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Genetics

Two-phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium*

SANDRA K. O. MANN AND RICHARD A. FIRTEL*

Department of Biology, Center for Molecular Genetics, M-034, University of California, San Diego, La Jolla, CA 92093

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ABSTRACT **Two classes of early genes in *Dictyostelium* are differentially regulated by extracellular pulses of cAMP interacting with its cell-surface receptor, conditions that also regulate chemotaxis and aggregation. The pulse-repressed genes, such as *K5*, are induced shortly after the onset of starvation and are repressed a few hr later during aggregation by cAMP pulses. The pulse-induced genes (including *D2*, *M3*, and those encoding contact sites A, the G_α protein subunit $G_\alpha 2$, and the cell-surface cAMP receptor) are maximally induced just prior to aggregation by pulses of cAMP and are subsequently repressed by sustained moderate levels of cAMP—conditions that exist sequentially in development. In this manuscript, we further analyze the requirement for cAMP pulses and characterize a requirement for protein synthesis for the expression of these two classes of genes. Our results indicate that the control of expression of both the pulse-induced and pulse-repressed genes requires other developmentally regulated factors in addition to starvation and cAMP pulses. We also identified another early gene, *F9*, whose expression is stimulated upon starvation, is not responsive to cAMP, and is hyperstimulated by cycloheximide, in a manner similar to the cycloheximide stimulation of c-*fos* and other serum-induced genes in mammalian cells. Examination of the kinetics of expression of the pulse-induced genes in a mutant blocked in the cAMP relay pathway indicates that their expression is controlled by a two-phase process. The first phase requires starvation and CMF, an extracellular conditioned medium factor, and results in a low level of expression. The second phase requires establishment of the cAMP signal-relay system and induces the genes to a high level. Both phases require prior and concomitant protein synthesis. Some of the members of the pulse-induced class encode elements of the cAMP signal-relay system that controls aggregation, indicating a feedback autoregulation. The two-phase process might allow the "fine-tuning" of the level of expression of genes involved in aggregation.**

The cellular slime mold *Dictyostelium discoideum* grows vegetatively as individual amoebae. When food is depleted, a multicellular developmental program is initiated. Approximately 10^5 cells stream together to form a tight aggregate that proceeds through a series of morphogenetic changes, culminating in a fruiting body comprised of a stalk bearing a sorocarp (ball) of spores. The 10-hr aggregation process is initiated when a cell functioning as an "aggregation center" begins to emit pulses of cAMP at 6- to 9-min intervals. The cAMP binds to cell-surface cAMP receptors on neighboring cells and transiently activates two intracellular signal transduction pathways. One pathway is believed to activate phospholipase C, resulting in the release of intracellular Ca^{2+} (1). This leads to the activation of guanylate cyclase and chemotaxis, activation of pulse-induced genes, and repression of one set of pulse-repressed genes (1–3). The second pathway activates adenylate cyclase, which results in the synthesis and release/secretion of cAMP as a "pulse" into the extracellular environment, thus relaying the cAMP signal. Once the cell-surface receptors bind cAMP, they rapidly change to an inactive state, and the intracellular biochemical pathways adapt (1, 4). As the extracellular environment is cleared of cAMP by extracellular cAMP phosphodiesterase (5, 6), the receptors return to an active state, readying the cell to respond to the next signal. Thus, the original pulse of cAMP is amplified and propagated outward from the aggregation center in waves. This signal relay system serves to establish a gradient of cAMP, which functions as the chemoattractant during aggregation. Cells move up the cAMP gradient toward the aggregation center, forming "streams" as they establish cell–cell contacts and move together (for a review, see ref. 4).

A number of classes of developmentally regulated genes in *Dictyostelium* whose expression is regulated by cAMP-activated signal transduction processes have been identified (2, 7). (*i*) *K5* (a pulse-repressed gene) is induced shortly after the initiation of starvation and is repressed during aggregation of pulses of cAMP. (*ii*) D2 and M3 (pulse-induced genes) are early genes whose expression is stimulated by nanomolar-level pulses of cAMP and repressed by moderate continuous cAMP levels. A number of other genes important for cAMP signal relay and aggregation are similarly regulated. (*iii*) Prestalk- and prespore-specific late genes are induced by 10 and 15 hr into development, respectively, and require moderate levels of cAMP for their expression (7–10). Changing levels of cAMP within the population of cells is used to regulate gene expression throughout *Dictyostelium* development.

In this manuscript, we further examine the requirements for expression of the pulse-repressed and pulse-induced genes, and we identify a gene induced immediately upon starvation whose expression is stimulated by cycloheximide. Our results support the hypothesis that the pulse-induced genes are controlled by a two-phase induction process. The first phase is initiated by starvation and the second is activated by cAMP pulses. Our results point to a general regulatory program required for the expression of early genes during *Dictyostelium* development.

MATERIALS AND METHODS

Strains NC-4, KAx-3, and Synag7 (clone PND7.2.2) were grown and developed as described (2, 3). Cells pulsed with cAMP received pulses at 6-min intervals to a final concentration of 25 nM. Cells developed in the presence of continuous cAMP were shaken in 500 μM cAMP initially, and additional cAMP was added every hour to 100 μM (see ref. 2 for details). In the designated experiments, cycloheximide was used at a 500 μg/ml level.

Cell samples were harvested at appropriate time points by centrifugation and RNA was purified, size-fractionated, blot-

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Abbreviations: CsA, contact sites A; CMF, conditioned medium factor.

*To whom reprint requests should be addressed.

ted, and probed as described (2). Nuclear run-on experiments were performed as described by Nellen *et al* (11). Identical aliquots of RNA samples were loaded in experiments shown in Figs. 2 and 4. All RNA samples were examined by gel electrophoresis for equal levels of 17S and 26S rRNAs.

RESULTS

Requirement for Developmentally Regulated Protein Synthesis. Our description of the expression of the early genes *K5, D2,* and *M3* under a variety of conditions has been presented (2) and is summarized along with that of gene *F9* (see below) and prestalk and prespore genes in Fig. 1. *K5* is induced upon starvation and is maximally expressed by 2.5–4 hr in the wild-type strain NC-4 (2, 3). To examine the kinetics of *K5* induction and requirements for prior protein synthesis, we developed cells in shaking culture with and without cycloheximide. Fig. 2 shows the developmental kinetics of *K5* with cycloheximide added at 30, 60, and 90 min. At the 30-, 60-, and 90-min time points before the addition of cycloheximide, there was no K5 mRNA at 30 min, a very low level at 60 min, and a moderate level at 90 min. Cycloheximide given before 60 min blocked *K5* expression. When it was given at 90 min or later, further K5 mRNA accumulation was blocked, and K5 RNA that had accumulated was rapidly lost, suggesting that prior and concomitant protein synthesis is required for K5 mRNA expression and possibly stability. When cells shaken with cycloheximide for 4 hr were washed and plated on filters, K5 RNA accumulation was rapidly induced and reached maximal levels within 1 hr; thereafter RNA levels declined rapidly (data not shown).

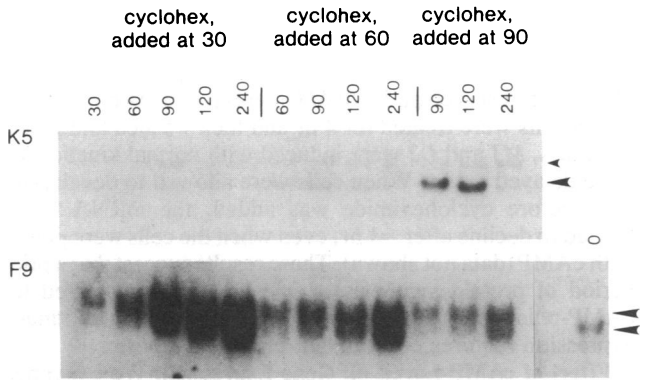
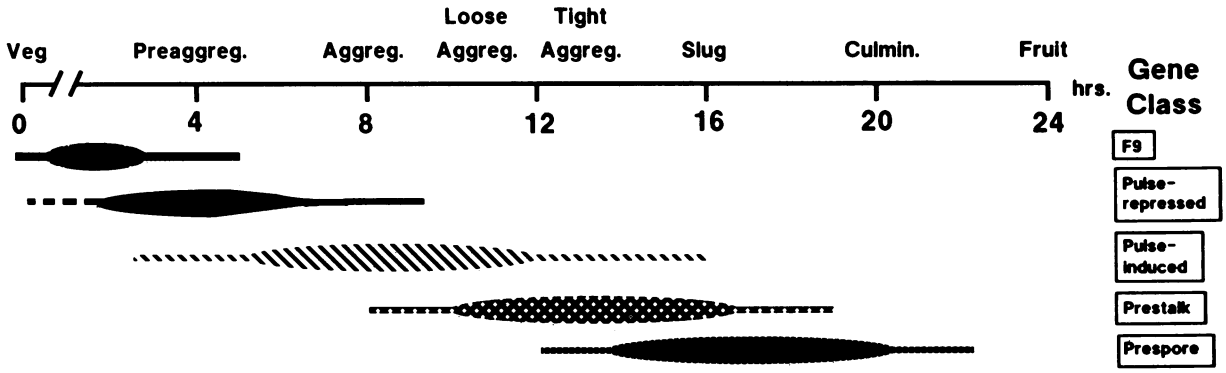


FIG. 2. Effect of cycloheximide and cAMP on early gene expression in NC-4 cells. The expression of *K5* and *F9* are shown in cells developed in shaking culture. Cycloheximide (cyclohex) was added at 500 μg/ml at the designated time.

Fig. 2 also shows the expression of gene *F9* under these conditions. The *F9* probe hybridized to two bands. During normal development, F9 RNA was expressed at low levels in vegetative cells and increased upon starvation, peaking at 90 min before rapidly decreasing (data not shown). Treatment with cycloheximide dramatically increased the accumulation of both F9 RNAs, with the smaller RNA showing a relatively larger increase in response to cycloheximide. The earlier the cycloheximide was added, the greater was the degree of stimulation, which suggests that a block of protein synthesis during the initial phases of development allows continued



Gene Class	cAMP Signal		Pathway Requirement		Cyclohex.
	pulse	continuous	A (cAMP relay)	B (PLC?)	
F9	-	-	-	-	stimulates
Pulse-repressed	represses	stimulates	no	yes	inhibits
Pulse-induced (D2, CsA, $G_\alpha 2$, receptor)	induces	represses	no	yes	inhibits
Prestalk	induces (low)	induces	no	yes*	inhibits
Prespore	-	induces	no	yes*	inhibits

*cAMPS-[Sp] is an antagonist for prespore and an agonist for prestalk gene induction.

FIG. 1. Summary of early cAMP-regulated gene expression. This figure shows a summary of the developmental kinetics of *F9*, the pulse-repressed gene *K5*, pulse-induced genes, the early class of prestalk genes, and the major class of prespore genes in the wild-type strain NC-4 and the axenic strain KAx-3. Data are from this manuscript (for *F9*), from references (2, 12, 14), and from unpublished data. The thickness of the bar indicates the relative level of mRNA for each class. The dashed line for *K5* in vegetative growth indicates a low but detectable expression of K5 mRNA in vegetative cells in strain KAx-3 and in some experiments with strain NC-4. The figure also presents the effects of cAMP levels and cycloheximide as well as the signal-transduction pathway involved in their regulation. Pathway A activates adenylate cyclase to establish the cAMP relay system. Pathway B is that involving activation of phospholipase C and inositol phospholipid turnover. Results on the effect of the slowly hydrolyzable analog cAMPS-[Sp] are from W. Spek, R. van Eigk, R.A.F., M. Wang, and P. Schaap (unpublished data). These results indicate that the cAMP-mediated pathways controlling prestalk and prespore gene expression are not identical.

expression and accumulation of F9 RNA, in contrast to our observations on *K5*.

When cells were treated with cycloheximide as described above, we observed no *D2* or *M3* expression (data not shown). When cells were treated for 4 hr and then washed and plated on filters, *M3* and *D2* were induced with normal kinetics but were delayed ≈4 hr. When cells were allowed to develop for 3 hr before cycloheximide was added, the mRNA levels started to decline after ≈1 hr, even when the cells were pulsed with cAMP (data not shown). These results suggest that a prior period of protein synthesis of 3–4 hr may be required for cAMP-pulse responsiveness of *D2* and *M3* and that continued expression requires concomitant protein synthesis.

Effect of cAMP Levels on Gene Expression. We examined the effects of cAMP levels on the three early genes in NC-4 cells. We previously have found that NC-4 shaking cultures establish endogenous cAMP oscillations, which induce *M3* and *D2* expression. Pulsing cells from the onset of starvation with cAMP results in repression of *K5* expression and a precocious higher level of *M3* and *D2* expression. Continuous cAMP, which down-regulates the receptors (see ref. 1), prevents *M3/D2* expression and allows continued *K5* expression (2, 3). When we pulsed cells for either 3 or 6 hr and then exposed them to continuous levels of cAMP, K5 RNA (which had been repressed) rapidly reaccumulated within 1.5 hr (data not shown). In contrast, when continuous cAMP was given at 3 or 6 hr *M3/D2* levels of expression rapidly declined (data not shown). These results suggest that the continued cAMP pulsing and the resultant oscillations of receptors between active and adapted states are required for repression of *K5* and continued expression of *M3/D2*.

To examine the molecular basis for this repression of *M3* and *D2* expression, we performed nuclear run-on experiments comparing the relative level of transcription in vegetative cells, 6-hr cells shaken without cAMP, 6-hr pulsed cells, 6-hr cells shaken in the presence of continuous cAMP, and cells pulsed for 6 hr followed by 1.5 hr of continuous cAMP. To avoid bacterial contamination of our nuclei, we used the NC-4-derived axenic strain KAx-3, which has the same pattern of pulse-induced gene expression as NC-4 (3). Expression in vegetative cells was at background levels, and 6-hr cells showed a high level of transcription (Fig. 3). When cells were pulsed with cAMP, the level was ≈50% higher. Cells pulsed for 6 hr followed by continuous cAMP addition for 1.5 hr showed low levels of expression. Addition of continuous cAMP from the onset of starvation resulted in near background levels of expression.

Two-Phase Induction of the "Pulse-Induced" Early Genes. A number of genes known to be expressed during aggregation and induced by cAMP pulses play an important role in aggregation and early development. These include genes encoding the cell-surface cAMP receptor (12), adenylate cyclase (15), $G_\alpha 2$ (the α subunit of the G protein coupling the cAMP receptor and phospholipase C; A. Kumagai, M. Pupillo, R. Gundersen, R. Miake-Lye, P. N. Devreotes, and R.A.F., unpublished data), contact sites A (CsA or gp80, an integral membrane protein associated with cell–cell adhesion; refs. 16 and 17), and D2 (a serine esterase required for normal aggregation; ref. 13). (The function of M3 is not known.) Our previous analysis of *D2* and *M3* expression in the aggregation-deficient *Dictyostelium* mutant Synag7 suggested that these genes are expressed at a low level in the absence of any cAMP pulses (3). This mutant strain lacks the cAMP relay pathway and does not activate adenylate cyclase in response to cAMP pulses (18, 19). However, the cells induce *D2* and *M3* expression and repress *K5* expression when pulsed with cAMP (3). Since Synag7 populations cannot establish the cAMP oscillations observed in cultures of wild-type cells, this represents an excellent strain to examine the relative importance of cAMP pulses versus starvation and other factors on pulse-induced gene expression.

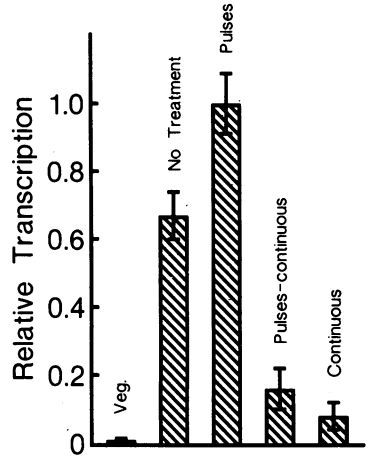


FIG. 3. Relative level of transcription of *D2* in the nuclear run-on experiments. The level of *D2* expression in nuclear run-on experiments was measured in nuclei isolated from vegetative cells, cells shaken 6 hr with no treatment (cells show oscillating levels of cAMP), cells given 25 nM cAMP pulses for 6 hr, cells pulsed for 6 hr then given 300 μM cAMP for 1.5 hr, and cells given 300 μM cAMP at 0 hr and then 100 μM every 2 hr. Relative levels of expression were determined by densitometry with an LKB densitometer. Each nuclear run-on transcription sample was hybridized to filters carrying cDNA inserts from pGEM3, *D2*, and gene *IG7*, which is expressed at the same level continuously through this stage of development and whose expression is unaffected by cAMP (20). Relative level of expression of *D2* to that of *IG7* was determined in each sample after "background" hybridization (<2% of intensity of D2 RNA pulse-induced levels) was subtracted. This ratio is plotted using the D2 RNA pulse-induced level as 1.0. IG7 RNA levels varied <20% between samples. Data shown are averages relative to IG7. Bars indicate the range in the two experiments.

To examine the responsiveness of gene expression to cAMP pulses, we starved Synag7 cells in shaking culture and transferred aliquots to "pulsed cAMP" conditions at 3 hr intervals, beginning at 0 hr. Cells were analyzed for RNA levels 6 hr later (Fig. 4). No detectable expression of D2, CsA, cAMP receptor, and M3 RNAs was observed in vegetative cells, whereas low levels of expression of $G_\alpha 2$ RNA were observed in these experiments and no expression in others (data not shown). Shaking cells in the absence of cAMP caused an induction of D2, M3, and CsA RNA levels by 3 hr that remained fairly constant through 15 hr. The relative level of D2, CsA, and M3 RNAs induced upon starvation compared with the level of full expression (see marker lane) was substantially different among the three genes (see *Discussion*). The $G_\alpha 2$ and receptor RNA levels increased through 9 hr, although they remained substantially below those observed in control NC-4 6-hr cells. The presence of cycloheximide from the time of starvation blocked this initial rise (data not shown).

When cells were pulsed with cAMP, regardless of the time that the pulsing was initiated, we saw induction to the maximum or close to the maximum level for all five genes. *M3* showed the lowest relative level of stimulation by the second inductive phase. *D2* and the genes encoding $G_\alpha 2$ and receptor were stimulated >10-fold by the pulse-induced phase. The relative induction of the gene encoding CsA was even greater.

DISCUSSION

Starvation initiates the multicellular developmental program in *Dictyostelium*. During the period between removal of nutrients and commencement of aggregation, the cellular machinery required for aggregation, including that for the

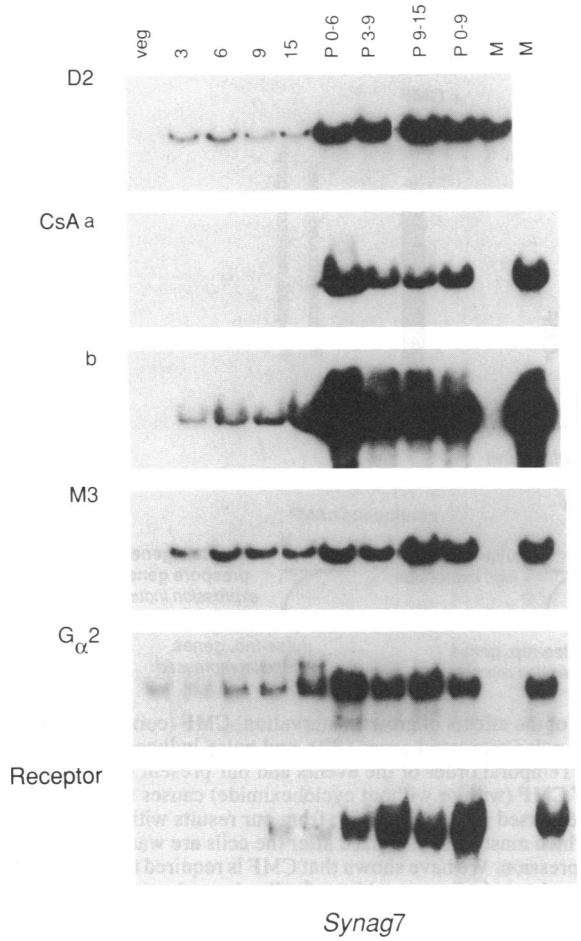


FIG. 4. Two-phase expression of pulse-induced genes. Expression of D2, CsA, M3, $G_\alpha 2$ subunit, and cell surface cAMP receptor mRNAs was measured in shaking cultures. Numbers indicate the time after starvation. An aliquot of cells was removed at times indicated and pulsed for the time period shown (i.e., P 3–9 = cells pulsed from 3 to 9 hr), at which time RNA was isolated from the cells. Marker (M) lanes contain RNA from NC-4 cells developed for 6 hr. For CsA, the blot was given a short (CsAa) and a long (CsAb) exposure to better show the relative expression of CsA mRNA in the absence of cAMP pulses (phase one).

signal transduction system, is synthesized. We have examined in this report some of the control mechanisms regulating several classes of genes, including many that encode elements of this machinery. We have examined the kinetics and requirements for expression of *F9*, the pulse-repressed gene *K5*, and five pulse-induced genes. A summary of our understanding of the temporal kinetics and signals regulating these genes is given in Fig. 5.

In addition to starvation, protein synthesis during at least the first 60–90 min after the onset of starvation is required for accumulation and maintenance of K5 RNA. When protein synthesis is inhibited for 4 hr and then allowed to proceed, K5 RNA accumulates much more rapidly than during normal development. These data suggest that synthesis of a protein factor is required, and the gene encoding it is induced by starvation. It is possible that in the presence of cycloheximide, a developmentally induced RNA is accumulated that is translated after release from cycloheximide. We note that *F9* is expressed at a low level during growth and induced in the first hour of development. F9 RNA accumulates to very high levels in the presence of cycloheximide in starved cells, reminiscent of the induction of c-*fos* in response to growth factors (22). Moreover, like c-*fos* and other serum-induced genes, the ability of cycloheximide to stimulate high levels of F9 RNA accumulation is restricted to that time immediately after the stimulus, which is starvation in *Dictyostelium*. A gene with a developmental pattern similar to that of *F9* may be involved in *K5* and/or subsequent gene induction.

An important observation from the experiments using Synag7 is that induction of the pulse-induced genes occurs by a two-phase process. The first phase is initiated upon starvation or, perhaps in some cases, in late logarithmic-phase cells. The second phase requires pulsatile binding of cAMP to the cell-surface receptor and results in a high level of expression. We expect that the initial phase requires the synthesis of both trans-acting DNA binding proteins and a secreted soluble protein factor, CMF (9) (Fig. 5). We have shown previously that this factor is required for the expression of *D2* and *M3* and the discoidin I gene family [which is not induced by cAMP (23)], and its action is required for the cAMP induction of prestalk and prespore cells (9, 21). CMF may be identical to a factor identified in growing suspensions of NC-4 that can also induce discoidin I expression (24). We believe that CMF functions *in vivo* as a cell-density-determining factor. When cells are starved at a density below a particular threshold, they do not develop. Under these conditions, the concentration of CMF in the extracellular environment is insufficient to allow gene expression and can be complemented by adding CMF (ref. 9; R. H. Gomer and R.A.F., unpublished data). Because *Dictyostelium* can successfully aggregate only if there are enough cells within a given "sphere of influence" to form an aggregate, CMF may be the organism's mechanism to assess the cell density prior to inducing genes whose products are required for aggregation. However, the addition of purified CMF to vegetative growing cells does not appear to be sufficient to induce the initial phase of pulse-induced gene expression (unpublished data).

The second phase of induction involves pulsatile binding of cAMP to the cell-surface receptor (see Fig. 5). A prerequisite for this is the establishment of the cAMP signal-relay system. Many of the genes apparently regulated by this two-phase process are involved in aggregation, including the genes for the receptor and $G_\alpha 2$, essential elements of the signal-relay system. Therefore, it is logical that the initial induction occurs by a "pulse-independent" mechanism, "priming" the cells so they can initiate and respond to a cAMP signal. After establishment of the rudiments of the relay system, rapid and complete induction of this class of genes can occur, the signal-relay system can be maximally activated, and aggregation can proceed optimally. Thus, genes such as those encoding $G_\alpha 2$ and the receptor show autoregulation in that the gene products are required for the cAMP induction of their own synthesis. This two-phase induction system allows autoregulation to "fine-tune" the expression of many genes involved in aggregation.

Prior protein synthesis is also required for both inductive phases of the pulse-induced genes, suggesting that expression of trans-acting factors may be necessary. *D2* expression is blocked by cycloheximide added at 3 hr, even if the cells are subsequently pulsed, indicating a requirement for continuous protein synthesis. Gene products with development kinetics similar to K5 or possibly F9 RNA may be required. Continued pulsing is also required for full and maintained expression. Adaptation of the receptor by continuous cAMP levels or cessation of exogenous pulses to Synag7 cells results in a decline of RNA already accumulated. Repression of the *D2/M3* class of genes occurs *in vivo* at the time that the multicellular aggregate has formed and extracellular cAMP levels have increased. Our results indicate that moderate sustained levels of cAMP, which maintain the cell-surface receptor in an adapted state, are involved in this transcriptional repression. These same conditions are required for

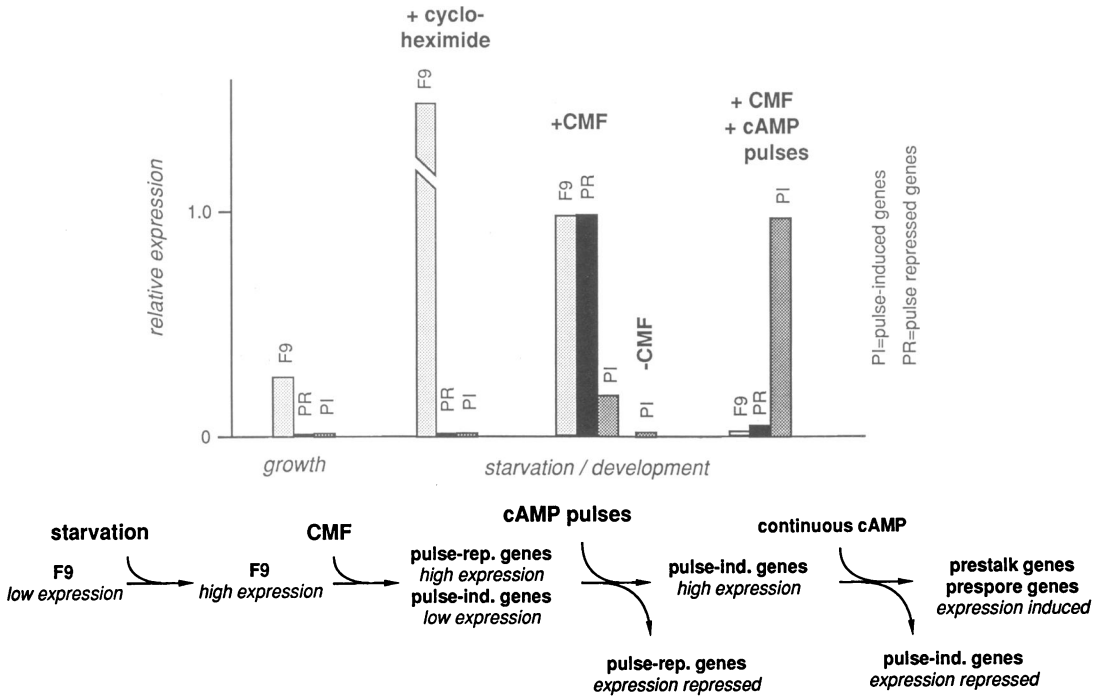


FIG. 5. Temporal pattern and regulation of early genes. (*Upper*) Summaries of the effects of growth, starvation, CMF (conditioned medium factor) (see text and refs. 9 and 21), and cAMP pulses on the expression of *F9*, pulse-repressed genes (PR), and pulse-induced genes (PI). For the relative quantitation of the pulse-induced genes, gene *D2* is used. (*Lower*) Temporal order of the events and our present understanding of the physiological signals controlling these events. Starvation in the presence of CMF (with or without cycloheximide) causes a rapid rise in F9 mRNA levels. We have not quantitated the effect of CMF on *F9* or the pulse-repressed genes. However, from our results with cycloheximide, we believe the increase in *F9* expression does not depend on CMF, a protein which must be synthesized after the cells are washed. We believe that CMF and other protein factors may be required for pulse-repressed gene expression. We have shown that CMF is required for pulse-induced gene expression (9). Repression of *F9* expression is cAMP pulse-independent and may be repressed by a feedback mechanism similar to that for c-*fos* (22). By the time cAMP pulses are initiated (3–4 hr of development), *F9* expression has turned off. See the text for the effect of CMF and cAMP pulses on the pulse-repressed and pulse-induced genes.

maximal induction of prestalk genes, the next known class of cAMP-induced genes expressed during development (9, 25).

We also notice that the relative levels of maximal expression induced by the two phases are different for each gene and may correlate with the function of the gene product. $G_\alpha 2$, the receptor, and D2 are required for aggregation, and the initial phase is higher than that for CsA, which is involved in cell–cell adhesion and not essential for aggregation. This two-phase process allows development in *Dictyostelium* to be responsive to a large number of environmental and physiological conditions, permitting flexibility while still maintaining a program composed of dependent pathways. In contrast, in many metazoans, the timing of the developmental program is very rigid because different parts of the complex organism must differentiate at the same rate. *Dictyostelium* has regulatory pathways controlled by extracellular factors similar to complex multicellular organisms, but its relative simplicity and varying environmental factors both permit and require substantial flexibility in the timing of various parts of the developmental program. We expect that further molecular characterization of the regulation of these genes will provide greater insight into the control mechanisms and the biological significance of these processes.

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