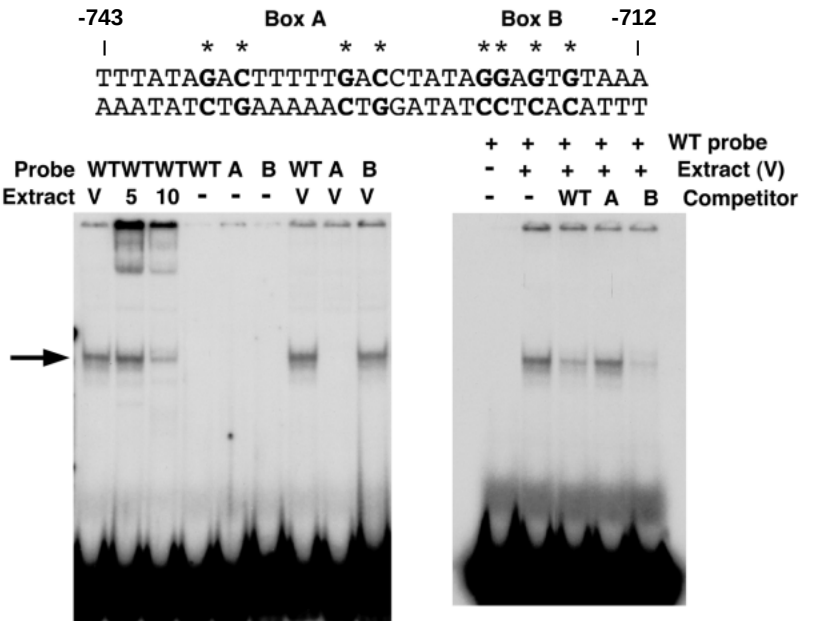

Fig. 4. Sequence-specific, DNA-protein binding within the early *CAR1* promoter. The WT double-stranded oligonucleotide (–743 to –712) was radiolabeled and incubated without nuclear extract (–) or with extracts from cells in growth (V) or at various hours of development. Reactions were separated on native polyacrylamide gels and mobilities of DNA fragments visualized by autoradiography. The predominant *CAR1*-specific complex is indicated. The *CAR1* complexes often migrate heterogeneously, but the lower bands are not as reproducibly observed. Mobility shift assays were also performed with radiolabeled Box A or Box B mutant (*) oligonucleotides (see Materials and Methods). In some cases, reactions included 100-fold molar excess of an unlabeled double-stranded oligonucleotide competitor.

is variable. The complexes at the top of the gel that differ with developmental stage form equally well with wild-type (WT) and either mutant oligonucleotide (see Fig. 5) and also with oligonucleotides unrelated to *CAR1* (see Gollop and Kimmel, 1997), suggesting that these reflect non-specific interactions.

To further define the sites for protein interaction, we performed a series of binding assays using extracts from growing cells preincubated with a 100- to 500-fold molar excess of unlabeled oligonucleotide competitors. Binding competition was only detected for the *CAR1*-specific complexes using WT and mutated Box B oligonuceotides (Fig. 4). When similar experiments were performed using a 500-fold molar excess of mutated Box A oligonucleotide as competitor, no inhibition of complex formation was observed. We also analyzed oligonucleotides containing single-site mutations (either position –737, –735, –729 or –727) in the GAC direct repeat (GAC-DR) of Box A, and none of these were able to form *CAR1*-specific complexes or to compete for protein binding of a WT sequence (data not shown). The mutation studies indicate that the GAC-DR may be a critical target sequence for a potential *CAR1* transcription factor.

While the data localize sequences from –740 to –725 of the early *CAR1* promoter as a potential interacting site for an essential transcription factor, the presence or relative abundance of this binding factor during development does not appear to dictate *CAR1* promoter activity. The *CAR1* early promoter has low activity in growing cells, is induced >20-fold by 5 hours, and is repressed by 10 hours of development (see Figs 1, 2A). Yet, we did not reproducibly observe a significant difference in relative binding activity in extracts prepared from growing or developing cells (Fig. 4). We partly attribute the apparent reduction in *CAR1* DNA-binding activity at 10 hours (see Fig. 4) to a decreased purity in nuclei obtained from multicellular structures in comparison with earlier stages of development. Furthermore, the relative levels of the binding activity did not vary in cells differentiated in suspension culture (Fig. 5) when *CAR1* gene expression (see Fig. 2C) was alternatively induced (lanes B-H) or repressed (lane I) by differential exposure to cAMP. If the nuclear *CAR1* DNA-binding factor were essential for the induction of early *CAR1* expression or involved in its repression, it must function independently of its ability to bind in vitro in a sequence-specific manner (see below).

Fig. 5. Regulation of *CAR1* DNA-binding activity during differentiation in suspension culture. Cells were grown in axenic media (A), harvested, washed, and shaken in culture with pulses (B-H) of cAMP to 20 nM at 6 minute intervals or with 300 μM cAMP after pulsing (I). At indicated times, nuclear extracts were prepared and incubated with radiolabeled WT or mutated Box A double-stranded oligonucleotides (–743 to –712; see Fig. 4). Reactions were separated on native polyacrylamide gels and mobilities of DNA fragments visualized by autoradiography. A, growth; B, 0.5 hour; C, 1.0 hour; D, 1.5 hours; E, 2.0 hours; F, 2.5 hours; G, 3.0 hours; H, 4.0 hours; I, 3 hours of pulses, followed by 3 hours with 300 μM cAMP.

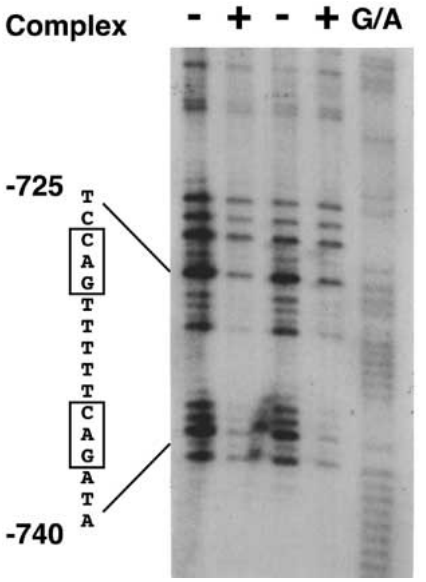

Fig. 6. DNase I protection in the *CAR1* DNA-protein complex. Mobility shift assays were performed using a 269 bp *CAR1* fragment, end-labeled on the 3′ strand at position −756 and extending to −487. The isolated *CAR1* complex (+) and uncomplexed DNA (−) were digested with a concentration series of DNase I and gel fractionated in parallel with chemically sequenced DNA, to align the G+A sites within the protected sequence. Note that the sequence listed (see Fig. 4) is complementary to the radiolabeled strand used to determine G/A alignments.

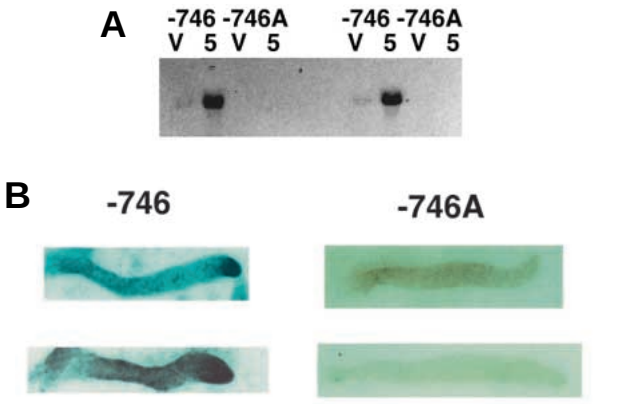

Fig. 7. The −746 mutated Box A promoter is not expressed during development. (A) Cells carrying wild-type (−746) or mutated Box A (−746A) *CAR1*/*lac*Z fusions were grown in axenic media (V), developed synchronously for 5 hours and total RNA isolated. The RNAs were size separated on denaturing gels and blotted for hybridization to *lac*Z. Two independent −746A isolates were compared to wild-type (−746) expression. (B) Cells carrying the −746 or −746A promoter fusions of *CAR1*/*lac*Z were developed, fixed and stained in situ for β-galactosidase activity.

Finally, we mapped some of the nucleotide sites involved in protein interaction within the −756 to −487 region of the early promoter by DNase I protection in isolated *CAR1* nucleoprotein complexes (Fig. 6). We encountered a major difficulty with the extreme A+T bias in the sequence of the element (see Fig. 1; Louis et al., 1993). The $(dT)_n$ and $(dA)_n$ runs were particularly resistant to DNase I digestion in naked DNA, making it impossible to map all potential sites for protein interaction. Nonetheless, by comparing digestion patterns of uncomplexed and complexed DNAs subjected to differing concentrations of DNase I, we see two protected regions centered near the GAC-DR (see Fig. 6) within sequences −748 to −712. The protections differed slightly at the two GAC sites. No other regions exhibited significant protection, nor did we observe DNase I hypersensitive sites.

Mutation of the GAC-DR prevents in vivo expression of the early *CAR1* promoter

Although the exact relationship between the GAC-DR-binding factor and early *CAR1* promoter activity is not fully defined, its activity may be required for transcription during early *Dictyostelium* development. To more directly examine the role of this sequence-specific protein interaction in the regulation of *CAR1*, we constructed the −746A variant of a full-length early *CAR1* promoter/*lac*Z fusion with the Box A mutations that prevent in vitro protein binding (see Figs 4, 5). Several transformants were analyzed for their ability to express *lac*Z mRNA during early development. As observed in Fig. 7, none of the cells carrying this early *CAR1* promoter variant (−746A) expressed *lac*Z mRNA or detectable β-galactosidase activity during development on solid substratum. Thus, mutations that prevent specific in vitro DNA-protein interaction also prevent in vivo expression of an otherwise wild-type promoter.

We have also examined the ability of a concatamerized 32 bp element containing the GAC-DR to activate a heterologous, minimal promoter. The *Act15ΔBam* promoter has been deleted of a central sequence essential for promoter activity, but retains its transcription initiation site. This inactive, minimal promoter has been used successfully to identify regulatory sequences required for cell-specific gene expression in *Dictyostelium* (Kawata et al., 1996; Powell-Coffman et al., 1994). Sequences −743 to −712 containing the wild-type (WT) GAC-DR or Box A mutations were multimerized as 6 direct repeats and inserted into the *Act15ΔBam* promoter in fusion with *lac*Z. As seen, β-galactosidase activity was only detected in mounds containing the WT GAC-DR construct (Fig. 8A). Cells carrying *Act15ΔBam* or *Act15ΔBam* with mutated Box A concatamers had no activity. It should be noted that we could only examine the ability of the *CAR1* GAC-DR promoter element to function in its wild-type orientation. Constructs placed in reverse polarity within *Act15ΔBam* appear to create multiple TATA-like elements and new transcription start sites.

To determine if the GAC-DR element mediates developmentally induced and/or repressed expression, we examined *lac*Z northerns of RNA from cells carrying *Act15ΔBam*/*lac*Z fusion with WT GAC-DR multimers that were differentiated in suspension culture. Two conditions of cAMP incubation were used. In one, differentiation was initiated with 40 nM pulses of cAMP at 6 minute intervals; after 3 hours, cells received a bolus of cAMP to 500 μM to promote later phases of differentiation. In a separate control, differentiation was inhibited by incubating cells with 500 μM cAMP through the entire culture period. As seen in Fig. 8B, growing cells (V) expressed *lacZ* mRNA to significant levels, but when 40 nM pulses of cAMP were added to initiate differentiation, expression was induced several fold beyond

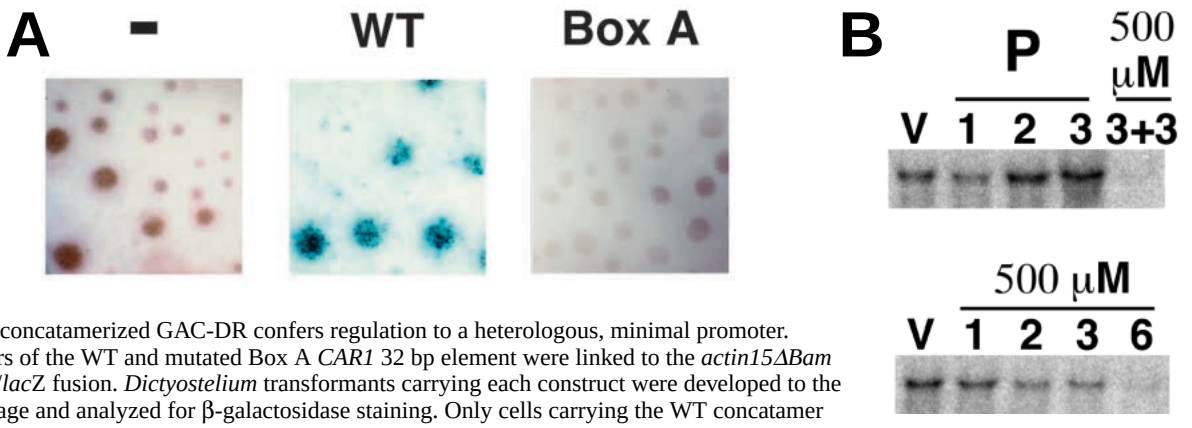

Fig. 8. A concatamerized GAC-DR confers regulation to a heterologous, minimal promoter. (A) 6-mers of the WT and mutated Box A *CAR1* 32 bp element were linked to the *actin15ΔBam* promoter/*lac*Z fusion. *Dictyostelium* transformants carrying each construct were developed to the mound stage and analyzed for β-galactosidase staining. Only cells carrying the WT concatamer expressed *lac*Z. The *actin15ΔBam* promoter (–) and the *actin15ΔBam* promoter fused with a mutated Box A concatamer (Box A) were inactive. Eosin was used as a counter-stain to visualize better these latter structures. (B) A *Dictyostelium* transformant carrying a 6-mer of the WT *CAR1* 32 bp element linked to the *actin15ΔBam* promoter/*lac*Z fusion was grown in axenic media (V), harvested, washed and incubated in suspension culture either with pulses of cAMP to 40 nM at 6 minute intervals (P) for 3 hours, followed by 500 μM cAMP for an additional 3 hours (3+3), or with continuous exposure to 500 μM cAMP. At indicated times (in hours) cells were collected and total RNA isolated, size separated on denaturing gels and blotted for hybridization to *lac*Z.

(~3-5 times, in various experiments). A nearly complete repression was observed within 3 hours after exposure of pulsed cells to 500 μM cAMP, conditions that were also sufficient to repress the endogenous early promoter (see Fig. 2). Very similar patterns of regulated expression was observed for cells developed on solid substrata (not shown). Controls that were continuously exposed to 500 μM cAMP exhibited no *lac*Z induction, only a gradual repression.

These data suggest that the GAC-DR element is involved in the developmentally regulated expression and repression of the early *CAR1* promoter. It should be emphasized that the level of induction observed with this heterologous fusion construct is not comparable to that of endogenous *CAR1*. This may result from the relatively higher levels of expression of the (GAC-DR)$_6$*Act15ΔBam*/*lacZ* fusion in growing cells and reflect an increased sensitivity of a concatamerized GAC-DR promoter, in contrast to the endogenous *CAR1* promoter with only a single GAC-DR element.

The CAR1 GAC-DR-binding factor is ~40 kDa with zinc-dependent activity

We have made a preliminary estimate of the molecular mass of the CAR1 (GAC-DR) factor and partially characterized its binding properties. Radiolabeled WT and mutated Box A oligonucleotides were incubated with nuclear extracts, electrophoresed in native agarose gels and subjected to UV cross-linking. The region of the gels corresponding to the major *CAR1*-specific nucleoprotein complex was excised and the WT and Box A samples were then size separated on SDS gels and DNA-protein complexes identified by autoradiography. As seen in Fig. 9, the WT DNA/protein band migrated with an apparent mobility of ~60 kDa. Size correction for the DNA component predicts a *CAR1* GAC-DR-binding protein of ~40 kDa. No specific band is observed for the mutated Box A sample, which had been treated identically.

Finally, in the initial steps to purify this *CAR1* DNA-binding component, we observed that its activity was acutely sensitive to dialysis against 1 mM EDTA (Fig. 10). Binding activity was restored with 1 mM $ZnCl_2$ but not with several other divalent cations, suggesting that the *CAR1*-binding factor is likely to form a 'zinc-finger'-dependent interaction with DNA.

DISCUSSION

The initial step required for the regulation of genes by extracellular cAMP in *Dictyostelium* occurs through receptor interaction at the cell surface (Gomer et al, 1986; Haribabu and Dottin, 1986; Kimmel, 1987; Oyama and Blumberg, 1986; Schaap and Van Driel, 1985). There is, however, a diversity of receptor-mediated intracellular events. Some, including G protein-dependent activations of adenylyl cyclase, guanylyl cyclase and phospholipase C, are adaptive and responsive to cAMP oscillations (Drayer and van Haastert, 1992; Johnson et al., 1992; Klein et al., 1985; Kuwayama et al., 1993), whereas others (e.g. post-aggregation gene expression) may be stimulated by a non-varying administration of cAMP and function independently of an apparent association with G proteins (Chen et al., 1996; Gomer et al, 1986; Haribabu and Dottin, 1986; Kimmel, 1987; Oyama and Blumberg, 1986; Schaap and Van Driel, 1985; Schnitzler et al., 1995).

The regulation of *CAR1* through the differential activation

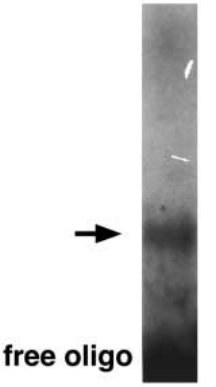

Fig. 9. Approximate molecular mass of the *CAR1* GAC-DR-binding protein. Mobility shifts were performed with radiolabeled WT and Box A oligonucleotides. After exposure to UV, the gel region with the *CAR1* complex was excised and re-fractionated on SDS gels relative to protein markers. The *CAR1* complex has a mobility corresponding to ~60 kDa (arrow). The protein portion is estimated at 40 kDa. A majority of the WT oligonucleotide is not cross-linked with protein and migrates as free probe.

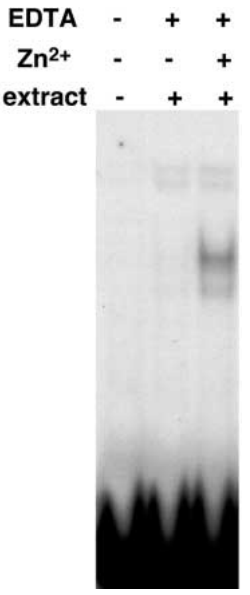

Fig. 10. Zinc-dependent protein interaction with *CAR1* DNA. The WT double-stranded oligonucleotide (−743 to −712) was radiolabeled and incubated without nuclear extract, with extract dialyzed against buffer with 1 mM EDTA, or with EDTA-dialyzed extract supplemented with 1 mM $ZnCl_2$. Reactions were separated on native polyacrylamide gels and mobilities of DNA fragments visualized by autoradiography.

of distinct promoters serves as a paradigm for the major transitions in gene expression observed during the development of *Dictyostelium* (Louis et al., 1993). The analyses of the 5′-deletions and internal mutations within the early *CAR1* promoter indicate that sequences between −740 and −720 relative to transcription initiation are required for the temporally, spatially and cAMP-regulated expression of *CAR1* in vivo and the interaction with an essential regulatory nuclear factor in vitro. In particular, the imperfect, direct repeat involving the two GACs may be a specific binding target. Although the multimerized GAC-DR element confers pulse-regulated expression and cAMP-dependent repression on a heterologous, minimal promoter, the extent of induction by cAMP pulses does not precisely match that of endogenous *CAR1*. Basal (growth) expression appears elevated with the multlmerized construct and other sequences or sequence context within the full-length promoter could also contribute a regulatory function.

The GAC-DR sequence shares some homology with a consensus motif for retinoid receptor-type binding (Minucii and Ozato, 1996), but we have been unable to confirm any direct relationship with the *CAR* motif and RXR-like activity in *Dictyostelium* using mobility shift assays and specific oligonucleotides (A. R. K. and M. Lazar, unpublished). The GAC-binding site, as a direct repeat, suggests protein dimer formation in DNA binding, but we have also not been able to determine a simple cooperative event using crude extracts. Although both GAC sites are essential, DNase I foot-prints indicate slightly different interactions at the two GAC sites. A single protein with two DNA-binding sites, possibly zinc-finger-like motifs, could recognize both GAC sites too.

Cells that do not express the early *CAR1* promoter still have GAC-DR-binding activity. The GAC-DR-binding protein may be constitutively expressed, but its function may be acutely sensitive to a CAR1-mediated intracellular signalling cascade. During growth or late development, when pulsed cAMP signalling does not occur, the GAC-DR-binding protein may be quiescent or an essential developmentally regulated cofactor may not be expressed. Recently it has been shown that paired G-Box sequences and their specific binding factor GBF are required for expression at high levels of *Dictyostelium* promoters induced after aggregation (Ceccarelli et al., 1992; Gollop and Kimmel, 1997; Haberstroh et al, 1991; Hjorth et al., 1990; Schnitzler et al., 1994, 1995). A pair of G-Boxes appears to be required for the expression of the late *CAR1* promoter (R. Gollop, J. M. L., B. L., and A. R. K., unpublished) and cells that lack GBF accumulate cAMP and repress the early promoter, but fail to induce the late promoter (Schnitzler et al., 1994). Early *CAR1* promoter repression is, thus, not dependent upon GBF or a coordinate induction of an alternative promoter, but appears directly related to alterations in intracellular signalling and may be mediated by an activity change in the GAC-DR-binding protein.

We have not been able to determine if the apparent, multiple *CAR1* DNA-protein complexes represent interactions with distinct factors. The relative distribution of *CAR1* DNA-protein complexes with different mobilities varied with extract preparation and did not reflect the transcriptional state of the *CAR1* locus. Although the DNase I foot-prints of major and minor *CAR1*-specific complexes appear to be identical, the apparent sizes of their associated proteins may differ (data not shown). While this could result from post-translational modification, we have not been able to interconvert the two forms or to alter DNA binding in vitro by treatment with phosphatase. The size diversity could likely result from small proteolytic differences during extract preparation that do not alter DNA-binding specificity.

The properties of the nuclear factor(s) that recognizes the selective sequence within the early *CAR1* promoter suggest that it may be a transcriptional effector of other, similarly regulated genes (Desbarats et al., 1992; Franke et al, 1991; Maniak and Nellen, 1990; Mann and Firtel, 1987, 1989). The essential elements in these genes have not been entirely characterized. It will be very interesting to compare their transcriptional regulatory components with those of *CAR1* and to understand the various pathways activated through pulse stimulation of the cAMP receptors. A detailed analysis of all of the *CAR1*-specific binding activities will resolve these questions and perhaps indicate the shared cAMP receptor-mediated signal transduction pathways that regulate gene expression during early development.

Finally, an additional, significant observation arises from analysis of the spatial localization of *CAR1* expression (see Fig. 3). *CAR1* is expressed at highest levels in the prestalk A cells that differentiate at the periphery of the mound and are fated to sort to the tip of the aggregate. It has been suggested that these cells are the most chemotactic to cAMP (Early et al., 1995). In our continuing studies on the function of the CAR gene family, we are interested to determine if these high levels of CAR1 are required for sorting and chemotactic response, or if cells that are fated to enter the tip and become the post-aggregation centers of cAMP signalling, specifically require elevated levels of CAR1.

We are most appreciative of the technical assistance and advice of Ms G. T. Ginsburg, of the *Act15ΔBam* /*lac*Z cells from Dr Peter Balint-Kurti, and of the many helpful discussions with Drs A. Dean, R. Gollop, R. A. Firtel, M. Lazar, S. Millar, C.L. Saxe, P.L. Schwartzberg, R. T. Simpson and A. Wolffe.

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