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The Cyclic Nucleotide Phosphodiesterase Gene of *Dictyostelium discoideum* Contains Three Promoters Specific for Growth, Aggregation, and Late Development

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The cyclic nucleotide phosphodiesterase (phosphodiesterase) plays essential roles throughout the development of *Dictyostelium discoideum*. It is crucial to cellular aggregation and to postaggregation morphogenesis. The phosphodiesterase gene is transcribed into three mRNAs, containing the same coding sequence connected to different 5′ untranslated sequences, that accumulate at different times during the life cycle. A 1.9-kilobase (kb) mRNA is specific for growth, a 2.4-kb mRNA is specific for aggregation, and a 2.2-kb mRNA is specific for late development and is only expressed in prestalk cells. Hybridization of RNA isolated from cells at various stages of development with different upstream regions of the gene indicated separate promoters for each of the three mRNAs. The existence of specific promoters was confirmed by fusing the three putative promoter regions to the chloramphenicol acetyltransferase reporter gene, and the analysis of transformants containing these constructs. The three promoters are scattered within a 4.1-kilobase pair (kbp) region upstream of the initiation codon. The late promoter is proximal to the coding sequence, the growth-specific promoter has an initiation site that is 1.9 kbp upstream of the ATG codon, and the aggregation-specific promoter has an initiation site 3 kbp upstream.

When *Dictyostelium discoideum* amoebae are deprived of nutrients, they initiate a developmental program that results in the formation of a multicellular organism composed of differentiated cell types. The aggregation process that takes place early in development is driven by chemotaxis toward cyclic AMP (cAMP) (6), and one of the earliest events in development is the elaboration of the biochemical apparatus required for chemotaxis toward cAMP (for reviews see references 7, 18, and 23). Soon after starvation, the cells synthesize an adenylate cyclase, a cell surface cAMP receptor, an extracellular phosphodiesterase, and a specific phosphodiesterase inhibitor. These proteins, among others, function coordinately to allow the cells to aggregate by chemotaxis. Cells secrete pulses of cAMP every 5 to 6 min, and neighboring cells respond by moving toward elevated concentrations of cAMP and by emitting cAMP to create a relay. The binding of cAMP to cell surface receptors activates second messenger cascades involving G proteins (27) that lead to a variety of cellular events (for reviews, see references 13 and 20), including the chemotactic response and the regulation of genes specific for development. The cAMP receptor is down regulated and phosphorylated upon constant stimulation by cAMP (25, 53), and this results in the adaptation of cellular responses (52). The cyclic nucleotide phosphodiesterase (phosphodiesterase) acts outside the cell to reduce cAMP levels, limiting saturation and down regulation of the receptor. The phosphodiesterase exists in membrane-bound and free extracellular forms (30, 47). Its activity is regulated at the gene level (16) and is controlled at the protein level by a specific inhibitor (15). A mutation affecting one of the signal transduction pathways, *fgdA* (3, 22), prevents the induction of the aggregation-specific form of the phosphodiesterase mRNA (16).

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The requirement for phosphodiesterase during aggregation is demonstrated by the fact that mutants deficient in phosphodiesterase are unable to aggregate unless the enzyme is provided exogenously (2, 4). Complete development can be restored by production of the enzyme from the cloned gene, introduced into the cells by transformation (11). The phosphodiesterase plays a role in late development when chemotaxis is involved in morphogenesis leading to fruiting body formation (43, 45). Overproduction of phosphodiesterase blocks late development (10), and local addition of phosphodiesterase to developing prespore cells reverses the differentiation of these cells (54).

We have cloned the cDNA (28, 38) and the genomic DNA (37) coding for the phosphodiesterase. During early development two distinct mRNAs (1.9 and 2.4 kilobases [kb]) are transcribed from the phosphodiesterase gene. The 1.9-kb mRNA is present at a low level in vegetative cells and in early development. The 2.4-kb mRNA is induced soon after starvation and accumulates during aggregation. The accumulation of the 2.4-kb mRNA is increased by cAMP treatment but is blocked by cycloheximide (16). To understand the origin of these two mRNAs and to identify the factors and the second messenger pathways involved in the induction of the 2.4-kb mRNA, we have studied the structure of the phosphodiesterase gene. We have reported the complete sequence of the gene and demonstrated the existence of two promoters responsible for the transcription of the 1.9- and 2.4-kb mRNAs (37). These two mRNAs have the same coding sequence but differ in their 5′ untranslated sequences, which are derived from two different exons. The promoters responsible for the transcription of the 2.4- and 1.9-kb mRNAs are located about 3 and 2 kilobase pairs (kbp) upstream of the initiation codon, respectively (37).

In this report, we complete our description of the structure

and the expression of the phosphodiesterase gene. We show that in late development, there is a third phosphodiesterase mRNA. This mRNA is 2.2-kb long and appears after 10 h of starvation on filters, at a time when the cells have completed aggregation and are organized in preslug structures. This 2.2-kb mRNA is transcribed from a distinct promoter that lies adjacent to the phosphodiesterase coding sequence, in contrast with the promoters of the 2.4- and 1.9-kb mRNAs, which are distal. By the polymerase chain reaction (PCR) technique for amplification of cDNAs, we have recovered and sequenced cDNA clones derived from the 5′ end of the 2.2-kb mRNA, and this allowed us to locate the start site of transcription. We show here that the phosphodiesterase gene is transcribed into three mRNAs that are expressed at different times during growth and development and we discuss the significance of this complex organization.

MATERIALS AND METHODS

Enzymes and chemicals. Most of the enzymes and chemicals were the same as those used in previous work (37), except that the *Taq* polymerase was purchased from Beckman Instruments, Inc.

Conditions for development and transformations. The axenic strain AX3-K was used for all of the experiments except for the separation of prestalk and prespore cells, where strain NC4 was used. Strain AX3-K was grown in HL/5 medium (14), and strain NC4 was grown on lawns of *Klebsiella aerogenes* as described by Sussman (50). The conditions for development in liquid and on Whatman no. 50 filters have been previously described (16). The Sörensen buffer used to starve the cells was 17 mM Na_2H–KH_2PO_4 (pH 6.0) supplemented with 50 μM $CaCl_2$. The separation of prestalk and prespore cells from slugs by density gradient centrifugation was performed as described by Ratner and Borth (39). Transformation experiments were performed as described by Nellen et al. (35). For the transformations with the pAV-CAT derivatives we performed the subsequent chloramphenicol acetyltransferase (CAT) assays on populations of transformants resulting from one transformation plate.

Probes and plasmid constructions. The various probes used for Northern (RNA) blot hybridizations were prepared by purification of the appropriate restriction fragments on low-melting-temperature agarose gels and were labeled by the random priming method (12). Probes A, D, and C were composed of the restriction fragments of the phosphodiesterase locus shown in Figure 1. Probe B was prepared from a cDNA clone derived from the 5′ end of the 1.9-kb mRNA cloned into pUC13 (37). This cDNA extends from primer A to the 5′ end of exon II (see Fig. 6). The plasmid was cut with *Bst*BI (an isoschizomer of *Asu*II) and *Hin*dIII (in the multiple cloning site sequence), liberating a fragment that contains all of exon II and 22 bp of exon III (from the *Asu*II site to the splice site).

Plasmid pGP-I has been described previously (37). Plasmid pBDE-1 is a derivative of the transformation vector pB10TP-1 (9) constructed by replacing the 0.7-kbp *Hin*cII-*Hin*cII fragment of pB10TP-1 with the 2.6-kbp *Eco*RV-*Hin*cII fragment of pGP-1 (Fig. 1A). The plasmid selected carries the phosphodiesterase gene in the counterclockwise direction (*Eco*RV-site of the insert near the *Hin*dIII-site of the vector). The reporter gene coding for CAT was carried by the vector pAV-CAT (32) that provides a polylinker and a transcription terminator flanking the CAT gene as well as a G418 resistance gene. All of the fragments to be tested for their promoter activity were inserted into pAV-CAT by using the *Pst*I and *Sst*I sites of the polylinker. The fragments were first subcloned into pUC vectors in such a way that the direction of transcription would be compatible with the CAT coding sequence. pVCAT carries the *Bcl*I-*Sca*I fragment of the phosphodiesterase locus (Fig. 1A and 6). This fragment was cut from pC34 (37) and ligated into pUC18, cut with *Bam*HI and *Hin*cII, prior to insertion into pAV-CAT. pACAT carries the 1.6-kbp *Bcl*I-*Bcl*I fragment of the phosphodiesterase locus (Fig. 1A and 6). This fragment, cut from pC34, was first subcloned into the *Bam*HI site of pUC18. After the proper orientation was selected, the fragment was transferred into pAV-CAT. pLCAT carries the *Eco*RV-*Asu*II fragment of the phosphodiesterase locus. The *Eco*RV-*Asu*II fragment from pGP-I was cloned into pUC19 cut with *Sma*I and *Acc*I and was then transferred to pAV-CAT. The plasmids used, and their construction, are summarized in Table 1.

RNA extraction and analysis. Total RNA was extracted as described by Franke et al. (16). Poly(A)$^+$ RNA was purified on oligo(dT)-cellulose columns (31). Northern blots were performed as described previously (37), except that 0.45 μM Nytran membrane (Schleicher & Schuell) replaced GeneScreen Plus (DuPont, NEN Research Products).

Primer extension analysis. The primer extension analysis was performed as described previously (38) with two oligonucleotides complementary to the deduced mRNA sequence. Primer A was 5′-dGTATATTTTTTGTAGTTAT TCGAACTATA-3′, and primer B was 5′-dCACAATCT TCTTGTTGATGGGAATTTAC-3′. The locations of these oligonucleotides within the phosphodiesterase locus are shown in Fig. 5 and 6. For the nontransformed strain AX3-K, we used 10 μg of poly(A)$^+$ RNA for the primer extension reaction and loaded 1/10 of it on a 6% acrylamide–7 M urea sequencing gel, except for the sample shown in Fig. 4, lane 1, where we used 100 μg of total RNA and loaded one-third of the reaction mixture. For the strains transformed with pGP-1 or pBDE-1 we used 12 μg of total RNA for the extension reaction and loaded 1/10 of the reaction mixture on a sequencing gel.

Synthesis of specific cDNAs by the polymerase chain reaction (PCR). To synthesize cDNAs derived from the 5′ end of the 2.2- and 2.4-kb mRNAs, we used a protocol similar to the one described previously for the 1.9-kb mRNA (37) with the following modifications. For the first strand synthesis, we used primers A and B described above; as a second primer for the PCR amplification we used either a d(pC)12-18 oligonucleotide or primer C (5′-dCCCACAAACGCCACA CACTCAC-3′ (see Fig. 5 and 6). For amplifications with d(pC) 12-18 and primers A or B, we performed two rounds of amplification. The products of the first round of amplification were ethanol precipitated before the second amplification. Each round of amplification was composed of 35 cycles (94°C, 1 min; 50°C, 2.5 min; 50°C to 65°C, 1.5 min; 65°C, 2.5 min), except that for the first and last cycles, the extension time at 65°C was increased to 15 min. For amplifications with primers B and C, one round of amplification produced enough DNA for cloning into pUC13. The program used was composed of 35 cycles (94°C, 1 min; 55°C, 2.5 min; 70°C, 2.5 min), except that for the first and last cycles, the extension time at 70°C was extended to 15 min. The cloning and sequencing of the amplified fragments was performed as described previously (37).

Nuclear runoff. The preparation of synthetically active nuclei, radiolabeling of transcripts, and the preparation and hybridization of blots were as described by Nellen et al. (35).

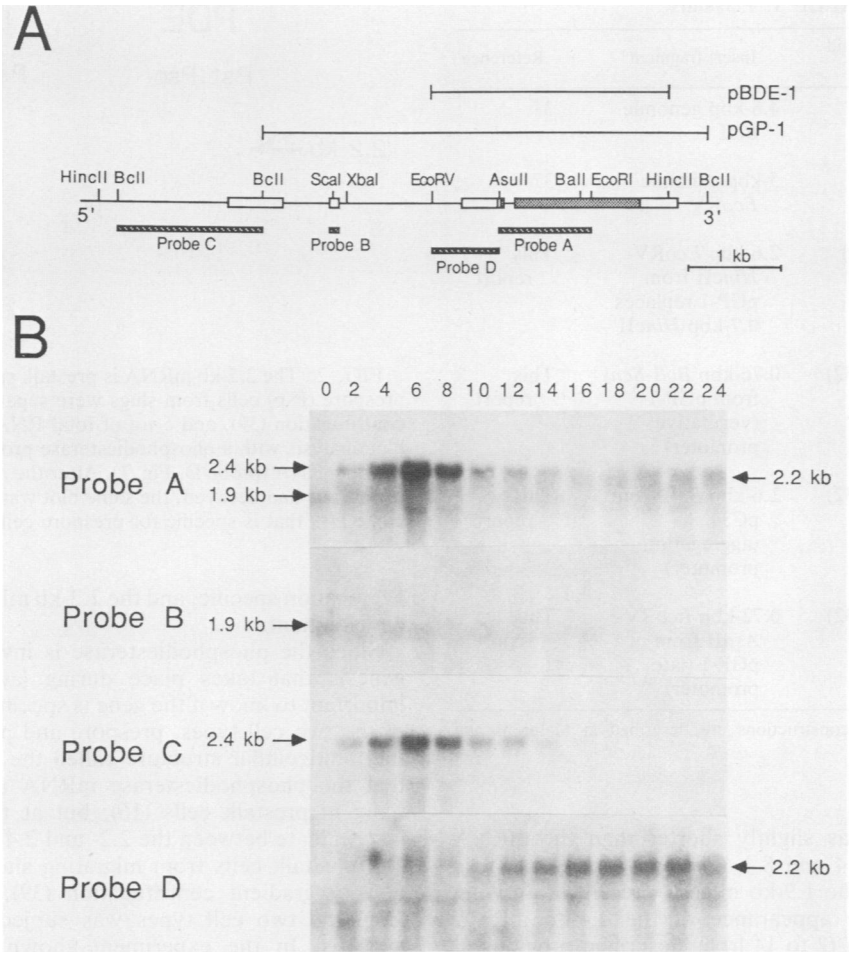

FIG. 1. The phosphodiesterase gene is transcribed into three mRNAs. (A) Restriction map of the phosphodiesterase locus. Symbols: ▭, transcribed nontranslated regions; ▩, translated regions. The probes used for the Northern blots and the genomic fragments carried by the plasmids used in the transformation experiments are indicated. (B) RNA samples extracted from cells developing on filters were subjected to Northern blot analysis (5 μg per lane) with the probes indicated in panel A. The time on filters is indicated in hours on the top of the figure. The sizes of the phosphodiesterase mRNAs are indicated on the side.

Nuclei were obtained from cells grown in HL/5 (5×10^6 cells/ml), from shaken cells starved in Sörensen buffer for 4 h and from shaken cells starved for 4 h in Sörensen buffer with addition of cAMP to a final concentration of 1 mM after 3 h of starvation. Conditions of growth and starvation were as described by Franke et al. (16). The plasmid pGP-1 was digested with restriction enzymes to separate the phosphodiesterase DNA sequence from the vector, and 2 μg of digested DNA was run in each lane of a 1% agarose gel. DNA fragments were blotted to GeneScreen Plus (DuPont, NEN Research Products). The membrane was hybridized overnight with the probe and washed by the method of Franke et al. (16) with the following modifications. Radiolabeled nuclear transcripts (3×10^6 cpm) were added to each membrane in the presence of 200 μg of herring sperm DNA per ml (Sigma Chemical Co.), 100 μg of yeast tRNA per ml (Bethesda Research Laboratories, Inc.), and 40 μg of polyadenylic acid per ml (Sigma Chemical Co.).

CAT assays. The amount of CAT protein produced by the strains transformed with derivatives of the pAV-CAT vector was measured by an enzyme-linked immunosorbant assay with a kit supplied by 5 Prime-3 Prime, Inc. (West Chester, Pa.). Cells from two filters were harvested in Sörensen buffer, pelleted, and suspended in 0.9 ml of CAT assay buffer (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, 3 mM KCl, 1% bovine serum albumin, 0.05% Tween 20 [pH 7.0]). Aliquots of 0.3 ml were flash frozen and stored at −80°C. Cells were lysed by three freeze-thaw cycles. The assays were performed according to the instructions provided by the manufacturer. The assay yielded a linear colorimetric response from 10 to 200 pg of CAT. The background was 0.8 ng/10^7 cells in the pAV-CAT vector (without any phosphodiesterase promoter).

RESULTS

The phosphodiesterase gene is transcribed into three mRNAs. We have shown that during early development, the phosphodiesterase gene is transcribed into the following two mRNAs: a 1.9-kb mRNA present in growing cells and a 2.4-kb mRNA induced shortly after starvation (16, 37). These two mRNAs are transcribed from distinct promoters and differ by their 5′ untranslated sequences (37). When RNA from cells developing on filters was subjected to Northern blot analysis with the phosphodiesterase coding region as a probe, we observed that the mRNA that accu-

TABLE 1. Plasmids

Plasmid	Parental plasmid (reference)	Insert fragment[a]	Reference
pGP-1	pBR322	4.8-kbp genomic *Bcl*I	37
pC34	pUC13	5-kbp genomic *Eco*RV	37
pBDE-1	pB10TP-1 (9)	2.6-kbp *Eco*RV-*Hin*cII from pGP-1 replaces 0.7-kbp *Hin*cII	This report
pVCAT	pAV-CAT (32)	0.76-kbp *Bcl*I-*Sca*I from pGP-1 (vegetative promoter)	This report
pACAT	pAV-CAT (32)	1.6-kbp *Bcl*I from pC34 (aggregation promoter)	This report
pLCAT	pAV-CAT (32)	0.72-kbp *Eco*RV-*Asu*II from pGP-1 (late promoter)	This report

[a] Details of the plasmid constructions are described in Materials and Methods.

mulated after 16 h was slightly shorter than the mRNA accumulated between 4 and 8 h (Fig. 1B, probe A). This Northern blot shows the 1.9-kb mRNA present in growing cells (zero hour), the appearance of the 2.4-kb mRNA shortly after starvation (2 to 14 h) as described previously, and a third mRNA, 2.2 kb long, present in late development.

To distinguish the different phosphodiesterase mRNAs, we hybridized the same RNA samples to probes specific for each transcript. Our knowledge of the gene structure (37) allowed us to design specific probes for the various mRNAs (Fig. 1A). The probe specific for the 1.9-kb mRNA (probe B) was a cDNA fragment derived from the 5′ end of the 1.9-kb mRNA (37) (see Materials and Methods for details). For the 2.4-kb mRNA, we used the 1.6-kbp *Bcl*I-*Bcl*I genomic fragment located at the 5′ end of the gene (probe C). For the 2.2-kb mRNA, we used the *Eco*RV-*Asu*II fragment (probe D). The choice of this fragment was dictated by the results described below. The 1.9-kb mRNA was present at a low level in vegetative cells, and its level decreased slowly during development (Fig. 1B, probe B). The 2.4-kb mRNA was not present in vegetative cells but was induced shortly after starvation and was present in the cells from 2 to 16 h, with a peak at 6 h (Fig. 1B, probe C). The 2.2-kb mRNA was present in the cells from 10 h of starvation to 24 h and accumulated to a level between those of the 1.9 and 2.2-kb mRNAs (Fig. 1B, probe D). The relative levels of the mRNAs can be judged from figure 1B, probe A, since all three mRNAs share the same coding sequence. There was a slight cross-hybridization between the 2.4-kb mRNA and probe D which is visible in the early time points. This experiment demonstrates that the phosphodiesterase gene is transcribed into three different mRNAs that accumulate at different times during *D. discoideum* development. The 1.9-kb mRNA is the only phosphodiesterase transcript present during the vegetative stage, the 2.4-kb mRNA is

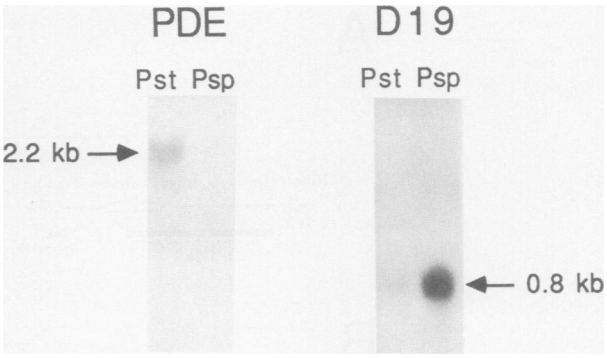

FIG. 2. The 2.2-kb mRNA is prestalk specific. Prestalk (Pst) and prespore (Psp) cells from slugs were separated by density gradient centrifugation (39), and 5 μg of total RNA was used for Northern blot analysis with a phosphodiesterase probe (PDE) specific for the 2.2-kb mRNA (probe D, Fig. 1). After the radioactivity bound to the membrane had decayed, the same blot was hybridized to the cDNA clone D19 that is specific for prespore cells (1).

aggregation specific, and the 2.2-kb mRNA is specific for late development.

Since the phosphodiesterase is involved in the morphogenesis that takes place during late development, it is important to know if the gene is specifically expressed in one of the two cell types, prespore and prestalk, that compose the multicellular structure called the slug. We have shown that the phosphodiesterase mRNA accumulates preferentially in prestalk cells (16), but at that time, we did not discriminate between the 2.2- and 2.4-kb mRNAs. Prespore and prestalk cells from migrating slugs were separated by density gradient centrifugation (39), and RNA extracted from the two cell types was subjected to Northern blot analysis. In the experiment shown in Fig. 2, we have hybridized equal amounts of RNA to a probe specific for the 2.2-kb mRNA (probe D, Fig. 1A). The 2.2-kb mRNA was detected only in the prestalk RNA preparation. We hybridized the same membrane with the D19 cDNA clone (1) as a probe. The D19 mRNA is specific for the prespore cells, and it showed a strong hybridization signal with the prespore RNA preparation (Fig. 2), indicating that the cell separation was good and that the prespore RNA sample was intact. Thus, the 2.2-kb mRNA is specific for late development and for prestalk cells.

Location of the promoter responsible for the transcription of the 2.2-kb mRNA. To locate the region of the phosphodiesterase locus responsible for the transcription of the 2.2-kb mRNA, we analyzed cell lines transformed with different fragments of the phosphodiesterase gene. We first analyzed a cell line cotransformed with the plasmids pGP-1 and pB10TP-1 (10). pB10TP-1 carries the marker for G418 resistance (9), and pGP-1 carries the 4.8-kbp *Bcl*I fragment of the phosphodiesterase locus (Fig. 1 and Table 1). Cells containing 100 to 200 copies of the plasmid pGP-1 overexpress the 1.9-kb mRNA in vegetative cells and early in development (10). We starved these cotransformed cells on filters, extracted RNA at different times, and subjected the samples to Northern blot analysis, with the phosphodiesterase coding region as a probe (Fig. 3). The RNA samples used in the Northern blot (Fig. 3) were from vegetative cells and cells developing on filters from zero to 22 h after starvation, but it should be noted that the development of this transformed cell line is blocked because of overproduction of phosphodiesterase (10). In this experiment, the three phosphodies-

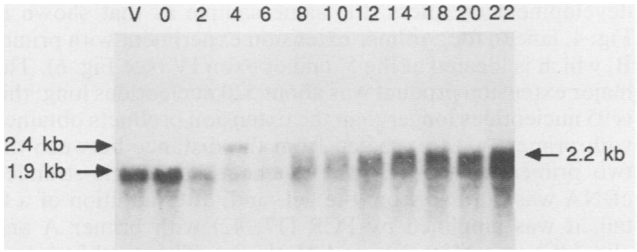

FIG. 3. pGP-1 directs the transcription of two mRNAs. Cells carrying 100 to 200 copies of the plasmid pGP-1 were prepared for development on filters, and RNA samples extracted at different times were subjected to Northern blot analysis (2 μg per lane). The blots were hybridized to probe A (see Fig. 1). V, Vegetative stage; this corresponds to exponentially growing cells. The numbers indicate hours of starvation on filters.

terase mRNAs were visible, but in contrast to the experiment performed on the parental strain AX3-K (Fig. 1), the 2.4-kb mRNA is the least abundant. The 1.9-kb mRNA was present at a high level in vegetative cells; its level decreased soon after starvation. The 2.4-kb mRNA was present at a level comparable to the level found in nontransformed cells during early development (2 to 8 h). The level of 2.2-kb mRNA present in the cells harvested after 8 h of starvation was increased compared with the level found in nontransformed cells. We knew from previous experiments that the 4.8-kbp *Bcl*I fragment contained the promoter for the 1.9-kb mRNA but not the promoter for the 2.4-kb mRNA (10, 37). The high level of 2.2-kb mRNA compared with that of 2.4-kb mRNA indicates that the promoter responsible for the transcription of the 2.2-kb mRNA is located within the 4.8-kbp *Bcl*I fragment.

Next, we constructed a plasmid carrying the entire phosphodiesterase coding region but with a shorter 5′ region than the one in pGP-1. This plasmid, pBDE-1, contains the *Eco*RV-*Hin*cII fragment of pGP-1 inserted into the transformation vector pB10TP-1 (Table 1). The transformed strain, carrying 100 to 200 copies of pBDE-1, did not overproduce phosphodiesterase during growth or early development in liquid, whereas the strain carrying pGP-1 did (Table 2). This is in agreement with the fact that the 1.9-kbp transcript is still present at high levels when growing cells are washed and suspended in buffer (10; Fig. 3). The 1.9-kbp mRNA rapidly disappears during early development but not before producing a lot of extracellular enzyme (10). During development on filters, the pBDE-1 transformant overproduced phosphodiesterase at a level similar to that of the pGP-1 transformant (Table 2). The slightly higher level of enzyme found in the AX3-pGP1 transformants is due to the high level of 1.9-kb transcript expressed during growth. Early in development, this transcript is still present (Fig. 3) and is responsible for the additional phosphodiesterase production. The high level of 2.2-kb mRNA in pBDE-1 transformants during late development was confirmed by Northern blot analysis (data not shown) and by primer extension (Fig. 4, lane 6). These results indicate that the promoter responsible for the transcription of the 2.2-kb mRNA is located between the *Eco*RV and the *Asu*II sites in the 5′ untranslated region of the phosphodiesterase gene. The overproduction of phosphodiesterase during late development in the pBDE-1 transformants blocks morphogenesis after aggregation, and these strains are unable to form fruiting bodies. They have a final morphology that is very similar to that of the pGP-1 transformants (10).

TABLE 2. Extracellular phosphodiesterase activity in wild type and transformed strains[a]

Time (h)	Phosphodiesterase activity (U/ml) in:		
	AX3	AX3-pGP1	AX3-pBDE1
Growth	11	760	7
Development in liquid			
2	14	80	22
4	35	140	45
6	53	300	75
8	107	670	92
10	128	1000	124
Development on filters			
16	18	109	60
22	15	230	132

[a] AX3-pGP1 and AX3-pBDE1 are strains transformed with plasmids pGP-1 and pBDE-1, respectively. For development in liquid, exponentially growing cells were harvested and starved in Sörensen buffer at a density of 2×10^7/ml. The cell suspension (20 ml in a 125-ml Erlenmeyer flask) was incubated at 22°C with shaking (110 rpm). The extracellular phosphodiesterase activity was assayed in the supernatant after inactivation of the specific inhibitor as described previously (15, 36) and is expressed in units per milliliter. 1 U is the amount of enzyme necessary to hydrolyze 1 nmol of cAMP in 1 min at 35°C in 50 mM Tris hydrochloride (pH 7.4) containing 50 μM cAMP. For development on filters, exponentially growing cells were washed in Sörensen buffer and 2×10^7 cells were deposited on 47-mm Whatman no. 50 filters supported by two Whatman 17 pads (16). The extracellular phosphodiesterase was collected from the cells, filters, and supporting pads. The cells were harvested in 2 ml of Sörensen buffer and pelleted. The supernatant was used to extract the enzyme from the pads by shaking them for 15 min before collecting the liquid. Phosphodiesterase activity was assayed as described above and is expressed in units per milliliter of extract.

Start site of transcription. We used the primer extension technique to find the initiation site of transcription and sequenced cDNA clones derived from the 5′ end of the 2.2-kb mRNA to determine its splicing pattern. In the primer extension experiment shown in Fig. 4, we used a primer located in exon III (primer A in Fig. 6). As described previously (37), this primer generated a cluster of extension products from 134 to 152 nucleotides long when it was hybridized to RNA extracted from vegetative cells (Fig. 4, lane 1) and extended. These products correspond to the 1.9-kb mRNA. When we used RNA that had been extracted from cells starving in liquid for 2.5 or 4 h in the presence of 1 mM cAMP (Fig. 4, lanes 2 and 3), the 134-to-152-nucleotide extension products were still visible, but a more abundant product appeared. This extension product is about 650 nucleotides long and corresponds to the 2.4-kb mRNA. A few additional bands are visible in lane 2 (Fig. 4) whose significance is not clear at the moment. Finally, when RNA extracted from cells in late development was used (Fig. 4, lane 4), the 650-nucleotide product became weaker, and a doublet of 430 and 422 nucleotides appeared. These products probably represent the 2.2-kb mRNA, because their presence in this lane is consistent with the fact that the 2.2-kb mRNA accumulates during late development.

To confirm that the 422- and 430-nucleotide extension products correspond to the 2.2-kb mRNA, we used RNA extracted either from cells transformed with pGP-1 that overproduce both the 1.9- and the 2.2-kb mRNAs or from cells transformed with pBDE-1 that overproduce only the 2.2-kb mRNA (see above). RNA extracted from growing pGP-1-transformed cells shows the 134-to-152-nucleotide extension products (Fig. 4, lane 5) and, after starvation, both the 134-to-152- and 422-to-430-nucleotide extension products (Fig. 4, lane 7). RNA extracted from cells transformed with

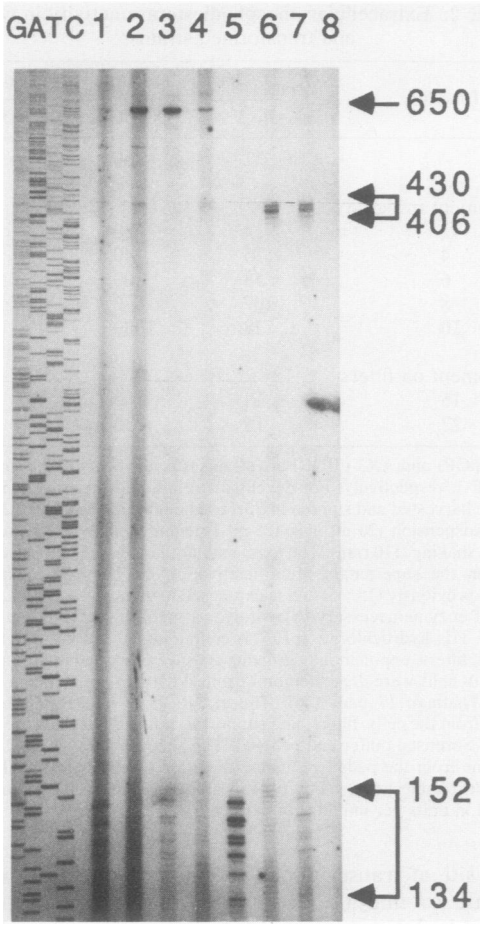

FIG. 4. Mapping of the transcriptional start sites by primer extension. Primer A was end labeled with [γ-^{32}P]ATP, annealed to various RNA samples, and extended with Moloney murine reverse transcriptase. Aliquots were loaded on a sequencing gel as described in Materials and Methods. The sequencing reaction of the phage M13 DNA shown on the left of the figure was used to determine the lengths of the extension products indicated on the right. Lanes: 1, RNA from growing AX3-K cells; 2, RNA from AX3-K cells starved for 2 h in Sörensen buffer containing 1 mM cAMP; 3, RNA from AX3-K cells starved for 4 h in Sörensen buffer containing 1 mM cAMP; 4, RNA from AX3-K cells starved on filters for 18 h; 5, RNA from growing AX3-K cells transformed with pGP-1; 6, RNA from cells transformed with pBDE-1 starved on filters for 16 h; 7, RNA from cells transformed with pGP-1 starved on filters for 22 h; 8, tRNA from calf liver.

pBDE-1 starved for 16 h shows the 422-to-430-nucleotide extension products (Fig. 4, lane 6). Because the signal is stronger in the transformants, we can see the 422- and 430-nucleotide extension products observed with the wild type as well as two other less abundant products of 406 and 414 nucleotides. The differences in size between the three groups of extension products (150, 430, and 650 nucleotides) match the size differences of the three mRNAs (1.9, 2.2, and 2.4 kb).

The primer extension experiment revealed the length of the 5′ untranslated region of the 2.2-kb mRNA but did not tell what region of the genomic DNA is transcribed. To answer that question, we isolated cDNA clones derived from the 5′ region of the 2.2-kb mRNA by the PCR technique that we also employed for the 1.9-kb mRNA (37). We used RNA extracted from cells transformed with pBDE-1 after 16 h of development on filters (the same sample as that shown in Fig. 4, lane 6) for a primer extension experiment with primer B, which is located at the 5′ end of exon IV (see Fig. 6). The major extension product was about 520 nucleotides long; this is 95 nucleotides longer than the extension products obtained with primer A, as expected from the distance between the two primers in the cDNA sequence. The single-stranded cDNA was purified from the gel, and, after addition of a G tail, it was amplified by PCR (17, 42) with primer A and oligo(dC) (see Materials and Methods). The amplified double-stranded product was ligated into pUC13 and sequenced. We discovered that the 5′ portion of the 2.2-kb mRNA is composed of exon III plus the adjacent 5′ 388 nucleotides in the genomic DNA (Fig. 5 and 6). We called this sequence exon III′. The 5′ portion of the 2.2-kb mRNA contrasts with the 5′ portions of the 1.9- and 2.4-kb mRNAs, which are each composed of a different exon located further upstream and are spliced onto exon III (Fig. 6).

Since there is no splicing between exon III and exon III′, and primer A used for the amplification resides in exon III, we could not rule out the possibility that the sequence we isolated in the experiment described above might have resulted from the amplification of genomic DNA present in the RNA used for the primer extension. To rule out that possibility, we performed an amplification with primer B located in exon IV and with primer C located on the opposite strand in exon III′. We used extension products made with primer B and RNA from cells transformed with pBDE-1 (same sample as that described above) or RNA from nontransformed cells starved on filters for 18 h. These extension products were amplified with primers B and C. The DNA fragments we isolated were bounded by primers B and C and had the expected cDNA sequence with the correct splicing pattern between exons III and IV. Amplification of genomic DNA would have shown a sequence containing the intron located between exons III and IV.

Previously, we located the 5′ end of the 2.4-kb mRNA by a combination of primer extension and S1 nuclease mapping experiments. We used the extension product shown in Fig. 4, lane 3, to recover a cDNA derived from the 5′ end of the 2.4-kb mRNA. After purification of the 650-nucleotide band, we amplified this cDNA sequence with primer A and oligo(dC) as described above. The cDNA clone we isolated was only 1 nucleotide shorter than the longest primer extension product described by Podgorski et al. (37).

Activity of the three phosphodiesterase promoters during development. We have shown that the phosphodiesterase gene is transcribed into three mRNAs that accumulate at different stages of development (Fig. 1). We wanted to know whether the level of each mRNA is regulated transcriptionally or posttranscriptionally. The 2.4-kb mRNA appears soon after starvation, and treatment with 1 mM cAMP increases the level of the 2.4-kb mRNA (16). Nuclear runoff experiments showed that the induction of the 2.4-kb mRNA and the increase in its abundance after treatment of cells with cAMP was due to increased transcriptional activity (data not shown).

To be able to analyze the transcriptional activity of each promoter individually, we prepared constructs containing the CAT gene linked to each of the three putative phosphodiesterase promoters. From our knowledge of the structure of the gene and previous transformation experiments, we decided to use the following restriction fragments to test their transcriptional activity. The *Eco*RV-*Asu*II fragment was expected to contain the 2.2-kb mRNA promoter, the *Bcl*I-*Sca*I fragment was expected to contain the 1.9-kb

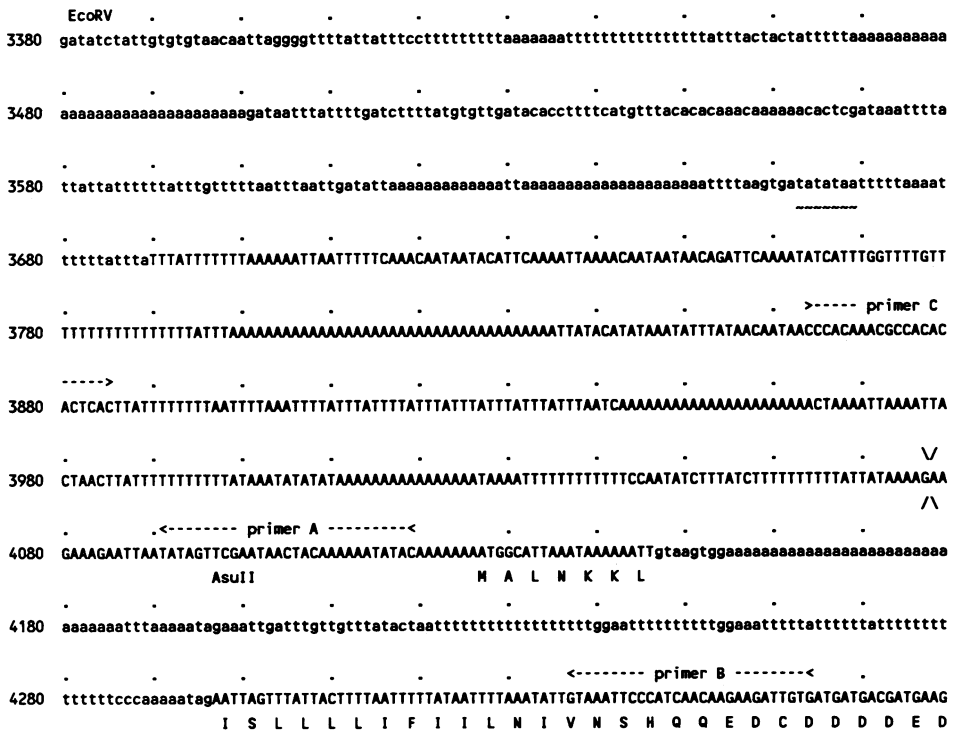

FIG. 5. Nucleotide sequence of the region containing the late development-specific promoter. The nucleotide sequence shown is from the *Eco*RV site to the beginning of the phosphodiesterase coding sequence. This is a portion of the sequence presented by Podgorski et al. (37); therefore, we have kept the same numbering for the nucleotides. Sequences that are part of a mature mRNA are shown in capital letters. The longest extension product obtained for the 2.2-kb mRNA is represented (position 3690). The locations and orientations of the oligonucleotides that we used for primer extension and PCR amplification are indicated above the sequence. The *Eco*RV and *Asu*II restriction sites are also indicated. Symbols: $\overset{V}{/\backslash}$, splice site that is used for the splicing of exons I and II onto exon III; - - -, TATA box.

promoter, and the upstream *BclI-BclI* fragment was expected to contain the 2.4-kb promoter (Fig. 6). The constructs were made as described in Materials and Methods and Table 1, with the pAV-CAT vector (32) that contains the CAT gene from the Tn*9* transposon (19) and a G418 resistance gene.

The three different constructs, as well as the pAV-CAT vector alone, were introduced into AX3-K cells by transformation, and, after selection of stable transformants, the amount of CAT protein produced during development was measured by enzyme-linked immunosorbent assay (see Materials and Methods). The results presented in Fig. 7 show the percentage of the maximum amount of CAT protein produced by each transformant and allow a comparison of the temporal regulation of the three promoters.

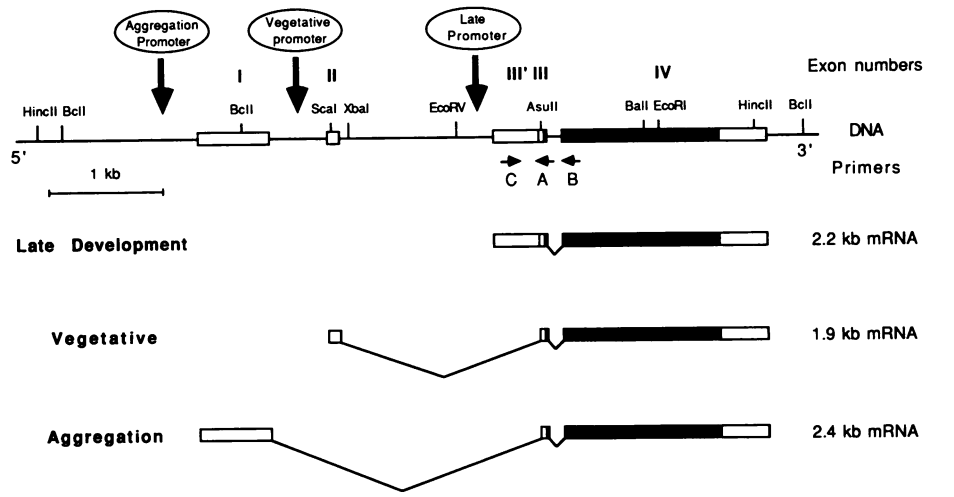

FIG. 6. Schematic representation of the phosphodiesterase locus. The restriction map of the phosphodiesterase locus is shown at the top. Symbols: ▭, transcribed but not translated regions; ▩, coding sequence. The locations of the oligonucleotides used for primer extension and PCR amplification are indicated, but the primers are not drawn to scale. The splicing patterns of the three mRNAs are shown.

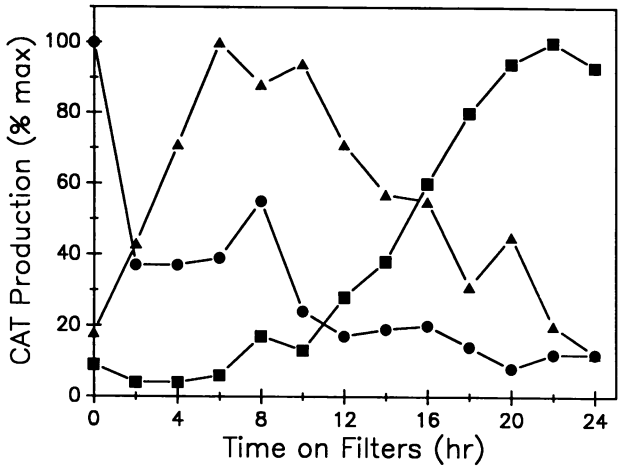

FIG. 7. Transcriptional activity of the three phosphodiesterase promoters. The DNA fragments containing the three phosphodiesterase promoters were fused to the CAT coding sequence carried by the vector pAV-CAT (Table 1), and the amount of CAT protein produced by transformants carrying these construct was measured during development. Cells were developed on filters as described previously (28). Two filters were harvested at each time point and were lysed and assayed as described in Materials and Methods. The data shown are from one typical experiment and are the averages of duplicate assays. The data are expressed as percentage of the maximum amount for each transformant. The maximum amount of CAT was 260 ng/10^7 cells in the pVCAT transformant, 50 ng/10^7 cells in the pACAT transformant, and 90 ng/10^7 cells in the pLCAT transformant. Symbols: ●, pVCAT; ▲, pACAT; ■, pLCAT.

The 1.9-kb mRNA accumulates to its highest level in vegetative cells and rapidly disappears in starving cells (Fig. 1). Its promoter activity, measured by the amount of CAT protein, is maximal in vegetative cells, decreases rapidly after starvation to about 40% of its activity and decreases even further during late development. It is apparent that the CAT protein is not stable in the cells, for it disappears when the promoter is not active anymore. The inclusion of the protease inhibitor phenylmethylsulfonyl fluoride (2 mM) during cell lysis did not increase the amount of CAT protein measured.

The 2.4-kb mRNA accumulates during aggregation (zero to 6 h; Fig. 1) and decreases thereafter. When linked to the CAT gene, its promoter shows the highest transcriptional activity between zero and 6 h. Between 6 and 10 h, the amount of CAT protein stays constant, while it decreases after 10 h.

The 2.2-kb mRNA accumulates during late development (12 to 22 h; Fig. 1). Its promoter shows the same pattern of activity when linked to the CAT gene, with low activity in vegetative cells and during the first 10 h of development, followed by an increase in activity during late development (Fig. 7). The CAT protein reaches maximum levels by 20 h.

All three promoters show exactly the pattern of expression that one would predict from the results shown in Fig. 1, and we conclude that each of the DNA fragments we used contains the necessary information for proper temporal regulation.

The absolute levels of expression do not follow the pattern observed in Fig. 1. The aggregation-specific promoter is the most active promoter as judged by the amount of phosphodiesterase mRNA transcribed, but it is the least-active promoter when linked to the CAT gene (50 ng of CAT per 10^7 cells). The CAT constructs with the vegetative promoter showed the highest level of transcription (260 ng of CAT per 10^7 cells). Since the plasmid copy number of the transformants is about the same (100 to 200 per cell), this indicates that expression is not proportional to copy number for each promoter, maybe because other elements are involved in the regulation of the level of expression of the various promoters.

DISCUSSION

The cyclic nucleotide phosphodiesterase plays a crucial role in the development of *D. discoideum*. It is one of the central elements of the chemotactic apparatus and during aggregation, it permits the cell surface cAMP receptor to regain sensitivity to cAMP stimulation. The absence of phosphodiesterase leads to an aggregation-deficient phenotype (2, 4). There is growing evidence that chemotaxis toward cAMP directs the movements of the multicellular structure that is formed after aggregation (43, 46) and that extracellular cAMP is involved in the differentiation of prespore cells (54). We have shown that the presence of phosphodiesterase is required for proper formation of fruiting bodies (11) and that an excess of phosphodiesterase during late development blocks morphogenesis (10). In this work, we complete the description of the structure of the phosphodiesterase gene previously reported (37) and show that the importance of the phosphodiesterase in *D. discoideum* development is reflected in the complex structure and regulation of the gene.

The phosphodiesterase gene is transcribed into three mRNAs that differ only in their 5′ noncoding sequences. Therefore, the specific role of each of these mRNAs is not determined by differences in their coding sequences. The 2.2-kb mRNA has the same coding sequence as the two others, and no open reading frame can be found upstream of the ATG codon located in exon III (Fig. 5). We have mapped the start site of transcription of the 2.2-kb mRNA by primer extension, which revealed two major start sites. A TATA box sequence is found 20 nucleotides upstream of the first initiation site. Two stretches of deoxythymidine residues are located between the TATA box and the start site of transcription (Fig. 5). These features are usually seen in *D. discoideum* genes transcribed by RNA polymerase II (24).

We have shown that the phosphodiesterase gene has three independent promoters located in a region that extends 4.1 kbp upstream of the ATG codon. The two distal promoters are linked to the coding sequence by the removal of introns (Fig. 6). By using the reporter gene CAT, we have shown that we could separate these three promoters and that each of them has the same temporal regulation as the mRNA it transcribes. These results indicate that the regulation observed at the mRNA level occurs primarily at the transcriptional level and that the phosphodiesterase gene has three promoters specific for growth, aggregation, and late development.

These results raise the question of the significance of this complex gene that is transcribed to produce three different mRNAs, two of them having long 5′ untranslated sequences, and all of them coding for the same protein. It appears that the temporal regulation and the tissue specificity of the promoters is an important part of the answer. The aggregation-specific promoter is inducible by cAMP, and the late development-specific promoter directs the synthesis of a mRNA that is detected only in prestalk cells. These two promoters allow a temporal and spatial regulation of phosphodiesterase production. This is critical, since an excess or

a lack of phosphodiesterase blocks late development (10, 11). The cAMP inducibility of the aggregation-specific promoter is consistent with the role of the enzyme during aggregation. The fact that the 2.2-kb mRNA is specific for prestalk cells, probably as a result of the specificity of the promoter, is relevant to our understanding of late development (for a review, see reference 55). cAMP is believed to induce prespore differentiation, whereas adenosine, a by-product of cAMP degradation by the phosphodiesterase, inhibits prespore differentiation (54). The fact that the phosphodiesterase mRNA is prestalk specific fits well in a model involving gradients of cAMP and adenosine to direct the morphogenesis of the slug (46, 54). Such a model relies on low cAMP and high adenosine levels in the prestalk region, which could be achieved by localization of the phosphodiesterase in the prestalk region. The enzyme has been shown to accumulate preferentially in the prestalk region of the slug (41). The inducibility of the phosphodiesterase late promoter by differentiation inducing factor (DIF), a prestalk morphogen (21, 34), is now being tested.

The structure of the phosphodiesterase gene could be the result of an evolutionary process in which additional promoters have been added to a preexisting gene having only the proximal promoter. There are species of cellular slime molds, such as *Dictyostelium minutum*, *Dictyostelium lacteum*, or *Polysphondylium violaceum*, that do not use cAMP for aggregation (8, 48, 51) but that use cAMP during late development (43–45). These species produce membrane-associated phosphodiesterase during culmination (44, 45). We postulate the existence of an ancestral phosphodiesterase gene having a promoter active only during late development. Later in the evolutionary process, species using chemotaxis toward cAMP during aggregation may have acquired a distal promoter that is active during aggregation and that is cAMP inducible.

It is possible that the long leader sequence of the 2.4-kb mRNA and the removal of a long intron are merely a way to connect a cAMP-inducible promoter to the phosphodiesterase coding sequence, although the length of the leader sequence could also influence the efficiency of translation of the mRNA (26). Of the numerous examples of genes transcribed into several mRNAs with different tissue specificities, it is interesting to note that one is the *Drosophila* dunce gene that also codes for a cAMP phosphodiesterase (5). Another example is the gene that encodes acetyl coenzyme A carboxylase in the rat; this gene has two promoters and, by use of alternate splicing, is transcribed into five mRNAs that have the same coding sequence but that show some tissue specificity (29). It appears that these mechanisms of developmental regulation are ancient because we find them in *D. discoideum*, an organism that diverged very early from the main branch of the eucaryotes (33, 49).

There is phosphodiesterase activity throughout the life cycle of *D. discoideum*, i.e., a low level during growth, a high level during aggregation, and an intermediate level during late development. The phosphodiesterase activity is regulated at multiple levels. First, there is transcriptional regulation as we have described above. Second, the protein can be localized to the membrane or secreted (47, 56). Third, the activity can be controlled at the enzymatic level with a specific inhibitor (15, 30) that inactivates the free extracellular enzyme but not the membrane-bound form. The balance between phosphodiesterase production and inhibitor production is achieved by virtue of the opposite effect that cAMP has on the expression of the phosphodiesterase and inhibitor genes. The phosphodiesterase is induced whereas the inhibitor is repressed by cAMP (40; L. Wu and J. Franke, submitted for publication). *D. discoideum* cells have evolved an elaborate system to regulate the phosphodiesterase activity and the level of extracellular cAMP. Regulation of the phosphodiesterase at the gene level is achieved by the use of three promoters which are probably sensitive to different signals. The next important problem is to identify those signals and the second messenger pathways that mediate their effects on the phosphodiesterase and inhibitor genes.

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