MOLECULAR AND CELLULAR BIOLOGY, Jan. 1987, p. 458–469
0270-7306/87/010458-12$02.00/0

Vol. 7, No. 1

Cyclic AMP Regulation of Early Gene Expression in *Dictyostelium discoideum*: Mediation via the Cell Surface Cyclic AMP Receptor

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We examined two sets of genes expressed early in the developmental cycle of *Dictyostelium discoideum* that appear to be regulated by cyclic AMP (cAMP). The transcripts of both sets of genes were not detectable in vegetative cells. During normal development on filter pads, RNA complementary to these genes could be detected at about 2 h, peaked around 6 to 8 h, and decreased gradually thereafter. Expression of these genes upon starvation in shaking culture was stimulated by pulsing the cells with nanomolar levels of cAMP, a condition that mimics the in vivo pulsing during normal aggregation. Expression was inhibited by caffeine or by continuous levels of cAMP, a condition found later in development when in vivo expression of these genes decreased. The inhibition of caffeine could be overcome by pulsing cells with cAMP. These results suggest that the expression is mediated via the cell surface cAMP receptor, but does not require a rise in intracellular cAMP. mRNA from a gene of the second class was induced upon starvation, peaked by 2.5 h of development, and then declined. In contrast to the other genes, its expression was maintained by continuous levels of cAMP and repressed by cAMP pulses. These and other results on a number of classes of developmentally regulated genes indicates that changing levels of cAMP, acting via the cell surface cAMP receptor, are involved in controlling these groups of genes. We also examined the structure and partial sequence of the cAMP pulse-induced genes. The two tandemly duplicated M3 genes were almost continuously homologous over the sequenced portion of the protein-coding region except for a region near the N-terminal end. The two M3 genes had regions of homology in the 5′ flanking sequence and showed slight homology to the same regions in gene D2, another cAMP pulse-induced gene. D2 showed extremely significant homology over its entire sequenced length to an acetylcholinesterase. The results presented here and by others suggest that expression of many early genes in *D. discoideum* is regulated via the cell surface cAMP receptor. We expect that many of these genes may play essential roles in early *Dictyostelium* development and could code for elements of the cAMP signal transduction pathway involved in aggregation.

Dictyostelium discoideum grows vegetatively as individual amoebae, which aggregate to form a multicellular organism when starved. The aggregate of approximately 10^5 cells then develops over a 26-h period into a fruiting body with two terminally differentiated cell types, spores and stalk cells (see references 24 and 25). We are interested in understanding the regulatory mechanisms controlling temporal and cell-type-specific gene expression during development.

Aggregation of amoebae is mediated by cyclic AMP (cAMP) pulsing. (For a detailed review, see reference 7.) cAMP is secreted by a cell at an aggregation center and binds to receptors of nearby cells. This transiently activates adenylate cyclase, resulting in a rapid increase in intracellular and secreted cAMP. This increase is followed by a refractory period during which the cell does not respond to additional cAMP and the amount of intracellular cAMP returns to original levels. Thus, a pulsatile response-secretion cycle is established to relay waves of cAMP through the population. Cells sense the change in cAMP levels and move up the cAMP gradient toward an aggregation center. In addition, the activation of the receptor by cAMP results in a transient rise in intracellular cGMP levels (26) and phosphorylation of myosin (3). These changes may be central in controlling chemotaxis. The chemotactic response to cAMP results in the formation of a multicellular organism.

In addition to this role, cAMP is known to be involved in the regulation of gene activity throughout the development of *D. discoideum*. In early development, this includes the induction of cAMP phosphodiesterase (20, 48, 51), cAMP receptors (16, 21), and Contact Sites A (a protein proposed to be involved in cell cohesion) (13). cAMP is known to affect discoidin I expression during aggregation (50; S. K. O. Mann and R. A. Firtel, unpublished observations) and, in later development, the specific induction of genes preferentially expressed in either prestalk or prespore cells (1, 30, 31, 37).

We have previously reported the construction and identification of a series of cDNA clones complementary to developmentally regulated mRNAs found in 6-h developing cells (41). In the present study we describe the regulation and characterization of a group of genes that are preferentially expressed during early development. We demonstrate that exposing *Dictyostelium* cells to low-level pulses of cAMP every 6 min, which mimics in vivo signaling, specifically stimulates the expression of a subset of these early genes. In contrast,the expression of another early gene is repressed by these signals. The cAMP-pulse-inducible genes are repressed by continuous low or high levels of cAMP, which results in an adaptation of the cell surface cAMP receptor (7), or by caffeine, which blocks the activation of adenylate cyclase (4). Transcripts of these genes are first

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detected during the interphase period between starvation and aggregation. We cloned and examined a duplicated gene in this set and an individual gene. We describe here their physical similarities and differences, as well as their regulation in relation to the overall importance of cAMP as a factor regulating gene activity throughout *Dictyostelium* development.

MATERIALS AND METHODS

Growth and development of *D. discoideum*. Wild-type NC-4 and a derived axenic strain, KAx-3, were used in these studies. NC-4 cells were grown on *Klebsiella aerogenes* on SM agar plates (46), and KAx-3 cells were grown axenically as described previously (12). NC-4 cells were harvested from agar plates with cold 8 mM 2-(*N*-morpholino)ethanesulfonic acid–2 mM KCl–0.6 mM $MgSO_4$, pH 6.5 (MES-PDF) (11) and freed of bacteria by repeated differential centrifugation in cold MES-PDF. KAx-3 cells were harvested by centrifugation and washed twice in cold MES-PDF. The cells of both strains were then suspended in MES-PDF containing 50 mg of ampicillin and 25 mg each of tetracycline and chloramphenicol per liter.

Cells (7×10^8) were spread onto 12.5-cm Whatman number 50 filters for development as described previously (11). At various times, developing cells were harvested from filters by washing with cold MES-PDF followed by centrifugation.

Cells were suspended in MES-PDF buffer at a concentration of 1×10^6 to 4×10^6 cells per ml for development. The suspension was distributed into three flasks (with volumes three to five times that of the suspension) and shaken at 180 rpm. One flask was used for each of the following conditions: (i) continuous high cAMP (started at 500 μM cAMP, additional cAMP added at 1-h intervals to 100 μM), (ii) pulsed cAMP (pulses of cAMP given every 6 min to a final concentration of 25 nM), and (iii) no cAMP added. cAMP solutions were made up in MES-PDF and neutralized to pH 6.5. Cell samples were harvested at particular time points throughout the experiment, and RNA was extracted.

To determine the effect of caffeine on cells developed in suspension, NC-4 cells were suspended in MES-PDF buffer at a concentration of 3×10^6 cells per ml in four flasks and received one of the following treatments: (i) caffeine (3 or 5 mM initially and then to 2 mM every 2 h), (ii) pulsed cAMP (pulses of cAMP given every 6 min to a concentration of 25 nM), (iii) caffeine plus pulsed cAMP (both as before), or (iv) no treatment.

RNA purification. RNA was extracted from cells harvested at 2.5-h intervals by lysing them in 50 mM Tris (pH 8.4) containing 1% diethylpyrocarbonate by addition of sodium dodecyl sulfate to 1% and then extracting four times with 1:1 phenol-chloroform solution. A 0.1 volume of 3 M sodium acetate, pH 4.7, was added prior to the last two extractions. The RNA was then precipitated once with ethanol, once by the addition of LiCl to 4 M, and twice more with ethanol.

Poly(A)$^+$ RNA was purified on poly(U)-Sepharose. RNA was bound in binding buffer (0.4 M NaCl, 10 mM Tris hydrochloride [pH 7.4], 0.2% sodium dodecyl sulfate, 10 mM EDTA, 10% deionized formamide), washed with 0.5× binding buffer, and then eluted with 80% formamide–2 mM EDTA, pH 7.2. The RNA was then ethanol precipitated and dissolved in H_2O.

RNA blots. Poly(A)$^+$ RNA was size fractionated on 1.5% agarose gels containing formaldehyde (23) and transferred to nitrocellulose or Biodyne transfer membrane (ICN, Irvine, Calif.) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Filters were baked, prehybridized, and hybridized at 37°C with ^{32}P-nick-translated inserts from the cDNA clones for 24 h. They were then washed, air dried, and exposed to X-ray film. Prehybridization, hybridization, and wash solutions were as described previously (31).

Isolation of genomic clones. Genomic fragments containing the M3 and D2 genes were isolated from a previously constructed λ Charon 13 *Eco*RI library (34) by the method of Benton and Davis (2).

cAMP assays. NC-4 cells were harvested and suspended in buffer under the four conditions described above: (i) caffeine, (ii) pulsed cAMP (pulses given every 6 min to a concentration of 25 nM), (iii) caffeine plus pulsed cAMP, or (iv) no treatment. After 7 h, 0.5-ml samples were taken from each culture every 30 s and boiled to denature the protein, liberate the cAMP, and destroy the cAMP phosphodiesterase activity. The samples were then centrifuged to pellet the cell debris. A 50-μl portion of the supernatant was assayed for cAMP with the Amersham cAMP assay kit (TRK.432) according to the instructions in the kit. Samples for RNA extraction were also taken every 2.5 h. These RNAs were size-fractionated, blotted, and probed as described above.

Sequencing. The M3 genes and cDNAs were sequenced by end labeling of appropriate fragments with [γ-^{32}P]ATP using polynucleotide kinase, digestion with a second enzyme so that only one end of each fragment was labeled, and gel purification of the appropriate fragment(s). Sequencing was performed by the method of Maxam and Gilbert (27) and Sanger et al. (43). The sequence for the D2 gene and cDNA was determined by the method of Sanger et al. (43). Appropriate restriction fragments were cloned into phage M13mp8 or -mp9. Single-stranded template was prepared according to the Bethesda Research Laboratories data sheet by using the modifications of Poole and Firtel (36).

All sequenced fragments were sequenced from both ends and an overlap of at least 30 base pairs (bp) was obtained in each case. All sequences were determined at least two times. The one exception was the genomic *Rsa*I-*Pst*I fragment of D2, which was also sequenced several times but from only one end.

S1 nuclease mapping. The 5′ ends of the transcription units of the M3 genes were determined by S1 mapping as described previously (18).

Quantitation of M3 gene expression. A gene-specific probe for the M3L gene was prepared by using the Promega riboprobe SP6 system. A 155-bp *Dde*I-*Ava*II fragment from the 5′ end of M3L was gel purified, blunt ended with the Klenow fragment of DNA polymerase I, and ligated into the *Hin*cII site of pSP64. The correct orientation was determined by subcloning into M13mp8 and sequencing as described above for D2. ^{32}P-labeled RNA probes were made and then hybridized in excess to RNA. The reaction mixture was then digested with RNase A, and the products were analyzed by gel electrophoresis and quantitated as described previously (32, 39), with the following changes: 15 μg of poly(A)$^+$ RNA or tRNA was used for each hybridization, and labeling was carried out with [α-^{32}P]UTP rather than CTP so that the RNA probe was labeled along its entire length.

Protein sequence homology searches. Protein sequence homology searches were performed against an updated version of the NEWAT data base. The significance of the alignments was determined against sequences of the same

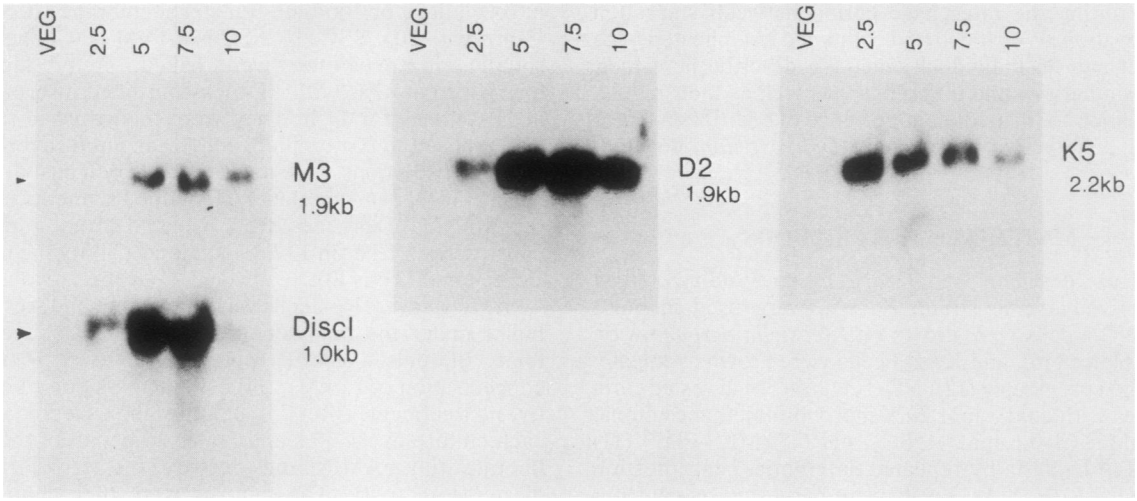

FIG. 1. Kinetics of developmentally regulated early genes. Poly(A)$^+$ RNA was isolated from developing NC-4 cells at 2.5 h time points, size fractionated on a denaturing formaldehyde gel, transferred to nitrocellulose, and probed with nick-translated insert from four cDNA clones. The sizes of the four mRNAs are indicated.

length and composition, but scrambled, with a program that weights similar amino acids (10).

RESULTS

Kinetics and regulation of early gene expression. We have previously reported the construction of a cDNA library made from RNA isolated from 6-h developing cells and the identification of inserts complementary to RNA present in 6-h developing cells but not in bacterially grown vegetative cells (41). Developmental RNA blots probed with labeled insert from several 6-h cDNA clones are shown in Fig. 1. There are four genes or gene families represented. None of the genes were detectably expressed in vegetative cells. RNA complementary to discoidin I, which is encoded by a three-gene family, is present by 2.5 h, reaches a maximum between 5 and 7.5 h, and falls off precipitously by 10 h (34, 35, 42). K5 expression peaks quickly (by 2.5 h) and gradually decreases thereafter, with RNA still detectable at 15 h. M3, a duplicated gene family (described here), and D2, a unique gene, appear to have similar if not identical developmental kinetics (longer exposure of the M3-probed blot indicated this more clearly [data not shown]). RNA was detectable at 2.5 h, reached a maximum between 5 and 7.5 h, and then gradually decreased. The results of RNA blots on discoidin I, M3, and D2 (Fig. 1) are in agreement with previous results for RNA excess hybridization kinetics (41, 42), where quantitation showed that there is less than one copy of mRNA for each gene per five vegetative cells. At their peaks, discoidin I, M3, D2, and K5 mRNAs represent approximately 1, 0.1, 0.3, and 0.02%, respectively, of the poly(A)$^+$ RNA as determined by RNA excess hybridization and comparison of RNA blots. By 15 h of development, the levels of RNA complementary to all four genes is less than 10% of their 10-h level (41; data not shown).

To examine the effects of cAMP on the expression of M3 and D2, vegetative wild-type NC-4 cells grown in association with *K. aerogenes* were washed and then placed in a shaking culture (see Materials and Methods). One such shaking culture was maintained in the absence of added cAMP, the second had cAMP added initially and then maintained at a moderate level (100 to 500 μM), and the third was given low-level pulses (25 nM) of cAMP every 6 min to mimic the normal in vivo pulsing during aggregation. As can be seen from the developmental blots in Fig. 2, M3 and D2 mRNAs accumulated with the same kinetics, though later in development they were lost at different rates, possibly due to a difference in mRNA half-lives. These genes were expressed in the absence of exogenous cAMP, although they were somewhat delayed relative to normal development on filters and were also at a reduced level. When the cells were pulsed with cAMP, the mRNA accumulated precociously and to a higher level, indicating that expression of the genes was stimulated. In contrast, maintaining a constant, moderate level of cAMP resulted in a very low level of gene activity. When a low level of cAMP (100 nM) was provided continuously, expression of the genes was also inhibited (data not shown). When 300 μM cAMP was added to cells pulsed with cAMP, there was a rapid loss of M3 and D2 mRNA, suggesting that constant moderate levels of cAMP repress the expression of these genes (data not shown). These results suggest that M3 and D2 are coordinately regulated by the same cAMP-mediated pathway and they have been designated class II genes.

The effect of cAMP on K5 expression was different from that described for M3 and D2 (see Fig. 2). Cells in shaking suspension showed RNA complementary to K5 present by 2.5 h. High, constant levels of cAMP did not effect K5 expression, but pulsing cells with low levels of cAMP resulted in a premature turn-off. Similar effects have been observed for a different gene (M4-1), which is expressed in vegetative cells and whose expression is repressed by cAMP pulses (17). K5 and genes similarly regulated have been designated class I genes. In contrast, the expression of the discoidin I genes is not affected by pulses of cAMP but is repressed by high constant levels of cAMP (50; data not shown).

The precocious and increased level of expression of M3 and D2 induced by pulses of cAMP suggests that the regulation of expression of these genes is mediated via the cAMP receptor and possibly through the activation of adenylate cyclase. Caffeine is known to inhibit the activation of adenylate cyclase in *D. discoideum* (4), thus preventing the cells from producing intracellular cAMP and an extracellular

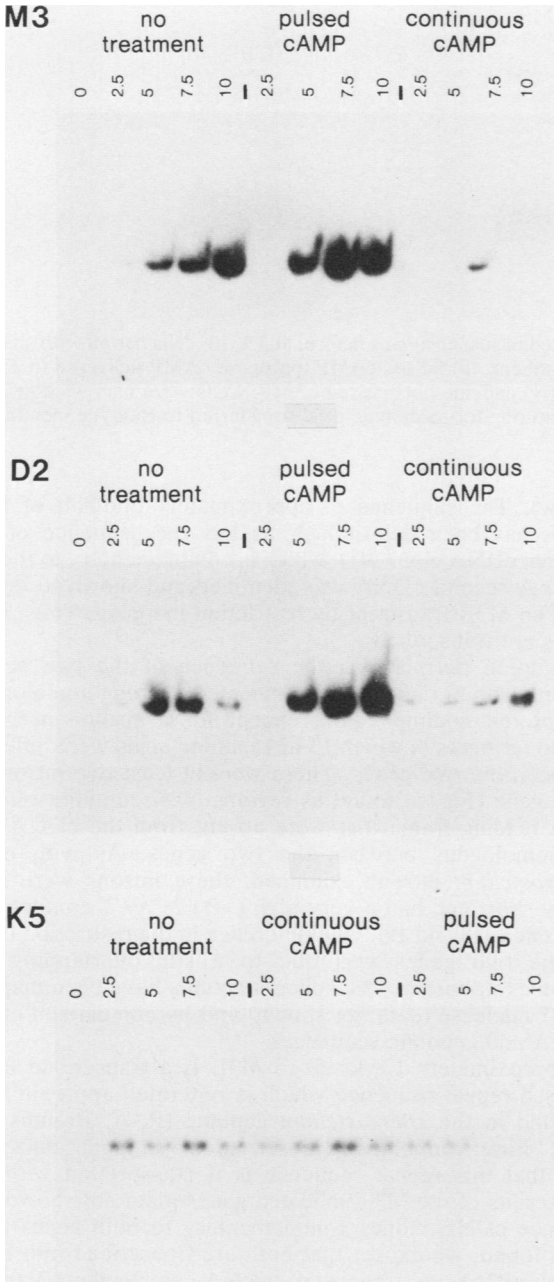

FIG. 2. Effects of cAMP on developmental gene expression. NC-4 cells were grown on bacteria, washed free of bacteria by repeated differential centrifugation, and then suspended in MES-PDF buffer at 4×10^6 cells per ml. Flasks were shaken at 180 rpm and each was given one of the following treatments: (i) high continuous levels of cAMP (initially at 500 μM cAMP, with additional cAMP added to 100 μM each hour), (ii) pulsed cAMP (pulses of cAMP delivered to 25 nM every 6 min), and (iii) no cAMP treatment (see Materials and Methods). Cells were harvested at 2.5-h intervals, and poly(A)$^+$ RNA was purified. This was size fractionated on denaturing formaldehyde gels, transferred to nitrocellulose, and probed with nick-translated insert from three cDNA clones (see Materials and Methods). Note that the order for the K5-probed blot is different than for the other two.

cAMP signal. To determine the effect of caffeine on the expression of the M3/D2 genes, cells were shaken in buffer (see Materials and Methods) and received one of the following treatments: (i) no additions, (ii) low-level pulses of cAMP delivered at 6-min intervals, (iii) caffeine added to a 3 or 5 mM concentration initially and then to 2 mM hourly, or (iv) caffeine added as before plus cAMP pulses. As can be seen from the developmental blots in Fig. 3, cells shaken in the absence of added cAMP expressed D2, as in the previous experiment, and cells pulsed with cAMP showed precocious induction of the gene and a somewhat higher level of expression. Treating the cells with caffeine virtually eliminated D2 expression, and pulses of cAMP in the presence of caffeine overcame the caffeine inhibition and resulted in a peak level of accumulation of RNA similar to that in untreated cells and ca. one-half to two-thirds the maximum level of pulsed cells. The slight reduction and delayed accumulation of D2 RNA may be due to the fact that the expression of the cAMP cell surface receptor is also regulated under these conditions (21; P. Devreotes and A. R. Kimmel, personal communication; see Discussion). M3 showed a similar pattern of regulation in this experiment (data not shown).

To draw conclusions from the above experiments, it was necessary to determine the cAMP oscillations in our cultures under the four different conditions. At 7 h, samples were taken from each of the four flasks at 30-s intervals for 10 min to be assayed for cAMP (see Materials and Methods). Figure 4 shows a graph of the results of one such cAMP assay. Cells given pulses of cAMP received and then quickly amplified this pulse. Cultures shaken in the absence of any external cAMP treatment did show oscillations of endogenous cAMP with a periodicity of ~6.5 min, although these oscillations did not reach the same level as those in the cAMP-pulsed cultures. Under these conditions, these cells had receptor levels that were ca. two-thirds that found in pulsed cells (Silan and Firtel, unpublished observation). Cultures shaken in the presence of caffeine showed no detectable cAMP levels, and cultures pulsed with cAMP in the presence of caffeine showed peaks corresponding to the amount of added cAMP, but, as expected, did not amplify that signal. The variability in the levels of cAMP in the caffeine plus cAMP pulses resulted from some scatter in the assay values. While the cAMP signal was not relayed via the activation of adenylate cyclase, the pulses of cAMP did transiently activate guanylate cyclase (see reference 7) (Silan and Firtel, unpublished observation). Between pulses, cAMP is hydrolyzed by the extracellular and membrane forms of the cAMP phosphodiesterase (7).

Structure of M3 and D2 genes. Hybridization of M3 and D2 cDNA probes to *Dictyostelium* genomic blots indicated that the genes were localized on unique *Eco*RI restriction fragments of 7.2 and 6.9 kilobases (kb), respectively (data not shown). To examine the structure of the M3 duplicated genes, a λ Charon 13 genomic library made from an *Eco*RI limit digest was screened to identify the recombinant phage carrying the 7.2-kb *Eco*RI fragment. An appropriate phage was identified, and the insert and various subfragments were then subcloned into pBR322. A restriction map of the M3 duplicated gene region is included in Fig. 5. There are two transcription units with the same polarity, designated M3L and M3R. The polarity of transcription was determined by using ^{32}P-5′-labeled probes and finding which strand was complementary to M3 mRNA by hybridization (42; data not shown). These results agreed with subsequent DNA sequencing and S1 nuclease mapping of the 5′ ends (see

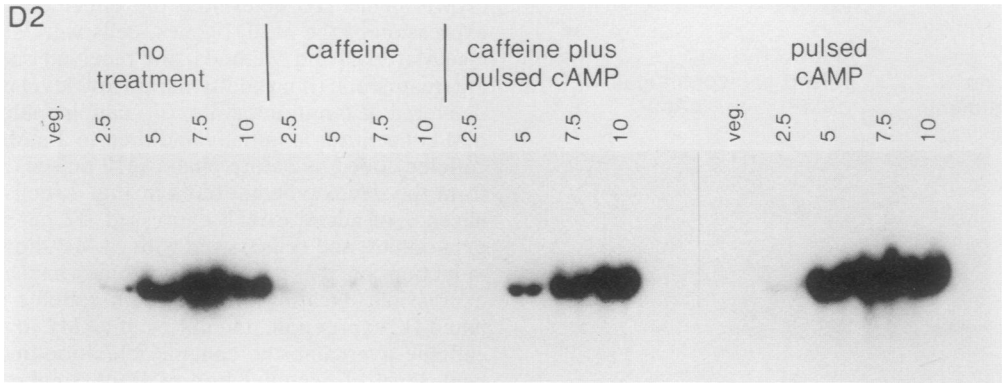

FIG. 3. Effects of caffeine on M3 gene expression. NC-4 cells were developed in suspension as before, at 3×10^6 cells per ml with shaking at 130 rpm. Each flask was used for one of the following conditions (i) no treatment, (ii) pulsed cAMP (pulses of cAMP delivered to 25 nM every 6 min), (iii) caffeine (initially at 5 mM and then to 2 mM hourly), and (iv) caffeine plus pulsed cAMP. Cells were harvested at 2.5-h intervals, and poly(A)$^+$ RNA was purified. This was size fractionated on denaturing formaldehyde gels, transferred to Biodyne membrane, and probed with nick-translated insert from the M3L cDNA clone.

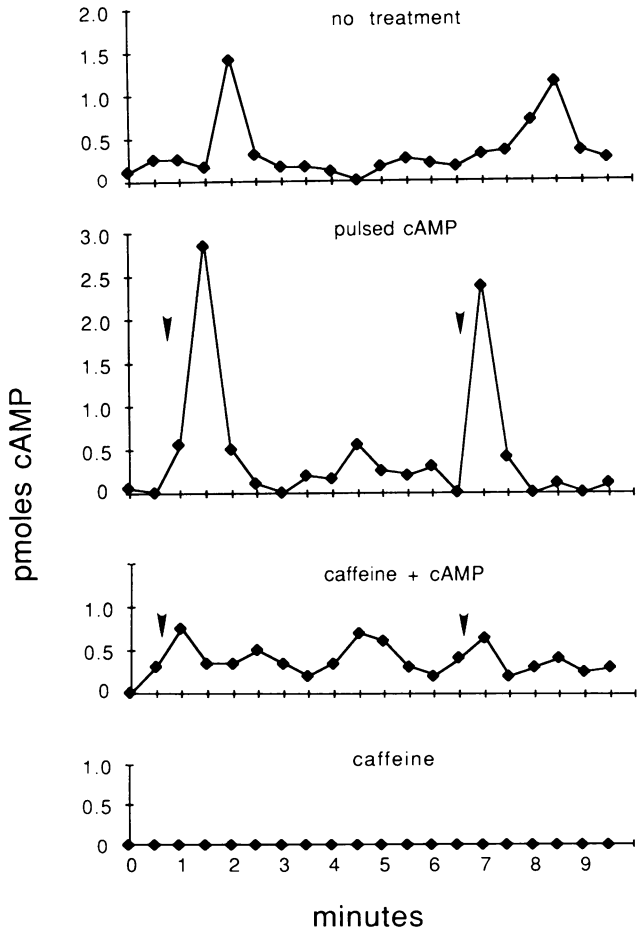

FIG. 4. cAMP signaling under shaking culture conditions. Cells were shaken in buffer under one of the following conditions: (i) no treatment, (ii) pulsed cAMP, (iii) caffeine, or (iv) cafeine plus pulsed cAMP. At 7 h, samples were taken at 30-s intervals for 10 min, boiled, centrifuged, and assayed for cAMP. The two cultures that were pulsed with cAMP received 25-nM pulses at the times indicated by arrowheads. A 25 nM solution corresponds to 1.25 pmol per culture volume containing 10^6 cells. Values given in the figure are per culture volume containing 10^6 cells. For convenience in plotting, a value of 0 cAMP was used for levels of cAMP below the detection level of the assay (<0.05 pmol).

below). The sequence of approximately one-half of both genes has been determined, as has the sequence of the original cDNA clone (41), which is complementary to the left gene. A second cDNA was identified and shown to derive from an M3R transcript by restriction mapping. Thus, both genes are transcribed.

Figure 6 (left) shows the sequence of the two genes. Comparison of the sequence indicated a strong homology in the protein-coding region, except for a section near the amino terminus in which 13 of 15 amino acids were different between the two genes. There were at least two introns in each gene (Fig. 5) found as regions of discontinuity in the open reading frame that were absent from the cDNA and nonhomologous between the two genes. As with other *Dictyostelium* introns examined, these introns were relatively short and had a very high (>90%) A+T content (see references 18 and 19). One difference in the restriction maps of the two genes was due to a site overlapping the intron/exon border. The splice junctions have been mapped by S1 nuclease (data not shown) and by comparison of the cDNA and genomic sequences.

Approximately 1.8 kb 5′ to M3L is a transcribed interspersed repeat sequence which is reiterated approximately 100-fold in the *Dictyostelium* genome (R. A. Brandis and A. R. Firtel, unpublished observation). Mapping data suggest that this repeat sequence is not associated with the transcripts of the M3 duplicated genes (data not shown).

Since cDNA clones complementary to both genes have been found, we expect that both are transcribed into functional mRNA and translated. mRNA complementary to M3 has been isolated by hybridization to immobilized DNA and translated in vitro. Analysis on one-dimensional gels of the products shows a protein of approximately 50 kilodaltons (Brandis and Firtel, unpublished observations).

The genomic clone for D2 was isolated from the same λ Charon 13 genomic library. D2 is carried on a 6.9 kb *Eco*RI fragment. A map of the gene is also shown in Fig. 5. Most of D2 has been sequenced and one 100-bp intron has been found, which is consistent with the 2.0-kb length of the coding region and the 1.9-kb length of the message. This intron was found as a region of discontinuity in the open reading frame with a very high (>85%) A+T content. The splice junctions have not been mapped precisely. The sequence of D2 is given in Fig. 6 (right). The location of the D2 cDNA is also shown in Fig. 5.

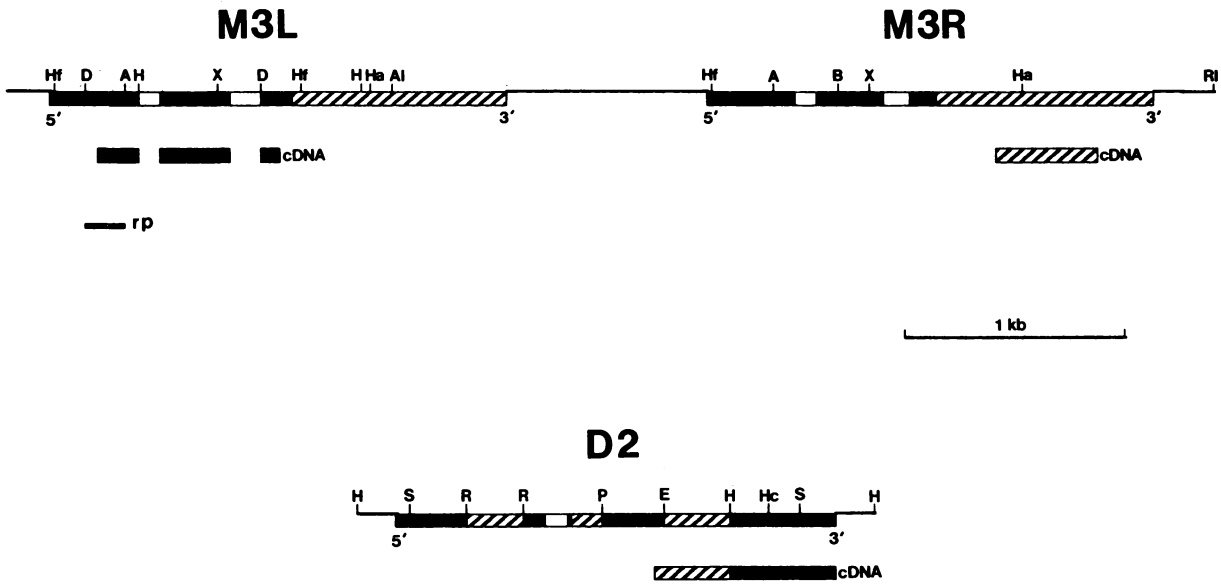

FIG. 5. Restriction maps of cloned developmentally regulated genes. Transcribed regions are indicated by a bar. Solid sections have been sequenced, white sections are introns (also sequenced), and hatched sections have not been sequenced. The location of the cDNAs are shown. The DNA fragment subcloned into the Riboprobe system(Promega Biotech) for determining the differential levels of the two M3 genes is labeled rp. Abbreviations: A, *Ava*II; Al, *Alu*I; B, *Bgl*II; D, *Dde*I; E, *Eco*RV; H, *Hin*dIII; Ha, *Hae*III; Hf, *Hin*fI; P, *Pst*I; R, *Rsa*I; RI, *Eco*RI; S, *Sau*3A; X, *Xba*I. (Note that *Sau*3A sites in M3 have not been mapped.)

5' Flanking sequence of M3 and D2. We analyzed the 5' sequences of the three genes (Fig. 7). The 5' ends of the M3 mRNAs were mapped using S1 nuclease (data not shown) and the cap sites are indicated by arrowheads. The TATA boxes are marked (box A), as are the oligo(dT) regions found between the TATA box and the start of transcription in all *Dictyostelium* pol II genes (box B) (18, 19). In the 5'-untranslated region of both M3 transcripts there is a shared 11-bp sequence (box C). Analysis of the 5' flanking sequences of the two genes indicates that there are several regions of homology, although M3L has an insertion of 48 nucleotides relative to M3R (or M3R has a deletion). The sequences are aligned in the figure to show homology, and identical sequences within the flanking region are underlined.

The 5' flanking region of D2 shows little homology to that of the M3 genes except for three copies of a 6-bp sequence ($^{GGTTTG}_{CCAAAC}$) that is also found in M3L (included in boxes labeled D in the figure). The GT-rich sequence is not found in the sequenced part of the M3L flanking region. There are also two GGGGG sequences (labeled box E) in D2 that are complementary to a CCCCC sequence found in both M3R and M3L. These last sequences are very unusual for *Dictyostelium* noncoding DNA, which is >85% A+T overall.

Relative level of expression of M3L and M3R. Identification of cDNAs derived from both M3L and M3R indicated both are expressed. We examined the relative transcription of the M3 left and right genes by using the SP6 Riboprobe system. The 155-bp *Dde*I-*Ava*II fragment at the 5' end of M3L (Fig. 5), which includes the protein-coding region of least homology, was cloned in the correct orientation into SP64. A ^{32}P-labeled single-stranded RNA probe complementary to M3 mRNA was made from the SP6 promoter of the vector and was hybridized in excess to a measured quantity of poly(A)$^+$ RNA (see Materials and Methods). After digestion with RNase A, the resulting double-stranded RNA fragments were purified, denatured, and sized on a sequencing-type polyacrylamide gel. The probe hybridized along its entire length to mRNA from the left gene, but only in fragments with message from the right gene. We quantitated the relative amount of each of the two messages by cutting the appropriate bands from the gel, eluting them, and determining the level of radioactivity (39). The results indicated that M3R is expressed (at the level of steady-state RNA) at an approximately threefold-higher level than M3L (data not shown). When S1 analysis of end-labeled single-stranded DNA probes is used (see reference 28) a similar quantitation is obtained.

Proteins coded for by the M3 and D2 genes. With the aid of Russell Doolittle (Chemistry Department, University of California at San Diego), a computer analysis was performed on the amino acid sequences derived from the known nucleotide sequences for the M3 and D2 genes (9, 10) (see Materials and Methods). This analysis has provided information regarding proteins from other systems with which these *Dictyostelium* proteins share homology.

Interestingly, the D2 protein shows 25% identity over all sequenced regions to the acetylcholinesterase from the electric ray fish *Torpedo californica* (45), suggesting that the protein encoded by D2 might be an esterase (Fig. 8A). Aligning the first 174 amino acids of the D2 protein to those of the *Torpedo* acetylcholinesterase yields a match score of +7.5, when expressed in standard deviations, above a set of comparisons of sequences of the same length and composition that are scrambled (see Materials and Methods). Statistically, the homology is quite significant and is probably biologically relevant considering the homologous domains lie in the same regions of the protein. The D2 protein, like the acetylcholine esterase, has a long, very hydrophobic region at the amino terminus which is followed by three basic amino acids, suggesting that this is a leader sequence

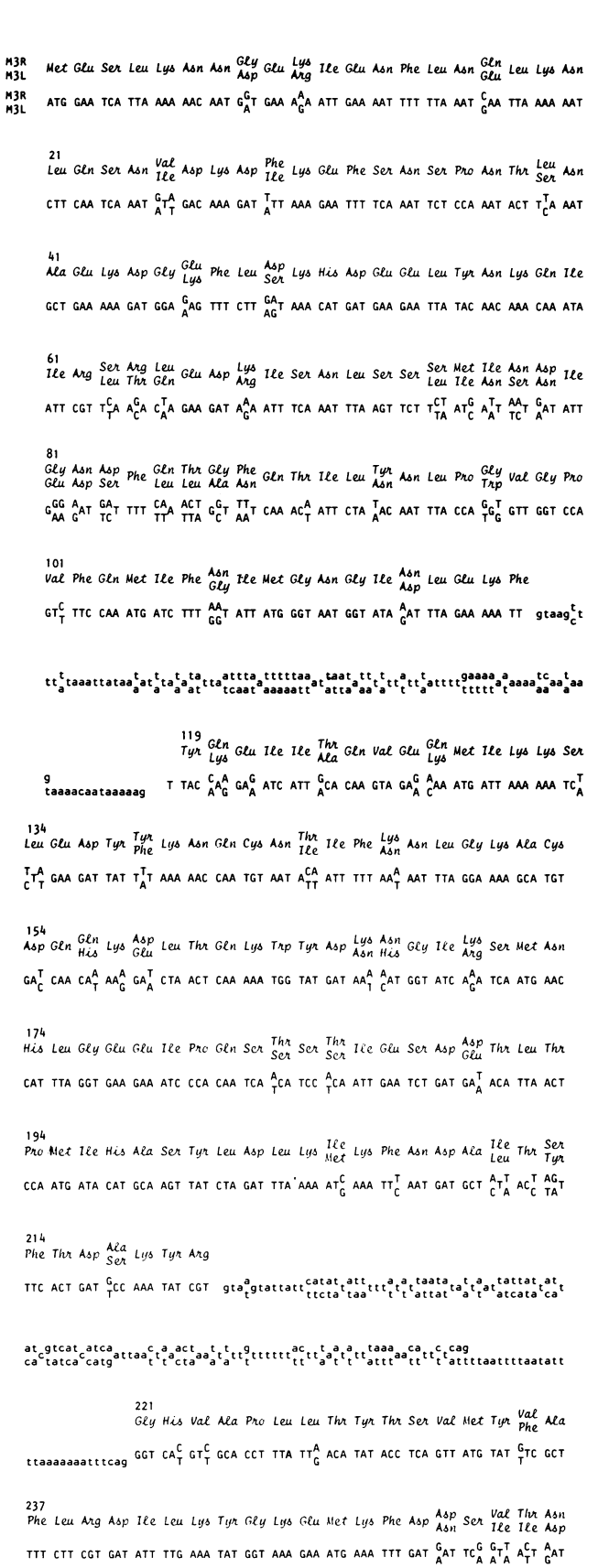

FIG. 6. Nucleotide sequence of M3 and D2 genes. (Left) Comparison of nucleotide and derived amino acid sequence for the two M3 genes. Sequences common to both genes are indicated in the center line. Nonhomologous nucleotides and amino acids are indicated, with sequences for M3R above the center line and those for M3L below the center. The sequence begins with the first codon of the protein-coding region. The nucleotide sequence of the known introns is indicated by lower-case type. The 3′ half of the gene has not yet been sequenced. (Right) Nucleotide and derived amino acid sequence for the D2 gene. The sequence begins with the first codon of the protein-coding region. The known intron is indicated by lower-case type. The three gaps in the sequence (Fig. 5) have not been sequenced.

and that the protein is secreted into a membrane vesicle. No other long hybrophobic regions that could be transmembrane domains are found in the regions sequenced.

With regard to the two M3 proteins, a 41-amino-acid region of both derived protein sequences shows homology to a domain of bovine lung cGMP-dependent protein kinase (Fig. 8B). The match score is +6.3 standard deviations

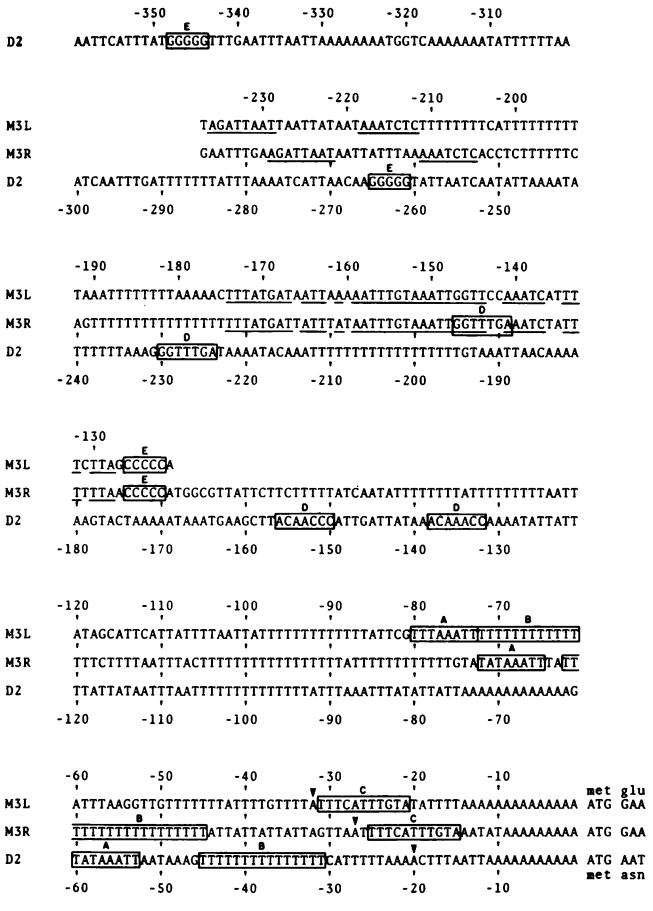

FIG. 7. 5′ Flanking and untranslated sequence for the M3 and D2 genes. The first nucleotide of the AUG start codon is designated as +1. M3L is presented discontinuously to align regions of homology with M3R. The TATA boxes are labeled box A; oligo(dT) runs are labeled box B (see text). Significant homologies between the flanking regions of the two M3 genes are underlined. Potentially interesting regions of homology, as discussed in the text, are in boxes labeled C, D, and E.

above the jumbled sequence, significant for a short sequence. However, this region is located just downstream from the section of least homology between the two M3 genes, while in the cGMP-dependent protein kinase, the region of homology lies on the carboxy-terminal side of the active site, which is part of the most highly conserved region between cGMP-dependent protein kinase and a series of other mammalian kinases (47). Because the domains do not lie in the same parts of the protein, the homologies may be less significant biologically.

DISCUSSION

cAMP regulation of early gene expression. Our analysis shows that there are at least three classes of early developmentally regulated genes in *D. discoideum*. All three classes, which are represented by the discoidin I multigene family, gene K5, and the M3/D2 genes, are not expressed in log-phase vegetative cells of the wild-type strain NC-4 or in axenically grown KAx-3 cells (data not shown). These genes are maximally expressed prior to the formation of aggregates, and the level of mRNA has decreased appreciably by the time culmination initiates.

Interestingly, our results show that the M3/D2 genes (class II genes) are induced by cAMP, but only when the cAMP is given in low-level pulses to mimic in vivo signaling. When cells are shaken in the absence of added cAMP, the M3/D2 genes are expressed, though at a lower level than that seen in cells developed on filter pads. It is known that autonomous pulsing of cAMP is established in shaking cultures (14; P. Devreotes, personal communication), which we have shown occurs in our cell cultures (Fig. 4). This endogenous pulsing probably accounts for the gene activity we see under these culture conditions. No expression is observed when the cells are shaken in the presence of caffeine, which inhibits the activation of adenylate cyclase, thus preventing the cells from emitting pulses of cAMP (4) (Fig. 4). When pulses of cAMP are delivered in the presence of caffeine, the M3/D2 genes are expressed, although at a slightly lower level than in untreated cells. As shown in Fig. 4, the cAMP pulse is not amplified in the presence of caffeine; however, other intracellular receptor-mediated events, such as the transient activtion of cGMP, occur normally. These observations suggest that regulation of class II genes is mediated, at least in part, via the cell surface cAMP receptor and that activation of adenylate cyclase and the concomitant rise in intracellular cAMP are not essential. One might expect the level of expression under these conditions to be the same as that observed in cells shaken in the absence of caffeine. However, the appearance of the receptor is also induced by cAMP pulsing and inhibited by caffeine (21; P. Devreotes and A. R. Kimmel, personal communication), possibly preventing a complete recovery of expression. In separate experiments using these cells, we found the level of receptor to be lower (by approximately 50%) in cells treated with caffeine and pulsed with cAMP (unpublished observation). We thus expect that the lower receptor levels and possibly lower levels of other components of the signal transduction system may explain the lower level of M3/D2 expression.

Low or high continuous levels of cAMP affect M3/D2 gene expression in a manner opposite to that of low-level pulses. We saw in shaking cells a very low level of M3/D2 mRNA accumulation which is retarded relative to cells developing on filters or cells shaken in the absence of added cAMP. This inhibitory effect is also seen in axenically grown KAx-3 cells (data not shown). Inhibition of expression by perfusion with a continuous level of cAMP is understandable for receptor-mediated regulation of early genes, considering that the cAMP receptor-mediated signal transduction system adapts, the receptor is down regulated under these conditions, and no intracellular signal is relayed (see references 7 and 17).

The remaining two classes of early genes also respond to cAMP. The discoidin I genes have previously been shown to be repressed by moderate to high cAMP levels, although pulsing with low levels has no effect (50; Mann and Firtel, unpublished observations). K5, a class I gene, is induced very early in development and seems to be one of the earliest classes of regulated genes. Its expression is prematurely turned off by cAMP pulses, but continues in the presence of high, continuous levels of cAMP. Interestingly, the opposite is true for class II genes, suggesting that K5 and possibly other class I genes play a role very early in development, perhaps as regulators of the expression of class II and other genes. Our results with cAMP suggest that the repression of class I gene expression may also be mediated through the cell surface cAMP receptor. This gene is induced very early in development, before aggregation begins. As the preaggregation interphase proceeds, receptor and probably other components of the cAMP signal transduction pathway accu-

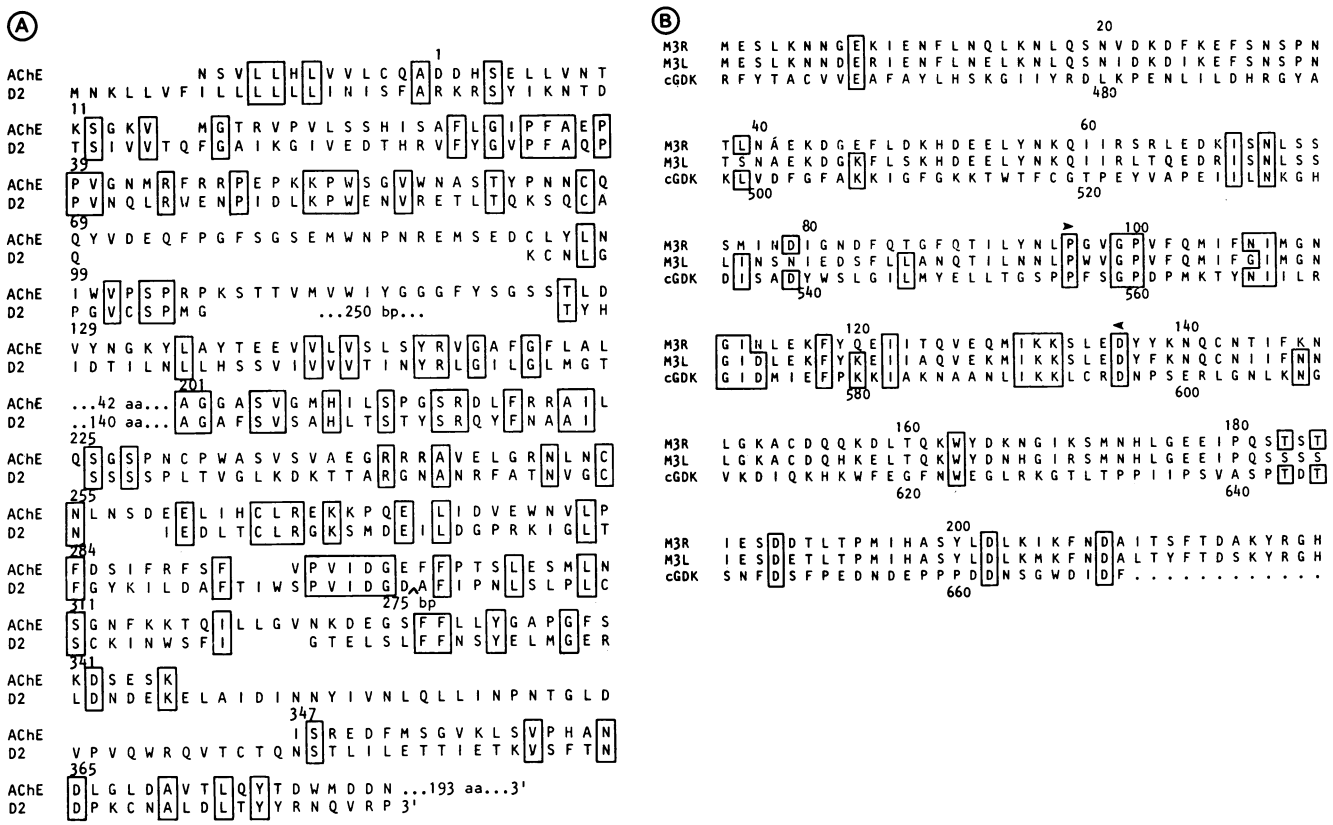

FIG. 8. Comparisons of derived amino acid sequences of M3 and D2. (A) The derived amino acid sequence for the D2 gene is compared with that for a cDNA of *T. californica* acetyl cholinesterase. The homologies are boxed. The three gaps in the D2 sequence indicate nonsequenced regions as indicated on the map for Fig. 4. The entire derived amino acid sequence for the *T. californica* acetylcholinesterase has been determined (43). The gaps in that sequence are to align the two proteins to show optimum homology. The first amino acid of the coding region is indicated by the number 1; preceding amino acids are thought to be part of a leader peptide. The number above the beginning of each line refers to the number of the acetylcholinesterase residue. (B) The amino acid sequence derived from the known nucleotide sequence for the two M3 genes is compared with the amino acid sequence of bovine lung cGMP-dependent protein kinase. Amino acid homologies are boxed. The protein-coding region of least homology in the two M3 genes (see text) is bracketed. The 41-amino-acid region of most significant homology is indicated by arrows. The homology includes 14 amino acids for M3L and 13 for M3R. Numbers above the line indicate the number of M3 residue, and numbers below the line indicate the number of the cGMP-dependent protein kinase residue.

mulate, cAMP oscillations are established, and the expression of this class of gene is then repressed while class II genes are being induced.

mRNAs complementary to class II genes accumulate with the kinetics expected for genes involved in aggregation or required for multicellular differentiation: M3 and D2 RNAs are detectable by 5 h in shaking cells, peak around 7 h into development, and then decrease after about 8 h, at a time when aggregates are forming. Continuous high levels of cAMP repress expression of these genes and discoidin I in shaking cultures (50; Mann and Firtel, unpublished observation). We postulate from these experiments that increasing levels of cAMP are produced by cells in aggregates and that this is the developmental signal that results in a decrease in M3/D2 gene expression. These experiments suggest that changing levels and timing of cAMP production appear to be important regulatory factors in controlling early gene expression in *Dictyostelium* spp.

It is interesting that cAMP receptors, cAMP phosphodiesterase, and Contact Sites A are induced by pulses of cAMP in early development (13, 16, 20, 21, 48, 51; Devreotes and Kimmel, personal communication) and may be other members of the class II developmentally regulated genes to which M3 and D2 belong. The cAMP receptor and cAMP phosphodiesterase, as well as possibly proteins encoded by M3 and D2, are part of the cAMP signaling-relay system responsible for mediating cAMP-dependent aggregation. It has been proposed that cAMP regulates the expression of these genes through interactions with its receptor via a signal transduction pathway that possibly includes activation of guanylate cyclase (22, 29); however, the intracellular signal responsible for regulating these genes is not known. Thus it appears that some if not all of the genes whose expression is stimulated by cAMP may be important components of the aggregation system and therefore may be involved in modulating their own expression. That is, more stimulation by cAMP leads to a higher level of expression of the genes and an increased capacity to respond to an additional cAMP signal. This type of autoregulatory response is reasonable considering the biology of the system; however, such a regulatory pathway is unusual for eucaryotes. It is worth noting that while cAMP pulses appear to be central in the regulation of these genes, it is not known whether the molecular mechanisms regulating the transcription of these genes is the same and whether these genes possess common *cis*-acting regulatory elements.

In various studies we and other researchers have shown that cAMP is also involved in late gene expression (1, 5, 30, 31, 37; R. H. Gomer, D. Armstrong, B. H. Leichtling, and R. A. Firtel, J. Cell. Biol., in press). We have shown that a moderate level of cAMP can induce the expression of prestalk genes in competent cells (30, 31). These genes are maximally expressed in cells allowed to develop for 6 to 10 h, which suggests that the prestalk genes are induced when the normal developmental levels of cAMP reach a threshold concentration in vivo at the time of aggregate formation, the signal that represses class II gene expression. Recent studies suggest this regulation is also mediated via the cAMP receptor (44; Gomer et al., in press). It is at this time in development that the M3/D2-type genes begin to be repressed, presumably by rising levels of cAMP in the aggregate. Thus *D. discoideum* makes use of changing levels of cAMP to modulate the expression of several sets of genes during different parts of its life cycle.

Analysis of amino acid homologies. We have examined the structure of the genes encoding the M3 and D2 mRNAs. Approximately 60% of the D2 gene has been sequenced. The amino acid sequence derived from the known nucleotide sequence has been analyzed and found to share very significant identity (25% of all residues) over all regions to the amino acid sequence derived from a *T. californica* acetylcholinesterase cDNA sequence. It should be noted, however, that the alignments are made for the best possible match and that there are three sizable gaps in the D2 sequence (Fig. 8A). These gaps correspond to regions of the gene that have not been sequenced. However, the evidence suggests that the encoded protein may be a choline esterase of as-yet-unidentified function in *D. discoideum*. The presence of a putative leader sequence suggests that the protein encoded by D2 is secreted or inserted into a membrane.

There are two M3 genes, both of which are transcribed, though the M3R mRNA accumulates to a threefold-higher level than the M3L mRNA. As expected for homologous genes, most of the protein-coding region that has been sequenced shows relatively little amino acid substitution. One major exception is a region near the amino terminus. It is possible that this region of amino acid change has some specific biological function that differentiates the two proteins.

The homology between the M3 proteins and bovine lung cGMP-dependent protein kinase extends over a 41-residue region and involves 14 amino acids for the M3L protein and 13 for the M3R protein. Thus 34 and 32%, respectively, of the residues are conserved. This degree of indentity over this short region, while apparently statistically significant, involves a region of the cGMP-dependent protein kinase that is not involved in cGMP or ATP binding, nor does it appear to be part of the catalytic domain (47; S. Taylor, personal communication) (Fig. 8B). The homologous region contains a Pro-X-X-Gly-Pro-X-Pro in the middle of the domain, which may be involved in a specific turn of the protein. In addition to the amino acid identities, other interchanges involve conservative amino acid replacements. Because of its high level of conservation, it is possible that this domain may be involved in a protein-protein interaction.

cGMP appears to be involved in the membrane transduction system between the cAMP receptors on the surface of developing *Dictyostelium* cells and the intracellular system responsible for chemotaxis. Evidence for this include the following: cAMP pulses lead to a rapid, transient rise in intracellular cGMP levels (26); there is an intracellular phosphodiesterase specific for cGMP (8); and "streamer" *Dictyostelium* mutants show abnormally high concentrations of cGMP that are not rapidly degraded (40). These mutants have chemotactic movement responses of up to 520 s in duration rather than the normal 100 s and therefore develop large aggregation streams. The importance of the M3 genes to cGMP metabolism is presently unknown. This is being examined by using anti-sense mutagenesis approaches to knock out M3 expression.

Analysis of 5′ nucleotide sequences. All three genes have a TATA box and an oligo(dT) sequence between the TATA box and the cap site, as do all other *Dictyostelium* pol II genes examined (18, 19). D2 and M3R both have a TATA sequence of TATAAATT, which differs from the *Dictyostelium* consensus sequence TATAAA$^{T}_{A}$A (19) at only the last nucleotide. The M3L gene, which is expressed at a lower level, has a T instead of the highly conserved A residue in position 2. Other workers examining the function of the TATA box in metazoan gene expression have shown that divergence at position 2 normally results in a lower rate of transcription (15, 49). Our results on the in vivo level of expression of members of the M3 gene family and two of the ~20 *Dictyostelium* actin genes, actins 7 and M6, suggest that this may also be true for *D. discoideum* (28, 39; this study). There are other differences between the 5′ sequences of the two genes that could possibly affect the relative levels of transcription. In M3L the oligo(dT) stretch adjoins the TATA box, rather than being separated by several nucleotides as in most other *Dictyostelium* genes. Also, there is a 48-nucleotide insertion in the 5′ flanking region of M3R (or an M3L deletion) that may have some effect on the relative levels of gene expression.

In looking for significant sequence homologies among the 5′-untranslated and flanking regions of the three genes, the analysis is greatly complicated by the fact that the noncoding *Dictyostelium* sequence is generally 90% A+T. There are several short regions homologous between the two M3 genes that are relatively GC-rich and potentially interesting, in particular the CCCCC sequence at positions −170 in M3R and −125 in M3L. There are fewer sequences shared among all three genes. There are two GGGGG sequences, at −265 and −345 in the D2 5′ flanking region. If the CCCCC sequence is involved in the regulation of M3, it is possible that one of the GGGGG sequences in D2 may be functionally equivalent. Moreover, the sequence GGTTTG is shared by two genes.

In the 5′-untranslated regions of the M3 genes, there is a shared 11-base-pair (bp) sequence containing the only G and C in the untranslated portion (labeled box C). All three genes have a stretch of at least nine A's immediately before the AUG initiation codon. This compares favorably with the coordinately regulated discoidin I gene family, whose members show strong sequence homology within their entire 5′-untranslated region (34), and contrasts with most of the noncoordinately regulated actin genes, which do not exhibit such strong homology (38).

In the 5′ flanking region, the two M3 genes have the most sequence homology between −122 and −174 in M3L and between −170 and −222 in M3R. The coordinately regulated discoidin I genes have a 12-nucleotide imperfect palindrome lying approximately 200-bp upstream from the cap site (36). In two of the three genes, this palindrome is flanked by inverted repeats. We have proposed that these sequences may well be involved in discoidin I regulation. A GC-rich region also appears to be involved in conferring cAMP regulation and cell-type specificity to the *pst-cath* and *Dd-ras* genes, both preferentially expressed in prestalk cells (5a,

6; P. K. Howard, C. D. Reymond, T. P. Spann, and A. R. Firtel, unpublished observation). The 53-bp region of homology upstream from the M3 genes is interesting because of its GC-richness and its length, particularly considering the relatively weak homology in the 5′ flanking region directly upstream from the TATA box. We feel this region may include sequences involved in the regulation of these genes.

To functionally determine the *cis*-acting sequences necessary for the regulation of the M3/D2 genes, we are preparing to use in vitro mutagenesis and DNA-mediated transformation of *D. discoideum* (33). It is expected that such studies will help us understand how these genes are regulated by cAMP and show whether they share a common *cis*-acting regulatory element.

ACKNOWLEDGMENTS

We are indebted to R. Doolittle for his computer analysis of the protein homologies and C. Silan for technical assistance. We also thank M. G. Rosenfeld, S. Taylor, P. Devreotes, and G. Gill for discussions on numerous occasions and for helpful suggestions.

S.K.O.M. was the recipient of a National Science Foundation predoctoral fellowship and was subsequently supported by Public Health Service predoctoral training grant GM07313 from the National Institutes of Health. This work was supported by Public Health Service grant GM30693 to R.A.F.

LITERATURE CITED

1. **Barklis, E., and H. F. Lodish,** 1983. Regulation of *Dictyostelium discoideum* mRNAs specific for prespore and prestalk cells. Cell **32**:1139–1148.
2. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt11 recombinant clones by hybridization to single plaques *in situ*. Science **196**:180–182.
3. **Berlot, C. H., J. A. Spudich, and P. N. Devreotes.** 1985. Chemoattractant-elicited increases in myosin phosphorylation in *Dictyostelium*. Cell **43**:307–314.
4. **Brenner, M., and S. D. Thoms.** 1984. Caffeine blocks activation of cyclic AMP synthesis in *Dictyostelium discoideum*. Dev. Biol. **101**:136–146.
5. **Chung, S., S. M. Landfear, D. D. Blumberg, N. S. Cohen, and H. F. Lodish.** 1981. Synthesis and stability of developmentally regulated *Dictyostelium* mRNAs are affected by cell-cell contact and cAMP. Cell **24**:785–797.

5a. **Datta, S., and R. A. Firtel.** 1987. Identification of the sequences controlling cyclic AMP regulation and cell-type-specific expression of a prestalk-specific gene in *Dictyostelium discoideum*. Mol. Cell. Biol. 149–159

6. **Datta, S., R. H. Gomer, and R. A. Firtel.** 1986. Spatial and temporal regulation of a foreign gene by a prestalk-specific promoter in transformed *Dictyostelium discoideum*. Mol. Cell Biol. **6**:811–820.
7. **Devreotes, P.** 1982. Chemotaxis, 117–168. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
8. **Dicou, E., and P. Brachet.** 1980. A separate phosphodiesterase for the hydrolysis of cGMP in growing *Dictyostelium discoideum* amoebae. Eur. J. Biochem. **109**:507–514.
9. **Doolittle, R. F.** 1981. Similar amino acid sequences: chance or common ancestry? Science **214**:149–159.
10. **Feng, D. F., M. S. Johnson, and R. F. Doolittle.** 1985. Aligning amino acid sequences: comparison of commonly used methods. J. Mol. Evol. **21**:112–125.
11. **Firtel, R. A., L. Baxter, and H. F. Lodish.** 1973. Actinomycin D and the regulation of enzyme biosynthesis during development of *Dictyostelium discoideum*. J. Mol. Biol. **79**:315–327.
12. **Firtel, R. A., and J. Bonner.** 1972. Characterization of the genome of the cellular slime mold *Dictyostelium discoideum*. J. Mol. Biol. **66**:339–361.
13. **Gerisch, G., H. Fromm, A. Huesgen, and U. Wick.** 1975. Control of cell contact sites by cAMP pulses in differentiating *Dictyostelium discoideum* cells. Nature (London) **255**:547–549.
14. **Gerisch, G., and B. Hess.** 1974. Cyclic-AMP-controlled oscillations in suspended *Dictyostelium* cells: their relation to morphogenetic cell interactions. Proc. Natl. Acad. Sci. USA **71**:2118–2122.
15. **Grosschedl, R., B. Wasylyk, P. Chambon, and M. L. Birnstiel.** 1981. Point mutation in the TATA box curtails expression of sea urchin H2A histone gene *in vivo*. Nature (London) **294**:178–180.
16. **Juliani, M. H., J. Brusca, and C. Klein.** 1981. cAMP regulation of cell differentiation in *Dictyostelium discoideum* and the role of the cAMP receptor. Dev. Biol. **83**:114–121.
17. **Kimmel, A. R., and B. Carlisle.** 1986. A gene expressed in undifferentiated vegetative *Dictyostelium* is repressed by developmental pulses of cAMP and reinduced during differentiation. Proc. Natl. Acad. Sci. USA **83**:2506–2510.
18. **Kimmel, A. R., and R. A. Firtel.** 1982. The organization and expression of the *Dictyostelium* genome, p. 233–324. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
19. **Kimmel, A. R., and R. A. Firtel.** 1983. Sequence organization in *Dictyostelium*: unique structure at the 5′-ends of protein coding genes. Nucleic Acids Res. **11**:541–552.
20. **Klein, C., and M. Darmon.** 1977. Effects of cyclic AMP pulses on adenylate cyclase and the phosphodiesterase inhibitor of *D. discoideum*. Nature (London) **268**:76–78.
21. **Klein, C., and M. H. Juliani.** 1977. cAMP-induced changes in cAMP binding sites on *D. discoideum* amoebae. Cell **10**: 329–335.
22. **Lappano, S., and M. B. Coukell.** 1982. Evidence that intracellular cGMP is involved in regulating the extracellular cAMP phosphodiesterase and its specific inhibitor in *Dictyostelium discoideum*. Dev. Biol. **93**:43–53.
23. **Lebrach, H., D. Diamond, J. M. Wozney, and H. Boedker.** 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical examination. Biochemistry **16**:4743–4751.
24. **Loomis, W. F.** 1975. *Dictyostelium discoideum*, a developmental system. Academic Press, Inc., New York.
25. **Loomis, W. F.** 1982. The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
26. **Mato, J., F. Krens, P. van Haastert, and T. Konijn.** 1977. 3′:5′-Cyclic AMP-dependent 3′:5′-cyclic GMP accumulation in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA **74**: 2348–2351.
27. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
28. **McKeown, M., and R. A. Firtel.** 1981. Differential expressionand 5′-end mapping of actin genes in *Dictyostelium*. Cell **24**:799–807.
29. **McRobbie, S. J., and P. C. Newell.** 1984. A new model for chemotactic signal transduction in *Dictyostelium discoideum*. Biochem. Biophys. Res. Commun. **123**:1076–1083.
30. **Mehdy, M. C., and R. A. Firtel.** 1985. A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*. Mol. Cell. Biol. **5**:705–713.
31. **Mehdy, M., D. Ratner, and R. Firtel.** 1983. Induction and modulation of cell-type specific gene expression in *Dictyostelium*. Cell **32**:763–771.
32. **Melton, D. A., P. A. Kreig, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12**:7035–7056.
33. **Nellen, W., C. Silan, and R. A. Firtel.** 1984. DNA-mediated transformation in *Dictyostelium discoideum*: regulated expression of an actin gene fusion. Mol. Cell. Biol. **4**:2890–2898.
34. **Poole, S., R. Firtel, E. Lamar, and W. Rowekamp.** 1981. Sequence and expression of the discoidin I gene family in *Dictyostelium discoideum*. J. Mol. Biol. **152**:273–289.
35. **Poole, S., R. A. Firtel, and W. Rowekamp.** 1982. Expression of the discoidin I genes in *Dictyostelium*, p. 795–803. *In* W. A. Frazier, L. Glazer, and D. I. Gottlieb (ed.), Cellular recognition. UCLA Symposia on Molecular and Cellualr Biology, vol. 3. Alan R. Liss, New York.

36. **Poole, S. J., and R. A. Firtel.** 1984. Conserved structural features are found upstream from the three coordinately regulated discoidin I genes of *Dictyostelium discoideum*. J. Mol. Biol. **172**:203–220.
37. **Reymond, C. D., R. H. Gomer, M. C. Mehdy, and R. A. Firtel.** 1984. Developmental regulation of a *Dictyostelium* gene encoding a protein homologous to mammalian *ras* protein. Cell **39**:141–148.
38. **Romans, P., and R. A. Firtel.** 1985. Organization of the *Dictyostelium discoideum* actin multigene family: flanking sequences show subfamily homologies and unusual dyad symmetries. J. Mol. Biol. **183**:311–326.
39. **Romans, P., R. A. Firtel, and C. L. Saxe.** 1985. Gene-specific expression of the actin multigene family of *Dictyostelium*. J. Mol. Biol. **186**:337–355.
40. **Ross, F., and P. Newell.** 1981. Streamers: chemotactic mutants of *Dictyostelium discoideum* with altered cyclic GMP metabolism. J. Gen. Microbiol. **127**:339–350.
41. **Rowekamp, W., and R. Firtel.** 1980. Isolation of developmentally regulated genes from *Dictyostelium*. Dev. Biol. **79**: 409–418.
42. **Rowekamp, W., S. Poole, and R. Firtel.** 1980. Analysis of the multigene family coding the developmentally regulated carbohydrate-binding protein discoidin I in *D. discoideum*. Cell **20**:495–505.
43. **Sanger, F., S. Nicklen, and A. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
44. **Schaap, P., and R. van Driel.** 1985. Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP. Exp. Cell Res. **159**:388–398.
45. **Schumacher, M., S. Camp, Y. Maulet, M. Newton, K. MacPhee-Quigley, S. S. Taylor, T. Friedmann, and P. Taylor.** 1986. Primary structure of *Torpedo californica* acetylcholinesterase deduced from cDNA sequence. Nature (London) **319**:407–409.
46. **Sussman, M.** 1966. Biochemical and genetic methods in the study of cellular slime mold development, p. 397–410. *In* D. Prescott (ed.), Methods in cell physiology 2. Academic Press, Inc., New York.
47. **Takio, K., R. Wade, S. Smith, E. Krebs, K. Walsh, and K. Titani.** 1984. Guanosine cyclic 3′,5′-phosphate dependent protein kinase, a chimeric protein homologous with two separate protein families. Biochemistry. **23**:4207–4218.
48. **Van Haastert, P. J. M., R. C. van der Meer, and T. M. Konijn.** 1981. Evidence that the rate of association of adenosine 3′,5′-monophosphate to its chemotactic receptor induces phosphodiesterase activity in *Dictyostelium discoideum*. J. Bacteriol. **147**:170–175.
49. **Wasylyk, B., R. Derbyshire, A. Guy, D. Molko, A. Roget, R. Teoule, and P. Chambon.** 1980. Specific *in vitro* transcription of conalbumin gene is drastically decreased by single-point mutation in T-A-T-A box homology sequence. Proc. Natl. Acad. Sci. USA **77**:7024–7028.
50. **Williams, J., A. Tsang, and H. Mahbubani.** 1980. A change in the rate of transcription of a eukaryotic gene in response to cAMP. Proc. Natl. Acad. Sci. USA **77**:7171–7175.
51. **Yeh, R. P., F. K. Chan, and M. B. Coukell.** 1978. Independent regulation of the extracellular cyclic AMP phosphodiesterase-inhibitor system and membrane differentiation by exogenous cyclic AMP in *Dictyostelium discoideum*. Dev. Biol. **66**: 361–374.