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Identification of a Signal Transduction Response Sequence Element Necessary for Induction of a *Dictyostelium discoideum* Gene by Extracellular Cyclic AMP

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The signal transduction pathways that lead to gene induction are being intensively investigated in *Dictyostelium discoideum*. We have identified by deletion and transformation analysis a sequence element necessary for induction of a gene coding for uridine diphosphoglucose pyrophosphorylase (UDPGP1) of *D. discoideum* in response to extracellular cyclic AMP (cAMP). This regulatory element is located 380 base pairs upstream of the transcription start site and contains a G+C-rich partially palindromic sequence. It is not required for transcription per se but is required for induction of the gene in response to the stimulus of extracellular cAMP. The cAMP response sequence is also required for induction of the gene during normal development. A second A+T-rich *cis*-acting region located immediately downstream of the cAMP response sequence appears to be essential for the basal level of expression of the UDPGP1 gene. The position of the cAMP response element coincides with a DNase I-hypersensitive site that is observed when the UDPGP1 gene is actively transcribed.

The mechanisms by which extracellular stimulatory molecules induce genes are not well understood. Extracellular cyclic AMP (cAMP) plays two important roles in the development of *Dictyostelium discoideum* (for a review, see reference 9): as an acrasin for the chemotaxis of aggregating cells and as a regulator of gene expression. Addition of exogenous cAMP to aggregation-competent cells causes precocious expression of some developmentally regulated genes (2, 6, 13, 19, 26).

Recent evidence strongly suggests that control of gene expression by cAMP is mediated by transmembrane signal transduction in which extracellular cAMP binds to cell surface receptors and elicits intracellular responses (11, 27, 34). These findings have since been confirmed in other laboratories (10, 15). The identity of the second messengers required for signal transduction and the manner in which they affect gene expression are now being investigated. Although exogenous cAMP appears to stabilize some mRNAs (17), it has also been shown to regulate the transcription of some genes (6, 12, 16, 18, 39). The focus of current research is identification of the *cis*-acting sites at which extracellular agonists regulate transcription.

A gene for uridine diphosphoglucose pyrophosphorylase (UDPGP1) is expressed in the absence of added cAMP, but its transcription is induced 5- to 15-fold by addition of cAMP to aggregation-competent cells (13). The fact that this gene is expressed in the absence of added cAMP but is stimulated by exogenous cAMP provides an attractive opportunity to distinguish between elements required for transcription per se and elements required for regulating transcription in response to a stimulus of extracellular cAMP. We have previously reported the isolation and sequence characterization of cDNA and genomic clones of the UDPGP1 gene and the mapping of the start of transcription of the gene to a 6-nucleotide region, suggesting that transcription occurs from a single promoter (8, 31). Although the gene is expressed in vegetative cells, it is also developmentally regulated. Its mRNA level declines in late-log-phase axenic cells and in early development but is then induced later in development at 9 to 10 h, when tight aggregates are formed. Its expression is then reduced twofold at 12 h and increases again at 15 to 20 h. Expression of this gene is regulated primarily at the transcriptional level during development (29). We now describe the identification of a sequence element necessary for induction of the UDPGP1 gene in response to stimulation with extracellular cAMP. The sequence, which we call a cAMP response sequence, is required for inducibility but not for transcription per se. A second *cis*-acting regulatory element located immediately downstream of the cAMP response sequence is essential for basal cAMP-independent transcription and appears to become nonfunctional late in development. The position of the cAMP response sequence coincides with a site that shows increased sensitivity to DNAse I when the gene is actively transcribed. Deletion of the cAMP response sequence also leads to the loss of inducibility of the gene during normal development. Therefore, the cAMP response sequence is required for developmental expression of the gene late in development.

MATERIALS AND METHODS

Growth and development of *D. discoideum*. Strain AX-3 was used in all experiments. Axenically grown transformed cells at 2×10^6 to 3×10^6/ml were harvested, washed, and developed on Millipore membrane filters as previously described (13). Cells were made aggregation competent in suspension at 5×10^6/ml by shaking in MES-PDF on a gyratory shaker at 150 rpm for 6 h as described previously (11). cAMP was then added to 200 μM, and the shaker speed was increased to 230 rpm to prevent the formation of agglomerates. cAMP was replenished every 2 h thereafter.

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Cells were harvested 6 and 12 h after the initial cAMP addition.

Construction of a plasmid containing the entire UDPGP1 gene. Three overlapping genomic clones, pUC125, MRP900, and pUC123, described previously (31), were used to reconstruct a 7-kilobase-pair (kb) genomic fragment containing the UDPGP1 mRNA-coding regions, 2 kb of the 5'-flanking region, and 2 kb of the 3'-flanking region (see Fig. 3A). The 5' noncoding region of the gene and part of the coding region were obtained from the *Hin*dIII-*Eco*RV fragment of pUC125. The middle part of the RNA-coding region of the gene was obtained from MPR900 by cutting within the gene with *Eco*RV and in the plasmid with *Pvu*II. This fragment was ligated to the *Eco*RV site of pUC125. The resulting plasmid was digested with *Hin*dIII to release a 3.5-kb *Hin*dIII fragment including the 5'-flanking region and 1.5 kb of the UDPGP1 gene. The plasmid was also cut with *Fsp*I to destroy vector sequences. This 3.5-kb *Hin*dIII fragment was ligated into the *Hin*dIII site of pUC123, which contained the 3' end of the UDPGP1 gene. The construct harboring the entire UDPGP1 gene was termed pUP1. Subsequently, the 6.5-kb *Hin*cII-*Sst*I fragment of pUP1 containing the UDPGP1 gene was inserted into the *Hin*cII- and *Sst*I-digested vector pTZ18U (U.S. Biochemical Corp.) to give rise to plasmid pTUP1 (see Fig. 3B).

Construction and sequencing of 5' deletions for transformation. Plasmid pTUP1 was linearized by partial digestion with *Sca*I and then digested with *Bgl*II to remove a 1-kb fragment of the UDPGP1 gene, which was replaced by a 270-base-pair (bp) *Bgl*II-*Sca*I fragment of the human creatine kinase M (hCK-M) cDNA clone, pJN2-CK-M (25). The resulting plasmid, pTUP1-CK, harbored a reporter gene, UDPG1–hCK-M, whose chimeric transcript contained hCK-M cDNA sequences and was 730 nucleotides shorter than the normal UDPGP1 mRNA. Deletions were introduced into the 5'-flanking region of this modified UDPGP1 gene by using exonuclease III and mung bean nuclease as follows. pTUP1-CK was digested with *Hin*cII and *Sph*I, cleaving sites in the polylinker at the 5' end of *D. discoideum* genomic sequences. The DNA of the 5'-flanking region was resected to various extents by treatment with exonuclease III. It was then digested with mung bean nuclease to create blunt ends. The resulting plasmids were recircularized by ligation and used to transform competent *Escherichia coli* cells. Individual 5'-deletion clones were isolated, and their deletion endpoints were determined by the dideoxy-chain termination sequencing method, using double-stranded DNA as templates (37).

DNase I-hypersensitive sites. Procedures described previously (24, 28) were used for isolation of nuclei, DNase I digestion, DNA extraction, gel electrophoresis, Southern transfer, and hybridization. Nuclei isolated from vegetative cells and slugs (1.5×10^9 cells per ml) were digested with 5 U of DNase I per ml for 15 s to 20 min at 25°C. As a control for endogenous nuclease activity, a sample of the nuclei was incubated for 20 min at 25°C in the absence of DNase I. Purified genomic DNA (20 μg) was treated with 1 U of DNase I per ml for periods of time ranging from 15 to 60 s at 0°C. The nuclear DNA was purified and cut to completion with *Hin*dIII and *Bgl*II, and 5-μg samples were size fractioned on 1.5% vertical agarose gels. The Southern blots (35) were hybridized with the 202-bp *Eco*RI-*Bgl*II fragment of MPR900 (31) labeled with [α-^{32}P]dATP by the random-priming method of Feinberg and Vogelstein (7).

DNA-mediated transformation of *Dicytostelium* amoebae. DNA-mediated transformation was performed essentially as described by Nellen et al. (21, 22). AX-3 cells were cotransformed with 5'-deletion derivatives of pTUP1-CK and B10SX (21), which contains the neomycin resistance gene as a selectable marker. The following modifications were incorporated into the experiments. The calcium phosphate precipitates of the DNA were left on the cells for 7 h, and the glycerol shock was carried out with an 18% glycerol solution for 7 min. Selection for transformed cells was started 16 h after glycerol shock, using 20 μg of G418 per ml. After 5 days, the cells were harvested, placed in 50 ml of HL-5 medium (13), and grown axenically in suspension culture. To select stable transformants, G418 was added to 10 μg/ml when the density was 2×10^5 cells per ml.

Nucleic acid analysis. Expression of the UDPGP1–hCK-M gene was measured by Northern (RNA) blot analysis of total cell RNA prepared as described by Hatch and Bonner (14) and Haribabu et al. (12). Hybridization probes were labeled with [α-^{32}P]dATP either by the random-priming method or by nick translation. Filters were hybridized by using the UDPGP1 cDNA or the 270-bp *Sca*I-*Bgl*II fragment of the hCK-M cDNA clone (25). The intensity of the bands was quantitated by densitometric scanning, using a Shimadzu cs 9000 scanner. The approximate copy number per cell of the pTUP1-CK DNA was determined by Southern blotting (35), for which genomic DNA was isolated as described by Reymond (33), and hybridization was performed with the UDPGP1 cDNA as a probe.

RESULTS

Sequence of the 5'-flanking DNA of the UDPGP1 gene. Our immediate goal was to identify and characterize *cis*-acting sequence elements required for the regulation of transcription of the UDPGP1 gene in response to the stimulus of extracellular cAMP and to determine whether these elements function during development. A genomic clone of the UDPGP1 gene, pUC125, that contains approximately 2,050 bp of 5'-flanking DNA (31) was used to determine the nucleotide sequence of the 5'-flanking region to −907. The 5'-flanking region has an A+T content of 90%, typical of *D. discoideum* noncoding DNA (Fig. 1). Within this A+T-rich region lie eight short G+C-rich sequence elements. Two of them, centered at −209 and −385, represent nearly perfect G+C-rich palindromes with A+T-rich cores. None of the short G+C-rich sequence elements upstream of the UDPGP1 gene show any significant homology with one another or with published sequence elements involved in the regulation of other genes in *D. discoideum* (3, 5, 6, 23, 30). Two approaches, mapping of DNase I-hypersensitive sites and deletion analysis, were used to determine the potential regulatory roles of these elements.

Mapping of DNase I-hypersensitive sites in the 5' end of the UDPGP1 gene. DNase I-hypersensitive sites are thought to be associated with regions at which regulatory proteins might act (for reviews, see references 4 and 32). To help in identifying putative *cis*-acting regulatory elements in the 5'-flanking region of the UDPGP1 gene, we looked for the presence of DNase I-hypersensitive sites before and after gene activation, using indirect end labeling (20, 40). We isolated nuclei from vegetative cells and from slugs and treated them with 5 U of DNase I per ml for 15 s to 20 min (see Materials and Methods). We next isolated nuclear DNA from the DNase I-treated nuclei and cut samples with *Hin*dIII and *Bgl*II, which gave a 3-kb fragment (+953 to −2040). Equal amounts of DNA were size fractioned in

FIG. 1. Nucleic acid sequence of the 5′-flanking region of the UDPGP1 gene. Horizontal arrows indicate the 5′ deletion endpoints, determined by sequencing, of pUTP1-CK 5′ clones that were used to stably transform AX-3 cells. The short G+C-rich regions are underlined. The position of the transcription start site is designated as nucleotide +1. The deletion endpoints of the plasmids used for transformation are indicated by arrows. The initiating ATG is indicated by Met.

agarose gels and blotted onto nitrocellulose membranes. Hybridization was performed with the terminal 202-bp *Eco*RI-*Bgl*II DNA fragment (from +751 to +953). For clarity only, the 60-s time points are shown in Fig. 2A (lanes 5 and 7). In vegetative cells grown axenically to late log phase, the gene is transcribed at a low level (29), and DNase I-hypersensitive sites were barely detectable (Fig. 2A, lane 5). However, when development proceeded to the slug stage and the gene was actively transcribed, several (six to eight) DNase I-hypersensitive sites appeared (Fig. 2A, lane 7). Their positions with respect to the transcription start site are indicated in Fig. 2B. There were five prominent sites at −95, −265, −395, −550 and −745. In this and other experiments, the −265 and −745 sites appeared to be doublets (at −265 and −215 and at −745 and −700). In addition, a weak site at approximately −850 was also evident but did not increase significantly during development. The positions of all of the hypersensitive sites coincided with the short G+C-rich elements (Fig. 1 and 2B). As a control to test for the presence of sequences sensitive to any endogenous nuclease activity, nuclei isolated from vegetative cells and slugs were incubated for 20 min in the absence of exogenous DNase I. Even when the gene was transcribed actively (slugs), no sites were detected that were preferentially susceptible to cleavage by an endogenous nuclease (Fig. 2A, lanes 4 and 6). Analysis of purified genomic DNA digested with DNase I at 0°C revealed no significant sequence-specific cleavage in the 5′-flanking region (Fig. 2A, lane 3).

Thus, sites hypersensitive to exogenous DNase I increased within the 5′-flanking region of the UDPGP1 gene when the gene was being transcribed vigorously, and they coincided with short G+C-rich sequence elements. This coincidence was previously observed in the 5′-flanking region of the cysteine proteinase I gene of *D. discoideum* (6, 28).

Identification of a cAMP response sequence. To test whether the short G+C-rich sequence elements that coincided with the position of DNase I-hypersensitive sites were involved in the regulation of UDPGP1 gene expression, we performed a deletion analysis of the 5′-end region. We reconstructed plasmid pUP1, containing a 7-kb *Hin*dIII-*Bgl*II genomic fragment that includes the UDPGP1 gene and approximately 2 kb of 5′- and 3′-flanking regions (Fig. 3A). Three overlapping genomic clones, pUC125, pUC123, and MPR900, characterized previously (31), were used to construct pUP1 (see Materials and Methods). Subsequently, a 6.5-kb genomic *Hin*cII-*Sst*I subfragment of pUP1 containing 1.5 kb of 5′-flanking sequences was inserted into the phagemid vector pTZ18 to give plasmid pTUP1 (Fig. 3B). To distinguish in transformation experiments between the transcript of the exogenous transforming UDPGP1 gene and the endogenous gene, we constructed pTUP1-CK, a derivative of pTUP1 containing a shortened exogenous gene and harboring an hCK-M reporter sequence (Fig. 3B). To do so, we removed a 1,000-bp *Bgl*II-*Sca*I fragment containing two short introns and 773 bp of coding sequences and replaced it with a 270-bp reporter fragment from the coding region of the hCK-M cDNA clone (25). The mRNA of the UDPGP1–hCK-M gene was, as expected, 1,200 nucleotides long rather than 1,700 nucleotides long, the size of the mRNA from the

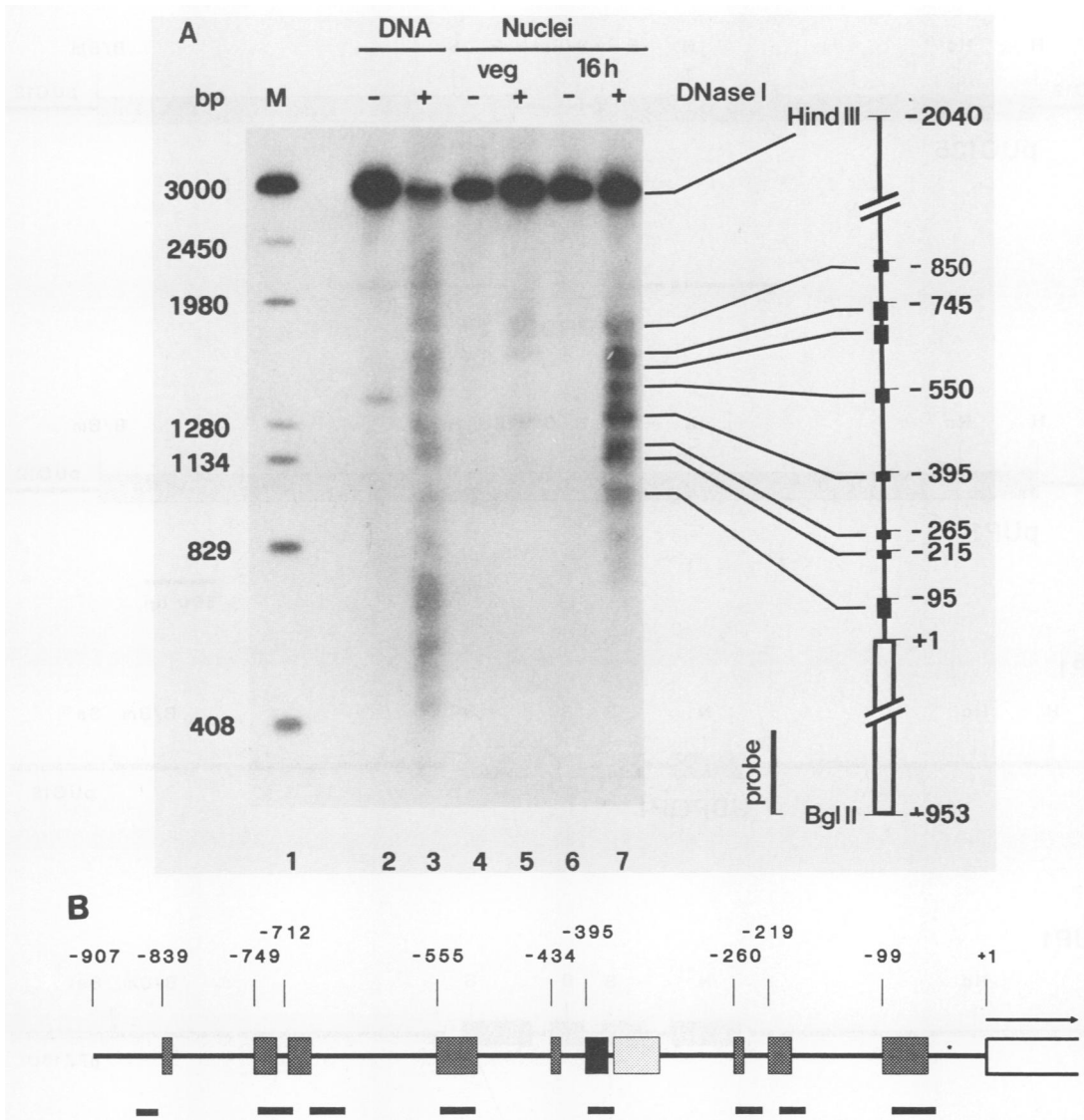

FIG. 2. DNase I-hypersensitive sites in the 5′-flanking region of the UDPGP1 gene. (A) Lanes: 1, pUC125 partially digested with *Dra*I and cut to completion with *Hin*dIII and *Sst*I (see Fig. 3 for the restriction map); 2, purified nuclear DNA digested with *Hin*dIII and *Bgl*II; 3, genomic DNA treated with DNase I (1 U/ml) for 15 s at 0°C; 4 and 6, nuclei isolated from late-log-phase vegetative cells and slugs incubated for 20 min at 25°C in the absence of DNase I; 5 and 7, nuclei digested with DNase I (5 U/ml) for 60 s. After the first incubation, the nuclear DNA was isolated and cut with *Bgl*II and *Hin*dIII. Samples of 5 μg were size fractionated on a 1.5% agarose gel, blotted onto a nitrocellulose membrane, and hybridized with the [α-^{32}P]dATP-labeled 202-bp *Eco*RI-*Bgl*II fragment of MPR900. Symbols: ■, position of a DNase I-hypersensitive site upstream of the transcription start site; □, transcribed region. (B) Map of the DNase I-hypersensitive sites in the 5′-flanking region of the UDPGP1 gene. Symbols: ▬, position of a DNase I-hypersensitive site observed at the slug stage; ▩, short G+C-rich sequence; ■, the cAMP response sequence defined in Results; □, position of the sequence element essential for the basal level of expression of the UDPGP1–hCK-M reporter gene.

endogenous gene. The difference in size (500 nucleotides) of the two mRNAs and the presence of the hCK-M reporter sequence in the transforming DNA allowed simultaneous monitoring, in Northern blots, of the transcripts from the exogenous transforming gene and the endogenous gene (Fig. 4B).

We have recently transformed AX-3 cells with pTUP1-CK and shown that, as in untransformed vegetative cells, the level of UDPGP1 mRNA in transformants is variable (11, 13, 29). Usually, untransformed vegetative cells harvested in early log phase have a high level of UDPGP1 mRNA, but cells in late log phase have a lower level. When transformed cells were starved in suspension, the UDPGP1 mRNA levels and transcription rates dropped three- to fivefold. Addition of cAMP or progress into tight aggregates caused a 3- to 15-fold rise in the mRNA levels or the rates of transcription above the levels in control cells. After transformation with plasmid pTUP1-CK, harboring the UDPGP1–hCK-M gene, both the endogenous UDPGP1 gene and the exogenous transformed gene were coordinately regulated in response to exogenous cAMP (29). These results indicated that 1,500 bp of 5′-flanking DNA is sufficient for proper regulation of the fusion gene. On the basis of these results, we used the plasmid pTUP1-CK as parental DNA for a deletion analysis of the 5′-flanking region of the UDPGP1 gene. We constructed a series of 5′ deletions from the unique *Hin*cII site,

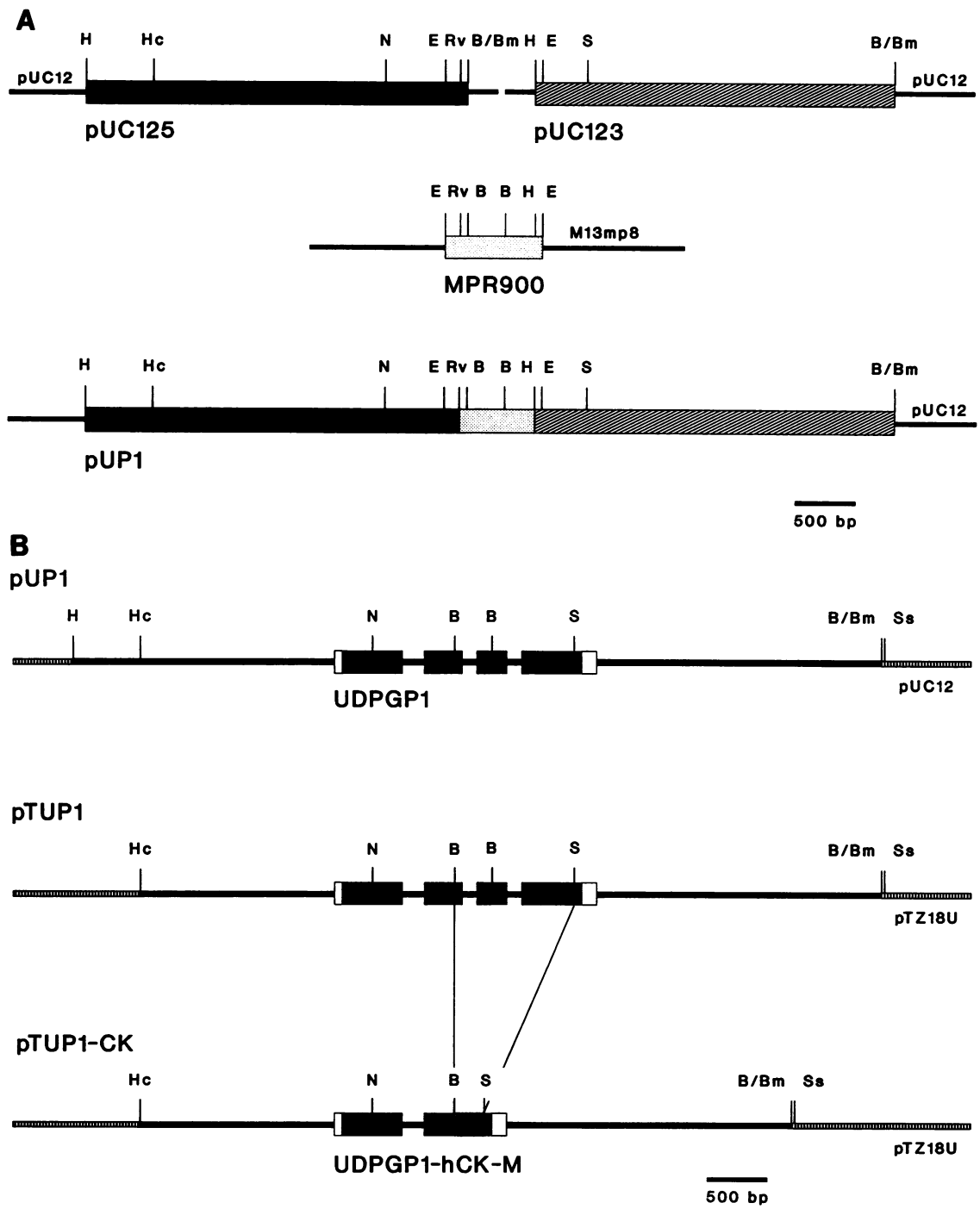

FIG. 3. (A) Reconstruction of the full-length genomic clone of the UDPGP1 gene. Restriction maps of the overlapping genomic clones pUC125, MPR900, and pUC123 and the reconstructed full-length genomic clone pUP1 are shown. Construction of pUP1 is described in Materials and Methods. Abbreviations: B, *Bgl*II; B/Bm, *Bgl*II-*Bam*HI fusion; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; N, *Nsi*; S, *Sca*I. (B) Structures of the UDPGP1 gene and the UDPGP1–hCK-M reporter gene. Restriction map of pUP1 and the intron-exon structure of the UDPGP1 gene are shown at the top. Symbols: ■, exons; □, untranslated regions; —, introns and intergenic regions. Plasmid pTUP1 consists of the 6.5-kb *Hin*cII-*Sst*I fragment that was inserted into pTZ18U. The restriction map of plasmid pTUP1-CK, containing the UDPGP1–hCK-M reporter gene, is shown at the bottom. In this gene, the 1,000-bp *Sca*I-*Bgl*II fragment of the UDPGP1 gene was removed and replaced with a 270-bp *Bgl*II-*Sca*I fragment (▩) from the coding region of the hCK-M cDNA clone (25). Details of the construction of pTUP1-CK are described in Materials and Methods. Restriction sites are abbreviated as for panel A. Ss, *Sst*I.

using exonuclease III and mung bean nuclease (see Materials and Methods). More than 100 individual clones containing deleted DNA were isolated, and their deletion endpoints were mapped by restriction enzyme analysis. The deletion endpoints of approximately 40 selected plasmids were determined by double-stranded DNA sequencing (37). AX-3 cells were cotransformed with plasmid B10SX and cloned plasmids containing 5′ deletions of the UDPGP1 gene (Fig. 5A)

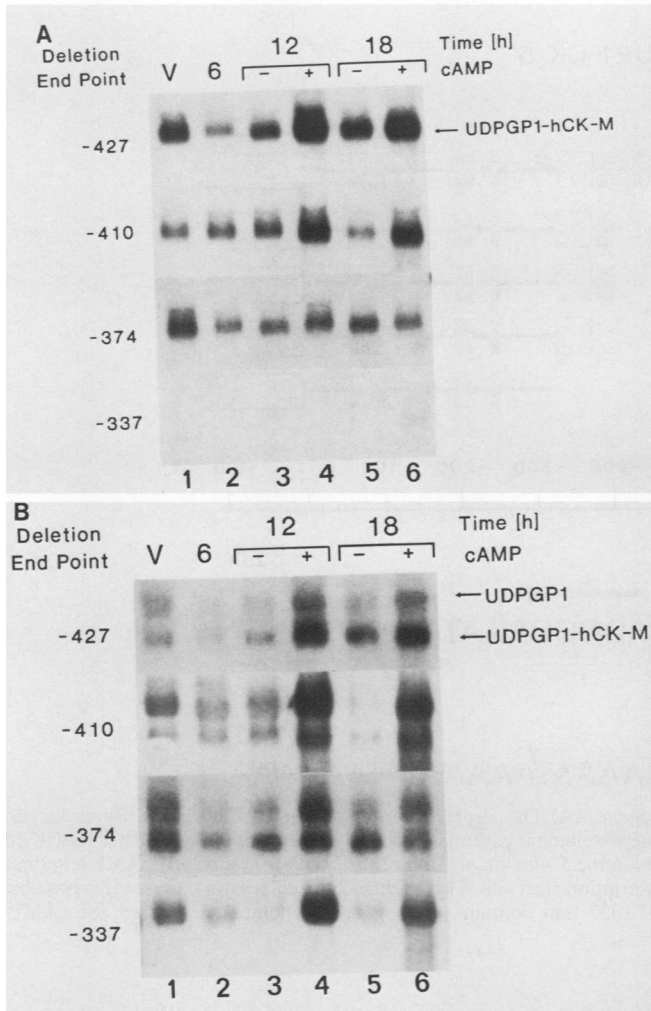

FIG. 4. Effect of the deletions in the 5'-flanking region on the cAMP-dependent expression of the UDPGP1–hCK-M reporter gene. Transformed AX-3 cells were treated with cAMP and harvested at the indicated times (see Materials and Methods). Total cell RNA was isolated and analyzed by Northern blots. Hybridizations were performed with the following [α-^{32}P]dATP-labeled probes: the 270-bp *Bgl*II-*Sca*I fragment of the hCK-M cDNA clone (A) or the UDPGP1 cDNA clone (B).

as described in Materials and Methods. Analysis by Southern blots of either single clones or transformed populations revealed that the numbers of copies of the plasmid integrated into the genome were fairly uniform: typically, 20 to 50 copies of the plasmids were integrated in tandem repeats (results not shown).

To examine expression of the UDPGP1–hCK-M fusion gene in response to exogenous cAMP, exponentially growing cells from transformed populations were harvested, washed, and starved to aggregation competence by shaking in suspension for 6 h. cAMP (200 μM) and was added at 6 h and every 2 h thereafter. Cells were harvested after 12 and 18 h, and total cell RNA was isolated and analyzed by Northern blots, using as probes labeled fragments of hCK-M cDNA (Fig. 4A) or UDPGP1 cDNA (Fig. 4B). The analysis in Fig. 4A shows transcripts of the exogenous transforming genes only because the hybridization probe was the hCK-M cDNA. However, Fig. 4B shows both the longer transcript of the endogenous gene and the shorter transcript of the exogenous gene because the probe was the UDPGP1 cDNA. Reduction of the 5'-flanking region of the UDPGP1–hCK-M construct from 1,500 to 410 bp had no effect on the basal levels of mRNA or on the ability of the gene to be induced after stimulation with exogenous cAMP. The −427 and −410 constructs were responsive to cAMP at 12 h (Fig. 4A, lanes 3 and 4) and 18 h (lanes 5 and 6). By contrast, a further deletion of 36 bp of 5'-flanking DNA to −374 severely reduced the cAMP inducibility of the transformed gene at 12 h (lanes 3 and 4) and abolished it at 18 h (lanes 5 and 6). Use of the UDPGP1 cDNA probe provided an internal control for variations in the inducibility of the endogenous gene (Fig. 4B). Each transforming gene, UDPGP1–hCK-M-427 or UDPGP1–hCK-M-410, was still coordinately regulated with the endogenous gene in the same population of cells and was inducible by addition of cAMP (Fig. 4B, −427 and −410). As expected from the results shown in Fig. 4A, deletion of the next 36 bp to −374 abolished inducibility of the transforming gene but not of the endogenous gene in the same cells. However, the basal level of expression of the −374 gene was similar to that of the endogenous gene in vegetative cells and in cells not treated with cAMP (Fig. 4B, lanes 1, 2, 3, and 5).

Densitometric scanning of the autoradiograms showed that the exogenous gene (UDPGP1–hCK-M) was induced to the same extent as the endogenous counterparts (UDPGP1) in the −427 and −410 transformants (Table 1). In the −374 transformant, in contrast, the exogenous gene was not induced by addition of cAMP (1.1-fold at 12 h and 0.56-fold at 18 h), whereas the endogenous gene was induced normally (5.7-fold at 12 h and 2-fold at 18 h).

TABLE 1. Quantitation of the effect of the 5' deletions on UDPGP1–hCK-M mRNA expression after cAMP stimulation and during development

mRNA	Relative mRNA level for given transformant[a]															
	After cAMP stimulation[b]								During development[c]							
	−427		−410		−374		−337		−427		−410		−374		−337	
	12 h	18 h	12 h	18 h	12 h	18 h	12 h	18h	10 h	20 h	10 h	20 h	10 h	20 h	10 h	20 h
UDPGP1–hCK-M	6.1	2.2	3.4	7.2	1.1	0.56	ND[d]	ND	2.4	3.4	2.1	6.9	0.49	0.17	ND	ND
UDPGP1	6.7	2.3	3.1	8.5	5.7	2.0	15.5	4.1	5.9	5.3	6.0	11.0	6.7	5.4	13.6	15.9

[a] Determined by densitometric scanning of autoradiogram of the data shown in Fig. 4 with a Shimadzu densitometer.
[b] Quantitated as the ratio of +cAMP to −cAMP at each time point with each transformant.
[c] Change in mRNA levels during development, quantitated relative to the level at 5 h because the mRNA levels of both genes were reproducibly low at this stage of development.
[d] ND, Not determined because no hybridization signals were detected.

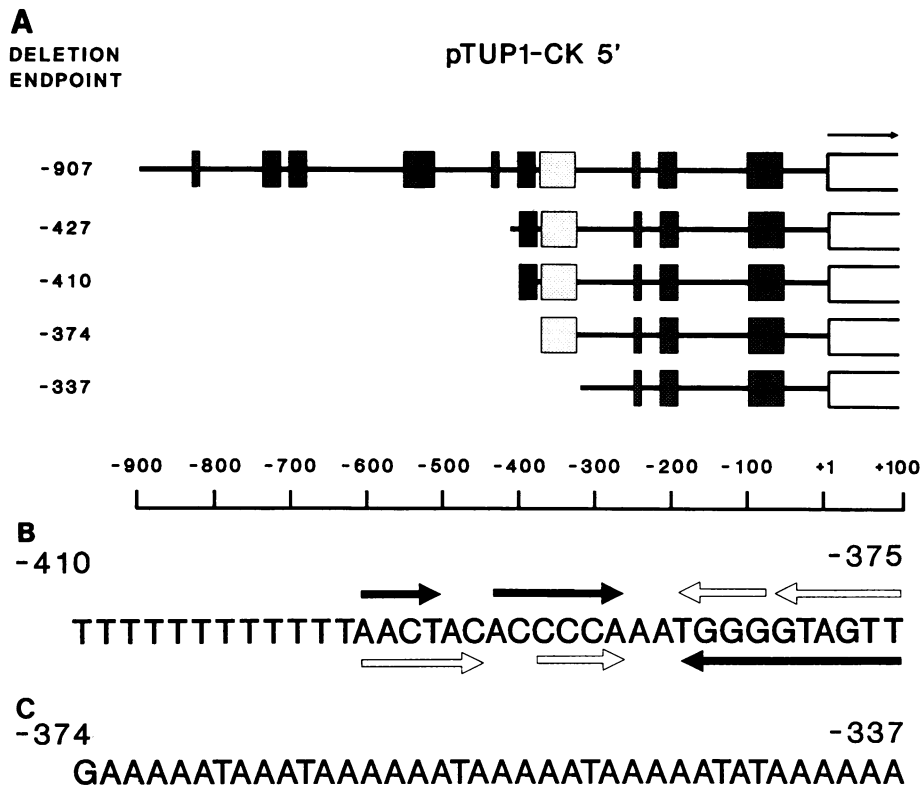

FIG. 5. Regulatory regions in the 5'-flanking region of the UDPGP1 gene. (A) The 5'-deletion derivatives of pTUP1-CK. Symbols: ▩, position of short G+C-rich region; ■, cAMP response sequence; □, sequence element essential for basal expression of the UDPGP1–hCK-M reporter gene. (B and C) Nucleotide sequences of the regulatory elements in the 5'-flanking region of the UDPGP1 gene. (B) cAMP response sequence that lies between positions −410 and −374 upstream of the transcription start site. The open and closed arrows indicate two possible types of inverted repeats. (C) Sequence between positions −374 and −337 that contains the regulatory element necessary for cAMP-independent expression of the UDPGP1 gene.

The conclusion that the UDPGP1 gene is transcriptionally regulated in response to cAMP has been independently confirmed by use of nuclear run-on experiments in which several other genes were also analyzed (B. Haribabu, J. Pavlovic, and R. P. Dottin, manuscript in preparation). These experiments also show that a sequence necessary for induction by cAMP resides between bp −410 and −374. This sequence, which we call a cAMP response sequence, consists of a 12-bp T-rich sequence and a palindromic region. Two possible palindromes can be identified (Fig. 5B). Either palindrome would contain 20 complementary nucleotides in a 24-bp region. Ten contiguous nucleotides preceding at the 3' end are complemented by nucleotides in the 5' half. Other features include (1) a G_4 stretch in the 3' half matched by a C_4 stretch in the 5' half and (2) an AA core at the axis of symmetry. Interestingly, the most prominent DNase I-hypersensitive site that appeared at the slug stage mapped to approximately the same position (Fig. 2). Deletion of the 37 bp adjacent to the cAMP response sequence in the −337 construct resulted in complete abolition of even the basal level of transcription, since no transcript from that exogenous transformed DNA was seen (Fig. 4, −337). The pattern of transcription of the endogenous gene in the same cells was unaffected (Fig. 4B, −337). We conclude that the 37-bp A-rich region extending from bp −374 to −337 (Fig. 5C) is necessary for basal expression of the gene. From one population to another, there are some differences in the basal level and therefore in the induction in response to cAMP. We attribute these differences to differences in the number and site of integration of the transforming DNA in different populations and to the fact that they grow and develop at slightly different rates. Therefore, it is most informative to compare unstimulated versus stimulated levels of expression within the same population rather than between populations. Expression of the endogenous gene provides a reliable internal control in these experiments. Deletion of the 36-bp region between −410 and −374 clearly prevents only the transforming gene from being induced, whereas the endogenous gene is regulated by cAMP addition. However, in the same population of cells, both genes are transcribed in the absence of the stimulus.

The cAMP response sequence is also required for gene induction during normal development. The previous experiments showed that exogenous cAMP exerts its effect on transcriptional induction of the UDPGP1 gene through a cAMP response sequence element located between −374 and −410. We also wished to test whether the same *cis*-acting element is required for induction of the gene during normal development. Cells transformed with the deletions shown in Fig. 5A were washed and placed on nitrocellulose filters to allow development. The cells were harvested after 5, 10, 15, and 20 h, and total cell RNA was isolated as described above and analyzed by Northern blots. Labeled cDNA fragments of the hCK-M gene (Fig. 6A) or the UDPGP1 gene (Fig. 6B) were used as hybridization probes.

As expected, deletion of the 5'-flanking region from bp 1500 to 427 or 410 bp upstream of the transcription start site did not severely affect the proper temporal regulation of the

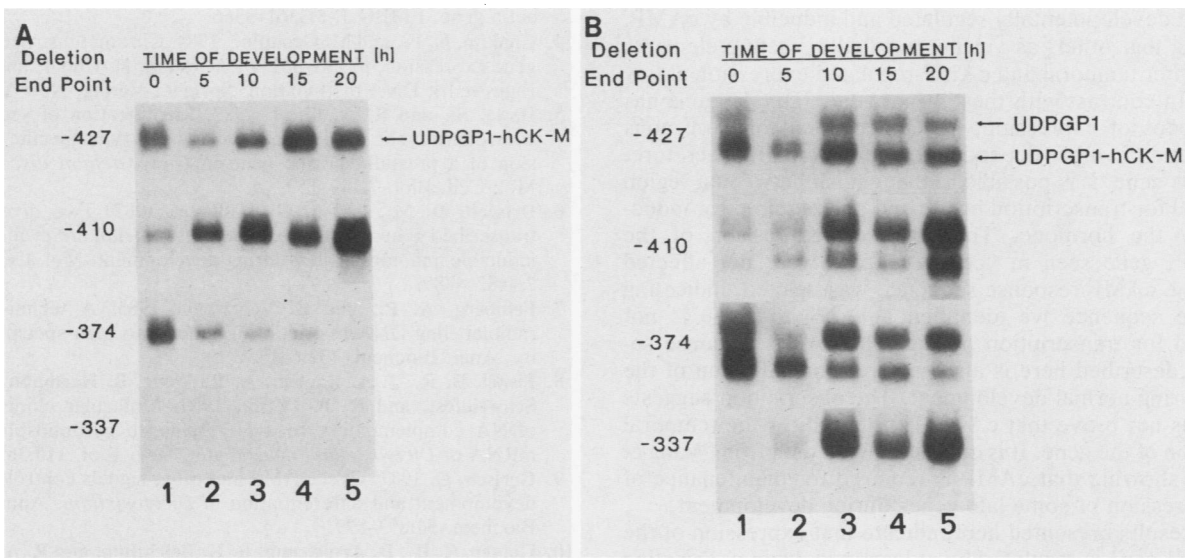

FIG. 6. Effect of deletions in the 5'-flanking region on developmental regulation of the UDPGP1–hCK-M reporter gene. Exponentially growing, transformed AX-3 cells were developed on Millipore membrane filters as described in Materials and Methods. Cells were harvested at the indicated times of development, and total cell RNA was isolated and analyzed by Northern blots as described in Materials and Methods. Hybridizations were performed with the following [α-^{32}P]dATP-labeled probe: the 270-bp *Bgl*II-*Sca*I fragment of the hCK-M cDNA clone (A) or the UDPGP1 cDNA clone (B).

UDPGP1–hCK-M fusion gene (Fig. 6). As previously reported, in vegetative cells the mRNA levels of the endogenous and the transformed genes were variable (11, 13, 29; Fig. 4B, −427 and −410, lane 1). Furthermore, the timing of development was variable between different transformed populations. For example, in the −427 transformants, both genes appeared to be slightly delayed in their patterns of expression. Nonetheless, the transformed genes were still coordinately regulated with the endogenous UDPGP1 gene in the same cells during development. At the culmination stage (20 h), the mRNA levels of −427 and UDPGP1 or of −410 and UDPGP1 increased 5- to 11-fold above the levels seen at 5 h (Fig. 6B, −427 and −410 lanes 2 and 5; Table 1). Further deletion of the 5'-flanking region from positions −410 to −374 did not affect expression of the transformed gene in vegetative cells (Fig. 6, −374, lanes 1). However, it did affect mRNA levels later in development, since that gene was no longer inducible at 10 h or at 20 h (Fig. 6, −374, lanes 3 to 5; Table 1). The UDPGP1–hCK-M mRNA levels seen in vegetative cells decreased steadily during late development until by the 20-h stage it was less than one-fifth the level seen at 5 h (Fig. 6, lanes 3 to 5; Table 1). Therefore, the cAMP response sequence is essential for induction of the gene in late development, and the basal promoter activity observed with the −374 deletion in vegetative cells appears to become nonfunctional in later development. No expression of the UDPGP1–hCK-M gene was observed in cells transformed with pTUP1-CK derivatives containing only 337 bp of 5'-flanking sequence (Fig. 6, −337). As before, the behavior of the endogenous UDPGP1 gene in the −337 transformants was not significantly affected and showed normal mRNA levels during development (Fig. 6B, −337).

These results show that the sequence element necessary for cAMP induction is also crucial for the proper induction and temporal regulation of the UDPGP1 gene during later development. Furthermore, a second, A+T-rich *cis*-acting sequence located immediately downstream of the cAMP-responsive element appears to be essential for any transcription of the gene.

DISCUSSION

Our studies attempted to identify and characterize regulatory sites associated with the UDPGP1 gene. The G+C-rich sequence elements in the 5' end all coincide with DNase I-hypersensitive sites, and in general their sensitivity to DNase I increases when the gene is transcriptionally active during development. A large body of evidence indicates that DNase I-hypersensitive sites are associated with *cis*-acting regulatory elements. In the region between the cysteine proteinase I gene and the DG17 gene of *D. discoideum*, four short G-rich regions that are putative regulatory elements coincide with the position of four DNase I-hypersensitive sites (6, 28). A DNase I-hypersensitive site that increases with gene activity has also been observed upstream of a developmentally regulated gene (SC253) by Ayres et al. (1). The appearance of the site was correlated with the activity of the gene. In the 5'-flanking region of the UDPGP1 gene, the most prominent DNase I-hypersensitive site is located within the cAMP response sequence approximately at −380 and is associated with a G+C-rich element centered at nucleotide −385 (Fig. 2B). However, the function of the other DNase-hypersensitive sites has not yet been determined. It is likely that the most distal sites beyond −500 are associated with a second gene starting at −1100 that is transcribed divergently from the UDPGP1 gene (unpublished data). The hypersensitive sites closer to the start of transcription of the UDPGP1 gene might more likely be involved in expression of the UDPGP1 gene.

The elements necessary for proper temporal expression of developmentally regulated genes in *D. discoideum* described so far are also essential for any transcription (3, 5, 6, 23, 30). In the cysteine proteinase 2 gene, a short G+C-rich region located approximately 230 bp upstream of the transcription start site is required for efficient expression both during development and in response to exogenous cAMP. Moreover, deletion of this G+C-rich region led to the loss of expression (5) or a 50-fold reduction in mRNA levels (30). The residual expression observed by Pears and Williams (30)

was still developmentally regulated and inducible by cAMP, implying that other, as yet unidentified *cis*-acting elements may confer temporal and cAMP-regulated expression at low levels. In contrast with these genes, the UDPGP1 gene has two modes of expression, one independent of exogenous cAMP and the other dependent on added cAMP. Therefore, with this gene, it is possible to distinguish between a region essential for transcription per se and one required for induction by the hormone. The levels of expression of the UDPGP1 gene seen in vegetative cells were not affected when the cAMP response sequence was deleted, indicating that the sequence we identified at −374 to −410 is not required for transcription per se. The cAMP response sequence described here is also required for induction of the gene during normal development. The observation suggests but does not prove that cAMP is required for the temporal induction of the gene. It is consistent with data from Wang et al. (38) showing that cAMP is required for maintenance of the expression of some late genes during development.

The results presented here indicate that expression of the UDPGP1 gene is regulated by at least two distinct *cis*-acting elements, a cAMP response sequence required for the transcriptional activation of the gene during later development and a A+T-rich region at −337 to −374 necessary but quite probably not sufficient for expression of the gene in vegetative cells. Since vegetative cells do not secrete or respond to cAMP, this sequence confers cAMP-independent expression to the gene in vegetative cells. Furthermore, because the −374 transformants do not continue to express the gene in late development, the element appears to become nonfunctional at that stage. Downstream from this sequence, at −301 to −327, lies a 27-bp sequence element containing 24 T residues. Similar sequences have been reported to be promoter elements necessary for constitutive transcription of several yeast genes (36). Previous results have shown that the UDPGP1 gene is transcribed from a single promoter (31). Therefore, the two regulatory sequences do not act independently at separate promoters. Although the cAMP response sequence is necessary for transcriptional induction, it may not be sufficient, and other regulatory sequence elements may act in conjunction with the two *cis*-acting elements identified to control expression of the UDPGP1 gene. Further studies are required to clarify this issue and to identify nucleotides within each sequence element that are essential for the activity of the gene.

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