MOLECULAR AND CELLULAR BIOLOGY, Oct. 1989, p. 4170–4178
0270-7306/89/104170-09$02.00/0

Vol. 9, No. 10

Expression and Organization of BP74, a Cyclic AMP-Regulated Gene Expressed during *Dictyostelium discoideum* Development

SUSAN B. HOPKINSON, RICHARD S. POLLENZ, IAIN DRUMMOND, AND REX L. CHISHOLM*

Department of Cell Biology and Anatomy, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611

Received 20 December 1988/Accepted 19 June 1989

We have characterized a cDNA and the corresponding gene for a cyclic AMP-inducible gene expressed during *Dictyostelium* development. This gene, BP74, was found to be first expressed about the time of aggregate formation, approximately 6 h after starvation. Accumulation of BP74 mRNA did not occur in *Dictyostelium* cells that had been starved in fast-shaken suspension cultures but was induced in similar cultures to which cyclic AMP pulses had been added. The BP74 cDNA and gene were characterized by DNA sequence analysis and transcriptional mapping. When the BP74 promoter region was fused with a chloramphenicol acetyltransferase reporter gene and reintroduced into *Dictyostelium* cells, the transfected chloramphenicol acetyltransferase gene displayed the same developmentally regulated pattern of expression as did the endogenous BP74 gene, suggesting that all of the *cis*-acting elements required for regulated expression were carried by a 2-kilobase cloned genomic fragment. On the basis of sequence analysis, the gene appeared to encode a protein containing a 20-residue hydrophobic sequence at the amino-terminal end and 26 copies of a 20-amino-acid repeat.

The cellular slime mold *Dictyostelium discoideum* is particularly well suited for studies of the cellular and molecular events responsible for cellular differentiation. As long as adequate nutrients are available, *Dictyostelium* cells exist as single, motile amoebae that reproduce by cell division. Depletion of available amino acids initiates a developmental program leading to the formation of multicellular aggregates (31). Early in starvation, a small number of the cells begin to secrete cyclic AMP (cAMP) pulses that are relayed outwardly in waves by surrounding cells (12). cAMP functions as a chemoattractant, and by 6 h of development the cells begin to stream together toward the signaling center. By 10 h, cells have formed cell aggregates or mounds. During aggregation, extracellular cAMP functions not only as a chemoattractant but also as a regulator for the expression of a variety of genes through interaction with the cell surface cAMP receptor (3, 17, 18, 22, 24, 25, 32, 36, 41, 43, 47). After formation of the mound, the aggregate undergoes a series of morphologic changes culminating in production of a mature fruiting body consisting of spores and stalk cells.

We and others have previously identified several extracellular signals that control the specific expression of genes that are temporally induced during development and expressed in a cell-type-specific manner. These signals include starvation, nanomolar cAMP pulses, differentiation-inducing factor (DIF), and some consequence of tight cell aggregate formation, perhaps an accumulation of high cAMP concentrations within the aggregates (3, 4, 7, 17, 18, 24, 43, 49, 50). Although *Dictyostelium* represents a well-defined system for study of gene regulation by extracellular signals, to date only a relatively small number of developmentally regulated genes have been subjected to detailed structural and functional characterization. One of the earliest genes to be induced during development is the cyclic nucleotide phosphodiesterase. The mRNA level is low in vegetative cells and increases within 80 min of the initiation of development. Accumulation of message then decreases and later accumulates to maximum levels late in aggregation (28). Discoidins I and II are carbohydrate-binding proteins synthesized early in development. Discoidin mRNA is not detectable in cells grown on bacteria but is present in low levels in vegetative cells grown axenically. By 4 h of development, however, the discoidin I mRNA constitutes 2% of the mRNA. The level of discoidin mRNA drops to undetectable levels by 8 h of development. Addition of cAMP to the *Dictyostelium* cells early in development prevents accumulation of the discoidin I message (11, 51). The cysteine proteinase 1 (CP1) and 2 (CP2) genes have been shown to be coregulated during development (44). mRNAs for both are absent in vegetative and early developing cells but accumulate by 10 to 12 h. Expression of CP1 and CP2 appears to be dependent on interaction of cAMP with the cell surface cAMP receptor. CP1 and CP2 messages are expressed at higher levels in prestalk cell fractions isolated by Percoll density gradient centrifugation. The upstream regulatory region of CP1 also directs transcription of the adjacent DG17, gene which shows similar temporal and spatial regulation (14). In the intergenic region between DG17 and CP1, there are four G-rich regions that may be important in regulating expression. Similarly, the CP2 gene (also called pst-cath) contains a G+C-rich sequence upstream of the transcriptional start site that has been shown to be important for proper expression of CP2 (8, 10). The expression of uridine diphosphoglucose pyrophosphorylase (UDPGP), an enzyme essential for *Dictyostelium* development, has been shown to be regulated by cell-cell contact and exogenous cAMP (1, 4, 16, 45). UDPGP is present in vegetative cells, but the message and protein levels increase during development such that during later stages there is a 5- to 10-fold rise in enzyme-specific activity due to increased expression of the UDPGP mRNA (16, 45).

To expand our understanding of the molecular mechanisms responsible for cAMP inducibility and temporal regulation of gene expression during *Dictyostelium* differentia-

* Corresponding author.

tion, we have examined the structure and regulation of BP74. In this report, we show that BP74 mRNA first accumulates in aggregating cells and that extracellular cAMP is necessary for BP74 expression. Using the BP74 cDNA, we have isolated a 4.6-kilobase (kb) genomic DNA fragment containing the entire coding region as well as 5′ and 3′ flanking regions. The nucleotide sequences of the BP74 gene and most of the cDNA have been determined, and the transcriptional start site has been located by S1 mapping. When reintroduced into *Dictyostelium* cells, this cloned fragment carries all of the *cis*-acting elements necessary to produce regulated expression of the transfected DNA.

MATERIALS AND METHODS

Cell lines and cultures. *D. discoideum* AX-3 strain RC3 (obtained from David Soll) grown axenically at 22°C in HL-5 medium was used for all RNA isolations (7). Cells were plated for filter development on Whatman no. 50 filters supported by several Whatman no. 3 filters saturated with DB (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgSO_4$ [pH 6.5]). Suspension cultures were fast shaken in DB at 250 rpm. cAMP pulses were generated by adding cAMP to a final concentration of 50 nM once every 6 min. For the disaggregation studies, cells developed for 15 h on filter pads were harvested, washed in PDF (20 mM KCl, 9 mM K_2HPO_4, 13 mM KH_2PO_4, 1 mM $CaCl_2$, 2.5 mM $MgCl_2$ [pH 6.4]) with 1 mM EDTA and vigorously vortexed to break up remaining aggregates. The cells were resuspended in PDF at 2.5×10^7 cells per ml, divided into two equal portions, and fast shaken at 250 rpm. cAMP at a final concentration of 1 mM was added to one flask initially, with a second dose of 0.3 mM added after 2 h. RNA was made from these cells as previously described (7).

Cloning and DNA analysis. A genomic library of 4- to 5-kb *Eco*RI fragments was prepared by completely digesting *Dictyostelium* nuclear DNA and size fractionating the digest by agarose gel electrophoresis. DNA in the 4- to 5-kb size range was eluted from the gel, ligated into lambda gt10, and packaged into bacteriophage particles. The resulting library was screened with a 700-base *Dra*I fragment isolated from the BP74 cDNA. For the genomic Southern analysis, *Dictyostelium* DNA was digested by a variety of restriction enzymes, and 3 μg of digested DNA was electrophoresed on a 0.7% agarose gel. The DNA was then transferred to GeneScreen Plus via alkaline blotting. The resulting blots were probed with DNA labeled by either nick translation or random oligonucleotide-primed synthesis.

RNA isolation and Northern (RNA) blotting. RNA was isolated from cell pellets by phenol-chloroform extraction. A solution of 10% sodium dodecyl sulfate–100 mM EDTA–10 mM Tris (pH 7.8) containing 0.1% diethyl pyrocarbonate (Sigma Chemical Co.) was added directly to a tube containing a cell pellet and vortexed. An equal volume of phenol-chloroform was added and vortexed. After heating to 55°C for 5 min and cooling on ice, the samples were spun in a microcentrifuge for 5 min. The aqueous phase was reextracted until no protein precipitate remained at the interface. Sodium acetate was added to 0.3 M, and the nucleic acids were precipitated with 2.5 volumes of ethanol. After suspension in diethyl pyrocarbonate-treated distilled water, 20 μg of each RNA sample was separated electrophoretically on a 1% denaturing agarose gel containing 6% formaldehyde and then transferred to nitrocellulose in 10× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Preparation of *Dictyostelium* nuclei and analysis of run-on transcription products. Nuclear run-on experiments were carried out essentially as described by Jacobson et al. (20) and Nellen et al. (40). Linearized plasmids (1 μg) containing BP74 cDNA or no insert were boiled in NaOH and filtered onto GeneScreen Plus by using a slotted manifold (Schleicher & Schuell, Inc.). In addition, the CZ22, D18, and actin 15 cDNAs were included on all filters as controls. The transcription data of these genes during development have been reported elsewhere (29) and are not shown. Filters were prehybridized (50% formamide, 3× SSC, 10 mM EDTA, 60 mM Na_2HPO_4, 0.2% sodium dodecyl sulfate, 4× Denhardt solution) for 16 h at 37°C. Hybridization was carried out in prehybridization solution that had been supplemented with 200 μg of salmon sperm DNA per ml. Typically, 0.6×10^6 to 1.0×10^6 cpm of probe per ml was used, and the filters were incubated for 72 h at 37°C. Equal counts were always added for every time point. After washing, filters were exposed to Kodak AR5 film, and the resulting autoradiographs were analyzed by using an LK13 imaging densitometer to determine relative intensities of bands. All DNAs were assayed in triplicate. Datum points were normalized by dividing the densitometry units by the total counts added to each filter. Total counts were determined by counting a portion of the hybridization solution immediately before it was added to the filters.

DNA sequence analysis. DNA sequence analysis was performed on clones generated by a variety of restriction digests, or deletions created by exonuclease III, and by specific priming with synthetic oligonucleotides. All sequencing reactions were performed by the dideoxy-chain termination technique. All sequences were determined on both strands.

Transcriptional mapping. The transcription start site was determined by using S1 nuclease. A ^{32}P-labeled riboprobe was made by using the 500-base *Ssp*I-*Hin*fI fragment as a template. For each sample, 60,000 cpm was hybridized to vegetative and 16-h filter-developed RNA at 65°C for 4 h. The hybridization was added to S1 buffer along with 100 U of S1 nuclease and incubated at 37°C for 30 min. Samples were electrophoresed on a DNA sequencing gel, using an M13 sequencing reaction as a size standard.

Transformation. *Dictyostelium* cells were transformed by using calcium phosphate coprecipitation and a glycerol shock and were selected in G418 as described by Knecht and Loomis (26).

Percoll cell fractionation. *Dictyostelium* cells were grown in shaking suspension in association with *Klebsiella aerogenes* to a density of 5×10^6/ml. Cells were harvested, washed, resuspended in DB, and plated (3×10^8 to 4×10^8 cells per plate) on SM (10 g of glucose, 10 g of proteose peptone, 1 g of yeast extract, 1 g of $MgSO_4$, 1.9 g of KH_2PO_4, 0.6 g of K_2HPO_4, and 2.0 g of Bacto-Agar [Difco Laboratories] per liter [pH 6.0 to 6.4]). Cells were developed to the finger stage, harvested, washed, and suspended in $NaKPO_4$ buffer. Cells were forced through a 21-gauge needle for 5 min to break up aggregates, pelleted and resuspended in 1 ml of 20 mM morpholineethanesulfonic acid (MES)–50 mM EDTA, and counted. Then 2×10^8 cells were layered over a discontinuous gradient (45-30-15% Percoll [Sigma]). The gradients were centrifuged at 12,000 rpm for 5 min. The cells, which were separated into two distinct regions of the gradient, were recovered and slowly diluted in 20 mM MES–50 mM EDTA. A portion of the developed cells was not fractionated and served as a standard to determined relative enrichment of BP74 in the Percoll gradient fractions. RNA was isolated as described above.

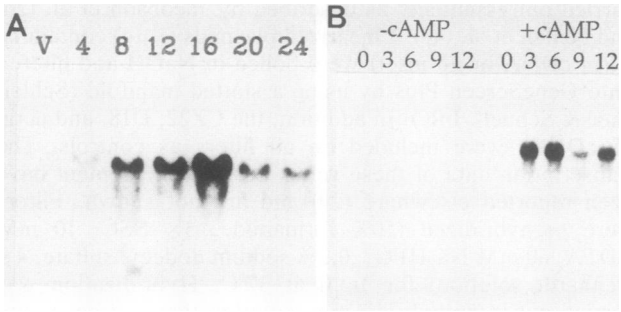

FIG. 1. (A) Developmental regulation of BP74 mRNA expression. Vegetative Ax3 cells were plated on filters (5×10^8 cells per filter), and cells were harvested at the indicated times (in hours) after plating. Total RNA was isolated, electrophoresed, and blotted to nitrocellulose. The filter was hybridized with a labeled BP74 genomic fragment. (B) Effect of cAMP pulses on expression of the BP74 message in starved suspension. Axenically grown Ax3 cells were harvested, washed, and suspended in duplicate in development buffer. One culture received pulses of cAMP, and one culture did not. At the times (in hours) indicated, cells from each suspension were harvested. Total RNA was extracted, blotted, and probed with a labeled BP74 genomic fragment.

RESULTS

The BP74 cDNA was isolated by plus-minus screening of a cDNA library constructed from cells that had developed for 15 h on filters (E. Barklis, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1984). This cDNA was used to screen a genomic library as described in Materials and Methods. The 4.6-kb *Eco*RI genomic fragment isolated was used to assess expression of the BP74 message on Northern blots of total RNA isolated from cells at various times during filter development. The BP74 fragment reacted with a 2.5-kb mRNA that appeared in low abundance 4 h after starvation. Message accumulation increased significantly by 8 h, the point in development when cells are in the process of streaming into mounds under the influence of cAMP. The level of BP74 mRNA accumulation was maximal around 16 h of development, which corresponded morphologically to the slug stage. After this point, BP74 mRNA levels decreased significantly (Fig. 1A). Since progression through these stages of development is largely dependent on cAMP signaling (17, 41, 43, 47, 50), we next asked whether cAMP influenced BP74 expression. *Dictyostelium* cells were developed in duplicate fast-shaken, starved suspension cultures; one received 50 nM cAMP pulses, and the other did not. The BP74 message was apparent at 3 h in the cAMP-pulsed culture but not in the untreated culture (Fig. 1B). The timing of this expression followed closely the appearance of the cell surface cAMP receptor in identically treated cell cultures (15, 22). BP74 mRNA could be induced to higher levels in fast-shaken suspension cultures by adding a high concentration of cAMP (1 mM) after 12 h of nanomolar cAMP pulses (data not shown). These results suggest that although nanomolar pulses of cAMP are sufficient to induce BP74 mRNA during aggregation, a higher, nonfluctuating level of cAMP may be required to maintain BP74 expression later in development.

It has been reported that accumulation of mRNAs for a number of developmentally regulated genes is regulated at a posttranscriptional level (34). The preceding results demonstrated that accumulation of the BP74 mRNA is dependent on the presence of cAMP, but they do not address whether

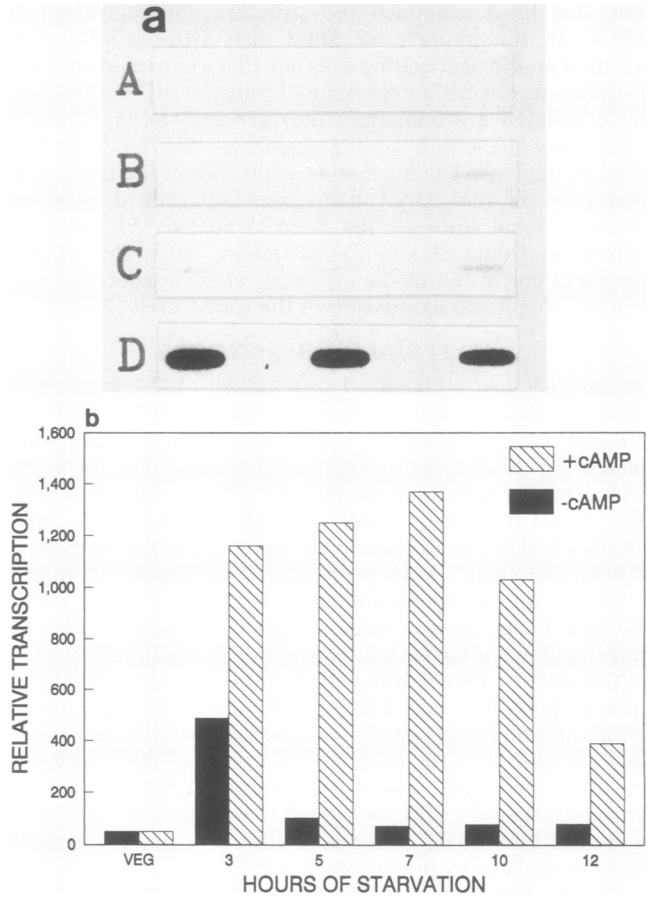

FIG. 2. Effect of cAMP pulses on BP74 transcription. (a) Nuclei from fast-shaken, starved suspensions with and without pulses of cAMP were isolated, and radioactive run-on transcripts were produced. Linearized plasmid DNA was immobilized, in triplicate, on nitrocellulose filters, and labeled transcripts from various time points were hybridized with the filters. These photos represent a portion of the data used to generate the graph in panel B. Lanes: A, pBR322 DNA hybridized with transcripts from vegetative nuclei; B, BP74 DNA hybridized with transcripts from vegetative nuclei; C, BP74 DNA hybridized with transcripts from a 10-h starved suspension without cAMP pulses; D, BP74 DNA hybridized with transcripts from a 10-h starved suspension treated with cAMP pulses. (b) Relative levels of transcription at various time points in cells shaken in starved suspension with and without cAMP pulses.

the induction is due to increased transcription of the gene or posttranscriptional stabilization of the message. To answer this question, nuclear run-on transcription assays were performed. Nuclei from fast-shaken cultures developed in the presence or absence of cAMP pulses were isolated at 0, 3, 5, 7, 10, and 12 h. Radioactive run-on transcripts were produced and hybridized to BP74 cDNA immobilized on filters. At 3 h after the initiation of starvation, the amount of BP74 transcription was induced dramatically in cells pulsed with cAMP (Fig. 2). By 7 h, BP74 transcription in cAMP-pulsed cells was 14 times that observed in cells developed in the absence of cAMP pulses. These results were consistent with the Northern blot data presented above and suggested that increased levels of BP74 message were the result of cAMP-mediated induction of transcription.

To examine the cell type specificity of expression of the BP74 mRNA, 16-h filter-developed cells were harvested and fractionated by centrifugation in a Percoll gradient. BP74

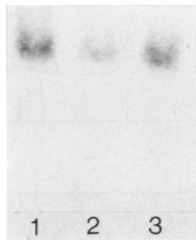

FIG. 3. Cell type specificity. RNA was prepared from cells fractionated over a Percoll gradient. Total RNA (15 μg) from each fraction and from developed, nonfractionated cells was electrophoresed and blotted to nitrocellulose. The Northern blot was probed with labeled BP74 DNA, and the resultant autoradiograph was densitometrically scanned. Lanes: 1, prestalk fraction; 2, prespore fraction; 3, nonfractionated cells.

mRNA was detected in both prespore and prestalk fractions of the gradient (Fig. 3). However, the message level was 50% higher in the prestalk fraction than the prespore fraction. For classification as a prestalk-specific message, Loomis suggested that genuine prestalk genes should show a minimum fivefold enrichment in the prestalk fraction in comparison with total unfractionated RNA (33). The BP74 mRNA was present in comparable amounts in both the prestalk fraction and total cells. Therefore, although BP74 may be somewhat enriched in prestalk cells, it cannot be classified as a prestalk-specific gene.

For a number of developmentally regulated *Dictyostelium* genes, it has been established that message levels are significantly decreased when late aggregates are dissociated (2, 30, 32, 38, 44, 46). If cAMP is added to these single-cell suspensions, message levels are maintained. To examine the effects of cAMP on BP74 in late development, cells were developed on filters for 15 h, dissociated completely, and fast shaken in duplicate starvation suspensions for 4 h. One of these cultures received 1 mM cAMP initially and then 0.3 mM cAMP after 2 h of shaking. The BP74 mRNA level was significantly increased in the suspension treated with cAMP in comparison with controls that did not receive cAMP (Fig. 4). This result supports the idea that cAMP is still necessary to maintain message levels during later time points in development. Whether this effect is due to an increase in the level of transcription or to message stabilization cannot be determined from these results. Nuclear run-on studies will be needed to answer this question.

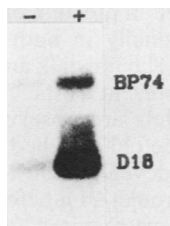

FIG. 4. Effect of cAMP on BP74 expression in disaggregated cells. Cells were developed for 16 h on filters, dissociated completely, and resuspended in starvation buffer. The suspension was divided; one half received a single 1 mM dose of cAMP, and one did not. After 4 h of rapid shaking, the cells were harvested and total RNA was isolated. The RNA was electrophoresed, blotted to nitrocellulose, and probed with labeled BP74 and D18 cDNAs. Hybridization to D18 was used as a control, since it had already been shown to have significantly higher message levels in disaggregated cells treated with cAMP than in untreated cells (7).

Organization of the BP74 gene. To determine the approximate number of BP74 genes and to begin to define the structure of the BP74 gene, the BP74 cDNA was hybridized to a Southern blot of digested *Dictyostelium* genomic DNA. The cDNA hybridized to a single 4.6-kb *Eco*RI fragment as well as to single bands in several other digests, suggesting that a single gene encodes the BP74 message. A diagram of the cloned 4.6-kb *Eco*RI fragment carrying the BP74 gene is shown in Fig. 5. The coding region of the genomic fragment was localized by hybridization with the cDNA, and the direction of transcription was identified by hybridization of 16-h mRNA with the strand-specific riboprobes transcribed from the cloned genomic DNA.

We next determined the complete nucleotide sequence of the genomic DNA starting at the *Bgl*II site and extending to the *Eco*RI site at the 3′ end of the gene (Fig. 6). The BP74 gene has an apparent coding region of 2,349 nucleotides on the strand consistent with that indicated to be the sense strand by hybridization of RNA with strand-specific RNA probes. This coding region is interrupted by two intervening sequences (Fig. 5). Comparison of genomic and cDNA sequences have identified the location of two A+T-rich introns in the 5′ end of the gene. Intron 1 is 309 bases long, flanked by the donor (GTATGT) and acceptor (AG) sequences. This intron is similar to other *Dictyostelium* introns, which are greater than 90% A+T and have a donor consensus sequence GTNNGT and acceptor sequence AG (23, 24). Intron 2 is 115 bases long and is flanked by donor (GT) and acceptor (AG) sequences. Although the donor GT conforms with the general consensus sequence for most eucaryotes, it deviates from the *Dictyostelium* consensus sequence of GTNNGT. The first two exons are quite small; exon 1 is 200 bases long, including the 5′ untranslated region, and exon 2 is only 91 bases long. The third exon contains the rest of the coding sequence of 2,084 bases. The clone also contains 178 bases of 3′ flanking sequence and 1,148 bases of upstream 5′ flanking sequence as well as an additional 500 bases extending 5′ of the *Bgl*II site to the *Eco*RI site.

Identification of the BP74 promoter region. We used S1 nuclease protection to localize the transcription start site of BP74 (Fig. 7). We used an antisense transcript corresponding to the 500-base *Ssp*I-*Hin*fI fragment of the genomic clone. After hybridization to mRNA and subsequent S1 treatment, a fragment with some microheterogeneity, representing the 5′ end of the first exon, was protected. There were four bands detected 32 to 35 nucleotides upstream of the initiation of translation. At position −23 (relative to the most 5′ transcriptional start site) there was a TATA box that matched exactly the *Dictyostelium* consensus sequence of TATAAAT. This was followed by a stretch a 10 thymidines and a short sequence resembling the CATT box described for other *Dictyostelium* transcription initiation sites (22, 23).

Finally, we wished to determine whether the cloned BP74 gene carried all of the *cis*-acting sequences required for proper developmental regulation. The 2-kb *Bgl*II fragment was cloned into the pAVNEO vector to drive chloramphenicol acetyltransferase (CAT) expression (Fig. 8A). This construct was then introduced into *Dictyostelium* cells by calcium phosphate coprecipitation. Stable transformants were selected by growth in G418. Transformed cell lines were established, and RNA was prepared from vegetative cells as well as from cells developed for 16 h on filter pads. Figure 8B shows a Northern blot probed with labeled CAT sequences. The CAT transcript was detected in 16-h developed cells; no message was seen in vegetative cells. As a control, the NPT1 gene was used to probe the same filter,

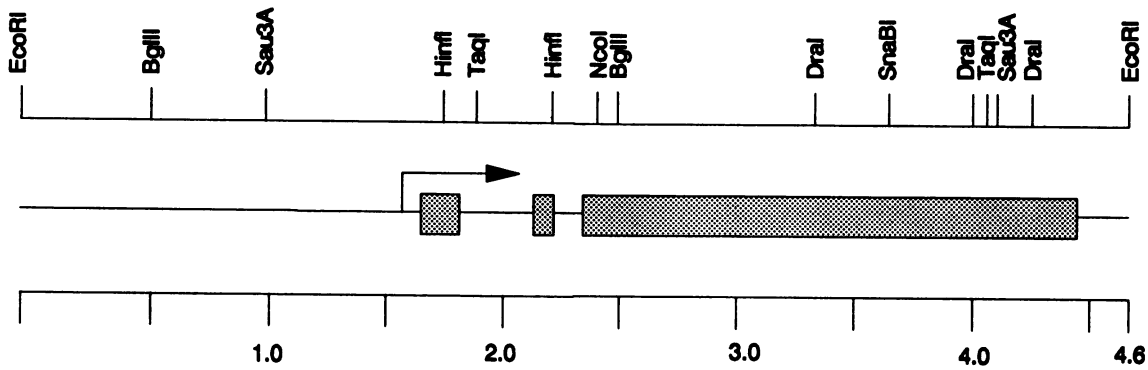

FIG. 5. Restriction map of a BP74 genomic clone. The clone was digested with a variety of restriction enzymes to identify the coding region and useful sites for further subcloning. Shaded areas represent the three exons. Direction of transcription is from left to right, as indicated by the arrow. Size is indicated in kilobases.

and hybridization was detected in both vegetative and 16-h developed cells. These results suggest that the *Bgl*II fragment contains all of the sequences necessary for proper developmental induction of BP74.

DISCUSSION

A key issue confronting biologists today concerns the identification of mechanisms controlling development and differentiation. An important component of this process is developmental gene regulation by extracellular stimuli and signal transduction. As a first step in dissecting the mechanisms responsible for correct temporal and spatial expression of genes during cellular differentiation, we have characterized the expression and structure of BP74, a gene induced during *D. discoideum* development. mRNA produced from this gene is induced during aggregation of starved cells in response to cAMP. In suspension starved cultures in which endogenous cAMP signaling is disrupted, the addition of exogenous pulses of cAMP is required for induction of BP74 mRNA accumulation. Significant accumulation of BP74 message is not seen in cells starved in the absence of cAMP pulses. Therefore, BP74 appears to be induced by cAMP pulses. In addition, using nuclear run-on transcription, we have shown that BP74 induction by cAMP is a transcriptional event, with cAMP pulses stimulating a 14-fold increase in the transcription rate of BP74 by 7 h of development in comparison with cells developed in the absence of cAMP pulses. Cells developed on filters show maximal accumulation of BP74 message of 16 h, when cells are at the slug stage of the developmental program. As slug migration ceases and fruiting body formation is initiated, BP74 mRNA levels decline.

Over the past few years, several developmentally regulated genes have been identified and studied by several laboratories. These genes have been categorized according to their temporal and spatial patterns of regulation. Cardelli et al. (5) described the patterns of protein synthesis observed during four distinct phases of development: early interphase (immediately after removal of nutrients), early aggregation, late aggregation, and culmination. On the basis of two-dimensional gel analysis, they were able to identify polypeptides that increased or decreased in expression during each of the developmental phases. Similarly, genes expressed during each of these phases have been described. Genes expressed early in development have been placed into at least three classes (36). One class, typified by discoidin, is absent in bacterially grown amoebae but is expressed by 2.5 h. Expression peaks by 5 to 7.5 h and has significantly decreased at 10 h of development. Discoidin transcription is down-regulated by cAMP (51). The mRNAs from class I and II early genes are absent in vegetative cells, peak by 2.5 h of development, and remain at low levels through 15 h. The expression of K5, a class I early gene, is decreased by cAMP pulses in starved suspension, whereas levels of class II early mRNAs M3 and D2 are enhanced by cAMP pulses, although expression is observed in the absence of added cAMP (36). The p8A7 gene produces two transcripts, p8A7L, which behaves like a heat shock message, and p8A7S, a non-cell-type-specific, developmentally expressed mRNA (35). The p8A7S message is also present in low levels in vegetative cells, is induced by starvation, and has no requirement for cAMP. UDPGP is expressed at low levels in vegetative cells, is induced to higher levels during later stages of development, and peaks at 20 h (1, 16, 19, 45). UDPGP is expressed in both cell types (16) and also appears to be enhanced by cAMP (19). Disaggregated cells depend on cAMP for continued expression of the message.

During late aggregation, *Dictyostelium* cells differentiate into two distinct cells types: spores and stalk cells. Genes expressed preferentially in one cell type or another have been placed into four classes: prestalk I and II and prespore I and II. Starvation, cAMP, and cell contact appear to have roles in inducing expression of genes in the various categories (7). In addition to these cell-type-specific genes, there are some genes whose products can be expressed to some degree in both cell types (2, 38). Regulation of mRNAs preferentially expressed in prestalk or prespore cells as well as genes expressed equally in both cell types have been investigated (2, 7, 38). The prestalk and non-cell-type-specific messages both are present as the cells enter late aggregation, and maximal levels are observed by 15 h. The CP2 message is two- to threefold enriched in prestalk cells and is first detected at 6 h of development. Message levels peak by 8 h but remain high through 14 h before declining (8, 10, 14, 43). Pulses of cAMP are required for induction of CP2 in shaking suspension. In contrast, non-cell-type-specific messages such as 10-C3 (38) do not require cAMP for induction, since they are present in shaking suspension without cAMP.

Jermyn et al. (21) have described two additional prestalk genes, pDd56 and pDd63, that are induced by DIF. These genes are induced after tip formation, in contrast to D11, CP1, and CP2, which are induced before tip formation. Several lines of evidence suggest that the early prestalk genes may actually be expressed in both prespore and

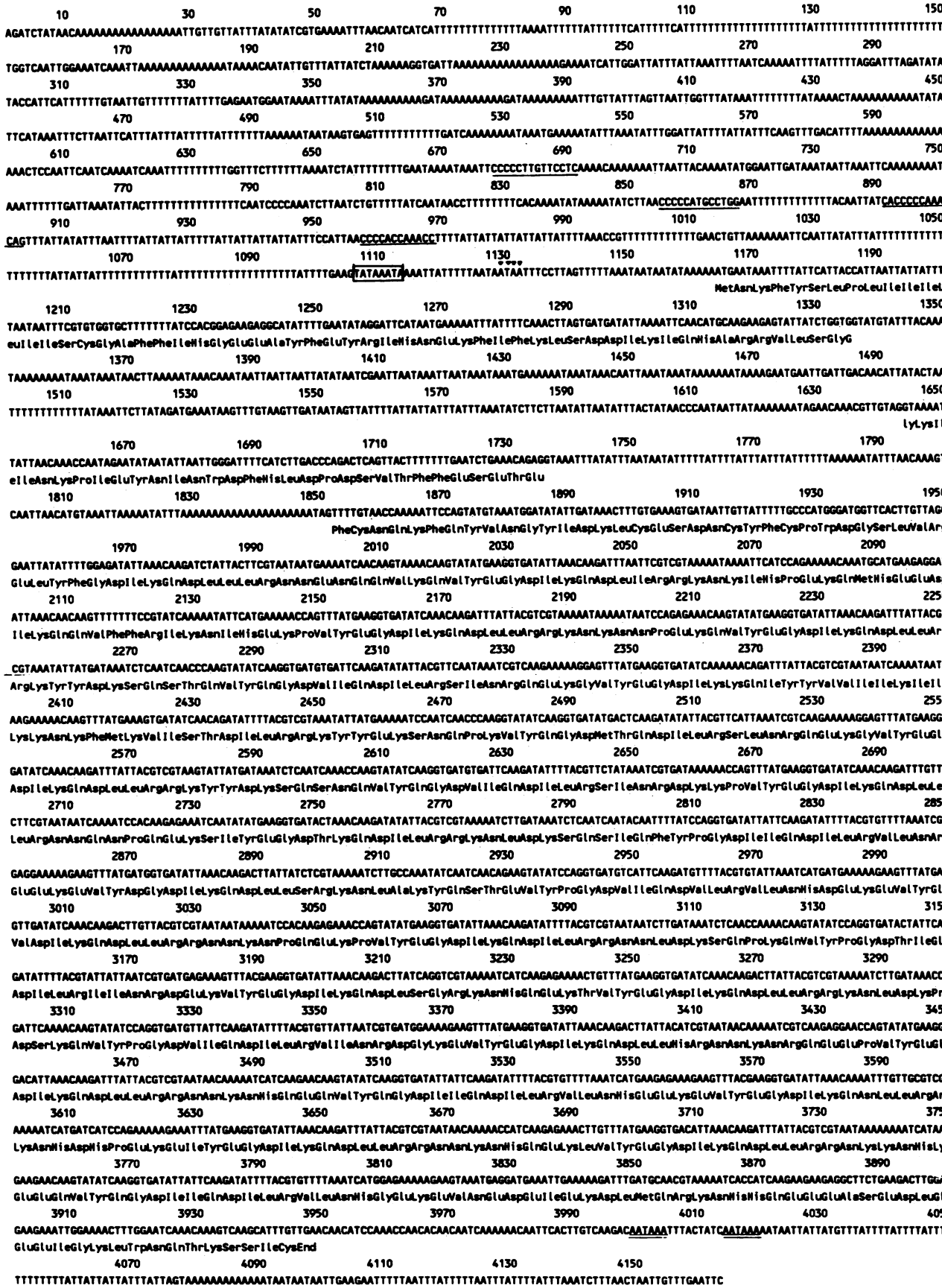

FIG. 6. Nucleotide and predicted amino acid sequences of the BP74 gene and flanking regions. A TATA box is identified as the boxed region beginning at −23. The four starts of transcription, as determined by S1 nuclease mapping, are marked with arrowheads. The four C-rich sequences in the upstream region are underlined. Two putative polyadenylation signals are indicated by a double underline.

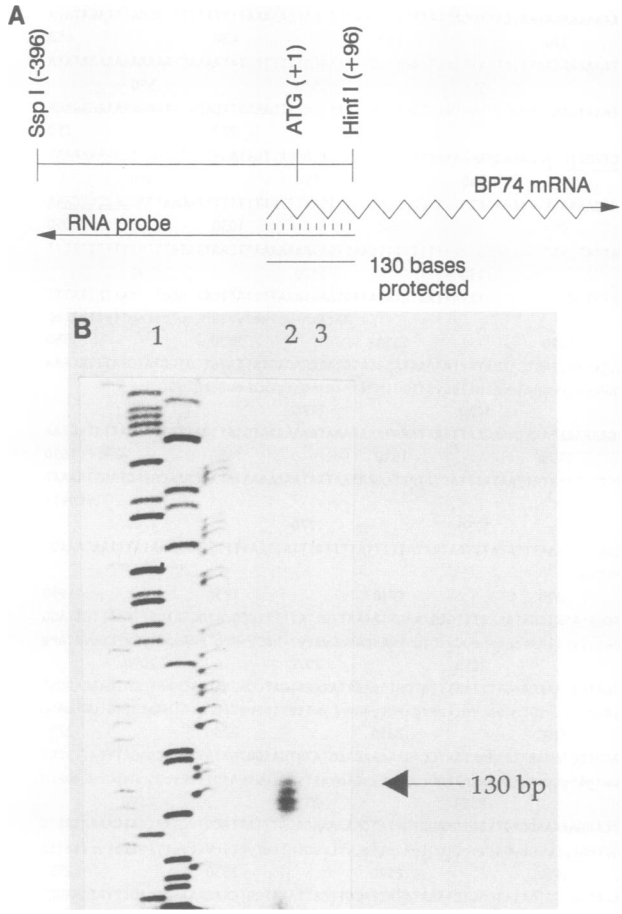

FIG. 7. S1 nuclease mapping of the 5′ coding region. (A) Identification of the transcription start site by using a labeled RNA transcript generated from the 500-base *Ssp*I-*Hin*fI fragment in the antisense direction. (B) S1 nuclease assay. Lanes: 1, M13 sequence (size marker); 2, riboprobe hybridized to 16-h developed RNA and treated with S1; 3, riboprobe hybridized to vegetative RNA and treated with S1 nuclease.

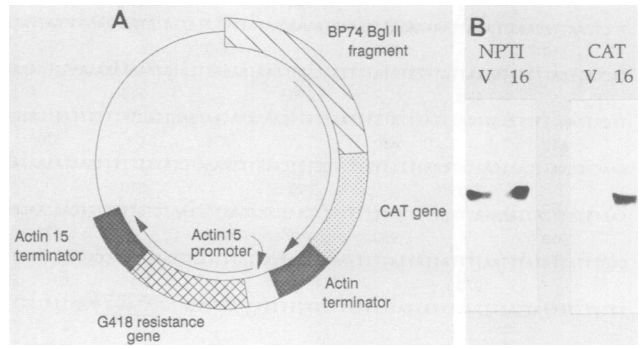

FIG. 8. Expression of the BP74-CAT construct in transformed cells. (A) Map of the pAVNEO vector with the 2.0-kb *Bgl*II fragment cloned into the *Bam*HI site. (B) Northern blot of RNA isolated from vegetative or 16-h developed transformed cells carrying the construct shown in panel A and hybridized with a probe for the CAT sequences.

prestalk cells but preferentially lost from prespore cells, whereas the DIF-inducible prestalk genes may be specifically transcribed in stalk cells (see reference 19 for a discussion). Like the DIF-inducible mRNAs, the late prespore messages are not detected until 15 h and appear to have requirements in addition to cAMP for induction (7).

BP74 message is present in both cell types, as determined by Percoll gradient fractionation, although message is expressed at a somewhat higher level in the prestalk fraction. According to the criterion suggested by Loomis (33), a prestalk-specific RNA should be enriched fivefold in the prestalk fraction of the gradient relative to the RNA isolated from whole slugs. Although BP74 is expressed at approximately 50% higher levels in stalk cell fractions than in spore cell fractions, when stalk cell expression is compared with that in unfractionated cells, similar levels are observed. Therefore, it seems unlikely that BP74 is stalk specific.

With respect to temporal and spatial regulation, BP74 does not appear to belong to any of the various classes of *Dictyostelium* genes that have been described. BP74 is not prestalk specific, yet it has cAMP requirements similar to those of some of the prestalk genes. It will be useful and interesting to compare the promoter region of BP74 with those of other genes and look for some shared sequences that may be responsible for the cAMP inducibility and temporal expression of this gene.

BP74 transcription initiates 32 to 35 nucleotides upstream of the ATG translation start site. A consensus TATA box is located at −23 (the first transcription start site is +1), followed by a short stretch of T's. The upstream region of BP74 contains four C-rich regions located approximately 180, 220, 290, and 470 bases upstream of the transcription start site. These C-rich boxes contain 12 to 15 nucleotides each; three of these regions contain five adjacent C nucleotides, whereas the fourth displays four adjacent C nucleotides. The cAMP-regulated UDPGP contains a C-rich region 100 bases upstream of the transcription start site (45). The structures of three other cAMP-regulated genes have been reported. Two of these, the CP1 and CP2 genes, which share sequence homology and appear to encode cysteine proteinases, both have two G-rich regions approximately 220 to 230 bases upstream of the start of transcription. Removal of these G-rich regions reduces transcription levels of the genes, suggesting that they may represent important regulatory elements (8, 9, 10). The third gene is DG17, which is linked to the CP1 gene but is transcribed in a divergent direction; the promoters of both genes are located in the 900 bases between the transcripts (14). Consequently, whereas the regions upstream of CP1 are G rich, the regions upstream of DG17 are C rich. The prestalk gene D11, which does not appear to be regulated primarily by cAMP, also contains a G-rich tract about 200 bases upstream of the transcription start site (2). The C-rich regions seen in BP74 share no sequence similarity with the elements seen in UDPGP, the cysteine proteinases, DG17, or D11. Therefore, it would appear that different genes, even those regulated by a common inducer, cAMP, have different promoters. Alternatively, the G-rich or C-rich sequences may be required by the transcriptional machinery but have no involvement in regulated expression. Further characterization of BP74 will contribute information necessary to obtain a clearer picture of the elements required for transcription and regulation of developmentally regulated *Dictyostelium* genes.

The DNA sequence of the BP74 coding region provides some possible clues regarding the nature of the BP74 gene product. BP74 encodes a protein of with a predicted molecular size of 94,115 daltons. The N-terminal sequence of the protein, beginning with Met, Asn, and Lys residues, consists

AMINO ACIDS	V	Y	E	G	D	I	K	Q	D	L	L	R	R	K	N	K	D	E	K	E
# IDENTICAL	23	26	16	25	26	20	17	25	24	14	23	21	16	8	22	7	10	8	12	13
# CONSERVED	26	26	17	25	26	25	17	25	24	26	24	22	16	9	24	18	12	8	12	14

FIG. 9. Structure of the BP74 repeat. The 20-amino-acid consensus sequence is shown on the top line. There are 26 repeats of this sequence, which have various degrees of homology along the 20 residues. The second line gives the number of identical amino acids at each position. The bottom line lists the number of identical or conserved amino acids at each position.

of a 22-amino-acid sequence containing 14 hydrophobic residues. This structure is frequently indicative of a signal sequence, suggesting that the BP74 polypeptide may be either a secreted or an integral membrane protein. The majority of the remaining protein sequence appears to be composed of tandem repeats. Starting at amino acid residue 140, there begins a 20-amino-acid sequence that is tandemly repeated 26 times. The consensus sequence of this repeat is shown in Fig. 9. As can be seen, several of the residues are conserved in all copies of the repeat. The least conserved amino acid is a glutamic acid at position 18 in the repeat, and it is present in 8 of the 26 repeats. The repeating sequence ends 39 residues from the C terminus. In this carboxy-terminal region, there is one possible site for N-linked glycosylation (Asn-Gln-Thr). There does not appear to be a membrane-spanning domain. These features suggest that BP74 may be a secreted extracellular protein.

Extracellular structural proteins with repeating motifs occur in a wide variety of organisms, from lower eucaryotes to humans (27, 39, 42, 48). Several examples of proteins with internal repeats of amino acids also exist in *D. discoideum*. The prestalk gene D11 codes for a cysteine-rich protein composed of a leader sequence and three types of repeats that alternate in a pattern. Although the function of this protein is unknown, it has many similarities to the *Dictyostelium* phosphodiesterase inhibitor protein (2). Two DIF-inducible prestalk genes, pDds56 and pDd63, also contain multiple repeats and have been shown to be extracellular proteins (6, 37, 50). The spore coat protein sp60 contains at least three repeats of a six-amino-acid sequence in the NH_2 terminus (13). Like many other of the proteins with periodicity, it functions as a structural protein.

Although the BP74 protein, with its putative leader sequence and series of repeats, shares many features with extracellular structural proteins, at present the function of the BP74 product is unknown. Comparison with the PIR data base, using the Bionet Resource, did not reveal any significant sequence similarity between BP74 and proteins in the data base. We have made a tryptophan E-BP74 fusion protein by using the pATH vector and are now making antibodies to this protein. An antibody specific for BP74 should allow us to begin to analyze the localization of BP74 polypeptides, which may provide additional clues regarding the role it plays during development.

ACKNOWLEDGMENTS

We thank other members of the laboratory, particularly Cecilia Warner, for helpful discussions.

This work was supported by Public Health Service grant GM34811 to R.L.C. from the National Institutes of Health.

LITERATURE CITED

1. **Ashworth, J. M., and M. Sussman.** 1967. The appearance and disappearance of uridine diphosphate glucose pyrophosphorylase activity during differentiation of the cellular slime mold *Dictyostelium discoideum*. J. Biol. Chem. **242**:1696–1700.
2. **Barklis, E., B. Pontius, and H. F. Lodish.** 1985. Structure of the *Dictyostelium discoideum* prestalk D11 gene and protein. Mol. Cell. Biol. **5**:1473–1479.
3. **Blumberg, D. D., J. Comer, and K. Higinbotham.** 1988. A Ca^{2+}-dependent signal transduction system participates in coupling expression of some cAMP-dependent prespore genes to the cell surface receptor. Dev. Gen. **9**:359–369.
4. **Bonner, J. T.** 1970. Induction of stalk cell differentiation by cyclic AMP in the cellular slime mold *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA **65**:110–113.
5. **Cardelli, J. A., D. A. Knecht, R. Wunderlick, and R. L. Dimond.** 1985. Major changes in gene expression occur during at least four stages of development of *Dictyostelium discoideum*. Dev. Biol. **110**:147–156.
6. **Ceccarelli, A., S. J. McRobbie, K. A. Jermyn, K. Duffy, A. Early, and J. G. Williams.** 1987. Structural and functional characterization of a *Dictyostelium* gene encoding a DIF inducible, prestalk enriched mRNA sequence. Nucleic Acids Res. **15**:7463–7476.
7. **Chisholm, R. L., E. Barklis, and H. F. Lodish.** 1984. Mechanisms of sequential induction of cell-type specific mRNAs in *Dictyostelium discoideum*. Nature (London) **310**:67–69.
8. **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. **7**:149–159.
9. **Datta, S., and R. A. Firtel.** 1988. An 80-bp cis-acting regulatory region controls cAMP and development regulation of a prestalk gene in *Dictyostelium*. Genes Dev. **2**:294–304.
10. **Datta, S., R. Gomer, and R. 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.
11. **Devine, J. M., Tsang, A. S., and J. G. Williams.** 1982. Differential expression of the members of the Discoidin I multigene family during growth and development of *Dictyostelium discoideum*. Cell **28**:793–800.
12. **Devreotes, P. N.** 1982. Chemotaxis, p. 117–168. *In* W. F. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
13. **Dowds, B., and W. F. Loomis.** 1986. Hexapeptide repeat structure in *Dictyostelium* spore coat protein. Biochem. Biophys. Res. Commun. **135**:336–339.
14. **Driscoll, D. M., and J. G. Williams.** 1987. Two divergently transcribed genes of *Dictyostelium discoideum* are cyclic AMP inducible and coregulated during development. Mol. Cell. Biol. **7**:4482–4489.
15. **Drummond, I., and R. L. Chisholm.** 1987. The effect of caffeine, adenosine, and buffer ionic composition on the induction of cell-surface cAMP binding during starvation of *Dictyostelium discoideum*. Dev. Genet. **9**:293–301.
16. **Fishell, B., J. Ragheb, A. Rajkovic, B. Haribabu, C. Scheinfest, and R. P. Dottin.** 1985. Molecular cloning of a cDNA complementary to a UDP glucose pyrophosphorylase mRNA of *Dictyostelium discoideum*. Dev. Biol. **110**:369–381.
17. **Gomer, R. H., D. Armstrong, B. H. Leichtling, and R. A. Firtel.** 1986. cAMP induction of prespore and prestalk gene expression in Dictyostelium is mediated by the cell-surface cAMP receptor. Proc. Natl. Acad. Sci. USA **83**:8624–8628.
18. **Haribabu, B., and R. P. Dottin.** 1986. Pharmacological characterization of cyclic AMP receptors mediating gene regulation in *Dictyostelium discoideum*. Mol. Cell. Biol. **6**:2402–2408.
19. **Haribabu, B., A. Rajkovic, and R. P. Dottin.** 1986. Cell-cell contact and cAMP regulate the expression of a UDP glucose pyrophosphorylase gene of *Dictyostelium discoideum*. Dev. Biol. **113**:436–442.
20. **Jacobson, A., R. A. Firtel, and H. F. Lodish.** 1974. Synthesis of messenger and ribosomal RNA precursors in isolated nuclei of the cellular slime mold *Dictyostelium discoideum*. J. Mol. Biol. **82**:213–230.
21. **Jermyn, K. A., R. R. Berks, and J. G. Williams.** 1987. Two distinct classes of prestalk-enriched mRNA sequences in *Dic-*

tyostelium discoideum. Development **100**:745–755.

22. **Kimmel, A. R.** 1987. Different molecular mechanisms for cAMP regulation of gene expression during *Dictyostelium* development. Dev. Biol. **122**:163–171.
23. **Kimmel, A. R., and R. A. Firtel.** 1983. Sequence organization in *Dictyostelium*: unique structure at the 5′ end of protein coding genes. Nucleic Acids Res. **11**:541–552.
24. **Kimmel, A., and R. Firtel.** 1982. The organization and expression of the *Dictyostelium* genome, p. 233–324. *In* W. R. Loomis (ed.), The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
25. **Klein, P., R. Vaughan, J. Borleis, and P. N. Devreotes.** 1987. The surface cyclic AMP receptor in *Dictyostelium*. J. Biol. Chem. **262**:358–364.
26. **Knecht, D. A., and W. F. Loomis.** 1987. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. Science **236**:1081–1086.
27. **Kochan, J., M. Perkins, and J. Ravetch.** 1986. A tandemly repeated sequence determines the binding domain for an erythrocyte receptor binding protein of P. falciparum. Cell **44**: 689–696.
28. **Lacombe, M., G. Podgorski, J. Franke, and R. Kessin.** 1986. Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum*. J. Biol. Chem. **261**:16811–16817.
29. **Landfear, S. M., P. A. Lefebvre, S. Chung, and H. F. Lodish.** 1982. Transcriptional control of gene expression during development of *Dictyostelium discoideum*. Mol. Cell. Biol. **2**:1417–1426.
30. **Landfear, S. M., and H. F. Lodish.** 1980. A role for cyclic AMP in expression of developmentally regulated genes in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA **77**:1044–1048.
31. **Loomis, W. F.** 1975. *Dictyostelium discoideum*: a developmental system. Academic Press, Inc., New York.
32. **Loomis, W. F.** 1982. The development of *Dictyostelium discoideum*. Academic Press, Inc., New York.
33. **Loomis, W. F.** 1987. Cell-type regulation in *Dictyostelium discoideum*, p. 201–218. *In* D. Hamer and M. Rosenberg (ed.), Genetic regulation of development. Alan R. Liss, Inc., New York.
34. **Mangiarotti, G., R. Giorda, A. Ceccarelli, and C. Perlo.** 1985. mRNA stabilization controls the expression of a class of developmentally regulated genes in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA **82**:5786–5790.
35. **Maniak, M., and W. Nellen.** 1988. Developmentally regulated membrane protein gene in *Dictyostelium discoideum* is also induced by heat shock and cold shock. Mol. Cell. Biol. **8**: 153–159.
36. **Mann, S. K. O., and R. A. Firtel.** 1987. Cyclic AMP regulation of early gene expression in *Dictyostelium discoideum*: mediation via the cell surface cyclic AMP receptor. Mol. Cell. Biol. **7**:458–469.
37. **McRobbie, S. J., R. Tilly, K. Blight, A. Ceccarelli, and J. G. Williams.** 1988. Identification and localization of proteins encoded by two DIF-inducible genes of *Dictyostelium*. Dev. Biol. **125**:59–63.
38. **Mehdy, M. C., D. Ratner, and R. A. Firtel.** 1983. Induction and modulation of cell-type-specific gene expression in *Dictyostelium*. Cell **32**:763–771.
39. **Muskavitch, M., and D. S. Hogness.** 1982. An expandable gene that encodes a Drosophila glue protein is not expressed in variant lacking remote upstream sequences. Cell **29**:1041–1051.
40. **Nellen, W., S. Datta, C. Reymond, A. Siversten, S. Mann, T. Crowley, and R. A. Firtel.** 1987. Molecular biology in *Dictyostelium*: tools and applications, p. 67–100. *In* J. A. Spudich (ed.), Methods in cell biology. Academic Press, Inc. New York.
41. **Oyama, M., and D. Blumberg.** 1986. Interaction of cAMP with the cell-surface receptor induces cell type-specific mRNA accumulation in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA **83**:4819–4823.
42. **Ozake, L. S., P. Svec, R. S. Nussenzweig, V. Nussenzweig, and G. N. Godson.** 1983. Structure of the Plasmodium knowlesi gene coding for the circumsporozoite protein. Cell **34**:815–822.
43. **Pavlovic, J., B. Haribabu, and R. Dottin.** 1988. Transmembrane signal transduction regulates gene expression in *Dictyostelium discoideum*. Dev. Genet. **9**:371–382.
44. **Pears, C. J., H. M. Mahbubani, and J. G. Williams.** 1985. Characterization of two highly diverged but developmentally co-regulated cysteine proteinase genes in *Dictyostelium discoideum*. Nucleic Acids Res. **13**:8853–8866.
45. **Ragheb, J., and R. Dottin.** 1987. Structure and sequence of a UDP glucose pyrophosphorylase gene of *Dictyostelium discoideum*. Nucleic Acids Res. **15**:3891–3906.
46. **Reymond, C., R. Gomer, M. Mehdy, and R. Firtel.** 1984. Developmental regulation of a *Dictyostelium* gene encoding a protein homologous to mammalian ras protein. Cell **39**:141–148.
47. **Schaap, P., and R. Van Driel.** 1985. Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP. Exp. Cell Res. **159**:388–398.
48. **Schwarzbauer, J., J. Tamkun, I. Lemischka, and R. Hynes.** 1983. Three different fibronectin mRNAs arise by alternative splicing within the coding region. Cell **35**:421–431.
49. **Wang, M., P. Van Haastert, and P. Schaap.** 1986. Multiple effects of differentiation-inducing factor on prespore differentiation and cyclic AMP signal transduction in *Dictyostelium*. Differentiation **33**:24–28.
50. **Williams, J. G., A. Ceccarelli, S. McRobbie, H. Mahbubani, R. R. Kay, A. Early, M. Berks, and K. A. Jermyn.** 1987. Direct induction of *Dictyostelium* prestalk gene expression by DIF provides evidence that DIF is a morphogen. Cell **49**:185–192.
51. **Williams, J. G., A. S. Tsang, and H. Makbubani.** 1980. A change in the rate of transcription of a eukaryotic gene in response to cyclic AMP. Proc. Natl. Acad. Sci. USA **77**:7171–7175.