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A change in the rate of transcription of a eukaryotic gene in response to cyclic AMP

(discoidin I mRNA/cyclic AMP inhibition/nuclear transcription)

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ABSTRACT **The plasmid pDd812 contains the DNA copy of an mRNA sequence from *Dictyostelium discoideum* that undergoes first an increase and then a decrease in concentration during the first few hours of differentiation. We have recently shown that the mRNA sequence complementary to pDd812 encodes discoidin I, a developmentally regulated lectin that may play a role in cellular cohesion. By using pDd812 as a hybridization probe, we found that addition of cyclic AMP during the first few hours of development inhibited the accumulation of discoidin I mRNA. By measuring the rate of transcription in isolated nuclei, we showed that, at least in part, this inhibition results from a rapid and specific reduction in the rate of transcription of the discoidin I gene. Addition of high external concentrations of cAMP is known to increase the intracellular concentration to a level normally found later in development. This natural increase in cAMP concentration occurs at the time during development when transcription of the discoidin I gene ceases. We suggest, therefore, that changes in the intracellular concentration of cAMP act at the level of transcription to control gene expression during development. This hypothesis is supported by our observation that several poly(A)$^+$RNA sequences that normally accumulate after transcription of the *discoidin I* gene has ceased are synthesized prematurely in cells exposed to exogenous cAMP.**

When deprived of a food source, individual amoebae of the cellular slime mold *Dictyostelium discoideum* aggregate to form multicellular structures in which individual cells differentiate to yield either stalk cells or spore cells. The process of aggregation occurs by directed movement of the amoebae toward centers that emit pulses of cyclic AMP (cAMP) (1–3). These pulses are received at the cell surface by cAMP receptors and result in a transitory activation of adenylyl cyclase that increases the intracellular concentration of cAMP (3).

In addition to its role in chemotaxis, such an increase in the intracellular concentration of cAMP appears to be required for, or at least to accelerate, changes in the pattern of developmental gene expression. Thus, exogenous cAMP is required for the differentiation of stalk and prespore cells under conditions where normal development is blocked—e.g., by plating the cells at low density (4) or under cellophane (5). When cells in the first few hours of development are exposed to either artifical pulses (6) or continuous high levels (7) of cAMP, the intracellular cAMP concentration increases and several proteins involved in aggregation appear prematurely (8–11). Because cAMP seems to have a role in controlling the expression of developmentally regulated genes, it is important to determine the level at which this control is exerted. Previous studies have shown that the induction of cAMP phosphodiesterase by cAMP is sensitive to protein and RNA synthesis inhibitors (10) and that increased activity of this enzyme is due to an increased rate of synthesis of the protein (12). An increased rate of synthesis of specific proteins was also observed in disaggregated cells treated with cAMP and, when mRNA was isolated and translated in a cell-free system, it was shown that elevated concentrations of several specific mRNA sequences were maintained in disaggregated cells treated with cAMP (13). However, none of these studies provide direct evidence for an effect of cAMP on gene transcription because the analysis of rates of specific gene transcription can only be performed by using nucleic acid probes complementary to individual RNA sequences.

The isolation of cloned copies of developmentally regulated mRNA sequences has been described (14, 15); in this study, we have used these clones to study the effect of cAMP on gene expression. Most of our results were obtained by using the plasmid pDd812 (14). The characterization of this plasmid shows that it contains a segment of the sequence encoding discoidin I. This is one of two carbohydrate binding proteins, discoidin I and discoidin II, that are synthesized in large amounts during aggregation. Discoidin I, the more abundant of the two, constitutes 1% of cellular protein at 10 hr of development but is virtually undetectable in vegetative cells (16, 17). The roles of discoidin I and discoidin II in development are unclear, although their carbohydrate binding properties, their times of synthesis, the fact that they can be detected at the cell surface (17, 18), and the phenotype of a supposed structural mutant in the *Discoidin I*-encoding gene (19) have led to speculation that they are involved in cell cohesion.

We have previously shown (14) that the mRNA complementary to pDd812 DNA (which we will term here discoidin I mRNA) occurs at very low concentrations in vegetative cells but increases in abundance such that at 3–4 hr of development, it constitutes 1.5% of the poly(A)$^+$RNA population. Over the next 4 hr of development, there is a rapid decrease in the concentration of discoidin I mRNA. By measuring transcription in isolated nuclei, we showed that these changes in the concentration of discoidin I mRNA were, at least in part, the result of changes in the rate of gene transcription (14). The effects of cAMP on gene expression led us to suspect that cAMP might also be involved in controlling the expression of the *Discoidin I* gene. Therefore, we studied the effect of addition of exogenous cAMP during early aggregation; we found that it blocks the accumulation of discoidin I mRNA. By measuring RNA synthesis in isolated nuclei, we were also able to show that exogenous cAMP reduces the rate of transcription of the *Discoidin I* gene. The effect of cAMP on gene expression is not restricted to the *discoidin I* gene because we find that exogenous cAMP also results in the premature accumulation of several developmentally regulated poly(A)$^+$RNA sequences synthesized later in development than discoidin I mRNA.

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Abbreviation: cAMP, cyclic AMP.

MATERIALS AND METHODS

Cell Culture and Development. *D. discoideum* strain AX2 (ATCC 24397 from J. Ashworth) was grown axenically and prepared for development as described (14). Strain NC4 was grown in association with *Escherichia coli* strain B/r and prepared for development by washing in 20 mM potassium phosphate buffer, pH 6.2/2 mM $MgSO_4$. Development was performed in the washing buffer in conical flasks shaken at 120 rpm and was monitored by assaying for cAMP phosphodiesterase and, in some cases, for glycogen phosphorylase (11). cAMP (Sigma) was dissolved in H_2O, neutralized with NaOH, filter sterilized, and stored at −20°C.

Cell Fractionation and Nucleic Acid Preparation. The techniques for purification of cytoplasmic poly(A)$^+$RNA and synthetically active nuclei were modified from those of Jacobson *et al.* (20) as described (14). In some of the experiments, total poly(A)$^+$RNA was prepared from cell pellets that had been "snap-frozen" and extracted as described by Margolskee and Lodish (21).

Hybridization Analysis of Nucleic Acids. Cytoplasmic or whole cell poly(A)$^+$RNA was quantitated by hybridization with [^{3}H]poly(U) (22), and 10-μg aliquots were denatured with glyoxal (23), purified by electrophoresis on agarose gels, and transferred to diazotized paper (24). The filters were hybridized with nick-translated plasmid DNA as described (14). When the filters were to be rehybridized with a new probe, they were washed at 65°C in 90% formamide for 3 hr.

Nuclear RNA was synthesized from nuclei in a 30-min labeling period, by using techniques modified from those of Jacobson *et al.* (20) as described (14). As before, the fraction of the transcript synthesized by RNA polymerase II was determined by measuring α-amanitin sensitivity, and the fraction of the RNA hybridizing to pDd812 DNA was determined by using plasmid DNA covalently bound to diazotized paper discs (14, 25).

RESULTS

Effect of Exogenous cAMP on Cytoplasmic Concentration of Discoidin I mRNA. The addition of cAMP during the first few hours of aggregation blocks the accumulation of discoidin I mRNA in both bacterially grown and axenically grown cells. The results of an experiment in which axenically grown cells (strain AX2) were used are shown in Fig. 1. As for most of our experiments, the concentration of discoidin I mRNA was determined by hybridization of nick-translated pDd812 DNA to poly(A)$^+$RNA transferred to diazotized paper from an agarose gel (24). The intensity of the autoradiographic signal for each sample was proportional to the abundance of discoidin I mRNA in the poly(A)$^+$RNA population (14).

In the absence of cAMP, discoidin I mRNA shows the following pattern of appearance and disappearance: there is a low concentration in vegetative cells, a rapid increase in concentration that reaches a peak at 3–4 hr of development, and then a rapid decrease in concentration over the next few hours (14). In the presence of cAMP, there is an inhibition of discoidin I mRNA accumulation; similar results were obtained in a series of experiments in which cytoplasmic or whole cell RNA from axenically grown AX2 cells that had been treated with 1 mM cAMP at 1–2 hr of development were used. Thus, exogenous cAMP prevents any increase in the concentration of discoidin I mRNA above that present in the cells at the time of addition, and the small amount of discoidin I mRNA initially present disappears rapidly. In one experiment, we confirmed the results shown in Fig. 1 by determining the RNA concentration by the more quantitative technique of hybridization of *in vitro* labeled

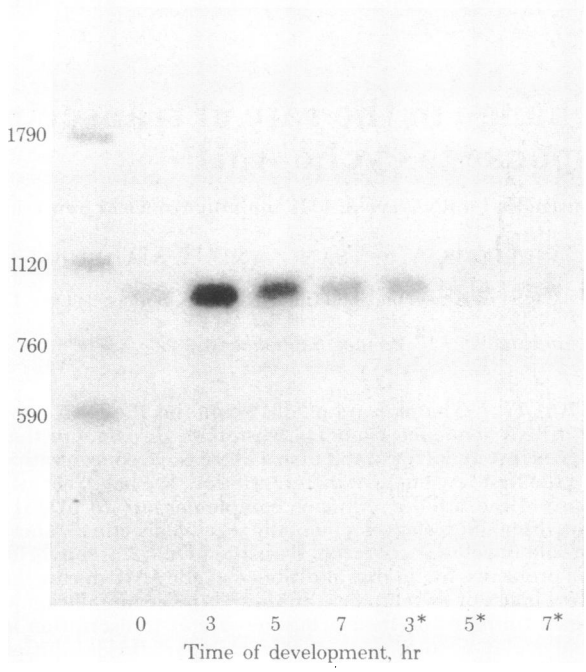

FIG. 1. Effect of exogenous cAMP on cytoplasmic concentration of *Discoidin I* mRNA in AX2 cells. Poly(A)$^+$RNA isolated from the cytoplasm of AX2 cells at the indicated stages of development was subjected to electrophoresis under denaturing conditions and then transferred to diazotized paper. After hybridization with nick-translated pDd812 DNA, the filter was washed and exposed for autoradiography. The marker is a *Hinf* digest of simian virus 40 DNA end labeled with ^{32}P. *, Sample received 1 mM cAMP at 1 hr of development.

RNA to plasmid DNA bound to filters (15). In the absence of cAMP, discoidin I mRNA composed 1.4% of the cytoplasmic poly(A)$^+$RNA population at 4 hr of development. In the presence of 1 mM cAMP added at 1 hr, discoidin I mRNA composed only 0.25% of the poly(A)$^+$RNA population at 4 hr of development. The observation that exogenous cAMP prevents accumulation of discoidin I mRNA suggests that the protein will not be synthesized in cells treated with cAMP. That this is indeed the case has been shown by pulse-labeling proteins synthesized during development (unpublished results).

Yeh *et al.* (26) have demonstrated that cAMP exerts its effect on developing cells via two independent mechanisms. On the one hand, the extracellular cAMP phosphodiesterase inhibitor system is modulated by a change in extracellular cAMP concentration. On the other hand, components required for aggregation competence—e.g., membrane-bound cAMP phosphodiesterase, cell surface cAMP receptors, and contact sites A—are regulated by the level of intracellular cAMP. Thus, continuous addition of cAMP at nanomolar concentrations—a treatment that does not cause an increase in the intracellular cAMP level—activates the extracellular cAMP phosphodiesterase inhibitor system but has no effect on the appearance of aggregation-specific components (26). An increase in the intracellular cAMP concentration can be brought about artificially by a single addition of 1 mM cAMP (7) or by activating the enzyme adenylyl cyclase by using 10-μM pulses of cAMP (26). To determine whether the extracellular or the intracellular mechanism operates on the accumulation of discoidin I mRNA, we examined the accumulation under different modes of addition of cAMP. Amoebae treated at 1 hr of development with 1 mM cAMP or with a 10-μM pulse of cAMP every 5 min had much lower levels of Discoidin I mRNA than control cells at 5 hr of development (Fig. 2). In contrast, the addition of a con-

tinuous flow of cAMP at a concentration equivalent to that given by the 10-μM pulses or the addition of 1 mM AMP had little effect on the accumulation of discoidin I mRNA, a result that suggests that the mechanism regulating the accumulation of discoidin I mRNA is similar or identical to that mediating aggregation competence—i.e., it occurs as a result of changes in the intracellular concentration of cAMP. It also shows that the effect of the elevation of the intracellular cAMP level is independent of whether it is caused by a single addition of 1 mM cAMP or by 10-μM pulses.

We next performed a series of experiments using bacterially grown cells (strain NC4) and found a somewhat different, and more variable, time course of accumulation of discoidin I mRNA. The concentration of discoidin I mRNA in vegetative NC4 cells was always much lower than that in axenically grown AX2 cells. In every experiment, the concentration of discoidin I mRNA started to decrease several hours later than that in AX2 cells but, in some experiments, the time at which maximal concentration was achieved was also delayed relative to that in AX2 cells. Thus, in one experiment, the peak of discoidin I mRNA accumulation was not achieved until after 6 hr of development (Fig. 3). A similar variability in the time of synthesis of the discoidin I protein has been observed (27). In initial experiments, we found that addition of 1 mM cAMP to NC4 cells at 1–2 hr of development delayed, but did not prevent, the accumulation of discoidin I mRNA. We feel that this is almost certainly due to degradation of the exogenous cAMP by extracellular cyclic AMP phosphodiesterase during the long interval between addition of the cyclic nucleotide and its time of action on discoidin I mRNA accumulation in NC4 cells [this is of course exacerbated by the induction of cAMP phosphodiesterase by cAMP (11)]. Accordingly, in the experiment shown in Fig. 3, we added a higher concentration of cAMP (5 mM) at a later time in development (4 hr). Under these conditions, the result was the same as obtained with AX2 cells—i.e., the further accumulation of discoidin I mRNA was blocked, and the pre-existing discoidin I mRNA disappeared. (Note: This figure shows the hybridization of the same filter to two separate probes—see below).

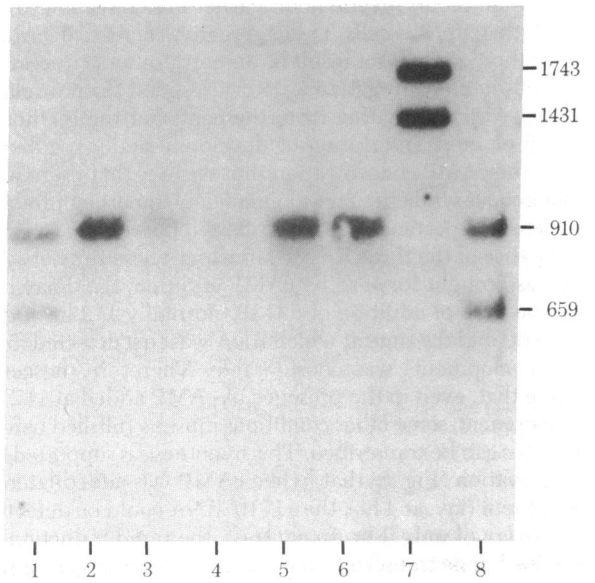

FIG. 2. Effect of AMP and cAMP applied in various modes on discoidin I mRNA accumulation in AX2 cells. Cells were grown axenically, washed, and shaken in phosphate buffer. After 1 hr of development, the cell suspensions were treated as follows: Control (lane 2), a single addition of 1 mM cAMP (lane 3), addition of cAMP at 5-min intervals to give a final 10-μM concentration in the cell suspension (lane 4), continuous addition of cAMP such that the amount of nucleotide delivered over a 5-min period was equivalent to a single 10-μM pulse (lane 5), and a single addition of 1 mM AMP (lane 6). Four hours after treatment, the cells were harvested and total cellular Poly(A)$^+$RNA was isolated and hybridized. The marker lanes contain pBR322 DNA digested with *Alu* 1 (lanes 1 and 8) and *Ava* 11 (lane 6).

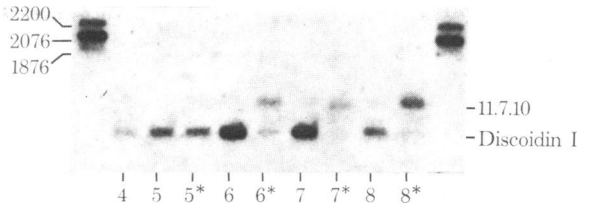

FIG. 3. Effect of exogenous cAMP on cytoplasmic concentration of discoidin I mRNA and 11.7.10 RNA in NC4 cells. Bacterially grown cells (strain NC4) were prepared for development and, at 4 hr, cAMP, at a final concentration of 5 mM, was added to half the cells. Total cellular poly(A)$^+$RNA was isolated from the cells, subjected to gel electrophoresis, and transferred to diazotized paper. The paper was hybridized with nick-translated pDd812 DNA, washed, and then exposed to X-ray film for 2 hr. After removal of most of the pDd812 DNA by exposure to high concentrations of formamide and high temperature, the paper was rehybridized with nick-translated pDd11.7.10 DNA. The paper was then washed as before and exposed to x-ray film for 2 days. This figure is a composite in which two autoradiographs were aligned. The size markers are a mixture of restriction fragments derived from pBR322 DNA. *, Cells incubated in the presence of cAMP.

Effect of Exogenous cAMP on Transcription of discoidin I RNA in Isolated Nuclei. Thus, exogenous cAMP, added early in development, prevents accumulation of the discoidin I mRNA sequence. This alteration in the rate of RNA accumulation can in principle be brought about by a change in cytoplasmic messenger RNA stability, by a change in the fraction of the primary transcript that is degraded within the nucleus, by an altered transcription rate, or by combinations of these possibilities. The increase and decrease in discoidin I mRNA concentration seen in normal development is brought about, at least in part, by changes in the rate of gene transcription (14). This has been shown by using a nuclear transcription system in which nascent chains initiated *in vivo* were extended *in vitro* in the presence of radiolabeled precursors (20). We have used this system to determine the effect of cAMP on the rate of discoidin I gene transcription (Fig. 4). Approximately 0.25% of the nuclear polymerase II transcript is complementary to pDd812 DNA at 2 hr of development. In the absence of cAMP, this fraction remains constant over the subsequent hour but, after only 15 min in the presence of 1 mM cAMP, the level of discoidin I RNA in the nuclear transcript decreases to 60% of the control and, after 1 hr, discoidin I RNA synthesis is reduced to 20% of the control value. This is a specific effect of cAMP on *discoidin I* gene transcription because the total amount of transcription by RNA polymerase II is not affected by prior exposure of the cells to cAMP. The average level of incorporation by RNA polymerase II in the four experiments summarized in Fig. 4 is given in Table 1; the addition of cAMP does not produce any significant effect.

Effect of Exogenous cAMP on Cytoplasmic Concentration of Other Developmentally Regulated mRNA Sequences. Thus, our results raise the possibility that the increase in intracellular cAMP concentration that occurs during aggregation in *Dictyostelium* is responsible for the cessation of *discoidin I* gene transcription. Under the conditions used here, [in contrast to those used by Landfear and Lodish (13)], the cells aggregate and synthesize developmentally regulated enzymes charac-

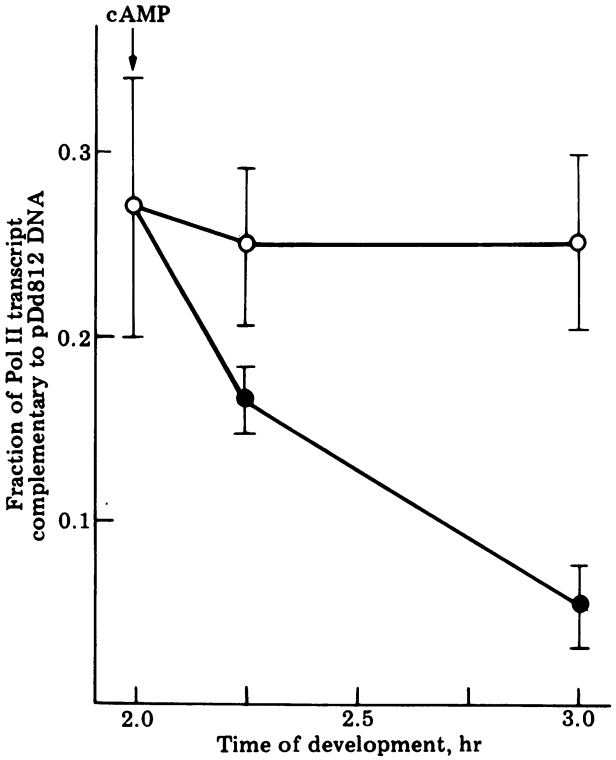

FIG. 4. Effect of exogenous cAMP on transcription of discoidin I RNA in isolated nuclei. Cells were starved in suspension for 2 hr. A sample of cells was removed for nuclear preparation, the remaining cells were divided into two portions, and one of these was treated with 1 mM cAMP. Aliquots for nuclear preparation were taken 15 and 60 min after the addition of cAMP. The *in vitro* nuclear RNA synthesis reaction was run for 30 min, and the fraction of RNA polymerase II transcript complementary to pDd812 was determined as described (14). Mean ± SD of results obtained in four separate experiments, two with NC4 cells and two with AX2 cells. In each case, triplicate determinations of the amount of RNA bound to pDd812 and duplicate determinations of the background hybridization to a heterologous plasmid were made. Pol II transcript in the RNA preparations was constant (50–60%) over the 1-hr period of the experiment and not affected by cAMP. Total RNA synthesized also was constant and not affected by cAMP. cAMP (1 mM) was used for both NC4 and AX2 cells because breakdown of the cyclic nucleotide extracellular cAMP phosphodiesterase is not a problem over such a short incubation period.

teristic first of the aggregative, and then of the postaggregative, stage of development (11, 15). A number of plasmids containing the DNA copies of poly(A)$^+$RNA sequences that are first detectable late in aggregation have been isolated (15). To determine the effect of exogenous cAMP on these RNA sequences, the diazotized paper sheets used for hybridization to pDd812 were rehybridized with several of them; for all those tested (pDd522, pDd10101, and pDd11710), the effect of exogenous cAMP was to *accelerate* the appearance of the specific RNA by several hours. Thus, the poly(A)$^+$RNA complementary to pDd11710 is barely detectable after 8 hr of development in the absence of cAMP (Fig. 3) but is almost fully induced by 6 hr of development when 5 mM cAMP is added at 4 hr of development. Thus, the increase in intracellular cAMP concentration during aggregation seems to be responsible for a reduction in the rate of accumulation of at least one mRNA sequence that is synthesized early in aggregation—discoidin I mRNA—and also for an increase in the rate of accumulation of at least three mRNA sequences that are synthesized later in aggregation. This suggests that the elevation of intracellular cAMP concentration is used to alter the pattern of gene expression.

Table 1. Effect of exogenous cAMP on subsequent nuclear transcription by RNA polymerase II

Time of development, hr	cAMP added at 2 hr	Activity*
2.25	−	8.1
2.25	+	9.6
3	−	7.2
3	+	6.4

Average level of incorporation obtained in four separate experiments.
* Activity is expressed as pmol of triphosphate per μg of DNA.

DISCUSSION

We have shown that the addition of cAMP to *D. discoideum* during the first few hours of development prevents accumulation of discoidin I mRNA. This developmental regimen also results in the premature accumulation of several other poly(A)$^+$RNA sequences that are synthesized later in development than discoidin I mRNA. Both the addition of high external concentrations of cAMP (7) and the pulsatile addition of low concentrations of cAMP (26) elevate the intracellular cAMP concentration to a level normally found only in cells late in aggregation. Thus, it seems reasonable to assume that the increase in intracellular cAMP concentration occurring during aggregation is responsible for the changes in the pattern of gene transcription that normally occur then (14, 15). A piece of indirect evidence, which is consistent with this hypothesis, is provided by correlating our observation that discoidin I mRNA accumulation in axenically grown AX2 cells ceases several hours earlier than that in NC4 cells with results obtained by Yeh *et al.* (26), who have shown that the intracellular increase in cAMP concentration occurs several hours earlier in axenically grown AX2 cells than in NC4 cells. The aggregative increase in cAMP concentration occurs as a result of an entrainment process in which cells in an aggregation territory respond to, and relay, pulses of cAMP emanating from the center of the territory. Coupling of the transcription of developmentally regulated genes to the cAMP concentration thus ensures that there will be synchronous synthesis of developmentally important proteins by all cells in a particular aggregation territory.

In the case of the three RNA transcripts whose time of synthesis was brought forward by cAMP addition, the delay between the time of addition of cAMP (normally 1–2 hr of development) and the time at which RNA was first detected (5–6 hr of development) was considerable. Therefore, it seems probable that, even in the presence of cAMP added at 1–2 hr of development, some other conditions must be fulfilled before these genes can be transcribed. This hypothesis is supported by our observation (Fig. 3) that, when cAMP was added later in development (i.e., at 4 hr), the 11710. RNA could be detected after a delay of only 2 hr. In contrast, the rapid reduction in *Discoidin I* gene transcription produced by cAMP (i.e., a significant effect within 15 min of addition) makes it highly unlikely that this effect is the result of a "cascade" involving the synthesis of new proteins. It seems more likely that cAMP interacts with a preexisting control protein to reduce the rate of *Discoidin I* gene transcription. Precedents from other cAMP-controlled systems in eukaryotes and recent studies of cAMP-binding proteins in *D. discoideum* may give some clue to the identity of this protein. In higher eukaryotes, all the known effects of cAMP on the enzymes involved in intermediary metabolism are mediated by cAMP-dependent protein kinases (28). These enzymes are composed of a catalytic and a regulatory subunit. On binding of cAMP to the regulatory subunit,

the enzyme dissociates and the catalytic subunit phosphorylates its substrate protein. There is no direct evidence that the effects of cAMP on gene expression in eukaryotes are mediated by cAMP-dependent protein kinases, but some indirect evidence has recently been provided by studies with mutants of the mouse lymphoma cell line S49 (29). In the case of *Dictyostelium*, there is evidence for both developmentally regulated cAMP-dependent protein kinases (30) and developmentally regulated cAMP-binding proteins that do not appear to be regulatory subunits of cAMP-dependent protein kinases (31, 32). The cAMP-dependent protein kinase and several of the cAMP-binding proteins are synthesized during early aggregation; it is tempting to speculate that interaction of cAMP with one or other of these proteins is responsible for the effects on gene expression presented in this study.

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