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Regulation of the Discoidin I γ Gene in *Dictyostelium discoideum*: Identification of Individual Promoter Elements Mediating Induction of Transcription and Repression by Cyclic AMP

FRANZ VAUTI, PIERO MORANDINI, JÜRGEN BLUSCH, ASTRID SACHSE, AND WOLFGANG NELLEN*

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

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We dissected the promoter of the developmentally induced and cyclic AMP-repressed discoidin I γ gene and identified a sequence element essential for developmental induction. Transfer of the element to an inactive heterologous promoter demonstrated that this sequence is sufficient to confer expression in axenically growing cells and to induce gene activity in development after growth on bacteria. A 16-base-pair sequence within this element was shown to be sufficient for induction in the discoidin promoter context and was used to reactivate different truncated promoter constructs. This led to the localization of an element necessary for down regulation of gene expression by extracellular cyclic AMP.

Upon deprivation of the food source, vegetatively growing *Dictyostelium discoideum* cells enter a developmental cycle which leads to the formation of multicellular aggregates. Cyclic AMP (cAMP) plays an important role not only in the chemotactic movement toward aggregation centers but also in the developmental regulation of gene expression. Both responses are apparently mediated via cAMP cell surface receptors (16; for reviews, see references 10 and 11).

During the early developmental processes, transcription of a number of genes is stimulated when cells start to secrete cAMP in a pulsatile manner (11, 12). Other genes, induced by different signals immediately with the onset of starvation, become repressed by the cAMP signal (19, 34).

To understand the molecular mechanisms of positive and negative regulation by cAMP, we have begun to define the promoter elements responsible for this control. Previously, we have identified a short sequence which is necessary for the developmental induction (21) and sufficient for cAMP-mediated stimulation (T. May, J. Blusch, and W. Nellen, submitted for publication) of the *Dictyostelium* alpha-fucosidase gene (22).

In this report, we chose the discoidin I γ gene promoter as an example for negative regulation by cAMP to identify sequences regulating the developmental expression of the gene. The availability of mutants specifically impaired in discoidin expression (1) makes this an attractive system.

The discoidin multigene family (31) is regulated on the transcriptional level (2, 38) and encodes lectins implied in cell substrate interactions of developing cells via the fibronectin-related RGDH (Arg-Gly-Asp-His) sequence (37). Functional analysis in mutants and antisense transformants (8) demonstrated that a lack of the gene product impairs the process of cell streaming in early development. Discoidin expression is induced two to three generations before deprivation of the food source by accumulation of a secreted factor (prestarvation factor) which apparently gives a measure for the ratio of cell density to the food supply (6, 7). Cells grown in bacterial suspension culture express discoidin when the density increases above 2×10^6 cells per ml; in axenically growing cells, induction of the genes begins at much lower densities. After the onset of development, discoidin mRNA accumulates and reaches a maximum at about 4 to 5 h. At this time, the cells begin to produce pulses of cAMP and transcription of the genes becomes repressed. When cells are starved in suspension cultures, discoidin expression can be repressed by the addition of cAMP (4, 38). Using 5′ deletions in the promoter analysis vector PAV-CAT (21) and different artificial gene fusions, we identified a sequence element which is necessary for developmental induction in the discoidin promoter context and sufficient for reactivating a silent heterologous promoter. This promoter element (discoidin induction element [dIE]), which possibly responds to prestarvation factor, is active in both orientations and thus displays some characteristics of an enhancer. In addition, we defined an element (discoidin negative cAMP element [dNCE]) located downstream of the positive sequence element which is necessary for down regulation of gene expression in response to cAMP. The identification of these promoter modules, which are the ultimate targets for extracellular signals, provides the basis for tracing back the transduction chain from the level of gene expression to the events at the membrane.

MATERIALS AND METHODS

Construction of promoter deletions in PAV-CATII. The 1.2-kilobase *Xho-Hin*dIII fragment of the discoidin I γ gene promoter (31) was cloned into the *Xho*I and *Hin*dIII sites of pIC20H (20). The fragment was excised with *Xba*I after transfer of the plasmid to a *dam* mutant host (*Escherichia coli* RHZ22) and then inserted into the *Xba* site of PAV-CATI. This plasmid was amplified in the *dam*$^+$ *E. coli* JM105, resulting in methylation of the upstream *Xba*I site. The reading frame between the *cat* gene and the residual coding region of the discoidin gene was adjusted by cutting at the accessible downstream *Xba*I site and the adjacent *Acc*I site, treating with Klenow polymerase, and religating. Deletions were introduced and analyzed as described previously by using the *Xho*I and *Sph*I sites in the polylinker for exonuclease III digestion and the SP6 promoter primer for sequencing (21).

Construction of hybrid promoters. For generating the dIE fragment, an *Eco*RI fragment containing the −411 deletion promoter and part of the *cat* gene was excised from the respective PAV-CAT construct and cloned into pUC18.

* Corresponding author.

Exonuclease III deletions were introduced from the *Xba*I site in the polylinker 3′ end to the fragment after protecting the vector by cutting with *Pst*I. A deletion containing sequences from −411 to −302 was selected, and the insert was excised with *Eco*RI and *Hin*dIII, blunt ended, and cloned into the blunt-ended *Bam*HI site of A15delBam. The same fragment was also cloned upstream of the promoter deletion −192. Plasmids containing both orientations of the insert were recovered and transformed into *Dictyostelium* cells.

To generate a TTG box fragment, two oligonucleotides of the sequences AATTAAAAATTGATTGTTTT and AATT AAAACAATCAATTTTT were synthesized. This corresponded to the TTG box in position −392, some adjacent nucleotides, and *Eco*RI-compatible ends. The two strands were hybridized, cloned into the *Eco*RI site of pIC20H, excised with *Sac*I, and cloned into the single *Sac*I site of deletions −271 and −192 and further downstream deletions. The fragment was also excised from pIC20H with *Bam*HI and *Bgl*II and cloned into the single *Bam*HI site of A15delBam, resulting in constructs containing a single and a triple insertion.

Other molecular methods. Isolation of DNA and RNA was performed as described previously (18, 23). RNA was separated on 1.2% agarose gels containing formaldehyde, blotted on Biodyne A membrane filters with a vacuum device, and hybridized with ^{32}P-labeled in vitro transcripts of the chloramphenicol acetyltransferase (CAT) or the discoidin I γ coding region which had been cloned into the GEM3Z vector (Promega Biotec, Madison, Wis.).

Restriction enzymes were used according to the recommendations of the manufacturer. Exonuclease III deletions were set with the Erase-a-base system (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany), and sequencing was done by the method of Sanger et al. (36) as modified by Chen and Seeburg (5) for double-stranded DNA.

Transformation of *Dictyostelium* cells. *Dictyostelium* strain AX2 cells were transformed either as described by Nellen et al. (26) with modifications as detailed by Nellen and Saur (25) or by electroporation (14). Primary clones were isolated and purified by one passage of growth on a bacterial lawn. For all 5′ deletion transformants, clones and populations were analyzed. For the hybrid promoters, populations from at least two independent transformation experiments were tested.

Preparation of food bacteria. NB (25 g standard nutrient broth; Merck, Darmstadt, Federal Republic of Germany) medium was inoculated with *Escherichia coli* B/r and incubated overnight. Oxygen was supplied by bubbling air through the culture. Bacteria were harvested by centrifugation at 3,000 × *g* for 30 min, washed twice with Soerensen buffer (17 mM potassium phosphate [pH 6.15]), adjusted to a concentration of 10^{10} cells per ml, and stored at 4°C for a maximum of 3 days. Bacteria stored for a longer time could still be used as a food source, but *Dictyostelium* cells would grow to lower concentrations before starting development in the suspension; also, the generation time was found to be longer when bacteria were not freshly prepared.

Developmental conditions. Transformed strains were kept in axenic medium containing 10 μg of G418 per ml in 24-well Costar plates. From the plates, precultures were inoculated into AX medium containing G418 (21) and grown to a density of 10^6 cells per ml (generation time about 8 h). Cells were then washed in Soerensen buffer and inoculated into a bacterial suspension at a density of 4.5 × 10^4/ml. This culture was grown up to 10^6 cells per ml (generation time about 3 h) and again used to inoculate a fresh suspension of bacteria. After growth to 10^6/ml, cells were harvested, washed three times with Soerensen buffer to separate food bacteria, and adjusted to 5 × 10^6/ml. Growth for at least eight generations on bacteria was necessary to reliably abolish the background of discoidin (or CAT) accumulated during growth in AX medium. The cell suspension (usually 15 to 30 ml) was divided into three parts to obtain equal samples of vegetative amoebae and cells developing in the absence or presence of cAMP. Development was done at 22°C with shaking at 150 rpm. If not otherwise stated, cAMP was added to a concentration of 1 mM in 1-h intervals starting at $t_{1.5}$. Pulses of 10 nM every 6 min and continuous flow delivering cAMP to a final concentration of 10 nM every 6 min were applied with a Braun perfusor. For CAT assays, cells were harvested at t_{11}; for RNA preparations, they were harvested at t_8. Except for Fig. 5, the data for all strains shown in one graph were obtained from cultures and preparations done in parallel. In all cases, vegetative cells and different developmental stages for one strain were derived from one culture and prepared at the same time.

CAT assays. CAT was assayed by the scintillation method (27) as described previously (21). Activities are given as arbitrary units of CAT activity since no calculations of enzyme quantity were done. The values are derived from counts per minute of [^{14}C]butyrylchloramphenicol detected in the scintillation cocktail phase of the assay. In all experiments, the kinetics of the transferase reaction were determined and the values shown in the graphs were taken from the linear part of the reaction.

RESULTS

Deletion series of discoidin promoter. When 1.2 kilobases of the discoidin I γ gene 5′-flanking region were inserted into the PAV-CATII promoter analysis vector (21), a developmental pattern of *cat* expression was obtained which mimicked the expression of the endogenous discoidin I genes. A series of deletions was introduced into the promoter by Exonuclease III digestion as described earlier (21). Deletion break points were determined by sequencing and are depicted in Fig. 1. Deletions down to −458 resulted in a dramatic decrease of promoter activity, but the pattern of regulation (see below) was not altered. Therefore, only this part of the promoter is shown. Minor differences with the previously published data (32) and with the more recent update in the EMBL database (accession number X00325) were found in the promoter sequence.

***cat* expression during growth in axenic medium.** We first examined gene expression from promoter deletions during growth in axenic medium. Discoidin is known to be highly expressed under these conditions, and indeed we detected high CAT activity. With progressive deletions, the activity gradually decreased (Fig. 2 and data not shown). In the experiment shown here, all deletion constructs were transformed in parallel and activities were determined from cultures grown in parallel. Independently transformed subsets of this deletion series always showed the same relative activity. Variability in copy number therefore does not contribute substantially to the measured CAT activity. Surprisingly, even the −135 construct (leaving only 73 base pairs [bp] upstream of the start of transcription) displayed levels of *cat* expression significantly above the background of the promoterless construct (PAV-CAT). In RNA prepared from these transformants, CAT mRNA could be detected at

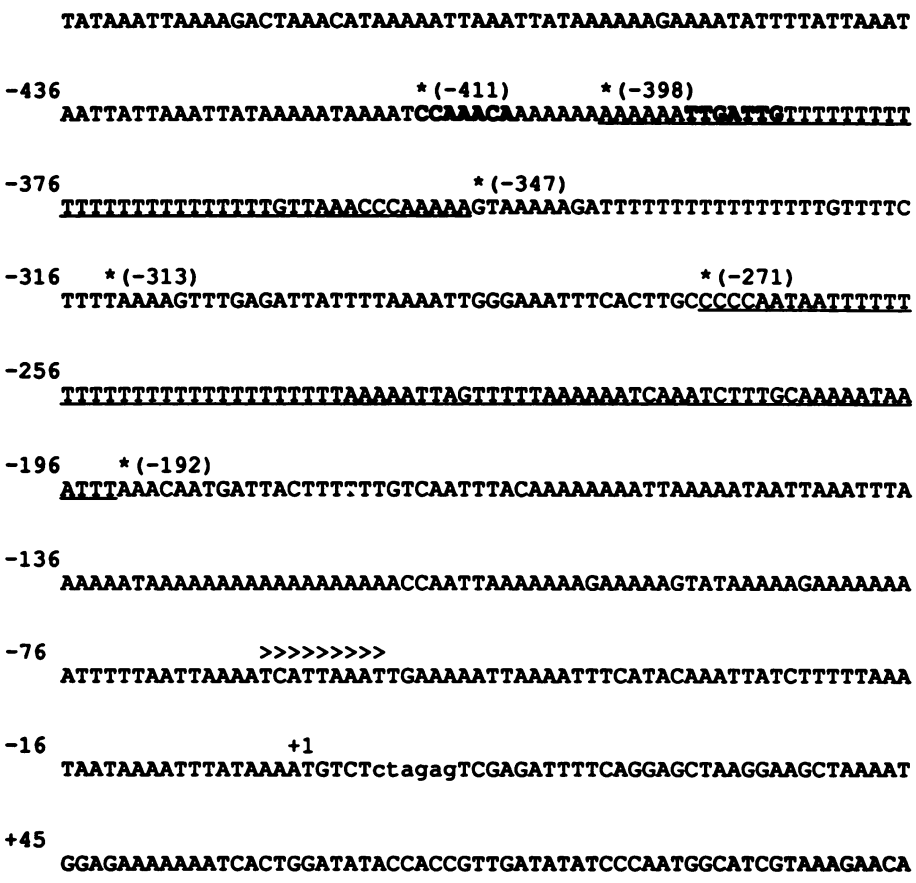

FIG. 1. Sequence of the discoidin I γ gene promoter. Deletion break points are indicated by numbers, the induction element (dIE) and the cAMP-responsive element (dNCE) are underlined, and the TTG and CAA boxes are shown in boldface.

levels roughly corresponding to the CAT enzyme activities (Fig. 2b). Interestingly, with deletions beyond −458, a smear and an additional large transcript appeared in Northern (RNA) blots (e.g., see Fig. 2b and 7b; also data not shown). This indicates that, as in several other *Dictyostelium* gene promoters, an upstream terminator is contained in the promoter region (24; M. Maniak and W. Nellen, unpublished data). The readthrough transcripts originating somewhere in the vector, however, did not significantly contribute to CAT activity in the PAV-CAT constructs (compare smear in lane PAV-CAT, Fig. 2b, and the corresponding CAT activity in Fig. 2a).

cAMP-mediated down regulation of discoidin promoter activity. In most published investigations on discoidin expression, cAMP was used at concentrations 4 to 5 orders of magnitude above physiological levels (e.g., see reference 4). Although this proved to be convenient and resulted in a clear-cut response of the cells, we wished to know whether more physiological conditions also had an effect. Figure 3 shows CAT activity in cells transformed with the correctly regulated −398 promoter construct (see below). During development, cAMP was added at different concentrations and in different ways. High concentrations of 1 mM and 150 μM resulted in efficient repression of enzyme activity. A clear, although reduced, effect was observed with pulses of 10 nM added every 6 min. Surprisingly, a constant flow yielding the same final concentrations had a similar reduced effect. This is unusual since the latter application has no effect at all on most early genes which are induced by cAMP (e.g., see reference 11).

Sequence element between −411 and −347 mediates developmental induction of discoidin promoter. A series of deletions was tested for developmental induction and the effect of cAMP on promoter activity. The data are shown as arbitrary units of CAT activity in Table 1. Deletions up to −411 displayed a developmental induction on the RNA (Fig. 4c) as well as on the CAT enzyme level (Fig. 4a and b). These constructs also showed cAMP-mediated down regulation (or inhibition of induction) on the RNA and CAT levels. Further deletions showed a dramatic decrease in developmental induction (in the absence of cAMP), while the residual expression in the presence of cAMP was almost the same. Consequently, the induction and repression factors (Fig. 4b) are close to 1 (Table 2). However, basal promoter elements which permit expression in axenically growing cells were still present (Fig. 2). From these results, we conclude that a sequence element between −411 and −347 is necessary for induction of gene expression during development but not for gene expression per se. With the loss of inducibility in deletion −347, we can no longer determine a repression factor and we cannot say whether sequences which mediate the negative response to cAMP are overlapping with this element or are located further downstream.

The −411 to −302 element is an autonomous inducer for developmental gene expression. Deleting the −411 to −347

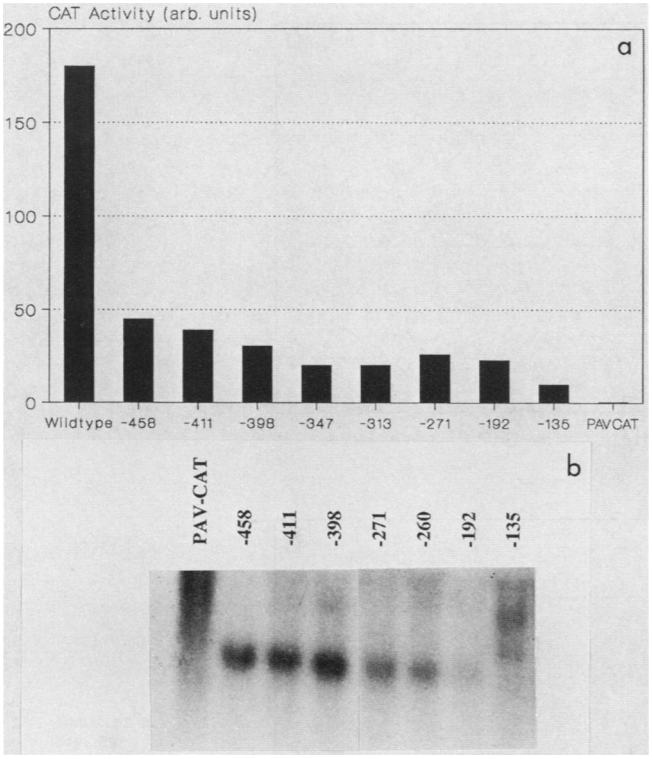

FIG. 2. (a) CAT activity in axenically growing cells transformed with various promoter deletion constructs. As a comparison, CAT activity directed by a PAV-CAT vector without a promoter is shown. (b) Northern blot of RNA prepared from some of the deletion transformants grown in axenic medium. The filter was hybridized with a *cat*-specific probe. The smear seen in lane PAV-CAT and in deletions below −458 is due to readthrough transcription originating in vector sequences. Growth of cells and preparation of samples were done in parallel for all transformants and with equal cell numbers. All cultures were grown up to a density of 2 × 10^6/ml. The figure shows data from a representative experiment. arb., Arbitrary.

element strongly reduced the induction of gene expression. If this element was autonomous, one could ultimately determine whether both functions reside in the same sequence by introducing the fragment into a heterologous promoter. This had been successfully done previously with the DCRE (*Dictyostelium* cAMP-responsive element), a positive cAMP element from the alpha-fucosidase promoter (May et al., submitted). A 109-bp fragment from −411 to −302 (dIE) containing this sequence was cloned into a deleted, nonfunctional actin 15 (A15delBam) promoter (29). Examination of transformed cell lines revealed that the dIE could strongly reactivate this silent promoter under axenic growth conditions. When cells were grown in bacterial suspension, the hybrid promoter was essentially inactive. Upon development, however, CAT activity was induced but no significant downregulation by cAMP was observed (Fig. 5 and Table 2). This experiment showed that the −411 to −302 element is sufficient to confer expression in axenically growing cells and at least some developmental induction. It also demonstrated that sequences mediating the strong down regulation by cAMP are not contained within this fragment. Consequently, sequences necessary for negative regulation by cAMP have to be located further downstream in the promoter.

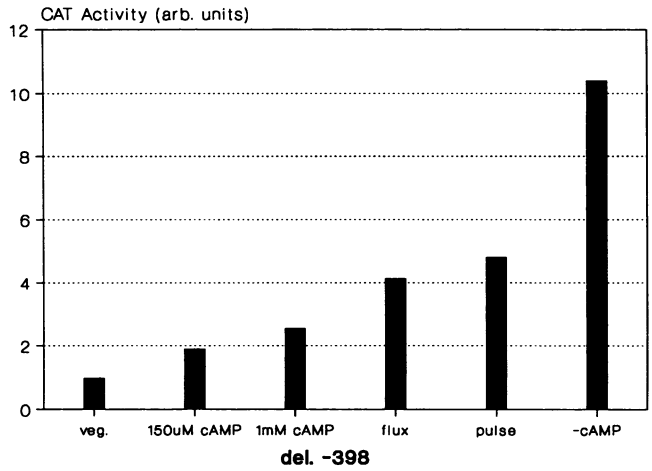

FIG. 3. CAT activity in cells transformed with the deletion (del.) −398 promoter construct after growth in a bacterial suspension (veg.) and after development without cAMP (dev.), with 1 mM cAMP added every hour, with 150 μM cAMP added every hour, with pulses of 10 nM cAMP every 6 min (pulse), and with a continuous flow of cAMP delivering 10 nM cAMP per 6 min (flux). Cells for the different developmental conditions were taken from one culture at a density of 10^6 cells per ml. After washing and resuspension in buffer, equal portions were taken for the different samples. arb., Arbitrary.

A 16-bp sequence (TTG box) is sufficient for mediating inducer activity. Upon close inspection of the −411 to −347 element, we noticed two short elements with the sequences CAAACAA (position −410) and TTGATTG (TTG box, position −392) which are palindromic. Comparison with other promoters of *D. discoideum* revealed the presence of at least one of these elements in many putative or established regulatory regions (Fig. 6). A 20-bp oligonucleotide containing the TTG box and some adjacent nucleotides (AATT AAAAATTGATTGTTTT) was synthesized and fused to deletion −271. The construction resulted in a hybrid promoter which had the oligonucleotide fused in the reverse orientation. This corresponded approximately to the CAAA CAA box in position −410. The fusion was thus equivalent to an internal 110-bp deletion of a −410 promoter between −400 and −271. The construct regained strong activity and was significantly down regulated by cAMP (Fig. 7). The repression factor of 2.7 (Table 2) was, however, much lower than that of the correctly regulated 5′ deletion promoters −458 and −411. When we examined the RNA in this transformant (Fig. 7b), the induction and repression was much more pronounced and similar to that of the −411 promoter. We assume that the CAT data to some extent are

TABLE 1. Expression levels in deletion transformants

Deletion	cpm of CAT activity[a]		
	Vegetative	−cAMP	+cAMP
Wild type (−1200)	9,375	200,100	27,385
−458	1,485	28,005	4,205
−411	1,805	29,365	2,785
−347	1,150	2,650	2,325
−271	2,155	2,890	2,720

[a] CAT activity generated by the 5′ deletion constructs in transformants under different conditions. The numbers give counts per minute of [^{14}C] butyrylchloramphenicol measured in the scintillation cocktail phase of the assay. The values are from the experiment shown in Fig. 4a.

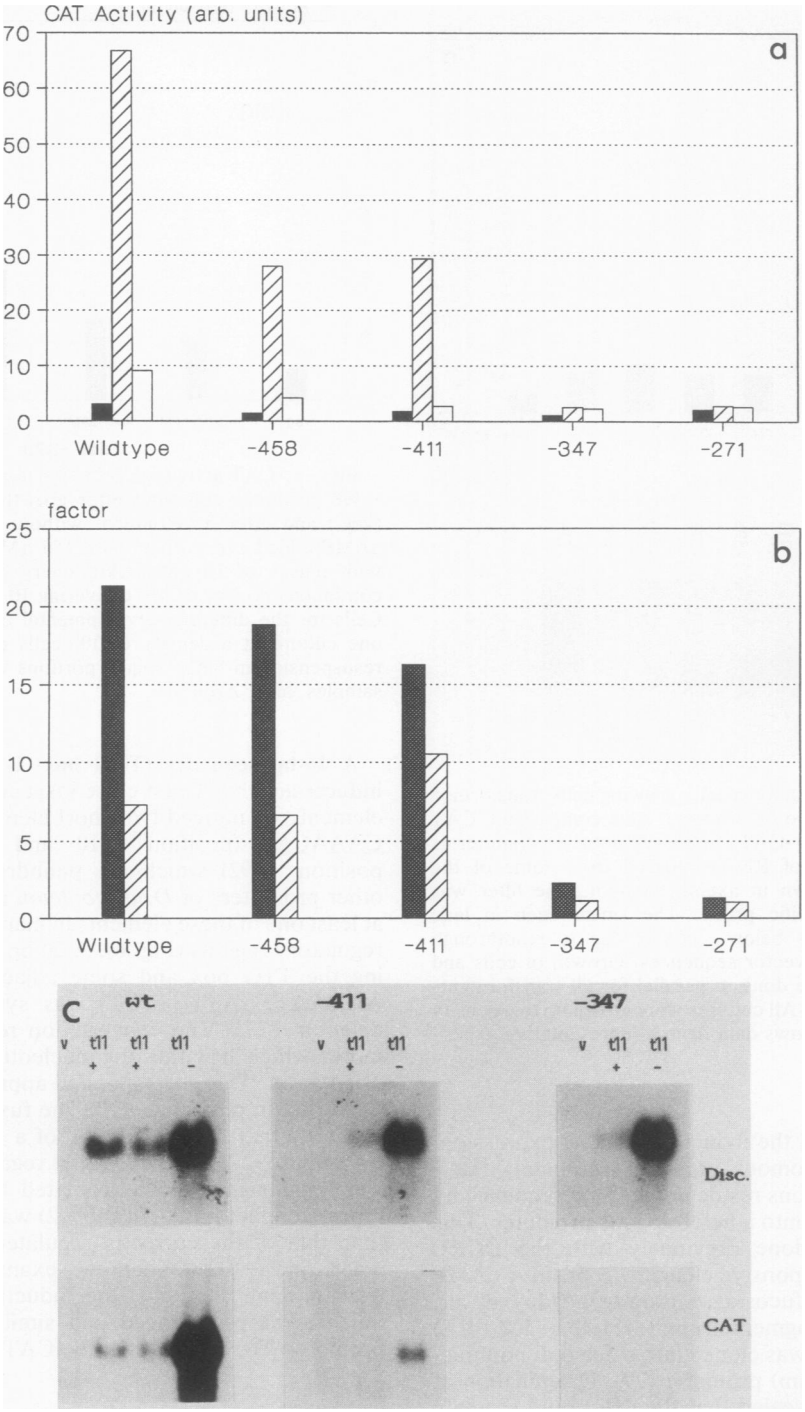

FIG. 4. (a) CAT activity in cells transformed with selected deletion constructs after vegetative growth (■) and after development in the absence (▨) or presence (□) of cAMP (1 mM every hour). For the wild-type promoter, one-third of the measured activity is shown. arb., Arbitrary. (b) The values from panel a are used to calculate the factor of developmental induction (Ind.) (■) (cpm dev. −cAMP/cpm veg.) and the factor of repression (Rep.) (▨) in response to cAMP (cpm dev. −cAMP/cpm dev. +cAMP) (cpm is counts per minute). (c) RNA was isolated from some of the cultures used for the CAT assays shown in panels a and b and probed for CAT transcripts (lower panel) and endogenous discoidin (Disc.) transcripts (upper panel). Growth and development of cells and preparation of samples were done in parallel for all transformants and with equal cell numbers. The figure shows data from a representative experiment. wt, Wild type; v, vegetative growth.

obscured by posttranscriptional mechanisms like differential protein stability (see Discussion).

Our observations showed that the oligonucleotide is sufficient for induction and also that, in combination with the TTG box, deletion −271 is still capable of responding to cAMP. We can exclude that the oligonucleotide itself confers cAMP responsiveness since transfer of the dIE, which contains the TTG and the CAA boxes, to a heterologous promoter (see above) did not result in a cAMP-sensitive hybrid promoter. Furthermore, deletion −398, which re-

TABLE 2. Induction-repression factors[a]

Deletion or construct	Induction (−cAMP/vegetative)	Repression (−cAMP/+cAMP)
Wild type (−1200)	21.3	7.3
−458	18.8	6.7
−411	16.3	10.5
−347	2.3	1.1
−271	1.3	1.1
A15 ax	1.2	1.2
A15 del. ax	0.67	0.59
A15 dIE ax	0.89	1.6
A15 dIE bact.	3.8	1.2
−271	2.9	1.4
−271/TTG	3.9	2.7
−192	2.1	0.96
−192/TTG	0.68	0.76

[a] Developmental induction and repression by cAMP was calculated by dividing CAT counts per minute from the −cAMP culture by CAT counts per minute from vegetative cells (induction) and by dividing CAT counts per minute from the −cAMP culture by CAT counts per minute from the +cAMP culture (repression). Therefore, repression is shown as a positive value. Lines 1 to 5 show factors from Fig. 4, lines 6 to 9 show factors from Fig. 5, and lines 10 to 13 show factors from Fig. 7. ax, Axenic; bact., bacterial; del., deletion.

moves the CAA box responsible for reactivation of the −271 deletion (though still containing the TTG element), is still cAMP regulated and indistinguishable from deletion −411.

Sequences between −271 and −192 are necessary for down-regulation by cAMP in conjunction with the TTG box. We used the TTG box oligonucleotide to construct fusions with promoters truncated downstream of −271 to see whether the oligonucleotide could also restore activity of these constructs. When ligated to deletion −192, corresponding to an equivalent of an internal 189-bp deletion of a −410 promoter, the construct was clearly activated in comparison with deletion −192. In contrast to the fusion on deletion −271, no repression by cAMP could be detected (Fig. 7 and Table 2). A similar fusion was made by ligating the entire dIE to deletion −192 (data not shown). Again, a small increase in activity but no effect of cAMP was observed. Since the oligonucleotide and the dIE cannot autonomously confer repression by cAMP (see above), we conclude that sequences required for down regulation are still contained in deletion −271 but are no longer functional in deletion −192. This 79-bp fragment (dNCE) therefore contains *cis*-acting sequences necessary for the negative regulation by cAMP.

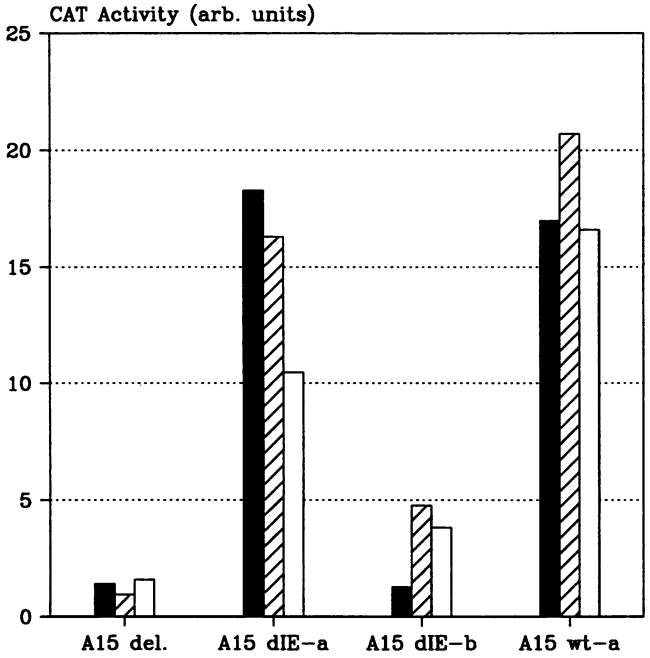

FIG. 5. Comparison of CAT activity obtained with the intact A15 promoter (A15 wt), the A15delBam construct (A15 del.), and the A15delBam construct after insertion of the −411 to −302 element (A15 dIE). Data are shown for cells grown in axenic medium (■) and developed in suspension culture with (□) or without (▨) pulses of cAMP. For the A15delBam-dIE construct, cells were also examined after growth in a bacterial suspension. CAT activities for A15 wt and A15 dIE-a were measured after 20 min of the enzyme reaction; CAT activities for A15 del. and A15 dIE-b were measured after 6 h. a, Axenic; b, bacterial; arb., arbitrary.

GENE	POSITION	SEQUENCE		REF.
DISC I ALFA	-419	AAGATTGAA		a
DISC I GAMMA	-388	TTGATTGTT	*	a
DISC I GAMMA	-406 R	TTGTTTGGA		a
DISC I BETA	-516 R	TAGATTGGA		a
DISC I BETA	-496 R	AAGATTGAC		a
DISC I BETA	-395 R	ATGATTGAA		a
DISC I BETA	-298 R	ATGATTGTA		a
PHOSPHODI-INH	-377	TTGATTGGA		b
PHOSPHODI-INH	-344	AAGATTGTC		b
PHOSPHODI-INH	-288 R	GTGATTGTT		b
PHOSPHODI-INH	-225 R	GAGATAGTA		b
pDG1 (PLASMID)	-349	TAGATTGTA		c
pDG1 (PLASMID)	-285 R	GAGATTGAT		c
pDG1 (PLASMID)	-257 R	GTGAATGAA		c
A11H2	-414 R	ATGATTGGG		d
A11H2	-339	ATGATTGGT	*	d
BP74	-559 R	TTGATTGAA		e
D2	-418 R	TTGTTTGA		f
D2	-141 R	TTGTTTGT		f
ACTIN 6	-595 R	TTGATTGAT		g
ACTIN 6	-589	TTGATAGAT	*	g
ACTIN 6	-180	TTGATTGAA		g
ACTIN 5	-280	ATGATTGT		g
ACTIN 15	-298	TAGATTG		h
CP1	-250 R	TTGATTGAA	*	i
Sc29 C	-469	GTGATTGG		l
Sc253	-253	ATGAATGT		l

FIG. 6. Comparison of sequence elements similar to the TTG box (TTGATTG oligonucleotide) in other *Dictyostelium* promoters. R, The reverse orientation of the sequence is given; *, the box is located in a region involved in gene regulation. References: a, 32; b, R. H. Kessin, personal communication; c, 28; d, 21; e, 13; f, 35; g, 33; h, 17; i, 30; l, 3. DISC, Discoidin; PHOSPHODI-INH, phosphodiesterase inhibitor.

DISCUSSION

Previously, we used the promoter analysis vector PAV-CAT to identify a regulatory element responsible for developmental induction of the *Dictyostelium* alpha-fucosidase gene (21). Now we investigated the regulation of the discoidin I γ gene promoter to identify sequence elements responsible for cAMP-mediated down regulation of gene expression (38). With defining conditions to monitor the cAMP response, we found that not only the extremely high cAMP concentrations used previously (1 mM/h [4]) but also lower concentrations (100 μM/h) result in the expected transcriptional repression. Pulses of cAMP most closely correspond to the physiological conditions and also mediate down

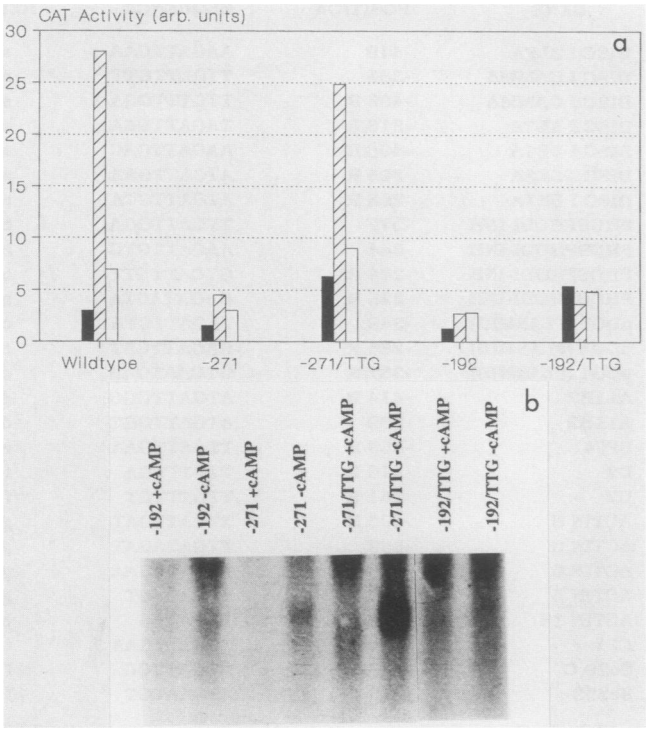

FIG. 7. (a) CAT activities obtained with deletions −271 and −197 with and without fusion of the TTG box oligonucleotide. For comparison, activity of the −411 promoter construct is shown. ■, Vegetative; ▨, without cAMP; □, with cAMP. arb., Arbitrary. (b) RNA isolated from the same cultures and probed for CAT transcripts. Most of the readthrough transcripts originating in vector sequences (Fig. 2b, lane PAV-CAT) have been cut off. The smear seen in most of the lanes is due to these readthrough RNAs or their degradation products. Growth and development of cells and preparation of samples were done in parallel for all transformants and with equal cell numbers. The figure shows data from a representative experiment.

regulation. Apparently, the pulsatile signal is transmitted via the cell surface cAMP receptors. The fact that the receptor-mediated signal transduction pathway becomes refractory for several minutes after cAMP binding explains why many cAMP-induced genes cannot be stimulated by a continuous flow of cAMP which leads to permanent occupation of the receptor. Interestingly, down regulation of the discoidin promoter can also be achieved by a flow. This suggests that negative regulation of the discoidin gene is not controlled by the same receptor-mediated mechanism as positive regulation of, e.g., the alpha-fucosidase gene. Alternatively, one could assume that the pulse-induced signal transduction pathway can be bypassed. Kimmel (15) has already suggested that at least two different mechanisms are involved in gene regulation by cAMP.

We defined two sequence elements in the discoidin I γ gene promoter: one which mediates expression under axenic growth conditions and most likely the developmental induction of the gene, and one which is required for repression by cAMP. The induction element (dIE) contains a short sequence, TTGATTG (TTG box), which can be found on either strand in putative or established regulatory regions of many *Dictyostelium* promoters. We first suspected this element to be involved in regulation since it was palindromic to the sequence CAAACAA also contained in our promoter deletion −411. Only later we found that elimination of one box (CAAACAA in deletion −398, not shown) had no effect on promoter activity. A 16-bp oligonucleotide containing the TTG box was sufficient to restore activity of a truncated discoidin promoter, and as mentioned above, a single copy had this effect. However, we were not successful in reactivating the silent A15delBam promoter (29) with one, two, or three TTG box oligonucleotides, indicating that in the context of a heterologous promoter, this sequence is not sufficient. However, the entire −411 to −302 fragment (dIE) could restore the activity of A15delBam, demonstrating autonomous function of this element. The hybrid promoter displayed developmental induction after vegetative growth of cells on bacteria and activity in vegetative cells grown in axenic medium. Both are characteristics of the discoidin promoter and can be, at least in part, assigned to the dIE. Examination of further promoter deletions showed, however, that sequences further downstream can also promote gene expression during axenic growth.

These observations demonstrated that two independent promoter regions confer expression in vegetative cells grown in axenic medium. The dIE is sufficient for this regulation in the context of a heterologous promoter. Deletions down to −135, however, are also functional under axenic growth conditions and show CAT activities significantly above the background of the promoterless PAV-CAT construct. An element downstream of −135, which we will provisionally term dAE (discoidin axenic element), has to be responsible for this expression and can function independently of the dIE and the dNCE. It is interesting that Williams and co-workers (9) found that one member of the otherwise coregulated discoidin I gene family is not or is only weakly expressed under axenic growth conditions. This could mean that one promoter element specifically enhances expression during growth in axenic medium and that another one is responsible for developmental activation of the promoter and can only provide low levels of transcription under axenic growth conditions.

Using a minimal sequence required for gene induction (TTG box oligonucleotide), we constructed equivalents to internal deletions of a −410 promoter which revealed that sequences between −271 and −192 are necessary for down regulation by cAMP. Although the activity of the TTG box/−192 fusion was low, it was clearly above the level of the −192 deletion promoter. We also tested TTG box fusions with deletions downstream of −192 and found expression levels above those with the deletion alone but never any effect of cAMP (data not shown). Thus, the TTG box could reactivate most of the deletions, but the dNCE (−271 to −192) was required for the cAMP response. An internal deletion of the dNCE region from a −411 promoter confirmed these results (data not shown). Although we could rule out that the TTG box by itself confers cAMP responsiveness, it is possible that interaction between this element (or a corresponding *trans*-acting factor) and the dNCE (or a corresponding *trans*-acting factor) is necessary for cAMP-mediated down regulation. Most of the promoter constructs described here were examined on the level of both RNA expression and CAT activity. Although in general both assays gave similar results, there are some discrepancies. In the CAT assay, the −271/TTG promoter, for example, showed repression by a factor of about 3 upon cAMP addition, while the RNA was down regulated to undetectable levels. Apparently, a posttranscriptional mechanism obscures some of the CAT data. A possible explanation is the extreme stability of the CAT protein under certain conditions (Maniak and Nellen, unpublished observations). Based

on our data, previously suggested putative regulatory elements like the I box (32) can now be examined in the light of functional tests. Most of the developmental induction is lost in deletions, which still contain the I box, and cAMP responsiveness can be reactivated by the TTG box in deletion −271, which has lost most of the I box. Therefore, it is unlikely that the I box is involved in developmental induction or in mediating the cAMP effect.

Apart from the two *cis*-acting sequences described here, there is evidence for a third element (dAE) downstream of deletion −135 which is sufficient for expression in axenically growing cells and might represent the basal promoter (Fig. 2). This means that at least three distinct promoter elements jointly regulate transcription of the discoidin I γ gene. Interestingly, Alexander and co-workers (1) have identified three regulatory genes by genetic analysis which are involved in the control of discoidin expression. Combination of the genetic data with our molecular analysis will help to unravel the regulatory mechanisms of discoidin expression and will ultimately shed light on the signal transduction pathway causing gene regulation by cAMP.

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Franz Vauti and Piero Morandini contributed equally to this work; the order of the first two authors is therefore arbitrary.

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