MOLECULAR MECHANISM OF GLYCOGEN PHOSPHORYLASE GENE REGULATION DURING *DICTYOSTELIUM* DEVELOPMENT

By

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(ABSTRACT)

Development of multicellular organisms is one of the most fundamental but least understood biological processes. Due to its simple life cycle, the lower eukaryote *Dictyostelium* has been used as a model system to study several basic biological problems, such as cell differentiation, cell motility, cell adhesion, signal transduction, and especially gene regulation. Glycogen phosphorylase is the enzyme that initiates one of the key biochemical pathways, glycogen degradation, during *Dictyostelium discoideum* development. Two forms of glycogen phosphorylase, gp1 and gp2, exist in *D. discoideum* with gp1 being active in vegetative cells and gp2 in differentiating cells. Study of glycogen phosphorylase gene regulation clearly will provide insight into the molecular mechanism of *D. discoideum* development and facilitate understanding of development in general. Two distinct genes that encode the two forms of glycogen phosphorylase were cloned. The nucleotide sequence analysis of the gp2 gene revealed an open reading frame of 2976 bp, that consists of three exons separated by two introns. An interesting feature in the gene is a 45 bp sequence in the second exon that contains 11 CAA trinucleotide repeats. The entire 5' and 3' non-coding regions of the gp2 gene and the whole 5' noncoding region

of the gp1 gene have also been cloned. The regulation of the gp2 gene by *Dictyostelium* developmental signals was studied. Both cyclic AMP (cAMP) and Differentiation Inducing Factor (DIF) were discovered to induce gp2 gene expression during differentiation. DIF was also found to inhibit the cAMP responsiveness of the gene. Both cAMP and DIF induction of the gene were repressed by NH_3. Another developmental signalling molecule, adenosine, was involved in gp2 gene regulation through the inhibition of the DIF-mediated expression. The cell-type-specificity of the gp2 gene were also investigated. The gene was found to be expressed in both prestalk/stalk and prespore/spore cells. This is in agreement with the cAMP and DIF inducibility of the gene since the former molecule is a spore-cell morphogen, while the latter is a stalk-cell morphogen. A model of gp2 gene regulation during development is proposed, based on these findings. The two gp2 introns and the 45 bp CAA repeat were studied by deletion of these elements. However, there were no alterations of gp2 gene expression observed after these deletions. Also investigated was genomic structural alteration in gp1$^-$ mutants that were obtained through homologous recombination and antisense RNA. Southern analysis revealed that the normal gp1 gene was disrupted in all homologous recombination transformants and in half of the antisense RNA transformants. Finally, for the first time, an extrachromosomal luciferase reporter vector has been established for the study of *cis*-acting regulatory elements in *D. discoideum*.

This dissertation is dedicated with great respect

to

my elementary and high school teachers

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Table of Contents

CHAPTER 5

List of Figures

Chapter 1. Introduction

Development and Gene Regulation.

Development of multicellular organisms, in most of the cases, begins with a single cell, the fertilized egg. In coordination with cell proliferation, cell differentiation initiated from this fertilized cell proceeds to provide all of the cell types of the body as embryonic development progresses. Cell differentiation generates cellular diversity of an organism with various cells with different structures and functions, such as neurons, muscle cells, blood cells, liver cells, lung cells, skin cells, and so on. During the period of cell differentiation, two other processes, morphogenesis (creation of structure) and growth (regulation of size) organize the cells, tissues and organs into highly ordered forms that characterize an organism. Many coordinating interactions, including cell adhesion, cell migration, contact inhibition, tissue interactions, body segmentation, growth control, and so on, are required to fulfill the whole process of development. Although certain specific developmental events have been described at the cellular level, many fundamental problems remain poorly understood, especially when a cascade of molecular processes is involved. For example, how is cell differentiation induced? How is a tissue formed from differentiated cells? How is an organ organized from tissues? How is a body constructed from organs? How is cell growth in an organ regulated throughout development? What are the molecular mechanisms underlying these complex developmental processes?

Every single cell of an organism possesses identical genetic information. How can cells be differentiated into different cell types even though they possess identical genetic information? Advances in molecular biology have greatly facilitated the understanding of development. Elucidation of differential gene expression in different cell types has established a base from which to investigate cell differentiation as well as pattern formation. The processes can be described in molecular terms. Considering the complexity of various processes during development, regulation of the differential gene expression must also be a highly controlled program with numerous but precise interactions. Gene regulation at different levels (DNA, RNA and protein) provides approaches to study the developmental events. Structural features in chromatin, such as nucleosomes, may control the accessibility of a gene to RNA polymerase and transcription factors. Transcriptional regulation enables a cell to turn on or off the gene through *trans*-acting transcription factors that bind to the promoter, enhancer and/or other *cis*-acting regulatory DNA elements of the gene. RNA processing generates alternative RNA molecules from one gene by splicing different combinations of exons together, which can then be translated into different proteins. Recently-discovered RNA editing creates more variations of RNA and protein molecules from a single gene. When mRNA is transported from the nucleus to the cytoplasm, translational regulation of gene expression can be used as a fine-tuning mechanism to adjust the amount of protein production. Control of RNA stability also contributes to the fine-tuning regulation. Once a polypeptide is made, post-translational regulation plays an important role in controlling the activity of proteins, by cleavage of a part of the peptide sequence, covalent modifications such as glycosylation and phosphorylation, subcellular targeting, and protein turnover.

These regulatory mechanisms provide the molecular basis for differential gene expression that results in cellular divergence and structural order during development. The

differential gene expression takes place when cells are exposed to specific signals. However, the nature of most of the signals remains unknown. And for those that are known, the signal transduction pathways and targeted genes are poorly understood. Several model systems have been employed to study the mechanisms involved in developmental processes. Mice, chicken, frogs, flies (Drosophila), worms (*C. elegans*) and the cellular slime mold (*Dictyostelium*) are well-established systems for such studies. Each of them provides an unique approach to the eventual understanding of multicellular development and represents a specific evolutionary state. Elucidation of principles in any of these systems will facilitate general understanding of development among organisms. While higher organisms provide opportunities to explore the complexity of numerous interactions during development, *Dictyostelium* offers a system to simplify the process.

Dictyostelium as a Model System.

The first cellular slime mold, *Dictyostelioum mucoroides*, was discovered by German mycologist O. Brefeld in 1869 (Brefeld, 1869). However, the modern era of *Dictyostelium* began with the discovery of another species, *D. discoideum*, by K. B. Raper in 1935 (Raper, 1935). This is because, over the past several decades, most of the work in *Dictyostelium* have been done with *D. discoideum.*

Dictyostelium is found in the surface layer of soil and forest litter in many geographic regions. It has been classified under both fungi (Raper, 1973) and protozoa (Olive, 1975), based on its characteristics at different phases of the life cycle. However, it may be more proper to consider this organism as a bridge between fungi and protozoa.

Two alternate life cycles, sexual and asexual, are observed in *D. discoideum*. The sexual cycle is difficult to reproduce in laboratory conditions. Almost all of the research is carried out using the asexual cycle. In this life cycle (Figure 1), haploid, free-living cells (amoebae) feed on bacteria and other organic matter during vegetative growth. At this stage, cells are homogeneous and undifferentiated. When food sources become limited, a population of the homogeneous amoebae (10^5-10^6 cells) enter a social state by aggregating towards a central point. The aggregation is directed by a chemo-attractant, cyclic AMP (cAMP). The emission of cAMP is initiated by a few cells, which then become the center for aggregation. When cells in the vicinity of the circular zone are exposed to cAMP through a cAMP receptor, they move towards the center in response to the signal. Meanwhile more intracellular cAMP molecules are synthesized through adenylate cyclase in these cells. Release of the cAMP molecules from these cells into the neighboring circular zone causes the cells in this zone move towards the aggregation center. The cAMP signal, thus is radiated outward in an oscillatory manner by a cell to cell relay mechanism. After each oscillatory movement, the cAMP molecules are degraded by extracellular and membrane-associated phosphodiesterase. The degradation ensures that the cells are capable of responding efficiently to the next cAMP stimulus. As aggregation progresses, the cells are arranged in a form composed of strings or streams radiating outwards from the aggregation center. At the end of aggregation, cells are organized into a ball-like aggregate. Then a small group of cells are sorted out to form a tip structure on the top of the aggregate. The tip guides the cells in the aggregate to extend upward and form a finger-like structure. Since the tip directs the morphogenetic movements and determines the polarity in pattern formation, it has been considered as an organizer, as seen in embryonic development and tissue regeneration. The standing finger structure assumes a horizontal orientation and forms a structure termed a pseudoplasmodium or simply a slug. A slug is sensitive to

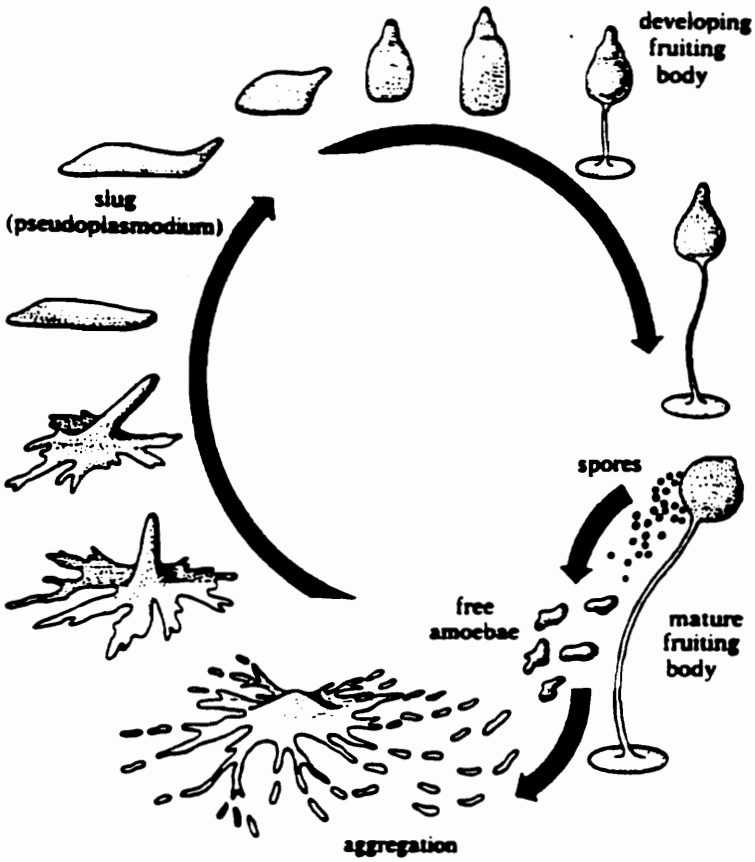


Figure 1. Asexual life cycle of *Dictyostelium discoideum.*

favorable physical conditions such as light, heat and moisture and will migrate towards them under the guidance of the tip. As it migrates, a slug secretes a slime sheath along the trail of migration. Starting at the finger stage, the cells are no longer homogeneous. A specific spatial pattern of precursors to two cell types is formed. The anterior 3/10 of a slug contains the precursors for the stalk (prestalk cells), the posterior 6/10 harbors the precursors for spores (prespore cells), and very end 1/10 is composed of precursors for the basal disc (characteristics of these cells resemble prestalk cells). Both position-dependent and position-independent factors have been proposed as the mechanism for prestalk / prespore pattern formation. In the position-dependent hypothesis, it is thought that the fate of the two cell types is determined by their initial position in a slug (Strandmann and Kay, 1990;), whereas the position-independent view hypothesizes that prestalk and prespore cells are pre-determined and sorted out based on the specific phases of cell cycle that they are in when development begins (Weijer et al, 1984; Gomer and Firtel, 1987; Zimmermann and Weijer, 1993). Evidence suggests that cells in S and early G2 phases differentiate preferentially into prestalk cells, while cells in late G2 phase undergo prespore differentiation. As soon as the slug migration stops, another process called culmination takes place. The culmination process is characterized by the elevation of prespore cells and the elongation of stalk. Cyclic AMP has been proposed to act as the signal to direct the cell movement during culmination (Sussman, 1982). Finally, a fruiting body is formed with a stalk supporting a spore head containing mature spores. When the environment becomes favorable for growth, spores will germinate and enter the free-living amoebae stage. A new life cycle starts over again.

The entire development process involves only two major cell types, and is completed in 24 hours under laboratory conditions. The proportion of the two major cell types and the size of the organism are well controlled. The organism is very easy to

manipulate in the laboratory and is accessible to almost all of the molecular genetic and biochemical techniques. Many developmental mutants have been isolated and a powerful new method has been developed to prepare developmental mutants by tagging developmental genes with restriction-enzyme-mediated integration of plasmid DNA (Kuspa and Loomis, 1992). *Dictyostelium* has seven chromosomes with a genome size of only 5 x 10^7 base pairs (bp), which is only about 10 times that of the *E. coli* genome. In general, in comparison to the complexity of metazoan embryogenesis, the simplicity of *Dictyostelium* provides an excellent system to investigate the essential nature of development.

Developmental Gene Regulation in *Dictyostelium*.

As seen in metazoan embryogenesis, the expression of developmental genes in *Dictyostelium* is also subject to both temporal and spatial regulation. During *Dictyostelium* development, fewer than 400 genes have been estimated to be specific and essential for development (Loomis, 1978). It has also been observed that 150-200 new proteins are synthesized through development (Loomis, 1985). The activities of these proteins increase at (1) the onset of development (0 hr), (2) the peak point of cAMP chemotactic signalling (8-10 hr), (3) during slug formation (16 hr), and (4) the stage of culmination (22 hr). Interestingly, some of the proteins appear earlier than their activities, suggesting that posttranslational regulation plays an important role in temporal control of cell differentiation. Both starvation and cell density signals contribute to the expression of the early developmental genes (Cardelli et al, 1985). The cell density requirement suggests that the cells are able to monitor the surrounding environment to ensure that sufficient numbers

of cells are available for multicellular development. At 8-10 hours of development more than 30 new proteins appear. This is the time point at which the greatest number of proteins are synthesized during development. One of these proteins, gp80, is believed to be an adhesion glycoprotein (Loomis et al, 1985). The mRNAs of a cAMP receptor, cAR1, and another recently reported cAMP receptor, cAR3, also appear in this time frame (Johnson et al, 1993). The next large increase of gene expression begins at 12 hours of development. About 20 prespore-specific proteins arise at this stage. Many genes are expressed in response to cAMP at this stage (Barklis and Lodish, 1983). At 22 hours of development, as the cells undergo terminal differentiation, the final peak of gene expression takes place and approximately 20 new proteins are synthesized. The majority of these proteins are stalk-cell-specific. A most interesting event occuring at this point is the dramatically rapid drop of NH_3 concentration in prestalk/stalk cell region (Wilson and Rutherford, 1978). This and many other reports on NH_3 effects suggest that the relief of NH_3 pressure may be responsible for the activation of the final set of developmentally regulated genes.

Promoters of some developmental genes have been analyzed. Three promoters have been discovered in the gene encoding for membrane-associated cAMP phosphodiesterase in *D. discoideum*. These promoters are responsible for gene expression during vegetative growth, aggregation and multicellular development (Podgorski et al, 1989; Hall et al, 1993). Also, many GC-rich DNA elements in the promoters have been suggested to be involved in temporal regulation (Pavlovic et al, 1989; Haberstroh et al, 1991; May et al, 1991; Fosnaugh and Loomis, 1993). However, no consensus sequences has been drawn from different genes.

The pattern of prestalk and prespore cells is established at the slug stage. Cell-type-specific gene expression can be detected in this and later stages (for reviews, see Loomis,

1985; Schaap, 1986). For some genes, the cell-specific expression is coordinated with temporal control. It has been observed that certain genes are only expressed in prestalk and prespore cells, but not in stalk and spore cells, and some others are induced to a much higher level only when the cells become terminally differentiated as stalk and spore cells. For example, expression of the genes that encode for UDP-galactose transferase and UDP-galactose epimerase are only detected in prespore cells, but not in spore cells (Lam et al, 1981; Telser and Sussman, 1971). There are genes that are expressed in undifferentiated amoebae, but become developmentally-regulated and cell-type-specific during cell differentiation. For example, both the membrane-associated cAMP phosphodiesterase gene and the ras gene are expressed in vegetative cells. As the cells proceed through the developmental program, the genes retain their transcriptional activities only in prestalk cells (Reymond et al, 1984). Some genes are not expressed until development is under way, but are active in both cell types (Loomis, 1985; Schaap, 1986). It is also interesting to note that majority of spore-specific proteins appear at tip formation stage (12 hours of development), which is about 10-12 hours before spore formation (maturation) (Cardelli et al, 1985; Morrissey et al, 1984). In contrast, many stalk-specific proteins are not synthesized before the onset of stalk formation at approximately 22 hours of development (Cardelli et al, 1985; Morrissey et al, 1984; Kopachik et al, 1985). When these proteins do exist in prestalk cells, they are usually also present in prespore cells.

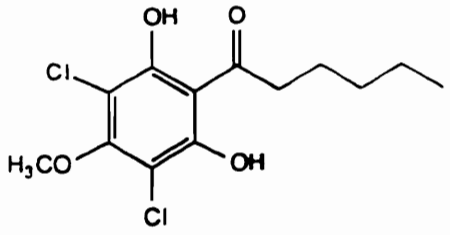
The temporal and spatial regulation of the developmental genes provides the underlying mechanism for differentiation and morphogenesis. The regulation is initiated and governed by developmental signals. Besides positional signals such as intercellular contact through adhesion molecules, four small molecules, cAMP, Differentiation Inducing Factor (DIF), NH_3, and adenosine, have been reported to function as diffusible developmental signals (Berks et al, 1991; Kay et al, 1989; Kimmel and Firtel, 1991; and

Williams, 1988). cAMP acts as a chemoattractant during aggregation through a cAMP receptor. After tip formation, a possible oscillatory emission of cAMP from the tip might direct the prestalk/prespore pattern formation (Sussman, 1982). An important function of cAMP during *Dictyostelium* development is that this molecule induces spore cell differentiation by activating and maintaining prespore-cell-specific genes (Barklis and Lodish, 1983; Kay et al, 1978; Oyama and Blumberg, 1986a; Town et al, 1976; and Weijer and Durston, 1985). It has also been observed that the presence of cAMP was necessary for the differentiation of a prestalk cell subtype, pstA, but was inhibitory for the differentiation of the other subtype, pstB (Berks and Kay, 1990). The involvement of cAMP in cell differentiation is very well established; however, the mechanisms that mediate the cAMP signal to direct different developmental processes have been controversial. Nevertheless, three cAMP signal transduction pathways have been proposed (Kimmel, 1987; Schaap, 1991). Different cell surface cAMP receptors and their associated various G proteins are presumably involved in transducing the signal to (1) adenylate cyclase, (2) phospholipase C (PLC), and (3) guanylate cyclase. In the first pathway, intracellular cAMP resulting from activated adenylate cyclase targets a cAMP-dependent-protein kinase (Rutherford et al, 1982, 1984) which in turn alters the phosphorylation state and activity of substrate proteins (Chambers et al, 1987). Spore cell maturation triggered by increased intracellular cAMP might be mediated with this pathway (Kay, 1989). Disruption of the kinase expression has been shown to alter normal development (Firtel and Chapman, 1990). The PLC pathway gives rise to secondary messengers, inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DG). These molecules can modulate gene expression possibly through protein kinase C (PKC) and calmodulin (Ginsburg and Kimmel, 1989). The cGMP-associated pathway may be important during chemotactic aggregation because it has been observed that cGMP can induce myosin polymerization (Liu and Newell, 1988).

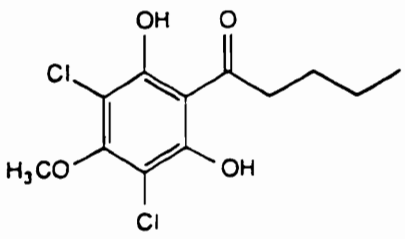
Recently, three more cell surface receptors for cAMP have been identified and two of them have been cloned (Johnson et al, 1992; Johnson et al, 1993; Saxe et al, 1993) in addition to cAR1, the one that is responsible for aggregation. Both mRNA and protein of cAR3 appear at early aggregation (6 hours of development) and are present in both cell types until culmination (22 hours of development). The cAR2 is expressed just before tip formation stage, then preferentially in prestalk cells. The presence of several cAMP receptors may account for the multiple roles of cAMP during *Dictyostelium* development.

The second developmental signal, DIF, is required for prestalk cell differentiation (Brookman et al, 1982; Kay and Jermyn, 1983; Town and Stanford, 1979; and Williams et al, 1987). The structure of three DIF molecules have been identified (Berks et al, 1991). They are closely related chlorinated alkyl phenones (Figure 2). The expression of prestalk-cell-specific marker genes emcA and emcB appear when DIF is present (Williams et al, 1987). DIF is also an inhibitor of cAMP-mediated prespore-specific gene expression (Berks and Kay, 1990). The DIF signalling system is poorly understood. It is not clear if DIF signal is transduced through a cell surface receptor or is directly transmitted into nucleus. A specific DIF binding protein and a DIF dechlorinase have been discovered (Insall and Kay, 1990; Nayler et al, 1992). A current report (Kubohara et al, 1993) described a molecule termed Differanisole A that was isolated from a soil microorganism, Chaetomium strain RB-001, and can induce the differentiation of Friend Leukemic Cells (mouse leukemia cells). Interestingly, this molecule has a similar structure to DIF-1 and can replace DIF-1 in inducing stalk cell differentiation.

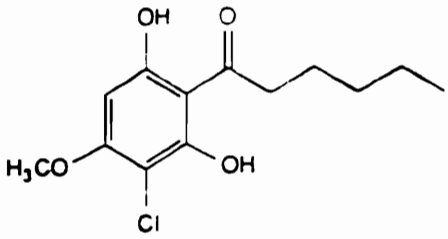
There is no evidence indicating that NH_3 alone can induce gene expression. However, it is thought that this molecule favors prespore cell differentiation, while inhibiting prestalk cell differentiation. For example, initial prespore cell differentiation could be facilitated by NH_3 (Gross et al, 1983), and the expression of some



DIF - 1



DIF - 2



DIF - 3

Figure 2. DIF structures.

prespore-specific genes could be enhanced by this molecule (Oyama and Blumberg, 1986b).

Adenosine is another molecule that has been shown to function as a developmental signal in *Dictyostelium*. Similar to NH_3, no gene has been reported to be activated by adenosine alone. Adenosine appears to antagonize the cAMP effect and prespore cell differentiation. It inhibits the binding of cAMP to its receptor (Theibert and Devreotes, 1984; and Van Lookeren Campagne et al, 1986). Depletion of adenosine in intact slugs induces conversion of prestalk cells to prespore cells (Schaap and Wang, 1986).

The protein products of over 100 developmentally regulated genes in *Dictyostelium* have been identified with cell-type-specificity (Williams, 1985; Schaap, 1986). A number of the corresponding genes have been cloned and some of their regulatory elements have been analyzed. The cellular developmental program has been well established and awaits its molecular description. The discovery of two key morphogens, cAMP and DIF, makes it possible to study a cascade of biochemical and molecular genetic processes from an original developmental signal to differential gene expression. Molecular behavior of a specific developmentally-regulated gene, therefore, can be integrated into the ultimate picture of development.

Glycogen Phosphorylase Genes in *Dictyostelium*.

Glycogen degradation is one of the major biochemical events during *Dictyostelium* development. The degradation of glycogen produces glucose precursors which are essential for synthesizing the structural end products of differentiated *Dictyostelium* cells

such as cellulose (Gustafson and Wright, 1972). The first step of the degradation is catalyzed by glycogen phosphorylase (1,4-a-D-glucan:orthophosphate-a-glucosyltransferase; EC 2.4.1.1). As characterized in early work (Firtel and Bonner, 1972), the activity of this enzyme, now termed glycogen phosphorylase 2 (gp2), appeared when cells begin to aggregate (~8 hours of development) and increased to the maximal level at culmination (Figure 3). This 5' AMP independent activity is not present during vegetative growth and pre-aggregation stage. It was also shown that the enzyme activity stopped increasing during development when the RNA synthesis and protein synthesis were inhibited, suggesting that the appearance of glycogen phosphorylase activity is due to *de novo* synthesis. In terms of temporal regulation, the glycogen phosphorylase may belong to the group of proteins which appear at 8-10 hours of development (see the section of **Developmental gene regulation in *Dictyostelium*** in this chapter).

About a decade ago, another glycogen phosphorylase activity, now termed glycogen phosphorylase 1 (gp1), was detected (Rutherford and Cloutier, 1986). In contrast to gp2, the new activity, gp1, was found to be 5'AMP dependent and present in vegetatively growing cells. As the development proceeds, gp1 activity decreases gradually (Figure 3). It becomes inactive at culmination stage when gp2 activity peaks. It is interesting that the two activities are complementary to each other, presumably to maintain constant level of glycogen phosphorylase activity throughout development. The regulation of the two forms of glycogen phosphorylase appeared to be similar to that seen in mammalian muscle, where the active (5' AMP independent) and inactive (5' AMP dependent) forms are reversible through phosphorylation and dephosphorylation of one protein. However, the evidence that was available suggested that the two forms of glycogen phosphorylase were two distinct proteins encoded by two separated genes (Naranan et al, 1988a; Naranan et al, 1988b). It is of interest to understand why

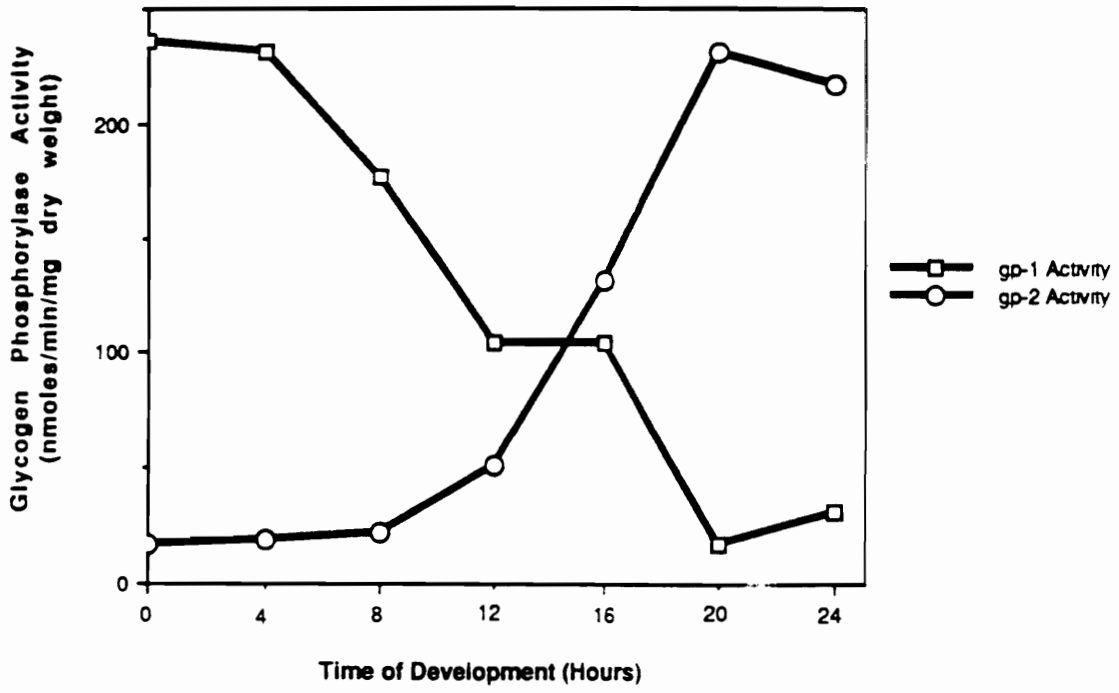


Figure 3. Glycogen phosphorylase activity during *Dictyostelium discoideum* development. Both gp1 (5' AMP-dependent) and gp2 (5' AMP-independent) activities were measured with the cells developed on non-nutrient buffer saturated filter for 0, 4, 8, 12, 16, 20 and 24 hours.

Dictyostelium uses two glycogen phosphorylase genes to fulfill one biochemical function and how these two genes are regulated and interact to meet the developmental requirements.

It has been well established biochemically that glycogen phosphorylase is crucial for terminal cell differentiation in Dictyostelium. In order to obtain a comprehensive understanding of the differentiation process, it is necessary to elucidate the genetic program of the glycogen phosphorylase gene. Knowledge from such a study will not only uncover the nature of a single gene, but also promote understanding of the molecular mechanism underlying developmentally regulated gene expression in general. In this dissertation, I will demonstrate the molecular basis underlying the expression of the glycogen phosphorylase gene during development, through a series of studies including gene cloning, nucleotide sequencing, temporal and spatial regulation of the gp2 gene by developmental signals, analysis of cis-acting regulatory elements in the gp2 gene, and related objectives.

Chapter 2. Cloning of Glycogen Phosphorylase Genes

Abstract

A *Dictyostelium discoideum* genomic DNA library was constructed and screened for glycogen phosphorylase-1 (gp1) and -2 (gp2) genes. A total of 18 kb genomic region containing the whole gp2 gene including entire 5' and 3' noncoding region, as well as adjacent genomic regions was cloned. A 4.4 kb fragment containing the 5' gp1 coding region and the entire 5' noncoding region was also cloned from the same library. DNA sequencing predicted that two introns separate the gp2 open reading frame into three exons that together are 2976 bp in length. A gp2 cDNA was cloned and sequenced. The result confirmed the existence of two introns in the gp2 gene. The first intron is 105 bp and the second is 109 bp. Sequencing data also revealed an open reading frame downstream of the 3' noncoding region of the gp2 gene. The peptide derived from this partial open reading frame has no significant similarity to any known protein sequence.

Introduction

Glycogen phosphorylase was characterized by earlier work as a developmentally regulated activity , which appears in the cells undergoing differentiation (Firtel and Bonner, 1972; Thomas and Wright, 1976; Higgins and Dahmus, 1982). About a decade ago, two forms of glycogen phosphorylase were recognized when a previously unknown glycogen phosphorylase activity was detected (Rutherford and Cloutier, 1986). The initially characterized activity is now known as gp2, while the newly found form is termed gp1. The gp1 activity is seen in undifferentiated cells during vegetative growth and diminishes graduately as the development progresses. At ~20 hours of normal development (culmination stage), gp1 becomes inactive. The activity of gp1 is 5'AMP-dependent. In contrast, the gp2 activity is undetectable in vegetative cells. This activity appears after cells have developed for 6-8 hours, then increases to its maximal level at 20 hours of development. In higher eukaryotic systems such as mammalian muscle, glycogen phosphorylase activity exists in two forms of one protein. The inactive "b" form (5'AMP-dependent) and the active "a" form (5'AMP-independent) are interconvertible through phosphorylation and dephosphorylation. Initially, it was thought that the gp1 and gp2 activities in *D. discoideum* were regulated in the same manner. However, a number of previous workers have concluded that the increase in gp2 activity during development is a result of *de novo* synthesis of the protein (Firtel and Bonner, 1972; Thomas and Wright, 1976; Higgins and Dahmus, 1982). Isozymes of glycogen phosphorylase that are the products of two different genes have been reported in rat heart tissue (Berndt et al, 1987). Also, it has been observed that two forms of UDP-glucose pyrophosphorylase might be encoded by two distinct genes (Fishel et al, 1982; Fishel et al, 1985; Haribabu, 1986).

Most importantly, a line of evidence strongly suggested that gp1 and gp2 are different protein molecules. For example, they differ in peptide maps, molecular weight on SDS-PAGE gels (gp1 of 92 kd and gp2 of 104 kd), affinity constants, and elution profiles from DE52 cellulose.

Cloning and sequencing of the gp2 gene showed that it is distinct from the gp1 gene (Rogers et al, 1992). The sequence analysis of the gp2 gene also revealed many interesting structural features of the gene, some of which might be important in regulating the gene during cell differentiation.

Results

Genomic cloning of the gp2 gene.

A partial EcoRI genomic library of *D. discoideum* was constructed using pBluescript II SK+ as the cloning vector. A total of 40,000 colonies were probed with a PCR-amplified gp2 DNA fragment (PCR primers were designed according to the partial peptide sequences of gp2). Twelve positive clones were identified and confirmed by sequencing the ends of the inserts (Figure 4). The DNA sequences in these clones cover the whole gp2 coding region and both 5' and 3' non-coding region. The cloned sequences also extend into the adjacent genomic region containing other genes. The orientation of the inserts in the vector were determined by DNA sequencing of the ends of the inserts.

Nucleotide sequencing of the gp2 gene.

A series of deletions of the gp2 gene were prepared by exonuclease III and mung bean nuclease digestions (Figure 5). These deletions then were used for sequencing the gp2 gene. Figure 6 shows the nucleotide sequence of the gp2 gene. The data revealed an open reading frame of 2976 bp that consists of three exons separated by two introns (see the section of **cDNA cloning and intron identification**). The gp2 gene sequence, like most other *Dictyostelium* genes, is flanked by extremely (A+T)-rich (~80%) noncoding sequences. The transcriptional start site was found 275 bp upstream of the AUG codon by primer extension. Directly upstream of the AUG codon for translational initiation is an

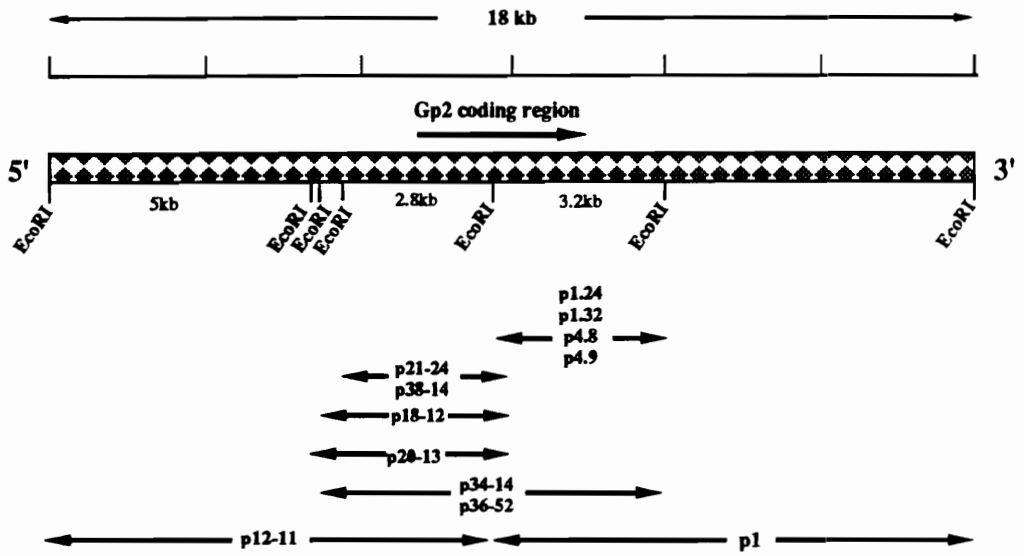
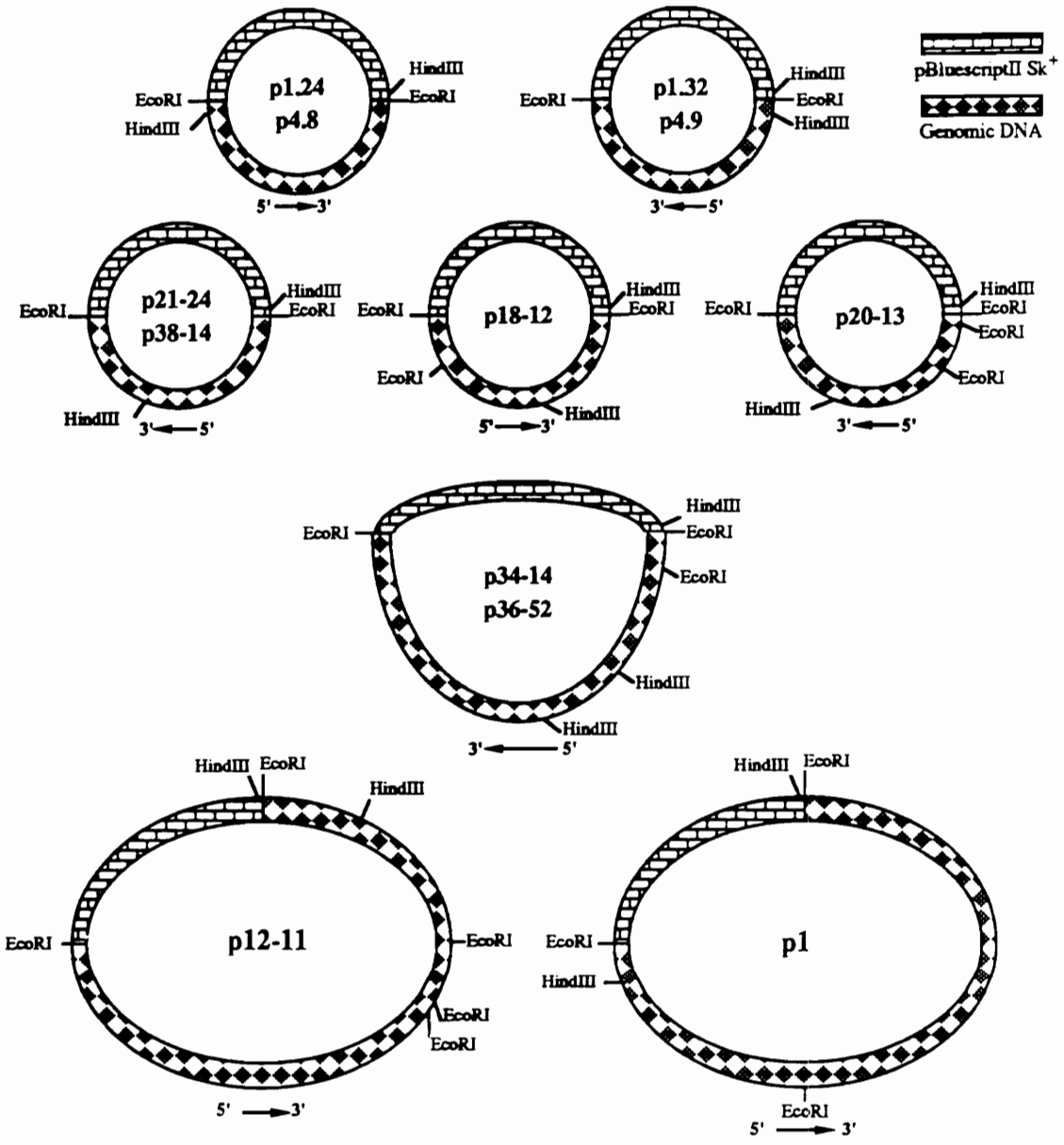


Figure 4. Maps of gp2 genomic clones. (A) Linear maps. A total of 18 kb genomic sequence is represented in 12 clones. The two biggest clones p1 and p12-11 span the entire 18 kb region with each containing 9 kb of the sequence. The sequences in clones p1.24, p1.32, p4.8 and p4.9 cover the same 3.2 kb region. The 3' ends of clones p21-24 and p38-14, p18-12, and p20-13 are identical, while the 5' ends of p21-24 and p38-14 are about 0.5 kb shorter than p18-12, and p20-13 is approximately 0.2 kb longer than p18-12 at the 5' end. Clones p34-14 and p36-52 contain the same 6.5 kb genomic sequence. (B) Circular (plasmid) maps. The circular maps indicate the orientations of the gp2 inserts in the vector. Clones containing identical genomic sequences and with the same orientation are presented in one map. These include p1.24 and p4.8, p1.32 and p4.9, p21-24 and p38-14, and p34-14 and p36-52.


p1.24
p4.8
EcoRI
HindIII
HindIII
EcoRI
5'→3'
p1.32
p4.9
EcoRI
HindIII
EcoRI
HindIII
3'←5'
pBluescriptII Sk+
Genomic DNA
p21-24
p38-14
EcoRI
HindIII
EcoRI
HindIII
3'←5'
p18-12
EcoRI
HindIII
EcoRI
EcoRI
5'→3'
HindIII
p20-13
EcoRI
HindIII
EcoRI
EcoRI
EcoRI
HindIII
3'←5'
p34-14
p36-52
EcoRI
HindIII
EcoRI
EcoRI
HindIII
HindIII
3'←5'
p12-11
HindIII
EcoRI
HindIII
EcoRI
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HindIII
EcoRI
5'→3'

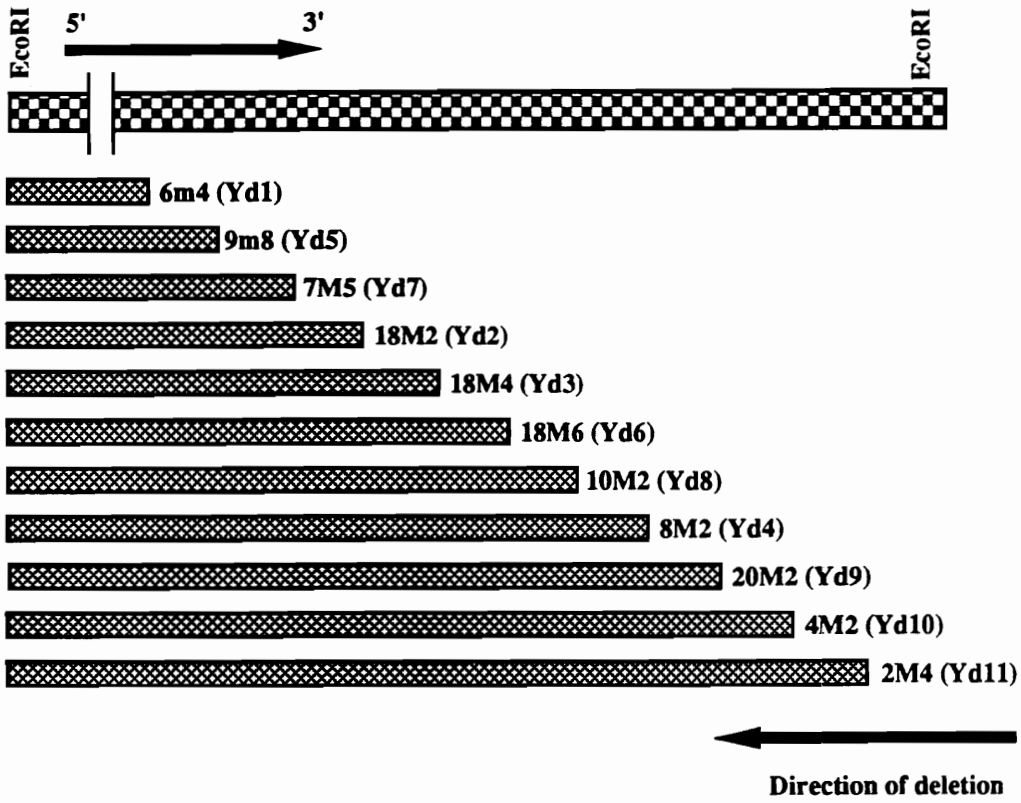
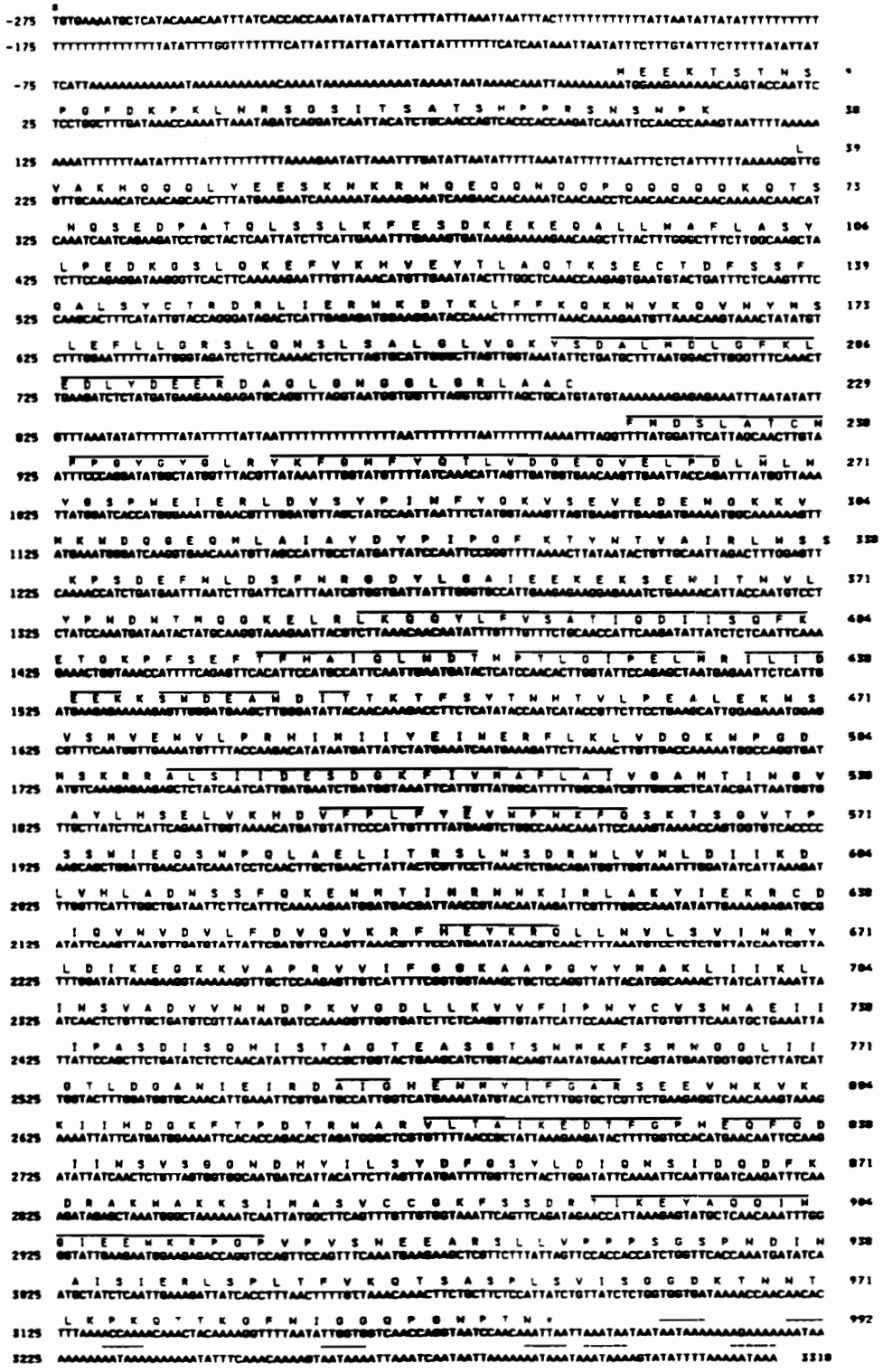


Figure 5. Deletion map of gp2 genomic clone p4.8. A total of 11 deletions was prepared by exo III nuclease and mung bean nuclease digestions. The gp2 fragment was deleted from the 3' to the 5' end. Each of the deletions is different in 100-200 bp.

Figure 6. Nucleotide sequence of gp2 gene (Rutherford et al, 1992). The nucleotide sequence of the gp2 gene is shown as well as the deduced amino acid sequence. Overlining indicates the segments of amino acid sequence that were confirmed by peptide sequencing. Also shown is the start site for transcription (#), stop codon coding sequence (*), and polyadenylation signal sequence (------). Intron 1 is from nucleotide 112 to 220, and intron 2 is from nucleotide 793 to 897. Nucleotide numbering is on the left; amino acid numbering is on the right.



oligo A run (8 residues) which is a typical structure at the translational start site of *D. discoideum* genes. The last exon of the open reading frame is terminated at the consensus *Dictyostelium* stop codon UAA. Seven polyadenylation signal sequences are located downstream of the stop codon. In the second exon, there is a 45 bp sequence containing 11 CAA trinucleotides with five of them repeated consecutively. Long stretches of repetitive CAA sequence have been also discovered in other *Dictyostelium* genes (see **Discussion**). The gp2 open reading frame encodes a peptide of 992 amino acids with a calculated molecular weight of 112,500 Dalton and an isoelectric point of 6.4.

Downstream of the gp2 gene, a sequence representing a part of an open reading frame was discovered in an opposite orientation to the gp2 gene (Figure 7). The TAA sites corresponding to the translational stop codons of the two open reading frames are 348 bp apart. Deduced amino acid sequence was used to search GeneBank database and no known peptide sequence was found with significant similarity.

cDNA cloning and intron identification of the gp2 gene.

Two extremely (A+T)-rich regions (nucleotides 112-220 and 793-897) were recognized by examining the genomic nucleotide sequence of the gp 2 gene. This suggested that the two regions represent two introns in the gene, since *Dictyostelium* non-coding sequences are extremely (A+T)-rich and both the regions are flanked by consensus 5' and 3' intron splicing sequences. To test this, gp2 cDNA was prepared by RT (reverse transcription) -PCR using a pair of oligo-nucleotide primers (Figure 8B) and total RNA from slug stage cells. The cDNA products generated from the primer pairs flanking each of the introns are about 100 bp smaller than the products amplified from genomic DNA

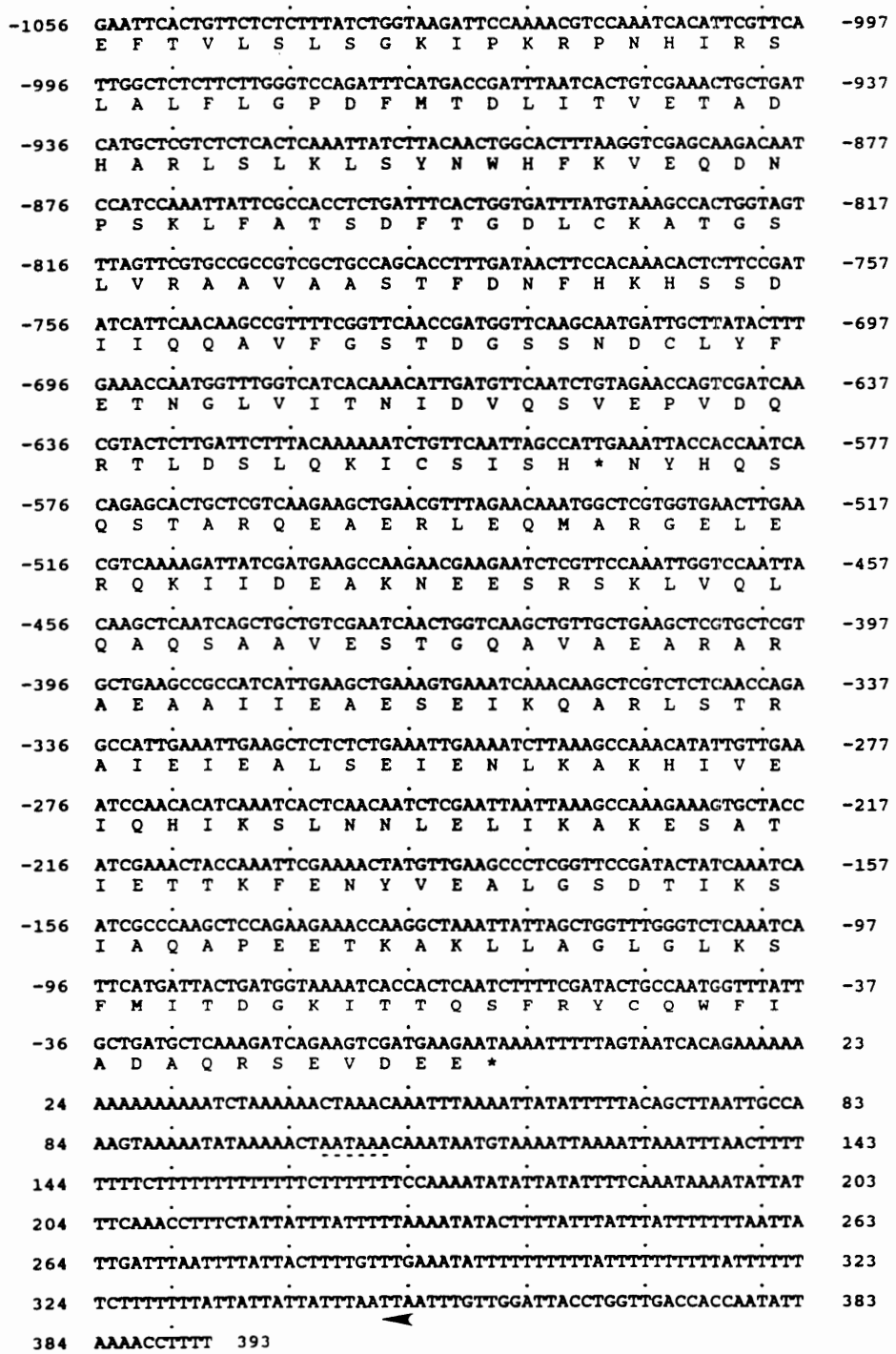


Figure 7. Nucleotide sequence at downstream of gp2 gene. The nucleotide sequence at downstream of the gp2 gene are shown as well as deduced amino acid sequence from a partial open reading frame. Stop codon site (*) and polyadenylation signal sequence (---) are also indicated. The complementary sequence of the gp2 stop codon site is marked with an arrow head.

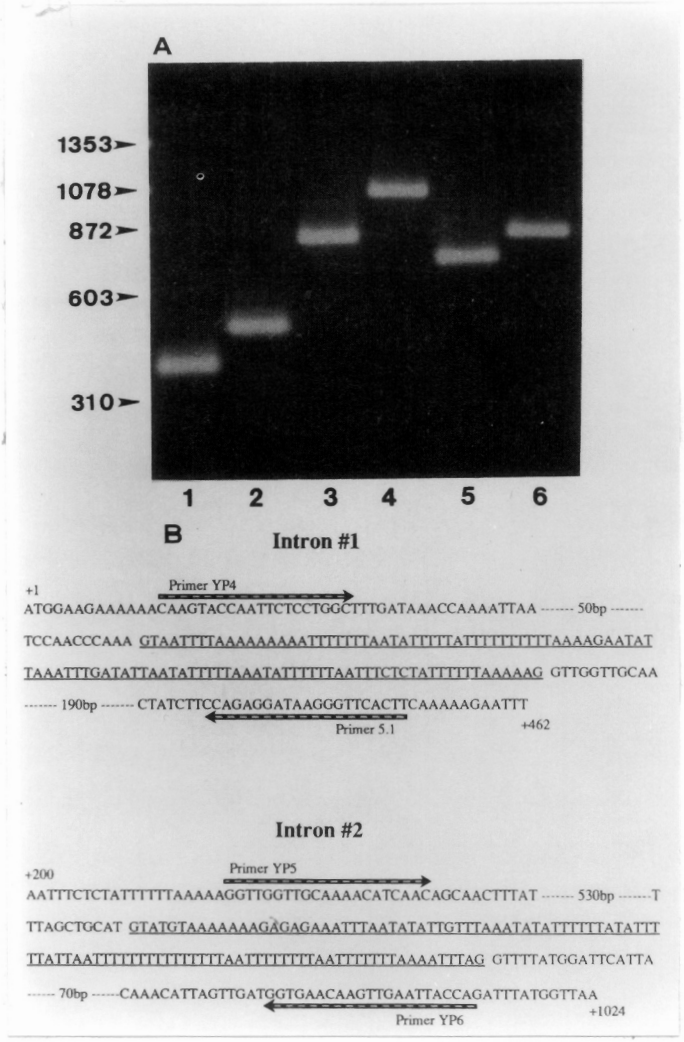


Figure 8. Gp2 cDNA synthesis and intron identification (Rutherford, et al, 1992). (A) Agarose gel of amplified cDNA. Lane 1, 3 and 5 are amplified cDNA fragments, while lane 2, 4 and 6 are parrellely-amplified genomic DNA fragments using the same pairs of primers. The primers used for lane1 and 2 flank intron 1, those for lane 3 and 4 flank both introns, and those for lane 5 and 6 flank intron 2. (B) Strategy for cDNA amplification. The primer pair of YP4 and 5.1 were used to amplify the regions flanking the first intron (lane 1 of A), and primer YP5 and YP6 to amplify the regions flanking the second intron (lane 5 of A). The cDNA containing entire exon 2 and a part of exon 1 and 3 were amplified by primers YP4 and YP6 (lane 3 of A). Sequences between the primers and the introns are indicated by dashes and a number.

(Figure 8A), and the PCR with the pair of primers that spanned both introns yielded a cDNA that is about 200 bp smaller than the genomic product (Figure 8A). The size differences between the products from mRNA and genomic DNA templates are due to the absence and presence of the intron sequences in the two templates. This was confirmed by nucleotide sequencing of the cDNA, which revealed precisely the absence of the two intron sequences in the cDNA.

Comparison of gp2 to gp1 and glycogen phosphorylases in other organisms.

The gp2 and gp1 genes were compared in their nucleotide sequences. No distinct similarity was observed at either their coding regions or at the 5' and 3' noncoding regions. However, examination of the deduced peptide sequences of gp1 and gp2 revealed 72.6% similarity and 55.6% identity (Figure 9). Figure 10 shows the amino acid sequence comparison of gp2 and glycogen phosphorylases from rabbit muscle and yeast. Gp2 exhibits 50% and 44% identity to the yeast and rabbit muscle enzymes, respectively. Comparison between gp2 and glycogen phosphorylases in *Escherichia coli* (Choi et al, 1989), potato (Nakano and Fukui, 1986), human liver (Newgard et al, 1986), and human brain (Newgard et al, 1988) revealed 47%, 47%, 47% and 44% identities, respectively. Figure 10 also shows several functional sites that have been derived from studies on the rabbit muscle enzyme. *Dictyostelium* gp2 possesses most of these sites, with several notable acceptions in terms of number of the conserved amino acid residues at these sites: (1) 1/9 at AMP binding site; (2) 0/3 at phosphorylation site; (3) 15/17 at active site; (4) 7/9 at pyridoxal phosphate binding site; (5) 3/4 at caffeine/purine inhibition site; (6) 6/8 at

glycogen storage site; and (7) 15/50 at dimerization site. For example, Asp-484 and Gly-677 in rabbit muscle glycogen phosphosrylase are replaced by Ser-567 and Ser-759 in gp2, respectively, at the active site. Also, at the pyridoxal phosphate binding site, Ileu-485 and Leu-683 in rabbit are substituted by Val-568 and Met-765 in gp2, respectively.

Genomic cloning of the gp1 gene.

The same partial EcoRI genomic library used for screening the gp2 gene was probed with a gp1 cDNA fragment. A total of 20,000 colonies were screened and one positive clone was identified and verified by nucleotide sequencing. This clone was found to contain a 4.4 kb insert that spans 1.4 kb 5' coding region and 3.0 kb upstream region (Figure 11). The entire 5' noncoding region of the gene has been sequenced and some regulatory sequences in this region have been studied (Sucic et al, 1993).

Figure 9. Amino acid sequence comparison of gp2 and gp1. Identical amino acid residues are indicated by lines. Conserved amino acid residues are indicated by two dots. Semi-conserved residues are indicated by one dot. The top line is gp1 and the lower line is gp2.

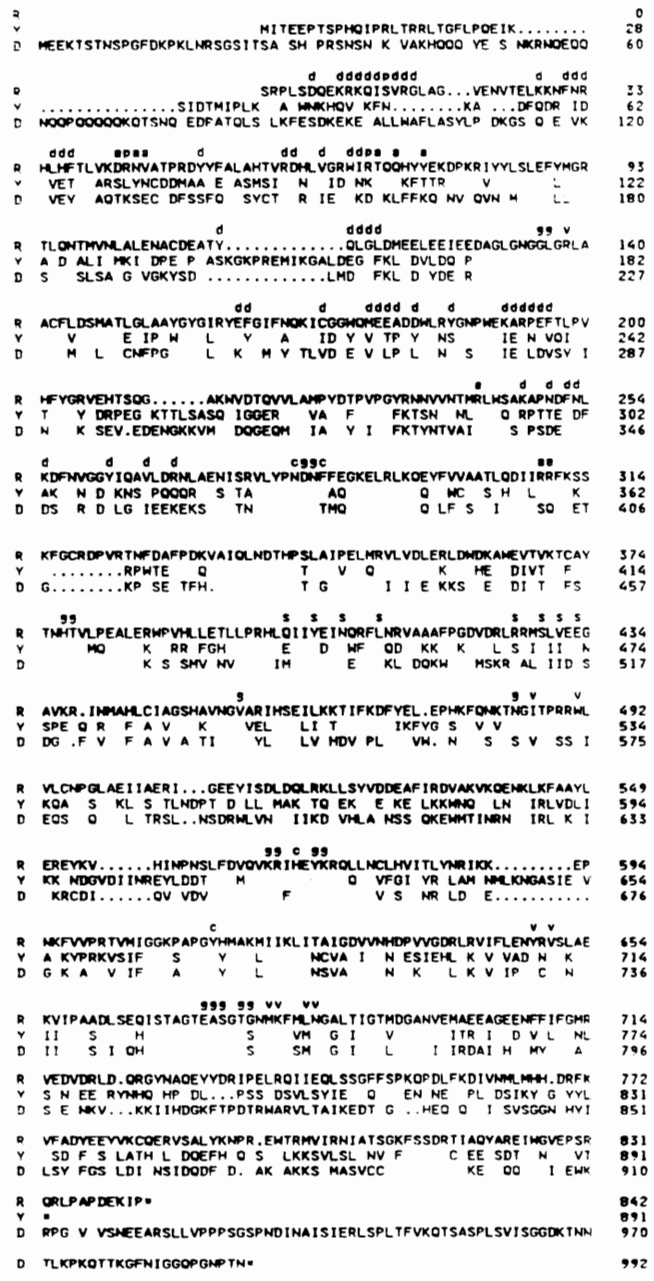


Figure 10. Primary structure comparison of *Dictyostelium* (gp2) (D), yeast (Y), and rabbit muscle (R) glycogen phosphorylases. Sequences were aligned sequentially using the gap/out subroutine of the Genetic Computer Group Program. Spaces indicate identity with the rabbit muscle protein. Gaps are shown by dots. Catalytic and regulatory sites of the rabbit muscle protein are marked above the sequence: a, AMP-binding site; c, coffeine/purine inhibition sites; d, subunit dimerization points; g, active sites; p, residues involved in phosphorylation; s, glycogen storage sites; v, cofactor (pyridoxal phosphate)-binding sites. *, stop codon.

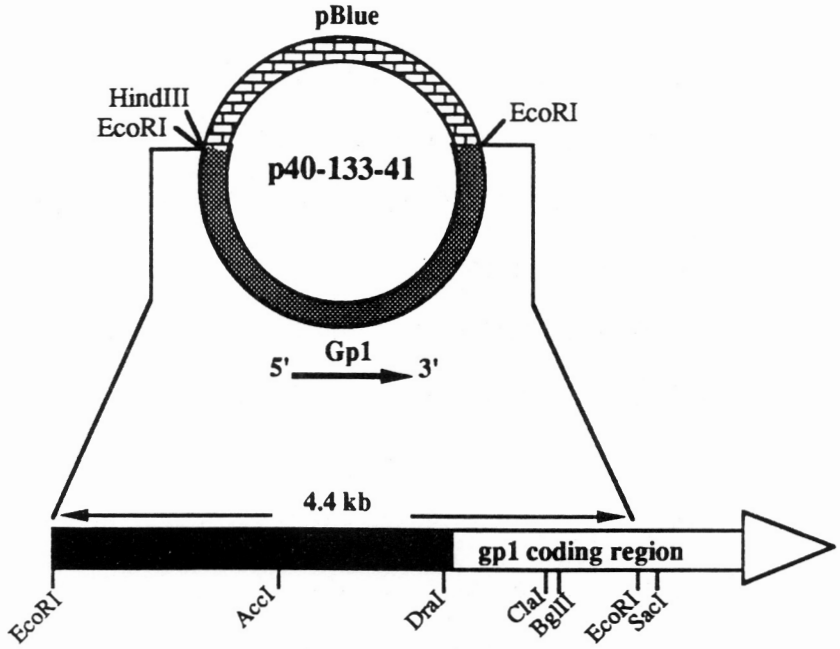


Figure 11. Map of gp1 genomic clone.

Discussion

Cloning and sequencing analyses of the gp2 gene revealed that the gene represents a structure typical of *Dictyostelium* genes. As seen in other *Dictyostelium* genes, the coding region of the gp2 gene is surrounded by extremely (A+T)-rich (>80%) upstream and downstream non-coding sequences. The sizes (~100 bp) and the extremely low G+C content (<20%) of the two gp2 introns are also typical of introns found in *Dictyostelium* genes. The majority of *Dictyostelium* introns are located near the 5' end of the coding region. This feature is also present in the gp2 gene. Whether or not this structure represents a regulatory signal awaits for further experimentation (see chapter 5). The 275 bp distance between the transcriptional start site and the translational initiation codon is also in the normal range for *Dictyostelium* genes. The most interesting feature in the gp2 gene is 11 CAA trinucleotide repeats in a 45 bp sequence that almost directly follow the first intron and resides in the second exon. CAA (or ACA, or AAC) repetitive sequence has also been discovered in other *Dictyostelium* genes in both coding and non-coding (5' and 3') regions (Kimmel and Firtel, 1985; Grant et al, 1990; Shaw et al, 1989; Andre et al, 1988). Possible developmental regulatory roles for the repeat have been suggested by several groups (for further discussion, see chapter 6)

Reports from the literatures are in agreement that the nondenatured *Dictyostelium* glycogen phosphorylase (gp2 form as identified now) exists as a homo-dimer (Thomas and Wright, 1972; Cloutier and Rutherford, 1987). However, the molecular weight of the subunit has been reported as 95,000 Da (Thomas and Wright, 1972), 90,000 Da (Cloutier and Rutherford, 1987), and 90,000-105,000 Da (Higgins and Dahmus, 1982). It was thought that the mature form (monomer) of the enzyme is 90,000 Da and is generated from

limited proteolysis. This was supported by the observation of a 110,000 Da glycogen phosphorylase from a *in vitro* translation system with poly(A)RNA (Rutherford et al, 1988). Since the calculated molecular weight of the deduced gp2 peptide (112,500 Dalton) is larger than any of the purified gp2 protein and is close in size to the one from *in vitro* translation system, gp2 may require a small peptide cleavage in order to activate the enzyme.

The nucleotide sequence of the gp2 gene is distinct from that of the gp1 gene, clearly indicating that the two forms of glycogen phosphorylase enzyme in *D. discoideum* are encoded by two different genes, and are not two interconvertible forms (phosphorylation and dephosphorylation) of one protein as seen in mammalian system. Sequence analysis showed the lack of a conserved phosphorylationsite in the amino acid sequence of gp2. This also explains the previously observed inability to phosphorylate or dephosphorylate gp2 *in vitro*, and to convert the enzyme from 5'-AMP-independent to 5'-AMP-dependent by dephosphorylation as observed in rabbit muscle (Naranan et al, 1988). In the rabbit muscle glycogen phosphorylase, there are 9 residues contributing to AMP-binding sites. However, only one of them (Arg-242) is conserved in gp2. Lack of the AMP-binding sites in gp2 is in agreement with the 5'-AMP independent property of the gp2 enzyme (Rutherford and Cloutier, 1986). The absence of the phosphorylation sites and AMP-binding sites in gp2 is characteristic of this glycogen phosphorylase in *D. discoideum*. Most of the residues for other functional sites, however, are conserved in gp2 including those for the active site, pyridoxal phosphate-binding site, caffeine/purine inhibition site, and glycogen storage sites.

A total of 12 gp2 genomic clones obtained from this study span 18 kb of the genomic region in which the gp2 gene resides. This 18 kb region might contain 4 or more genes assuming an average size of a *Dictyostelium* gene is 4 kb (including non-coding

region). The other genes in the vicinity might have certain structural relationship with the gp2 gene such as DNA-looping/coiling and DNA-phasing. These clones could be useful for such types of study. Most importantly, elements that are involved in both gp2 and gp1 gene regulation can be studied with the availability of needed genomic sequences.

Materials and Methods

Growth of *Dictyostelium* cells.

Cultures of *D. discoideum* AX3 cells were started on DM agar (Podgorski et al, 1982) plates in association with *Escherichia coli* b/r cells at 21°C. A large scale preparation of the cells was obtained from a HL5 (Firtel and Bonner, 1972) broth culture inoculated with vegetative cells from the DM agar plates, and incubated on a rotary shaker (180 rpm) at room temperature.

DNA preparations

Nuclear DNA was prepared from AX3 cells according to a previously described procedure (Welker et al, 1985), with modifications. Vegetative cells were lysed with NP-40 lysis buffer (Firtel and Lodish, 1973) followed by differential centrifugation at 12,000 x g for 10 minutes to recover nuclei. The nuclei then were lysed using a EDTA (0.2 M) / Sarcosyl (2% w/v) solution, pH 8.4 at 65°C for 5-15 minutes. The lysate was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and the DNA precipitated with EtOH and was purified using a CsCl-ethidium bromide density gradient (Welker et al, 1985).

For plasmid isolation from *E. coli* cells, the standard procedures were used (Sambrook et al, 1989).

Construction and screening of genomic library

For construction of the genomic library, 1 μg nuclear DNA from *D. discoideum* AX3 cells was partially digested with 10 units of the restriction enzyme EcoRI at 37°C for 5 minutes. The partially digested nuclear DNA then was ligated with 0.1 μg EcoRI-digested and Calf Alkaline Phosphatase (CIP)-treated vector pBluescript II SK+ (Strategene). The ligation was carried out using T4 DNA ligase at 4°C for 16 hours. Transformation of *E. coli* XL1-Blue cells with the ligation mixture was performed according to standard procedures (Sambrook et al, 1989).

For screening gp2 clones, a total of 40,000 colonies were blotted onto nitrocellulose filters (Nitroplus 2000, MSI), prehybridized (50% deionized formamide, 5X SSPE, 10X Denhardt's, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA) at 42°C for 2 hours, and hybridized (50% deionized formamide, 5X SSPE, 1X Denhardt's, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA) with two ^{32}P-labeled PCR-amplified-gp2 DNA probes (nucleotides 711-1474 and1767-2561) at 42°C for 16 hours. The blots were washed twice with 2X SSC + 0.1% SDS at room temperature for 15 minutes each, and once with 0.2X SSC + 0.1% SDS at 65°C for 30 minutes. Gp1 clone screening was carried out in the same way as for gp2, but using a 1.4 kb gp1 cDNA fragment as probe.

Nucleotide sequencing

Prior to sequencing, a set of nested deletions of cloned gp2 fragments in pBluscript II SK+ were prepared by exonuclease III and mung bean nuclease digestion (Strategene).

The experiment was performed according to the protocol given by the manufacturer. The chain termination method was employed for sequencing using a procedure provided by United States Biochemicals, Inc (USB). The sequenase version 2 sequencing kit (USB) was used for all of the sequencing reactions. Both single- and double-stranded template DNA were used. Sequences were analyzed using the Genetics Computer Group Program, version 5.0.

Gp2 cDNA cloning

An antisense cDNA strand was obtained by reverse transcription using total RNA prepared from slug stage cells (Rutherford et al, 1992) and a primer corresponding to nucleotides 993-1012. The synthesis of the sense strand cDNA as well as the following amplification of the cDNA were carried out in a thermal cycler with Taq polymerase. Three pairs of primers, flanking nucleotides 14-450, 221-1012, and 14-1012, were used to generate 3 segments of gp2 cDNA, covering the 1st intron, the 2nd intron, and both introns. The amplified cDNA was then cloned into pBluescript II SK$^+$ (Strategene) at the EcoRI site through EcoRI recognition sequences linked to the ends of the primers.

Chapter 3. Genomic analysis of the Disrupted gp1 Gene

Abstract

Availability of glycogen phosphorylase gene defects and mutants is of great interest for understanding the relationship between the gene and the developmental program. Gene-disruption based on homologous recombination and antisense RNA has been widely used to obtain targeted mutants. In the study described in this chapter, the nuclear DNA from cell lines that lacked gp1 activity were analyzed by restriction digestions followed by Southern hybridization. Four of them, which were transformed by a homologous recombination construct, showed alteration in the native gp1 gene. The other six were transformed by an antisense-RNA-producing construct. Genomic disruption of the gp1 gene was observed in three clones, whereas the other three showed no alteration.

Introduction

Studies using genetic mutants have been demonstrated as an effective approach to understand many fundamental biological processes throughout the history of experimental biology. The two forms of glycogen phosphorylase, gp1 and gp2, in *D. discoideum* are regulated differently during the life cycle of this organism (see chapter 1) and encoded by two separate genes (Rutherford et al, 1992; Rogers et al, 1992). Studies on the defects and mutants from any one of them clearly will provide insights to understand the regulation of the two genes during development. Advances in DNA technology enable us to obtain a mutation at a targeted site. Homologous recombination between a native gene sequence and a segment of its homolog located in a transformation vector has been shown to be an effective way to disrupt the gene in many systems from bacteria to mammals. This method has been successfully used in *Dictyostelium* to disrupt myosin heavy chain (DeLozanne and Spudich, 1987), a-actinin (Witke et al, 1987), and cAMP receptor cAR2 (Johnson et al, 1993) genes. Another commonly used method is to make a DNA construct that can produce a fragment of antisense RNA with respect to the targeted gene. Hybridization of the antisense and sense RNAs blocks translation from the sense RNA, thus, disrupts the gene expression. Inactivation of discoidin (Crowley et al, 1985) and myosin heavy chain (Knecht and Loomis, 1987) genes have also been demonstrated using this approach. Both gp1$^-$ and gp2$^-$ mutants have been prepared by homologous recombination and antisense RNA disruption (Rogers et al, 1993). This chapter demonstrates the genetic alteration in the gp1$^-$ mutants by Southern analysis of the genomic DNA from these mutants.

Results

Two DNA constructs, based on a transformation vector carrying a G418 resistance gene, were used to disrupt the gp1 gene (Rogers et al, 1993). The first one, pDd14F, was designed for homologous recombination and the second one was for antisense RNA. In the first construct, a 1.4 kb gp1 fragment was inserted between the actin 6 gene promoter and actin 8 gene terminator with the 5' end fused to the promoter and the 3' end fused to the terminator. The construct for producing antisense RNA, pDd14R, was made by fusing the 3' end of the same 1.4 kb fragment to the actin promoter and the 5' end to the actin terminator. This reversed gp1 fragment was transcribed under the direction of the actin promoter to produce an antisense RNA strand.

Nuclear DNA prepared from a total of ten gp1 mutants that lacked gp1 activity was analyzed by restriction digestion and Southern hybridization. Figure 12 shows a Southern blot of the nuclear DNA that was double-digested with AccI and SacI. AccI cuts at the 5' noncoding region of the gp1 gene and SacI cleaves the gp1 gene at the 3' end of the coding region. Because the sequence corresponding to the transforming 1.4 kb gp1 fragment resides between the two restriction sites, a disruption by homologous recombination in this region can be detected by an altered band on the Southern blot in comparison to the intact band from untransformed cells. Lane 13 shows a 3 kb band generated from the undisrupted gp1 gene (indicated by an arrow). Each of the four pDd14F transformants (9-2, 9-3, 9-4 and 9-5) gave the same banding pattern, and lacked the 3 kb gp1 band. Instead, a lower band appeared at the 2 kb position. The 3 kb native gp1 band was also missing in three of the pDd14R transformants, 11-3, 11-4 and 11-5. Clone 11-3 showed a 2.2 kb gp1 band and the other two gave a 2.8 kb gp1 band. The results indicate that the gp1 gene was disrupted in these clones by both pDd14F and pDd14R constructs. In clone

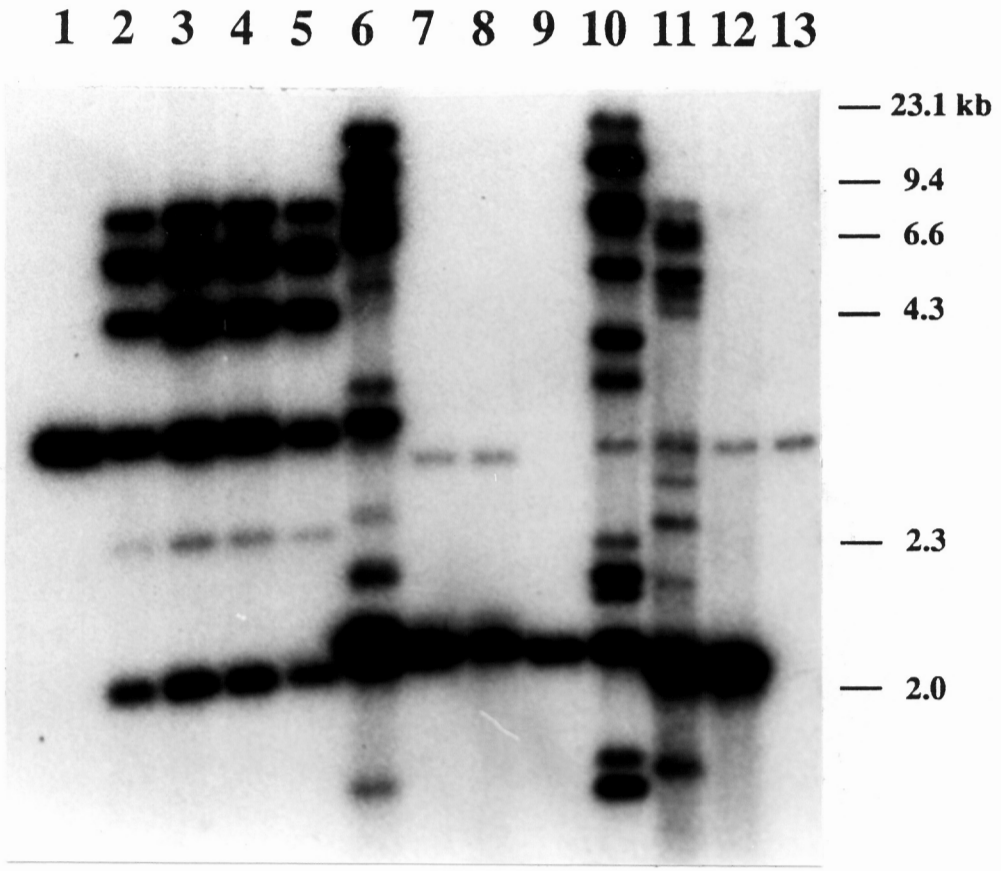


Figure 12. Southern analysis of genomic DNA from gp1⁻ mutants. One μg of genomic DNA and 1 ng pDd14F and pDd14R plasmid DNA were double-digested with AccI+SacI. The Southern blot was hybridized with ^{32}P-labeled 1.4 kb gp1 fragment (the same gp1 sequence as in pDd14F and pDd14R constructs). Lane 1 (pDd14F) and 9 (pDd14R) contain plasmid DNA. Lane 2 to 5 are pDd14F transformants 9-2, 9-3, 9-4 and 9-5, respectively. The pDd14R transformants are in lane 6 (11-3), 7 (11-4), 8 (11-5), 10 (13-5), 11 (15-2), and 12 (15-5). Lane 13 is the genomic DNA from untransformed *D. discoidem* AX3K cells. The gp1 genomic band from untransformed cell is marked by an arrow.

13-5, 15-2 and 15-5 which were also pDd14R transformants, the 3 kb band was present on the Southern blot, revealing that the gp1 gene was intact in these clones. This suggests that lack of gp1 activity in these clones was due to antisense RNA disruption.

On the same blot, all four pDd14F transformants showed the same pattern of genomic integration for the pDd14F vector DNA, while five different patterns were observed in the six pDd14R transformants (clone 11-4 and 11-5 gave identical patterns). Except for clones 11-4 and 11-5, the observed pattern does not indicate a single integration site in the genome, but instead suggests that multiple integration events occured during transformation. One of the integrations appeared to be in a tandem array pattern as indicated by the band showing the same migration as AccI+SacI double-digested pDd14F (lane 1) and pDd14R (lane 9) plasmid DNAs.

Discussion

The Southern analysis of the genomic DNA from the gp1$^-$ clones revealed that the native gp1 genomic structure was altered in all four pDd14F clones (9-2, 9-3, 9-4 and 9-5) and three (11-3, 11-4 and 11-5) of the six pDd14R clones. With the four pDd14F clones, it is clear that the absence of gp1 enzymatic activity resulted from the genomic disruption by homologous recombination. Lack of the gp1 activity in the pDd14R transformants was also likely due to the homologous recombination, since the wild type gene was altered. However, these transformants contained a pDd14R construct that was designed to produce antisense RNA, thus, antisense RNA disruption might also contribute to the lack of gp1 activity in 11-3, 11-4 and 11-5 clones.

Studies with the gp1$^-$ cells has revealed that the mutants grew normally in the vegetative phase and proceeded through development with no detectable differences from untransformed cells (Rogers et al, 1993). However, glycogen level increased 17-28 fold in amoebae, suggesting that there is no alternative glycogen degradation pathway in vegetative cells (Rogers et al, 1993). Also, in gp1$^-$ cells, no altered regulatory pattern was observed for gp2 activity during both vegetative growth and development (Rogers et al, 1993). In contrast, in gp2$^-$ cells, gp1 activity was retained without the normal decrease from vegetative growth to the end of development. This suggests that the expression of gp2 activity might be regulated by a cross-gene-communication mechanism (Rogers et al, 1993), which is important to insure proper regulation of glycogen degradation during *Dictyostelium* development.

Materials and Methods

Cell growth

Gp1$^-$ transformants were grown in HL5 medium (Firtel and Lodish, 1973) containing 20 μg/ml G418 at 21°C.

DNA preparation and Southern blotting

The isolation of nuclear DNA and preparation of Southern blot was performed using the same procedures as described in Chapter 2 of this dissertation.

Chapter 4. Establishment of an Extrachromosomal Luciferase Reporter Vector

Abstract

A reporter gene is usually employed to study the function of *cis*-acting regulatory elements. In order to introduce a regulatory element into cells and to select the cells containing the DNA element, a vector is used. To date, all of the reporter gene-containing vectors for *Dictyostelium* lack an origin of replication from this organism. Such vectors integrate into the host chromosomes and may generate potential dysfunctions of the reporter genes. An extrachromosomally-maintained reporter vector can overcome the possible disadvantages that result from using an integrating reporter vector. We have constructed an extrachromosomal luciferase reporter vector for *Dictyostelium discoideum*. This vector was stably retained in *D. discoideum* AX3K cells without alteration. The vector molecule was also found to exist in a relatively low copy number in the transformed cells.

Introduction

Dictyostelium discoideum is a lower eukaryote that has been a useful model system to study cell differentiation. To elucidate the mechanisms that lead to cell differentiation in this organism, it is essential to have an understanding of temporal and spatial expression of developmentally regulated genes. Analysis of *cis*- and *trans*- acting regulatory elements of these genes has been a focusing point to uncover the molecular machinery that controls the genetically programmed cell differentiation process. For *cis* element analysis, fusion of a segment of a DNA sequence with a reporter gene, such as luciferase or LacZ genes, is frequently used to test for the regulatory function of the segment. Thus, the expression of the reporter gene is under the control of the sequence to be tested (if it is indeed a *cis-acting* element). In order to introduce the fusion DNA into a recipient cell and maintain it in the cell for functional analysis, the fusion DNA needs to be carried in a plasmid vector. However, all of the currently available reporter vectors for *Dictyostelium* undergo integration into the chromosomes of a recipient cell after transformation. This is apparently due to the lack a *Dictyostelium* origin of replication in these vector. The integration appears to occur randomly at multiple sites on the chromosomes. This results in several potential problems. For example, the presence of an endogenous regulatory region that is adjacent to the vector integration site might influence the expression of the reporter gene. In addition, random and multiple integration events might generate various recombined structures at different integration sites. This type of integration pattern will complicate the expression pattern of the reporter gene. Moreover, inability to recover the vectors from *Dictyostelium* transformants makes it difficult to analyze certain phenomena which might be due to the genetic recombination between a sequence in the vector and a sequence in the

genome. An extrachromosomally-maintained vector containing a *Dictyostelium* origin of replication will overcome these problems. A number of nuclear-associated plasmids have been found in *Dictyostelium* (Metz et al, 1983; Noegel et al, 1985; Orii et al, 1989; Hughes et al, 1988). These plasmids provide useful sequences for construction of various vectors that can be used to target a DNA fragment of interest into the nucleus. Most importantly, no sequence identity was detected between the plasmids and *D. discoideum* genome (Noegel et al, 1985; Hughes et al, 1988), including chromosomal DNA, extrachromosomal rDNA and mitochondrial DNA. The lack of sequence identity ensures stable maintenance of the vectors whose construction is based on the plasmids. The origin of replication from one of these nuclear plasmids, Ddp2 (*Dictyostelium discoideum* plasmid 2), has been localized within a 626 bp fragment (Leiting et al, 1990; Chang et al, 1990; Slade et al, 1990). This fragment can be used to provide an autonomous replication sequence for the construction of various extrachromosomally-replicating vectors. This chapter demonstrates the construction of an extrachromosomally-maintained luciferase reporter vector for *Dictyostelium discoideum*, that is based on the 626 bp Ddp2 origin of replication.

Results

Plasmid construction.

The pVTL2 extrachromosomal luciferase reporter vector was constructed by fusion of a multiple-cloning-site-flanked luciferase gene with the backbone of plasmid p71d2-SB which is a derivative of plasmid p71d2 (Hughes et al, 1992). Figure 13A shows the construction of the pVTL2 vector that contains: (1) a luciferase reporter gene whose transcription is terminated by a *Dictyostelium* terminator from the *sp70* gene, (2) a *Dictyostelium* origin of replication and the *REP* gene from *Dictyostelium* plasmid Ddp2, (3) a G418 resistance gene whose expression is under the control of *Dictyostelium actin 6* promoter and *actin 8* terminator, and (4) an ampicillin resistance gene and an origin of replication for use in *Escherichia coli* cells, which are derived from plasmid pGEM3Z (Promega). Both the *REP* gene and the origin of replication from Ddp2 are necessary for extrachromosomal maintenance of the Ddp2 derivatives (Chang et al, 1990; Leiting et al, 1990). The pVTL2 vector has two multiple cloning sites; one at the 5' end and the other at the 3' end of the luciferase reporter gene. There are four unique restriction sites in the 5' region and five in the 3' region. This feature enables us to insert either a 5' or 3' *cis*-acting regulatory element into the vector at a site that is either 5' or 3' of the reporter gene. Thus, the vector is useful for studies with both 5' and 3' regulatory elements. Figure 13B shows the nucleotide sequence of the region that flank the luciferase gene. T7 and T3 promoters are present in the 5' and 3' multiple cloning sites, respectively. The presence of these promoter sequences provides primer (T3 or T7) binding sites for DNA sequencing, and thus enables confirmation of a correct fusion of an inserted DNA element. The pVTL2

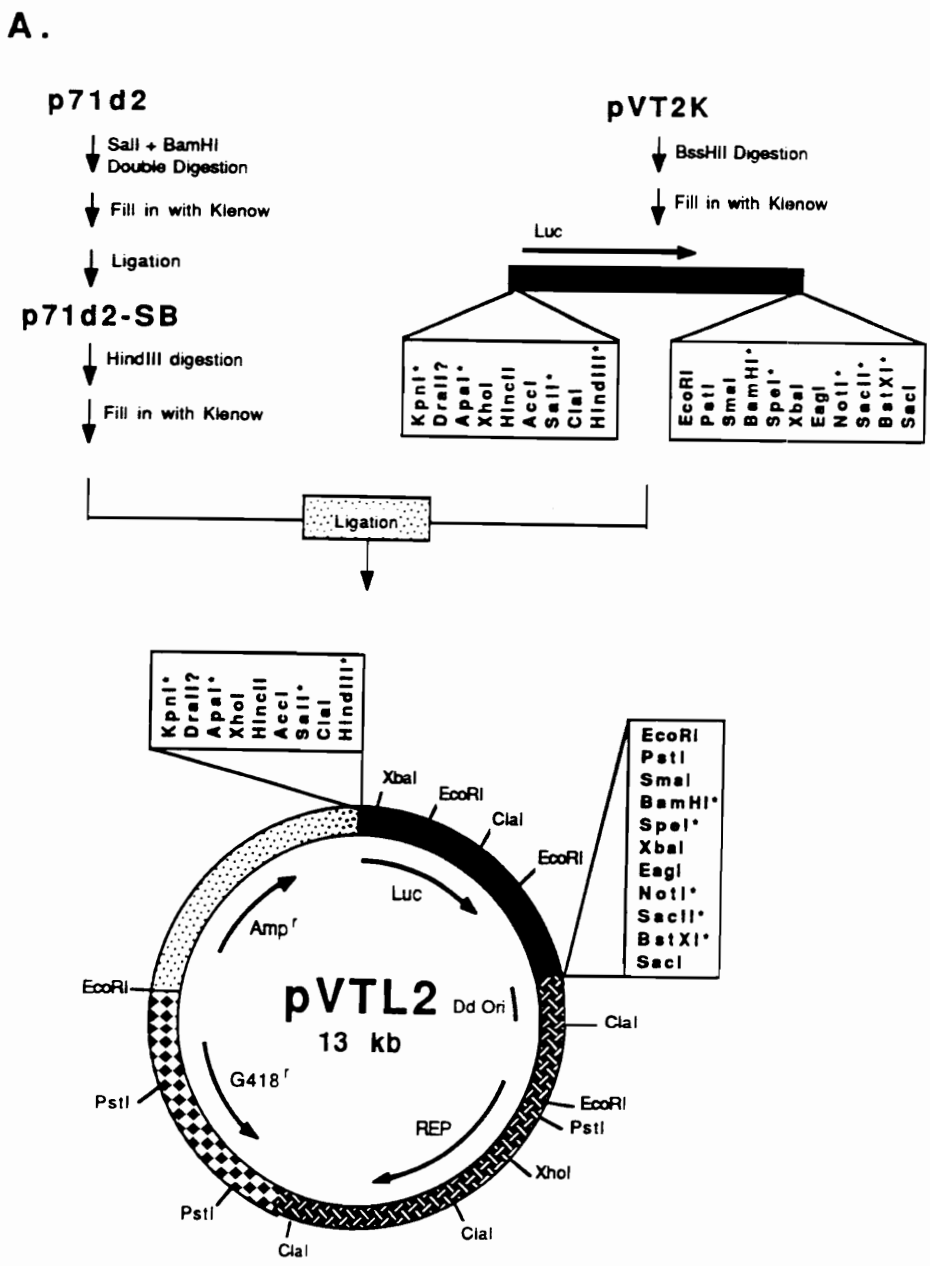
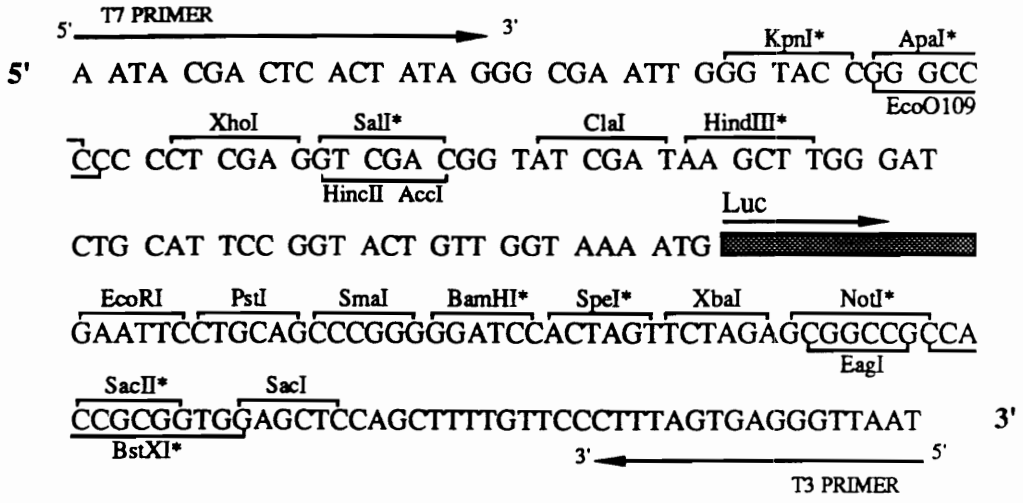


Figure 13. Maps of the pVTL2 vector. (A) A restriction map of the pVTL2 plasmid is shown. The restriction enzymes marked with * have an unique site in the multiple cloning regions of pVTL2. (B) Sequence surrounding the luciferase gene is also shown. The 5' region contains the T7 primer binding sequence, while the 3' region has the T3 primer binding sequence. The translation initiation codon (AUG) of the luciferase gene is present in the construct.

B.


T7 PRIMER
5'
3'
KpnI*
ApaI*
5' A ATA CGA CTC ACT ATA GGG CGA ATT GGG TAC CGG GCC
EcoO109
XhoI
SalI*
ClaI
HindIII*
CCC CCT CGA GGT CGA CGG TAT CGA TAA GCT TGG GAT
HincII AccI
Luc
CTG CAT TCC GGT ACT GTT GGT AAA ATG
EcoRI
PstI
SmaI
BamHI*
SpeI*
XbaI
NotI*
GAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCA
EagI
SacII*
SacI
CCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAAT
3'
BstXI*
3'
5'
T3 PRIMER

construct is a translational vector due to the presence of the initiation codon for translation (AUG) from the luciferase gene. This enables us to study regulatory elements that do not contain an ATG of their own, at translational level.

Extrachromosomal maintenance.

We have used a calcium phosphate-precipitation method (Nellen et al, 1984) to transform *D. discoideum* AX3K cells with the pVTL2 vector DNA. The transformation efficiency was 10^{-5} with 15 μg DNA, which is the typical range of transformation efficiency using this method. Therefore, the relatively large size of the pVTL2 vector (13 kb) does not lower the expected transformation efficiency.

The extrachromosomal maintenance of the vector was demonstrated by Southern hybridization of plasmid DNA prepared from the AX3K transformants. A *Cla*I-*Hind*III fragment containing the Ddp2 origin of replication was used as probe. Figure 14A shows the results from a representative experiment in which mini-preparations of pVTL2 plasmid from six independent *D. discoideum* AX3K transformants (lane 1-6) gave the same banding pattern as the pVTL2 plasmid DNA prepared from *Escherichia coli* host cells (lane 8). No hybridization was detected in the DNA prepared from untransformed AX3K cells (lane 7). The results indicate that the pVTL2 vector was maintained extrachromosomally in AX3K cells. The slowest migrating band in each of the six clones is likely the open-circle form of the plasmid. The faint bands between the open-circle band and the supercoiled band can be regarded as the linear form and partially-coiled forms of the plasmid resulting from incomplete neutralization during the preparation of the vector plasmid DNA. To further demonstrate the pVTL2 vector was retained in an unaltered form in *D. discoideum*

AX3K transformants, plasmid DNA prepared from the AX3K transformants was cleaved with restriction enzyme *Xho*I followed by Southern analysis using the same Ddp2 origin of replication sequence probe as described above. *Xho*I digestion of pVTL2 should produce two fragments of 5 kb and 8 kb. Because the 5 kb segment contains the origin of replication sequence, a corresponding band of 5 kb should hybridize with the probe. Figure 14B shows the expected result, a 5 kb band from XhoI digestion of DNAs prepared from AX3K cells and *E.coli* host cells. The AX3K cells were passed through 50 generations before they were used for the preparation of plasmid DNA. Therefore, the pVTL2 vector was quite stable in the AX3K transformants as an extrachromosomally-replicating element. The plasmid DNAs from both the AX3K transformants and the *E. coli* host cells were also examined in the same way with another restriction enzyme *Xba*I. Identical banding patterns were observed in both DNA preparations (data not shown).

Copy number of pVTL2 vector in AX3K cells.

The copy number of Ddp2 in its native strain is ~300 copies per cell (Hughes et al, 1990). The Ddp2 derivatives have been shown to have copy numbers between 100-300 copies per cell (Hughes et al, 1988; Chang et al, 1990; Leiting and Noegel, 1988). The copy numbers of these derivatives are somewhat lower than the native plasmid, but still relatively high. For a reporter vector, low copy number is preferred, simply because most of the elements to be tested are associated with genes that have low copy numbers themselves. We thought it was possible that the relatively large size of the pVTL2 construct (13 kb) might result in a low copy number vector plasmid. To determine the copy number of the pVTL2 vector, both nuclear DNA (containing the pVTL2 vector) and

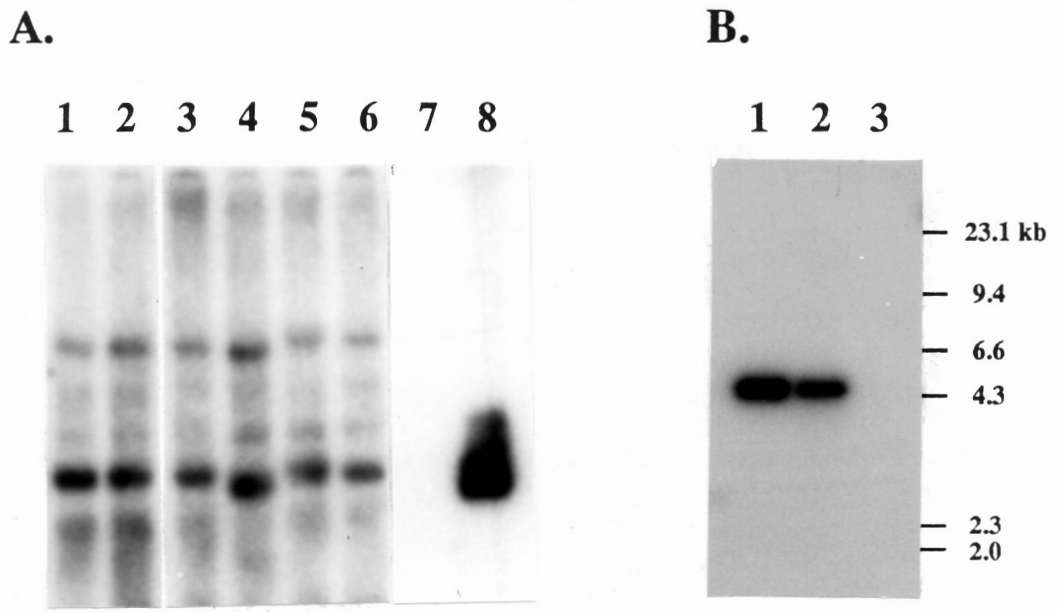


Figure 14. Southern analysis of the pVTL2 vector. (A) Undigested mini-prepared pVTL2 plasmid from 6 independent AX3K transformants (lane 1-6). was compared with the same plasmid prepared from *E. coli* host cells (lane 8). Lane 7: DNA from untranformed *D. discoideum* AX3K cells. (B) Large-scale prepared pVTL2 plasmid DNAs from both AX3K transformants and *E. coli* host cells were analyzed with restriction digestion. Lane 1: *Xho*I-digested pVTL2 DNA from the *E.coli* host cells. Lane 2: *Xho*I-digested plasmid DNA from an AX3K/pVTL2 transformant. lane 3: *Hind*III-digested λ DNA marker.

the pVTL2 plasmid DNA were separately prepared from the AX3K transformants and the *E. coli* host cells. The two DNAs were probed with the Ddp2 origin of replication and the resulting hybridization densities were compared. Because the size of pVTL2 (13 kb) is 2.6 x 10^{-4} as large as the size of *D. discoideum* genome (5 x 10^4 kb), 1 μg nuclear DNA from the AX3K transformants should give the same hybridization density as 0.26 ng pVTL2 plasmid DNA from the *E. coli* host cells, if there is only one copy of pVTL2 per cell. A dot blot (Figure 15A) was prepared with three amounts of (1) the pVTL2 plasmid DNA (0.26, 2.6 and 26 ng), (2) nuclear DNA (0.01, 0.1 and 1.0 μg) from four independent clones of the AX3K transformants named LV103, LV104, LV121 and LV122, and (3) nuclear DNA (0.1, 1.0 and 10 μg) from untransformed AX3K cells. The Ddp2 origin of replication sequence was again used as the probe in this experiment. The density of each hybridization dot was determined by a densitometer, and the densities from three dilution determinations were averaged for comparison. For the plasmid DNA, density readings from 0.26, 2.6 and 26 ng were divided by 1, 10 and 100, respectively. The sum of the three resulting readings was divided by 3 to give the average density for 0.26 ng plasmid DNA. For each of the four nuclear DNAs, the density readings from 0.01, 0.1 and 1.0 μg were multiplied by 100, 10 and 1, respectively. The resulting readings then were averaged to obtain the density for 1.0 μg nuclear DNA. Figure 15B presents a graphic version of relative densities of 1.0 μg nuclear DNA to 0.26 ng plasmid DNA whose density was designated as one unit. The results revealed that the copy numbers of LV103, LV104, LV121 and LV122 were 18, 38, 38 and 26 copies per cell, respectively, which are lower than all reported Ddp2-based plasmids (Hughes et al, 1992; Chang et al, 1990; Leiting and Noegel, 1988)

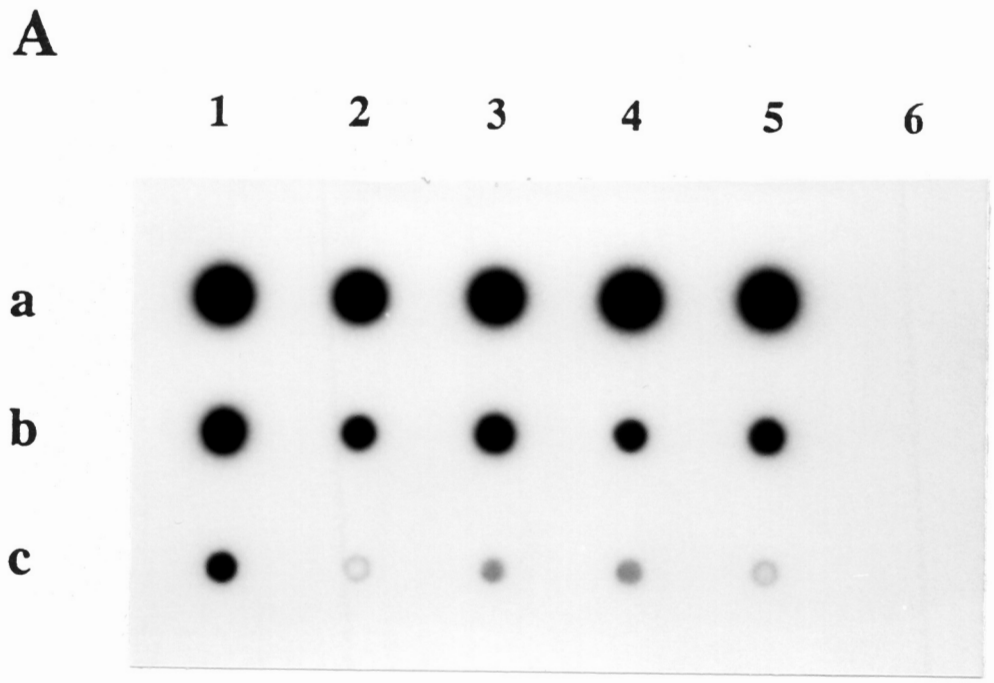
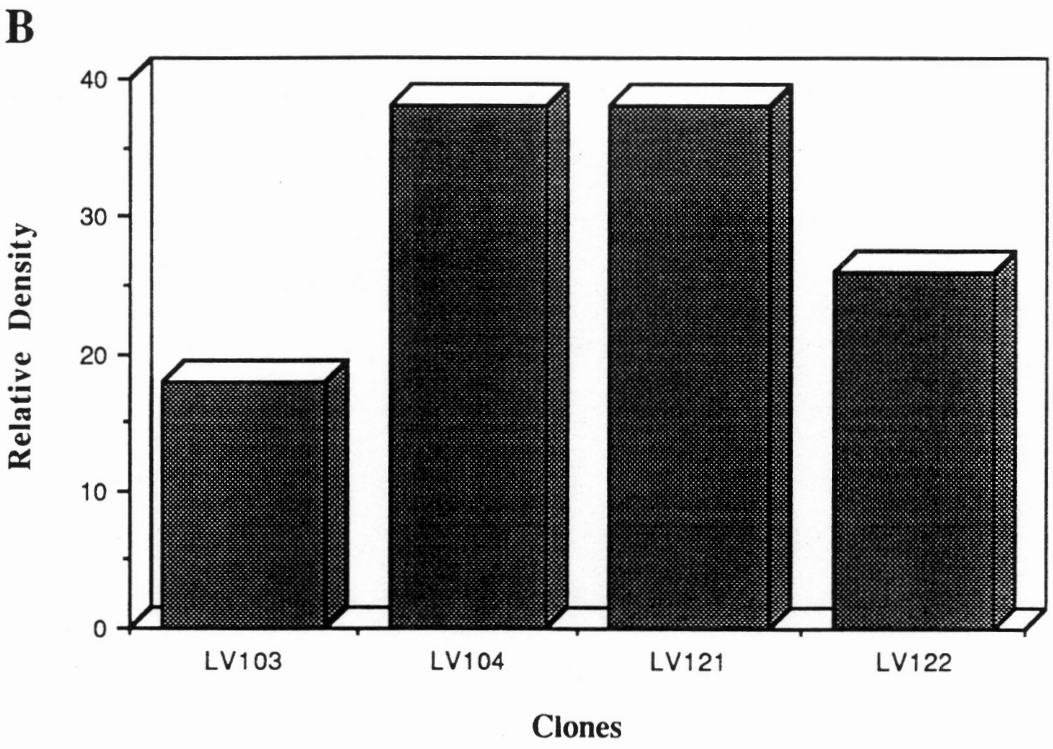


Figure 15. Copy number of the pVTL2 vector. (A) Southern hybridization of a dot blot. In the blot, column 1 contains 26 (a), 2.6 (b) and 0.26 (c) ng of plasmid pVTL2, and column 2, 3, 4 and 5 were loaded with 1 (a), 0.1 (b) and 0.01 (c) µg nuclear DNA from four independent clones, LV 103, LV104, LV121 and LV122, respectively. Row a, b and c of column 6 contain 1, 0.1 and 0.01 µg nuclear DNA, respectively, from untransformed AX3K cells. (B) Relative density of nuclear DNA to plasmid DNA. The average density of 1.0 µg nuclear DNA from each clone was divided by the average density of 0.26 ng plasmid DNA. The resulting relative densities represent the copy numbers of the pVTL2 vector molecule in a cell for each of the clones.


B
Relative Density
40
30
20
10
0
LV103
LV104
LV121
LV122
Clones

Discussion

An extrachromosomal luciferase reporter vector for *D. discoideum* has been established. This is the first extrachromosomal reporter vector that has been reported for the *Dictyostelium* system. The availability of such a vector will be beneficial for studying *cis*-acting regulatory elements in *Dictyostelium* genes. The stable extrachromosomal maintenance of the pVTL2 reporter vector has been shown in *D. discoideum* AX3K transformants. This demonstrated the usefulness of the vector, since the stable maintenance of the vector is crucial in order for regulatory elements to function correctly without alterations. Homologous recombinations have been observed in *D. discoideum* between a transforming DNA in an integrating vector and its complementary sequence in the chromosome (De Lozanne and Spudich, 1987; Witke et al, 1987; Rogers et al, 1993). The presence of *Dictyostelium actin 6* promoter and *actin 8* and *sp70* terminators (see the section of **Plasmid construction**) in the pVTL2 vector provides potential features for homologous recombination between the vector and *Dictyostelium* genome. However, our Southern analysis of the pVTL2 vector plasmid from AX3K transformants revealed that the vector was stably maintained in an unaltered form for 50 generations.

Copy number of the pVTL2 vector was determined to be between 18-38 copies per cell, which is the lowest number available among all reported *Dictyostelium* vectors. This increases the usefulness of the vector, because the low copy number is more close to the natural dosage found in most *Dictyostelium* genes (usually single copy). The average copy number of the four clones is less than 1/3 of the lowest copy number of the previously constructed vectors based on Ddp2 (Chang et al, 1990). It is possible that the copy numbers of the pVTL2 vector could be further reduced by decreasing the G418 selective pressure for the transformants. For the growth of the cells in this study, 5 μg/ml G418

was used. However, we have successfully maintained transforming DNA in AX3K cells with the same G418 selective marker by reducing the G418 concentration (in HL5 medium) to 1 μg/ml (Yin and Rutherford, unpublished observation). Many Ddp2 derivatives can be maintained stably in transformants without G418 selective pressure (Hughes et al, 1992; Chang et al, 1990). However, the pVTL2 vector is larger than the Ddp2-based plasmids, and therefore, it may be more difficult to maintain the pVTL2 vector for many cell generations in the absence of G418 selective pressure.

Materials and Methods

Plasmid construction.

A luciferase gene, which was fused to *Dictyostelium sp70* gene terminator, was prepared from plasmid pVT2K (Yin and Rutherford, unpublished data) by restriction digestion with *Bss*HII. The ends of the *Bss*HII-digested fragment were filled with Klenow enzyme. To prepare other parts of the vector, the plasmid p71d2 (Hughes et al, 1992, a generous gift from Dr. D.L. Welker) was digested with the restriction enzymes *Sal*I and *Bam*HI, filled in with Klenow enzyme, and religated. This digestion eliminated the *Sal*I and *Bam*HI sites in the resulting plasmid which is termed p71d2-SB. The elimination of these sequences was necessary for the final construct to have unique *Sal*I and *Bam*HI sites in the multiple cloning region. The p71d2-SB then was digested with *Hind*III, filled in with Klenow enzyme, and ligated with the *Bss*HII-digested and Klenow-filled luciferase gene-containing fragment. The fusion resulted in the final construct pVTL2.

Transformation.

D. discoideum AX3K cells were employed as recipient cells for the pVTL2 transformation. A previously described transformation procedure (Nellen et al, 1984) was used with a few modifications. A lower concentration of glycerol (12%) was used for the glycerol shock instead of 18%. After 2 days of treatment with 20 μg/ml G418, cells were plated onto a G418-containing (50 μg/ml) DM agar (Podgorski et al, 1982) in association

with G418 resistant *E.coli* B/r cells (Hughes et al, 1992). Before culturing the transformants for further analysis, colonies were streaked on new DM/G418 plates to ensure an isolation of pure independent clones.

DNA preparation and Southern blotting.

A previously described procedure (Hughes and Welker, 1988) of mini-scale *Dictyostelium* plasmid preparation was used to isolated plasmid DNA from cells grown on DM/G418 plates (50 μg/ml). The DNA was electrophoresed on a 0.8% agarose gel, transferred onto a nitrocellulose membrane (nitroplus 2000, MSI), hybridized (conditions as described in chapter 2) with a ^{32}P-labeled ClaI-HindIII fragment containing the Ddp2 origin of replication sequence, and exposed to an X-ray film.

For large scale plasmid DNA preparation, cells were cultured in HL5 (Firtel and Bonner, 1972) containing 5 μg/ml G418. Preparation of nuclei was performed according to a procedure described previously (Welker et al, 1985). Qiagen plasmid preparation columns (Qiagen) were used to isolate plasmid DNA from the nuclei according to the manufacturer's instructions. The plasmid DNA was digested with the restriction enzyme XhoI and analyzed in the same way as described for the undigested DNA.

Dot blot preparation.

Cells were grown in HL5 medium with the transformants under the G418 selective pressure of 5 μg/ml. A previously described procedure (Welker et al, 1985) was used to

isolate nuclear DNA. The dot blot was prepared using a Hybri-Dot Filtration Manifold from BRL. Before blotting, DNA was denatured with 0.1 volume of 3M NaOH at 65°C for 30 minutes, followed by a neutralization step with 0.1 volume of 3M NaAc, pH 4.8, at room temperature. A final volume of 0.5 ml DNA solution in 6X SSC was loaded onto a nitrocellulose membrane (nitroplus 2000, MSI). The membrane then was hybridized with ^{32}P-labeled Ddp2 origin of replication sequence.

The hybridization desity of the dot blot was determined by a video densitometer (version 620, BioRad).

Chapter 5. Dual Regulation of the gp2 Gene

Summary

Cell differentiation in *Dictyostelium* results in the formation of two cell types, stalk and spore cells. The stalk cells undergo programmed cell death, whereas spore cells retain viability. The current evidence suggests that stalk cell differentiation is induced by Differentiation Inducing Factor (DIF), while spore cell differentiation occurs in response to cAMP. We have discovered the first developmentally regulated *Dictyostelium* gene, the glycogen phosphorylase gene 2 (gp2) gene, that can be induced by both DIF-1 and cAMP, suggesting the possibility of a new group of developmentally regulated genes that have DIF-1 and cAMP dual responsiveness. The gp2 gene was found to be expressed in both prestalk/stalk cells and prespore/spore cells. The DIF-1-competence of the gp2 gene required uninterruped development, whereas the cAMP-competence for the gene required only starvation. Both DIF-1 and cAMP induction of the gene could be inhibited by NH_3, a factor that is thought to act as a developmental signal in *Dictyostelium*. Another developmental signal, adenosine, was found to repress the DIF-1 induction of the gp2 gene. Two introns in the gp2 gene were examined for their involvement in the regulation of the gene, but no regulatory function was detected. A model for the regulation of the gp2 gene during development is proposed.

Introduction

Dictyostelium discoideum is a simple eukaryote that has been used extensively as a model system to study development. The life cycle of this organism can be divided into two phases, unicellular growth and multicellular development. The switch from the growth phase to the development phase takes place when nutrients become limited. The developmental process commences when a population of homogeneous free-living cells (amoebae) aggregate, and then differentiate into two major cell types, stalk cells and spore cells. The stalk cells are programmed to die, while spore cells remain viable. Four diffusible molecules, cyclic AMP (cAMP), Differentiation Inducing Factor (DIF), NH3, and adenosine, have been shown to function as developmental signals (Berks et al, 1991; Kay et al, 1989; Kimmel and Firtel, 1991; and Williams, 1988). In addition to a chemoattractant role during aggregation, cAMP has also been demonstrated as a signal favoring prespore cell differentiation (Barklis and Lodish, 1983; Kay et al, 1978; Oyama and Blumberg, 1986a; Town et al, 1976; and Weijer and Durston, 1985). Recently, a family of cell surface receptors for cAMP have been identified (Johnson et al, 1992; Johnson et al, 1993; Saxe et al, 1993). Through these receptors, the cAMP signal regulates the activities of adenylate cyclase, guanylate cyclase, and phospholipase C. The resulting intracellular messengers, cAMP, cGMP, and IP3/diacylglycerol, carry the signals to downstream effectors such as cAMP-dependent protein kinase, cGMP-binding protein, protein kinase C, and Ca++/calmodulin (For a review, see Schaap, 1991).

In contrast to cAMP, DIF has been demonstrated to induce stalk cell differentiation (Brookman et al, 1982; Kay and Jermyn, 1983; Town and Stanford, 1979; and Williams et al, 1987). Five different species of DIF have been separated by fractionation, and three of

the molecular structures have been fully characterized (Kay et al, 1983; Masento et al, 1988; and Morris et al, 1987; Morris et al, 1988). DIF-1 is the most active form and accounts for 95% of the total DIF activity (Brookman et al, 1987). DIF-2 has been shown to be more active in inducing the conversion of prestalk cells to stalk cells, whereas DIF-1 is more active in converting vegetative cells to prestalk cells (Xie et al, 1991). DIF-1 also appears to be able to inhibit the expression of prespore cell marker genes (Early and Williams, 1988; Kopachik et al, 1985). A DIF-1 binding protein has been detected (Insall and Kay, 1990), whose maximal concentration occurs shortly before the rise of DIF, and before the prestalk/prespore pattern has been established. In addition, studies on two prestalk-cell-marker genes, ecmA (pDd63) and ecmB (pDd56), revealed that cAMP enhanced the DIF-1-induced ecmA gene expression, but repressed the DIF-1 induction of the ecmB gene. This suggests that cAMP might be involved in facilitation of the prestalk A cell differentiation, but inhibition of the prestalk B cell differentiation (Berks and Kay, 1990). A current report (Kubohara et al, 1993) described a molecule termed Differanisole A that was isolated from a soil microorganism, Chaetomium strain RB-001, and can induce the differentiation of Friend Leukemic Cells (mouse leukemia cells). Interestingly, this molecule has a similar structure to DIF-1 and can replace DIF-1 in inducing stalk cell differentiation.

As a byproduct of protein and nucleic acid degradation, NH_3 is known to function as a developmental signal in *Dictyostelium*. Depletion of NH_3 has been shown to induce the culmination process (Schindler and Sussman, 1977a). It has also been observed that initial prespore cell differentiation could be facilitated by NH_3 (Gross et al, 1983) and the expression of some prespore-specific genes could be enhanced by this molecule (Oyama and Blumberg, 1986b). However, it has also been reported that NH_3 did not appear to

preferentially facilitate the expression of either the prespore marker gene D19 or the prestalk marker genes pDd63 and pDd56 (Berks and Kay, 1990).

Adenosine is another molecule that has been identified as a developmental signal in *Dictyostelium*. Adenosine appears to play an antagonistic role to cAMP, in that during aggregation, it inhibits the binding of cAMP to the cAMP receptor (Theibert and Devreotes, 1984; and Van Lookeren Campagne et al, 1986). Depletion of adenosine in intact slugs induces conversion of prestalk cells to prespore cells (Schaap and Wang, 1986).

In this report we test the effect of DIF-1, cAMP, NH_3, and adenosine on the glycogen phosphorylase-2 gene. Glycogen degradation is essential for synthesizing the structural end products of differentiated *Dictyostelium* cells (Gustafson and Wright, 1972). The first step of the degradation is catalyzed by glycogen phosphorylase. Two glycogen phosphorylase isozymes (Brickey et al, 1990; Cloutier and Rutherford, 1987), which are encoded by two distinct genes (Rogers et al, 1992; and Rutherford et al, 1992), have been found in *D. discoideum*. During vegetative growth, only glycogen phosphorylase-1 (gp1) is active. Gp1 enzyme activity decreases during differentiation and becomes inactive after 20 hours of development. The activity of glycogen phosphorylase-2 (gp2), on the contrary, is undetectable in vegetative cells, but appears at 8 hours and reaches a maximal level at 20 hours of development. Previous studies have shown that the gp2 gene can be induced by cAMP in a shaking cell suspension (Rutherford et al, 1992; Sucic et al, 1993). Involvement of other developmental signals in the gp2 gene regulation is expected, due to the assumption that proper temporal and spatial gene expression triggers are required during development. Due to the lack of data on gene regulation through the interactions of DIF, cAMP, NH_3, and adenosine, and lack of direct evidence that NH_3 and adenosine participate in gene regulation in *Dictyostelium*, investigating the effects of the four developmental signals on the gp2 gene was especially interesting. This chapter shows: (1)

the gp2 gene is both DIF-1- and cAMP-inducible, (2) the DIF-1 induction of the gp2 gene requires prior cell-cell contact, while the cAMP induction does not, (3) DIF-1 inhibits the cAMP induction of the gp2 gene, (4) NH_3 inhibits both DIF-1 and cAMP induction of the gp2 gene, and (5) adenosine inhibits the DIF-1 induction but not the cAMP induction of the gp2 gene. A model of the gp2 gene regulation during the development is proposed.

Results

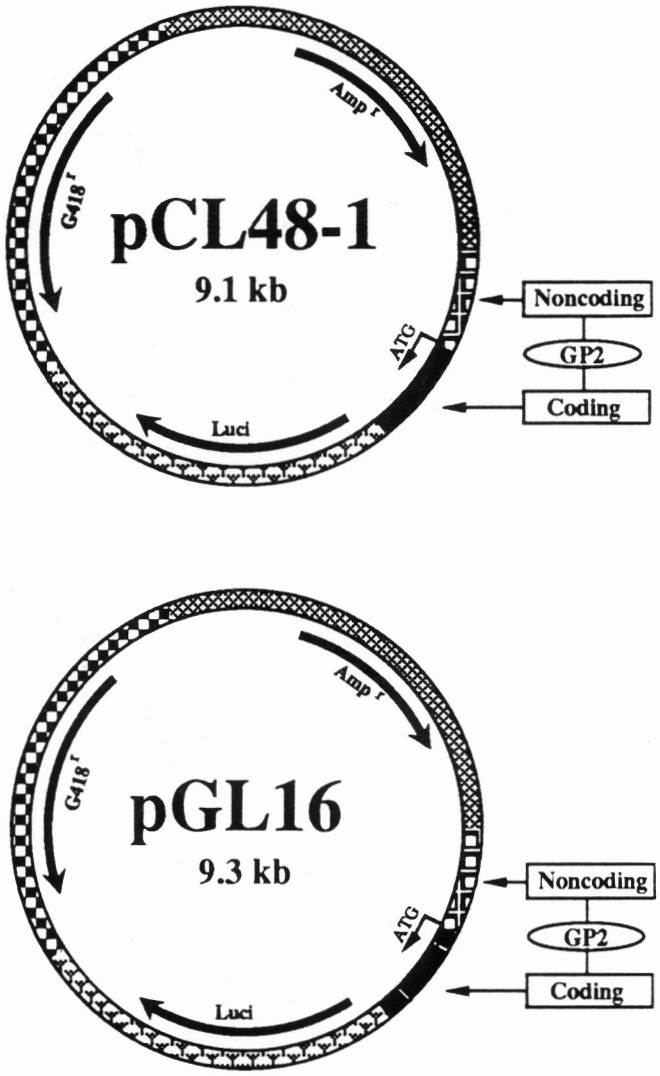
Plasmid constructions

A fragment containing the entire 5' non-coding region and 0.8 kb of the 5' coding region of the gp2 gene from a gp2 cDNA segment was fused in frame to the luciferase reporter gene in the vector backbone of the plasmid Pha1.4L00F (a generous gift from R. Firtel). This resulted in the plasmid pCL48-1 (Figure 16A & B). A parallel plasmid, pGL16, was constructed in the same way except that the gp2 coding region segment was incorporated. The only difference between pCL48-1 and pGL16 is that the latter construct contains two native gp2 introns. Both plasmid constructs contain the intact 5' non-coding region of the gp2 gene. Two gp2-LacZ gene fusion plasmids, pCZ28 and pGZ27 (Figure 17C & D), were constructed using the same gp2 gene fragments as found in the gp2-Luciferase gene fusions. The only difference between the two constructs is that pCZ28 lacks all introns while pGZ27 contains both native introns of the gp2 gene. The availability of these plasmids allows us to study the regulatory sequences that direct the expression of the gp2 gene during cell differentiation.

The constructs with/without introns in both gp2-luciferase fusion and gp2-LacZ fusion, enabled us to examine the two gp2 introns for their possible roles as enhancers. The gp2 introns, like many other *Dictyostelium* introns, are located in the 5' region of the gene. The close proximity of the introns to the 5' non-coding regulatory region and the extreme AT richness (80%) in both the non-coding region and the introns, make them candidates for possible regulatory sequences. However, to date there has been no documented attempt to examine any *Dictyostelium* intron for a possible regulatory function.

Figure 16. Maps of the plasmids. (A) Construction of pCL48-1 and pGL16. A 5' gp2 gene fragment, including the entire 5' non-coding region and a portion of the 5' coding region, was fused, in frame, to the luciferase reporter gene in the backbone of a luciferase reporter vector. The only difference between pCL48-1 and pGL16 is that the former contains a part of the gp2 coding region derived from a cDNA that has no intron, while the latter contains the same coding region but was derived from a genomic DNA that contained the two gp2 gene introns. The G418^r gene is driven by a *Dictyostelium* actin 6 gene promoter and terminated by a *Dictyostelium* actin 8 terminator. The *Dictyostelium* sp70 terminator was fused to the 3' end of the luciferase gene. (B) Sequences around the conjunction site of the gp2 and luciferase genes. The HindIII site fusion between the two genes puts both the gp2 gene ATG and the luciferase gene ATG in the same reading frame. (C) Construction of pCZ28 and pGZ27. A 5' gp2 gene fragment, including the entire 5' non-coding region and a portion of the 5' coding region, was fused, in frame, to the LacZ reporter gene in the LacZ reporter vector pDdGal16 (a generous gift from A. Harwood and R. Kessin). The only difference between pCZ28 and pGZ27 is the former contains a part of the gp2 coding region derived from a cDNA that has no intron while the latter contains the same coding region but was derived from a genomic DNA that has the two gp2 gene introns. (D) Sequences around the conjunction site of the gp2 and LacZ genes. The BamHI site fusion between the two genes puts both the gp2 gene and the LacZ gene in the same reading frame.

A.



B.

GP2~Luciferase Gene Fusion:

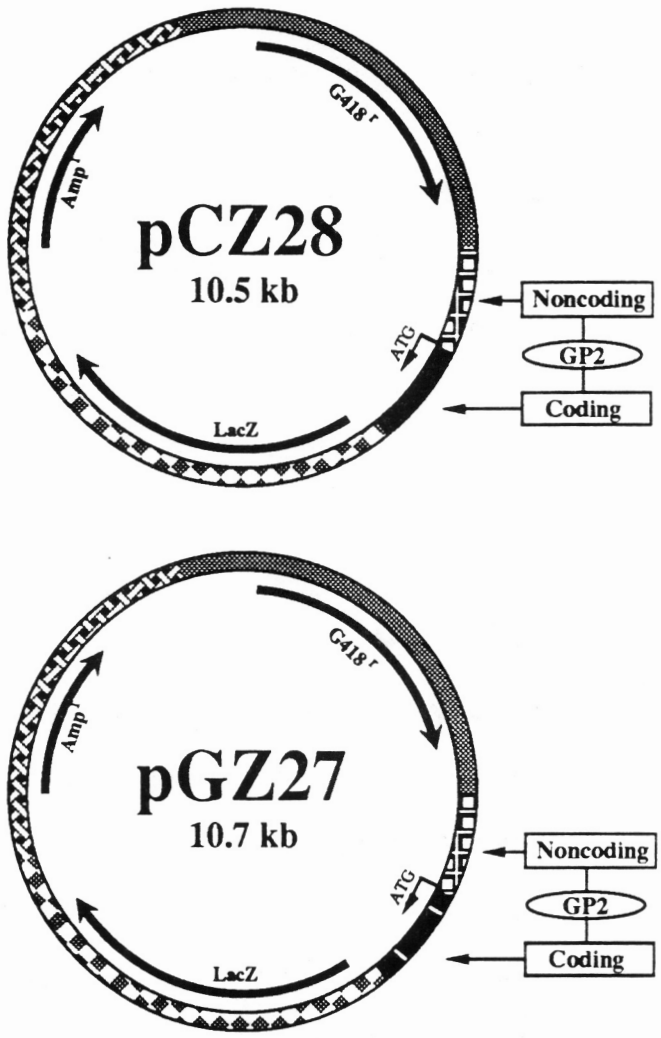
CAA GTT GAA TTA CCA GGA ATT CGA TAT CAA GCT TGG

GP2 Coding Region — EcoRI — EcoRV — HindIII

GAT CTG CAT TCC GGT ACT GTT GGT AAA ATG

ATG — Luciferase Gene

C.



D.

GP2~LacZ Gene Fusion:

CAA GTT GAA TTA CCA GGA AAT CCT GCA GCC CGG GGG
GP2 Coding Region EcoRI PstI SmaI

SmaI
ATC CCG GGC AAT TCA
BamHI → LacZ Gene, Starting at the 7th Codon.

Introns have been reported to act as transcriptional enhancers in mammals (Bornstein et al, 1988), Drosophila (Schultz et al, 1991), and C. elegans (Andrew Fire, personal communication). The constructs shown in Figure 16 in which a portion of the gp2 coding region was fused to the luciferase gene, allowed us to test the stability of the luciferase mRNA and protein in *Dictyostelium* cells, as compared to gp2-luciferase fusion constructs without native sequences attached to them. Also, the N-terminal region might contain information for correct compartmentalization and/or processing of the native gp2 protein and if so, the fusion protein would retain these signals.

DIF-1 induces gp2 gene expression

It has been shown previously that both gp1 and gp2 genes are cAMP-inducible (Sucic et al, 1993, Rutherford et al, 1992). Gp1 protein is present only in prestalk cells of the early culmination stage (Rogers et al, 1993) and is enzymatically inactive. The gp2 enzymatic activity is not detectable in vegetative cells but appears during the later stages of development where it reaches the maximal activity at 20 hour of development. The gp2 mRNA and protein appears earlier at the aggregation stage. Proteolytic cleavage of the protein at 18 hours of development is required for activation (Rutherford et al, 1992). Because both cell types appear to require active glycogen phosphorylase for terminal differentiation, we thought it is possible that the gp2 gene is expressed in both cell types and perhaps regulated by both cAMP and DIF, the spore and stalk morphogen, respectively. We examined the gp2 gene for DIF inducibility using the gp2-luciferase gene fusion, and we employed the gp2-LacZ gene fusion to observe the expression pattern in the two cell types. To test the possible DIF induction of the gp2 gene, AX3K cells were

transformed with either the pCL48-1 (without introns) or the pGL16 (with introns) construct. Four independent clones from each transformation were used; CL15-18 from the pCL48-1 transformation and GL15-18 from the pGL16 transformation. Cells that had reached early aggregation stage (streaming) on non-nutrient MES-LPS agar were dispensed at 2 x 10^6 cells /ml in MES-LPS buffer, either with or without 100 nM of DIF-1. The cell suspension was then incubated at 21°C on a rotary shaker at 180 rpm. A portion of the cell suspension was harvested at 2, 4, and 6 hours and assayed for luciferase activity. Figure 17 shows the results from a representative experiment in which the CL clones are shown in panel A and the GL clones in panel B. Little or no luciferase activity was observed in the cells from T_0 or 2 hour time points either in the presence or absence of DIF-1. However, after 4 hours, clearly higher luciferase activities were seen in the +DIF-1 cells as compared to the -DIF-1 cells, and by 6 hours, 7-30 fold higher activities were observed in the +DIF-1 cells. There was no significant difference found between the CL and GL clones, suggesting that the introns had no effect on the DIF-1-mediated induction of the gp2 gene at the tested conditions.

It is noteworthy that the cells used in the experiments were not pre-treated with exogenous cAMP prior to the exposure to DIF-1. It has been reported that the pre-treatment of *Dictyostelium* cells (Strain V12M2) with cAMP was necessary for the induction of some prestalk cell-specific genes by DIF-1 (Williams et al, 1987; Berks and Kay, 1990). We did find, however, that the cells had to reach the streaming aggregate stage (6-8 h) before exposing the cells to DIF-1 in order to observe the DIF-1 induction.

Prerequisites for gp2 gene expression by DIF-1 and cAMP

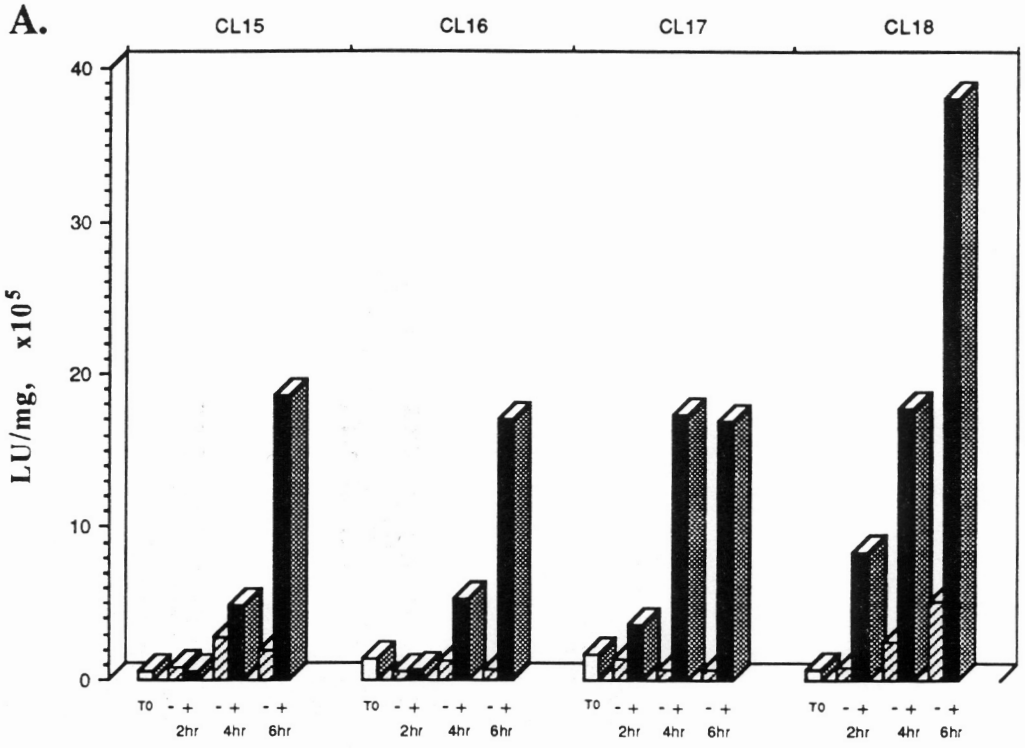
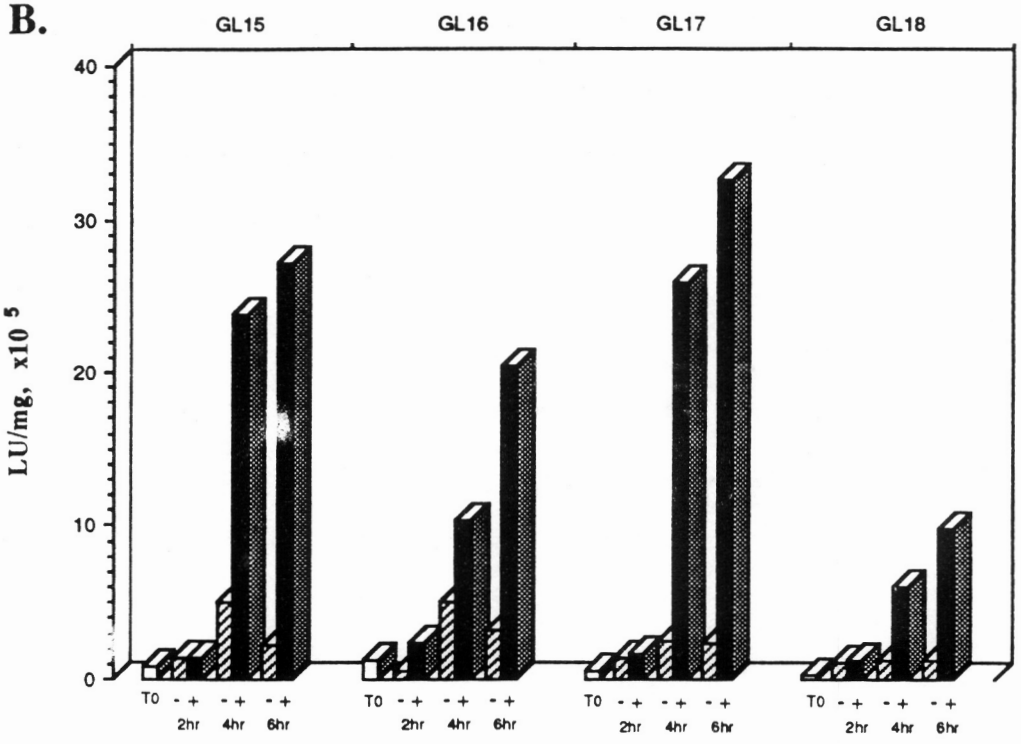


Figure 17. DIF-1 induces gp2 gene expression. Cells were developed to early aggregation stage (streaming), then harvested in MES-LPS buffer, and disaggregated by passing them through a 18.5G needle 5 times. The cells were then dispensed at 2 x 10^6 cells/ml in fresh MES-LPS buffer containing 100 nM DIF-1 (+) or 0.1% EtOH (-). The ethanol was included as a control because DIF-1 is dissolved in EtOH. The cell suspensions were shaken at 180 rpm for 2, 4 and 6 hours at 21°C. T0 cells were allowed to reach the early aggregation stage but were not shaken in liquid suspension. LU/mg = light units per mg protein. CL and GL clones were the transformants that containing the pCL48-1 and pGL16 plasmid constructs, respectively. Panel A shows the luciferse activities of 4 CL clones (without introns) and panel B shows the luciferse activities of 4 GL clones (with introns).


B.
GL15
GL16
GL17
GL18
40
30
20
10
0
LU/mg, x10 5
T0 - + - + - +
2hr 4hr 6hr

In contrast to the DIF-1 induction requirements mentioned above, cAMP-mediated induction of the gp2 gene in cell suspension could be demonstrated with the cells that had previously developed for a shorter period of time on non-nutrient agar (16 hours at 4°C or 4 hours at 21°C). T_0 determine if the different time length requirement for the DIF-1 and cAMP responsiveness was due to the length of time that the cells were starved, or to the formation of stable cell-cell contact on a solid surface, vegetative cells from two GL clones, GL17 and GL18, were starved in shaken flasks of MES-LPS buffer at 180 rpm for 10 or 14 hours. Under these conditions the cells are unable to form stable cell contacts. The cells then were pelleted, resuspended in fresh MES-LPS buffer containing either 100 nM DIF-1 or 1 mM cAMP, then shook for another 6 hours. Figure 18 shows the activation of the luciferase gene by cAMP under these conditions, but not by DIF-1. This indicates that starvation is sufficient for the cAMP-mediated gp2 gene induction, while cell-cell contact appears to be necessary for DIF-1 induction of the gp2 gene.

DIF-1 inhibits the cAMP induction of the gp2 gene

DIF-1 and cAMP are believed to function as antagonists during the *Dictyostelium* cell differentiation (Williams, 1988). The DIF-1 induction of a prestalk B cell marker gene, ecmB (pDd57), has been shown to be repressed by cAMP. A prespore cell marker gene, D19, is cAMP inducible and the induction is strongly inhibited by DIF-1 (Berks and Kay, 1990). An exception is the prestalk marker gene ecmA (pDd63). The DIF-1 induction of this gene was enhanced in the presence of cAMP (Berks and Kay, 1990). The gp2 gene, as described in this report, presents an unique situation because of its dual inducibility by both DIF-1 and cAMP. This provided the opportunity to test current ideas regarding the

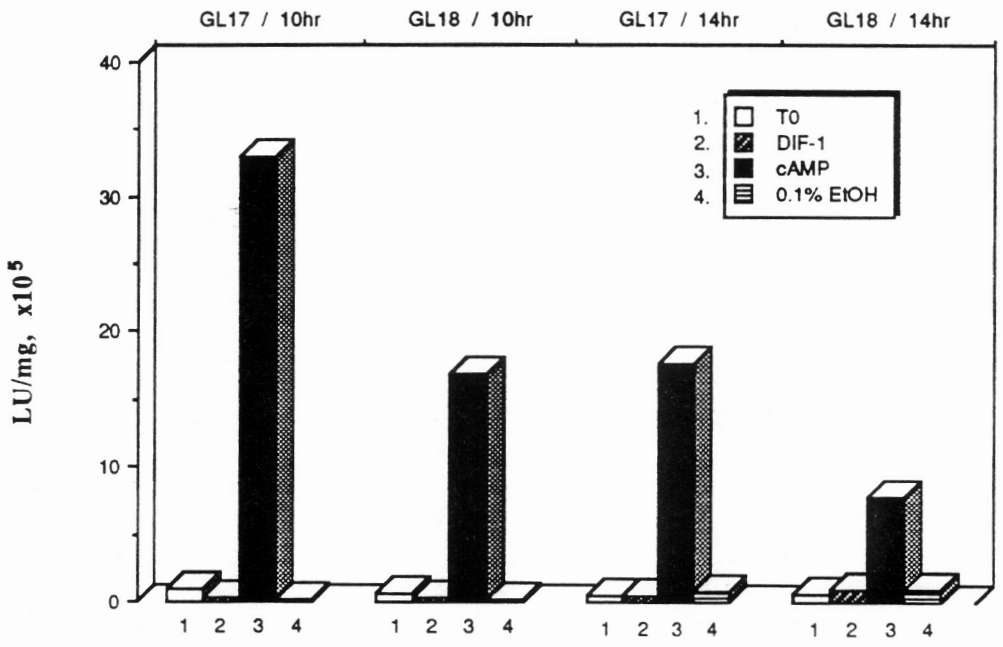


Figure 18. Prerequisites for gp2 gene expression in response to DIF-1 and cAMP. Vegetative cells were shaken at 180 rpm in MES-LPS buffer for 10 or 14 hours instead of being developed on MES-LPS non-nutrient agar as described in Figure 17 legend. These conditions prevent stable cell-cell contacts. The cells were then pelleted and dispensed at 2 x 10^6 cells/ml in fresh MES-LPS buffer, containing 100 nM DIF-1, or 1 mM cAMP, or 0.1% EtOH (see Figure 17 legend). The cell suspensions then were shaken at 180 rpm for an additional 6 hours. T_0 cells were shaken for 10 or 14 hours in MES-LPS and frozen without the additional 6 hours of treatment with DIF-1 or cAMP. See Figure 17 legend for LU/mg.

antagonistic nature of cAMP and DIF. Shaking experiments as described above were performed again, but included both DIF-1 and cAMP in the cell suspension. Figure 19A shows the results from a representative experiment in which 4 CL (no introns) and 4 GL (with introns) clones were tested. Compared to the cells treated with cAMP alone, ten fold inhibition was observed from the cells that were given a combination of DIF-1 and cAMP. The 10-fold inhibition was very consistent in 5 replications of the experiment. It should be noted, however, that this was only a partial inhibition of the luciferase activities of the DIF-1+cAMP cells, in that there were 5-10 x 10^5 LU/mg.

The concentration of DIF that was required for the inhibition of cAMP induction was also investigated. Three concentrations of DIF-1 and equivalent amounts of EtOH alone (the DIF solvent) were tested on two clones, CL17 and GL17 (Figure 19B). For clone CL17, 10 nM, 100 nM, and 500 nM of DIF-1, resulted in 4-, 15-, and 15-fold decrease in cAMP induction, respectively. A similar pattern was seen in clone GL17 with 2.5, 10, and 15 fold inhibition. Thus, concentrations as low as 10 nM are effective, but 100-500 nM are necessary for maximal inhibition of the cAMP induction. Figure 19B also shows that DIF-1, not EtOH, is the inhibitor of the cAMP-mediated induction of the gp2 gene.

Although there was variability from one experiment to the next, we saw no consistent differences between the response of CL and GL clones, suggesting that the introns had no effect on the DIF-1-mediated inhibition at the conditions tested.

Effects of NH_3 on gp2 gene expression

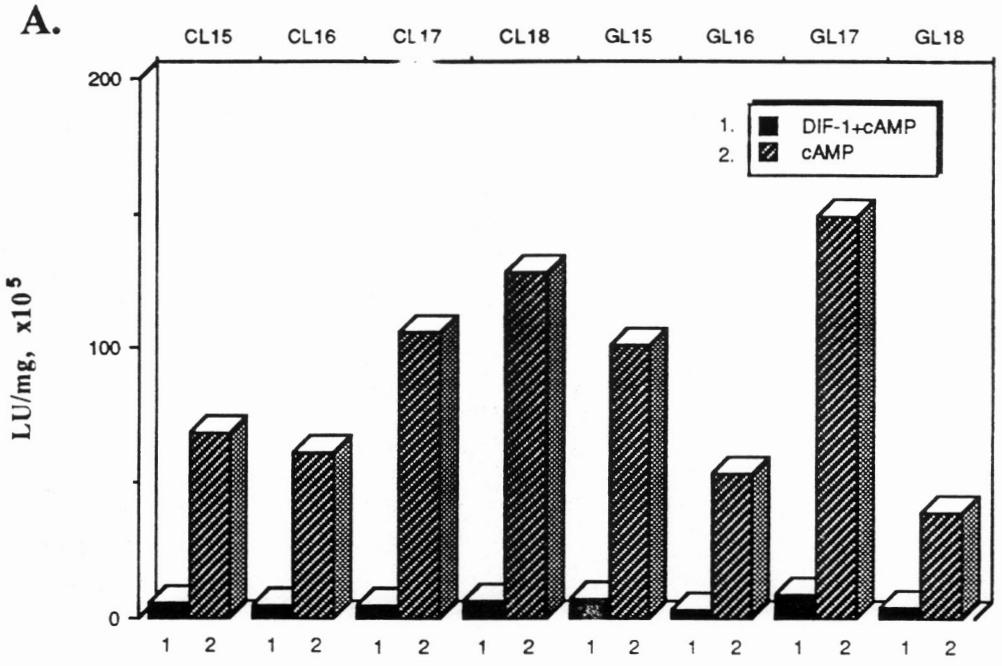
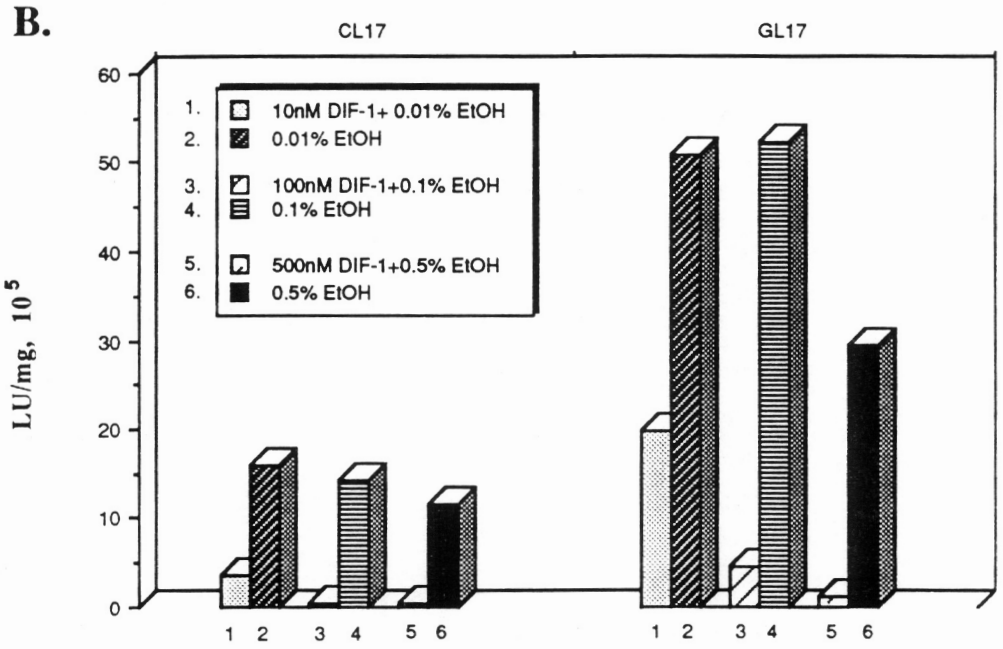


Figure 19. DIF-1 inhibits the cAMP-mediated gp2 gene induction. The cells were prepared as described in the legend of Figure 14. The cells were then dispensed at 2 x 10^6 cells/ml in fresh MES-LPS buffer containing: (Figure 19A) 100 nM DIF-1 + 1 mM cAMP or 1 mM cAMP; (Figure 19B) (1) 10 nM DIF-1 + 0.01% EtOH + 1 mM cAMP or 0.01% EtOH + 1 mM cAMP, (2) 100 nM DIF-1 + 0.1% EtOH + 1 mM cAMP or 0.1% EtOH + 1 mM cAMP, (3) 500 nM DIF-1 + 0.5% EtOH + 1 mM cAMP or 0.5% EtOH + 1 mM cAMP. The cell suspensions were shaken at 180 rpm for 6 hours. CL clones contain pCL48-1 construct (no introns) and GL clones contain pGL16 construct (with introns). See Figure 17 legend for LU/mg.


B.
CL17
GL17
LU/mg, 10^5
60
50
40
30
20
10
0
1. 10nM DIF-1+ 0.01% EtOH
2. 0.01% EtOH
3. 100nM DIF-1+0.1% EtOH
4. 0.1% EtOH
5. 500nM DIF-1+0.5% EtOH
6. 0.5% EtOH
1 2 3 4 5 6
1 2 3 4 5 6

NH_3 is thought to play an important role during *Dictyostelium* development in that it has been shown to favor spore cell differentiation in monolayer cell assay (Town, 1984; Bradbury and Gross, 1989), and to repress prestalk cell differentiation (Inouye, 1988; Wang and Schaap, 1989). Thus, NH_3 is thought to antagonize DIF during cell differentiation. However, NH_3 has also been reported to repress the cAMP effect during the aggregation stage (Schindler and Sussman, 1977b; Schindler and Sussman, 1979; Williams and Sussman, 1984). This raises question as to how NH_3 facilitates prespore cell differentiation while it also inhibits the effect of the prespore cell morphogen cAMP (Williams, 1988). Is the NH_3 facilitation of spore cell differentiation due to an enhancement of a cAMP effect or an inhibition of a DIF effect? Does NH_3 regulate DIF- and cAMP-mediated gene expression differently? Due to the fact that the gp2 gene can be induced by both DIF-1 and cAMP, the effects of NH_3 on both DIF-1 and cAMP inductions of the gene can be studied in one system.

Figure 20 shows that NH_3 inhibits the cAMP induction of the gp2 gene. Almost no cAMP induction appeared in any of the clones in the presence of 20 mM NH_4Cl in TS buffer at pH 7.5, indicating that the cAMP induction of the gp2 gene can be repressed by NH_3. However, when MES-LPS, pH 6.5 was used instead of TS buffer, pH 7.5, there was no effect of NH_3 on cAMP induction of the gp2 gene (data not shown). This pH-dependence of NH_3 effects has been observed in previous studies and has been demonstrated to be due to decreased NH_3 concentration at the lower pH (Berks and Kay, 1990; Schindler and Sussman, 1977b; Schindler and Sussman, 1979; Williams and Sussman, 1984). At 20 mM NH_4Cl, the concentrations of NH_3 at pH 6.5 and 7.5 are 40 μM and 400 μM, respectively (pK=9.2 for $NH_4 \leftrightarrow NH_3 + H+$). Thus, a 10 fold change in the level of cellular NH_3 results in major changes in the ability of cAMP to regulate the gp2 gene expression.

We also found that NH_3 inhibits the DIF-1 induction of the gp2 gene. In Figure 21, 20 mM NH_4Cl at pH 7.5 inhibited the DIF-1 induction of the gp2 gene. No NH_3 inhibition of the DIF-1-response was detected with MES-LPS buffer, pH 6.5, as seen above for the effect of NH_3 on the cAMP induction.

Again, there was no significant difference found between the CL and GL clones, suggesting that the introns had no effect on the NH_3-mediated inhibitions at the tested conditions.

Effects of adenosine on gp2 gene expression

It has been proposed that the prespore cell differentiation can be repressed by adenosine due to a decreased cell responsiveness to cAMP (Weijer and Durston, 1985; Schaap and Wang, 1986; Van Lookeren Campagne et al, 1986). Thus, adenosine is thought to be one of the factors that favor prestalk cell differentiation. The gp2 gene provided a unique opportunity to test for adenosine enhancement of the DIF-1 induction, and/or repression of cAMP induction of the gp2 gene, as might be predicted from previous studies at the cellular level.

We found that adenosine has no effect on the cAMP induction of the gp2 gene. Figure 22 shows the results from a representive experiment in which each of four clones, 2 CL and 2 GL clones, was treated separately with adenosine (2 mM), adenosine+cAMP (2 mM+1 mM), cAMP (1 mM), and none (negative control). No repeatable difference was observed between the luciferase activity from cells treated with adenosine+cAMP and cAMP alone, indicating that the cAMP induction of the gp2 gene was not affected by 2 mM exogenous adenosine. In contrast, Figure 23 showed that adenosine inhibited the DIF-1

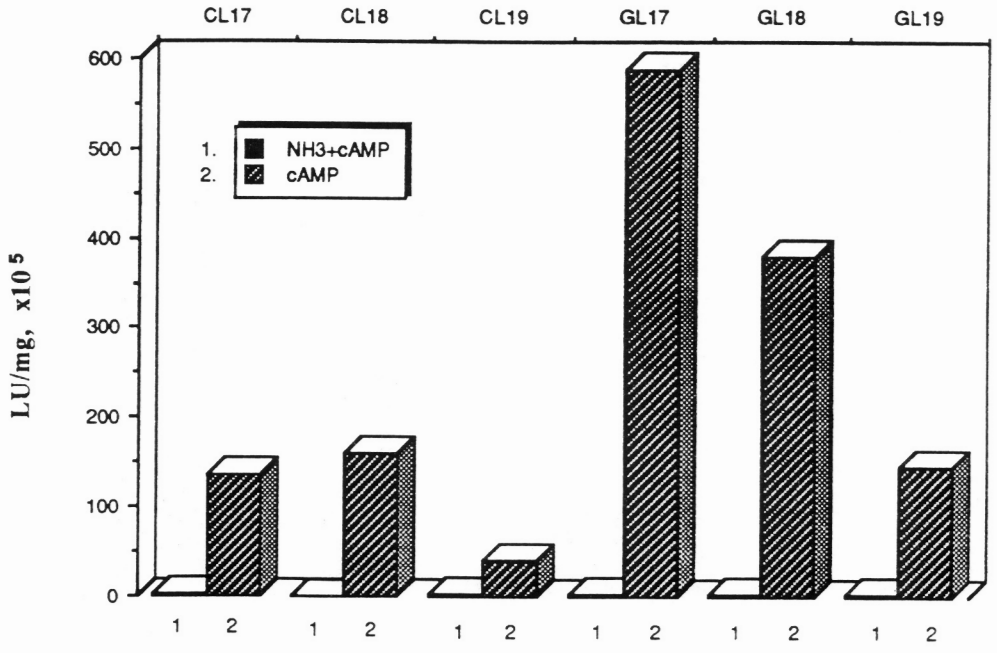


Figure 20. NH_3 inhibits the cAMP-mediated gp2 gene induction. The cells were prepared as described in the legend of Figure 14. The cells were then dispensed at 2 x 10^6 cells/ml in fresh TS buffer, pH 7.5, containing 20 mM NH_4Cl + 1 mM cAMP or 20 mM NaCl + 1 mM cAMP. The cell suspensions were shaken at 180 rpm for 6 hours. The use of 20 mM NaCl was to ensure that the same osmotic pressure was present in both + and - NH_3 suspensions. CL clones contain pCL48-1 construct (no introns) and GL clones contain pGL16 construct (with introns). See Figure 17 legend for LU/mg.

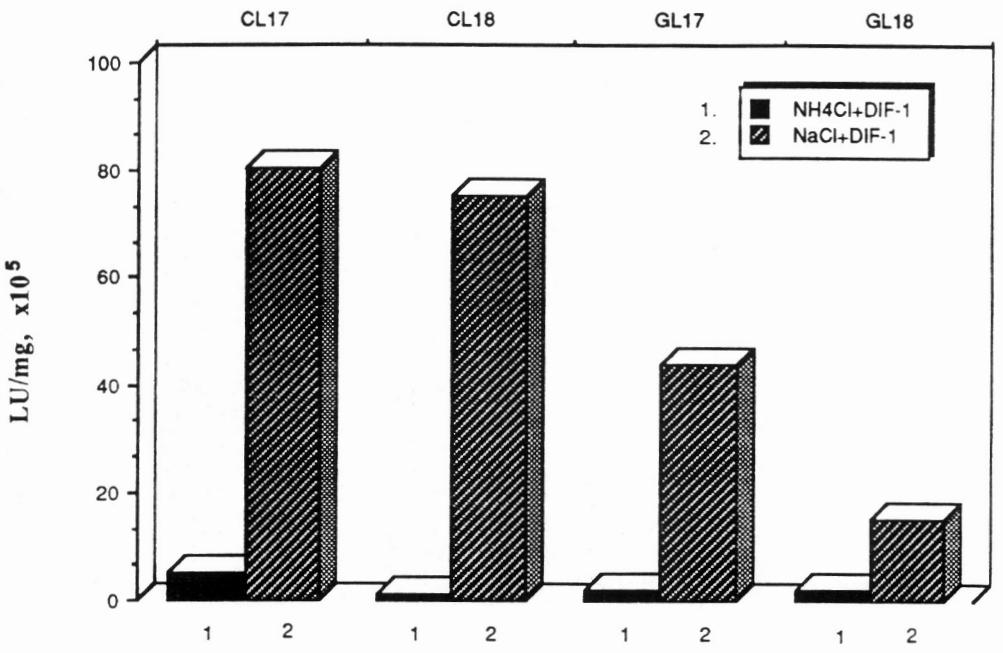


Figure 21. NH_3 inhibits DIF-1-mediated gp2 gene induction. The cells were prepared as described in the legend of Figure 17. The cells were then dispensed at 2 x 10^6 cells/ml in fresh TS buffer, pH 7.5 containing 20 mM NH_4Cl + 100 nM DIF-1 or 20 mM NaCl + 100 nM DIF-1. The use of 20 mM NaCl was to ensure that the same osmotic pressure was present in both + and - NH_3 suspensions. CL clones contain pCL48-1 construct (no introns) and GL clones contain pGL16 construct (with introns). See Figure 17 legend for LU/mg.

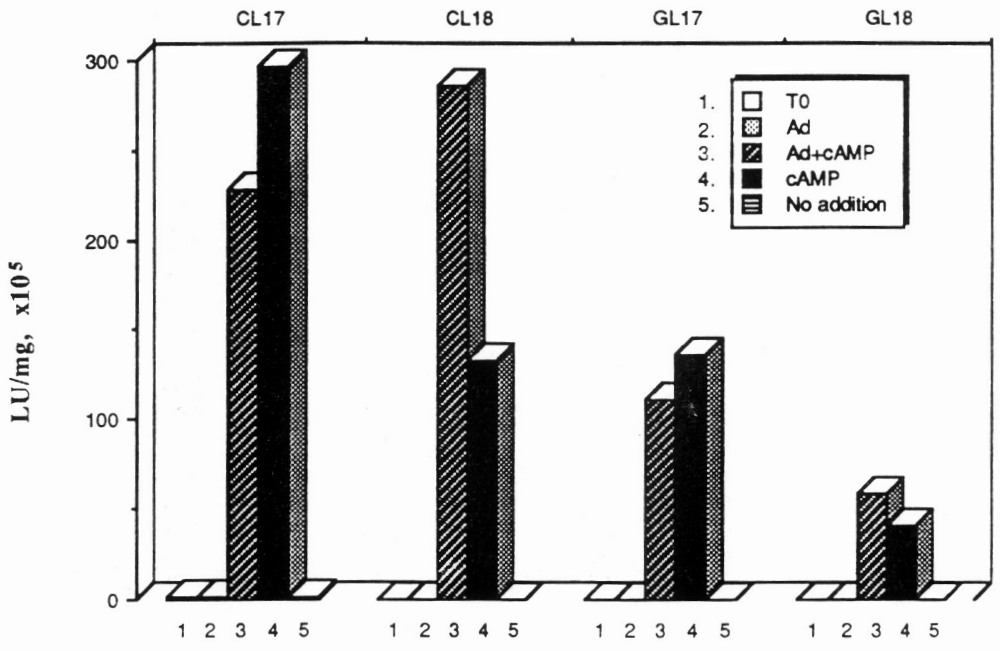


Figure 22. Adenosine does not inhibit the cAMP-mediated gp2 gene induction. The cells were prepared as described in the legend of Figure 17. The cells were then dispensed at 2 x 10^6 cells/ml in fresh MES-LPS buffer containing 2 mM adenosine, 2 mM adenosine + 1 mM cAMP, 1 mM cAMP, or no additions (negative control). CL clones contain pCL48-1 construct (no introns) and GL clones contain pGL16 construct (with introns). See Figure 17 legend for T_0 and LU/mg. Ad: Adenosine.

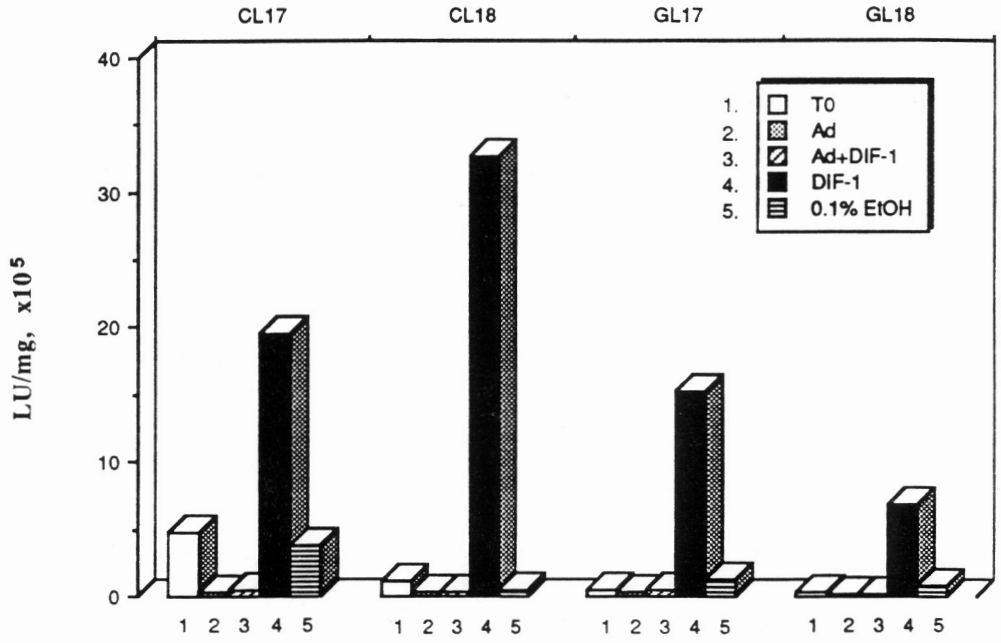


Figure 23. Adenosine inhibits the DIF-1-mediated gp2 gene induction. The cells were prepared as described in the legend of Figure 17. The cells were then dispensed at 2 x 10^6 cells/ml in fresh MES-LPS buffer containing 2 mM adenosine, 2 mM adenosine + 100 nM DIF-1, 100 nM DIF-1, or 0.1% EtOH (negative control, see Figure 2 legend). CL clones contain pCL48-1 construct (no introns) and GL clones contain pGL16 construct (with introns). See Figure 17 legend for T_0 and LU/mg. Ad: Adenosine.

induction of the gp2 gene. The same four clones as those for the adenosine vs. cAMP experiment were tested. The typical DIF-1 induction was again observed, but interestingly, if both DIF-1 and adenosine were present, there was no induction by DIF-1. Thus adenosine can repress the DIF-1-mediated induction of the gp2 gene, but has no effect on cAMP induction of the gene.

Again, there was no significant difference found between the CL and GL clones, suggesting that the introns had no effect on the adenosine-mediated inhibition in the experimental conditions.

The gp2 gene is expressed in both cell types

Elucidation of the DIF-1 and cAMP dual inducibilities for the gp2 gene suggested the possibility that the gene is expressed in both cell types since the two molecules are stalk and spore cell morphogens, respectively. AX3K cells were transformed with two parallel gp2:LacZ gene fusions, pCZ28 (no intron) and pGZ27 (with two gp2 introns), resulting in CZ and GZ clones. Cells from CZ and GZ clones were developed to various stages on filters before applying histochemical stain with X-Gal. Figure 24 shows examples of the staining pattern observed at the slug, culmination, and fruiting body stages of development. In numerous stages that have been studied, both prestalk/stalk and prespore/spore cells were stained, with the prespore region staining heavier than the prestalk region. The basal part of the stalk appeared to be stained heavier than the upper part of the stalk. Four independent CZ and GZ clones were studied, and all of the clones stained in the same fashion. These results show that the gp2 gene is expressed in both cell types. This agrees with the dual inducibility of the gp2 gene by both DIF-1 and cAMP (see Discussion). It is

Figure 24. The gp2 gene is expressed in both cell types. Cells were developed on Whatman #1 filters saturated in MES-LPS, then treated with 0.1% NP-40 for 15 minutes and 1% glutaraldehyde for 15 minutes, followed by staining with X-Gal for 15-60 minutes at 37°C. The stages of development shown are: (a) slug; (b) early culmination; (c) mid culmination; and (d) fruiting body. Panel A and B show the cells transformed with pCZ28 (without introns) and pGZ27 (with introns), respectively.

A.

B.

noteworthy that the cells in these experiments were stained for a very short period of time (from 15 minutes to an hour at 37°C). With longer staining periods, both cell types appeared to be stained to the same degree. Also, it cannot be ruled out that the relative intensity of the stain in different cell types might be due to the difference of permeability to X-Gal between the two cell types. We have observed that staining in the prespore zone was much slower if the detergent NP-40 was not used.

There was no significant difference found between the CL and GL clones, suggesting that the introns had no effect on the cell-type-specific expression of the gp2 gene at the tested conditions.

Discussion

In this study, the gp2 gene was discovered to be induced by both DIF-1 and cAMP. There is no report of a *Dictyostelium* gene that can be induced by both DIF and cAMP. The gp2 gene is the first one found to date to possess a dual responsiveness to DIF-1 and cAMP inductions. We also determined that the gp2 gene was expressed in both prespore/spore and prestalk/stalk cells. This finding is in agreement with the dual inducibility of the gp2 gene, and suggests that the gp2 gene expression in prestalk/stalk cells is induced by DIF-1 and the expression in prespore/spore cells is induced by cAMP. This type of duality might also exist in other developmentally regulated *Dictyostelium* genes whose transcriptional or translational products have been previously detected in both cell types (Loomis, 1985; Schaap, 1986).

DIF-1-induced expression of the gp2 gene did not require pre-treatment with cAMP or the presence of exogenous cAMP. Instead, cells become DIF-1 responsive if they are subjected to uninterrupted cell-cell contact for 6 hours (or more) at 21°C or 24 hours (or more) at 4°C. This finding differs from all previously-reported DIF-1-inducible genes; DIF-1 induction of other genes requires pre-treatment with cAMP or the presence of cAMP during the DIF-1 induction (Williams et al, 1987; Kay, 1990). Thus, different genes or gene groups might have distinct prerequisites for their DIF-1-induced expression in order to exhibit precise temporal and spatial expression along the program of development. For the gp2 gene, cell-cell contact, rather than exogenous cAMP, appears to be a prerequisite for the DIF-1 responsiveness. However, it cannot be ruled out that cAMP which accumulated from an endogenous source during the 6 hour-incubation on the rotary shaker, might have participated in the DIF-1-mediated induction.

It was found that the gp2 gene responded to cAMP earlier in the developmental cycle than to DIF-1 (see Materials and Methods). Also, starvation of the cells in shaking suspension was found to be sufficient to induce the cAMP-competence for the gp2 gene induction, but was not sufficient to induce the DIF-1-competence for the gene. Therefore, it is likely that the first appearance of gp2 gene expression at 6-hour (Brickey et al, 1990) is due to cAMP induction. Cyclic AMP might be the primary trigger for the gene through the aggregation stage, since the major rise in the level of DIF does not appear until formation of the tipped aggregates (Brookman et al, 1982). According to this model, as the development proceeds to the tip formation stage, the gp2 gene in the tip (prestalk cells) would switch from cAMP-responsive to DIF-1-responsive, while the gene in prespore cells would continue to be induced by cAMP.

As mentioned previously, cAMP and DIF-1 are considered to function as antagonists during *Dictyostelium* cell differentiation (For a review, see Williams, 1988). We have also found in this study that DIF-1 partially inhibited the cAMP-mediated induction of the gp2 gene. The partial inhibition pattern between DIF-1 and cAMP has also been observed with the cell type-specific marker genes D19 and pDd56 (Berks and Kay, 1990). These results might indicate that DIF-1 or cAMP is unable to completely turn off a cell-type-specific gene. However, a second factor may be required for complete inhibition. Also, it is still in question as to whether the DIF-1 signal directly inhibits the cAMP-mediated gp2 gene expression or whether it inhibits the gene in an indirect way. For example, if DIF-1 were to induce a phosphodiesterase gene, the resulting enzyme would cause hydrolysis of cAMP, thus inhibiting the cAMP effect. In addition, we do not know if cAMP can inhibit the DIF-1 induction of the gp2 gene, due to the fact that when the cells are competent for DIF-1 induction of the gp2 gene, they are also competent for cAMP

induction of the gene. Thus, even the inhibition does occur, it is impossible to differentiate the DIF-1 induction from the cAMP induction in terms of the reporting luciferase activity.

As a catabolic product of protein and nucleic acid degradation, NH_3 has been shown to inhibit both the intracellular and extracellular accumulation of cAMP (Schindler and Sussman, 1977b; Schindler and Sussman, 1979) and to inhibit the cAMP relay (Williams et al, 1984). In the case of the gp2 gene, our data showed that 20 mM NH_4Cl, pH 7.5, repressed the cAMP-mediated induction. Due to the presence of a high concentration of exogenous cAMP (1 mM) in these experiments, reduced extracellular cAMP level and inhibited cAMP relay probably were not the factors that caused the gp2 gene inhibition. Although NH_3 inhibition of the cAMP-mediated gp2 gene induction has been found in the cells from early aggregation stage, it is possible that the inhibition is diminished in prespore cells during slug and culmination stages if the number of cAMP receptors increase during late development. This lower ratio of NH_3 to cAMP receptor at late stages may allow NH_3 to act as a down-regulator instead of a complete inhibitor. We have shown previously, that, at the initial stages of the culmination process, endogenous NH_3 drops dramatically, then accumulates very quickly in prestalk/stalk cells, and more slowly in prespore cells (Wilson and Rutherford, 1978). The delayed NH_3 accumulation in prespore cells would maintain high levels of cAMP and thus result in continued cAMP activation of the gp2 gene. In the prestalk cell region, the cAMP-mediated gp2 gene induction is probably repressed due to a high NH_3 concentration and a possible low level of cAMP receptor sites.

The NH_3 inhibition of the DIF-1-mediated gp2 gene induction agrees with proposed antagonism between DIF-1 and NH_3 (Inouye, 1988; Wang and Schaap, 1989). The question arises, however, as to how gp2 gene expression and prestalk cell differentiation can be possible, if the DIF-1-mediated induction is inhibited by the high

concentration of NH_3 in prestalk zone. It should be emphasized again that the cells used in the study were from early aggregation stage where the DIF-1-responsive components may just begin to appear and are at very low concentrations. Thus the NH_3 inhibition shown in the study was very strong due to the high ratio of inhibitor:target. But, as the prestalk and prespore pattern is established, it is possible that the DIF-1-responsive components in prestalk cells reach a high concentration. At this lower inhibitor:target ratio, the NH_3 is unable to completely shut down the DIF-1 induction. Thus, NH_3 may act as a down-regulator to balance the DIF-1 effects in the prestalk cells.

There are a number of reports concerning the effects of NH_3 on development in *Dictyostelium*. For example, It has been shown that NH_3 facilitated spore cell differentiation in a monolayer assay (Town, 1984; Bradbury and Gross, 1989). This agrees with the studies showing an antagonistic relation between DIF-1 and NH_3 (Inouye, 1988; Wang and Schaap, 1989), because as mentioned previously, DIF-1 is stalk cell morphogen. However, it has also been shown that NH_3 inhibited cAMP accumulation and relay (Schindler and Sussman, 1977b; Schindler and Sussman, 1979; Williams et al, 1984). This result is in apparent conflict with the induction of spore cell differentiation in the monolayer assay, since cAMP is considered as the spore cell morphogen. In our study with the gp2 gene, NH_3 was found to inhibit both DIF-1 and cAMP induction of the gene. One interpretation for all these finding could be that NH_3 antagonizes both DIF-1-induced stalk cell differentiation and cAMP-induced spore cell differentiation. Thus, in the monolayer assay, the large excess of exogenous cAMP (5 mM) is able to overcome the NH_3 inhibition of cAMP relay. The reduced stalk cell differentiation might be due to inhibited DIF-1 signalling by NH_3, since no exogenous DIF-1 was added to the assay.

Adenosine has been shown to act as an antagonist to cAMP in regulating tip formation and in down-regulating the cAMP effect for prespore cell differentiation. This is

due to the inhibitory effect of adenosine on the binding of cAMP to cAMP receptor (Schaap and Wang, 1986; Van Lookeren Campagne et al, 1986). Thus, adenosine was thought to favor prestalk cell differentiation in concert with DIF-1. However, there has been no direct evidence to support this idea, and the hypothesis was suggested before it was known that there are several developmentally regulated cAMP receptors. In our study, adenosine was found to inhibit the DIF-1-mediated gp2 gene induction, but had no effect on the cAMP-mediated gp2 gene induction with aggregation stage cells. This result seems to be in apparent contradiction with the proposed role for adenosine as a stalk cell morphogen. The observation that depletion of adenosine caused increased formation of tips (prestalk cells) (Schaap and Wang, 1986) might be due to the relief of adenosine-repressed DIF-1 effect rather than an effect of adenosine on cAMP-directed pathways. For the gp2 gene, adenosine had no inhibitory effect on the cAMP induction. This also seems to conflict with previous observations of the antagonistic relation between cAMP and adenosine. However, multiple cAMP receptors have recently been discovered in different developmental stages (Johnson et al, 1992; Johnson et al, 1993; Saxe et al, 1993), and it is possible that the signal for the cAMP-regulated gp2 gene induction is mediated through a cAMP receptor that is insensitive to adenosine inhibition. For example, adenosine might only have an inhibitory effect on cAR1, which is the cAMP receptor responsible for chemotaxis during aggregation. Other receptors may be involved with the events that regulate cell differentiation. For the gp2 gene, cAR3 is the most likely candidate in the signalling pathway, due to the coincident expression of cAR3 and gp2 (8 to 12 hour).

In addition, depletion of adenosine in an intact slug could cause conversion of prestalk cells into prespore cells (Schaap and Wang, 1986). However, our result showed that adenosine antagonized the DIF-1 induction of the gp2 gene. It perhaps can be interpreted as a small number of a cAMP receptor that is responsible for expression of

some prespore-specific genes are present in prestalk cells to keep its reversible potential. But in normal condition, all of the few receptor sites are satuarated by adenosine. When adenosine is depleted and the repression is relieved, cAMP takes over and induces the expression of these spore-specific genes, which marks the cells with certain spore cell characteristics observed in the previous studies. One might argue why the cAMP effect can overcome the DIF-1 effect since the adenosine depletion would also relief the repression on the DIF-1 in prestalk cells. But there might not be sufficient adenosine-mediated repression on DIF-1 at slug stage because it is reasonable to expect that the DIF-1-responsive components are abundant enough in prestalk cells so that adenosine can not block the DIF-1 signal sufficiently. A similar interpretation can apply to prespore region where a large number of the cAMP receptor (not the one for the gp2 gene) are present, so that the effect of adenosine inhibition is slight enough not to repress the prespore cell differentiation. It would strengthen the above interpretation if the affinity of adenosine to the cAMP receptor is high in prestalk cells and low in prespore cells, and the affinity to DIF-1 effector is low in prestalk cells and high in the prespore cells due to the difference in ambient pH environment in two cell types.

Finally, a model for the gp2 gene regulation during *Dictyostelium* development is proposed, based on the facts that: (1) both DIF-1 and cAMP can induce the gp2 gene expression, (2) the gp2 gene is expressed in both cell types, (3) DIF-1 reduces the cAMP-mediated gp2 gene induction, (4) NH_3 inhibits both DIF-1- and cAMP-mediated gp2 gene induction, (5) adenosine inhibits DIF-1-mediated gp2 gene induction, but does not inhibit the cAMP-mediated gp2 gene induction, and (6) NH_3 is enriched in prestalk/stalk zone over prespore/spore zone during culmination (Wilson and Rutherford, 1978). The model also makes the following assumptions: (1) the cAMP receptor sites that are responsible for the gp2 gene induction are limited in the prestalk region, but are abundant in the prespore

cell region, (2) the concentration of the DIF-1-responsive components that function in gp2 gene induction is very low in prespore cell region, but is very high in prestalk cell region, and (3) adenosine is enriched in prespore/spore zone over prestalk/stalk zone. In this model (Figure 25), DIF-1 induces the gp2 gene expression in prestalk/stalk cells while cAMP induces the gp2 gene expression in prespore/spore cells. NH_3 down-regulates DIF-1- and cAMP-mediated gp2 gene inductions in prestalk/stalk cells and prespore/spore cells, respectively. It is important to note that neither of the two responses is completely shut down or even significantly repressed, but is only down-regulated to a certain level to keep it from over-expressing. The rate of the gp2 gene expression is controlled by the ratio of NH_3:cAMP receptor in prespore/spore cells and by NH_3:DIF-1-responsive components in prestalk/stalk cells. Thus, the cAMP-mediated gp2 gene induction is repressed in prestalk cells due to the high NH_3:cAMP receptor ratio in that region. The DIF-1-mediated gp2 gene induction is turned off in prespore/spore cells due to the high NH_3/adenosine:DIF-1-responsive components ratio in these cells. During aggregation when the prestalk/prespore pattern has yet to be formed, cAMP is the sole inducer of the gp2 gene expression. The signal for the cAMP-mediated gp2 gene expression might be passed through cAR3, or another receptor, to which adenosine can not compete with cAMP for binding. After pattern formation, cAMP retains its inducing role in prespore cells, whereas DIF-1 becomes the inducer for the gp2 gene induction in prestalk cells. Just prior to the culmination stage, both cAMP and DIF effects push the culmination process towards maturation of both cell types, due to the significant drop of NH_3 concentration in both cell types (Wilson and Rutherford, 1978).

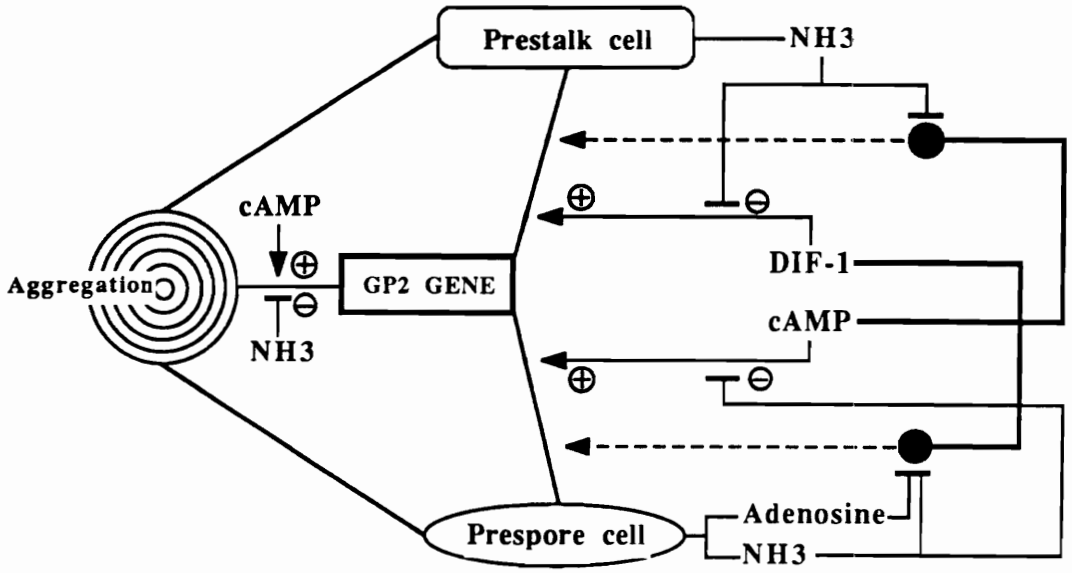


Figure 25. A model for gp2 gene regulation. A model is proposed for the gp2 gene regulation with four Dictyostelium developmental signals, DIF-1, cAMP, adenosine, and NH_3. In this model, DIF-1 induces the gp2 gene expression in prestalk/stalk cells while the gp2 gene expression in prespore/spore cells is induced by cAMP. NH_3 down-regulates both DIF-1 and cAMP inductions of the gp2 gene, but does not completely inhibit the expression. The cAMP induction of the gene is repressed in prestalk/stalk cells due to a high NH_3:cAMP receptor ratio in this region. The DIF-1 induction of the gene in prespore/spore cells is inhibited due to the high adenosine/NH_3:DIF-1-responsive components ratio in this zone. Cyclic AMP is the sole inducer of the gp2 gene during aggregation. After the formation of prestalk/prespore pattern, the gp2 expression in prestalk/stalk cells is induced by DIF-1, whereas cAMP induces the expression in prespore/spore cells. Positive sign: Gene induction signal; Negative sign: Gene down-regulation signal; Black dot: Signal blocking point; Dashed line: Blocked signal pathway.

Materials and Mathods

Plasmid construction

A multistep process was used to construct fusions of gp2 cDNA/or genomic region to a luciferase reporter gene. A cDNA fragment of the gp2 gene (corresponding to the genomic regions of +14 to +1012 bp from the translational initiation codon) was obtained by synthesizing the antisense strand of the cDNA by reverse transcription. Total RNA from slug stage cells and a primer corresponding to the region of +14 to +34 were used in this process. The synthesis of the sense strand cDNA was done with Taq polymerase and a primer corresponding to the region of +991 to +1012 bp in a thermal cycler. The PCR amplification of the double-stranded cDNA was performed using the same two primers described above. The amplified cDNA was then cloned into pBluescript II SK^+ (Strategene) at the EcoRI site through EcoRI recognition sequences on the primers. A 5' gp2 gene DNA fragment (-1216 to +20 bp) from a gp2 genomic clone was fused to the cDNA fragment at the RsaI site (+16 to +20 bp) so that the connection between the promoter and the coding region is exactly the same as in the native gp2 gene. The orientation of the gp2 insert was then reversed at the flanking EcoRI sites. This provided a BamHI (5')-HindIII (3') gp2 fragment for the in-frame fusion with the luciferase reporter gene. The fusion was done with the BamHI + HindIII double digested vector backbone of the plasmid Pha1.4L00F (a generous gift from R.Firtel). The final construct was named pCL48-1. To construct a parallel plasmid to pCL48-1, but with the coding region containing the two gp2 introns, a gp2 genomic DNA fragment was amplified by PCR using the same pair of the primers as described for the gp2 cDNA amplification above. The

amplified genomic DNA then was processed exactly as described above for pCL48-1. This generated a final plasmid construct named pGL16, which is exactly the same as pCL48-1 except it contains two introns.

For the gp2-LacZ fusion, a KpnI (5') - BamHI (3') fragment containing the same gp2 gene fragment as described for the two gp2-luciferase constructs was fused in-frame to the E.coli LacZ reporter gene in the KpnI-BamHI double digested LacZ reporter vector pDdGal-16 (a generous gift from A. Harwood and R. Kessin). The resulting two parallel plasmids pCZ28 and pGZ27 are exactly the same except the former lacks introns and the latter contains the two gp2 introns.

Transformation

Transformation was performed as described previously (Nellen et al, 1984), with a few modifications. After 2 days of G418 selection at 20 μg/ml in HL5, the recipient AX3K cells were plated on DM agar (Padgoski and Deering, 1980) containing 60 μg/ml of G418 in association with *E.coli* B/r 1-1 cells (a G418 resistant strain) (Hughes et al, 1992). Single colonies of transformants that formed on the agar plates were streaked on the same DM/G418 agar to ensure obtaining pure, independent clones.

Cell growth

All transformants were first grown on DM plates (containing 60 μg/ml of G418) on *E. coli* B/r 1-1 cell lawn. The vegetative cells then were transferred to axenic medium HL5

(Firtel and Bonner, 1972) containing 5 μg/ml of G418. The growth temperature for all cultures was 21°C.

Shaking conditions

Cells used for shaking experiments were pre-developed on MES-LPS (7 mM MES, 20 mM KCl, 5 mM MgSO4, pH 6.5) non-nutrient agar until early aggregation stage (streaming) which was either 4 hours at 21°C or 16 hours at 4°C. For the experiments with DIF including parallel samples with cAMP, NH_3, and adenosine, cells were pre-developed on the MES-LPS agar until the loose mound stage, which was either 6-8 hours at 21°C or 24-30 hours at 4°C. After this pre-development, the cells were harvested in MES-LPS, passed through a 18.5G needle to disassociate cell aggregates, pelleted, and resuspended at 2 x 10^6 cells/ml in MES-LPS. A 10 ml sample of the cell suspension, with appropriate concentrations of cAMP, DIF, NH_4Cl, and adenosine as mentioned in figure legends was shaken at 180 rpm at 21°C. TS buffer (10 mM Tris, 2 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.5) was used for some of the shaking experiments with NH_4Cl.

Luciferase assay

Cells harvested from shaken suspension were pelleted and resuspended in 100 μl of 30 mM glycylglycine (pH 8.3) containing PMSF, pepstatin A, and leupeptin at 5 μg/ml each. The cells were lysed by freezing at -80°C and thawing at room temperature. The cell

debris was pelleted at 14,000 x g for 10 minutes. The supernatants were used to measure the luciferase activity in a reaction, containing 0.3 mM luciferin, 2 mM ATP, 4 mM $MgCl_2$ in 0.5 M glycylglycine (pH 8.3), with a Berthold luminometer (Model Lumat LB 9501). Protein concentrations were determined by the Bradford method (Bradford, 1976).

Histochemical stain

Cells were spread on Whatman #1 filters supported by absorbent pads saturated with MES-LPS and developed to different stages at 21°C in the dark. Staining was performed as described previously (Dingermann et al, 1989) except the cells were treated with 0.1% NP40 for 15 minutes before the fixation step with 1% glutaraldehyde. The color development was completed in 15 to 60 minutes at 37°C.

Chapter 6. Conclusions

I. Cloning of Glycogen Phosphorylase Genes.

1. A 18 kb genomic region containing the gp2 gene has been cloned and the gp2 gene has been sequenced.
2. A gp2 cDNA containing the first, the second and a part of the third exon has been cloned and sequenced.
3. The gp2 gene contains an open reading frame of 2976 bp that is composed of three exons separated by two introns (105 bp and 109 bp).
4. Both 5' and 3' non-coding regions, as well as the two introns, of this gene are typical of *Dictyostelium* non-coding sequence in that are extremely (A+T)-rich (>80%).
5. The gp2 amino acid sequence exhibits 55.6% identity and 72.6% similarity to gp1, and 44% and 50% similarity to rabbit muscle and yeast glycogen phosphorylase, respectively.
6. The cloned gp1 gene fragment covers half of the coding region and the entire 5' non-coding region as well as extra-upstream genomic sequence.

II. Genomic Analysis of gp1$^-$ Mutants.

1. The lack of gp1 activity in homologous recombination transformants was due to disruption of the gp1 gene.
2. Antisense RNA disruption was responsible for the lack of gp1 activity in the transformants with unaltered gp1 gene structure.

III. Establishment of an extrachromosomal reporter vector.

1. The first extrachromosomal luciferase reporter vector has been established for *Dictyostelium*.
2. The vector is stably maintained in *D. discoideum* transformants with relatively low copy number.

IV. Dual Regulation of the gp2 Gene.

1. The gp2 gene is the first gene found to date, that can be induced by both cAMP and DIF-1 in differentiating cells.
2. DIF-1 inhibits the cAMP-mediated gp2 gene induction.
3. NH_3 represses both the cAMP- and DIF-1-mediated gp2 gene expression.
4. Adenosine inhibits the DIF-1-mediated gp2 gene expression.
5. Cell-cell contact is a prerequisite for the DIF-1-mediated gp2 gene induction.
6. The gp2 gene is expressed in both prestalk/stalk and prespore/spore cells.
7. No regulatory roles were detected for the two gp2 introns.
8. A model of gp2 gene regulation during development is proposed.

Literature Cited

Andre, E., F. Lottspeich, M. Schleicher, and A. Noegel. 1988. Severin, gelsolin, and villin share a homologous sequence in regions presumed to contain F-actin severing domains. J. Biol. Chem. 263:722-727.

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

Berks, M., and H. F. Kay. 1990. Combinatorial control of cell differentiation by cAMP and DIF-1 during development of *Dictyostelium discoideum*. Development 110: 977-984.

Berks, M., D. Traynor, I. Carrin, R. H. Insall, and R. R. Kay. 1991. Diffusible signal molecules controlling cell differentiation and patterning in *Dictyostelium*. Development Supplement 1: 131-139.

Berndt, N., H. P. Neubauer, and R. Rosen. 1987. Rat heart glycogen phosphorylase II is genetically distinct from phosphorylase I. Biochem. Biophys. Acta. 880:78-79.

Bornstein, P., J. McKay, D. J. Liska, S. Apone, and S. Devarayalu. 1988. Interactions between the promoter and first intron are involved in transcriptional control of a1(I) collagen gene expression. Mol. Cell. Biol. 8: 4851-4857.

Bradbury, J. M., and J. G. Gross. 1989. The effect of ammonia on cell-type-specific enzyme accumulation in *Dictyostelium discoideum*. Cell Diff. and Dev. 27: 121-128

Bradford, M. 1976. A rapid and sesitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brickey, D. A., V. Narana, J. F. Sucic, and C. L. Rutherford. 1990. Regulation of the two forms of glycogen phosphorylase by cAMP and its analogs in *Dictyostelium discoideum*. Mol. Cell. Biochem. 97: 17-33.

Brookman, J. J., K. A. Jermyn, and R. R. Kay. 1987. Nature and distribution of the morphogen DIF in the *Dictyostelium* slug. Development 100: 119-124.

Brookman, J. J., C. D. Town, K. A. Jermyn, and R. R. Kay. 1982. Developmental regulation of a stalk-cell differentiation-inducing factor in *Dictyostelium discoideum*. Dev. Biol. 91: 191-196.

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

Chambers, T. C., J. Song-Nichols, D. S. Campbell, E. Spitz, B. H. Leichtling, and H. V. Rickenberg. 1987. Identification of nuclear subtracts of the cyclic AMP-dependent protein kinase in *Dictyostelium discoideum*. Cell Diffrentiation 20:217-230.

Chang, A. C. M., M. B.Slade, and K. L. Williams. 1990. Identification of the origin of replication of the eukaryote *Dictyostelium discoideum* nuclear plasmid Ddp2. Plasmid 24: 208-217.

Choi, Y., M. Kawamukai, R. Utttsumi, H. Sakai, and T. Komano. 1989. Molecular cloning and sequencing of the glycogen phosphorylase gene from *Escherichia coli*. FEBS Lett. 243:193-198.

Cloutier, M. J., and C. L. Rutherford. 1987. Glycogen phosphorylase in *Dictyostelium*: developmental regulation of two forms and their physical and kinetic properties. J. Biol. Chem. 262:9486-9493.

Crowley, T. E., W. Nellen, R. H. Gomer, and R. A. Firtel. 1985. Phenocopy of discoidin minus mutant by antisense transformation in *Dictyostelium*. Cell 43:633-641.

De Lozanne, A., and J. A. Spudich. 1987. Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. Science 236:1086-1091.

Dingermann, T., N. Reindl, H. Werner, M. Hildebrandt, W. Nellen, A. Harwood, J. G. Williams, and K. Nerke 1989. Optimization and in situ detection of *Escherichia coli* β-galactosidase gene expression in *Dictyostelium discoideum*. Gene 85: 353-362.

Early, A. E., and J. G. Williams. 1988. A *Dictyostelium* prespore-specific gene is transcriptionally repressed by DIF in vitro. Development 103: 519-524.

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

Firtel, R. A., and A. L. Chapman. 1990. A role for cAMP-dependent protein kinase A in early Dictyostelium development. Gens Dev. 4:18-28.

Firtel, R. A., and H. F. Lodish. 1973. A small nuclear precursor of messenger RNA in the cellular slime mold *Dictyostelium discoideum*. J. Mol. Biol. 79: 295-314.

Fishel, B. R., R. E. Manrow, and R. P. Dottin. 1982. Developmental regulation of multiple forms of UDP-glucose pyrophosphorylase of *Dictyostelium*. Dev. Biol. 92:175-187.

Fishel, B. R., J. A. Ragheb, A. Rajkovic, B. Haribabu, C. Schweinfest, and R. P. Dottin. 1985. Molecular cloning of a cDNA complimentary to a UDP-glucose pyrophosphorylase mRNA of *Dictyostelium discoideum*. Dev. Biol. 110:369-381.

Ginsburg, G., and A. R. Kimmel. 1989. Inositol triphosphate and diacylglycerol can differentially modulate gene expression in *Dictyostelium*. Proc. Natl. Acad. Sci. USA 86:9332-9336.

Gomer, R. H., and R. A. Firtel. 1987. Cell-autonomous determination of cell-type choice in *Dictyostelium* development by cell-cycle phase. Science 237:758-762.

Grant, C. E., G. Bain, and A. Tsang. 1990. The molecular basis for alternative splicing of the CABP1 transcripts in *Dictyostelium discoideum*. Nuc. Acids Res. 18:5457-5463.

Gross, J. D., J. Bradbury, R. R. Kay, and M. J. Peacey. 1983. Intracellular pH and the control of cell differentiation in *Dictyostelium discoideum*. Nature 303: 244-245.

Gustafson, G. L., and B. E. Wright. 1972. Analysis of approaches used in studying differentiation of the cellular slime mold. CRC Crit. Rev. Microbiol. 1: 453-478.

Haribabu, B., A. Rajkovic, and R. P. Dottin. 1986. Cell-cell contact and cAMP regulate the expression of a UDP glucose pyrophosphorylase gene of *Dictyostelium discoideum*. Dev. Biol. 113:436-442.

Higgins, R. C., and M. E. Dahmus. 1982. Glycogen phosphorylase synthesis in *Dictyostelium discoideum*. J. Biol. Chem. 257:5068-5076.

Hughes, J. E., H. Ashktorab, and D. L. Welker. 1988. Nuclear plasmids in the *Dictyostelium* slime molds. Dev. Genet. 9: 495-504.

Hughes, J. E., G. J. Podgorski, and D. L. Welker. 1992. Selection of *Dictyostelium discoideum* transformants and analysis of vector maintenance using live bacteria resistant to G418. Plasmid 28: 46-60.

Inouye, K. 1988. Induction by acid load of the maturation of prestalk cells in *Dictyostelium discoideum*. Development 104: 669-681.

Insall, R., and R. R. Kay. 1990. A specific DIF binding protein in *Dictyostelium*. EMBO J. 9: 3323-3328.

Johnson, R. L., C. L. Saxe III, R. Gollop, A. R. Kimmel, and P. N. Devreotes. 1993. Identification and targeted gene disruption of CAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development. Gene and Dev. 7: 273-282.

Johnson, R. L., P. J. M. VanHaastert, A. R. Kimmel, C. L. Saxe III, B. Jastorff, and P. N. Devreotes. 1992. The cyclic nucleotide specificity of three cAMP receptors in *Dictyostelium*. J. Biol. Chem. 267: 4600-4607.

Kay, R. R. 1989. Evidence that elevated intracellular cyclic AMP triggers spore maturation in *Dictystelium*. Development 105:753-759.

Kay, R. R., M. Berks, and D. Traynor. 1989. Morphogen hunting in *Dictyostelium*. Development 1989 Supplement: 81-90.

Kay, R. R., B. Dhokia, and K. A. Jermyn. 1983. Purification of stalk-cell-inducing morphogens from *Dictyostelium discoideum*. Eur. J. Biochem. 136: 51-56.

Kay, R. R., D. Garrod, and R. Tilly. 1978. Requirement for cell differentiation in *Dictyostelium discoideum*. Nature, 271: 58-60.

Kay, R. R., and K. A. Jermyn. 1983. A possible morphogen controlling differentiation in *Dictyostelium*. Nature, 303: 242-244.

Kimmel, A. L., and R. A. Firtel. 1985. Sequence organization and developmental expression of an interspersed, repetitive element and associated single-copy DNA sequences in *Dictyostelium*. Mol. Cell. Biol. 5:2123-2130.

Kimmel, A. R., and R. A. Firtel. 1991. cAMP signal transduction pathways regulating development of *Dictyostelium discoideum*. Curr. Opin. Genet. Dev. 1: 383-390.

Knecht, D. A., and W. F. Loomis. 1987. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum*. Science 236: 1081-1086.

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

Kubohara, Y., K. Okamoto, Y. Tanaka, K. Asahi, A. Sakurai, and N. Takahashi, 1993. Differanisole A, an inducer of the differentiation of Friend Leukemic Cells, induces stalk cell differentiation in *Dictyostelium discoideum*. FEBS Lett. In press.

Lam, T. Y., G. Pickering, J. Geltovsky, and C. H. Siu. 1981. Differential cell cohensiveness expressed by prespore and prestalk cells of *Dictyostelium discoideum*. Differentiation 20: 22-28.

Leiting, B., I. J. Lindner, and A. A. Noegel. 1990. The extrachromosomal replication of *Dictyostelium* plasmid Ddp2 requires a *cis*-acting element and a plasmid-encoded *trans*-acting factor. Mol. Cell. Biol. 10: 3727-3736.

Leiting, B., and A. Noegel. 1988. Construction of an extrachromosomally replicating transformation vector for *Dictyostelium discoideum*. Plasmid 20: 241-248.

Liu, G., and P. C. Newell. 1988. Evidence that cyclic GMP regulates myosin interaction with the cytoskeleton during cheotaxis of Dictyostelium. J. Cell Sci. 90:123-129.

Loomis, W. F. 1985. Regulation of cell-type-specific differentiation in *Dictyostelium*. Cold Spring Harbor Symposia on Quantitative Biology, Volume L: 769-777.

Loomis, W. F., S. Wheeler, W. Springer, and S. Barondes. 1985. Adhesion mutants of *Dictyostelium discoideum* lacking the determinant recognized by two monoclonal adhesion blocking antibodies. Dev. Biol. 109: 111

Masento, M. S., H. R. Morris, G. W. Taylor, S. J. Johnson, A. C. Skapski, , and R. R. Kay. 1988. Differentiation-inducing factor from the slime mould *Dictyostelium*

discoideum and its analogues: synthesis, structure and biological activity. Biochem. J. 256: 23-28.

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

Metz, B. A., T. E. Ward, D. L. Welker, and K. L. Williams. 1983. Identification of an endogeneous plasmid in *Dictyostelium discoideum*. EMBO J. 2: 515-519.

Morris, H. R., M. S. Masento, G. W. Taylor, K. A. Jermyn, and R. R. Kay. 1988. Structure elucidation of two differentiation factors (DIF-2 and DIF-3) from the cellular slime mould *Dictyostelium discoideum*. Biochem. J. 249: 903-906.

Morris, H. R., G. W. Taylor, M. S. Masento, K. A. Jermyn, and R. R. Kay. 1987. Chemical structure of the morphogen differentiation inducing factor from *Dictyostelium discoideum*. Nature 328: 811-814.

Morrissey, J. H., K. M. Devine, W. F. Loomis. 1984. Timing of cell-type-specific differentiation in *Dictyostelium discoideum*. Dev. Biol. 103:414-424.

Nakano, K., Y. Tashiro, Y. Kikumoto, M. Tagaya, and T. Fukui. 1986. Amino acid sequence of cyanogen bromide fragments of potato phosphorylase. J. Biol. Chem. 261: 8224-8229.

Naranan, V., J. F. Sucic, D. A. Brickey, and C. L. Rutherford. 1988a. The relationship between the two forms of glycogen phosphorylase in *Dictyostelium discoideum*. Differentiation 38:1-10.

Naranan, V., D. A. Brickey, and C. L. Rutherford. 1988b. Glycogen phosphorylase "b" in *Dictyostelium*: stability and endogenous phosphorylation. Mol. Cell. Biochem. 83: 89-104.

Nellen, W., C. Silan, and R. A. Firtel. 1984. DNA-mediated transformation in *Dictyostelium discoideum*: regulated expression of an actin gene fusion. Mol. Cell. Biol. 4: 2890-2898.

Newgard, C. B., D. R. Littman, C. Van Genderer, M. Smith, and R. J. Fletterick. 1988. Human brain glycogen phosphorylase: cloning, sequence analysis, chromosomal mapping, tissue expression, and comparison with the human liver and muscle isozymes. J. Biol. Chem. 263: 3850-3857.

Newgard, C. B., K. Nakano, P. K. Hwang, and R. J. Fletterick. 1986. Sequence analysis of the cDNA encoding human liver glycogen phosphorylase reveals tissue-specific codon usage. Proc. Natl. Acad. Sci. USA. 83: 8132-8136.

Noegel, A., D. L. Welker, B. A. Metz, and K. L. Williams. 1985. Presence of nuclear plasmids in the lower eukaryote *Dictyostelium discoideum*. J. Mol. Biol. 185: 447-450.

Orii, H., Y. Tanaka, and K. Yanagisawa. 1989. Sequence organization and gene expression of pGD1, a plasmid found in a wild isolate of *Dictyostelium*. Nucleic Acid. Res. 17: 1395-1408.

Oyama, M., and D. D. Blumberg. 1986a. Changes during differentiation in requirements for cAMP for expression of cell-type specific mRNAs in the cellular slime mould *Dictyostelium discoideum*. Dev. Biol. 117: 555-556.

Oyama, M., and D. D. Blumberg. 1986b. Cyclic AMP and NH4+ both regulate cell type specific mRNA accumulation in the cellular slime mould *Dictyostelium discoideum*. Dev. Biol. 117: 557-566.

Podgorski, G. J., and R. A. Deering. 1980. Quantitation of induced mutation in *Dictyostelium discoideum*: characterization and use of a methanol resistance mutation assay. Mutat. Res. 74: 459-468.

Reymond, C. D., R. H. Gomer, M. C. Mehdy, and R. A. Firtel. 1984. Developmental regulation of a *Dictyostelium* gene encoding a protein homologous to mammalian ras protein. Cell 39: 141-148.

Rogers, P. V., S. Luo, J. F. Sucic, and C. L. Rutherford. 1992. Characterization and cloning of glycogen phosphorylase 1 from *Dictyostelium discoideum*. Biochim. Biophys. Acta 1129:262-272.

Rogers, P. V., J. F. Sucic, Y. Yin, and C. L. Rutherford. 1993. Disruption of glycogen phosphorylase gene expression in *Dictyostelium*: Evidence for altered glycogen metabolism and developmental co-regulation of the gene products. Differentiation, in press.

Rutherford, C. L., and M. J. Cloutier. 1986. Identification of two forms of glycogen phosphorylase in *Dictyostelium*. Arch. Biochem. Biophys. 250:435-439.

Rutherford, C. L., R. B. Peery, J. F. Sucic, Y. Yin, P. V. Rogers, S. Luo, and O. Selmin. 1992. Cloning, structural analysis, and expression of the glycogen phosphorylase-2 gene in *Dictyostelium*. J. Biol. Chem. 267: 2294-2302.

Rutherford, C. L., R. D. Taylor, L. T. Frame, and R. L. Auck. 1982. A cyclic AMP-dependent protein kinase in *Dictyostelium discoideum*. Biochem. Biophys. Res. Commun. 108:1210-1220.

Rutherford, C. L., R. L. Vaughan, M. J. Cloutier, D. K. Ferris, and D. A. Brickey. 1984. Chromatographic behavior of cyclic AMP-dependent protein kinase and its subunits from *Dictyostelium discoideum*. Biochemistry 23:4611-4617.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Saxe, C. L. III, G. T. Ginsburg, J. M. Louis, R. Johnson, P. N. Devreotes, and A. R. Kimmel. 1993. CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. Gene and Dev. 7: 262-272.

Schaap, P. 1986. Regulation of size and pattern in the cellular slime molds. Differentiation 33: 1-16.

Schaap, P. 1991. Intercellular interactions during *Dictyostelium* development. In Microbial Cell-Cell interactions, pp. 147-166. M. Dwokin, ed. American Society for Microbiology, Washington, DC.

Schaap, P., and M. Wang. 1986. Interactions between adenosine and oscillatory cAMP signalling regulate size and pattern in *Dictyostelium*. Cell 45: 137-144.

Schindler, J., and M. Sussman. 1977a. Ammonia determines the choice of morphogenic pathways in *Dictyostelium discoideum*. J. Mol. Biol. 116: 161-170.

Schindler, J., and M. Sussman. 1977b. Effect of NH_3 on cAMP associated activities and extracellular cAMP production in *Dictyostelium discoideum*. Biochem. Biophys. Res. Comm. 79: 611-617.

Schindler, J., and M. Sussman. 1979. Inhibition by ammonia of intracellular cAMP accumulation in *Dictyostelium discoideum*: its significance for the regulation of morphogenesis. Dev. Genet. 1: 13-20.

Schultz, J. R., T. Tansey, L. Gremke, and R. V. Storti. 1991. A muscle-specific intron enhancer required for rescue of indirect flight muscle and jump muscle function regulates Drosophila tropomysin I gene expression. Mol. Cell. Biol. 11: 1901-1911.

Shaw, D. R. H. Richter, R. Giorda, T. Ohmachi, and H. L. Ennis. 1989. Nucleotide sequences of *Dictyostelium discoideum* developmentally regulated cDNAs rich in (AAC) imply proteins that containclusters of asparagine, glutamine, or threonine. Mol. Cell. Genet. 218:453-459.

Slade, M.B., A. C. M. Chang, and K. L. Williams. 1990. The sequence and organization of Ddp2, a high-copy-number nuclear plasmid of *Dictyostelium discoideum*. Plasmid 24: 195-207.

Strandmann, R. P., and R. R. Kay. 1990. Position-dependent regulation of the prestalk-prespore pattern in *Dictyostelium* slugs. Dev. Genet. 11:447-452.

Sucic, J. F., S. Luo, B. W. Williamson, Y. Yin, and C. L. Rutherford. 1993. Developmental and cyclic-AMP mediated regulation of glycogen phosphorylase 1 in *Dictyostelium*. J. General Microbiol., in press.

Sucic, J. F., O. Selmin, P. V. Rogers, and C. L. Rutherford. 1993. Regulation of the *Dictyostelium* glycogen phosphorylase-2 gene by cAMP. Dev. Genet., in press.

Sussman, M. 1982. Morphogenetic signaling, cytodifferentiation, and gene expression. Chapter 9, pp 353-385, in The development of *Dictyostelium discoideum*, ed W. F. Loomis. Academic Press, Inc, New York

Telser, A., M. Sussman. 1971. Uridine diphosphate galactose-4-epimerase, a developmentally regulated enzyme in the cellular slime mold *Dictyostelium discoideum*. J. Biol. Chem. 246: 2252-2257.

Theibert, M., and P. N. Devreotes. 1984. Adenosine and its derivatives inhibit the cAMP signalling response in *Dictyostelium discoideum*. Dev. Biol. 106: 166-173.

Thomas, D. A., and B. E. Wright. 1976. Glycogen phosphorylase in *Dictyostelium discoideum*. Part I: Purification and properties of the enzyme. Part II: Synthesis and degradation during differentiation. J. Biol. Chem. 251:1253-1263.

Town, C. D. 1984. Differentiation of *Dictyostelium discoideum* in monolayer cultures and its modification by ionic conditions. Differentiation 27: 29-35.

Town, C. D., J. D. Gross, and R. R. Kay. 1976. Cell differentiation without morphogenesis in *Dictyostelium discoideum*. Nature 262: 717-719.

Town, C. D., and E. Stanford. 1979. An oligosaccharide-containing factor that induces cell differentiation in *Dictyostelium discoideum*. Proc. Natl. Acad. Sci. USA 76: 308-312.

Van Haastert P. J. M. 1983. Binding of cAMP and adenosine derivatives to *Dictyostelium discoideum* cells. Relationships of binding, chemotactic and antagonistic activities. J. Biol. Chem. 258 9643-9648.

Van Lookeren Campagne, M. M. V., P. Schaap, and P. J. M. Van Haastert. 1986. Specificity of adenosine inhibition of cAMP-induced responses in *Dictyostelium* resembles that of the P site of higher organisms. Dev. Biol. 117: 245-251.

Wang, M., and P. Schaap. 1989. Ammonia depletion and DIF trigger stalk cell differentiation in intact *Dictyostelium discoideum* slugs. Development 105: 569-574.

Weijer, C. J., and A. J. Durston. 1985. Influence of cAMP and hydrolysis products on cell type regulation in *Dictyostelium discoideum*. J. Embryol. Exp. Morph. 86: 19-37.

Weiger, C. J., G. Duschl, and C. N. David. 1984. Dependence of cell-type proportioning and sorting on cell cycle phase in *Dictyostelium discoideum*. J. Cell Sci. 70:133-145.

Welker, D. L., K. P. Hirth, and K. L. Williams. 1985. Inheritance of extrachromosomal ribosomal DNA during the asexual life cycle of *Dictyostelium discoideum*: examination by use of DNA polymorphisms. Mol. Cell. Biol. 5: 273-280.

Williams, B. G., E. M. Elder, and M. Sussman. 1984. Modulation of the cAMP relay in Dictyostelium discoideum by ammonia and other metabolites: possible morphogenetic consequences. Dev. Biol. 105: 377-388.

Williams, J. G. 1988. The role of diffusible molecules in regulating the cellular differentiation of *Dictyostelium discoideum*. Development 103: 1-16.

Williams, J. G., M. M. Berks, R. R. Kay, and K. A. Jermyn. 1987. Direct induction of *Dictyostelium* prestalk gene expression by DIF provides evidence that DIF is a morphogen. Cell 49: 185-192.

Wilson, J. B., and C. L. Rutherford. 1978. ATP, trehalose, glucose and ammonia ion localization in the two cell types of *Dictyostelium discoideum*. J. Cell. Physiol 94: 37-46.

Witke, W., W. Nellen, and A. Noegel. 1987. Homologous recombination in the *Dictyostelium* α-actinin gene leads to an altered mRNA and lack of the protein. EMBO J. 6:4143-4148.

Xie, Y., L. Kwong, G. Weeks. 1991. A possible role for DIF-2 in the formation of stalk cells during *Dictyostelium* development. Dev. Biol. 145: 195-200.

Zimmermann, W., and C. J. Weiger. 1993. Analysis of cell cycle progression during the development of *Dictyostelium* and its relationship to differentiation. Dev. Biol. In press.

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Academic degrees

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Professional experiences

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Publications

1. **Yin, Y.**, and D. L. Welker. 1992. *Dictyostelium giganteum* plasmid Dgp1 is a member of the Ddp2 plasmid family. Plasmid 28:37-45.
2. Rutherford, C.L., R.B. Peery, J.F. Sucic, **Y. Yin**, P.V. Rogers, S. Luo, and O. Selmin. 1992. Cloning, structural analysis, and expression of the glycogen phosphorylase-2 gene in *Dictyostelium*.. Journal of Biological Chemistry, 267:2294-2302.
3. Sucic, J.F., S.Luo, B.D.Williamson, **Y.Yin**, and C.L.Rutherford. 1993. Developmental and cAMP Mediated Regulation of Glycogen Phosphorylase 1 in *Dictyostelium*. J. of General Microbiology, in press.
4. Rogers, P.V., J.F.Sucic, **Y.Yin**, and C.L.Rutherford. 1993. Disruption of glycogen gene expression in *Dictyostelium*: evidence for altered glycogen metabolism and development co-regulation of the gene products. Differentiation, in press..

5. **Yin, Y.**, and C.L.Rutherford. 1993. Dual regulation of the glycogen phosphorylase-2 gene in *Dictyostelium discoideum*:: The effects of DIF-1, cAMP, NH_3, and adenosine. Manuscript submitted.
6. **Yin, Y.**, and C.L.Rutherford. 1993. Establishment of an extrachromosomal luciferase reporter vector for *Dictyostelium discoideum*. Manuscript submitted.

Abstracts and presentations at professional meetings

1. Glycogen Phosphorylase in Dictyostelium: Regulation and Transfection, International Dictyostelium Conference, September, 1990.
2. Regulation of glycogen phosphorylase genes in *Dictyostelium discoideum*, Virginia Academy Sciences, May, 1991.
3. Characterization of the Dgp1 plasmid from the cellular slime mold *Dictyostelium giganteum*, The Third CAASS Conference, June, 1991
4. Glycogen phosphorylase gene 2 in the lower eukaryote *Dictyostelium discoideum*, The third CAASS Conference, June, 1991.
5. PCR analysis of 5'-upstream regulatory regions of glycogen phosphorylase-1 gene in a simple differentiating eukaryote *Dictyostelium discoideum*, The Third CAASS Conference, June, 1991.
6. Characterization and regulation of glycogen phosphorylase genes in *Dictyostelium discoideum*, International Dictyostelium Conference, August, 1991.
7. Glycogen phosphorylase-1 and -2 gene expression in Dictyostelium discoideum, International Dictyostelium Conference, August, 1991.
8. Molecular mechanism of the glycogen phosphorylase gene regulation in *Dictyostelium discoideum*. 1992 Graduate Research Symposium, Virginia Tech, April, 1992.
9. Molecular mechanism of the glycogen phosphorylase gene regulation in *Dictyostelium discoideum*. International SCBA conference, June, 1992.
10. Regulation of the glycogen phosphorylase gene 2 by DIF-1, cAMP, NH_3, and adenosine during *Dictyostelium* development. Graduate Research Symposium, March, 1993.
11. Regulation of glycogen phosphorylase genes in *Dictyostelium*. International *Dictyostelium* Conference, Noordwijkerhout, the Netherlands. May, 1993.

12. Dual inducibilities and combinatorial regulation of the glycogen phosphorylase gene 2 during *Dictyostelium* development. The 5th SCBA international Symposium, June, 1993.
13. One gene, dual inducibility: DIF-1- and cAMP-mediated regulation of the gp2 gene in *Dictyostelium discoideum.* The 33rd annual meeting of The American Society for Cell Biology, December, 1993.

Honors and Grant awards

1. Grant award from Graduate Research Development Program, Virginia Tech. August, 1991,
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5. First place award from the Graduate Research Symposium, Virginia Tech. March, 1993.
6. Tuition Scholarship from Graduate School / Department of Biology, Virginia Tech. 1993.
7. Travel fellowship award from the 5th SCBA international Symposium from SCBA. May, 1993.
8. Distinguished Achievement in poster presentation at the 5th SCBA International Symposium. June, 1993.
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Memberships in Professional Organizations

1. American Society for Microbiology.
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