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Identification of a *cis*-Acting Element Controlling Induction of Early Gene Expression in *Dictyostelium discoideum*

THOMAS MAY, HEDI KERN, ANNETTE MÜLLER-TAUBENBERGER, AND WOLFGANG NELLEN*

Max-Planck-Institut für Biochemie, 8033 Martinsried, Federal Republic of Germany

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Using a new promoter analysis transformation vector for *Dictyostelium discoideum* (PAV-CAT), we have defined *cis*-acting elements in the promoter of the cyclic AMP-induced early-expressed gene A11H2, which encodes an α-fucosidase-related protein (A. Müller-Taubenberger, M. Westphal, A. Noegel, and G. Gerisch, FEBS Lett. 246:185–192, 1989). Sequences responsible for developmentally regulated gene induction could be separated from the basal promoter that conferred low levels of transcriptional activity. By gel shift experiments, we present evidence that the *cis*-acting element is the target of a *trans*-acting factor that by itself is subject to developmental regulation.

Developmental regulation of gene expression has been subject to extensive investigations in recent years. The lower eucaryote *Dictyostelium discoideum* is a relatively simple system in which to study these processes: upon starvation, cells enter a developmental cycle accompanied by morphological differentiation and by dramatic alterations in the pattern of gene expression. With the onset of development, some sets of genes are activated (9) and others become repressed (2). Components involved in triggering these developmental changes have been identified: deprivation of specific amino acids can induce the starvation response; PSF, a factor apparently measuring the cell density-to-food source ratio, leads to the activation of early developmental genes (5); and cyclic AMP (cAMP) is known to influence gene expression via a cell surface cAMP receptor (10, 12, 28). How these signals are finally transduced to the level of gene expression is, however, not understood.

Promoter elements related to cAMP responsiveness and to temporal regulation have been proposed (7, 8, 29) for CP2 (Pst Cath), a gene induced by cAMP in late development, and for Dd*ras* (30). Until now it was, however, not possible to delete the *cis*-acting sequences without losing all promoter activity. This fact suggested that the basal promoter and control elements subject to developmental regulation are very closely spaced or even reside in the same DNA fragment.

The *Dictyostelium* A11H2 gene, which encodes an α-fucosidase, is strictly regulated during development: on the RNA level, expression is not detectable in vegetative cells; induction is observed at about 4 h of development, and maximal amounts of the message are seen at 6 h. Apparently, cAMP is involved in the differential regulation of the gene (21). Because of the strong developmental induction, we have used the A11H2 promoter to investigate the regulation of early gene expression in *D. discoideum*. Our goal was to identify *cis*-acting sequence elements responding to signals involved in the control of early development. This determination would provide the prerequisites for the isolation of the corresponding *trans*-acting factors and could ultimately unravel the pathway of an external signal to the level of transcriptional gene regulation.

Using a new promoter analysis vector for *D. discoideum* that allows for sensitive measurements of promoter activity, we present the identification of a short DNA sequence necessary for temporal regulation of early gene expression but not for basic levels of transcription. We also show that this element specifically binds a *trans*-acting factor from developing but not from vegetative cells, suggesting that this regulatory protein itself is subject to developmental regulation.

MATERIALS AND METHODS

Construction of the promoter analysis vector. For the construction of the transformation vector PAV-CAT, the actin 8 terminator fragment (23) as described by Witke et al. (34) was cut from pIC20H (19) with *Hin*dIII and *Xho*I. The *Xho*I site was blunt ended with S1 nuclease, and the fragment was directionally cloned into the blunted *Pst*I site and the *Hin*dIII site of pGem4Z (Promega Biotec), resulting in plasmid Gem4Z-A8T, containing the terminator fragment in duplicate and in the correct orientation for the later constructions. The actin 15-Tn*903* cassette from vector NeoIT (16) was cut out with *Xba*I, blunt ended with S1 nuclease, and cloned into the blunt-ended *Hin*dIII site of Gem4Z-A8T. This construct, Gem4Z-A8T-A15Tn903, was digested with *Sal*I, blunt ended with S1 nuclease, and used to insert the blunt-ended *Hin*dIII chloramphenicol acetyltransferase (CAT) cassette (Pharmacia, Inc.). The correct orientation of the CAT gene was determined by digestion with *Eco*RI. This final construct was named PAV-CAT I. Ends of the 567-base-pair (bp) *Mbo*II fragment from the genomic subclone A11-1200 (see below) were repaired with T4 polymerase and ligated into the blunt-ended *Eco*RI and *Sal*I sites of pIC20H. The fragment was then cut out with *Hin*dIII, and ends were repaired with Klenow fragment and cloned into the blunt-ended *Xba*I site of PAV-CAT I. The orientation of the promoter fragment was determined by digestion with *Bam*HI and *Pst*I. This construction resulted in the correct reading frame between the short coding region contained on the promoter fragment and the CAT gene. The vector was named PAV-CAT I-A11H2; it is also referred to hereafter as del. −567.

For easier handling and more general application, we inserted additional restriction sites in PAV-CAT I. A representative of this vector family, PAV-CAT II, is shown in Fig. 1. The general features are (1) the Tn*903* cassette as a selectable marker in *D. discoideum*; (2) origin of replication and ampicillin resistance for cloning in *Escherichia coli*; (3) the CAT gene as a reporter for promoter activity; (4) a multiple cloning site adjacent to the reporter gene for the

* Corresponding author.

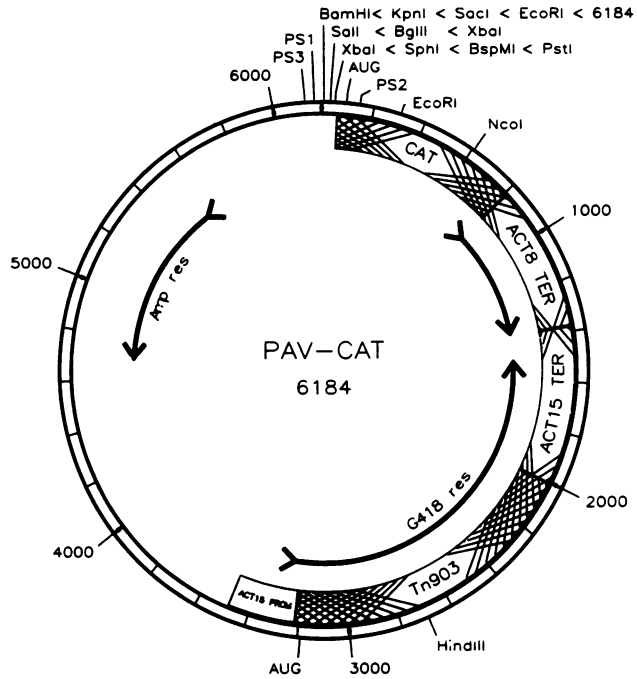

FIG. 1. Map showing the essential parts of the PAV-CAT II vector. Abbreviations: Act8 Ter, actin 8 terminator, duplicate fragment in tandem; Act15 Prom, actin 15 promoter; Act15 Ter, actin 15 terminator; Tn903, neomycin phosphotransferase I gene (arrows indicate orientations of the genes); P1 (SP6 promoter) and P3, priming sites for plasmid sequencing to determine deletion breakpoints; P2, priming site for plasmid sequencing to determine the gene-promoter junction. AUG initiation codons for the CAT and Tn*903* genes are indicated. The sequence of the actin 8 terminator is not entirely known; therefore, the size of the vector may not be exactly 6,184 bp.

insertion of promoter fragments, introduction of unidirectional deletions, and reading frame adjustment; (5) the SP6 promoter as a site for annealing a commercially available primer to determine deletion break points by plasmid sequencing; and (6) use of a primer complementary to the CAT gene to confirm the reading frame. Sequencing of PAV-CAT vectors can be done with miniprep DNA (1) purified by one additional phenol-chloroform extraction.

Identification and subcloning of A11H2 promoter fragments. The genomic lambda clone (21) was cut with *Sal*I and *Eco*RI, and the resulting fragments were cloned into pGEM3Z. Subclones were probed with upstream and downstream fragments of the A11H2 cDNA clone (9), and an approximately 1,200-bp fragment hybridizing only with the 5′ fragment of the cDNA was further analyzed (clone A11-1200). A 567-bp *Mbo*II subfragment was cloned into PAV-CAT I (see above) and used for promoter analysis.

The 178-bp *Ssp*I fragment (A11-178; positions −380 to −202; see Fig. 3) was subcloned into pIC20H, a vector containing a polylinker flanked by *Hin*dIII sites (19), and used for gel shift experiments.

Cell growth and development. Cells of the axenic strain AX2 (clone 214) were grown in AX2 medium (33). Transformed strains were always kept in the presence of 10 to 20 μg of G418 per ml. For development, cells were harvested at a density of 2×10^6, washed in 17 mM Soerensen buffer (Na_2H-KH_2PO_4), and shaken in Soerensen buffer at a density of 10^7/ml. Pulsing was done by adding cAMP to a concentration of 20 nM in 6-min intervals, using a Braun Perfusor VI.

Transformations. Transformations were generally done by the standard procedure (25), with modifications described previously (24). Single clones were picked from the transformation plate, grown in 24-well microdilution plates, and recloned on a lawn of *E. coli* B/2. For each deletion, several independent clones were analyzed for CAT activity.

CAT assays. CAT assays were carried out as described by Gorman et al. (11), with minor modifications: cells were harvested and washed in 100 mM Tris (pH 7.8). The cell pellet was resuspended at 10^8 cells per ml of 100 mM Tris (pH 7.8), and cells were lysed by three cycles of freezing (dry ice) and thawing (65°C). Cell debris was spun down, and the supernatant was used for the enzyme assay. For this assay, 30 μl of the lysate was combined with 10 μl of Tris (pH 7.8) and 1 μCi of [^{14}C]chloramphenicol (Amersham Corp.) and preincubated for 5 min at 37°C; 20 μl of acetyl coenzyme A (4 mM) was added, and incubation was continued for an additional 30 min. The reaction products were analyzed by thin-layer chromatography.

For quantitative evaluation of CAT activity, the scintillation assay described by Neumann et al. (27) was used. Extracts were mixed with 0.1 μCi of [^{14}C]butyryl coenzyme A (Dupont, NEN Research Products) and 15 μl of chloramphenicol (15 mM) and overlaid with 4 ml of Econofluor (Dupont, NEN) in a scintillation vial. Background was determined immediately after the reaction was set up on ice, and the preparation was incubated at 37°C; conversion of chloramphenicol to butyryl chloramphenicol was measured as ^{14}C counts per minute by scintillation counting in 10-min intervals. Plotting of counts per minute versus time on a linear scale gave a measure for the velocity of the reaction and thus for the concentration of enzyme in the extract.

For experimental convenience and since the overall protein content of *Dictyostelium* cells decreases during development, extracts for CAT assays were standardized on equal numbers of cells and not on equal amounts of protein. Equal samples of vegetatively growing cells were washed and developed as described above or immediately processed for extracts. This procedure ensured that at least within one transformed strain, enzymatic activities under different developmental conditions could be reliably compared.

DNA-protein-binding experiments. For gel shift assays, 10^9 cells were harvested at a density of 1×10^6 to 2×10^6/ml, washed, and suspended in 15 ml of ice-cold 0.4 STK (0.4 M sucrose, 10 mM Tris [pH 7.5], 70 mM KCl). The suspension was forced through one and then two layers of Nuclepore filters (pore size, 5 μm). The sucrose concentration was adjusted to 0.9 M with 2.5 STK (2.5 M sucrose); the suspension was layered over 1.1 STK (1.1 M sucrose) and centrifuged for 30 min at 4,000 rpm and 2°C. The pellet was resuspended in 0.4 STK, the sucrose concentration was adjusted to 1.5 M with 2.5 STK, and the suspension was layered over a cushion of 1.7 STK. After centrifugation for 1 h at 72,000 × *g* and 2°C in a Kontron TST 28 rotor, the pellet was suspended in 1.5 ml of NE-500 (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 1.5 mM $MgCl_2$, 500 mM NaCl, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol) and kept at room temperature for 10 min to extract nuclear proteins. Nuclei were spun down for 10 min in an Eppendorf centrifuge at 4°C. The supernatant (crude extract) was immediately used for binding experiments, or proteins were stepwise precipitated with $(NH_4)_2SO_4$. Precipitation was done for 10 min on ice, followed by spinning at 9,000 rpm in a Sorvall HB4 rotor. Pellets were resuspended in NE-0 (NE-500 without NaCl) and passed over a Sephadex G-25 spin column. The flowthrough was supplemented with 1/10 volume of TKMM

(20 mM Tris [pH 7], 20 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol). Extracts were either quick-frozen in liquid nitrogen and stored at −70°C or used immediately for gel shift assays.

Binding reactions were set up with 20 μl of 2× binding buffer (10 mM Tris [pH 8], 20 mM $MgCl_2$, 200 mM NaCl, 0.1 mM EDTA, 6 mM β-mercaptoethanol), 5 to 10 μl of extract, 3 μl of ^{32}P-labeled DNA (2 ng), competitor DNA as indicated, and water to a final volume of 40 μl. The reaction was carried out at room temperature for 30 min, glycerol was added to a final concentration of 25%, and samples were loaded on a 5% acrylamide gel (acrylamide-bisacrylamide ratio, 55:1) in 750 mM Tris (pH 8.8). Electrophoresis was performed overnight at 4°C in 385 mM glycine-50 mM Tris (pH 8.8). Voltage was limited to 150 V, and amperage was limited to 25 mA. Gels were dried on Whatman 3MM paper on a vacuum gel dryer and exposed to X-ray film for 4 to 15 h. As target DNA, fragment A11-178 and a synthetic double-stranded oligonucleotide comprising the sequence from −380 to −324 were used. As nonspecific competitors, poly(dI-dC) (Sigma Chemical Co.), *Sau*3A-digested pBR322, *Sau*3A-digested pIC20H, and a synthetic double-stranded 43-bp oligonucleotide of the sequence AATTCCTTATTT TGGATTGAAGCCAATATGATAATGAGGGATC were used.

Other molecular methods. Restriction enzymes and DNA-modifying enzymes were used as suggested by the manufacturers. Exonuclease III deletions were introduced by using the Promega-Biotec Erase-A-Base system.

Nuclear run-on reactions were carried out as described previously (22). A 900-bp actin cDNA fragment, a 800-bp fragment containing the CAT gene coding region (d), and a 1,100-bp genomic fragment from the A11H2 gene sharing 130-bp homology with the A11H2-CAT fusion gene transcript (u) were separated on an agarose gel and transferred to Biodyne A. Hybridization (16 h, 50% formamide, 37°C) was carried out with run-on transcripts generated by 10^8 nuclei from vegetative and developing cells, respectively; filters were washed in 0.2× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and exposed on X-ray film for 20 h.

Sequencing was done by the chain termination method (31) as modified for double-stranded DNA (3). For sequencing the deletion breakpoints, the SP6 promoter primer (Promega-Biotec) was used; for sequencing the promoter-CAT junction, a primer complementary to 24 nucleotides of the nontranscribed strand of the CAT gene was synthesized. RNA was isolated by the method of Chomczynski and Sacchi (4), with minor modifications (18). Northern (RNA) blots were prepared as described earlier (22). However, ethidium bromide was added to the sample buffer at a concentration of 0.1 μg/ml, and gels were blotted to Biodyne A (Pall) with a vacuum device at 40- to 60-cm water column, using a Pipetus pipette pump. RNA was cross-linked to the membrane by UV irradiation for 180 s on a Herolab UVT 2040 lamp. In vitro-synthesized SP6 or T7 transcripts (22) were used as probes. Hybridization was carried out in 50% formamide at 55°C.

RESULTS

The A11H2-CAT fusion gene is developmentally induced like the endogenous A11H2 gene. Accumulation of A11H2 mRNA could first be detected on Northern blots after 4 h of development. Expression levels reached a peak at 6 h and then slowly declined during the later developmental stages (21). As determined by run-on transcription assays, the A11H2 gene is regulated on the transcriptional level. There are no detectable nascent RNAs in nuclei of vegetative cells, but the gene is activated in the early hours of development (A. Müller-Taubenberger, unpublished results; S. Bozzaro, personal communication). To test whether the A11H2-CAT fusion gene in transformants was regulated similarly to the endogenous gene, we performed run-on assays with nuclei from cells transformed with the del. −567 construct (see below). In nuclei isolated from vegetative cells, no transcripts could be detected, whereas after 6 h of development, RNA hybridizing to the CAT probe was present in the nuclei. In contrast, actin mRNA was transcribed at high levels in vegetative cells and at lower levels in developing cells, as described earlier (20; Fig. 2A). On Northern blots, a similar pattern of expression was seen: both the endogenous A11H2 transcripts and the CAT mRNA derived from the gene fusion could be detected in developing cells but not during vegetative growth. Pulsatile addition of cAMP to developing cells had no additional stimulatory effect, since apparently strain AX2 produces sufficient endogenous cAMP (21; Fig. 2B). To determine whether this regulation was also reflected in the level of CAT activity, enzyme assays were carried out with extracts from vegetative and developed del. −540 transformants. Figure 2C shows the induction of enzyme activity. Surprisingly, however, vegetative cells expressed the gene at levels well above background. We assume that this apparent discrepancy with the results of run-on experiments and Northern blots is attributable to very low level transcription, high sensitivity of the assay, and high stability of the enzyme in vegetative cells (see Discussion). Nevertheless, these data show that transcriptional regulation was reflected on the level of CAT enzyme activity and that the del. −540 construct contained all of the information necessary for proper developmental induction of the gene.

Structure of the A11H2 gene promoter. The primary structure of the promoter was determined by sequencing different restriction fragments and all of the deletion constructs shown in Fig. 3. Like other *Dictyostelium* promoters, the sequence is very A+T rich. A TATAAA box is positioned at −150 (upstream from the ATG), and several G-rich elements, previously shown to be involved in gene regulation (6, 7, 26, 29) are located within the promoter sequence; the one at −354 is very similar to the upstream activating sequence in the actin 6 and actin 15 genes, and the G box at −286 is reminiscent of a promoter element found in the Pst Cath gene. In contrast to other *Dictyostelium* promoters, there is no T run (15) close to the transcriptional start site (21), and the 5′ untranslated region is unusually rich in G and C residues. The 5′ end of the fragment containing the promoter displays an open reading frame terminated by a TAA stop codon and the AATAAA sequence that is used as a polyadenylation signal in most other eucaryotes. This finding suggests that the fragment contains the end of a closely spaced neighboring gene transcribed in the same orientation.

Essential promoter elements are located between −370 and −238. PAV-CAT-A11H2 deletion vectors were transformed into *D. discoideum* AX2 cells, and CAT assays were performed with extracts from isolated, stably transformed clones. Samples were always processed in parallel to minimize experimental variations. Enzyme activities in cells transformed with representative deletion constructs are shown in Fig. 4. In each case, expression in vegetative cells was compared with expression after 6 h of development. Since position effects of vector integration into the genome and variations in copy number cannot be excluded, we did not attempt to quantitatively compare expression levels in

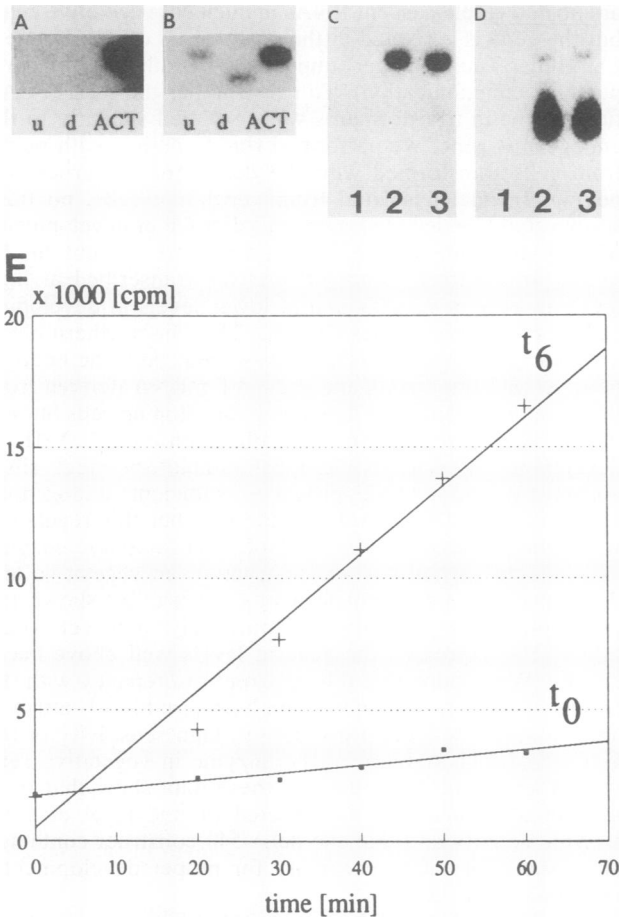

FIG. 2. (A and B) Run-on transcription assay of del. −567 transformants. Different DNA fragments homologous to the A11H2-CAT hybrid gene transcripts (u and d; see Material and Methods) and to actin mRNA were immobilized on Biodyne A filters and hybridized with run-on transcripts generated by nuclei from vegetative (A) and developing (B) cells of del. −567 transformants. (C and D) Northern blots of RNA from del. −567 transformants. RNA was prepared from vegetative cells (lanes 1) and after development in suspension culture for 6 h with (lanes 2) or without (lanes 3) pulsatile addition of cAMP. The filter was hybridized first with an A11H2 cDNA probe (C) to show expression of the endogenous gene and then with a CAT-specific probe (D) to show expression of the reporter gene. Residual hybridization to A11H2 RNA is seen. (E) CAT scintillation assay of del. −540 transformants. Extracts were prepared from vegetative cells (●) and after 6 h of development (+). Enzyme activities were determined by measuring accumulation of [^{14}C]butyryl chloramphenicol in 10-min intervals.

different deletion constructs. These factors can be neglected when testing for developmental induction of the fusion gene in a clonal isolate of a transformant. Enzyme activity increased with development in del. −567 (not shown), −540, and −370. In del. −305, no induction above the level in vegetative cells was observed, and in del. −238 promoter function was essentially lost. For better quantitation, CAT assays were also performed by the scintillation method (Fig. 4B). Assays with other deletion transformants were consistent with these results, and RNA isolated from several cell lines displayed the developmental pattern expected from the CAT assays (data not shown). From these results, we conclude that an element necessary for developmental gene induction is located between positions −370 and −305 and that an element supplying basic promoter activity in vegetative cells is located between positions −305 and −238.

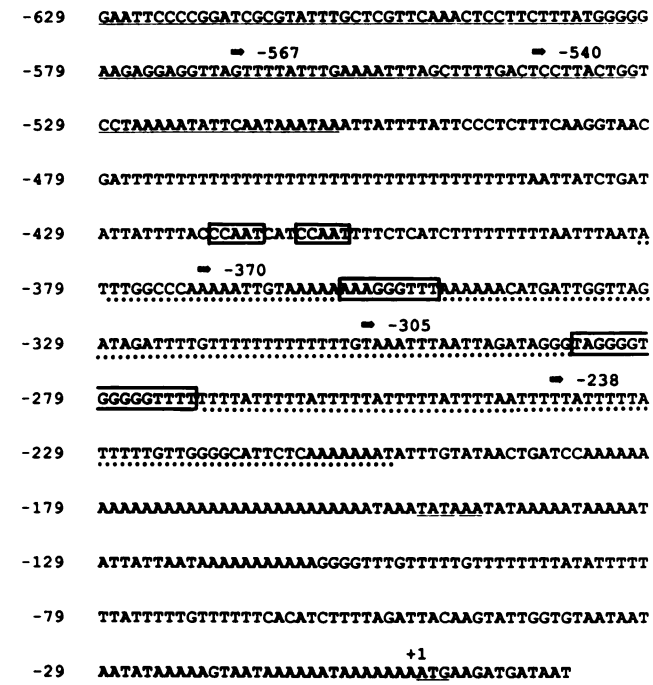

FIG. 3. Sequence of the A11H2 promoter region. Deletions used in this study are marked by arrows over the last nucleotide and by numbers indicating the position upstream from the ATG codon (+1). The upstream open reading frame is underlined. Putative regulatory sequences (two CCAAT motifs and two G-rich motifs) are boxed. The fragment used for gel shift experiments is marked by dots.

The −370/−305 element is a target for a *trans*-acting factor. Most likely, the −370/−305 element contains a binding site for a regulatory protein that confers developmental regulation on the promoter. To demonstrate the existence of a *trans*-acting factor and to gain initial insight into the mode of its regulation, we performed gel shift assays, using the A11-178 DNA fragment (Fig. 3) and nuclear extracts isolated from vegetative and developing AX2 cells. After differential ammonium sulfate precipitation, binding activity was detected only in the 30 to 60% fraction (data not shown). In the absence of competitors, two about equally strong bands were detected with this fraction from nuclear extracts of developing cells, but only the upper band was seen with extracts from vegetative cells (Fig. 5). Competition with a 150-fold weight excess of *Sau*3A-digested pBR322 resulted in loss of the slower-migrating complex with both extracts but did not significantly reduce the lower band formed with nuclear protein from developing cells. Poly(dI-dC) also abolished the upper complex with both extracts but also reduced the lower complex formed with extracts from developing cells. It is not unusual that increasing amounts of nonspecific competitor also decrease specific binding (e.g., a 750-fold weight excess of *Sau*3A-digested pBR322 DNA). The complete loss of the complex with a 500-fold weight excess of the specific competitor (A11-178) does, however, demonstrate that binding resulted not only from random DNA-protein interaction.

To confirm that the complex was formed on the element conferring developmental regulation and not on some other part of the A11-178 fragment, we also used a synthetic oligonucleotide of 58 bp containing the sequence from −380 to −324. This fragment was also capable of complex formation (Fig. 5B). In the presence of nonspecific competitor DNA, no complex was observed with extracts from vegeta-

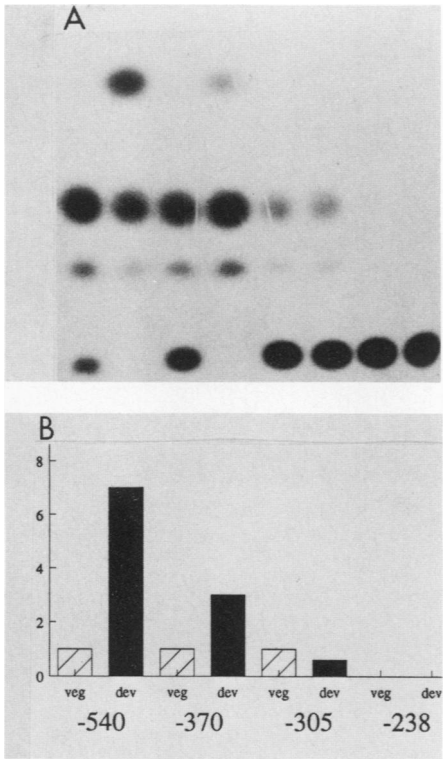

FIG. 4. CAT activity in deletion transformants. (A) Extracts prepared from vegetative and developing cells of various deletion transformants were analyzed for CAT activity by thin-layer chromatography. Spots on the chromatogram (from bottom to top): chloramphenicol, 1-acetyl chloramphenicol, 3-acetyl chloramphenicol, and 1,3-diacetyl chloramphenicol. (B) Equal portions from the same extracts were used for quantitative scintillation assays. After subtraction of background, activity in vegetative cells was set to 1; activity in developing cells is expressed relative to this value.

tive cells, whereas a clear band was seen with extracts from developing cells. Competition with a nonspecific oligonucleotide did not interfere with the binding, whereas excess of the specific DNA resulted in a significant reduction of the complex. These results indicate that the element necessary for developmental gene induction is also the target for a developmentally regulated DNA-binding protein.

DISCUSSION

We have constructed a new promoter analysis transformation vector for *D. discoideum* and have shown that promoter activity is faithfully reflected by CAT activity, which was used as a reporter. Promoter fragments can be cloned immediately into the multiple cloning site upstream of the CAT gene such that restriction sites producing 5′ and 3′ overhangs are located upstream and downstream of the promoter. The first set is used for unidirectional deletions, and the downstream sites may be used to adjust the reading frame between the CAT gene and the promoter fragment. The *Bsp*MI site is particularly convenient for reading frame adjustment since it allows the construction of all three frames by two subsequent rounds of digestion and repair (13). To confirm the reading frame, an oligonucleotide primer hybridizing to the 5′ end of the CAT gene is used for plasmid sequencing. Recombinant plasmids obtained from the Exonuclease III deletion procedure can first be analyzed by *Eco*RI digestion, which generates a fragment containing 244 bp of the CAT gene and the promoter. The SP6 promoter primer, which anneals upstream of the polylinker, is used for sequencing the deletion breakpoint. (A cotransformation vector containing some of the features of PAV-CAT had been independently proposed by Singleton [32].)

We have successfully used transient expression assays with PAV-CAT vectors containing strong *Dictyostelium* promoters (M. Maniak, P. Morandini, and W. Nellen, unpublished data). Transient activity of the A11H2-CAT fusions was, however, not sufficient for promoter analysis.

Using PAV-CAT, we have identified a *cis*-acting element in the promoter of the A11H2 gene that is responsible for temporal regulation of the gene during development. In contrast to earlier studies on late genes in *D. discoideum* (7, 8, 29), this element could be removed without losing promoter activity: whereas basal levels of enzyme activity in vegetative cells were maintained, the induction of expression during development was completely lost.

The significant CAT activity seen in extracts from vegetative cells was surprising, since we have not been able to detect mRNA by run-on transcription or by Northern blot in this stage. We assume that four factors contribute to this unexpected result: (1) very low level transcription takes place in vegetative cells (some primer extension products indicating the same transcription start site as in development have indeed been seen at this stage [Müller-Taubenberger, unpublished data]); (2) the high copy number of the gene fusion present in the transformed cells leads to an increase in basal RNA levels; (3) the high sensitivity of the enzyme assay allows for detection of low amounts of the gene product; and (4) the stability of the enzyme in vegetative cells results in a relative accumulation of activity. It could also be that in addition to being subject to transcriptional regulation as shown in this paper, expression is also subject to translational control. We could rule out that vegetative enzyme levels resulted from an endogenous activity, since the values were clearly above the background in untransformed cells. Furthermore, there is a strict correlation with an at least partially functional promoter: deletion of sequences between −305 and −238 abolished CAT activity in our assays. Therefore, transcription of the gene from a cryptic promoter, e.g., somewhere in the vector or in genomic flanking sequences, could be excluded.

It is possible that only the high sensitivity of the enzyme assay or the stability of CAT in vegetative cells enabled us to distinguish between basal and developmentally induced promoter activity. Basal promoter activity would have most likely escaped our attention in Northern blots (Fig. 2B). It therefore cannot be excluded that the Pst Cath gene, whose activity had been studied by using Northern blots for RNA detection (7, 29), also contains an element subject to temporal regulation separable from basal promoter function (see below).

The 65-bp element that confers developmental control contains a G box similar to the ones found in the actin 6 and actin 15 genes (6, 26). This might be an important element of the regulatory sequence, but it has to act in conjunction with other parts of the promoter because the actin genes show a different pattern of developmental regulation. To this end, we cannot say whether the −370/−305 element is subject to cAMP regulation. In contrast to AX3 cells, AX2 cells apparently produce endogenous cAMP pulses in suspension development (21; this report), and therefore we cannot distinguish between cAMP responsiveness and other putative developmental factors.

The fact that this element binds a protein in gel shift assays in a developmentally regulated way suggests interaction with a regulatory *trans*-acting factor responsible for developmen-

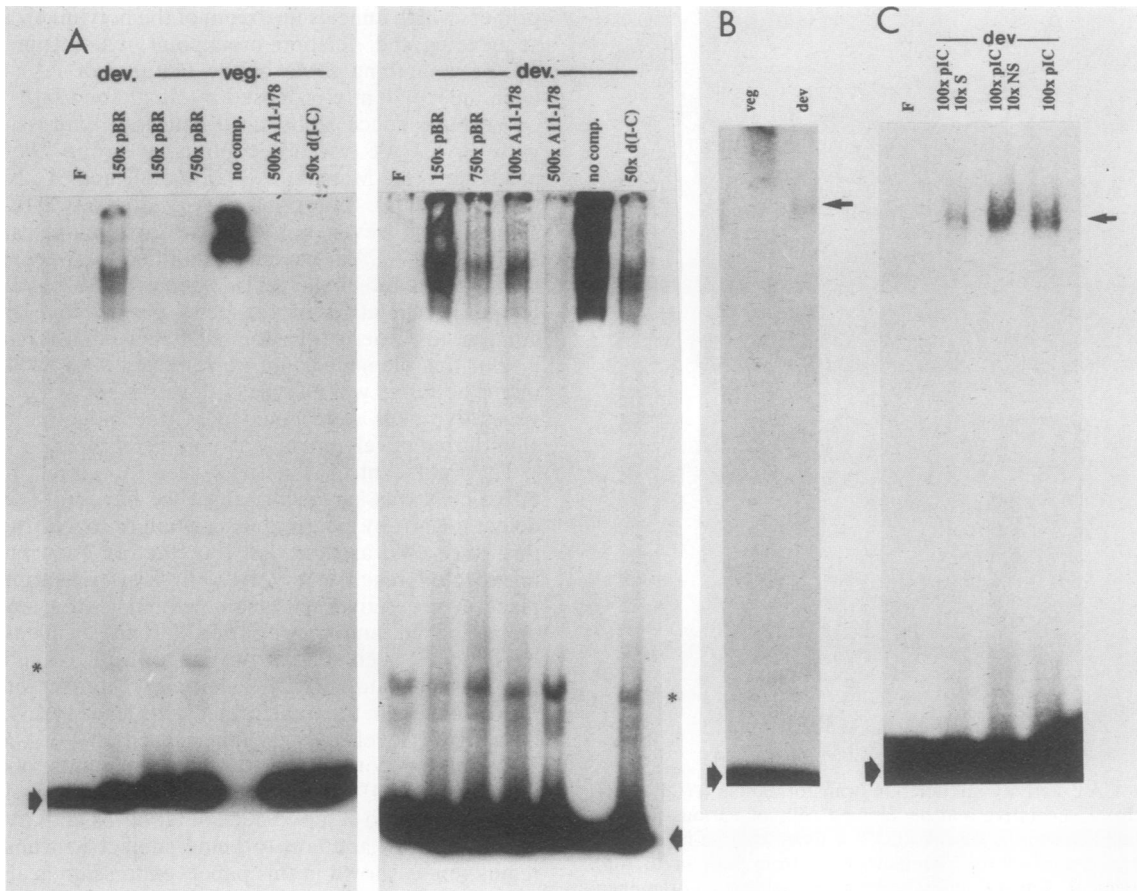

FIG. 5. (A) Gel shift assay, A11-178. Binding assays were performed with the fragment indicated in Fig. 3 and with nuclear extracts prepared from vegetative and developing cells. Competitors were added in weight excess as indicated above the lanes; 150× and 750× of pBR322 are equimolar to 100× and 500×, respectively, of A11-178. Abbreviations and symbols: F, DNA fragment without extract; veg., extract from vegetative cells; dev., extract from cells developed from 6 h; no comp., no competitor; d(I-C), poly(dI-dC); pBR, *Sau*3A-digested pBR322 DNA (nonspecific competitor); A11-178, *Hin*dIII/*Sau*3A-digested A11-178 vector (specific competitor); arrow, position of the DNA fragment; *, bands with higher mobility, probably due to secondary-structure formation of the DNA fragment. (B) Gel shift assay, 58-bp oligonucleotide. Binding assays were performed with the 58-bp oligonucleotide described in Materials and Methods. (B) Nuclear extracts prepared from vegetative (veg) and 5-h developing (dev) cells were tested in the presence of a 100-fold weight excess of *Sau*3A-digested pIC20H DNA. (C) Extracts from developing cells were tested in the presence of competitor DNA as indicated: 100× pIC, 100-fold weight excess of nonspecific competitor DNA (*Sau*3A-digested pIC20H); 10× NS, 10-fold molar excess of the nonspecific competitor 43-bp oligonucleotide; 10× S, 10-fold molar excess of the specific competitor 58-bp oligonucleotide. Lane F contains the fragment without addition of extract.

tal regulation of the gene. It is unlikely that this factor is identical to the DNA-binding proteins described by Hjorth et al. (14), since the factors were identified in extracts from different developmental stages and the competition characteristics were different. In the absence of competitors, the A11-178 fragment formed two major complexes with extracts from developing cells, one of which was easily competed out, had a mobility similar to that of the complex formed with extracts from vegetative cells, and probably resulted from nonspecific DNA-protein interaction. The other complex did not form with extracts from vegetative cells and was more resistant to nonspecific competitors but was completely lost in the presence of equimolar amounts of the specific competitor. We assume that this shift results from specific binding of a *trans*-acting factor. Since similar results were obtained when we used the 58-bp oligonucleotide in gel shift assays, we conclude that in both cases sequences within the −370/−305 element are the target for the DNA-binding activity. The observation that poly(dI-dC) and pBR322 also reduced the shifted band is difficult to interpret. It could mean that other nonspecific complexes running in the same position in the gel were removed or that these sequences really have some affinity to the binding protein(s) and could compete when present in large excess. We do not find sequence similarities between the −370/−305 element of the A11H2 gene and the putative cAMP-responsive element in the Pst Cath gene, but the 69-bp element sufficient for basal activity of the A11H2 gene contains a G box (TAGGGGTGGGGGTTTT) that is very similar to an essential promoter element defined by others (8, 29) (TAGGGGTGGGTTTT).

We do not yet know whether basal promoter activity is restricted to vegetative cells or is also present in development. The observation that the amounts of CAT produced by del. −305 stayed on the same level or even slightly decreased during development suggests that this truncated promoter is active only in vegetative cells. This result could, however, also be explained by an additional control on the level of translation or protein stability. It is interesting in this context that A11H2, like many other developmentally regulated genes in *D. discoideum*, appeared to be inactive during vegetative growth. The finding that transcriptional activity

can be shown with use of a sufficiently sensitive assay suggests that developmental induction of gene expression represents stimulation over vegetative expression and not a strict on-off mechanism.

Although we have not attempted to quantitate absolute expression levels with the different deletion constructs, our data suggest that transcriptional activity changes before inducibility is lost. It is therefore most likely that additional promoter elements are located upstream of −370. The two CCAAT boxes, found in positions −419 and −411, could be involved in a fine tuning of transcription. It should be noted that three CCAAT boxes are also located at a similar spacing upstream of the essential promoter elements of the Pst Cath gene. In mammalian cells, this sequence element is a target for the transcription factor C/EBP (17); to date there is, however, no evidence for a similar protein in *D. discoideum*.

The −370/−305 element is the first separable promoter sequence identified in *D. discoideum* that is necessary for developmental regulation of gene expression. Transfer of this element to other promoters will show whether it is also sufficient for temporal control of gene expression.

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