NUTRITIONAL AND CHEMOTACTIC SIGNALS INACTIVATE THE EXPRESSION OF A GROWTH-SPECIFIC GENE EARLY IN DEVELOPMENT IN *Dictyostelium discoideum*

by

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ABSTRACT

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The cellular slime mold, *Dictyostelium discoideum*, is a useful model for studying the regulation of growth-specific gene expression because cells cease division while they differentiate on an agar surface. A cDNA library made to vegetative cell transcripts was used to identify transcripts preferentially expressed in vegetative cells. We are studying the mechanism whereby the mRNA for one of these (D2) falls during aggregation when starvation, aggregation specific cell contacts and diffusible factors like cAMP are present. *In Vitro* transcription reactions performed with nuclei isolated from vegetative amoebae and from cells developing in suspension indicates that starvation, the trigger for the developmental program, inactivates the D2 gene transcription early in development. However, the D2 transcript levels stay high during suspension development and do not fall as a result of starvation or aggregation-specific cell contacts. Rather cells must be deprived of amino acids and cAMP administered to 50nM periodically in pulses, to mimic cAMP signal-relay in aggregation. This effect can be blocked with reagents that prevent cAMP from binding to the cell surface

receptor in aggregateless mutants known to be defective in a $G\alpha_2$ protein. Experiments with membrane-permeable cAMP analogues suggest that the loss of D2 transcript levels does not require a rise in the intracellular cAMP levels. In addition proper regulation of the D2 gene depends on cell proliferation, because blockage of the cell cycle with caffeine or incubation of temperature sensitive growth mutants at a restrictive temperature caused an inappropriate reduction in D2 mRNA levels.

I found evidence for both non-specific and specific degradation of D2 mRNA by use of inhibitors: non-specific degradation does not require protein synthesis but specific degradation requires new RNA synthesis. Overall these results indicate that the D2 gene is regulated at the transcriptional and possibly the translational level. Although some of the extracellular signals have been found, the intracellular signal and mechanisms that trigger a specific degradation of D2 transcripts remain to be determined.

DEDICATION

This Dissertation is dedicated to my wife, Fatma, and my mother, Amena, for their support, sacrifices, patience and understanding throughout my doctorate degree work.

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INTRODUCTION

In order to study the regulation of gene expression, it is important to have model systems. Dictyostelium discoideum, is one such favorable system for studies on the regulation of growth cell-specific gene expression using available molecular biology and molecular genetic techniques. The organism has a small haploid genome and exhibits an experimentally useful transition from proliferating vegetative cells to non-proliferating differentiating cells.

The life cycle

D. discoideum is a soil amoebae which grows on bacteria as the food supply (Bonner, 1967). Although it is difficult to grow the wild-type isolates in a defined medium in the absence of bacteria, several derivatives of D. discoideum NC-4 (i.e Ax-2 and Ax-3) were selected for axenic growth in liquid medium (Ashworth and Watts, 1970; Watts and Ashworth, 1970; Firtel and Bonner, 1972 a&b; Loomis, 1971; Rossomando and Sussman, 1973). Food exhaustion and starvation for amino acids (Marin, 1976) trigger biochemical and morphological differentiation in Dictyostelium. Upon starvation, the cells secrete cAMP which orients chemotaxis of the amoebae into multicellular aggregates of 10^5 cells and these pass through other distinct morphological stages: mound, mound with tip,

slug and mature fruiting bodies which contain two cell types, spore and stalk cells (Loomis, 1975). Under optimal environmental conditions, and with continued starvation, the differentiation process is completed within 24 hours (Fig.1). These morphological changes are closely linked to specific changes in cellular enzyme and protein composition and correlate with changes in gene expression (Alton and Lodish, 1977; Firtel, 1972; Sussman and Osmond, 1964 and 1976; Newell and Sussman, 1970). Completion of the differentiation process requires the expression of early and late developmental genes. Genes expressed during the pre-aggregation stage (i.e. discoidin I and cAMP receptor genes) are called early developmental genes, while those expressed after aggregation (i.e. prespore and prestalk genes) are late developmental genes.

Another chemoattractant of D. discoideum cells is folic acid. It is believed to be used by amoebae to sense the bacterial food sources (Pan et al., 1972, 1975; De Wit and Bulgakov, 1986a,b,c,d). Folic acid is a more effective attractant of preaggregation amoebae, attracting vegetative cells and early developing cells until cAMP signals are initiated (Pan et al., 1972 and 1975). When supplied in pulses, folic acid induces biochemical oscillations accompanied by periodic changes in cAMP concentration and stimulation of the development of preaggregation amoebae to

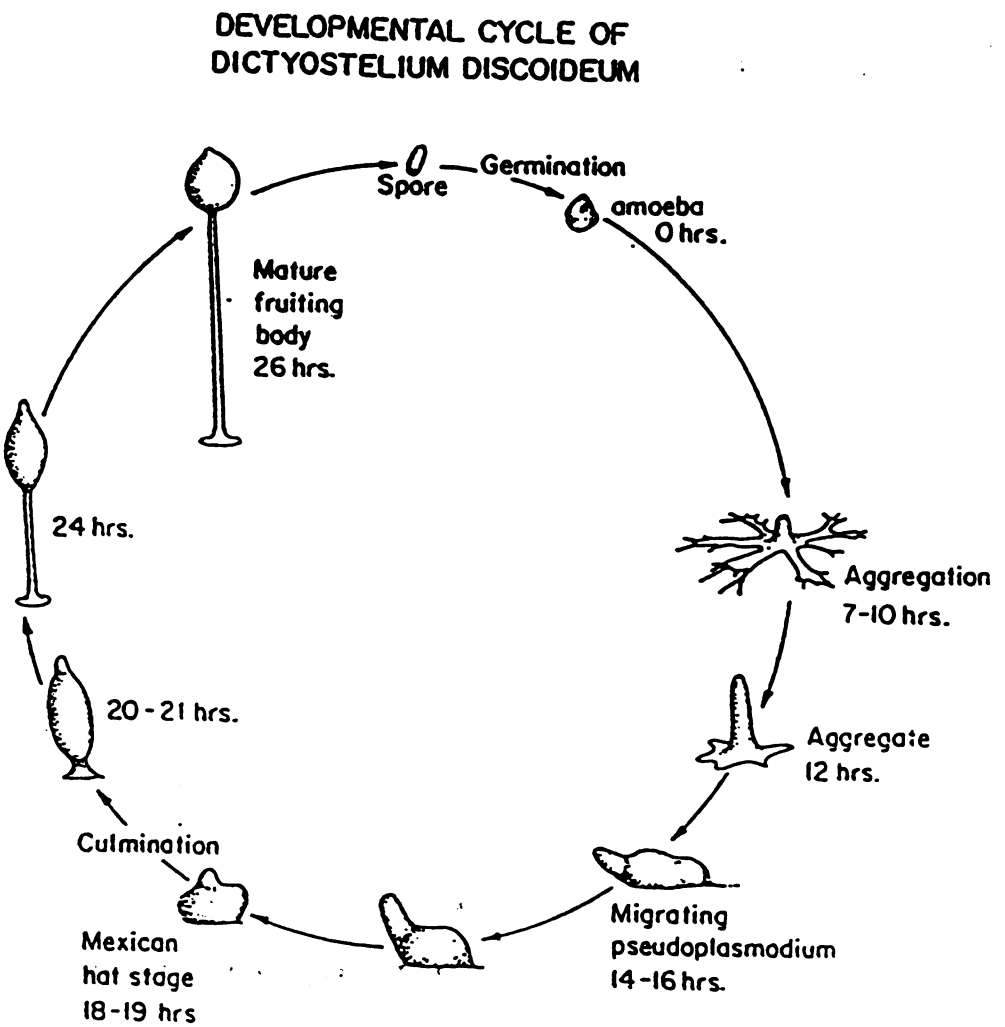

DEVELOPMENTAL CYCLE OF
DICTYOSTELIUM DISCOIDEUM
0
Spore
Germination
amoeba
0 hrs.
Mature
fruiting
body
26 hrs.
24 hrs.
Aggregation
7-10 hrs.
20-21 hrs.
Aggregate
12 hrs.
Culmination
Migrating
pseudoplasmodium
14-16 hrs.
Mexican
hat stage
18-19 hrs

FIGURE 1

aggregation-competent ones (Wurster and Schubiger, 1977). If these aggregation competent cells are removed from the suspension culture and transferred onto a coverslip, they immediately aggregate into streams (Gerisch, 1968).

The organization of the genome

D. discoideum has a genome size only about 1% that of mammalian cells. A single haploid cell contains about 4.5×10^7 base pairs of DNA and is about seven times the sequence complexity of *E. coli*, three times that of yeast, and one sixth that of *Drosophila* (Firtel and Bonner, 1972a). The genome has A-T regions (~ 72% of the genome) and exhibits similar gene organization and transcription as higher eucaryotes. It contains repetitive and single copy sequences interspersed (Firtel *et al.*, 1976 a&b), and the combination of coding, non-coding and untranscribed (Minty and Newmark, 1980) sequences is also found (Kimmel and Firtel, 1983). If all the genome coded for mRNA, there could be up to 20,000 genes. However, about half of the DNA present as A-T rich sequences is not transcribed. RNA hybridization analyses indicate that growing cells express only about 5,000 genes. Many of these genes are also expressed throughout development (Firtel, 1972). The genes of *Dictyostelium* have structural features common to other eucaryotes like the Goldberg-Hogness box (the "TATA" box) and the "CAAT" box, found, respectively,

about 31 and 80 nucleotides upstream from the eucaryotic transcription initiation sites (Benoist et al., 1980). These conserved sequence elements (the TATA homology and the CAAT homology) are believed to determine the fidelity and efficiency of transcription in vivo and in vitro in most eucaryotic systems studied (Minty and Newmark, 1980; McKnight and Kingsbury, 1982; Breathnach and Chambson, 1981). Unlike other eucaryotes, many but not all, Dictyostelium genes contain an oligo (dT) sequence, 5′ to the transcription site. The 5' upstream region contains two long homopolymers, respectively, of T and A and the 3' flanking regions also contain almost homopolymers of A and T. Dictyostelium has also a consensus sequence AAUAAA following the stop codon. This sequence is a poly-adenylation signal in higher eucaryotes and is found at the 3' ends of all the D. discoideum genes analyzed (Kimmel and Firtel 1983; Proudfoot and Brownlee 1976). Dictyostelium mRNA molecules, as in other eucaryotes, contain a 3′ terminal polyadenylic acid sequence (Adesnik and Darnell, 1972; Darnell et al., 1973) and a 5′ terminal cap structure, the unusual nucleotide, 7-methyl guanosine, linked to a 5′-5′-triphosphate bridge (Shatkin, 1976). Both are added post-transcriptionally.

Gene expression during growth and development

Global analyses of gene expression during growth and development have resulted from solution hybridization

analysis of RNA. Growing cells synthesize about 4000-5000 different mRNA-sized, polyadenylated sequences found in the cytoplasm. The same value is obtained from the polysomes which indicates that the corresponding genes (4000-5000) are actively expressed. An additional 9000 transcripts are found in the nucleus, but are transferred very poorly, if at all, to the cytoplasm (Blumberg and Lodish., 1980). During aggregation, abundant RNA species of vegetative cells were found to drop to low levels (Jacquet et al., 1981). New species of mRNAs, however, are induced before and after aggregation. During the preculmination stage (~ 18 hour of development), the newly synthesized mRNAs are estimated to be 700-900 species. The number of expressed genes during growth and development (~400 proteins) estimated from two-dimensional (2-D) gel electrophoresis (Alton and Lodish 1977; Cardelli et al., 1985) represents a minimum estimate since proteins from mRNAs present at less than 10-20 copies per cell would not be detected.

Physiological signals regulate gene expression

Gene expression in Dictyostelium is affected by cell associated developmental or environmental stimuli such as starvation, cell-cell contacts, cAMP and other factors (Marin, 1976 and 1977; Landfear and Lodish, 1980; Chung et al., 1981; Mangiarotti et al., 1982; Mangiarotti et al.,

1983; Chisholm et al., 1984; Mehdy and Firtel, 1985).

Starvation

Marin (1976, 1977) showed that the transition from growth to development is initiated by amino acid starvation. Amino acids are the only nutrients that specifically inhibit the initiation of development. There are two classes, essential and conditionally essential. The essential amino acids are: methionine, glycine, leucine, isoleucine, valine, arginine, histidine, tryptophan, phenylalanine, threonine and lysine. These amino acids have the following properties: (i) each is required to inhibit the initiation of development maximally, even when all other amino acids are present; and (ii) each is specifically required for maximal growth, even when all other nutrients are present. D. discoideum is either deficient in the endogenous synthesis or the retention of these amino acids, therefore, an adequate exogenous source is required to maintain growth. The conditionally essential amino acids (Glutamic acid, Glutamine, Aspartic acid, Asparagine, Alanine, Serine and Proline) have the following properties: (i) individually they are not essential for inhibiting the initiation of development; (ii) individually they are not essential for growth; (iii) as a group, they are essential for the amino acid-mediated inhibition of the initiation of development in the absence of, but not in the presence of, glucose; and (iv) as a group they are essential for growth in the absence of, but not in the presence of,

glucose. D. discoideum can synthesize the conditionally essential amino acids from Krebs' cycle and glycolytic precursors. These conditionally essential amino acids required for growth in the absence of glucose can also serve as a source of "Krebs" cycle and glycolytic intermediates or be utilized as carbon and energy sources for cell metabolism. Without glucose, available pools of the conditionally essential amino acids would be depleted more rapidly than the essential amino acids. The resulting starvation for the conditionally essential amino acids would then initiate development. Adequate endogenous levels of the conditionally essential amino acids are maintained in the cells by the addition of glucose or those amino acids to the medium. Under these conditions, the essential amino acid pools become the limiting factor for starvation. The role of glucose in enhancing the amino acid-induced inhibition of development, or slightly inhibiting when present alone may spare the conditionally essential amino acids. There is no positive evidence to support any direct role of glucose in the primary regulation of the transition from growth to development.

Chisholm (1987) found starvation to be sufficient for the induction of cAMP receptors whereas cAMP pulses might further increase numbers of receptors (Chisholm et al.,1987; Schaap et al., 1986). Receptor accumulation during development might therefore be a two-step process. An

additional prestarvation response occurs as exponentially growing cells gradually deplete their food supply (Clarke et al., 1988). Synthesis of certain proteins, discoidin I and the lysosomal enzymes alpha-mannosidase-1 and beta-galactosidase-2 (Clarke et al., 1987), was detected approximately three cell generations before the food supply became limiting for growth. Dictyostelium amoebae produce a soluble substance (prestarvation factor, PSF) that accumulates in the medium in direct relation to cell density and can induce these proteins. The cell density required for induction of these proteins is also affected by the concentration of food bacteria. This cellular response to environmental conditions during growth is distinct from starvation responses and was termed the prestarvation response, to distinguish it from the starvation response that occurs in the absence of bacteria or axenic broth.

Cell contacts

Cells acquire cell adhesion surface molecules (contact sites A) which enable them to adhere to other cells during aggregation and in the multicellular aggregates. Evidence has been presented that the appearance of these contact sites on the cell surface requires exposure to pulses of cAMP which the cells normally encounter during chemotactic signaling in aggregation (Gerisch et al., 1975). The immunological studies suggested at least three glycoproteins, with molecular

weights of 80,000, 95,000 and 150,000 daltons (Parish et al., 1978; Steineman and Parish., 1980; Geltosky et al., 1976), plays some role in developmentally regulated cell-cell adhesion. Many studies suggested that the interaction between cell-to-cell surface molecules also controls cell differentiation and the regulation of developmental gene expression. The induction of ~ 2500 new mRNA species (aggregation-stage mRNAs) occurs concomitant with formation of tight-cell-cell aggregates (Mangiarotti et al., 1981b; Chung et al., 1981). Furthermore, disruption of cell-cell contact results in decay of these mRNAs and in rapid cessation of synthesis of postaggregation-stage proteins.

Finney and his co-workers proposed that the 7 hour period between the initiation of development and the onset of multicellular morphogenesis, is composed of two rate limiting components which are identified by a lack of, or dependency on, cell-cell contacts and de novo protein synthesis (Finney et al., 1985). The first component, which includes the initial 4.5 hours, will progress in the absence of close cell-cell contacts as well as new protein synthesis. In contrast, the second one that includes the final 2.5 hours depends on cell-cell contacts and protein synthesis. They also tested the developmentally associated changes in protein synthesis during the first and the second rate-limiting period. They found significant increases and decreases in the

rates of protein synthesis of approximately 262 polypeptides. The majority of changes (74%) that occur during the first rate-limiting component are independent of close cell-cell contacts, while the majority of changes (66%) accompanying the second period occur in the present of cell-cell contacts. These results might suggest that many genes expressed or repressed during the first 4.5 hours of development do not need cell-cell contacts or protein synthesis for their expression or repression.

Cyclic AMP

Cylic AMP is an important second messenger and it affects many processes in other organisms (Robison et al., 1971) Its role in growth gene expression is, however, poorly understood (Paul and Robison, 1984).

In D. discoideum cAMP serves as a chemotactic agent and also influences the expression of large numbers of genes during early and late development. Aggregation is coordinated through a developmentally regulated cAMP signaling system by periodic synthesis and secretion of cAMP approximately at 6 minute intervals. The two responses essential to this coordinated aggregation are chemotaxis (cells move up a cAMP gradient) and signal relay (i.e. the synthesis and secretion of cAMP in response to extracellular cAMP). Both are mediated by cell-surface cAMP receptors and provide excellent models for transmembrane signaling in eukaryotic cells (Gerisch,

1987). Van Haastert and others classify cAMP receptors into two classes depending on the rate of dissociation of cAMP (Van Haastert, 1987 and Van Haastert et al., 1989). The fast receptor present at ~ 50-100 $X10^3$/cell is probably involved in the activation of adenylate cyclase, while the slow receptor (~$4X10^3$/cell) is associated with the chemotaxis response. Activation of the chemotaxis pathway is accompanied by a 2-3 fold increase in inositol triphosphate (1,4,5-IP_3) levels as well as a transient 5-10 fold increase in cGMP levels. The elevation of IP_3 levels might result in an increase in the intracellular Ca^{2+} levels since the addition of IP_3 to permeabilized cells results in a release of Ca^{2+} from mitochondrial stores (Newell et al., 1988; Van Haastert et al., 1989). A cAMP receptor gene was cloned by Klein (1988), but it is not known if it represents the cAMP receptor class associated with one or both receptor classes. The two cAMP receptor classes could be the products of two distinct genes or different products of one gene resulting in two classes of receptors.

Spontaneous oscillations of the fast cAMP receptor begin at ~ 3.5 hours of development and continue through 7 hours. The periodic cAMP signaling, in turn, regulates early gene expression as well as proper morphogenesis. Oscillation-defective mutants termed "Synag" strains fail to

differentiate unless a periodic exogenous cAMP is added. However, a constant level of extracellular cAMP, which is necessary for prespore gene expression, does not rescue these mutants, and additionally, suppresses early gene expression in wild-type cells. Oscillations of cAMP require the surface receptor, adenylate cyclase, and cAMP phosphodiesterase. When cAMP binds to its receptor, adenylate cyclase is activated and cAMP synthesized. If the cells are stimulated with a higher cAMP concentration more cAMP is synthesized until the receptors are saturated (or desensitized). After several minutes of continuous occupancy, the receptor will be phosphorylated and the cells will adapt and be unable to respond to cAMP. Thereafter, cAMP-phosphodiesterase will hydrolyze cAMP to effect a gradual loss of receptor phosphorylation and the return of the cells to the sensitized state. The cAMP receptor, identified by photoaffinity labeling (Juliani and Klein, 1981), oscillates between two interconvertible forms designated R (40Kb),and D (43 Kb) in parallel with the oscillations in cAMP. Stimulation with cAMP will phosphorylate and convert the R form to the D form. Devreotes and his co-workers propose a model in which there are seven transmembrane domains, the NH2-terminus is extracellular, and the serine-rich COOH-terminal tail is intracellular and is the site of ligand-induced phosphorylation (Klein et al., 1988).

Another important factor involved in transmembrane signaling are the G proteins. The biochemical and genetic evidence indicates that the cAMP-mediated signal transduction pathways are regulated through heterotrimeric G proteins with alpha, beta and gama subunits as in mammalian cells (Theibert and Devreotes 1986; Janssens and Van Haastert, 1987; Snaar-Jagalska et al., 1988; Kumagai et al., 1989; Pupillo et al., 1989).

Firtel and his group discovered and cloned two G-alpha protein subunits, $G\alpha_1$ and $G\alpha_2$ (Pupillo et al., 1989; Kumagai et al., 1989). These proteins have ~45% amino acid sequence identity with each other as well as with G alpha protein subunits from yeast and mammalian cells. Furthermore, the GTP/GTPase binding domains show 100% amino acid sequence identity with those of mammalian cells. The $G\alpha_1$ protein is expressed in vegetative cells through aggregate stages while $G\alpha_2$ is inducible by cAMP pulses and preferentially expressed in aggregation. The $G\alpha_1$ appears to function in both the cell cycle and development. Overexpression of $G\alpha_1$ results in large multinucleated cells that develop abnormally. The expression of $G\alpha_2$ is highly developmentally regulated. Rece88888888888nt results suggest that the $G\alpha_2$ protein subunit is coupled to the chemotaxis receptors that activate phospholipase C that hydrolyzes the membrane bound protein phosphoinositides into

inositol triphosphate (1,4,5 IP3) and diacylglycerol (DAG) (Kesbeke et al., 1988; Snaar-Jagalska et al., 1988; Kumagai et al., 1989).

The aggregateless mutant strains termed frigid (fgd) strains have been well characterized genetically and biochemically with respect to chemotactic ability (Coukell et al., 1983). These mutants are unable to respond to exogenous cAMP signals. Parasexual genetic studies indicate that the mutants fall in five complementation groups, fgd A, B, C, D and E. The fgd B ,D and E mutants fail to produce detectable levels of cAMP receptors, cAMP phosphodiesterase or extracellular phosphodiesterase inhibitor and the cells continue to respond chemotactically to folic acid. Therefore, these strains are probably arrested at the vegetative stage or in early development. Strains of groups fgd A and C produce low levels of cAMP receptors and secrete phosphodiesterase inhibitor; some of these mutants elicit a weak chemotactic response to cAMP. The Frigid A and C mutants initiate development when starved but the process is blocked at an early stage (Coukell et al., 1983). Present data suggest that the $G\alpha_2$ protein coupled to the chemotaxis receptor is encoded at the Frigid A locus. Strains HC85 and HC112 of the Frigid A group and strain HC317 of Frigid C group were used in our studies.

Although it is well known now that the cell surface receptor mediates gene regulation, the second messengers that might be involved in this type of regulation are not clear yet. Several studies indicate that intracellular cAMP and/or cGMP might not be involved in the gene regulation. For example, although the synthesis of intracellular cAMP and cGMP becomes insensitive to continuous stimulation by cAMP (Devreotes and Steck, 1979) the expression of many late developmental genes requires constant high levels of extracellular cAMP (Mehdy et al.,1983; Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Kimmel, 1987). Furthermore, the proper regulation of late gene expression is still observed in response to exogenous cAMP, despite the inhibition of adenylate cyclase (Kimmel, 1987; Oyama and Blumberg, 1986; Schaap et al., 1986). Recent studies show that the addition of IP_3 to permeabilized cells results in mobilizing calcium from intracellular stores and in a rapid transient elevation of cGMP (Europe-Finner and Newell, 1985). Similar responses can be evoked by addition of Ca^{2+} ions to permeabilized cells. The IP_3 and diacylglycerol formed by hydrolysis of phosphoinositides were identified as second messengers in the control of several physiological processes (Berridge, 1984). In D. discoideum, as well as in higher organisms, IP3 stimulates the liberation of Ca^{2+} from cellular, non-mitochondrial stores, while diacylglycerol

activates protein kinase C. To determine whether these second messengers are involved in the cAMP receptor-mediated gene regulation, the effect of various agonists and antagonists that modulate the pathways regulated by 1,4,5 PI_3 and DAG was studied (Pavlovic et al 1988; Blumberg et al., 1988). They show that the addition of the dihydropyridines, a highly specific class of Ca^{2+} channel blockers, or TMB-8, a putative inhibitor of calcium release, caused a complete inhibition of cAMP-regulated expression of prespore specific-genes and prevented the repression of a growth gene. Their results strongly suggest that calcium may play a role in the signal transduction pathway.

Control of gene expression

Gene expression in Dictyostelium, as in other eucaryotes, is controlled at both transcriptional and translational levels. Transcriptional control of eukaryotic genes means that increases or decreases in the synthesis of a primary RNA transcript in the cell nucleus are the main cause of changes in the rate of synthesis of a particular protein. Transcriptional initiation and transcriptional termination are both potentially subject to control, but control of initiation appears to be most important (Platt, 1986). Post transcriptional control can occur at different levels; RNA processing, RNA transport and message stability. Messenger

RNA decay (message stability) is now recognized as a major control point in the regulation of gene expression. The changes in the turnover rates of various mRNAs are important to the ability of a cell to replicate and differentiate normally and to respond quickly to changes in its environment. The wide diversity in mRNA decay rates seen both in procaryotes and eucaryotes is evidently due to recognition of some structural features in individual mRNAs. Special structures at the 3′ termini of many mRNAs appear to provide protection against rapid exonucleolytic digestion (Brawerman, 1987), but the selectivity of the decay process appears to be determined to a large extent by interaction between endonucleases or other factors and internal mRNA structures. The regulated decay of histone mRNA in mammalian cells requires the presence of a stem-loop structure at the 3′ end (Levine et al., 1987). Histone mRNA stability probably is achieved by a factor that protects this 3′ structure from nucleases. Transferrin receptor mRNA also is predicted to form a stem-loop structure which is iron-responsive: the mRNA decays in the presence of iron (Mullner and Kuhn., 1988). This particular sequence, inserted at the 5′ non-coding region in another mRNA, promotes translation of the mRNA in the presence of iron (Casey et al., 1988). Thus, probably there is an iron dependent factor that can control either the translation initiation or mRNA decay depending on the location of this recognition sequence on the mRNA molecule.

Furthermore, it was shown that there is a group of AU-rich sequences in the 3'non-coding region in many mRNAs that have short half-lives (Brawerman, 1987). The AU-rich sequence probably causes destabilization of the poly (A) tail whereas a short poly (A) tail often results in accelerated mRNA decay (Wilson and Treisman, 1988). However, the decay of some mRNAs seems to require more than one structural element; for example the decay of c-fos mRNA is determined by the AU-rich recognition sequence as well as another sequence in its coding region (Kabnick and Housman, 1988). Translation of mRNA plays an important role in controlling the degradation of some mRNAs as well. The addition of the cycloheximide, an inhibitor of polypeptide chain elongation, causes stabilization of many unstable mRNAs. Several lines of evidence indicate that mRNA degradation requires the progression of the ribosomes through at least part of the coding region (Graves et al., 1987). For example, it was found that the signal for the degradation of tubulin mRNA lies in the first four amino acids of the growing polypeptide chain. Therefore, the translation of a portion of the coding region of that mRNA is necessary for its degradation. Although different decay processes were identified, still little is known about how the cellular factors affect the susceptibility of mRNA to degradation.

The analysis of mRNA stability in growing and developing Dictyostelium cells has received considerable attention as well (Casey et al., 1983., Chung et al., 1981). The effects of several features of mRNA structure (i.e. 5' caps, 3' poly (A) tails, mRNA size and specific sequences within the transcript) in determining mRNA stability were examined (Manrow and Jacobson, 1988; Manrow et al., 1988). There is a good agreement that an intact 5' cap is essential for the stability of mRNAs, but the destabilization varies significantly between different mRNAs (Drummond et al., 1985; Furuichi et al., 1977; Green et al., 1983; Kreig and Melton 1984; 1978; McCrae et al., 1981). Studies of mRNA with coding region deletions indicate that there is no correlation between the size of an individual mRNA and its stability (Gay et al., 1987; Graves et al., 1987). The hypothesis that poly (A) tail lengths determine the mRNA stabilities was suggested by microinjection experiments using adenylated and deadenylated mRNAs (Drummond et al., 1985; Huez et al., 1974; Marbaix et al., 1975; Nudel et al., 1976). However, similar experiments with different poly $(A)^+$ and poly $(A)^-$ mRNAs do not support this hypothesis (Deshpande et al., 1979; Huez et al., 1983; McCrae et al.,1981; Sehgal et al., 1978). Recent data suggest that there is no direct correlation between the mRNA stability and the length of the poly (A) tail in D. discoideum (Shapiro et al., 1988). Furthermore, there is

strong evidence that UT rich regions located at the non-coding 5' or 3' end of mRNAs might play important roles in mRNA stability (Ross and Pizarro, 1983). Recent experiments show that the deletion or replacement of these UT rich regions results in substantial changes in the decay rate of individual mRNAs (Graves et al., 1987; Luscher et al., 1985; Morris et al., 1986; Rabbitts et al.,1985; Shaw and Kamen, 1986; Simcox et al., 1985). The UT rich sequence is particularly important because of the extremely high A+T content of noncoding regions in D. discoideum mRNAs (Romans and Firtel, 1985).

OBJECTIVES AND ORGANIZATION OF THE THESIS

Cell proliferation is a complex process that requires many genes, however, few of those genes have been identified. In eucaryotes, even a good genetic estimate of the number of genes involved in proliferation is unavailable. Because of the unusual life cycle of D. discoideum some genes whose expression is proliferation-specific were isolated (Kopachik et al., 1985). A cDNA library made to vegetative cell transcripts was constructed in the mammalian expression vector pc-D developed by Okayama and Berg (Okayama and Berg, 1982). A subset of 950 clones of the library were differentially screened with ^{32}p-labeled cDNA probes made to vegetative and slug stage cell transcripts. The results show

that most of these cloned sequences (646 clones) represent a class of "vital " genes. These are probably genes encoding gene products necessary for basic cellular metabolic processes or cell structure and the cDNA, therefore, hybridized to both probes. Another class representing 304 genes are termed "proliferative" because they are preferentially expressed in vegetative and cells early in development. We chose ten of those clones that gave the strongest signals of expression in the vegetative cells. These vegetative genes exhibit coordinate regulation; steady state mRNA levels as measured by northern blot analysis show a drastic decrease during aggregation beginning 4 hours into the developmental phase (Kopachik et al., 1985). The major objective in this project was to determine the nature of the mechanism regulating proliferation-specific gene expression. The hypothesis that differentiating cells have the ability to repress specifically expression of vegetative genes was examined.

At present there is a limited understanding of genetic and physiological signals responsible for the regulation of proliferation-specific gene expression. Whereas little is known about the physiological signals triggering the repression of vegetative gene expression during early development, the induction of differentiation -specific gene expression is mediated by a variety of signals such as

starvation for amino acids, cell to cell contacts, cAMP, differentiation inducing factor (DIF), and other low molecular weight factors (Mehdy and Firtel, 1985). These signals might also affect the expression of vegetative genes. In this work, an examination was made on the effect of these signals on the expression of two genes: one is a gene highly expressed in vegetative amoebae and whose corresponding cDNA sequence was cloned in the insert of the plasmid pcD-D2 and the other is a differentiation-cell-specific gene, pcD-D29, whose expression begins in the life cycle when D2 gene expression begins to be inactivated. Therefore, the analysis of the D29 gene expression provides a useful comparison.

The specific objectives of the project were:

1. To determine the role of starvation in the repression of the D2 gene during development.
2. To determine the role of cell-cell contacts in the inactivation of the D2 gene during development.
3. To test the hypothesis that cAMP might play a role in regulating the selective expression of this gene. Since a current hypothesis implicates the involvement of cAMP in the induction and stabilization of some differentiation specific genes. Mutants strains defective in the cAMP chemotaxis will be used comparatively.
4. To determine if the physiological signal(s) affect the D2 gene expression at the transcriptional or post-transcriptional levels.

MATERIALS AND METHODS

Strains

I. NC-4

Dictyostelium discoideum NC-4 is a wild type strain from which all the following strains were derived (Raper, 1935):

1. XP55 and XP95

XP55 is developmentally competent which carries cycloheximide resistance. XP95 is a thermosensitive mutant that grows at 22 ^{o}C but the growth is arrested at 27 ^{o}C. (Ratner and Newell, 1978).

2. KAX-3

This strain carries the genetic markers axe A and axe B. It was selected for growth in liquid HL5 medium in the absence of bacteria (Loomis, 1971 and Soll et al., 1976).

3. HC85, HC112 and HC317

These are aggregation deficient mutants termed "frigid" (fgd) strains; they were characterized genetically and biochemically with respect to chemotactic ability (Coukell et al., 1983; Kesbeke et al., 1988). The strains are chemotactically unable to respond to exogenous cAMP signals. The HC85 and HC112 strains were derived from the parental strains HC6 and HC91, while HC317 was derived from the XP55 strain.

Parasexual genetic techniques indicate that HC85 and HC112 strains fall in the complementation group fgd-A, and the HC317 in the complementation group fgd-C. Strains of both groups produce low levels of cAMP receptors but only HC317 shows some chemotactic response to cAMP. The fgd strains appear to initiate development when starved but become blocked at an early stage.

II. V12M2

This is a developmentally competent strain (Kopachik et al., 1983).

Growth conditions

All the D. discoideum strains, except for KAX-3, were grown in association with Klebsiella pneumoniae either on 1/2 SM agar plates (per liter: Bacto peptone 5 g, yeast extract 0.5 g, glucose 5 g, $MgSo_4.7\ H_2O$ 1g, KH_2PO_4 2.2 g, Na_2HPO_4 1 g and agar 20 g; Sussman, 1966) or on a suspension culture shaken at 150 rpm. The concentration of amoebae was determined using a hemocytometer and 2X106 cells were dispensed on plates or $5X10^5$ cells/ml in a suspension with pregrown bacteria. The cells were incubated at 22^oC. The plates were harvested within ~ 40 hours when the amoebae started to run out of bacteria; or when the cells growing in suspension reached the concentration of $5X10^6$ cells/ml. Cells multiply with a generation time of 4-5 hours. A new amoebae culture from spores stored on silica gel was started every month.

For axenic strains AX-3 and KAX-3, the spores were inoculated in ~ 2ml of HL5 medium (per liter: Thiotone 14 g, yeast extract 7 g, glucose 14 g, Na_2PO_4 0.5 g and KH_2PO_4 0.5 g; pH 6.5) at the concentration of $1X10^6$ spores/ml until they germinated. This culture was used to inoculate 50 ml of HL5 medium in a 500 ml flask which was shaken at 150 rev./minute at 22°C. When the cell concentration reached about $5X10^6$ cells/ml, it was diluted into fresh medium at the concentration of $5X10^5$ cells/ml. The axenic cells multiply with a doubling time of 10-12 hours.

Developmental conditions

The growing cells were harvested when the plates start to clear in NC-4 and XP55 strains or when the axenically growing cells reached the density of $2-3X10^6$ cells/ml. The bacteria cells were removed from amoebae by differential centrifugation in the buffer KK_2 at 1000 rpm; at that speed the amoebae will pellet out while bacteria stay in suspension. The amoebae were washed 3 times in cold KK_2 buffer(16.6 mM-KH_2PO_4, 3.8 mM-K_2HPO_4, 2 mM-$MgSO_4$; pH ~ 6.1). For normal development, about $2X10^8$ cells were placed on KK_2 agar plates and incubated at 22°C. The cells aggregate within 5 hours and took about 24 hours to form fruiting bodies. For suspension development, the cells were suspended at the concentration of $2X10^7$ cells/ml in development buffer (DB)

(5mM Na_2HPO_4, 5mM NaH_2PO_4, 2mM $MgSO_4$, 200 uM $CaCl_2$; Marin and Rothman, 1980). The total volume of the medium was 1/10 of the flask volume. The cells were shaken at 230 rpm to prevent cell-to-cell contacts or at 70 rpm to allow contacts (Mehdy et al., 1983).

Pulsing with cAMP

Cells developed in suspension were given cAMP at a 50 nM final concentration at 10 minute intervals. The pulsing began at 3 or 4 hours of development and continued until 12 hours of development.

Plasmid preparation

Large scale plasmid preparation

Amplification of the plasmid

1. A single bacterial colony was picked from an L-agar plate (per liter: Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g and 2% agar; medium pH is 7.5) containing ampicillin (50 ug/ml) and inoculated in 5 ml of L-broth with ampicillin allowed to grow overnight at 37°C.
2. The culture was transferred to 1 liter of L-broth containing ampicillin and grown at 37°C until the optical density 595 nm (OD_{595}) reached 0.5-0.7 which is the mid-log phase (about $5X10^7$ cells/ml).
3. Chloramphenicol (375 mg) was added to amplify the plasmid.

The cleared lysis

1. The cells were spun down in GSA rotor at 5,000 rpm for 5 minutes and the cell pellet resuspended in 50 mM tris buffer pH 7.4. Then the suspension was spun again.

2. The pellet was resuspended in 15 ml of 20% sucrose/ 50 mM tris pH 7.4 then 1 ml of 15mg/ml lysozyme was added.

3. Three ml of 250 mM EDTA pH 8 was added and the tube was placed on ice for 30 minutes.

4. Twelve ml of Triton X-100 solution (0.4% Triton X-100, 50 mM tris pH 8, 25 mM EDTA) was added slowly while stirring and the mixture was left at room temperature for ~ 10 minutes until it was clear and viscous.

5. The tube was centrifuged at 18,000 rpm in a SS-34 rotor for 45 minutes at $4^{o}C$ with no brake or was spun in an ultracentrifuge in 30 ml poly-carbonate tubes in a T-865 rotor at 20,000 for 30 minutes.

<u>Isolation of plasmid in CsCl gradient</u>

1. The supernatant was decanted into a 50 ml centrifuge tube. The chromosomal DNA was strained out with a tea strainer or mesh.

2. The volume was measured and 1 g. CsCl per ml of supernatant was added and dissolved.

3. For every 1 ml of the supernatant, 0.1 ml of 5 mg/ml ethidium bromide (EtBr) solution was added.

4. The supernatant was poured into a 35 ml polyallomer tube and the tube was balanced within 0.1 g with mineral oil or

a CsCl solution of the same density to fill up any extra volume.

5. The tubes were centrifuged at 40,000 rpm for at least 36 hours at 20°C.

6. Two bands were evident under UV light (366 nm, long wave length); the lower band contains the plasmid DNA and the upper one is chromosomal DNA.

7. The tube cap was removed and tube was punctured just below the plasmid to withdraw with an 18 gauge needle and a 1 ml syringe.

8. The EtBr was removed with salt saturated isopropanol (250 ml of isopropapol with 250 ml of 5M NaCl/10 mM tris pH 8.5/1mM EDTA to saturate). One volume of saturated isopropanol was added to the plasmid solution (invert to mix), then the tube was undisturbed for ~ 2 minutes to allow phase separation. The EtBr layer was removed and the extraction was repeated until no more EtBr was visible in the CsCl plasmid solution.

<u>Removal of CsCl and precipitation of the plasmid DNA</u>

1. The plasmid containing solution was poured into prepared dialysis tubing and dialyzed overnight against cold 10 mM tris pH 8/1mM EDTA (~ 4 liters).

2. The contents of the bag were removed to 30 ml Corex tube, then 1/10 volume of 3 M NaAoc and 2 volumes of cold Ethanol

was added. The tube contents were mixed well and stored at 20°C overnight or at -70°C for at least 1 hour.

3. The tube was spun down in HB-4 rotor at 10,000 rpm for 20 minutes at 4°C to pellet out the plasmid, then the pellet was dried under the vacuum.

4. The plasmid DNA was resuspended in a small volume (200 ul to 1 ml) of TE buffer (10 mM tris pH 8/1mM EDTA). 5 ul of DNA was placed in 1 ml of H_2O; the OD_{260} was measured to determine the concentration (1.0 OD unit = 50 ug/ml).

Small scale plasmid preparation

Vertical rotor preparation of plasmid

1. Bacterial cells were grown in 100 ml L-broth with 50 ug/ml ampicillin.

2. The cells were spun down in the HB-4 rotor at 7,000 rpm for 5 minutes and resuspended in 2 ml of cold sucrose buffer (25% sucrose, 50mM tris pH 8 and 10 mM EDTA).

3. To the cell suspension, 0.6 ml of lysozyme was added (5mg/ml lysozyme in 50 mM tris pH8/ 10mM EDTA buffer) and the contents were placed on ice for 5 minutes. Then 1.2 ml of 50mM tris/10mM EDTA buffer and 25 ul of 10 mg/ml RNase were added and the tube was returned to ice temperature for another 5 minutes.

4. Five ml of Triton X-100 solution (50 mM tris pH 8, 10 mM EDTA and 2% Triton X-100) was added slowly and the contents

were mixed by tipping.

5. To separate supernatant from cell debris, the tube was spun in an HB-4 rotor at 12,000 rpm at 4°C for 10 minutes.

6. The supernatant was diluted to 10 ml with H_2O containing 0.4 ml of 5 mg/ml EtBr, then 9.6 g CsCl was dissolved and the tube was spun at 2,500 rpm for 10 minutes to remove the protein particles that were formed as a thin film on the top of the supernatant.

7. The supernatant was transferred to 15 ml polyallomer tubes and the tubes were spun in VT-865 rotor at 50,000 rpm at 20°C for 18 hours.

8. The DNA plasmid was collected, dialyzed, precipitated and the concentration was determined as mentioned before.

Isolation of D2 and D29 DNA inserts from an agarose gel

1. The cDNA inserts of the D2 gene (0.45 kb.) and the D29 gene (0.4 kb.) cloned in the pcD-V1 plasmid (Fig. 2) were released by cutting the plasmid with BamH1 restriction enzyme (~4 units of the enzyme/ug of the plasmid DNA). The digestion reaction was run overnight at 37°C to make sure that the cutting of the plasmid was complete, then the reaction was inactivated by incubating the tubes at 70°C for 10 minutes.

2. The digested pcD-D2 or pcD-D29 plasmid was run on a 1.2% agarose gel for about 3 hours, along with the molecular weight markers, to separate the insert. The gel was stained

with ethidium bromide containing H_2O (0.5 ug/ml) for ~ 30 minutes.

3. The band of interest was localized using the long wave length UV lamp (366 nm) to minimize the damage to the DNA.

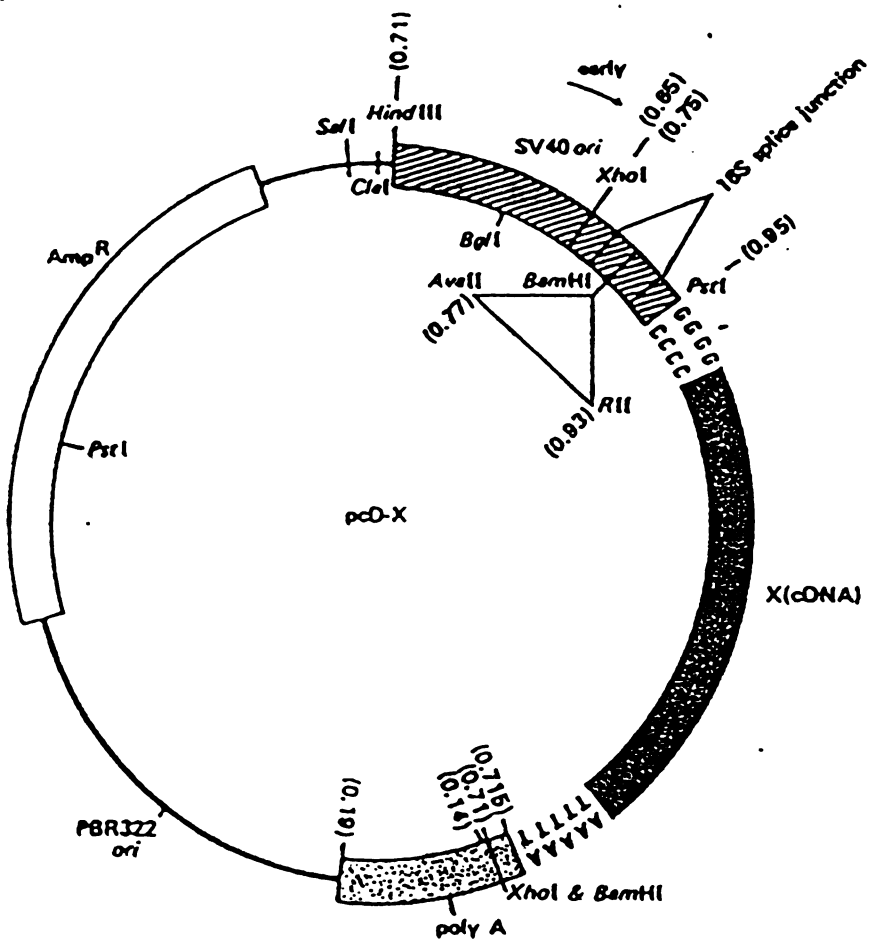
4. The band was cut with a sharp razor blade and placed into a dialysis bag filled with 0.5X TBE buffer then most of the buffer was removed leaving only ~ 2ml or less and the bag was tied.

5. The bag was immersed in a shallow layer of 0.5X TBE in an electrophoresis tank and with a voltage of 100 volt for 30 minutes at which time the current was reversed for ~ 30 seconds to release the DNA from the wall of the dialysis bag.

6. The bag was opened carefully and the buffer surrounding the gel slice was collected. The DNA insert was recovered by ethanol precipitation as mentioned before.

FIGURE 2

The cDNA cloning vector. The plasmid has the SV40 early region promoter upstream of the cDNA cloning site and an SV40 late polyadenylation sequence downstream of the cDNA insert.


(0.71)
early
(0.85)
(0.75)
SalI
HindIII
SV40 ori
16S splice junction
ClaI
XhoI
BglI
(0.95)
AvaII
BamHI
PstI
(0.77)
RII
(0.83)
AmpR
PstI
pcD-X
X(cDNA)
(0.19)
(0.715)
(0.74)
PBR322
ori
AAAAA
TTTTT
XhoI & BamHI
poly A

RNA isolation

Total cellular RNA was isolated according to Chirgwin's method (Chirgwin et al., 1979) by centrifugation of cells lysed in guanidinium thiocyanate placed over a cesium chloride cushion, or by using the phenol/chloroform extraction method (Jacobson, 1976). All glassware were baked at 350^{o}C for at least 3 hours and most of the reagents were treated with the RNase inhibitor, diethyl-pyrocarbonate (dep). Wherever possible, the solutions were treated with 0.1% diethylpyrocarbonate for at least 12 hours and then autoclaved for 20 minutes.

The guanidinium thiocyanate method

1. The cells (~ 2X10^{8} cells) were dissolved in 10 ml of 4M guanidinium/ 0.1M 2-mercaptoethanol (pH5) and frozen at -70C.
2. To isolate total RNA, the samples were thawed and spun at 3000 rpm/20 minutes to pellet the insoluble materials.
3. In an RNAse free AH-629 polyallomer tube 2.5 ml of 5.7 M CsCl/0.1 M EDTA (pH5) was added to form a CsCl cushion; the sample was layered over the cushion up to ~ 1 millimeter from the top of the tube using 4M guanidinium to balance if necessary.
4. The tube was centrifuged for 24 hours at 25,000 rpm at 15^{o}C.

5. All of the sample was removed by aspiration down to the CsCl layer and the tube walls were washed with 3 ml of dep'd water. The wash of H_2O and most of the CsCl were aspirated so that ~ 1 ml is left in the bottom. The tube was inverted and its bottom was cut with a sharp razor blade.

6. The area around the pellet was washed again with 0.5 ml of dep'd H_2O and drained with a pipette.

7. The pellet was jabbed with a blue tip in order to break it up then 0.5 ml of dep water was added and the RNA pieces were transferred to a microfuge tube.

8. Another 0.5 ml of dep H_2O was added and RNA was broken to small pieces by pipetting up and down several times then vortexed continuously until the RNA was dissolved completely.

9. The microfuge tube was spun for ~ 5 minutes to pellet the insoluble materials, if any, and the supernatant was transferred to a baked corex tube. To the supernatant was added 2 ml of dep H_2O, 75 ul of 4M NaCl and 7.5 ml of cold 100% Ethanol. After mixing well the tube was stored at -20°C overnight or at -70°C for at least an hour.

10. The tube was spun at 13,000 in HB-4 rotor/20 minutes at 4°C to pellet RNA. The supernatant was aspirated and the RNA pellet was washed gently with ~ 0.5 cold 80% ethanol to remove NaCl residues.

11. The RNA pellet was dried in a vacuum until the ethanol and the H_2O were evaporated. The pellet was dissolved in

small volume of dep'd H_2O (~200 ul).

The phenol/chloroform method

1. The cell pellet was suspended in 10 ml of cold HMK buffer (50mM Hepes, 40mM $MgCl_2$ and 20mM KCl) and to this was added, with vortexing, 200 ul 10% SDS, 10 ml phenol saturated with 1M tris pH 7.5, 0.5 ml 4M NaCl and 10 ml chloroform.
2. The tube was centrifuged at 3000 rpm for 10 minutes.
3. The aqueous phase was removed and extracted alternately with an equal volume of phenol and chloroform until the interphase disappeared.
4. RNA was precipitated by the addition of NaCl to 0.3M and 2 volumes of ethanol to the aqueous phase.
5. The tube was centrifuged at 13,000 rpm in an HB-4 rotor for 30 minutes, at 4°C and the RNA was resuspended at a concentration of 1 mg/ml in dep'd H_2O.

Northern blot analysis

1. The denatured RNA was sized-fractionated on 1.3% agarose gels containing formaldehyde (Lehrach et al., 1977) and transferred to Gene Screen (New England Nuclear) according to the manufacturer's directions.
2. The filters were baked at 80°C for 2 hours.
3. The blots were prehybridized in hybridization buffer containing (50% Formamide, 10% Denhardt's solution 50X,

50 mM tris pH 7.5, 1% of ultra-pure SDS, 10% Dextran Sulfate, 1 M NaCl, 1% denatured Salmon DNA and ~ 4% or less H_2O) at 42°C overnight.

4. Oligolabeled plasmid probes (~ $5X10^8$ to $1X10^9$ cpm/ug) were hybridized to the blots ($2X10^6$ cpm/blot) in the hybridization buffer for 2 days at 42°C.

5. The blots were washed twice (for 10 minutes each) in 2X SSC (0.3M Nacl/.003 M trisodium citrate) at room temperature, and then in 0.5X SSC with 1.0% SDS for 30 minutes at 65°C and in 0.1X SSC for 30 minutes at room temperature.

6. The blots were visualized by autoradiography at -70°C overnight using intensifying screens.

Oligolabeling

This technique was used for radiolabeling DNA restriction fragments to high specific activity (Feinberg and Vogelstein, 1983). The technique is very good for radiolabeling small DNA fragments (less than 500 bp). A brief description of this technique is as follows:

1. Sixty to 100 ng of D2 or D29 insert DNA was diluted to 11 ul in distilled H_2O in a 0.5 ml microfuge tube (if the entire plasmid was used, it was linearized with the appropriate restriction enzyme).

2. The tube was boiled for 5 minutes and cooled quickly in ice in order to denature DNA (single stranded DNA), then the following reagents were added to the tube:

A. 18 ul LS (LS is a mixture of 1M Hepes buffer pH 6.6: TM : OL at the ratio of 25: 25: 7 respectively; TM contains 250mM tris pH 8, 25 $MgCl_2$ and 50mM B-Mercaptoethanol; OL contains 90 O.D. units/ml Hexamers, 1mM tris pH8 and 1mM EDTA pH 8).

B. 1 ul of 16 mg/ml BSA.

C. 3 ul of 100uM dNTPs minus dCTP.

D. 4 ul of (^{32}P) dCTP (3000 Ci/mmol).

E. 1/2 ul of DNA polymerase I, Klenow fragment (9 units).

3. The tube was incubated at room temperature overnight.

4. The unincorporated ^{32}P was removed using the spun column procedure.

5. A one ml disposable syringe plugged at the bottom with glass wool was used as a column.

6. The column was filled with Sephadex G-50 equilibrated in SDS buffer (100mM NaCl, 10mM tris pH 8, 1mM EDTA and 0.4% SDS), then it was spun at about 1,500 rpm for 5 minutes in a bench centrifuge in order to pack down the Sephadex (the packed column volume was about 0.9 ml).

7. The DNA sample was applied to the column and the column was recentrifuged at the same speed and for the same time as before. The effluent from the column was collected in a

decapped eppendorf tube which represents the labeled DNA, while the unincorporated (^{32}P) dNTPs remain in the syringe.

8. The counts per minute (cpm) of the labeled DNA (probe) was measured using a scintillation counter and the probe specific activity was determined.

Nuclear transcription assays

Nuclear run-on transcription assays were performed essentially as described by Nellen (et al., 1987; Landfear et al., 1982; Williams et al., 1980).

I. Nuclei isolation

1. Axenic cells were grown in the rich medium (HL5) until they reached the concentration of $4X10^6$ cells/ml. Developing cells were shaken on suspension in DB under the conditions mentioned before.

2. Upon harvesting (3000 rpm/10 minutes), cells were washed 3 times with KK_2 buffer.

3. Cells were resuspended in 1/10 of the original volume of lysis buffer II (lysis buffer I + 10% percoll).

4. Cells were lysed by the addition of NP-40 detergent at the final concentration 1%.

5. Nuclei were collected by centrifugation at 5000-6000 rpm for 5 minutes.

6. The pellet was resuspended in 1/10 of the original volume of lysis buffer II, then unlysed cells were spun down at 1000

rpm for 5 minutes.

7. The nuclei were collected again from the supernatant as before, and washed once in 1/20 original volume of lysis buffer I (50 mM Hepes-pH 7.5, 40 mM $MgCl_2$, 20 mM KCl, 0.15 mM Spermidine, 5% Sucrose, 14 mM Mercaptoethanol and 0.2 mM Phenyl methyl sulfonyl fluoride 'PMSF'), then they were precipitated again.

8. As soon as the nuclei were isolated, they are used in the <u>in vitro</u> transcription reaction.

II. In vitro transcription

1. The reaction was set up as follows:

A. 34 ul H_2O

B. 20 ul 5X transcription buffer (200 mM Tris pH 7.9, 1.25 M KCl, 50 mM $MgCl_2$, 25% glycerol and 0.5 mM DDT)

C. 5 ul of 4mM ATP, 4mM GTP and 4mM CTP.

D. 1 ul (30 units/ul) of the RNase inhibitor (RNasin).

E. 10 ul (100 uCi) of a fresh ^{32}P UTP (3000 Ci/mmole).

F. 20 ul nuclei (from $2X10^7$ cells)

Incubation was at 23 ^oC for 30 minutes.

2. The reaction was stopped by the addition of:

A. 10 ul 1M tris, pH 8.4.

B. 10 ul 0.2M EDTA.

C. 10 ul 20% SDS.

D. 80 ul H_2O).

E. 200 ul phenol-chloroform.

3. Unincorporated nucleotides were removed by passing the aqueous phase over a Sephadex G-50/SDS spun column.

4. Equal numbers of incorporated counts from each time point were hybridized to filter-immobilized DNA for 48 hours 42^{0}C as mentioned above.

Chemicals

Unless otherwise stated all the chemicals used in this work were purchased from Sigma Chemical Company. Adenosine 3′:5′-monophosphothioate cyclic Sp-isomer (cAMP-S) was obtained from Boehringer Mannheim Biochemicals. Formamide and guanidinium thiocyanate was from Fluka. ^{32}P-CTP and ^{32}P-UTP was from New England Nuclear.

RESULTS

I. The expression of the vegetative specific D2 and the differentiation specific D29 genes in vegetatively growing and differentiating cells

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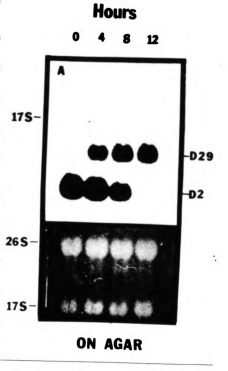
The vegetative XP55 cells, a derivative of the wild type strain NC-4, were grown with Klebsiella pneumoniae on 1/2 SM agar plates (Kay and Trevan, 1981) and were collected for RNA isolation before being starved. For normal development with cell aggregation, the vegetative amoebae were harvested, washed three times with KK2 buffer, placed on KK2 plates ($2X10^8$ cells/ plate) and observed to aggregate in response to cAMP. At the end of the aggregation stage tight aggregates with tips had formed by 12 hours.

Total RNA isolated from vegetative cells and developing cells (at 4 hour intervals) was size-fractionated on 1.3% formaldehyde agarose gels and electroblotted onto "Gene Screen" membrane. The blot was hybridized with the oligolabelled pcD-D2 and pcD-D29 plasmids that have the cDNA inserts for these genes. The D2 and D29 cDNA inserts hybridized to small transcripts on northern blots (Fig. 3A). The ethidium bromide stained blot of (A) is shown in (Fig. 3B) as a control for RNA analysis to indicate that equal

FIGURE 3

The expression pattern of the growth gene (D2) and the early developmental gene (D29) in the developmentally competent strain XP55 during normal development on agar (A). RNA isolation and northern blot analysis were performed as described in the materials and methods section. The autoradiogram shows that the D2 transcripts are abundant in growing cells and early in non-growing differentiating cells but are lost when cells begin to aggregate. The levels of D29 transcripts, in contrast, rise during this period. The ethidium bromide stained blot of (A) is shown in (B) as a control for RNA analysis to indicate that equal amounts of RNA (10 ug) as judged by the equality of the amount of RNA in the 26S and 17S ribosomal bands were loaded in the lanes. All blots shown in this work were stained with ethidium bromide and observed to have an equal RNA loading by this method.


Hours
0 4 8 12
A
17S
D29
D2
26S
17S
ON AGAR

amount of RNA (10ug) were loaded in the lanes as judged by equality of the amount of RNA in the 26S and 17S ribosomal bands. D2 transcript levels were high in growing cells but fell dramatically by 8 hours of differentiation. D29 transcript levels, however, were undetectable in growing cells but increased by 4 hours of differentiation (Fig. 3A). Similar results were obtained with V12M2 and NC-4 strains (data not shown). Thus, D2 gene expression is inactivated while the D29 gene expression is activated.

The D2 gene might be an important growth gene because its expression is reduced in the life cycle when cells are no longer proliferating. The D2 transcript levels are high during cell proliferation, decline dramatically to undetectable levels during development when the cells are arrested at the G2 phase (Weijer et al., 1984; Katz and Bourguignon., 1974) and increase again upon the resumption of growth after the germination stage (Kopachik et al., 1985). To investigate if cessation of cell proliferation alone affects D2 transcript levels, we used two mutant strains which are thermosensitive for growth (tsg), XP95 and HM27. The XP95 mutant, whose defect maps to tsg A on linkage group IV, and HM27 (unmapped mutant) grow normally at 22^{o}C but cease proliferation at 27^{o}C (Ratner and Newell, 1978; Kopachik et al., 1983). RNA isolated after 18 hours of incubation at

these temperatures was analyzed on a northern blot to determine the D2 and D29 mRNA levels (Fig. 4A). The interruption of the cell proliferation caused a loss of D2 transcript levels in both strains whereas D29 transcripts were detected only in the HM27 strain. However, the transcript levels of another gene, pLK 326, were unchanged which indicates that the loss of D2 transcripts was specific and it was not due to a heat shock response. In the control experiments, where cells grew at 22^{0}C for 18 hours, the D2 transcript levels were high, while D29 transcript levels were not detected. We also blocked the cell proliferation of an axenically growing strain AX-3 with caffeine (Hagmann, 1986) to investigate whether D2 transcript levels can be affected in a strain without a thermosensitive growth mutation. In order to determine the appropriate dose of caffeine that arrests cell proliferation, we grew the cells in the presence of different caffeine doses (i.e. 0.0, 0.625, 1.25, 2.5, 5 and 6.25 mM). The 5 mM dose inhibited cell division completely, the 2.5 mM caffeine had an intermediate effect and the 1.25 mM dose or less had no effect on the growing cells. Thus, caffeine affects the growing cells in a dose dependent manner (Fig. 4B). These results were confirmed by northern blot analysis of RNA isolated from cells under different concentrations (Fig. 4C) Caffeine has a reversible inhibitory effect on cell growth. When the caffeine arrested

cells were washed thoroughly with KK_2 buffer and resuspended in fresh medium, they recovered from the inhibitory effect of caffeine (Fig. 4 D). Furthermore xanthine, a caffeine precursor, was found to have a similar effect as caffeine (Fig. 4 E). Different strains might vary in their responses to the addition of caffeine. For example, the XP55 strain is less responsive to the caffeine addition in lowering the D2 transcript levels than AX-3 strain (data not shown).

When caffeine (5mM) was added to the growing cells, D2 transcript levels were lowered, but interestingly D29 transcript levels increased as if the early developmental events were induced as occurred in the HM27 strain at $27^{o}C$ (Fig. 4 F). The increase in D29 transcript levels were comparable to that of normal developing cells. Thus blockage of cell proliferation resulting from caffeine or from incubation of some growth mutants at a restrictive temperature lowers D2 and raises D29 transcript levels. These results suggest that the cell cycle might play a role in the regulation of D2 gene expression. On the other hand, when we added to growing cells the DNA synthesis inhibitor, hydroxyurea (1mM), known to arrest the cell cycle at the S-phase, there was no effect on the D2 transcript levels (Fig. 4G).

FIGURE 4A

Blockage of the cell proliferation lowers D2 mRNA levels. The expression pattern in vegetative amoebae of two tsg mutants (XP95 and HM27) incubated at 21^{o}C and after shifting an identical culture to 27^{o}C for 18 hours. The autoradiograms show that the interruption of the cell cycle caused a loss in the D2 transcript levels whereas D29 transcripts in the HM27 strain were induced. The transcript levels of the control gene, pLK326, were unchanged.

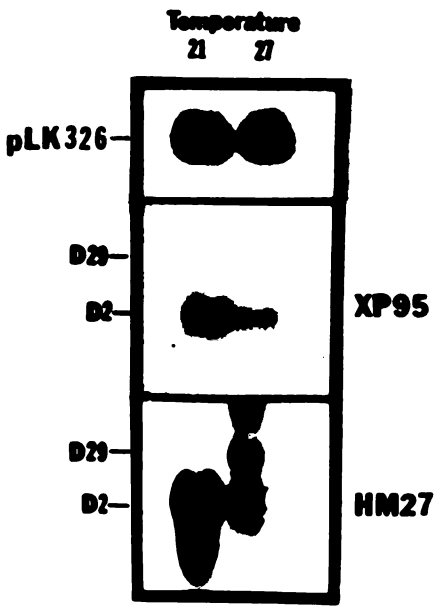

Temperature
21
27
pLK326
D29
D2
XP95
D29
D2
HM27

FIGURE 4B

Caffeine affects cell proliferation in a dose dependent manner. Caffeine was added to axenically growing cells at different concentrations,0, 1.25, 2.5 and 5 mM.

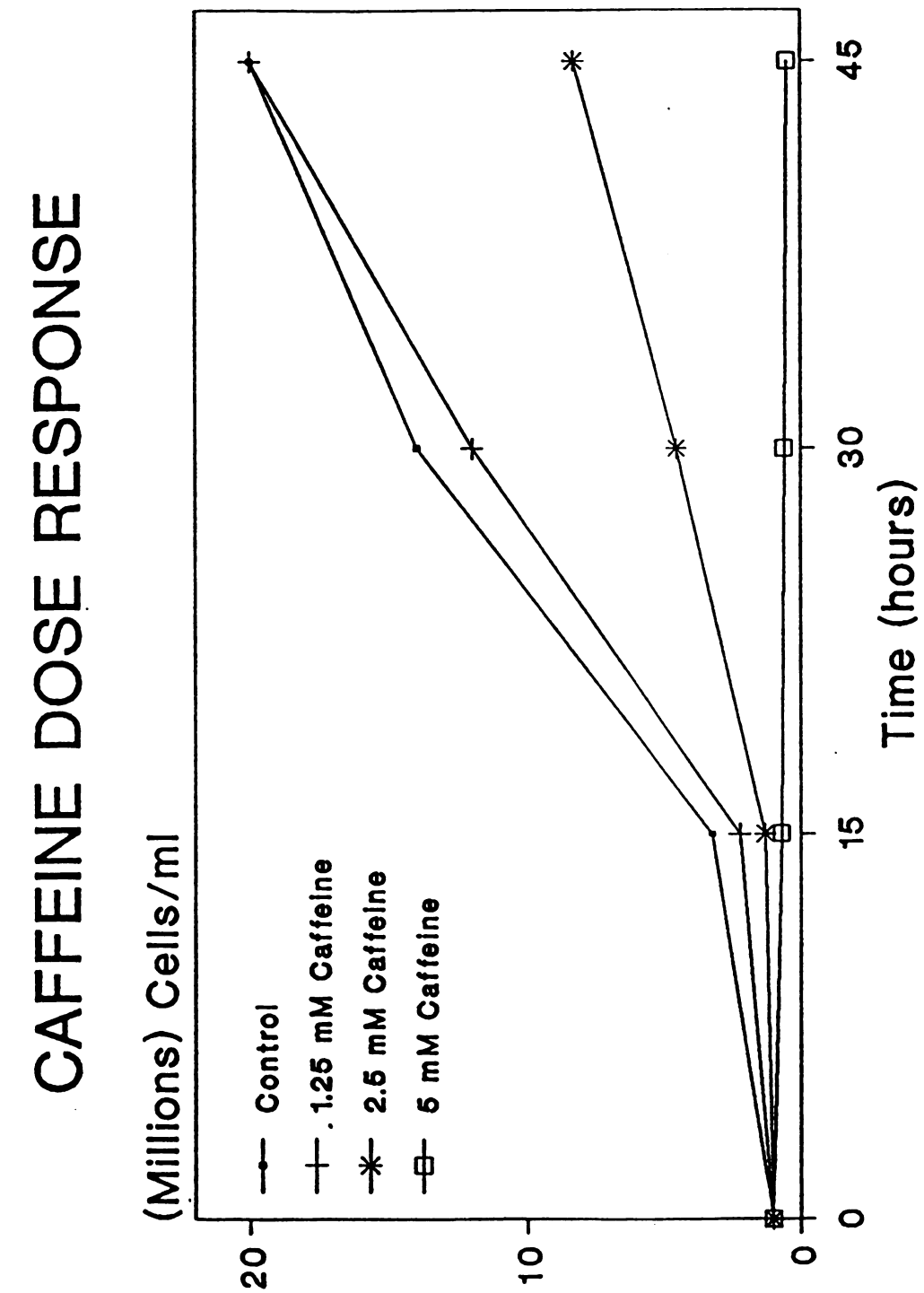

CAFFEINE DOSE RESPONSE
(Millions) Cells/ml
Control
1.25 mM Caffeine
2.5 mM Caffeine
5 mM Caffeine
20
10
0
0
15
30
45
Time (hours)

FIGURE 4C

Caffeine lowers D2 mRNA levels. RNA isolated from cells treated with various concentrations of caffeine was analyzed for D2 mRNA levels.

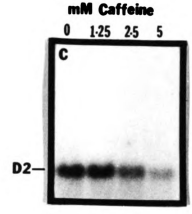

mM Caffeine
0
1·25
2·5
5
C
D2—

FIGURE 4D

The inhibitory effect of caffeine is reversible. AX-3 cells were incubated in the presence of 5mM caffeine, then half of the 5mM caffeine treated cells were removed from caffeine, and resuspended in fresh medium. These cells recovered from the caffeine inhibition effect.

CAFFEINE RECOVERY EXPERIMENT

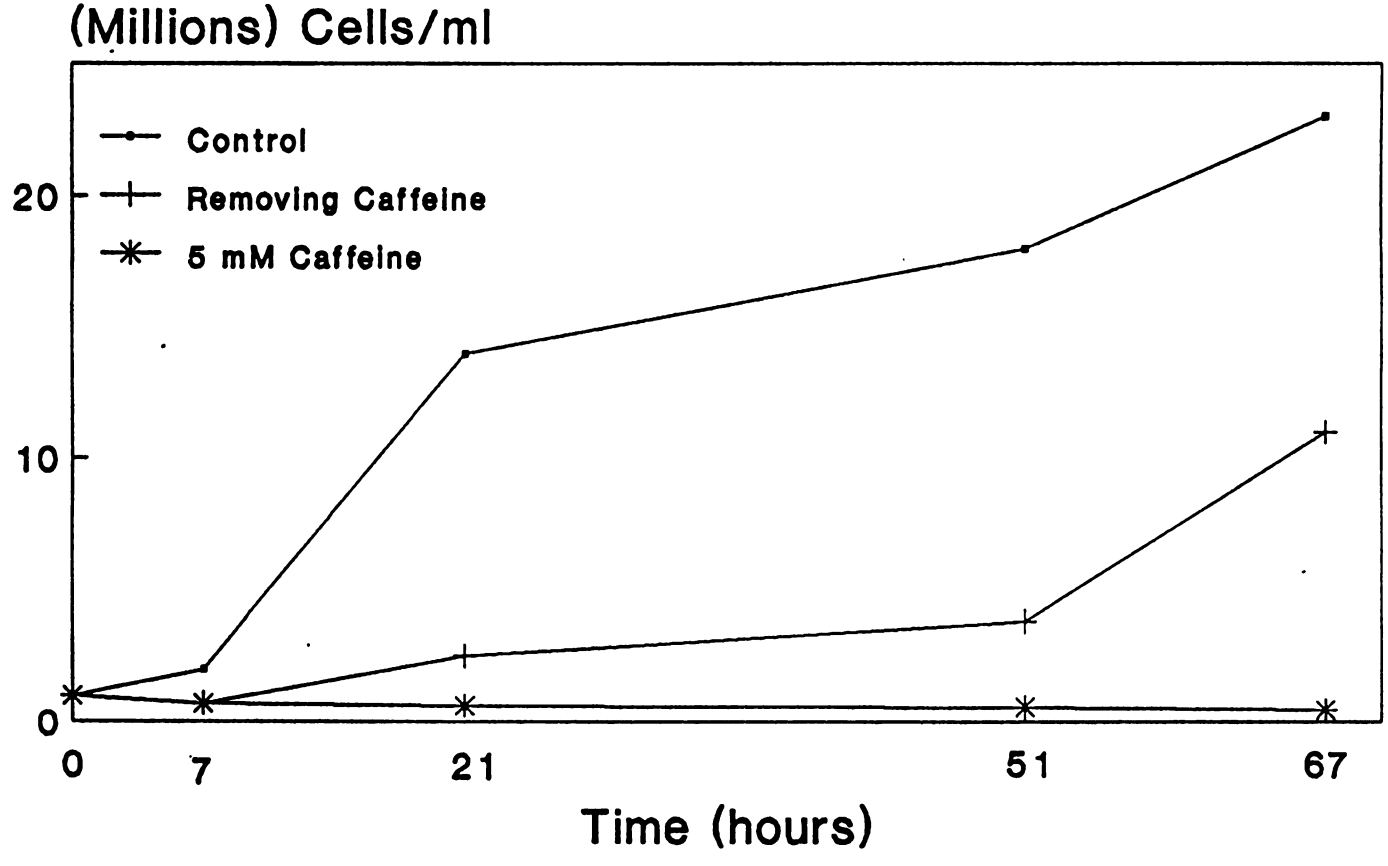


FIGURE 4E

Xanthine inhibits cell growth. AX-3 cells were grown in the presence of different concentrations of xanthine, 0, 2.5 and 5mM. Xanthine at 5mM is as effective as caffeine at the same concentration.

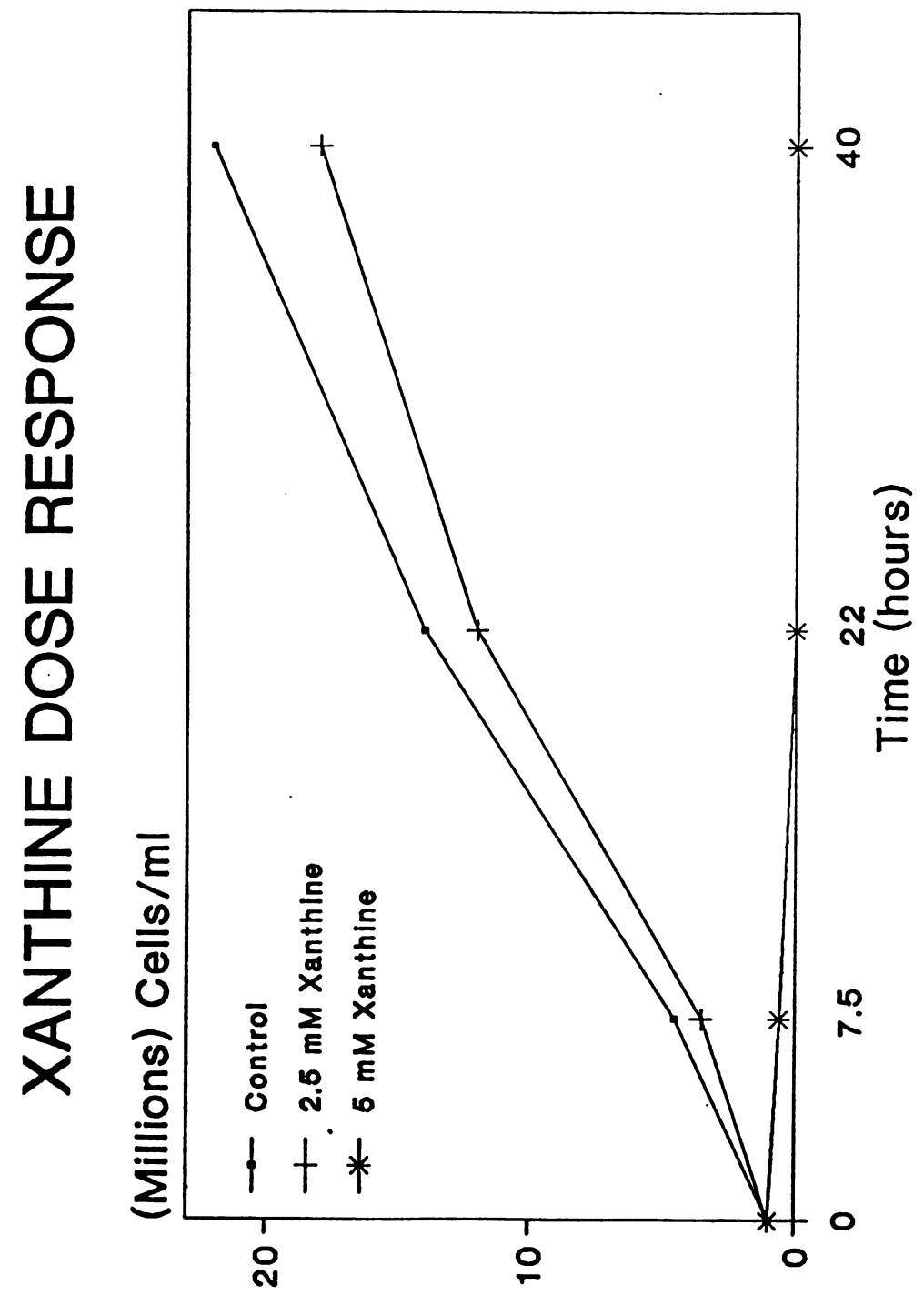
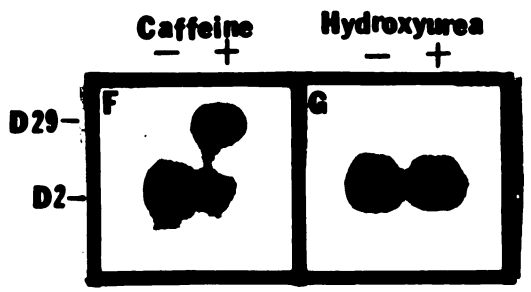

XANTHINE DOSE RESPONSE
(Millions) Cells/ml
Control
2.5 mM Xanthine
5 mM Xanthine
20
10
0
0
7.5
22
40
Time (hours)

FIGURE 4 F AND G

F. Caffeine reduced D2 transcript levels, but induced D29, gene expression. AX-3 strain was grown in nutrient broth (HL5) for 12 hours in the presence (+) or absence (-) of caffeine. The autoradiogram shows that when the cell proliferation was blocked with caffeine (5mM), the D2 mRNA levels were lowered, but the D29 mRNA levels increased.

G. Hydroxyurea does not affect D2 mRNA levels. AX-3 cells were grown in the presence (+) or absence (-) of the DNA synthesis inhibitor, hydroxyurea (1mM). The autoradiogram shows that the addition of hydroxyurea did not change the D2 transcript levels.


Caffeine
− +
Hydroxyurea
− +
F
G
D29
D2

Thus, arresting the cell cycle at the S might not affect the D2 transcript levels.

II. The physiological signal(s) that regulates D2 gene expression

---

The loss of D2 transcripts during normal development might be due to (1) starvation for nutrients, (2) the effect of cell-to-cell contacts which develop during differentiation, (3) cAMP which is an essential signal for both chemotaxis and morphogenesis during the developmental program, (4) the cell cycle effect just described, or (5) a combination of these signals. Development of cells in a suspension was used to study the effects of these signals on the expression of the D2 and D29 genes. Development in suspension allows for close monitoring of the concentration of extracellular cAMP and the easy introduction of drugs or analogues that might affect signaling. Amoebae will develop and retain aggregation competence in shaken suspension but will proceed no further with development (Gerisch, 1968). Although suspension development prevents normal morphogenesis, cells do initiate the early developmental events. Several studies indicate that cAMP receptors are induced in Dictyostelium cells shaken in suspension (Henderson, 1975; Chisholm et at., 1984) and cell-cell contacts termed contact sites A form (Gerisch et al., 1975). The addition of cAMP in pulses to cells in

suspension was shown to stimulate maximal cAMP binding activity (Chisholm et al., 1987; Schaap et al., 1986). Kimmel showed that cells shaken at a high density ($2X10^7$ cells/ml) and low speed (90 rpm) in suspension culture may emit pulses of cAMP and stimulate the entire culture to produce endogenously generated pulses of cAMP (Kimmel and Carlisle, 1986; Kimmel, 1987).

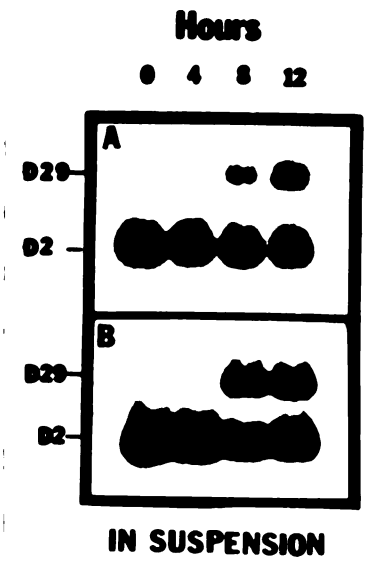
1. The effect of suspension development and cell-cell contacts

---

Starvation was initiated by washing cells three times with phosphate buffer (KK_2), and placing in flasks shaken in suspension in development buffer (DB) at the concentration $2X10^7$ cells/ml at 230 rpm to prevent cell-cell contacts or at 70 rpm to allow contacts. Cells shaken at high speed remain substantially as single cells while cells shaken at a low speed form cell masses (Mehdy et al., 1983). RNA isolated at 4 hour intervals (0, 4, 8 and 12) was analyzed on northern blots. In the slow and fast shaking experiments, the D2 transcript levels did not fall whereas the D29 transcript levels rose by ~8 hours of development in suspension (Fig 5 A and B). Thus, starvation alone, which might be an effective physiological signal in the activation of the D29 gene, is not a sufficient signal for the inactivation of D2

FIGURE 5

Starvation or cell-cell contacts are not sufficient signals for lowering the D2 transcript levels. XP55 cells were developed in suspension in the presence (slow shaking) or absence (fast shaking) of cell-cell contacts. The autoradiogram shows that the levels of D2 transcripts remained high during development in suspension.


Hours
0 4 8 12
A
D29
D2
B
D29
D2
IN SUSPENSION

gene expression. Moreover, cell-cell contacts, which play an important role in the induction of several thousands of genes during aggregation (Firtel, 1972; Landfear and Lodish, 1980; Finney et al., 1985), had little effect on the D2 transcript levels. Nevertheless, cell-cell contacts or probably cAMP signals spontaneously emitted from cell aggregates on suspension did enhance the expression of D29 gene. The D29 gene expression was induced by 4 hours in cells developing on agar but by 8 hours in suspension. Additionally, the D29 transcript levels were higher in slow shaken cells, where small aggregates are formed, than in fast shaken cells, where cells remain substantially as single cells. Athough cell-cell contacts appear to play a role in inducing the expression of D29 gene, the induction of this gene in vegetative cells by caffeine or in the tsg mutant disagrees with the cell contact hypothesis. Presumably another signal other than cell-cell contacts activates D29 gene expression.

2. The effect of elevating the extracellular or the intracellular cAMP levels

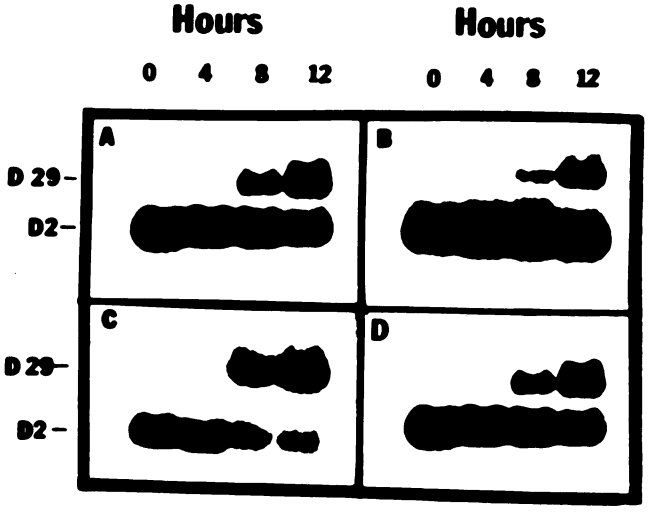
---

Extracellular cAMP is known to induce some differentiation specific transcripts and repress other transcripts (see Introduction, P.9) when added to cells shaken in suspension cultures (Barklis and Lodish, 1983; Bozzone and Berger, 1987 and Chung et al., 1981). To study the effect of cAMP on D2 gene expression, cAMP (1mM) was

added four hours after the cells were placed in suspension at slow or fast shaking speed and the cells were allowed to differentiate for another 8 hours. RNA isolated from aliquots of cells collected at 4 hour intervals (0, 4, 8, and 12) was analyzed as before. The D2 transcript levels remained high and did not respond to the addition of cAMP (Fig. 6 A). We also tried the cAMP thioanalogue cAMP-S which inhibits aggregation in NC-4 derived strains (Rossier et al., 1978). Cyclic AMP-S is about 10,000 times more slowly hydrolyzed by cAMP-phosphodiesterases but its affinity for the cAMP receptor is only about 10 fold less than cAMP. The cAMP-S added at a lower physiological level (5uM) also was ineffective (Fig 6 B). In the meantime, there was no significant change in the D29 transcript levels with the addition of cAMP or cAMP-S. Therefore, high levels of cAMP or cAMP-S do not affect D2 and D29 mRNA levels. On the other hand, cells of the V12M2 strain differentiating under the same conditions lowered D2 transcript levels (Fig 6 C). However, the levels of D2 transcript remained high in V12M2 cells differentiating in suspension in the absence of cAMP (Fig.6 D). The difference in the response between XP55 and V12M2 strains to 1 mM cAMP might be due to the ability of the V12M2 strain to develop in the presence of cAMP-S concentrations which prevent aggregation in NC-4 derived strains.

FIGURE 6 A,B,C,D

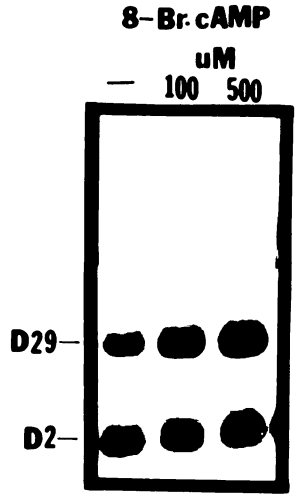
Non-oscillating extracellular cAMP levels do not affect D2 gene expression. The expression pattern of XP55 during suspension development with the addition of 1mM cAMP (A), 5uM cAMP-S (B) or V12M2 in the presence (C) or absence (D) of 1mM cAMP. The autoradiograms show that although D2 mRNA levels fall in V12M2, they do not in XP55.


Hours
0 4 8 12
Hours
0 4 8 12
A
B
C
D
D 29
D2
D 29
D2

Possibly high intracellular but not high extracellular levels of cAMP will lower D2 gene expression. The 8-bromo cAMP analogue, which is capable of entering the cells (Kwong et al., 1988; Kay, 1989), added at a 50 and 100uM to XP55 cells developing in suspension did not affect D2 gene expression (Fig. 6 E). Another cAMP analogue, 8-chlorophenylthio cAMP (200 uM), added under the same conditions also was ineffective (data not shown). These cAMP analogues are known to affect gene expression for some genes (Oyama and Blumberg 1986; Theibert and Palmisano, 1986; Van Haastert and Klein, 1983; De Wit, 1982). Therefore, if those analogues are membrane permeable and capable of acting like intracellular cAMP, they should have lowered D2 transcript levels if an increase in intracellular levels of cAMP is required for lowering D2 mRNA levels. In conclusion, increasing the extracellular or the intracellular cAMP levels does not affect D2 gene expression.

FIGURE 6 E

Elevating the intracellular cAMP levels does not affect the D2 gene expression. The expression pattern during suspension differentiation with the addition of the cAMP membrane permeable analogue (8-bromo cAMP) at the concentration of 100uM and 500uM. The autoradiogram shows that increasing the intracellular cAMP was ineffective in lowering the D2 mRNA levels.


8-Br. cAMP
uM
—
100
500
D29
D2

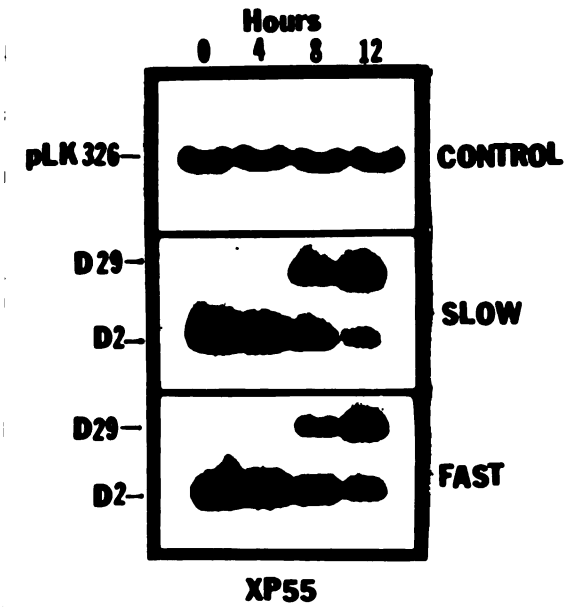
3. Oscillation of extracellular cAMP levels is required for D2 gene expression

---

Chemotaxis activation as well as the induction and repression of different classes of genes expressed early in development requires that a cell surface cAMP receptor receives an intermittent cAMP signal. This will result in an oscillation of the receptor between the active and the inactive forms, and thus activate the intracellular signal transduction process that leads to chemotaxis and regulation of gene expression (see Introduction, p11). Furthermore, recent studies (Klein et al., 1988) indicate that the oscillation of extracellular cAMP levels is required for the induction of the receptor gene expression. We, therefore, tried to mimic the normal developmental conditions by applying cAMP in pulses to cells shaken in suspension. Pulses of cAMP were applied (at 50nM final concentration) to XP55 cells in 10 minute intervals starting after 3 hours in suspension culture and continuing for another 9 hours. Total RNA isolated at 4 hour intervals was analyzed on a northern blot. Surprisingly, cAMP pulses caused a gradual loss of D2 transcript levels by 8 and 12 hours of development in suspension, but no significant change was detected in the D29 transcript levels (Fig. 7A). In the KAX-3 strain, the 200 nM cAMP pulses were the most effective in lowering D2 transcript levels (2-3 fold less than the control).

FIGURE 7A

Oscillation of extracellular cAMP levels is necessary to lower the D2 mRNA levels. The expression pattern during suspension differentiation in the presence of cAMP pulses. XP55 cells were developed in suspension at a slow or fast shaking speed and with the addition of cAMP pulses. Pulsing was begun after 3 hours and continued for another 9 hours at a 50 nM final concentration at 10 minute intervals. The autoradiograms (a and B) show a gradual loss of the D2 mRNA within 8 and 12 hours of development, while pLK326 mRNA levels remained unchanged.


Hours
0
4
8
12
pLK 326
CONTROL
D29
D2
SLOW
D29
D2
FAST
XP55

Higher doses of pulses (more than 50 nM with the XP55 strain or more than 200 nM with the KAX-3 strain) were not as effective (data not shown). The extracellular cAMP-phosphodiesterase will effectively degrade a cAMP pulse within few minutes and before the addition of another one; therefore as in normal development cAMP-phosphodiesterase prevents the cAMP level from building up (see Introduction, p11). However, the level will be built up quickly with the addition of high cAMP doses because high concentrations of cAMP may saturate the system and inhibit the phosphodiesterase activity. Therefore, the oscillation of the extracellular cAMP level is required for the inactivation of the D2 gene expression.

The vitamin folic acid, which is given off by bacterial food sources, is another chemoattractant of Dictyostelium cells. It attracts vegetative cells and continues to attract developing cells until cAMP signals are initiated early in development. Folic acid induces biochemical oscillations, accelerates development to the aggregation stage and elevates intracellular cAMP levels when applied in pulses (Wurster and Schubiger, 1977). XP55 cells were shaken in suspension (in development buffer at the concentration of $5X10^7$ cells/ml) for 2 hours, then the oscillations were induced by means of 11 pulses of folic acid (at 200 nM final

concentration) supplied at intervals of 8 minutes. RNA was isolated and analysed on a northern blot as before. Folic acid pulses had no effect on the D2 transcript levels during development (Fig. 7B). This reinforces the idea that increasing the intracellular levels of cAMP is not required for the repression of the D2 gene. On the other hand, when 1 mM folic acid is added to cells developing in suspension in the presence of cAMP pulses, the effect of cAMP pulsing was blocked (Fig. 8B). Therefore a high concentration of folic acid, as a chemoattractant or as a nutrient, probably is a signal to continue the expression of the D2 gene. Similarly, we found that the addition of 1 mg/ml of a mixture of essential and non-essential amino acids (at the beginning of development) to cells developing in suspension in the presence of cAMP pulses blocked the pulsing effect and prevented the loss of the D2 gene mRNA (Fig. 8A). The effect of amino acids may be due to the retardation of starvation response. The results indicate that both starvation and cAMP oscillations are necessary for the inactivation of D2 gene expression.

In another experiment we tried to study if the protein synthesis is required to lower the D2 transcript levels. There are probably two major mechanisms for the deactivation of gene expression during early development, one dependent upon and one independent of protein synthesis (Singleton, et al., 1988). We found that when the protein synthesis

inhibitor, cycloheximide, was added (500 ug/ml) to NC-4 cells developing on suspension in the presence of cAMP pulses, it accelerated the loss of D2 transcripts (Fig. 8 C). These results suggest that, unlike other genes, the loss of D2 transcript levels does not require protein synthesis (Singleton et al., 1988).

FIGURE 7B

Folic acid pulses do not affect the D2 transcript levels. The expression pattern during suspension differentiation in the presence (+) or absence (-) of folic acid pulses. XP55 cells were developed in suspension at the concentration of $5X10^7$ cells/ml for 2 hours, then 11 pulses of folic acid (at 200nM final concentration) were added at intervals of 8 minutes. The autoradiogram shows the D2 transcript levels did not change.

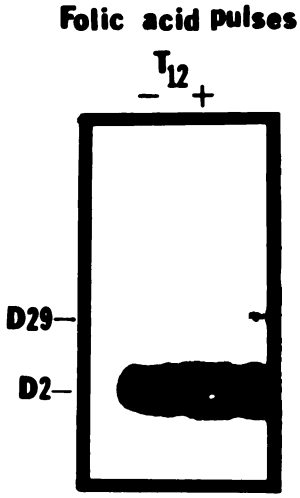
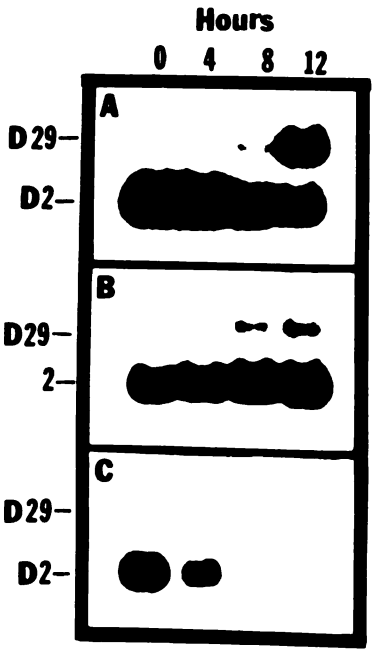

Folic acid pulses
T12
−
+
D29
D2

FIGURE 8 A,B,C

The expression pattern during suspension development for strain XP55 with the addition of 1mg/ml amino acids (A), 1mM folic acid (B) or cycloheximide (C) in the presence of cAMP pulses. The autoradiograms show that the nutrients blocked the pulsing effect, while cycloheximide lowered the D2 and D29 mRNA levels.


Hours
0
4
8
12
A
D29–
D2–
B
D29–
2–
C
D29–
D2–

III. Cyclic AMP signals that inactivate D2 gene expression are transduced through cell surface cAMP receptors

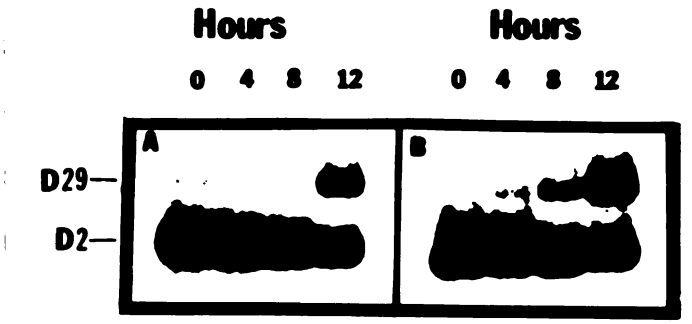
The cAMP receptors in Dictyostelium, similar to other cell-surface receptors in eukaryotes, respond to extracellular signals and activate an intracellular signal transduction mechanism by interaction with GTP-binding regulatory proteins. The intracellular signal regulates the expression of a number of genes during early development and it may also control the D2 gene expression.

1. Activation and deactivation of the cell surface receptor is necessary for D2 gene inactivation

If the pulsatile signal that results in lowering D2 transcript levels requires the phosphorylation and the dephosphorylation of the cell surface cAMP receptor, blocking the oscillation of the receptor should prevent the loss of D2 transcripts. We, therefore, used reagents such as cAMP-S and adenosine that are capable of blocking the signal transduction. XP55 cells were developed with cAMP pulses and the addition of 5uM cAMP-S (A) and 5mM adenosine (B). RNA isolated from aliquots of cells collected at 4 hour intervals was analyzed on northern blots. The D2 transcript levels did not respond to cAMP pulses and remained high under all cases (Fig. 9 A and B). Cyclic AMP-S probably antagonizes the effect of cAMP pulses by occupying the

FIGURE 9 A AND B

Activation and deactivation of a cell surface cAMP receptor is necessary for lowering the D2 mRNA levels. The expression pattern during suspension differentiation for XP55 strain developing with cAMP pulses and the addition of 5uM cAMP-S (A) or 5mM adenosine (B). The autoradiograms show that the D2 mRNA levels remained high under these conditions.


Hours
0 4 8 12
Hours
0 4 8 12
A
B
D29—
D2—

cell surface cAMP receptors continuously. Adenosine competitively inhibits binding of cAMP to cell-surface cAMP receptors (Theibert and Devreotes, 1984). Thus, the oscillation of cAMP receptors is necessary for the regulation of the D2 gene.

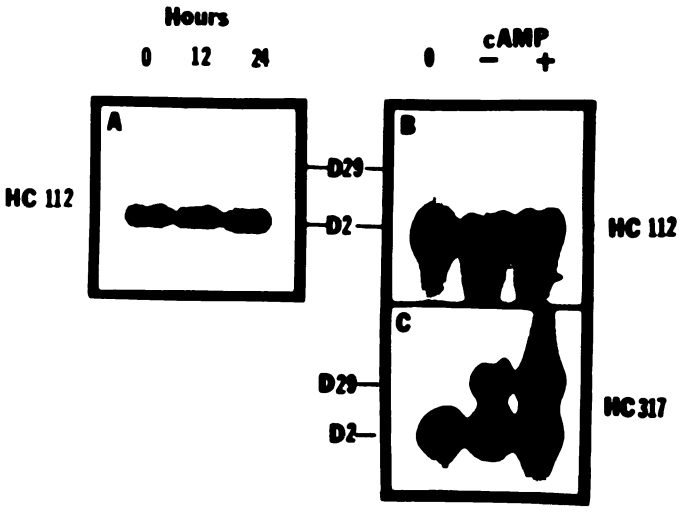
2. Signal transduction mutants show defective responses

We also sought genetic evidence to show that cAMP signals are transduced through the cell surface cAMP receptor. We used aggregation-deficient mutants termed " Frigid" strains (fdg A and fdg C). These mutants accumulate cAMP receptors but they do not respond chemotactically to cAMP (except the HC 317 mutant that has a weak response to cAMP) and they have no detectable activation of intracellular adenylate and guanylate cyclase (Coukell et al, 1983; Kesbeke et al., 1988). The HC112 mutant has a missense mutation in G alpha-2 (Pupillo et al., 1989; Kumagai et al., 1989). The fdg C HC317 mutant has low levels of cAMP receptors but shows some chemotactic response to cAMP. HC112 cells developed on agar and RNA was isolated at 12 hour intervals. The HC112 strain did not lower the D2 transcript levels (Fig. 10 A) but the HC317 cells did (data not shown). When these mutants were allowed to develop in suspension cultures for 4 hours and then pulsed with cAMP, both strains failed to lower D2 transcript levels, however, the D29 mRNA levels increased only in the HC 317 strain (Fig. 10 B and C). Thus, the cAMP

signal is transduced through the cAMP receptor and the G alpha-2 protein might be involved in the intracellular response.

FIGURE 10

Signal transduction mutants show a defective response to cAMP pulses. The expression pattern during development on agar for strain HC112 defective in a $G\alpha_2$ protein (A) and during suspension development for HC112 (B) and HC317 (C) in the presence (+) or absence (-) of cAMP pulses. The autoradiograms show that HC112 and HC317 cells do not lower the D2 transcript levels.


Hours
0 12 24
0 cAMP
− +
A
B
C
HC 112
D29
D2
HC 112
D29
D2
HC 317

IV. Levels of D2 gene regulation

The regulation of the D2 gene might reflect its importance as a putative cell proliferation dependent gene. The gene is regulated by a combination of transcriptional and post-transcriptional mechanisms as most of the cell cycle dependent genes (i.e. histones, thymidine kinase or c-fos, c-myc proto-oncogenes; Rittling and Baserga, 1987).

1. Transcriptional and post-transcriptional regulation of the D2 gene

We investigated if the effect of cAMP pulsing on the inactivation of D2 gene expression occurs by shutting off the transcription machinery or by shortening the half life of D2 transcripts. In vitro transcription reactions (Nellen et al., 1987; Landfear et al., 1982; Williams et al., 1980) were performed with nuclei isolated from vegetative cells and from cells developing in suspension for 3, 5, 6, 8 and 12 hours in the absence of cAMP pulses. Equal counts of labeled transcripts at each time point were hybridized to 1 ug of these target DNAs: the D2 gene insert, the D29 gene insert, and pcD-V1 plasmid as a control. The D2 gene was transcribed during the growth phase. However, the transcription declined during suspension development and almost ceased by 5 hours. In contrast, the D29 gene transcription was inactive during vegetative growth and then activated during development (Fig.

11). Since the previous results showed that high transcript levels of the D2 gene can be detected by northern blot analysis at 12 hours of suspension development (and up to 24 hours in another experiment), the D2 mRNA might have a very unusual long half life in the absence of cAMP pulses. Therefore, starvation alone might be the signal that inactivates D2, and activates D29, gene transcription while the loss of D2 transcripts when cAMP is administered is probably due to selective destablization of the D2 mRNA.

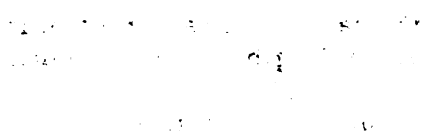
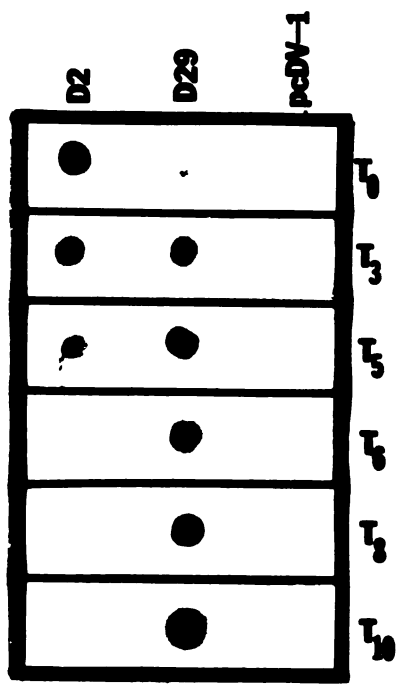


FIGURE 11

Starvation inactivates the D2 gene transcription, but activates the transcription of the D29 gene. In Vitro transcription reactions were conducted with nuclei from vegetative cells and from cells developing in suspension in the absence of cAMP pulses as described in the materials and methods section. The labelled transcripts from vegetative cells or from developing cells were hybridized to 1 ug of pcD-V1 plasmid control DNA or 1 ug of D2 and D29 inserts cDNA. The autoradiograms show that the D2 gene transcribed during the growth phase was inactivated during suspension development by about 5 hours. In contrast, the D29 gene was activated during development.


D2
D29
pcDV-1
T1
T3
T5
T6
T8
T10

DISCUSSION

The goal of this work was to uncover cell signals that regulate the loss of growth-specific transcripts during early development. Using starved amoebae, triggered to initiate the developmental cycle, we were able to investigate the effects of the cell cycle, starvation, cell-cell contacts and cAMP on the deactivation of D2 gene expression.

D2 seems to be an important gene because D2 mRNA levels correlate well with changes in cell proliferation in the life cycle. We used thermosensitive (tsg) growth mutations and found blockage of cell proliferation reduced D2 transcripts both in HM27 and XP95 mutants, however D29 transcripts were induced only in HM27. The transcript levels of the control pLK 326 gene remained unchanged under the same conditions. These results indicate that the loss of D2 transcripts in growing cells incubated at a restrictive temperature is specific and is not simply due to a heat shock response. The observation that HM27 and XP95 differ in their responses to the restrictive temperature suggests that they might be blocked in the same phase but have different defective genes or blocked at different phases in the cell cycle when the restrictive temperature was imposed. Similarly, a genetic analysis of tsg mutants in yeast, Saccharomyces cerevisiae,

indicates that they are blocked at various points in the cell cycle (Pringle and Hartwell, 1981).

Another agent that can block the cell cycle is caffeine. Caffeine exhibits a variety of modifying effects in mammalian cells; it delays or arrests cell division depending on the concentration being used. The cell cycle in mammalian cells is viewed as an interdependent sequence of biochemical events with the completion of DNA replication a prerequisite for the initiation of mitosis (Baserga, 1976). Nuclei undergo mitosis only after the S-phase nucleus has completed DNA replication. Addition of caffeine to BHK mammalian cells (Syrian hamster fibroblast), arrested at the S-phase, causes premature chromosome condensation, nuclear envelope breakdown, morphological "rounding up" and mitosis-specific phosphoprotein synthesis (Schlegel and Pardee, 1986, 1987). Caffeine (5 mM) could also induce multiple entries into mitosis while cells are blocked in DNA synthesis (Schlegel and Pardee, 1987). Inhibitors of protein synthesis (i.e 1 ug/ml cycloheximide) block these caffeine-induced events, while inhibitors of RNA synthesis (i.e. 2 ug/ml actinomycin D) have little effect. These results suggest that caffeine induces the translation or stabilizes the protein products of mitosis-related RNA that accumulates in S-phase cells when DNA replication is suppressed. Similar results were obtained from the studies on the cyclin gene, the auxiliary subunit

of DNA polymerase whose synthesis correlates with the proliferative state of the cells. The levels of cyclin protein fluctuate during the cell cycle, with a clear increase during the S phase (Bravo and Celis, 1980 & 1985).

Furthermore, caffeine might activate mitotic factors and uncouple them from the G1 phase and S-phase controls of the normal cell cycle. In D. discoideum, the drug affects cell proliferation in a dose dependent manner. Addition of 5mM caffeine to growing cells will completely block cell proliferation (Hagmann, 1986). The mechanism by which caffeine arrests cell proliferation is not known yet. Nevertheless, in our studies caffeine seems to be a useful drug to study the cell cycle in D. discoideum because it arrests cell division without affecting cell viability. Brenner found that when cells on a filter are perfused with caffeine, cAMP synthesis is inhibited. However, when caffeine is removed from the perfusate, normal cAMP synthesis is restored (Brenner and Thoms, 1984). We also found that the caffeine inhibition effect on cell growth is reversible. Xanthine is a caffeine precursor and was found to have a similar inhibition effect on growing cells. Both xanthine and caffeine could be converted to purine and the effects of xanthine or caffeine on growth in D. discoideum might occur because of purine rather than these drugs. In fact, our studies showed that the addition of 5 mM purine to growing

cells arrested cell proliferation. However, the inhibition effect was reversible once the cells were washed from purine and resuspended in a fresh medium (data not shown).

It is known that caffeine, as well as xanthine, inhibit cAMP phosphodiesterase in mammalian cells (Butcher and Sutherland, 1962), while in D. discoideum caffeine has no effect on phosphodiesterase (Brenner and Thoms, 1984). Instead, caffeine activates adenylate cyclase in vegetative amoebae and increases the basal cAMP levels but inhibits the cAMP mediated activation of adenylate cyclase during development (Brenner and Thoms, 1984; Devreotes., 1986; Hagmann, 1986). When developing cells are pulsed with cAMP in the presence of caffeine, the relay mechanism is not activated, and the cells see only the exogenous cAMP signal (Brenner and Thoms, 1984). There is also indirect evidence that caffeine might also increase cytosolic calcium levels in D. discoideum. Brenner found that caffeine has similar effects on cell shape changes as measured by light scattering and cAMP and cGMP accumulation, to the cation ionophore A23187 known to increase the intracellular Ca^{2+} levels (Brenner and Thoms, 1984). Hagmann showed that when growing cells were treated with caffeine then washed and developed in starvation buffer, their development was accelerated by 2 hours as judged by stream formation and the appearance of

contact sites A. Thus, he suggested that caffeine stimulates the expression of early developmentally regulated genes (Hagmann, 1986). We found that the D2 transcript levels decrease after caffeine addition but D29 transcript levels increase as if early developmental events were induced in spite of nutritional signals from the food source. Since caffeine increases the basal cAMP levels in vegetative amoebae, therefore, the intracellular cAMP or Ca^{2+} might be responsible for the inactivation of the D2, and the activation of the D29, genes. These possibilities will be discussed further later on in this chapter.

The important point is that the cell cycle control can bypass the nutritional control and induce developmental events during vegetative growth. We also found that D. discoideum strains have different responses to the caffeine addition. For example the AX-3 strain was less responsive to caffeine in lowering D2 transcripts than the XP55 strain. The different responses are probably attributed to the genetic differences from one strain to another or to the nutritional differences since the XP55 strain grows in the presence of bacteria, while the AX-3 strain grows axenically.

The G2 phase is the longest phase of the Dictyostelium cell cycle and almost all cells are in G2 during both growth

and development (Weijer, et al., 1984). Furthermore, starvation causes a decline in the rate of cell division to negligible levels and blocks the cells in the G2 phase (Katz and Bourgignon, 1974; Weijer et al., 1984). Therefore, the G2 phase might play an important role in the regulation of proliferation genes as well as developmental genes. Since no G2 blocking drugs are available, we blocked the cell cycle at the S phase by the addition of hydroxyurea or at mitosis with nocadazole (data not shown), but there was no effect on D2 transcript levels. These results suggest that the S or M phases do not play a role in the regulation of the D2 gene expression. However, hydroxyurea inhibits protein synthesis as well. Therefore, we cannot conclude that the S phase is not involved because the addition of cycloheximide also lowered D2 mRNA levels.

Dictyostelium cells have a very short G1 phase, if any, and most of the cell cycle consists of the G2 phase which is longer in axenically growing cells than in cells growing in the presence of bacterial food sources (Weijer et al., 1984). By this evidence and the fact that aggregating cells are blocked in the G2 phase some regulatory events might occur in the G2 phase which requires protein synthesis. Consequently addition of cycloheximide to vegetative cells might arrest some of the cells at the G2 phase. The possibility that the G2 phase plays a role in the regulation

of the D2 can be tested by arresting growing cells at the G2 phase and adding cAMP pulses which was found to inactivate the D2 gene during development. Cells could be synchronized in the stationary phase at which time they accumulate at a point late in the G2 phase of the cell cycle (Zada-Hames & Ashworth, 1978; Yarger et al., 1974). In the control experiment the exponentially growing cells will be pulsed with cAMP. This particular experiment was done before and it was found that cAMP pulses had no effect on the D2 transcript levels when applied to growing cells (data not shown). However, pulsing the stationary phase cells with cAMP might lower the D2 mRNA levels. Thus, our working model is that there are probably two important events to be completed in order to inactivate D2 gene expression: the first one is the cells must arrest in the cell cycle possibly in the G2 phase; the second one is an appropriate physiological signal that will act secondarily to lower the D2 transcript levels.

Starvation for amino acids is a necessary trigger for the developmental program (Marin, 1977). Here it was found that starvation is a necessary but not sufficient physiological signal to lower D2 transcript levels. The starving cells in suspension shaken culture did not inactivate D2 gene expression while developing cells at the same age on an agar plate did inactivate D2 gene expression. These results again reinforce the idea that starvation alone or blocking cells

in the G2 phase is not enough. Another signal must be present which is generated at the onset of development but disrupted in suspension culture. I found the main signal to be oscillating extracellular levels of cAMP. It is now of interest to see if nutritional controls acting to increase D2 mRNA levels can reverse the effect of cAMP to decrease D2 mRNA levels.

We found that addition of a 1 mg/ml mixture of essential and non-essential amino acids (added at the beginning of development) to cells pulsed with cAMP prevented the loss of D2 transcript levels. This concentration of amino acids prevents growing cells, when they are starved, from aggregation (data not shown). Moreover, the addition of amino acids to starved cells might stimulate cell proliferation again (Marin, 1977). Starvation is the stimulus that induces the formation of cAMP receptors, therefore, the addition of amino acids will probably prevent the appearance of cAMP receptors. Thus, it is not surprising that amino acids antagonize the cAMP effect. Nevertheless, if the amino acids were added by 4 hours of development when the cAMP pulsing started, probably the amino acids will not prevent the loss of D2 transcripts because by that time cAMP receptors already had formed.

Similarly, we might expect folic acid to play a role in

the regulation of transcript levels because vegetative amoebae detect folic acid given off by bacterial food sources. We found that 1mM folic acid added to cells developing in the presence of cAMP prevented the loss of transcript levels. Therefore, folic acid probably as a chemoattractant or as a nutrient, is a signal to continue the expression of vegetative genes. Furthermore, it is known that folic acid administered in pulses can induce cAMP oscillations in amoebae mimicking those induced by cAMP pulses (Wurster & Schubiger, 1977; Devreotes, 1983; Gerisch et al., 1975). The connection between the two chemotactic systems might occur by the membrane bound adenyl cyclase (Devreotes, 1983). It was found, however, that folic acid pulses had no effect on the loss of D2 transcript levels. Perhaps cAMP oscillations induced by folic acid pulses were not sufficient to trigger the intracellular signal responsible for the inactivation of the D2 gene expression or cAMP is not an intracellular signal.

Although some studies suggest that cell-cell contacts rather than cAMP can control gene expression, it was found that cell-cell contacts do not affect the D2 transcript levels and that cAMP, administered in pulses, is the primary signal that lowers the D2 transcript levels. As mentioned earlier in the introduction the preaggregative period of D. discoideum (~ 7 hours) is composed of two rate limiting

components in terms of their dependency on cell-cell contacts and de novo protein synthesis. The first component of the preaggregative period (the first 4.5 hours) is independent of cell-cell contacts as well as new protein synthesis, while the second one (2.5 hours) depends on cell-cell contacts and protein synthesis. Cells developing in suspension will progress completely through the first rate-limiting component, but will make no progress through the second one even with the addition of cAMP (Finney et al., 1988). These results might suggest that the actual effect of cell-cell contacts or the signal that might result from contacts is absent in suspension culture.

Although cell-cell contacts play an important role in developmental gene expression by stabilizing 2000-3000 mRNA species the induction of expression of prespore genes requires cAMP (Chung et al., 1981). Mehdy found that cAMP can stimulate isolated cells in suspension to express mRNAs preferentially accumulated in prestalk cells . Thus, it appears that cAMP is necessary and sufficient under the culture conditions used for the expression of these genes, while the establishment of sustained cell-cell contacts is not. However, the results on four different prespore genes indicate that sustained cell association is necessary for induction of expression of these genes (Mehdy, et al., 1983). Barklis' studies indicate that there is coordinate control

of prespore and prestalk gene expression. All prespore mRNAs they studied are comparable in all aspects of regulation. Prespore mRNAs begin to accumulate at 8-12 hours, during or just after the onset of aggregation. The level of prespore mRNAs is drastically reduced by disaggregation. Addition of cAMP partially or completely prevents the loss of these mRNAs in disaggregated cells. Prestalk clones can be subdivided into two classes on the basis of their temporal patterns of accumulation. The messages complementary to clones of class 1 are present at low levels in vegetative and early developing cells but accumulate to 10-20 fold higher levels during later stages of development. The mRNAs encoded by clones of class 2 accumulate only between 8 and 15 hours and decrease thereafter. Disaggregation causes a decrease in the abundance mRNAs in class 2 of at least 30 fold, whereas it stimulates the accumulation of mRNAs in class 1. Furthermore, in postaggregation cells, the half-life of developmentally regulated mRNA as well as the constitutive mRNAs present throughout growth and differentiation is about 4 hours. After disaggregation, the developmentally regulated mRNA sequences but not constitutive RNAs are degraded and decay with half lives of 25-45 minutes. Addition of cAMP to disaggregated cells restored levels of these mRNA (Mangiarotti et al., 1981a; Landfear and Lodish, 1980). These findings might suggest that cell-cell contact is nonessential for the maintenance of late gene expression in the late stage

aggregates. Instead, it appears that the levels of extracellular cAMP is the critical parameter at this stage of development.

Our results showed that cAMP pulses are a necessary signal for the inactivation of D2 gene expression. Finney and coworker's data might represent indirect evidence besides ours that there is no role of cell-cell contacts in the inactivation of our D2 gene. On the other hand, the D29 gene was expressed by about 4 hours in normal development and by 8 hours during suspension development either in slow or fast shaking culture. However, the D29 transcript levels were always higher in slow shaking cells than on fast shaking cells whether or not cAMP pulses were added. These results suggest that cell-cell contacts might play a role in the expression D29 expression. However, the induction of the D29 gene in a tsg mutant or by caffeine addition to growing cells is not in agreement because cell contacts do not form under these conditions. Perhaps the signal(s), responsible for the induction of D29 in vegetative cells as well as in developing cells is not cell-cell contact per se, but a intracellular signal(s) induced by contact. In addition, caffeine or the tsg mutant might activate this signal(s) bypassing the normal requirement for cell-cell contact. It is also possible that the tsg defect, as well as caffeine, causes the cell to act as though it is starving or is blocked in the cell cycle.

Development in suspension is a convenient system that allows for close monitoring of the effect of different physiological signals. However, development in suspension is not completed to form spore and stalk cells (Gerisch, 1968). In addition the cAMP signalling system might not work. Kimmel found that the endogenous cAMP signaling is inhibited in cells developing in suspension culture at a high shaking speed (200 rpm) and low cell density ($1X10^6$ cells/ml). However, when cAMP was added to 25 nM at 6 minutes intervals to cells developing under the same conditions, the cAMP signal-relay system was expressed normally. Furthermore, cells, developing in shaking culture at a low speed (90 rpm) and at a high density ($2X10^7$ cells/ml), were able to express the cAMP signal-relay system (Kimmel and Saxe, 1986). In our studies cells were developed in shaking culture at a fast (230 rpm) or a low speed (70 rpm) and at a high density ($2X10^7$ cells/ml). Although cells developing at a low speed and at a high density might express the cAMP-signal relay system, our results showed that it has no effect on the inactivation of the D2 gene.

Cyclic AMP is known to induce some differentiation specific transcripts and repress other transcripts when added at high levels (i.e. 1 mM) to cells shaken in suspension cultures (Barklis and Lodish, 1983; Bozzone and Berger,

1987; Chung et al., 1981), but D2 gene expression was unaffected. We also tried the poorly hydrolyzable analogue of cAMP (cAMP-S) to avoid the possibility that the 1mM cAMP dose was degraded too quickly by phosphodiesterases. Cyclic AMP-S is about 10,000 times more slowly hydrolyzed by cAMP phosophodiesterases but the affinity for the cAMP receptor is only reduced about 10 fold. Cyclic AMP-S added at the concentration of 5uM to cells developing in suspension was ineffective as well. Perhaps adding such high doses of cAMP and cAMP-S will saturate the system and inactivate the cAMP receptors by continuously occupying them. Higher concentrations of cAMP induce a decrease in the number of cAMP binding sites, a phenomenon termed down-regulation (Klein and Brachet 1979; Klein and Juliani, 1977; Sibley and Lefkowitz, 1985). Van Haastert found that a half-maximal loss of cAMP binding sites was induced by exposing cells for 15 minutes at 20^{0}C to 50 nM cAMP (Van Haastert, 1987). These results suggest that exposing the cells to 25 nM of cAMP is completely sufficient to occupy the cAMP receptors. The 5 uM cAMP-S dose added to the cells would be about 10 times higher than what is needed to occupy the receptors.

Several studies suggest that aggregation as well as the expression of some genes during early development require the oscillations of cAMP signals released by cells at 6 minutes

intervals. When mRNA was extracted from cells developing in suspension in the presence or absence of cAMP pulses translated in vitro and the polypeptides separated by two dimensional gel electrophoresis, of about 400 polypeptides visualised, 8 decreased and 15 increased (Hassanain and Kopachik, 1989). These results reflect a significant number of specific reductions and inductions in mRNA levels in cells pulsed with cAMP. When cAMP was added in 50 nM pulses to cells shaken in suspension, the D2 transcript levels decreased. The cells of KAX-3 required a higher concentration of cAMP pulses (200nM) to lower D2 mRNA levels and, interestingly, high concentrations (more that 50nM in XP55 or more that 200 nM in KAX-3) resulted in less repression which reinforces our idea that high cAMP levels result in the inactivation of cAMP receptors. Apparently the oscillation in cAMP levels is necessary for the loss of D2 transcripts in NC-4 derived strains. The different response to cAMP pulses between different strains might be due to a different number of cAMP receptors or different cAMP binding affinities. The reasons why the V12M2 strain is able to inactivate D2 expression in the absence of cAMP pulses or in the presence of high levels of cAMP are not known. It may be relevant that this strain is largely insensitive to the inhibition of aggregation by cAMP-S (Rossier et al., 1978). V12M2 might have uncoupled the inactivation of vegetative gene expression from the cAMP receptor oscillation or

perhaps the receptors are irreversibly modified and become responsive only to a high concentration of cAMP.

The affinity of the cAMP receptor to higher levels of cAMP can be explained by understanding that the cell surface cAMP receptor can interconvert between multiple affinity forms (receptors with different dissociation constant, K_d values) and that the different cellular events mediated through the receptor occur at different cAMP concentrations. While chemotaxis and cAMP relay are induced at a concentration as low as 0.1 nM in the aggregated mounds, the concentration is as high as 1-2uM. Such high concentrations will enable the cells to express the prespore mRNAs (Shaap and Spek, 1984; Schaap and Driel, 1985). Those mechanisms that activate late developmental changes will suppress some of the growth and early aggregation stage events. For example, the nanomolar cAMP pulses that activate chemotaxis and signal relay will also inactivate the expression of the growth gene, D2 (Hassanain and Kopachik, 1989). A similar conclusion was reached by Kimmel and Carlisle who showed that the M4-1 gene is repressed by low level oscillations of cAMP early in development (Kimmel and Carlisle, 1986).

In general, D. discoideum genes probably fall into four classes regarding their responses to cAMP: (i) some genes

respond to non-oscillating cAMP levels by increasing their mRNAs (i.e. the prespore gene, PL3); (ii) in the second class, genes respond to non-oscillating cAMP levels and decrease their mRNAs (i.e. the early developmental gene, discoidin-I) and growth genes, D3, C3 and C5 (Blumberg et al., 1988); (iii) in the third class, genes respond to oscillating levels of cAMP by increasing their mRNAs (i.e. the early developmental gene, K5); and (iv) in the fourth class which is rare, genes respond to oscillating levels of cAMP by lowering their mRNAs (i.e. the M4-1 gene as well as our D2 gene).

The studies were extended to find if the cell surface receptor and its two interconvertible forms are involved in this signal transduction. Cyclic AMP-S, capable of inhibiting aggregation in NC-4 derived strains and adenosine, a competitor of cell-surface cAMP binding were added, but the D2 transcript levels remained high. These reagents have a low turnover rate. They prevent cAMP from binding to its receptor and thus inhibit the cAMP mediated repression of the D2 gene. These results suggest that the cAMP receptor must oscillate in parallel with the oscillations of cAMP levels and that cAMP signals are transduced through cell surface cAMP receptors. Binding of cAMP to the receptor (R form) will induce the receptor phosphorylation and convert it to the phosphorylated D form. The phosphorylated form continues to

have cAMP bound which prevents another pulse of cAMP within a few minutes from triggering activation of adenylate cyclase and the subsequent release of newly synthesized cAMP. After several minutes the R form is reformed and another cAMP signal can be relayed. Thus, adding these reagents will prevent the oscillations of receptor between the two interconvertable forms which are necessary in order to trigger the intracellular signal(s) and inactivate the D2 gene expression (Juliani and Klein, 1981; Klein and Saxe, 1988).

Present data suggest two classes of cAMP receptors (see the model Figure 12), the signal relay receptor which might be associated with the activation of adenylate cyclase and the chemotaxis receptor that is probably linked to the enzyme phospholipase C (Newell et al., 1988; Firtel et al., 1989). The $G\alpha_2$ protein is probably coupled to the chemotaxis receptor and is encoded by the Frigid A gene (Kesbeke et al., 1988; Snaar-Jagalska et al., 1988; Kumagai et al., 1989). The observation that cAMP stimulates the production of 1,4,5 IP_3 in permeabilized wild type, but not in fgd A mutant cells, suggests that the chemotaxis receptor might be coupled to phospholipase C by the $G\alpha_2$ protein (Newell et al., 1988; Firtel et al.,1989; Janssens and Van Haastert., 1987; Snaar-Jagalska et al., 1988; Kumagai et al., 1989). Moreover, the $G\alpha_2$ protein might be indirectly coupled to adenylate cyclase

because neither the chemotaxis pathway nor the signal relay pathway can be activated in Frigid A mutants in vivo. Nevertheless, other studies show that the non-hydrolyzable GTPγS analogue can activate adenylate cyclase *in vitro* (in isolated membranes) in wild type strains as well as in fgd A mutants, which suggests that a G protein other than $G\alpha_2$ is linked to adenylate cyclase (Kesbeke *et al.*, 1988; Snaar-Jagalska, 1988). Therefore, the chemotaxis and the signal relay pathways are probably interconnected and the activation of the signal relay pathway is dependent on the activation of chemotaxis pathway.

The aggregation defective mutants provide strong evidence of the mode of action of cAMP pulses in D2 gene expression. The HC112 and HC317 cells accumulate cAMP receptors but do not efficiently induce the intracellular responses of adenylate cyclase and guanylate cyclase activation and they are unable to aggregate (Coukell *et al.*, 1983., Keskebe *et al.*, 1988). These strains can neither inactivate D2 transcripts during development on agar nor when pulsed with cAMP in shaken suspension. However, we found that the HC112 strain is capable of responding to cAMP pulses in part by studying the proteins synthesized in comparison to other strains that respond to cAMP pulses, using the two dimensional gel electrophoresis technique. Poly (A) mRNA

isolated from HC112 or KAX-3 cells developing on suspension in the presence of cAMP pulses was translated *in vitro*. The translated polypeptides were separated by two-dimensional gel electrophoresis and the flourographs compared. It was found that some of the polypeptides induced by cAMP pulses and visualized in the KAX-3 fluorographs were also visualized in the HC112 fluorographs (Hassanain and Kopachik, 1989). The results indicate that the HC112 strain can synthesize some of the proteins induced in other strains when cAMP pulses are applied. The hypothesis is that a new gene product(s) is needed in order to lower the D2 mRNA levels and that cAMP pulses, which might increase the intracellular Ca^{2+} levels and activate protein kinase C, will induce the expression of the gene(s) that results in destabilizing the D2 messagess (Figure 12).

All the mutants in the Frigid A complementation group affect receptor mediated processes and G α_2 protein levels (Lo *et al.*, 1978; Coukell *et al.*, 1983; Kumagai *et al.*, 1989). However, the actual defect in the intracellular response of the Frigid C mutant, HC317, is not yet known. Since the HC112 strain is in the Frigid A class and the stimulatory effect of cAMP on GTP δS binding to membranes is lost in this strain, it is believed to be defective in interaction between the chemotaxis receptor and a G α_2

protein. In conclusion, our results suggest that the chemotaxis receptor as well as the G α_2 protein are probably involved in the inactivation of the D2 gene as mentioned before (Figure 12).

Our studies went further in trying to find the intracellular signal that causes the inactivation of D2 gene expression. We used cAMP analogues that are capable of entering the cells, 8-bromo cAMP and 8-chlorophenylthio cAMP, to elevate the intracellular levels. These analogues were ineffective in lowering D2 transcript levels. Furthermore, the addition of folic acid in pulses to developing cells, known to induce intracellular cAMP oscillations, did not lower the D2 mRNA levels. Although intracellular cAMP appears to modulate expression of some genes regulated during early development possibly by activating a cAMP-dependent protein kinase, intracellular cAMP signaling might not be required to inactivate the expression of D2 gene. The inactivation of D2 gene expression can be functionally independent of adenylate cyclase activation and a consequent increase in intracellular cAMP levels. Blumberg's group found that different signal transduction systems are utilized for cAMP receptor mediated accumulation of prespore (PL3 and D2) and prestalk (D11) mRNA (class 1). They showed that cAMP can induce prespore and prestalk mRNAs accumulation even under

the conditions, where the receptor-associated adenylate cyclase is inactivated by conditions of high osmolarity or by placing the cells into a medium containing 5% glucose and 2% albumin (Darman and Klein, 1978; Oyama and Blumberg, 1986). Firtel and his group reached similar conclusions. They studied the expression of two genes expressed during aggregation; cAMP pulses induce the expression of one class 3 gene and repress another class 4 gene. They found that pulsing the developing cells with cAMP in the presence of caffeine to inhibit the activation of adenylate cyclase or pulsing the cells of Synag mutants, unable to activate adenylate cyclase, will repress and induce these genes as usual. Thus, they concluded that the full induction, of or full repression, of these genes requires cycles of activation and deactivation of the cAMP receptor but does not require a rise in the intracellular cAMP (Mann et al., 1988). However, since caffeine appears to have different effects on vegetative and developing cells (see results section and discussion pages 2,3 and 4), caffeine might not be a proper drug to inhibit the activation of adenylate cyclase. On the other hand, Firtel's results on the Synag mutants provide some evidence that the intracellular cAMP is not required for the regulation of these genes. Similary, the extracellular cAMP administered in pulses is a necessary signal to inactivate the D2 gene expression, but intracellular cAMP does not seem to be the second messenger involved in the

inactivation. Therefore, it must be another second messenger induced by the extracellular cAMP signal that inactivates the expression of the D2 gene. The binding of the extracellular cAMP to its receptor promotes the accumulation of other intracellular second messengers such as 1,4,5 inositol triphosphate (IP_3) and diacyglycerol (DAG) (Europe Finner and Newell, 1986, 1987). Production of IP3 permits mobilization of intracellular Ca2+, which is suggested to promote cGMP synthesis (Europe Finner and Newell, 1985). Furthermore, a rapid influx of Ca^{2+} occurs immediately following cAMP binding to the cell surface receptor, and an efflux of Ca^{2+} out of the cells is observed following removal of cAMP (Bumann et al., 1984, 1986; Europe-Finner & Newell, 1985).

In mammalian cells these second messengers can activate a family of protein kinases, including protein kinase C. These results suggest that Ca^{2+} is the possible second messenger that inactivates our gene. Kimmel showed that cAMP, in part, regulates the expression of the postaggregation gene, 2-H6 (class 1), by activating the 1,4,5 inositol triphosphate (IP_3) synthetic pathway (Kimmel and Eisen, 1988). Blumberg's group also believe that Ca^{2+} is involved in the signal transduction pathway and thus in the regulation of gene expression. In order to examine this possibility they tested the effects of four Ca^{2+} antagonists (TMB-8,

nifedipine, nitrendipine and W_7) on cAMP-mediated expression. Although TMB-8 is a relatively nonspecific Ca^{2+} antagonist, it was shown to block Ca^{2+} uptake and intracellular Ca^{2+} mobilization (Chiou and Malagodi, 1975; Brand and Felber, 1984; Europe-Finner and Newell, 1985). The nifedipine and nitrendipine compounds specifically bind to the proteins comprising Ca^{2+} channels and thereby block the influx of Ca^{2+} into cells (Murphy and Snyder, 1982). The W_7 compound is an anticalmodulin agent that binds to calmodulin and inhibits Ca^{2+}/calmodulin regulated enzyme activities (Kanamori, et al., 1981). However, one should be careful about interpreting these data because the effects of these drugs have not been attributed to Ca^{2+} in D. discoideum. Blumberg's group found all four antagonists inhibit the cAMP-dependent accumulation of prespore mRNAs (class 1) as well as the cAMP dependent repression of D3 (class 2), a growth-phase gene. However, the antagonists have only a small effect on the accumulation of cAMP-dependent prestalk mRNA (D11, class 1). Therefore, they suggest that a Ca^{2+} dependent signal transduction system possibly plays a role in cAMP-dependent accumulation of these prespore mRNAs as well as in the cAMP-mediated suppression of the group of growth phase mRNAs (Blumberg et al., 1988). Our results showing intracellular cAMP to not be involved in the inactivation of D2 gene expression could be explained if Ca^{2+} is the intracellular signal. Moreover, the effect of 5

mM caffeine on growing cells causing inactivation of D2, and activation of D29, gene expression, can now be explained if caffeine increases the intracellular $Ca2^{+}$ levels, therefore, we think the intracellular second messenger that inactivates D2 expression possibly is Ca^{2+}. Caffeine has a similar effect on other organisms, in mammalian cells the drug is known to raise cytosolic Ca^{2+} levels in the skeletal and heart muscle by releasing Ca^{2+} from the sarcoplasmic reticulum (Katz et al., 1977), and in the acellular slime mold Physarum polycephalum caffeine triggers release of Ca^{2+} from intracellular vesicles (Matthews, 1977). Since caffeine might increase Ca^{2+} as well as cAMP when added to growing amoebae, it could have multiple effects on the cells.

Some experiments can be done to support our speculations. The Ca^{2+} ionophore A23187, which increases the intracellular Ca^{2+} levels (Pfeiffer et al., 1978), can be added to cells developing in suspension to see if that will inactivate D2 expression. Furthermore, nifedipine and nitrendipine that block Ca^{2+} channels could be used as well. In conclusion, our model, states that, in order to inactivate D2 gene expression, the cells have to be arrested at the G2 phase which is initiated by starvation that induces development. The next step is to elevate the intracellular Ca^{+} level which is mediated by pulsing developing cells with cAMP or by

adding caffeine to growing cells.

Analysis of inactivation of the D2 gene suggests the repression of the D2 gene does not require the activation of adenylate cyclase and the accompanying rise in the intracellular cAMP levels. However, it might require a rise in the intracellular Ca^{2+} levels. Thus the inactivation of D2 gene expression might be mediated through the chemotaxis receptor and protein kinase C. In contrast, the pulse-repressed gene, M4-1 (class 4), appears to require a rise in the intracellular cAMP in order to be inactivated (Kimmel and Saxe, 1986; Kimmel, 1987). The M4-1 gene can be repressed in wild type cells by cAMP pulses. However, this gene can not be repressed by cAMP pulses in Synag mutants, which are unable to activate adenylate cyclase by cAMP (Theibert and Devreotes, 1986; Van Haastert et al., 1987; Kumagai et al., 1989). These results suggest that the repression of the M4-1 gene requires a rise in intracellular cAMP, which might be mediated through the signal relay receptor and cAMP dependent protein kinase.

Our in vitro run-off transcription experiments showed that the transcription of the D2 gene was inactivated by ~ 5 hours in cells shaken in suspension. The repression was probably initiated by starvation because it occurs with or without addition of cAMP in pulses. High levels of D2

transcripts, however, can be detected by northern blot analysis up to 24 hours in cells developing in suspension in the absence of cAMP. When cAMP is added in pulses to the cells in suspension, we observed a significant loss in the D2 mRNA levels. This observation raises the question of how the D2 messages are protected from degradation in the absence of pulses.

Amara and Lodish (1987) found that disaggregation induces the instability of the prespore mRNAs (class 1). Incubating disaggregated cells with the RNA synthesis inhibitors, daunomycin and actinomycin D, prevented the loss of the prespore mRNAs. Furthermore, the addition of cycloheximide prevented the loss of these mRNAs as well (Amara and Lodish, 1987). These results indicate that the destabilization of specific prespore mRNAs requires RNA and protein synthesis. In contrast, Mangiarotti and his group reported that destabilization of sensitive prespore mRNAs is induced by cell dissociation even in the presence of concentrations of nogalamycin that inhibit RNA synthesis (Mangiarotti, et al., 1989). The nogalamycin results indicate that RNA synthesis is not required to trigger the degradation event. The disagreement between Lodish and Mangiarotti's conclusions probably is due to a secondary effect of actinomycin D and daunomycin other than the inhibition of transcription. On the other hand, there is a remote possibility that nogalamycin

failed to inhibit the synthesis of a minor species of RNA essential for the induction of mRNA decay, which is inhibited by actinomycin D and daunomycin. In recent studies, Manrow and Jacobson (1988) used a ^{32}P pulse-chase protocol to analyze the rate of decay of individual mRNA species in aggregated cells and in cells disaggregated in the presence and absence of cAMP. They found that in aggregated cells, the amount of ^{32}P label decreased very slowly in some of the mRNAs studied while it remained constant in other mRNAs. In cells disaggregated without cAMP, the cell-type-specific mRNAs decayed quickly with half-lives of 20-30 minutes but with cAMP the half-lives were about 4 hours. We found that the addition of nogalamycin (300 μg/ml)to cells developing in suspension culture in the presence of cAMP pulses, prevents the loss of D2 mRNA (data not shown). These results indicate that new gene product(s) is needed to specifically lower the D2 transcript levels. Cyclic AMP pulses that might increase the intracellular Ca^{2+} and activate protein kinase C could induce the production of a specific RNAse that degrades the D2 transcripts (specific degradation). On the other hand, blockage of protein synthesis with cycloheximide might exclude the D2 as well as D29 mRNAs from the polysomes and expose them for degradation with non-specific RNAse (non specific degradation; Figure 12).

The results with cycloheximide showed that the drug lowered the D2 mRNA levels with or without cAMP pulses. The results suggest that, unlike other genes, the loss of D2 transcript levels by cAMP does not require protein synthesis. There are probably two major mechanisms for the deactivation of gene expression during early development; one dependent upon and one independent of protein synthesis. Therefore, our gene seems to fall in the second class (Singleton et al., 1988). In addition, the results also indicate that the D2 gene is under the translational control; the D2 mRNA might be protected from degradation by being in the polysomes and as soon as the message is translated or the protein synthesis is inhibited by cycloheximide the D2 mRNA will be excluded from the polysomes and degraded with non-specific RNAse (Figure 12). Thus our results indicate that protein synthesis is not required to non-specifically destabilize the D2 mRNA but it is probably necessary to specifically destabilize D2 mRNA by cAMP pulses but RNA synthesis is required. Dictyostelium probably has mechanisms in place for shutting down the expression of certain genes while maintaining the expression of others when development is initiated. Genes repressed early in development need RNA synthesis to decay their mRNAs while the decay of late gene mRNAs do not require RNA synthesis. Furthermore, extracellular cAMP may play a double role, it stabilizes late developmental mRNAs but destabilizes vegetative as well as early developmental mRNAs.

A question that remains to be answered is how cAMP pulses trigger the degradation of D2 transcripts. As mentioned in the introduction, there is a 5' cap sequence that seems to be important for mRNA stability in D. discoideum (Shapiro et al., 1988; Casey et al., 1983; Chung et al., 1981). Cyclic AMP which is the primary signal might stimulate nucleases to remove the 3' or 5' terminal sequences and may lead to degradation of the RNA molecules at a rapid rate. Moreover, these messages might be protected by polysomes and the destabilization occurs by the exclusion of these messages from polysomes. In this case, the primary control would be at the level of mRNA translation rather than mRNA stability per se (non specific action of cycloheximide). Similarly, the degradation of histone mRNA has been shown to depend on the translatability of histone mRNA itself (Sive, et al., 1984), and the destabilization of tubulin mRNA depends upon the recognition of the first amino acids of the alpha-tubulin as they emerge from the ribosome (Yen et al., 1988). Steel and Jacobson (1988) reached similar conclusion regarding ribosomal protein mRNAs. The expression of these genes was drastically and specifically reduced by a block to translational initiation at the start of development. While the r-protein mRNAs were almost fully loaded on polysomes during vegetative growth, they were specifically excluded from polysomes early in development.

To investigate if there are 5'or 3' specific sequences targeting the degradation of D2 mRNA after the addition of cAMP pulses, the D2 genomic DNA could be cloned into a Dictyostelium transformation vector and different sequences can be deleted or replaced at the 5' or 3' non-coding regions of the D2 gene in order to define putative regulatory sequences. The stability of the altered D2 mRNAs could be studied in the vegetative amoebae as well as in developing cells in the presence or absence of cAMP pulses.

In summary, as shown in the model (Fig. 12) the D2 gene is regulated at transcriptional and translational levels. Starvation alone is a sufficient signal to inactivate the D2 gene transcription. Starvation for amino acids and cAMP pulses are the primary signals that destabilize D2 mRNAs. The loss of the D2 mRNA levels mediated through the chemotaxis receptor and the $G\alpha_2$ protein does not require the activation of adenylate cyclase and the accompanying rise in the intracellular cAMP
levels. Instead it might require a rise in the intracellular Ca^{2+} levels.

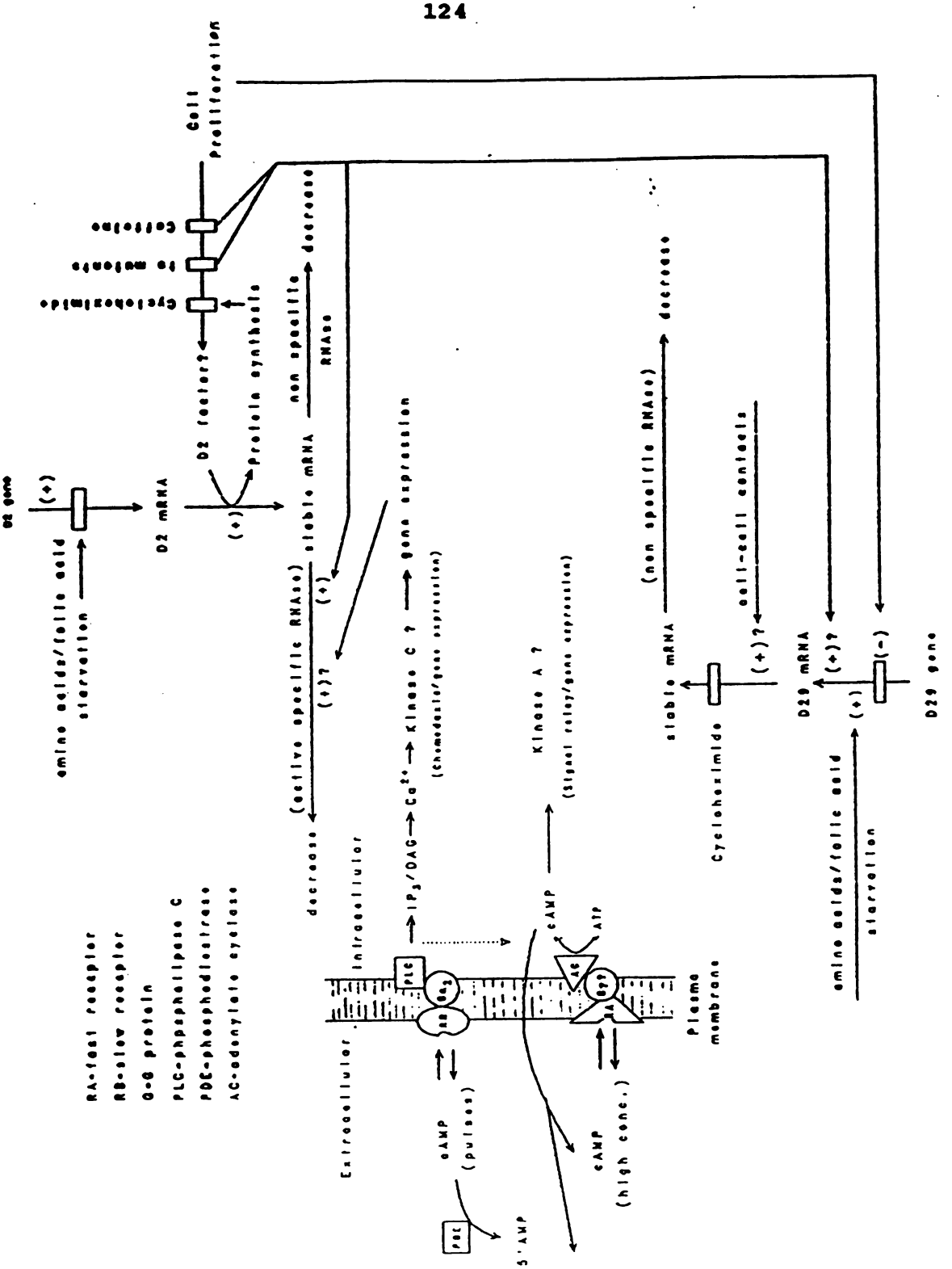


FIGURE 12

A model for the regulation of D2 gene expression.

REFERENCES

Adesnik, M. and Darnell, J. E. (1972). Biogenesis and characterization of histone messenger RNA in Hela cells. J. Mol. Biol. 67, 397-406.

Alton, T. H. and Lodish, H. F. (1977). Developmental changes in messenger RNA and protein synthesis in Dictyostelium discoideum. Dev. Biol. 60, 180-206.

Amara, J. F. and Lodish, H. F. (1987). Specific mRNA destabilization in Dictyostelium discoideum requires RNA synthesis. Mol. Cell. Biol. 7(12), 4585-4588.

Ashworth, J. M., and Watts, D. J. (1970). Metabolism of the cellular slime mold Dictyostelium discoideum in the axenic culture. Biochem. J. 119, 175-182.

Barklis, E. and Lodish, H. F. (1983). Regulation of Dictyostelium discoideum mRNAs specific for prespore or prestalk cells. Cell 32, 1139-1148.

Baserga, R. (1976). Multiplication and division in mammalian cells (Dekker, New York), pp. 39-40.

Benoist, C., and Chambon, P. (1981). In vivo sequence requirements of the early promoter region. Nature 290, 304-310.

Benoist, C., O'Hare, K., Breathnach, R. and Chambon, P. (1980). The ovalbumin gene sequence of putative control region. Nucl. Acid Res. 8, 127-142.

Berks, M. and Kay R. R. (1988). Cyclic AMP is an inhibitor of stalk cell differentiation in Dictyostelium discoideum. Dev. Biol. 126, 108-114.

Berridge, M. J. (1984). Inositol triphosphate and diacylglycerol as second messengers. Biochem. J. 220, 345-360.

Blumberg, D. D., and Lodish, H. F. (1980). Complexity of nuclear and polysomal RNAs in growing Dictyostelium discoideum cells. Dev. Biol. 78, 268-284.

Blumberg, D. D., Comer, J. F. and Higinbotham, K. G. (1988). A Ca^{2+}-dependent signal transduction system participates in coupling expression of some cAMP-dependent prespore genes to

the cell surface receptor. Dev. Genet. 9, 359-369.
Bonner, J. T. (1967). The cellular slime molds. 2nd ed. Princeton university Press, Princeton, N. J.

Bozzone, D. M. and Berger, E. A. (1987). Distinct developmental regulation and properties of the responsiveness of different genes to cyclic AMP in Dictyostelium discoideum. Differentiation 33, 197-206.

Brand, M. D. and Felber, S. M. (1984). The intracellular calcium antagonist TMB-8 inhibits mitochondrial ATP production in rat thymocytes. Biochem. J. 224, 1027-1030.

Bravo, R. and Celis, J. E. (1980). A search for differential polypeptide synthesis throughout the cell cycle of HeLa cell. J. Cell Biol. 84, 795-802.

Bravo, R. and Celis, J. E. (1985). Changes in the nuclear distribution of cyclin (PCNA) during S-phase are not triggered by post-translational modifications that are expected to moderately affect its change. FEBS Lett. 182, 435-440.

Brawermann, G. (1987). Determinants of messenger RNA stability Cell 48, 5-6.

Breathnach, R. and Chambon, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. Ann. Rev. Biochem. 50, 349-384.

Brenner, M. and Thoms, S. D. (1984) Caffeine blocks activation of cAMP synthesis in Dictyostelium discoideum. Dev. Biol. 101, 136-146.

Bumann, J. Wurster, B. and Malchow, D. (1984). Attractant-induced changes and oscillations of the extracellular Ca^{2+} concentration in suspension of differentiating Dictyostelium cells. J. Cell Biol. 98, 173-178.

Butcher, R. W. and Southerland, E. W. (1962). Adenosine 3',5'-phosphate in biological materials. J. Biol. Chem. 237, 1244-1250.

Cardelli, J. A., Knecht, D. A., Wunderlich, R. and Dimond, R. L. (1985). Major changes in gene expression occur during at least four stages of development of Dictyostelium discoideum. Dev. Biol. 110, 147-156.

Casey, J. L., Hentze, M.W., Koeller, D. M., Caughman, S. W., Rouault, T. A., Klausner, R. D., and Harford, J. B. (1988). Iron-responsive elements: regulatory RNA sequences that

control mRNA levels and translation. Science 240, 924-928.

Casey, L., Palatnik, C. M., Jacobson, A. (1983). Messenger RNA half-life in Dictyostelium discoideum. Dev. Biol. 95, 239-243.

Chiou, C. Y. and Malagodi, H. M. (1975). Studies on the mechanism of action of a new Ca^{2+} antagonist, TMB-8, in smooth and skeletal muscles. Br. J. Pharmacol. 53, 279-285.

Chirgwin , J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-9299.

Chisolm, R. L., Barklis, E. and Lodish, H. F. (1984). Mechanism of sequential induction of cell-type specific mRNAs in Dictyostelium discoideum. Nature 310, 67-69.

Chisholm, R.L., Hopkinson, S. and Lodish, H. F. (1987). Superinduction of the Dictyostelium discoideum cell surface cAMP receptor by pulses of cAMP. Proc Natl. Acad. Sci. U.S.A. 84, 1030-1034.

Chung, S., Landfear, S. M., Blumberg, D. D. cohen, N. S. and Lodish, H. F. (1981). Synthesis and stability of developmentally regulated Dictyostelium discoideum mRNAs are affected by cell-cell and cAMP. Cell. 27, 785-797.

Clarke, M., Yang, J. and Kayman, S. C. (1988). Analysis of the prestarvation response in growing cells of Dictyostelium discoideum. Dev. Gen. 9, 315-326.

Clarke, M., Kayman, S. C., Riley, K. (1987). Density-dependent induction of discoidin-I synthesis in exponentially growing cells of Dictyostelium discoideum. Diff. 34, 79-87.

Coukell, M. B., Lappano, S., and Cameron, A. M. (1983). Isolation and characterization of cAMP unresponsive (Frigid) aggregation deficient mutants of Dictyostelium discoideum. Dev.
Genet. 283-297.

Darman, M. and Klein, C (1978). Effects of amino acids and glucose on adenylate cyclase and cell differentiation of Dictyostelium discoideum. Dev. Biol. 63, 377-389.

Darnell, J. E., Jelinek, W. R. and Molloy, G. R. (1973). Biogenesis of mRNA: Genetic regulation in mammalian cells. Science 181, 1215-1221.

Deshpande, K. A., Chatterjee, B., and Roy, A. K. (1979). Translation and stability of rat liver messenger RNA for alpha$_2$ u-globulin in Xenopus oocytes. The role of terminal poly (A). J. Biol. Chem. 245, 8937-8942.

Devreotes, P. N. (1983). The effect of folic acid on cAMP-elicited cAMP production in Dictyostelium discoideum. Dev. Biol. 95, 154-162.

Devreotes, P. N., Fontana, D., Klein, P., Sherring, J. and Theibert, A. (1987): Transmembrane signalling in the Dictyostelium. in Spudich, J. (ed): "Methods in Cell Biology." Vol. 28. New York: Academic Press, pp 299-329

Devreotes, P. N., Steck, T. L. (1979): cAMP relay in Dictyostelium discoideum. II. Requirements for the initiation and termination of the response. J. Cell Biol. 80, 300-309.

De Wit, R., Arents, J. and Driel, R. (1982). Ligand binding properties of the cytoplasmic cAMP-binding protein of Dictyostelium discoideum. FEBS Lett. 145, 150-154.

De Wit, R. J. W., and Bulgakov, R. (1986a). Folate chemotactic receptors in Dictyostelium discoideum. I. ligand-induced conversion between four receptor states. Biochm. Biophys. Acta 886, 76-87.

De wit, R. J. W., and Bulgakov, R. (1986b). Folate chemotactic receptors in Dictyostelium discoideum. II. Guanine nucleotides alter the rates of interconversion and the proportioning of four receptor states. Biochim. Biophys. Acta 886, 88-95.

De Wit, R. J. W., and Bulgakov, R. (1986c). 2-Deamino folic acid elicits desensitization without excitation of the cyclic GMP response in Dictyostelium discoideum. Biochim. Biophys. Acta 887, 242-247.

De Wit, R. J. W., Bulgakov, R., Rinke De Wit, T. F., Konijn (1986d). Developmental regulation of the pathways of folate-receptor-mediated stimulation of cAMP and cGMP synthesis in Dictyostelium discoideum. Differentiation 32, 192-199.

Drummond, D. R., Armonstrong, J., and Colman, A. (1985). The effect of capping and polyadenylation on the stability, movement, and translation of synthetic messenger RNAs in Xenopus oocytes. Nucleic Acids Res. 13, 7375-7394.

Europe-Finner, G. N. and Newell, P. C. (1985). Inositol 1,4,5-triphosphate induces cyclic AMP formation in Dictyostelium discoideum. Biochem. Biophys. Res. Commun. 130,

115.

Europe-Finner, G. N. and Newell, P. C. (1986). Inositol 1,4,5, triphosphate induces calcium release from a non-mitochondrial pool in amoebae of Dictyostelium. Biochem. Biophys. Acta 887, 335-340.

Europe-Finner, G. N. and Newell, P. C. (1987). Cyclic AMP stimulates accumulation of inositol triphosphate in Dictyostelium. J. Cell Sci. 87, 221-229.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling endonuclease fragments to high specific activity. Analytical Biochemistry 132, 6-13.

Finney, R. E., Langtimm, C. J. and Soll, D. R. (1985). Regulation of protein synthesis during the preaggregative period of Dictyostelium discoideum development: involvement of close cell associations and cAMP. Dev. Biol. 110: 171-191.

Firtel, R. A. (1972). Changes in the expression of single copy DNA during development of cellular slime mold Dictyostelium discoideum. J. Mol. Biol. 66, 363-377.

Firtel, R. A., and Bonner, J. T. (1972a). Characterization of the genome of the cellular slime mold Dictyostelium discoideum. J. Mol. Biol. 66, 339-361.

Firtel, R. A., and Bonner, J. T. (1972b). Developmental control of alpha 1-4 glucan phosphorylase in the cellular slime mold Dictyostelium discoideum Develop. Biol. 29, 85-103.

Firtel, R. A., Jacobson, A. and Lodish, H. F. (1972). Isolation and hybridization kinetics messenger RNA from Dictyostelium discoideum. Nature New Biol. 239, 255-228.

Firtel, R. A., Cockburn, A., Frankel, G. and Hershfield, v. (1976a). structural organization of the genome of Dictyostelium discoideum: analysis of EcoRI restriction endonuclease. J. Mol. Biol. 102, 831-852.

Firtel, R. A., Kindle, K. and Huxley, M. P. (1976 b). Structural organization and processing of the genetic transcript in the cellular slime mold Dictyostelium discoideum. Fed. Proc. 35, 13-22.

Firtel, R. A., Van Haastert, P. J. M., Kimmel, A. R. and Devreotes, P. N. (1989). G protein linked signal transduction pathways in development: Dictyostelium as an experimental system. Cell 58, 235-239.

Furuichi, Y., LaFinandra, F., and Shatkin, A. J. (1977). 5' terminal structure and mRNA stability. Nature (london) 266, 235-239.

Gay, D. A., Yen, T. J., Lau, J. T. Y., and Cleveland, D. W. (1987). sequences that confer beta-tubulin autoregulation through modulated mRNA stability reside within exon 1 of a beta-tubulin mRNA. Cell 50, 671-679.

Geltosky, J. E., Siu, C. H., and lerner, R. A. (1976). Glycoproteins of the plasma membrane of Dictyostelium discoideum during development. Cell 8, 391-396.

Gerisch, G. (1968). Cell aggregation and differentiation in Dictyostelium. Current Topics in Dev. Biol. (Academic Press, N.Y.). 3, 159-195.

Gerisch, G. (1987). Cyclic AMP and other signals controlling cell development and differentiation in Dictyostelium. Ann. Rev. Biochem. 56, 853-879.

Gerisch, G., Fromm, H., Huesgen, A., and Wick, U. (1975). Control of cell contact sites by cyclic AMP pulses in differentiating Dictyostelium cells. Nature (London) 255, 547-549.

Graves, R. A., Pandey, N. B., Chodchoy, N. and Marzluff, W. F. (1987). Translation is required for regulation of histone mRNA degradation. Cell 48, 615-626.

Green, M. R., Maniatis, T. and Melton, D. A. (1983). Human beta-globin pre-mRNA synthesized in vitro is accurately spliced in Xenopus oocyte nuclei. Cell 32, 681-694.

Hagmann, J. (1986). Caffeine and heat shock induce adenylate cyclase in Dictyostelium discoideum. The EMBO J. 5(13), 3437-3440.

Haribabu, B. and Dottin, R. P. (1986). Pharmacological characterization of cAMP receptors mediating gene regulation in Dictyostelium discoideum. Mol. Cell Biol. 6, 2402-2408.

Hassanain, H. H. and Kopachik, W. (1989). Regulatory signals affecting a selective loss of mRNA in Dictyostelium discoideum. J. Cell Sci. 94, 501-509.

Henderson, E. J. (1975). The Cyclic adenosine 3:5'monophosphate receptor of Dictyostelium discoideum. Binding characteristics of aggregation competent cells ana variation of binding levels during the life cycle. J. Biol. Chem. 250, 4730-4636.

Huez, G., Cleuter, Y., Bruck, C., Van Vloten-Doting, L., Goldbach, R., and Verduin, B. (1983). Translation stability of plant viral RNAs microinjected into living cells: influence of a 3' Poly (A) segment. Eur. J. Biochem. 130, 205-209.

Huez, G., Marbaix, G., Hubert, G., Leclercq, M., Nudel, U., Soreq, H., Salomon, R., Lebleu, B., Revel, M., and Littauer, Z. (1974). Role of the polyadenylate segment in the translation of globin messenger RNA in Xenopus oocytes. Proc. Natl. Acad. Sci. U.S.A. 71, 3143-3146.

Jacobson, A. (1976). Analysis of mRNA transcription in Dictyostelium discoideum, or slime mold messenger RNA: how to find it and what to do with it once you've got it. Meth. molec. Biol. 8, 161-209.

Jacquet, M., Part, D. and Felenbok, B. (1981). Changes in the polyadenylated messenger RNA population during development of Dictyostelium discoideum Dev. Biol. 81, 155-166.

Janssens, P. M. and Van Hasstert, P. J. M. (1987). Molecular basis of transmembrane signal transduction in Dictyostelium discoideum. Microbiol Rev. 51, 396-418.

Juliani M. H., Klein, C. (1981). Photoaffinity labeling of the cell surface adenosine 3': 5'-monophosphate receptor of Dictyostelium discoideum and its modification in down-regulated cells. J. Biol. Chem. 256, 613-619.

Kabnick, K. S. and Housman, D. E. (1988). Determinants that contribute to cytoplasmic stability of human c-fos and ß-globin mRNAs are located at several sites in each mRNA. Mol. Cell. Biol. 8, 3244-3250.

Kanamori, M., Naka, M., Asano, M. and Hidaka, H. (1981). Effects of N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide and other calmodulin antagonists (calmodulin interacting agents) on calcium-induced contraction of rabbit aortic strips. J. Pharmacol. Exp. Ther. 217, 494-499.

Katz, E. R. and Bourguignon, L. Y. W. (1974). The cell cycle and its relationship to aggregation in the cellular slime mold,
Dictyostelium discoideum. Dev. Biol. 36, 82-87.

Katz, E. R., Repke, D. I. and Hasselbach, W. (1977). Dependence of ionophore- and caffeine-induced calcium relase from sarcoplasmic reticulum vesicles on external and internal calcium ion concentrations. J. Biol. Chem. 252, 1938-1949.

Kay, R. R. (1989). Evidence that elevated intracellular cAMP

triggers spore maturation in Dictyostelium. Development 105 (4), 753-759.

Kay, R. R. and Trevan, D. (1981). Dictyostelium amoebae can differentiate into spores without cell contact. J. Embryol. Exp. Morphol. 62, 369-378.

Kesbeke, F., Snaar-Jagalska, B. E., and Van Haastert, P. J. M. (1988). Signal transduction in Dictyostelium discoideum fgd A mutants with a defective interaction between surface cAMP receptor and a GTP-binding regulatory protein. J. Cell Biol. 107, 521-528.

Kimmel, A. (1987). Different molecular mechanisms for cAMP regulation of gene expression during Dictyostelium development. Dev. Biol. 122, 163-171.

Kimmel, A. R. and Carlisle, B. (1986). A gene expressed in undifferentiated vegetative Dictyostelium is repressed by developmental pulses of cAMP and reinduced during differentiation. Proc. Natl. Acad. Sci. U.S.A. 83, 2506-2510.

Kimmel, A. R. and Eisen, M. (1988). Regulation of gene expression by the intracellular second messengers IP_3 and diacylglycerol. Dev. Genet. 9, 351-358.

Kimmel, A. R. and Firtel, R. A. (1982). The organization and expression of the Dictyostelium genome. In Loomis WF (ed). "The Development of Dictyostelium discoideum." New York. Academic Press, pp233-324.

Kimmel, A.R. and Firtel, R. A. (1983). Sequence organisation in Dictyostelium: unique structure at the 5'-end of coding genes. Nucleic Acids Res. 11, 541-552.

Kimmel, A. R. and Saxe, C. L. III (1986). Cyclic AMP-controlled gene expression in Dictyostelium mutants exhibiting abnormal patterns of developmental cAMP metabolism. Dev. Biol.117, 209-214.

Klein, C. P., Brachet, P. and Darmon, M. (1977). Periodic changes in adenylate cyclase and cAMP receptors in Dictyostelium discoideum. FEBS Lett. 76, 145-147.

Klein, C. P. and Juliani, M. H. (1977). Cyclic AMP-induced changes in cAMP-binding sites on Dictyostelium amoebae. Cell 10, 329-335.

Klein, P., Sun, T. J., Saxe, C. L., Kimmel, A. R., and Devreotes, P. N. (1988). A chemoattractant receptor controls development in Dictyostelium discoideum. Science 241, 1467-

1472.

Kopachik, w., Bergen, L. G., and Barclay, S. L. (1985). Genes selectively expressed in proliferating Dictyostelium amoebae. Proc. Natl. Acad. Sci. U.S.A. 85, 8540-8544.

Kopachik, W., Oohata, A., Dhokia, B., Brookman J. J., and Kay, R. R. (1983). Dictyostelium discoideum mutants lacking DIF, a putative morphogen. Cell 33, 3947-403.

Kreig, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. 12, 7057-7070.

Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N., and Firtel, R. A. (1989). Regulation and function of G protein subunits in Dictyostelium. Cell 57, 265-275.

Kwong, L., Sobolewski, A. and Weeks, G. (1988). The effect of cAMP on differentiation inducing factor (DIF)-mediated formation of stalk cells in low-cell-density monolayers of Dictyostelium discoideum. Differentiation 37, 1-6.

Landfear, S. M., Lefebvre, P., Chung, S. and Lodish, H. (1982). Transcriptional control of gene expression during development of Dictyostelium discoideum. Mol. and Cell. Biol. 2(11), 14171426.

Landfear, S. M. and Lodish, H. F. (1980). A role of cyclic AMP in the expression of developmentally regulated genes in Dictyostelium discoideum. Proc. Natl. Acad. Sci. U.S.A. 77, 1044-1048.

Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker (1977). RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry 16, 4743.

Levine, B. J., Chodchoy, N., Marzluff, W. F., and Skoultchi, A. I. (1987). Coupling of replication type histone mRNA levels to DNA synthesis requires the stem-loop sequence at the 3' end of the mRNA. Proc. Natl. Acad. Sci. U.S.A. 84, 6189-6193.

Lo, E. K. L., Coukell, M. B., Tsang, A. S. and Pickering, J. L. (1978). Physiological and biochemical characterization of aggregation-deficient mutants of Dictyostelium discoideum: detection and response to exogenous cAMP. Can. J. Microbiol. 24, 455-465.

Loomis, W. F., Jr.(1971). Sensitivity of Dictyostelium

discoideum to nucleic acid analogues. Exp. Cell Res. 64, 484-486.

Loomis, W. F. (1975). In Dictyostelium diocoideum: a developmental system. Academic Press, New York.

Luscher, B., Stauber, C., Schindler, R., and Schumperli, D. (1985). Faithfull cell-cycle regulation of a recombinant mouse histone H^4 gene is controlled by sequences in the 3' terminal part of the gene. Proc. Natl. Acad. Sci. U.S.A. 82, 4389-4393.

Mangiarotti, G., Altruda, F. and Lodish, H. F. (1981a): Rates of synthesis and degradation of rRNA during differentiation of Dictyostelium discoideum. Mol. Cell. Biol. 1, 35-42.

Mangiarotti, G., Bulfone, S., Giorda, R., Morandini, P. and Ceccarelli, A. (1989). Analysis of specific mRNA destabilization during Dictyostelium discoideum development. Development 106, 473-481.

Mangiarotti, G., Ceccarelli, A. and Lodish, H. F. (1983). Cyclic AMP stabilizes a class of developmentally regulated Dictyostelium discoideum mRNAs. Nature 301, 616-618.

Mangiarotti, G., Chung, S., Zuker,, C., and Lodish, H. F. (1981b). Selection and analysis of cloned developmentally-regulated Dictyostelium discoideum genes by hybridization-competition. Nucl. Acids Res. 9, 947-963.

Mangiorotti, G., Lefebvre, P. and Lodish, H. F. (1982). Differences in stability of developmentally regulated mRNA in aggregated and disaggregated Dictyostelium discoideum cells. Dev. Biol. 89, 82-91.

Mann, S. K. O., Pinko, C. and Firtel, R. A. (1988). Control of early gene expression in Dictyostelium. Dev. Genet. 9, 337-350.

Manrow, R. E., Jacobson, A. (1988). Messenger RNA decay rates in late developing Dictyostelium cells are heterogeneous and cyclic AMP does not act directly to stabilize cell-type specific mRNAs. Mol. Cell. Biol. 8, 4088-4097.

Manrow, R. E., Shapiro, R. A., Herrick, D., Steel, L. F., Blinder, D. and Jacobson A. (1988). Regulation of mRNA stability and the poly(A) and the poly(A) problems in Dictyostelium discoideum. Dev. Genet. 9, 403-419.

Marbaix, G., Huez, G., Burny, A., Cleuter, Y., Hubert, E., Leclercq, M., Chantrenne, H., Soreq, H., Nudel, U., and

Littauer, E. (1975). Absence of polyadenylate segment in globin messenger RNA accelerates its degradation in Xenopus oocytes. Proc. Natl. Acad. Sci. U.S.A. 72, 3065-3067.

Margolskee, J. P., Lodish, H. F.(1980). Half-lives of messenger RNA species during growth and differentiation of Dictyostelium discoideum. Dev. Biol. 74, 37-49.

Marin, F. (1976). Regulation of development in Dictyostelium discoideum: I. Initiation of the growth to development transition by amino acid starvation. Dev. Biol. 48, 110-117.

Marin, F. (1977). Regulation og development in Dictyostelium discoideum: II. Regulation of early cell differentiation by amino acid starvation and intercellular interaction. Dev. Biol. 60, 389-395.

Marin, F. T., and Rothman (1980). Regulation of development in Dictyostelium discoideum. IV. Effects of ions on the rate of differentiation and cellular response to cAMP. J. Cell Biol. 87, 823-827.

Matthews, L. M. (1977). Ca^{2+} regulation in caffeine-derived microplasmodia of Physarum polycephalum. J. Cell Biol. 72, 502-505.

McCrae, M. A., and Woodland, H. R. (1981). Stability of non-polyadenylated viral mRNAs injected into frog oocytes. Eur. J. Biochem. 116, 467-470.

Mcknight, S. L. and Kingsbury, R. (1982). Transcriptional control signals of a eucaryotic protein-coding gene. Science 217, 316-324.

Mathis, D. J. and chambson, P. (1981). The SV40 early region TATA box is required for accurate in vitro initiation of transcription. Nature, 290, 310-315.

Mehdy, M. C. and Firtel, R. A. (1985). A secreted factor and cAMP jointly regulate cell-type-specific gene expression in Dictyostelium discoideum. Mol. Cell. Biol. 5, 705-713.

Mehdy, M. C., Ratner, R. A., and Firtel, R. A. (1983). Induction of cell type specific gene expression in Dictyostelium. Cell 32, 763-771.

Minty, A. and Newmark, P. (1980). Gene regulation: old and remote controls. Nature 288, 210-211.

Morris, T., Marashi, F., Weber, L., Hickey, E., Greenspan, D., Bonner, J., Stein, J., and Stein, G. (1986). Involvement

of the 5' leader sequence in the coupling the stability of a human H3 histone mRNA with DNA replication. Proc. Natl. Acad. Sci. U.S.A. 83, 981-985.

Mullner, E. W. and Kuhn, L. C. (1988). A stem-loop in the 3' untranslated region mediates iron-dependent regulation of transferrin receptor mRNA stability in the cytoplasm. Cell 53, 815-825.

Murphy, K. M. M. and Snyder, S. H. (1982). Calcium antagonist receptor binding sites labeled with [^{3}H] nitrendipine. Eur. J. Pharmacol. 77, 201-202.

Nellen, W., Datta, S., Reymond, C., Sivertsen, A., Mann, S., Crowley, T. and Firtel, R. A. (1986). Molecular biology in Dictyostelium: Tools and application."Methods in Cell Biology." Vol. 28. New York: Academic Press, pp 67-99.

Newell, P. C., Europe-Finner, G. N., Small, N. V. and Liu, G. (1988). Inositol phosphate of Dictyostelium. J. Cell Sci. 89, 123-127.

Newell, P. C. and Sussman, M. (1970). Regulation of enzyme synthesis by slime mold cell assemblies embarked upon alternative developmental programmes. J. Mol. Biol. 49, 627-637.

Nudel, U., Soreq, H., Littauer, U. Z., Marbaix, G., Huez, G., Leclerq, M., Hubert, E., and Chantrenne, H. (1976). Globin mRNA species containing poly (A) segments of different lengths. Their functional stability in Xenopus oocytes. Eur. J. Biochem. 64, 115-121.

Okayama, H. and Berg, P. (1982). High-efficiency cloning of full-length cDNA. Mol. Cell. Biol. 2(2), 161-170.

Oyama, J., and Blumberg, D. D. (1986). Interaction of cAMP with the cell-surface receptor induces cell-type-specific mRNA accumulation in Dictyostelium discoideum. Proc. Natl. Acad. Sci. U. S. A. 83, 4819-4823.

Pan, P., Hall, E. M. and Bonner, J. T. (1972). Folic acid as a second chemotactic substance in the cellular slime moulds. Nature (London) New Biol. 237, 181-182.

Pan, P., Hall, E. M. and Bonner, J. T. (1975). Determination of the active portion of the folic acid molecule in the cellular slime mold chemotaxis. J. Bacteriol. 122, 185-191.

Parish, R., Schmidlin, S., and Parish, C. R. (1978). Detection of developmentally controlled plasma membrane

antigens of Dictyostelium discoideum cells in SDS-polyacrylamide gels. FEBS Lett. 95, 366-370.

Paul, G. and Robison, G. A. (1984) The role of cAMP in cell proliferation: A critical assessment of the evidence. Advances in Cyclic nucleotide Research. 15, 193-294.

Pavlovic, J., Haribabu, B., and Dottin, R. P. (1988). Transmembrane signal transduction regulates gene expression in Dictyostelium discoideum. Dev. Genet. 9, 371-382.

Pfeiffer, D. R., Taylor, R. W. and lardy, H. A. (1978). Ionophore A23187: cation binding and transport properties. Ann. N. Y. Acad. Sci. 307, 402-423.

Platt, T. (1986). Transcription termination and the regulation of Gene expression. Ann. Rev. Biochem. 55, 339-372.

Pringle, J. and Hartwell, L. (1981). In, The molecular biology of the Yeast Saccharomyces. P. 87.

Proudfoot, N. J. and Brownlee, G. G. (1976) 3' non coding region sequences in eucaryotic messenger RNA. Nature 263, 211-214.

Pupillo, M., Kumagai, A., Pitt, G. S., Firtel, R. A., Devreotes, P. N. (1989). Multiple alpha subunits of guanine nucleotide-binding proteins in Dictyostelium. Proc. Natl. Acad. Sci. U.S. 86, 4892-4896.

Rabbitts, P. H., Forster, A., Stinson, M. A., and Rabbitts, T. H. (1985). Truncation of exon 1 from the c-myc gene results in prolonged c-myc mRNA stability. EMBO J. 4, 3727-3733.

Raper, K. B. (1935). Dictyostelium discoideum, a new species of slime mold from decaying forest leaves. J. Agr. Res. 55, 135-147.

Ratner, D. I., and Newell, P. C. (1978). Linkage analysis in Dictyosteliun discoideum using multiply marked tester strains: establishment of linkage group VII and reassessment of earlier linkage data. J. gen. Microbiol. 109, 225-236.

Rittling, S. R. and Baserga, R. (1987). Regulatory mechanisms in the expression of cell cycle dependent genes. Anticancer Research 7, 541-552.

Romans, P., and Firtel, R. A. (1985). Organization of the Dictyostelium discoideum actin multigene family: flanking sequences show subfamily homologies ans unusual dyad

symmetries. J. Mol. Biol. 183, 311-326.

Ross, J., and Pizarro, A. (1983). Human beta and delta globin messenger RNAs turn over at different rates. J. Mol. Biol. 167, 607-617.

Rossier, C., Gerisch, C. and Malchow, D. (1978). Action of a slowly hydrolysable cyclic AMP analogue on developing cells of Dictyostelium discoiseum. J. Cell Sci. 35: 321-338.

Rossomando, E. F., and Sussman, M. (1973). A 5′-adenosine monophosphate-dependent adenylate cyclase and an adenosine 3′:5′-cyclic monophosphate-dependent adenosine triphosphate pyrophosphohydrolase in Dictyostelium discoideum. Proc. Natl. Acad. Sci. U.S. 70, 1254-1257.

Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1971). Cyclic AMP,. Academic Press (New York and London).

Schaap, P. and Spek, W. (1984). Cyclic AMP binding to the cell surface during development of Dictyostelium discoideum. Differentiation 27, 83-87.

Schaap, P., Van Driel, R. (1985). Induction of post-aggregative differentiation in Dictyostelium discoideum by cAMP. Evidence of involvement of the cell surface receptor. Exp. Cell Res. 159, 388-398,

Schaap, P., Van Lookeren Campagne, M.M., Van Driel, R., Speck, W., Van Hasstert, P. J. M. and Pinas, J. (1986). Postaggregative differentiation induction by cyclic AMP in Dictyostelium intracellular transduction pathway and requirement for additional stimuli. Dev. Biol. 118, 52-63.

Schlegel, R. and Pardee, A. B. (1986). Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. Science 232, 1264-1266.

Schlegel, R. and Pardee, A. B. (1987). Periodic mitotic events in the absence of DNA replication. Proc. Natl. Acad. Sci. U.S.A. 84, 9025-9029.

Shatkin, A. J. ((1976). Capping of eucaryotic mRNA. Cell 9, 645-653.

Shaw, G., and Kamen, R. (1986). A conserved AU sequence from the 3\` untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46, 659-667.

Sehgal, P. B., Soreq, H., and Tamm, I. (1978). Does 3\`-terminal poly (A) stabilize human fibroblast interferon mRNA in oocytes of Xenopus laevis. Proc. Natl. Acad. Sci. U.S.A.

75, 5030-5033.

Shapiro, R. A., Herrick, D., Manrow, R. E., Blinder, D., Jacobson, A. (1988). Determinants of mRNA stability in Dictyostelium discoideum amoebae: differences in poly (A) tail length, ribosome loading, and mRNA size cannot account for the heterogeneity of mRNA decay rates. Mol. Cell. Biol. 8, 1957-1969.

Sibley, D. R. and Lefkowitz, R. J. (1985). Molecular mechanisms of receptor desensitization using the ß-adrenergic receptor-coupled adenylate cyclase system as a model. Nature (London) 317,124-129.

Simcox, A. A., Cheney, C. M., Hoffman, E. P., and Shearn, A. (1985). A deletion of the 3' end of the Drosophila melanogaster hsp70 gene increases stability of mutant mRNA during recovery from heat shock. Mol. Cell. Biol. 5, 3397-3402.

Singleton, C. K., Manning, S. S. and Feng, Y. (1988). Effect of protein synthesis inhibition on Gene expression during early development of Dictyostelium discoideum. Mol. Cell. Biol. 8(1), 10-16.

Sive, H. L., Heintz, N. and Roeder, R. G. (1984). Regulation of human histone gene expression during the HeLa cell cycle requires protein synthesis. Mol. Cell. Biol. 4, 2723-2734.

Snaar-Jagalska, B. E., Kesbeke, F., and Van Haastert, P. J. M. (1988). G-protein in the signal-transduction pathways of Dictyostelium discoideum. Dev. Genet. 9, 215-226.

Soll, D. R., Yarger, J. and Mirick, M. (1976). Stationary phase and the cell cycle of Dictyostelium discoideum in liquid nutrient medium. J. Cell Sci. 20, 513-523.

Spudich, J. A. (1987). Dictyostelium discoideum. Molecular approaches to cell biology. Methods in Cell Biology. Vol. 28. Academic Press.

Steel, L. F. and Jacobson, A. (1988). Post-transcriptional regulation of ribosomal protein gene expression during development in Dictyostelium discoideum. Dev. Genet. 9, 421-434.

Steineman, C., and Parish, R. W. (1980). Evidence that a developmentally regulated glycoprotein is target of adhesion blocking Fab in reaggregating Dictyostelium. Nature (London) 286, 621-623.

Sussman, M. (1966). Biochemical and genetic methods in the

study of cellular slime mold development . In "Methods in Cell Physiology" (D. Prescott, ed.). 2, 397-410. Academic Press, New York.

Sussman, M. and Brackenbury, R. (1976). Biochemical and Molecular-genetic aspects of cellular slime mold development. Ann. Rev. Plant. Physiol. 27, 229-265.

Sussman, M. and Osmond, M. J. (1964). UDP-galactose polysaccharide transferase in the cellular slime mold Dictyostelium discoideum: appearance and disappearance of the during differentiation. Proc. Natl. Acad. Sci. U.S. 52, 81-87.

Theibert, A. and Devreotes, P. N. (1984). Adenosine and its derivatives inhibit the cAMP signaling response in Dictyostelium discoideum. Dev. Biol. 106, 166-173.

Theibert, A., and Devreotes, P. N. (1986). Surface receptor-mediated activation of adenylate cyclase in Dictyostelium discoideum: regulation by guanine nucleotides in wild-type cells and aggregation deficient mutants. J. Biol. Chem. 261, 15121-15125.

Theibert, A., Palmisano, M., Jastorff, B. and Devreotes, P. (1986). The specificity of the cAMP receptor mediating activation of adenylate cyclase in Dictyostelium discoideum. Dev. Biol. 114, 529-533.

Tomchik, K. J. and Devreotes, P. N. (1981). cAMP waves in Dictyostelium discoideum: demonstration by a novel isotope dilution fluorography technique. Science 212, 443-446.

Van Haastert, P. J. M. (1987). Down-regulation of cell surface cyclic AMP receptors and desensitization of cyclic AMP-stimulated adenylate cyclase by cyclic AMP in Dictyostelium discoideum. Kinetics and concentration dependence. J. Biol. Chem. 262, 7700-7704.

Van Haastert, P. J. M., de Vries, M. J., Penning, L. C., Roovers, E., Van der Kaay, J., Erneux, C. and Van lookeren Campagne, M. M. (1989). Biochem. J. 258, 577-586

Van Haastert, P. J. M. and Kien, E. (1983). Binding of cAMP derivatives to Dictyostelium discoideum cells: Activation mechanism of the cell surface cAMP receptor. J. Biol. Chem. 258, 9636-9642.

Watts, D. J., and Ashworth, J. M. (1970). Growth of myxamoebae of the cellular slime mold Dictyostelium discoideum in axenic culture. Biochem. J. 119, 175-182.

Weijer, C., Duschl, G. and David, C. N. (1984). A revision of the Dictyostelium discoideum cell cycle. J. Cell Sci. 70, 111-131.

Werner, B., Lichtenstein, L. M., and Parker, C. W. (1974). Cyclic AMP, cell growth and immune response. (Springer-Verlag, New York).

Williams, J. G., Tsang, A. S. and Mahbubani, H. (1980). A change in the rate of transcription of a eukaryotic gene in response to cyclic AMP. Proc. Natl. Acad. Sci. U.S.A. 77, 7171-7175.

Wilson, T., and Treisman, R. (1988). Chemoattractant and guanosine 5'-[γ-thio] triphosphate induce the accumulation of inositol 1,4,5, triphosphate in Dictyostelium cells that are labelled with [^{3}H] inositol by electroporation. Nature 336, 396-399.

Wurster, B. and Schubiger, K. (1977). Oscillations and cell development in Dictyostelium discoideum stimulated by folic acid pilses. J. Cell Sci. 27, 105-114.

Yarger, J., Stults, K. and Soll, D. R. (1974). Observations on the growth of Dictyostelium discoideum in axenic medium: evidence for an extracellular growth inhibitor synthesized by stationary phase cells. J. Cell Sci. 14, 681-640.

Zada-Hames, I. and Ashworth, J. M. (1978). the cell cycle during the vegetative stage of Dictyostelium discoideum and its response to temperature change. J. Cell Sci. 32, 1-20.

APPENDIX

Regulatory signals affecting a selective loss of mRNA in *Dictyostelium discoideum*

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Summary

We identified signals that affect mRNA levels complementary to a gene that is highly expressed in vegetative *Dictyostelium discoideum* cells. This gene has been cloned as cDNA in the plasmid pcD-D2. The level of transcripts homologous to pcD-D2 fell dramatically in strain XP55 during the aggregation stage of development when cells differentiate on agar. The level, however, did not fall simply as a result of starvation or aggregation-specific cell contact. Rather, before the level is reduced cells must be deprived of amino acids and cyclic AMP administered in amounts and at intervals in pulses to mimic cyclic AMP signal-relay in aggregation. This effect can be blocked either with cyclic AMP-S (a non-hydrolysable cyclic AMP analogue) or adenosine, both of which prevent cyclic AMP binding to the cyclic AMP cell surface receptor. It is also blocked in 'frigid' aggregation-deficient mutants HC85 and HC112 known to be defective in a Gα protein. We conclude that the transcript level is balanced by positive nutritional signals acting against negative signals transduced in part through a cell surface cyclic AMP receptor.

Key words: gene expression, *Dictyostelium discoideum*, cyclic AMP.

Introduction

Amoebae of the cellular slime mould *Dictyostelium discoideum* exhibit an experimentally useful transition from proliferating vegetative cells to non-proliferating developing cells, which become fully differentiated spore or stalk cells. Although the induction of transcripts and proteins during development is well studied, much less is known about the mechanisms governing the loss of transcripts and proteins. A number of mRNAs and proteins are lost from cells during the period of aggregation. Two-dimensional gel electrophoresis studies of proteins synthesized during vegetative growth or early in development show that some previously synthesized proteins disappear during development (Alton and Lodish, 1977; Cardelli *et al.* 1985). Transcripts that decrease in abundance have been detected using DNA solution hybridization methods (Firtel and Jacobsen, 1977; Blumberg and Lodish, 1980; Jacquet *et al.* 1981) and some of these transcripts have been cloned in genomic or cDNA libraries (Kopachik *et al.* 1985*a*; Steel and Jacobsen, 1986, 1987; Faurer *et al.* 1988; Singelton *et al.* 1988). Genes whose expression is high in vegetative, but undetectably low in developing, cells have been identified using a cDNA library made from vegetative cell mRNA (Kopachik *et al.* 1985*a*). Although high levels of the transcripts are present in vegetative amoebae and early in development there is a drastic loss during aggregation of the amoebae beginning four hours into the developmental phase. Transcripts are barely detectable by twelve hours of development but reappear during spore germination when vegetative amoebae are released and resume cell proliferation.

Whereas little is known about the physiological signals triggering an inactivation of vegetative gene expression in early development, the induction of differentiation-specific gene expression is mediated by a variety of signals: starvation for amino acids (Marin, 1976, 1977); cell-to-cell contacts (Chung *et al.* 1981; Mehdy *et al.* 1983); cyclic adenosine monophosphate (Town and Gross, 1978; Chung *et al.* 1981; Mehdy *et al.* 1983; Barklis and Lodish, 1983; Oyama and Blumberg, 1986; Bozzone and Berger, 1987) differentiation inducing factor (Kopachik *et al.* 1983, 1985*b*; Williams *et al.* 1987) and other low molecular weight factors (Medhy and Firtel, 1985). It is conceivable that these signals and others may also play roles in repressing vegetative gene transcription. Here we examine the effect of physiological signals on the expression of two genes: one is a gene highly expressed in vegetative amoebae and whose corresponding cDNA sequence is cloned in the insert of the plasmid pcD-D2 and the other is a differentiation-cell-

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specific gene whose corresponding cDNA sequence is cloned in the insert of the plasmid pcD-D29 (Kopachik *et al.* 1985*a*).

Materials and methods

Strains and growth of amoebae

NC-4 is a wild-type isolate of *D. discoideum* from which the following strains were derived with the relevant markers indicated: XP55 (Ratner and Newell, 1978) and HC317 derived from XP55 and carrying fgdC for aggregate-deficient development (Coukell *et al.* 1983); KAX4 with axeA and axeB for ability to grow in axenic media (Loomis, 1971); HC6 and HC91 which are parental strains of fgdA carrying strains HC85 and HC112 (Coukell *et al.* 1983). V12M2 is a developmentally competent strain and the parent of HM27 from which HM44, a strain that lacks accumulation of Differentiation Inducing Factor, was derived (Kopachik *et al.* 1983). Amoebae, except for KAX4, were grown in association with *Klebsiella pneumoniae* and harvested as described by Kay and Trevan (1981). KAX4 was grown in HL-5 broth as described by Soll *et al.* (1976).

Developmental conditions on agar and in suspension

Harvested amoebae were allowed to develop on 2% Bacto-agar buffered with KK_2 (20 mM-potassium phosphate, pH 6.2) as described by Kopachik *et al.* (1985*b*). Conditions for development in suspension were according to the methods described by Medhy *et al.* (1983), except that cells were shaken in development buffer (5 mM-Na_2HPO_4, 5 mM-NaH_2PO_4, 2 mM-$MgSO_4$ and 200 μM-$CaCl_2$, pH 6.5). Fast and slow shaking cultures were maintained at 230 and 70 revs min^{-1}, respectively.

RNA isolation and analysis

Total cellular RNA was isolated by the method previously described (Chirgwin *et al.* 1979; Kopachik *et al.* 1985*a*), by centrifugation of a guanidinium thiocyanate extract through a cesium chloride cushion or by extraction of a cell pellet with phenol and chloroform (Jacobsen, 1976). Procedures for Northern blotting followed the methods previously described (Kopachik *et al.* 1985*a*).

Two-dimensional gel electrophoresis

Proteins were separated by the two dimensional gel electrophoresis procedures previously described (Kopachik *et al.* 1985) except that pH 5–7 ampholytes were used.

Chemicals and materials

Adenosine, adenosine cyclic monophosphate, 8-bromoadenosine cyclic monophosphate, folic acid, and BME amino acids were obtained from Sigma Chemical Co. Adenosine 3′:5′-monophosphothioate cyclic Sp-isomer (cyclic AMP-S) and 8-(4-chlorophenylthio) adenosine cyclic monophosphate were obtained from Boehringer Mannheim Biochemicals. Rabbit reticulocyte lysates and HYBOND-mAP paper was from Amersham Corporation.

Results

Gene expression during growth and differentiation

For normal development harvested starving amoebae were placed on agar buffered with KK_2 and observed to aggregate in response to cyclic AMP signals within 5 h. At the completion of aggregation by 12 h tight aggregates with tips had formed. In contrast, harvested starving cells placed in flasks containing development buffer (DB) and shaken as suspensions of cells at 230 revs min^{-1} remain substantially as single cells or if shaken at 70 revs min^{-1} form multicellular cell masses within 12 h (Medhy *et al.* 1983). Total RNA was isolated and then analyzed by Northern blotting to determine whether differentiation on agar or in a suspension affected the expression of the pcD-D2 and pcD-D29 genes (Kopachik *et al.* 1985*a*).

Levels of transcripts homologous to pcD-D2 fell dramatically during the period that pcD-D29 transcript levels rose during normal development on agar (Fig. 1A). Thus expression of the pcD-D2 gene during this early development period is inactivated when expression of the pcD-D29 gene is activated.

It is conceivable that pcD-D2 transcript levels are lowered solely in response to starvation from removal of the bacterial food source. We therefore examined the mRNA levels in cells placed in suspension cultures, which prevents normal aggregation. The pcD-D2 transcript levels, unexpectedly, did not fall whereas the pcD-D29 transcript levels rose in cells differentiating in suspension cultures (Fig. 1C and D). Thus starvation alone cannot be a sufficient physiological signal for inactivation of pcD-D2 gene expression but may be sufficient for activation of the pcD-D29 gene. Further support for this can be seen in Fig. 7, below, which presents results using an aggregation-deficient mutant strain HC112. Starving cells of this strain plated out on agar for normal development never complete aggregation and do not inactivate the pcD-D2 gene. Cell-to-cell contacts that develop during differentiation may be in part responsible for the induction of many transcripts that appear at the tight aggregate stage of development (Chung *et al.* 1981). These contacts, however, had little effect on the pcD-D2 transcript levels in cells that formed large adhesive cell masses with extensive cell–cell contacts (Fig. 1D).

Other differentiation-specific transcripts are induced in cells after cyclic AMP is added to suspension cultures (Chung *et al.* 1981; Barklis and Lodish, 1983; Medhy *et al.* 1983; Bozzone and Berger, 1987) whereas some transcripts are repressed after cyclic AMP addition (Williams *et al.* 1980; Bozzone and Berger, 1987). To investigate the possiblity that cyclic AMP might similarly affect gene expression from these two genes, cyclic AMP (1 mM) was added 4 h after cells were placed in DB buffer. The results in Fig. 2A show that the pcD-D2 mRNA did not fall after the addition of cyclic AMP or of a poorly hydrolysable analogue of cyclic AMP, adenosine 3′:5′-monophosphothioate cyclic Sp-isomer (cyclic AMP-S) added at 5 μM (Fig. 2B).

Not all developmentally competent strains were observed to be insensitive to cyclic AMP. Cells of the strain V12M2 differentiating under the same conditions responded to cyclic AMP addition by substantially lowering pcD-D2 mRNA levels (Fig. 2C). This difference in response between XP55 and V12M2 may be related to the

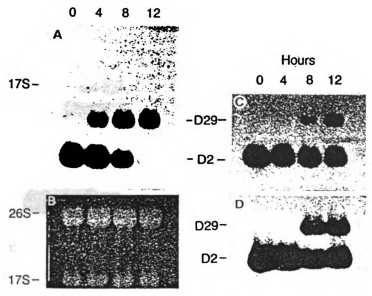

Fig. 1. The expression of the pcD-D2 and pcD-D29 genes in the developmentally competent strain XP55 during normal development on agar (A) and during differentiation in suspension cultures at fast-shaking (C) or slow-shaking (D) speeds. Total cellular RNA isolated from cells at the indicated hours of development was electrophoretically size-separated on formaldehyde–agarose gels (1.3%) and electroblotted onto hybridization transfer membranes (GeneScreen). The membrane was hybridized with oligolabelled (Feinberg and Vogelstein, 1983) plasmid DNA, washed, and exposed to X-ray film. Hybridization of the cDNA plasmids pcD-D2 and pcD-D29 to homologous mRNA is shown (A,C and D). The ethidium bromide-stained blot of (A) is shown in (B) as a control for RNA analysis to indicate that equal amounts of RNA (10 μg) as judged by equality of the amount of RNA in the 26S and 17S ribosomal bands were loaded in the lanes. All blots shown in this work were stained with ethidium bromide and used further if they had equal RNA loading.

latter strain's ability to develop normally on agar in the presence of concentrations of cyclic AMP-S that prevent aggregation of NC-4-derived strains (Rossier *et al.* 1978). No difference was observed between these two strains during development on agar. When developing normally V12M2 cells show the same loss of pcD-D2, and gain of pcD-29, transcripts (Kopachik *et al.* 1983*a*).

Cyclic AMP delivered in pulses lowers pcD-D2 transcript levels

The expression of some genes is affected by the application of cyclic AMP in amounts and in intervals that mimic the extracellular oscillations of cyclic AMP levels caused by its release from cells and degradation by extracellular cyclic AMP-phosphodiesterase during normal aggregation (Kimmel and Carlisle, 1986; Kimmel and Saxe, 1986; Kimmel, 1987; Mann and Firtel, 1987). However, only one gene (M4–1) expressed in vegetative cells has been shown to be repressed by pulses of cyclic AMP (Kimmel and Carlisle, 1986). We estimated the extent of changes in transcript abundance by translating mRNA extracted from cells developing with or without addition of cyclic AMP in pulses. The translated polypeptides were separated by two-dimensional gel electrophoresis and the fluorographs compared. Of the ≈400 polypeptides visualized eight decreased and 15 increased in levels consistently in several experiments (Fig. 3). Since these changes reflect altered mRNA abundance, we conclude that there are a significant number of specific reductions and inductions in mRNA levels in cells pulsed with cyclic AMP.

To check for an effect of cyclic AMP pulses on the expression of the pcD-D2 gene XP55 cells were given 50 nM (final concentration) pulses of cyclic AMP in 10-min intervals starting after 3 h in suspension culture. Expression of the pcD-D2 gene was repressed by pulses of cyclic AMP (Fig. 4A). Cells of another strain, KAX4, were found to require a higher concentration of cyclic AMP (200 nM) for optimal reduction of pcD-D2 mRNA levels and, interestingly, higher concentrations resulted in less repression (Fig. 4B).

The cyclic AMP signal received by cells chemotactically responding to cyclic AMP is transduced through a cell surface cyclic AMP receptor, which has recently been shown to be required for cyclic AMP chemotaxis (Klein *et al.* 1988). If the transduction of the pulsatile signal that results in lowered pcD-D2 transcript levels requires the activation and deactivation oscillations of the cyclic AMP receptor (Juliani and Klein, 1981; Klein *et al.* 1987) the addition of too large an amount of cyclic AMP to be

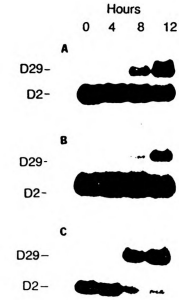

Fig. 2. The expression pattern during suspension differentiation for strain XP55 with the addition of 1 mM-cyclic AMP (A) or 5 μM-cyclic AMP-S (B) or V12/M2 with the addition of 1 mM-cyclic AMP (C). RNA blot hybridizations were conducted as described in the legend to Fig. 1.

effectively degraded before the next pulse might cause the receptor oscillations to cease. Further preliminary support for the idea that receptor oscillations are required might be obtained by pulsing cells with the optimal concentration of cyclic AMP in the presence of cyclic AMP-S. Cyclic AMP-S is an effective cyclic AMP analogue for chemotaxis (Rossier *et al.* 1978) and cyclic AMP-S (5 μM) did inhibit the cyclic AMP-mediated loss of pcD-D2 transcripts (Fig. 5A). Perhaps the increased occupancy of the cyclic AMP receptor, which might be expected to occur from cyclic AMP-S at this concentration, prevented signal transduction. Adenosine, albeit at very high concentrations (5 mM), competitively inhibits binding of cyclic AMP to the receptor (Newell and Ross, 1982; Theibert and Devreotes, 1984) and when added at the start of cyclic AMP pulsing prevented the cyclic AMP-mediated loss of transcripts (Fig. 5B).

We found that some nutrients can also inhibit the effect of cyclic AMP pulses. When cells are placed in DB buffer supplemented with a 1 mg ml^{-1} mixture of essential and non-essential amino acids (Fig. 5C) or 1 mM folic acid (Fig. 5D) the effect of cyclic AMP pulses is nullified. These observations reinforce the theory that both starvation *and* cyclic AMP receptor oscillations are necessary for the inactivation of the pcD-D2 gene expression. Interestingly, folic acid pulses, which accelerate development to the aggregation stage (Wurster and Schubiger, 1977) and raise intracellular cyclic AMP levels, had no effect on pcD-D2 transcripts levels in developing cells (Fig. 6A).

One response to cyclic AMP binding to the cell surface receptor is an increase in intracellular cyclic AMP levels. To determine if an increase in intracellular cyclic AMP is a sufficient signal to cause pcD-D2 transcript levels to fall the membrane-permeable cyclic AMP analogues 8-bromo

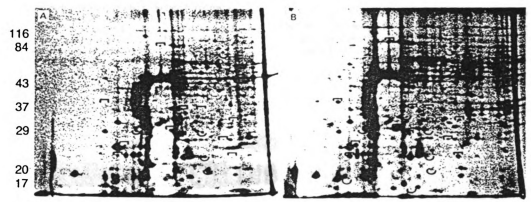

Fig. 3. Autofluorograms of polypeptides incorporating L-[^{35}S]methionine during *in vitro* translation of mRNA from KAX4 cells developing with (B) or without (A) addition of cyclic AMP in pulses. Amoebae (5×10^6 ml^{-1}) were allowed to develop in DB buffer in flasks shaking at 150 revs min^{-1} at 21 °C. After 4 h of development cyclic AMP (final concentration 200 nM) was added every 10 min for the next 8 h. RNA was extracted from the cells after a total of 12 h of development. Poly A$^+$ mRNA was selected with HYBOND-mAP paper and the mRNA was translated *in vitro* in rabbit reticulocyte lysates. The polypeptides were separated by two-dimensional gel electrophoresis as described in detail previously (Kopachik *et al.* 1985*b*). A line was drawn above spots that were darker in (B) and below spots that were darker in (A).

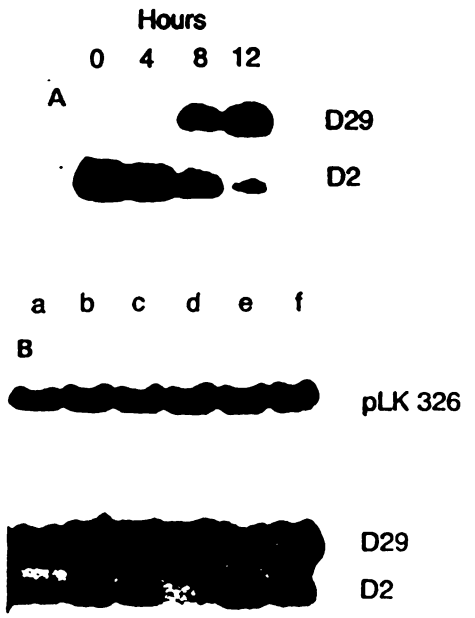

Fig. 4. The expression pattern during suspension differentiation with the addition of cyclic AMP pulses. Pulsing of XP55 cells (A) was begun after 4 h and continued for another 8 h at 50 nM final concentrations at 10 min intervals. The timing of cyclic AMP addition was similar for KAX4 cells (B) but RNA was extracted only after 12 h of development and the cyclic AMP concentration was varied: lanes a, no cyclic AMP; b, 10 nM; c, 50 nM; d, 200 nM; e, 500 nM; f, 1000 nM. RNA blot hybridizations were conducted as described in the legend to Fig. 1. The blot shown in B was probed with oligolabelled plasmid pLK326 in addition, to show the constituitive expression of the pLK326 gene.

cyclic AMP (Fig. 6B) and 8-chlorophenylthio-cyclic AMP (not shown) were added to developing cells. The pcD-D2 transcript levels, however, did not fall in response to these analogues, which are capable of affecting *Dictyostelium* differentiation (Kwong *et al.* 1988; R. R. Kay, unpublished data).

Defective signal transduction in aggregation-deficient mutant strains

Certain aggregation-deficient mutants termed 'frigid' (fgd) strains have been well characterized genetically and biochemically with respect to chemotactic ability (Coukell *et al.* 1983; Kesbeke *et al.* 1988). The fgdA strains, HC85 and HC112, produce low levels of cyclic AMP receptors but are unable to respond chemotactically to cyclic AMP. The defect in signal transduction may reside in a Gα protein-mediated step (Kumagai *et al.* 1989). Cells of the strain HC317 (fgdC) also possess low levels of receptors but show some chemotactic response to cyclic AMP. An analysis of the ability of these frigid strains to lower pcD-D2 transcript levels is shown in Fig. 7: HC112 cells when developing on agar did not lower pcD-D2, or raise pcD-D29, transcript levels (A) but HC317 cells did (data not shown).

When amoebae were starved in suspension cultures for 4 h and then pulsed with cyclic AMP, both HC112 and HC317 cells failed to inactivate pcD-D2 expression (Fig. 7B and C). HC85 amoebae were similar in response to HC112 amoebae in their lack of response (data not shown). Not all cyclic AMP-mediated changes in gene expression are defective in the fgdA mutants. Poly(A)$^+$ mRNA was extracted from HC112 cells taken from cultures in the absence of or with cyclic AMP pulsing and translated *in vitro*. The translated polypeptides were separated by two-dimensional gel electrophoresis and the flourographs compared. Some polypeptides were visualized in the HC112 fluorographs only if the cells from which the RNA was obtained were pulsed with cyclic AMP and these polypeptides were visualized also in the fluorographs from the experiment in which KAX4 cells were pulsed with cyclic AMP (Fig. 3).

Fig. 5. The expression pattern during suspension differentiation for strain XP55 developing with cyclic AMP pulsing and the addition of 5 μM-cyclic AMP-S (A), 5 mM adenosine (B), 1 mg ml^{-1} amino acids (C) and 1 mM-folic acid (D). Addition of cyclic AMP was conducted as described in the legend to Fig. 4.

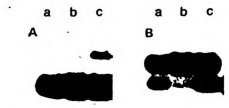

Fig. 6. The expression pattern in cells developing with the addition of folic acid (A) and 8-bromo cyclic AMP (B).
A. RNA from vegetative XP55 cells (lane a); RNA from cells developing for 12 h with 1 mM-folic acid added at the start of development (lane b); RNA from cells treated with folic acid pulses (folic acid was added to XP55 cells after 2 h of development for eight additions at 12-min intervals with cells at 5×10^7 per ml^{-1}) (lane c). B. RNA from KAX4 cells after 12 h of development (lane a); with 200 nM-cyclic AMP added in pulses as described in the legend to Fig. 3 (lane b); with 8-bromo cyclic AMP added at a final concentration of 1 mM after 4 h of development (lane c). RNA blot hybridizations were conducted as described in the legend to Fig. 1.

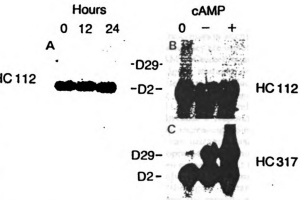

Fig. 7. The expression pattern during development on agar for strain HC112 (A) and during suspension development for HC112 (B) and HC317 (C) with (+) and without (−) the addition of cyclic AMP pulses. Pulsing was conducted as described in the legend to Fig. 4.

Discussion

It is interesting to contrast the regulatory mechanisms guiding the loss of transcripts with the mechanisms found to promote increases in transcripts and gene products. Marin (1976, 1977) used morphological markers of aggregation and showed that starvation for amino acids is a necessary trigger for the appearance of aggregates and the continuation of the developmental program. Although amino acids will prevent aggregation we could not assume that amino acids would also prevent pcD-D2 mRNA levels from falling and pcD-D29 mRNA levels from rising. Since we found that amino acids prevented the loss of pcD-D2 transcripts, it is conceivable that the regulation of genes expressed in vegetative cells and early in development might be coordinated with nutrient levels. Essential amino acids could make good candidates for substances whose levels might be monitored by cells. Similarly, one might expect the vitamin folic acid to play a role in regulation of transcript levels because vegetative amoebae detect folic acid given off by bacterial food sources (Pan *et al.* 1972). Folic acid is a chemoattractant for feeding amoebae and continues to attract developing amoebae until cyclic AMP-mediated chemotaxis takes over early in development. Our finding that folic acid (1 mM) given to developing cells prevented the loss of vegetative transcripts, indicates that elevated concentrations of folic acid may, as a chemoattractant or as a nutrient, be a signal to continue expression of this gene. It is well known that folic acid delivered in pulses can induce intracellular oscillations of cyclic AMP in amoebae that mimic the oscillations triggered by cyclic AMP pulses (Gerisch and Wick, 1975; Wurster and Schubiger, 1977; Devreotes, 1983). We, however, found

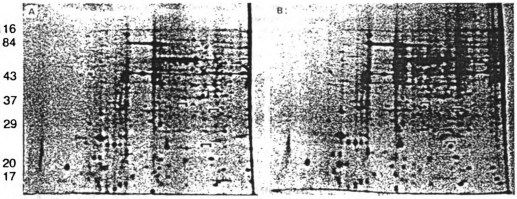

Fig. 8. Autofluorograms of polypeptides incorporating L-[^{35}S]methionine during *in vitro* translation of mRNA from HC112 cells developing with (B) and without (A) addition of cyclic AMP in pulses. The autofluorograms were prepared as described in the legend to Fig. 2. The mRNA was extracted from cells after 12 h of development.

no effect of folic acid pulses on the loss of vegetative transcripts. In addition 8-bromo cyclic AMP and 8-chlorophenylthio cyclic AMP, analogues capable of entering some cells, were without effect on pcD-D2 gene expression, whether added to growing or developing cells. This preliminary evidence suggests that pcD-D2 transcripts do not respond to elevation of intracellular cyclic AMP concentrations. Mann and Firtel (1987) have reached a similar conclusion to account for the absence of intracellular cyclic AMP signals in the regulation of several genes repressed or stimulated by cyclic AMP pulses early in development.

Aggregation depends on the induction of mRNAs encoding many new proteins, some of which are dependent on oscillations consisting of cyclic AMP signals released by cells at about 6-min intervals (Gerisch *et al.* 1979; Devreotes and Steck, 1979). This coordination of gene expression may be necessary for gene products that are essential for aggregation and post-aggregation development. It is, however, less clear why the loss of some transcripts would be controlled by oscillations of extracellular cyclic AMP. Nevertheless, as our data indicate a number of transcripts are lost in cells after pulsing with cyclic AMP. Hitherto, only Kimmel and Carlisle (1986) had reported a gene, M4-1, expressed in vegetative cells and repressed by low-level oscillations of cyclic AMP. Since we have confirmed this interesting discovery in one other gene, it might be possible to compare the sequence and structure of the two genes in an effort to find common, possibly regulatory elements responsible for this control. Here we have extended the analysis to ask whether the cell surface receptor and its two interconvertible forms are involved in this signal transduction. The cyclic AMP analogue cyclic AMP-S is capable of inhibiting aggregation in NC-4-derived strains (Rossier *et al.* 1978). Cyclic AMP-S is about 10000 times more slowly hydrolysed by cyclic AMP phosphodiesterases but the affinity for the cyclic AMP receptor is reduced only about 10-fold. Thus cyclic AMP-S added at 5 μM might be expected to occupy the receptor almost continuously. The capacity of cyclic AMP-S to inhibit suggests if the cyclic AMP receptor oscillates in parallel with the extracellular cyclic AMP oscillations this oscillation may be necessary for the loss of pcD-D2 in NC-4-derived strains. If this model is correct, then the absence of an effect resulting from the addition of high levels of cyclic AMP, and the inhibition of cyclic AMP pulsing by cyclic AMP-S and adenosine is easily explained: the receptor cannot oscillate under these conditions. The reasons why the V12M2 strain is able to inactivate pcD-D2 expression in the absence of cyclic AMP pulses or in the presence of high levels of cyclic AMP are obscure. V12M2 cells are, however, largely insensitive to the inhibition of aggregation by cyclic AMP-S (Rossier *et al.* 1978) and may uncouple the inactivation of pcD-D2 gene expression from the cyclic AMP receptor oscillations, or the cyclic AMP receptor oscillations continue to occur in spite of cyclic AMP or cyclic AMP-S binding.

Another approach to understanding the mode of action of cyclic AMP pulses on pcD-D2 mRNA levels is the use of well-characterized aggregation-defective mutants. The frigid aggregation mutants are excellent for this purpose. These strains accumulate cyclic AMP receptors but do not efficiently induce the intracellular responses of adenylate and guanylate cyclase activation, and they are unable to respond chemotactically to cyclic AMP (Coukell *et al.* 1983; Keskebe *et al.* 1988). The fgdA mutants are believed to be defective in interaction between the surface receptor and a Gα protein (Keskebe *et al.* 1988; Kumagai *et al.* 1989). As shown here, HC112 is not entirely defective in response to cyclic AMP because it responds to cyclic AMP pulses by selectively increasing some transcript levels. Because the strains can neither inactivate pcD-D2 transcripts during development on agar nor when pulsed with cyclic AMP in shaken suspensions, it is possible that the Gα protein is involved in the signal transduction. The insensitivity of the fgdA mutant strains also confirms that starvation and the capacity to undergo some early developmental changes is not sufficient for pcD-D2 gene inactivation. This suggests that the switch to reduce vegetative mRNA levels may not be made until cells are aggregation competent. In wild type cells the loss begins during aggregation after 4 h of development and continues throughout completion of aggregation after 12 h. Thus before cells become committed to reducing vegetative mRNA levels they must also be able to respond to cyclic AMP signals from the surrounding cells. Other mutants in fgdB, -D and -E, which are blocked earlier than the fgdA and -C classes, would be expected not to inactivate pcD-D2 gene expression, but these strains have not been used. Two other mutants, TS2 a strain blocked early in development when developing at 26.5 °C (Loomis *et al.* 1976), and HM44, which is unable to accumulate the morphogen DIF (Kopachik *et al.* 1983), were able to inactivate pcD-D2 gene expression when the amoebae were developing on agar (data not shown). TS2 at 26.5 °C and HM44 must be blocked after the switch to inactivate the genes is made and DIF accumulation is not necessary for the inactivation in HM44.

It is conceivable that many of the losses of mRNA in early development could occur by accelerated turnover of the mRNA using existing RNases, and cyclic AMP pulses may stimulate this specific degradation. It is of course possible that pcD-D2 transcripts have short half-lives and the transcript levels could drop quickly. It will be interesting to determine the mechanism of action of cyclic AMP oscillations and whether gene transcription is repressed, or transcripts are degraded, or both.

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References

ALTON, T. AND LODISH, H. F. (1977). Developmental changes in messenger RNAs and protein synthesis in *Dictyostelium discoideum*. *Devl Biol.* **60**, 180–206.

Barklis, E. and Lodish, H. F. (1983). Regulation of *Dictyostelium discoideum* mRNAs specific for prespore or prestalk cells. *Cell* **32**, 1139–1148.

Blumberg, D. D. and Lodish, H. F. (1980). Changes in the messenger RNA population during differentiation of *Dictyostelium discoideum*. *Devl Biol.* **78**, 268–284.

Bozzone, D. M. and Berger, E. A. (1987). Distinct developmental regulation and properties of the responsiveness of different genes to cyclic AMP in *Dictyostelium discoideum*. *Differentiation* **33**, 197–206.

Cardelli, J. A., Knecht, D. A., Wunderlich, R. and Dimond, R. L. (1985). Major changes in gene expression occur during at least four stages of development of *Dictyostelium discoideum*. *Devl Biol.* **110**, 147–156.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.

Chung, S., Landfear, S. M., Blumberg, D. D., Cohen, N. S. and Lodish, H. F. (1981). Synthesis and stability of developmentally regulated *Dictyostelium* mRNAs are affected by cell-cell contact and cAMP. *Cell* **24**, 785–797.

Coukell, M. B., Lappano, S. and Cameron, A. M. (1983). Isolation and characterization of cAMP unresponsive (Frigid) aggregation-deficient mutants of *Dictyostelium discoideum*. *Devl Genet.* **3**, 283–297.

Devreotes, P. N. (1983). The effect of folic acid on cAMP-elicited cAMP production in *Dictyostelium discoideum*. *Devl Biol.* **95**, 154–162.

Devreotes, P. N. and Steck, T. L. (1979). Cyclic 3',5' AMP relay in *Dictyostelium discoideum*. II. Requirements for the initiation and termination of the response. *J. Cell Biol.* **80**, 300–309.

Faure, M., Kalekine, M., Boy-Marcotte, E. and Jacquet, M. (1988). Developmental control of the expression of the dihydrooratate dehydrogenase and UMP synthetase genes in *Dictyostelium discoideum*. *Cell Differ.* **22**, 159–164.

Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analyt. Biochem.* **132**, 6–13.

Firtel, R. A. and Jacobson, A. (1977). In *Biochemistry of cell differentiation*. II (ed. Paul, J.), vol. 15, pp. 377–429. University Park Press, Baltimore.

Gerisch, G., Malchow, D., Roos, W. and Wick, U. (1979). Oscillations of cylic nucleotide concentrations in relation to the excitability of *Dictyostelium*. *J. exp. Biol.* **81**, 33–47.

Gerisch, G. and Wick, U. (1975). Intracellular oscillations and release of cyclic AMP from *Dictyostelium* cells. *Biochem. biophys. Res. Commun.* **65**, 364–370.

Jacobson, A. (1976). Analysis of mRNA transcription in *Dictyostelium discoideum*, or slime mold messenger RNA: how to find it and what to do with it once you've got it. *Meth. molec. Biol.* **8**, 161–209.

Jacquet, M., Part, D. and Felenbok, B. (1981). Changes in the polyadenylated messenger RNA population during development in *Dictyostelium discoideum*. *Devl Biol.* **81**, 155–166.

Juliani, M. and Klein, C. (1981). Photoaffinity labeling of the cell surface adenosine 3':5'-monophosphate receptor of *Dictyostelium discoideum* and its modification in down-regulated cells. *J. biol. Chem.* **256**, 613–619.

Kay, R. R. and Trevan, D. (1981). *Dictyostelium* amoebae can differentiate into spores without cell contact. *J. Embryol. exp. Morph.* **62**, 369–378.

Kesbeke, F., Jagalska, B. E. S. and Van Haastert, P. J. M. (1988) Signal transduction in *Dictyostelium* fgd A mutants with a defective interaction between surface cAMP receptors and a GTP-binding regulatory protein. *J. Cell Biol.* **107**, 521–528.

Kimmel, A. R. (1987). Different molecular mechanisms for cAMP regulation of gene expression during *Dictyostelium* development. *Devl Biol.* **122**, 163–171.

Kimmel, A. R. and Carlisle, B. (1986). A gene expressed in undifferentiated vegetative *Dictyostelium* is represented by developmental pulses of cAMP and reinduced during dedifferentiation. *Proc. natn. Acad. Sci. U.S.A.* **83**, 2506–2510.

Kimmel, A. R. and Saxe, III, C. L. (1986). cAMP-controlled gene expression in *Dictyostelium* mutants exhibiting abnormal patterns of developmental cAMP metabolism. *Devl Biol.* **117**, 209–214.

Klein, P., Knox, B., Borleis, J. and Devreotes, P. (1987). Purification of the surface cAMP receptor in *Dictyostelium*. *J. biol. Chem.* **262**, 352–357.

Klein, P. S., Sun, T. J., Saxe, III, C. L., Kimmel, R. L. and Devreotes, P. N. (1988). A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science* **241**, 1467–1472.

Kopachik, W., Bergen, L. G. and Barclay, S. L. (1985*a*). Genes selectively expressed in proliferating *Dictyostelium* amoebae. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8540–8544.

Kopachik, W., Dhokia, B. and Kay, R. R. (1985*b*). Selective induction of stalk-cell-specific proteins in *Dictyostelium* *Differentiation* **28**, 209–216.

Kopachik, W., Oohata, A., Dhokia, B., Brookman, J. J. and Kay, R. R. (1983). *Dictyostelium* mutants lacking DIF, a putative morphogen. *Cell* **33**, 397–403.

Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N. and Firtel, R. A. (1989). Regulation and function of *G*α protein subunits in *Dictyostelium*. *Cell* **57**, 265–275.

Kwong, L., Sobolewski, A. and Weeks, G. (1988). The effect of cAMP on differentiation inducing factor (DIF)-mediated formation of stalk cells in low-cell-density monolayers of *Dictyostelium discoideum*. *Differentiation* **37**, 1–6.

Loomis, W. F. (1971). Sensitivity of *Dictyostelium discoideum* to nucleic acid analogues. *Expl Cell Res.* **64**, 484–486.

Loomis, W. F., White, S. and Dimond, R. (1976). A sequence of dependent stages in the development of *Dictyostelium discoideum*. *Devl Biol.* **53**, 171–177.

Mann, S. K. and Firtel, R. A. (1987). Cyclic AMP regulation of early gene expression in *Dictyostelium discoideum*: mediation via the cell surface cyclic AMP receptor. *Molec. cell. Biol.* **7**, 458–469.

Marin, F. T. (1976). Regulation of development in *Dictyostelium discoideum*. *Devl Biol.* **48**, 110–117.

Marin, F. T. (1977). Regulation of development in *Dictyostelium discoideum*: II. regulation of early cell differentiation by amino acid starvation and intercellular interaction. *Devl Biol.* **60**, 389–395.

Mehdy, M. C. and Firtel, R. A. (1985). A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*. *Molec. cell. Biol.* **5**, 705–713.

Mehdy, M. C., Ratner, D. and Firtel, R. A. (1983). Induction and modulation of cell-type specific gene expression in *Dictyostelium discoideum*. *Cell* **32**, 763–771.

Newell, P. C. and Ross, F. M. (1982). Inhibition by adenosine of aggregation centre initiation and cyclic AMP binding in *Dictyostelium*. *J. gen. Microbiol.* **128**, 2715–2724.

Oyama, M. and Blumberg, D. D. (1986). Interaction of cAMP with the cell-surface receptor induces cell-type-specific mRNA accumulation in *Dictyostelium discoideum*. *Proc. natn. Acad. Sci. U.S.A.* **83**, 4819–4823.

Pan, P., Hall, E. M. and Bonner, J. T. (1972). Folic acid as a second chemotactic substance in the cellular slime mold. *Nature, new Biol.* **237**, 181–182.

Ratner, D. I. and Newell, P. C. (1978). Linkage analysis in *Dictyostelium discoideum* using multiply marked tester strains: establishment of linkage group VII and reassessment of earlier linkage data. *J. gen. Microbiol.* **109**, 225–236.

Rossier, C., Gerisch, G. and Malchow, D. (1978). Action of a slowly hydrolysable cyclic AMP analogue on developing cells of *Dictyostelium discoideum*. *J. Cell Sci.* **35**, 321–338.

Singleton, C. K., Gregoli, P. A., Manning, S. S. and Northington, S. J. (1988). Characterization of genes which are transiently expressed during the preaggregative phase of development of *Dictyostelium discoideum*. *Devl Biol.* **129**, 140–146.

Soll, D. R., Yarger, J. and Mirick, M. (1976). Stationary phase and the cell cycle of *Dictyostelium discoideum* in liquid nutrient medium. *J. Cell Sci.* **20**, 513–523.

Steel, L. F. and Jacobson, A. (1986). Ribosomal proteins are encoded by single copy genes in *Dictyostelium discoideum*. *Gene* **41**, 165–172.

Steel, L.F. and Jacobson, A. (1987). Translational control of

ribosomal protein synthesis during early *Dictyostelium discoideum* development. *Molec. cell. Biol.* 7, 965–972.

THIERERT, A. AND DEVIBOTES, P. N. (1984). Adenosine and its derivatives inhibit the cAMP signaling response in *Dictyostelium discoideum*. *Devl Biol.* 106, 166–173.

TOWN, C. D. AND GROSS, J. D. (1978). The role of cyclic nucleotides and cell agglomeration in post-aggregative enzyme synthesis in *Dictyostelium discoideum*. *Devl Biol.* 63, 412–420.

WILLIAMS, J. G., CROCCARELLI, A., MCROBBIE, S. J., MAHBUBANI, H., KAY, R. R., EARLY, A., BERKS, M. AND JERMYN, K. (1987). Direct induction of *Dictyostelium* prestalk gene expression by DIF provides evidence that DIF is a morphogen. *Cell* 49, 185–192.

WILLIAMS, J. G., TSANG, A. S. AND MAHBUBANI, H. (1980). A change in the rate of transcription of a eucaryotic gene in response to cyclic AMP. *Proc. natn. Acad. Sci. U.S.A.* 77, 7171–7175.

WURSTER, B. AND SCHUBIGER, K. (1977). Oscillations and cell development in *Dictyostelium discoideum* stimulated by folic acid pulses. *J. Cell Sci.* 27, 105–114.

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