Regulation of Glycogen Phosphorylase Genes in *Dictyostelium discoideum*

by

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(ABSTRACT)

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study eukayotic development, cell differentiation, and aging. A crucial developmental event in *Dictyostelium* is glycogen degradation. The degradation of glycogen provides glucose monomers that are used to synthesize structural components necessary for cellular differentiation. Glycogen degradation is catalyzed by glycogen phosphorylase, and two developmentally regulated glycogen phosphorylase activities have been discovered in *Dictyostelium*. Glycogen phosphorylase 1 (gp-1) activity is predominant early in development, and is dependent upon 5′ AMP as a positive allosteric modifier; glycogen phosphorylase 2 (gp-2) activity peaks late in development and is independent of 5′ AMP. I showed that these two glycogen phosphorylase activities are associated with unique proteins that are the products of two distinct, but related, genes. Both genes were observed to be typical *Dictyostelium* genes in a number of respects. The gp-1 and gp-2 enzymes were also found to be similar to glycogen phosphorylases from other organisms. I also examined the developmental expression of these genes and found that both mRNAs are developmentally regulated; gp-1 mRNA levels fluctuate during development, while gp-2 mRNA levels increase late in development. The expression of the gp-1 and gp-2 genes is regulated by exogenous cAMP. Exogenous cAMP enhances the level of gp-1 mRNA, apparently through a mechanism that requires intracellular cAMP signaling. Specific DNA sequence elements appear to be required for maximal vegetative and late developmental expression of gp-1. Exogenous cAMP induces the appearance of gp-2 mRNA via a mechanism that appears to be independent of intracellular cAMP signaling. Repeated TA-rich sequences located between nucleotides 193 and 305 upstream of the transcriptional start site are necessary for maximal cAMP induction of gp-2. I also examined the cell type specific expression of gp-1 and gp-2. gp-1 is expressed predominantly in pre-stalk cells. gp-2 is expressed in both cell

types in a temporally regulated fashion; this type of expression has not been reported for other *Dictyostelium* genes, but, given the importance of glycogen degradation in both stalk and spore cells, it is not inconceivable that such regulation is necessary.

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Table of Contents

List of Illustrations

List of Tables

Chapter 1: Introduction

The processes of cellular differentiation and development in multicellular organisms involve a multitude of remarkable and complex events. Obviously, the progression from a unicellular fertilized egg to a complex organism containing a variety of different cell types requires elaborate and precise biochemical control. Thus, central to the understanding of organismic development is the elucidation of the molecular mechanisms that trigger developmental events such as cell differentiation. Recent experimentation suggests that cellular aging and even death are simply a continuation of the developmental program that is initiated upon fertilization. Researchers refer to this as programmed cell death, and insight into this process will undoubtedly be gained if the molecular basis for cell differentiation can be understood. Additionally, developmental anomalies such as cancer, birth defects, and premature aging can only be understood if the normal regulatory mechanisms of differentiated or differentiating cells are determined.

Model systems are essential to our understanding of the molecular basis for cell differentiation, development, and aging. The cellular slime mold, *Dictyostelium discoideum*, provides an elegant and widely used model to study the processes of development and aging. Many aspects of this organism make it well suited to act as a developmental model. For example, *Dictyostelium* undergoes a short, simple, and well documented life cycle in which one intial cell type differentiates into two cell types; one of the differentiated cell types actually undergoes a programmed cell death.

Also, *Dictyostelium* is easily cultured and manipulated in the laboratory. Finally, this organism is readily amenable to the genetic manipulations required to carry out modern molecular biology experimentation.

The Life Cycle of *Dictyostelium discoideum*

The life cycle of *Dictyostelium discoideum* is shown in Figure 1. *D. discoideum* was first isolated and characterized by Raper (1935). Vegetative cells of *D. discoideum* are ameboid in appearance and are found living in the soil of most temperate forests; in this environment they feed on bacteria and particulate organic matter. When the local supply of nutrients becomes depleted the behavior of the amoebae changes dramatically. Individual amoebae begin streaming together, in a chemotactic response to cyclic adenosine monophosphate (cAMP), and form a loose aggregate of cells. Approximately 12 hours after the initiation of nutrient depletion, the aggregate becomes slug-like in appearance and is called a pseudoplasmodium, or simply a slug. The slug migrates along the substratum, and secretes a slime sheath. The cells making up the slug are no longer a homogeneous mixture; rather, two distinct cell types are seen. This was first observed by Raper (1940), who noticed that the slug seemed to be composed of two distinct regions of cells, and Bonner (1952), who reported that, in response to vital dyes, the cells making up the anterior portion of the slug exhibited different staining than those in the posterior portion of the slug. Raper and subsequent investigators have shown that the cells at the anterior portion of the slug ultimately form a slender stalk upon which rests a fruiting body containing mature spores derived from the posterior portion of the slug (Raper, 1940; Bonner, 1952; Bonner et al., 1955); thus, the anterior-most cells in the slug are called pre-stalk cells and the cells from the posterior region of the slug are called pre-spore cells. The transformation of the slug into a mature fruiting body begins when slug migration ceases and a stage called culmination begins. At the culmination stage, the anterior-most of the pre-stalk cells begin to differentiate into mature stalk cells; these cells begin to secrete cellulose, which is the major component of the stalk, and also begin to form the stalk structure.

The stalk continues to elongate due to the movement of stalk cells from the apex to the base of the stalk in what Bonner calls a "reverse fountain" pattern of movement (Bonner, 1944; Raper and Fennell, 1952); the elongation of the stalk ultimately causes the mass of pre-spore cells from the posterior of the slug to be lifted upward. As this lifting occurs the pre-spore cells differentiate into spores and the mature fruiting body is thus formed. Each spore in the fruiting body is capable of developing into an amoeba under suitable conditions. Fruiting bodies are usually seen within 24 hours of the onset of nutrient depletion.

Clearly, *D. discoideum* is an ideal model system to study cell differentiation and development. The developmental cycle is well characterized, rapid, and simple; indeed, this organism represents one of the the simplest developmental models that can can be imagined, in which one initial cell type (vegetative amoebae) differentiates into two cell types (stalk cells and spore cells).

The Role of cAMP in the Developmental Cycle of *Dictyostelium discoideum*

An important regulatory molecule in all eukaryotic systems is cAMP. Not surprisingly, this molecule has been implicated as an important regulatory agent in several different aspects of the life cycle of *Dictyostelium*. As mentioned above, cAMP is the chemoattractant molecule responsible for the aggregation of amoebae (Konijn, et al., 1968; Robertson et al., 1972). Chemotaxis begins when certain amoebae begin secreting wave-like pulses of cAMP (Devreotes, 1983; Gerisch, 1987). The cAMP binds to cell surface cAMP receptors of other amoebae, inducing the chemotactic response and activating adenylate cyclase, which results in an increase in intracellular cAMP levels. Secretion of intracellular cAMP by these amoebae amplifies the chemotactic signal (Shaffer, 1975; Roos et al., 1975; Gerisch and Wick, 1975), and aggregation eventually results. cAMP levels are decreased by cAMP phosphodiesterase activity; three different phosphodiesterase

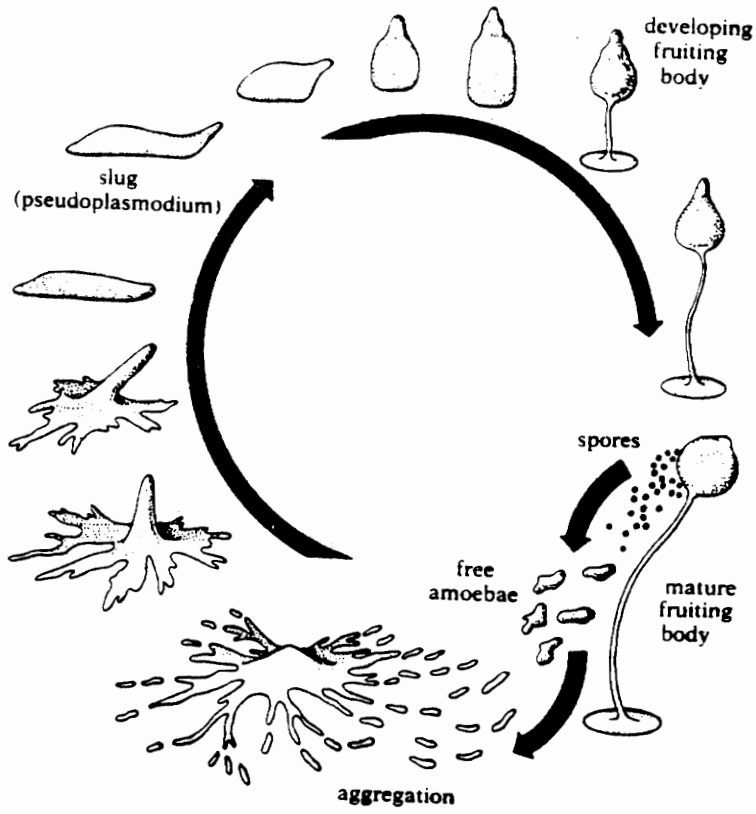

Figure 1. The life cycle of *Dictyostelium discoideum*

enzymes (intracellular, membrane bound, and extracellular) appear to be present in *D. discoideum* (Devreotes, 1982).

In addition to acting as the chemotactic agent in the development of *Dictyostelium*, exogenous cAMP is necessary for the expression and stabilization of many developmentally regulated mRNA species (Sampson et al., 1978; Town and Gross, 1978; Landfear and Lodish, 1980; Landfear et al., 1982; Barklis and Lodish, 1983; Mangiarotti et al., 1983; Mehdy et al., 1983; Williams et al., 1984; Driscoll and Williams, 1987). Several of the genes under cAMP regulation have recently been identified, cloned, and sequenced, and recent research has concentrated on identifying DNA sequence elements and DNA binding proteins involved in the cAMP mediated control of these genes (Datta and Firtel, 1987; Driscoll and Williams, 1987; Pears and Williams, 1987; Datta and Firtel, 1988; Pavlovic et al., 1989; Haberstroh and Firtel, 1990; Hjorth et al., 1990; Haberstroh et al., 1991).

While the importance of cAMP in regulating some developmentally regulated genes is well established, the mechanism or mechanisms by which cAMP-mediated mRNA expression and stabilization occurs has been the focus of much research and some controversy. At least two different molecular mechanisms appear to be involved in the cAMP mediated regulation of gene expression in *Dictyostelium* (Europe-Finner and Newell, 1987; Gerisch, 1987; Janssens and Van Haastert, 1987; Kimmel, 1987). These two mechanisms probably function through different cell surface cAMP receptors which activate alternate sets of G-proteins and intracellular second messengers (Johnson et al., 1989; Saxe et al., 1991). One of these pathways appears to require an intracellular increase in cAMP. Fluctuations in intracellular cAMP levels are important in regulating many different biochemical processes in both eukaryotes and prokaryotes. An intracellular increase in cAMP results from activation of adenylate cyclase, usually in response to extracellular stimuli such as hormones. In *Dictyostelium*, activation of adenylate cyclase occurs in response to exogenous cAMP; thus, cAMP can act as both a first and second messenger in this organism. The physiological effects of elevated intracellular cAMP levels are often the result of a cAMP-dependent protein kinase, an enzyme which is activated by cAMP and which alters the phosphorylation state and activity of substrate enzymes (Walsh et al., 1968). Intracellular cAMP levels can be decreased

through the action of phosphodiesterase; decreased cAMP levels inactivate cAMP-dependent protein kinases and may activate phosphatase enzymes which dephosphorylate, and alter the activity of, enzymes which were phosphorylated by the cAMP-dependent kinases. The discovery of cAMP-dependent protein kinases in *Dictyostelium* (Sampson, 1977; Rutherford et al., 1982, 1984; De Gunzburg and Veron, 1982; Leichtling et al., 1984; Majerfield et al., 1984) raised the possibility that some biochemical processes in this organism are also regulated through a series of cAMP-dependent phosphorylations and dephosphorylations. The cAMP-dependent protein kinase may also play a direct role in gene expression; Woffendin et al. (1986) have shown that the kinase enters the nucleus of developing cells of *D. discoideum*, and Chambers et al. (1987) have identified several nuclear proteins as substrates of the enzyme. Also, Firtel and Chapman (1990) and Harwood et al. (1992) have shown that normal development will not occur if the expression of a cAMP dependent kinase is disrupted. Clearly, intracellular cAMP fluctuations and the activity of cAMP dependent protein kinases are important in the development of *Dictyostelium.* However, some genes that are regulated by cAMP appear to be controlled through a second mechanism that does not require an intracellular increase in cAMP. By using cAMP analogs with high affinity for either the kinase or the cell surface receptors, some researchers have accumulated evidence which suggests that this type of cAMP mediated gene expression occurs directly through the cell surface cAMP receptor in a mechanism independent of the cAMP-dependent protein kinase (Haribabu and Dottin, 1986; Oyama and Blumberg, 1986; Kimmel, 1987; Mann and Firtel, 1987). In this mechanism, interaction of cAMP with cell surface receptors leads to the production of intracellular second messangers inositol-tris-phosphate (IP_3), diacylglycerol (DAG), and calcium (Europe-Finner and Newell, 1986; Schaap et al., 1986; Europe-Finner and Newell, 1987; Janssens and Van Haastert, 1987; Milne and Coukell, 1988; Ginsburg and Kimmel, 1989).

Other factors in addition to exogenous cAMP have been implicated in cell differentiation, gene expression, and mRNA stabilization in *Dictyostelium.* For example, cell-cell contact is known to play a role in these these processes (Alton and Lodish, 1977; Chung et al., 1981; Mangiarotti et al., 1981; Blumberg et al., 1982). Also, it has been shown that the differentiation inducing factors (DIFs), a group of low molecular weight, hydrophobic compounds, can induce pre-stalk specific

mRNAs (Williams et al., 1987; Jermyn et al., 1987), transcriptionally repress pre-spore genes (Early and Williams, 1988), as well as act as stalk cell morphogens (Town et al., 1976; Morris et al., 1987; Williams, 1988). Recent reports suggest that the DIFs and cAMP modulate at least four classes of gene regulation in *Dictyostelium*: prespore specific, prestalk A, prestalk B, and vegetative (Jermyn et al., 1989; Williams et al., 1989; Berks et al., 1991). Still other reports have implicated intracellular pH (Gross et al., 1983; Aerts, 1988) and ammonia (Schindler and Sussman, 1977; Oyama et al., 1988) as important factors in cell differentiation; intracellular pH changes may be yet another second messenger system activated by exogenous cAMP (Van Lookeren Campagne et al., 1989).

Glycogen Phosphorylase in *Dictyostelium discoideum*

A key event in the development of *Dictyostelium discoideum* is glycogen degradation. The degradation of glycogen provides glucose precursors which are used to synthesize components of differentiated cells; one such component is cellulose, a major component of the stalk (Wright et al., 1968; Marshall et al., 1970; Gustafson and Wright, 1972). Glycogen degradation is catalyzed by the enzyme glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyl transferase; EC 2.4.1.1). Early research on this enzyme in *Dictyostelium* showed it to be 5′ AMP independent and developmentally regulated; little or no activity was detected early in development, but activity increased as development progressed and ultimately peaked at culmination (Firtel and Bonner, 1972). Several different studies suggested that the increase in phosphorylase activity during development was due to *de novo* synthesis of the enzyme. Firtel and Bonner (1972) showed that the increase in phosphorylase activity during development would not occur if RNA and protein synthesis were inhibited. Thomas and Wright (1976) found that ^{35}S-methionine incorporation into the phosphorylase protein during development correlated with the increase in enzyme activity, suggesting that the enzyme was being synthesized during development. Higgins and Dahmus (1982) also presented evidence that the enzyme was synthesized during development, and suggested that the developmental regulation of phosphorylase was at the level of mRNA transcription. In light

of the work described above, it was generally accepted that glycogen phosphorylase in *Dictyostelium* was developmentally regulated and accumulated due to *de novo* protein synthesis.

New questions on the developmental regulation of glycogen phosphorylase in *Dictyostelium* were raised when Rutherford and Cloutier (1986) identified a previously unknown glycogen phosphorylase activity. Unlike the phosphorylase activity described above, this new form was dependent upon 5′ adenosine monophosphate (5′ AMP) for activity; in this respect it was analogous to the "inactive", or 'b' form of glycogen phosphorylase seen in higher eukaryotes. Cloutier and Rutherford (1987) showed that this new glycogen phosphorylase activity was present in vegetative amoebae and in the early developmental stages; this activity decreased as development progressed. Since this activity was observed early in development, it was called gp-1. The glycogen phosphorylase described by Firtel and Bonner (1972), which resembled the mammalian active, or 'a', form of glycogen phosphorylase in being independent of 5′ AMP, was called gp-2 since its activity peaked late in development. The developmental change in the activities of these two forms of glycogen phosphorylase is shown in Figure 2. Interestingly, the combined specific activity of gp-1 and gp-2 was observed to be constant throughout development. In other eukaryotic systems where "active" (5′ AMP independent) and "inactive" (5′ AMP dependent) forms of glycogen phosphorylase have been identified, the inactive form is converted to the active, 5′ AMP independent form through a cAMP mediated phosphorylation. The discovery of gp-1 activity in *D. discoideum* has raised the possibility that the gp-1 and gp-2 activities are developmentally regulated in this manner. Indeed, reports have suggested that gp-2 activity can be induced by cAMP (Okamoto and Takeuchi, 1976; Takemoto et al., 1978; Okamoto et al., 1982; Schaap and Van Driel, 1985). Alternately, it is possible that the two forms represent the products of different genes. Were this the case it would explain the results obtained by Firtel and Bonner (1972), Thomas and Wright (1976), and Higgins and Dahmus (1982). Recall that all of these researchers concluded that gp-2 activity increased due to the synthesis of gp-2 molecules; however, gp-1 activity was unknown when these experiments were carried out. It may seem unlikely that two enzymes which catalyze the same reaction would be the products of distinct genes. However, there are reasons to believe that this could be the case in *Dictyostelium*. For example, developmentally regulated glycogen de-

gradation in *Dictyostelium* does not provide the cells with an energy source; rather, glycogen degradation, and the appearance of gp-2 activity, are part of a developmental program. Also, some evidence suggests that two developmentally regulated forms of UDP-glucose pyrophosphorylase, another enzyme involved in glycogen metabolism, are the products of separate genes (Fishel et al., 1982; Fishel et al., 1985; Haribabu et al., 1986). Glycogen phosphorylase isozymes in other eukaryotes have also been shown to result from distinct genes (Berndt et al., 1987).

The discovery of gp-1 prompted investigations into two areas: 1) the relationship between gp-1 and gp-2--that is, are these two activities associated with a single protein and modulated by covalent alterations or are they associated with two different proteins; and 2) what is the developmental function of these two activities. Investigations into the relationship between these two phosphorylase activities quickly began to produce evidence that the two activities were not associated with the same protein. For example, the two activities appeared to be associated with purified proteins of different molecular weights. The gp-1 activity was associated with a 92 kd protein. The gp-2 activity appeared to be associated with a protein of 104 kd (Naranan et al., 1988a), although there have been some discrepancies in the literature regarding the molecular weight of this enzyme. Thomas and Wright (1976) reported a molecular weight of 95 kd for gp-2, but Higgins and Dahmus (1982) reported a molecular weight of 105 kd. However, the apparent higher molecular weight of gp-2 does not necessarily mean that gp-2 activity is associated with a unique protein, since phosphorylation of a protein can increase that protein's apparent molecular weight on SDS-PAGE (Dahmus, 1981).

The purified glycogen phosphorylases were used to generate polyclonal antibodies and these antibodies were used to carry out an extensive series of immunochemical experiments designed to study the relationship between gp-1 and gp-2 (Naranan et al., 1988a). In these experiments, the two forms of glyocogen phosphorylase were found to exhibit little antigenic similarity. Figure 3 shows Western analysis of protein extracts from different developmental stages. The membrane in Figure 3A was stained with anti-gp-1 antiserum, while the membrane in Figure 3B was stained with anti-gp-2 antiserum. The anti-gp-1 detects a band (or doublet) of 92 kd. Curiously, this band is present throughout development even though gp-1 activity decreases during

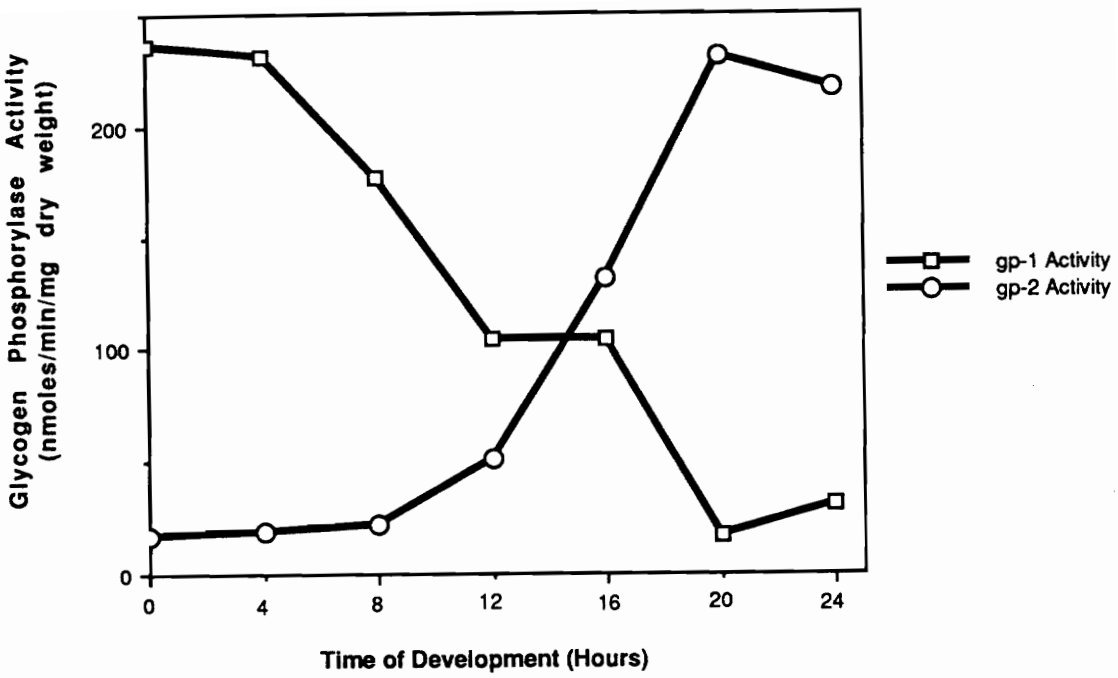

Figure 2. **Glycogen phosphorylase activity during the development of *Dictyostelium discoideum*:** Cells were allowed to develop on filters until harvested at progressive stages of development. Glycogen phosphorylase activity was assayed at the indicated times of development. Open circles show the activity in the absence of 5′ AMP (gp-2 activity), and open squares show gp-1 activity.

development. The anti-gp-2 detects a band (or doublet) of about 104-110 kd. This doublet is detected only later in development when gp-2 activity is observed. The doublet detected by anti-gp-1 is likely an artifact due to non-specific proteolysis. The doublet seen on the anti-gp-2 Western blot may represent the proteolytic processing of an inactive precursor gp-2 molecule; this possibility is discussed later (Chapter 2). Note that essentially no cross-reactivity is seen between the two antibodies. Table 1 shows the results of immunoinhibition experiments done using the two antibodies. Again, little or no cross-reactivity is seen. Immunoprecipitation analyses also showed essentially no immunological similarity between gp-1 and gp-2 (Naranan et al., 1988a). These results all suggest that the the two forms of glycogen phosphorylase are unique proteins.

Other experimentation done to examine the relationship between gp-1 and gp-2 included immunotitration and *in vitro* translation (Rutherford et al., 1988). The immunotitration data suggested that the developmentally regulated increase in gp-2 activity was due to synthesis of new gp-2 molecules rather than to covalent modification of existing molecules. The *in vitro* translation results are shown in Figure 4. The results shown in Figure 4 suggest that two distinct mRNA species exist for the two forms of glycogen phosphorylase; interestingly, the anti-gp-2 antiserum immunoprecipitated a protein of about 110 kd--larger than the 104 kd molecular weight of gp-2 observed in other experiments. The possible significance of this molecular weight discrepancy is discussed later (Chapter 2). Peptide mapping experiments (Naranan et al., 1988a) showed that peptide maps of purified gp-1 and gp-2 were markedly different. Also, the antibodies raised to gp-1 and gp-2 recognized only the proteolytic fragments of their respective antigens; that is, the anti-gp-1 recognized only proteolytic fragments of the gp-1 protein, while the anti-gp-2 recognized only proteolytic fragments of the gp-2 protein. All of these results are consistent with the gp-1 and gp-2 activities resulting from two different proteins. Other experiments were carried out to examine the potential phosphorylation of gp-1 and the possible effects that this modification would have on the activity and molecular weight of the gp-1 protein. Naranan et al. (1988b) were able to show *in vitro* and *in vivo* phosphorylation of the 92 kd gp-1 protein. This phosphorylation did convert the activity of gp-1 to 5′ AMP independent but did not alter the molecular weight of gp-1 on SDS gels. A phosphorylated 104 kd protein was never seen in these experiments.

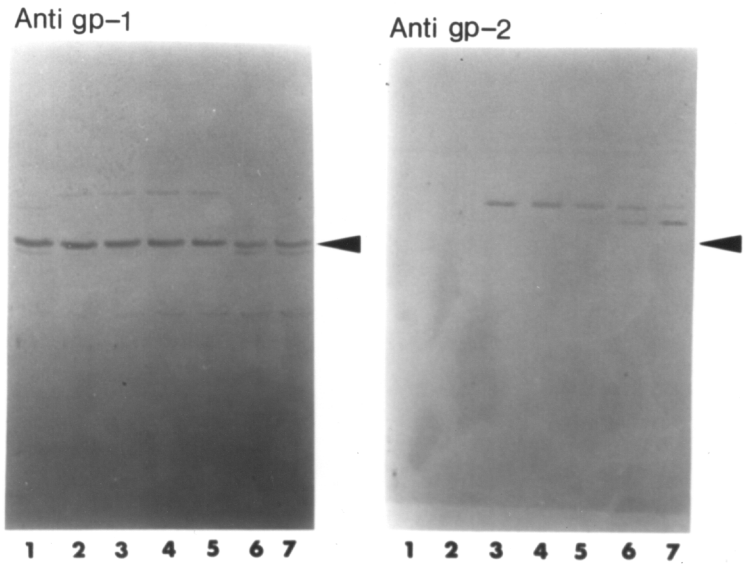

Figure 3. Western blot analysis of gp-1 and gp-2 from seven developmental stages: Cells at each developmental stage were harvested and lysed by freeze-thaw. Lysates were centrifuged at 14,500 x g for 6 minutes and prepared for SDS-PAGE and Western blotting. Lane 1: Amoebae. Lane 2: Rippled Amoebae. Lane 3: Aggregates. Lane 4: Early Slugs. Lane 5: Migrating Slugs. Lane 6: Early Culmination. Lane 7: Late Culmination. The arrows indicate 92 kd on both blots. Headings indicate staining with anti-gp-1 or anti-gp-2.

Table 1. Immunoinhibition analyses of gp-1 and gp-2 activities

	% inhibition of	
Antiserum Used	gp-1 activity	gp-2 activity
anti-gp-1	97%	15%
anti-gp-2	0%	70%

Partially purified preparations of gp-1 or gp-2 protein were incubated for 1h in the presence of the respective antisera. The mixtures were then centrifuged at 12,000 x g for 10 min; the supernatant fluid was then assayed for gp-1 or gp-2 activities. These activities were compared to the activities in supernatants derived from pre-immune incubations; percent inhibition was calculated using these values.

All of the data summarized in the previous paragraphs are consistent with gp-1 and gp-2 being unique proteins and, most likely, the products of distinct genes. However, these data were not without ambiguities. For example, the low cross reactivity between the anti-gp-1 and anti-gp-2 antibodies could be due to drastic alterations in conformation that might occur because of phosphorylation/dephosphorylation. Also, the immunotitration data can only be interpreted with considerabel caution because of the low cross reactivity between Given these ambiguities, it became obvious that the only way to obtain a definitive answer to the question regarding the relationship between gp-1 and gp-2 was to clone the gene or genes for these proteins. In the chapters that follow I will show that the gp-1 and gp-2 proteins are indeed the products of two different but related genes, and will also describe experimentation designed to examine the developmental regulation of these two genes, the regulation of these genes by cAMP, and the cell type specific regulation of both the gp-1 and gp-2 genes.

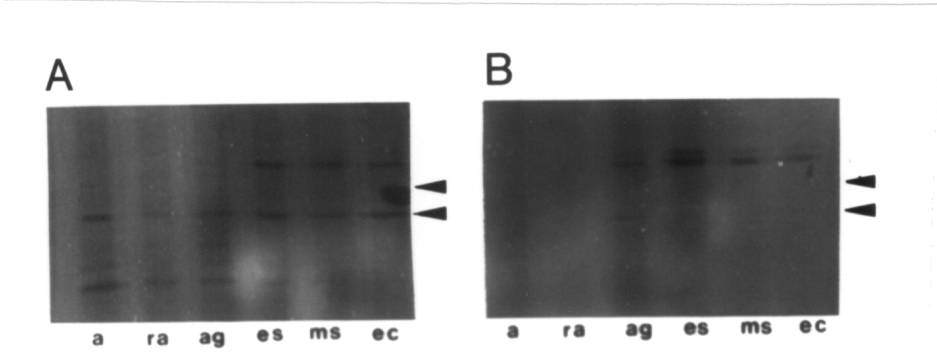

Figure 4. ***In vitro*** **translation analysis of RNA from six developmental stages:** Poly(A) RNA was obtained from six developmental stages and translated *in vitro*. The translation products were then immunoprecipitated with either anti-gp-1 (Figure 4A) or anti-gp-2 (Figure 4B) antisera. RNA used was from amoebae (am), rippled amoebae (ra), aggregates (ag), early slugs (es), migrating slugs (ms), and early culmination (ec) stage cells. The arrows indicate 92 kd and 104 kd. Note that the immunoprecipitated gp-2 protein appears larger than 104 kd; the possible significance of this is discussed later (Chapter 2).

Chapter 2: Cloning of the gp-1 and gp-2 Genes

Abstract

The molecular cloning of the gp-1 and gp-2 genes are described in this chapter. Genomic and cDNA clones were both obtained for the gp-1 gene. This gene was found to encode two exons specifying 853 amino acids; the two exons were found to be separated by a 139 base pair (bp) intron flanked by consensus *Dictyostelium* intron splice sites. The 5′ and 3′ flanking sequences were observed to be extremely rich in A + T composition, similar to the flanking sequences of other *Dictyostelium* genes. The deduced amino acid sequence of the gp-1 protein was compared to the published sequences of other glycogen phosphorylases and demonstrated a high degree of amino acid conservation at a number of crucial active and regulatory sites. The gp-1 transcript was found to be developmentally regulated, but the pattern of gp-1 mRNA expression did not match either the changes seen in gp-1 activity during development or the levels of gp-1 protein observed on Western blots. PCR amplified and genomic clones of the gp-2 gene revealed a 2975 bp open reading frame (ORF) interrupted by two short introns. The gp-2 gene was similar to, but definitely different than, the gp-1 gene. The *Dictyostelium* gp-2 deduced amino acid sequence was also similar to glycogen phosphorylases of other organisms, and the 5′ and 3′ flanking sequences exhibited the characteristic A + T enrichment seen in most *Dictyostelium* genes. Northern analysis of the gp-2

transcript showed it to be developmentally regulated, but the gp-2 mRNA accumulated well before gp-2 activity and protein levels increased during development. These discrepancies may indicate that the gp-2 enzyme is synthesized as a larger, inactive precursor molecule that is cleaved to yield active gp-2.

Introduction

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study the molecular processes involved in eukaryotic cell differentiation and aging. This organism proceeds through a well documented developmental cycle during which a homogeneous population of free living, vegetative cells undergoes differentiation to produce two distinct cell types (Loomis, 1982). A crucial step in cellular differentiation in *Dictyostelium* is glycogen degradation. The degradation of glycogen provides glucose precursors that are used to synthesize components of differentiated cells (Wright et al., 1968; Marshall et al., 1970; Gustafson and Wright, 1972). Glycogen degradation is catalyzed by the enzyme glycogen phosphorylase. Two developmentally regulated glycogen phosphorylase activities have been identified in *Dictyostelium* (Rutherford and Cloutier, 1986; Cloutier and Rutherford, 1987). These two activities are designated gp-1 and gp-2. The activity of gp-1 is 5′ AMP dependent, is predominant early in development, and is associated with a 92 kd protein that is present throughout development (Chapter 1, this dissertation; Naranan et al., 1988a). The gp-2 activity is 5′ AMP independent, peaks late in development, and is associated with a 104 kd protein that is detected on Western blots late in development (Chapter 1, this dissertation; Naranan et al., 1988a). Given the importance of glycogen degradation in cellular differentiation in *Dictyostelium*, the regulation of these two glycogen phosphorylase activities is likely to be of considerable developmental significance. Two obvious regulatory possibilities exist. First, the gp-1 and gp-2 activities could reside on the same protein molecule and be interconverted by covalent modification such as phosphorylation; this regulation is seen in several other eukaryotic systems that utilize 5′ AMP dependent and 5′ AMP independent glycogen phosphorylases. A second possibility

is that the gp-1 and gp-2 activities are associated with two different proteins. Evidence suggested that the 92 kd and 104 kd molecules are distinct proteins, rather than the 104 kd molecule representing phosphorylated 92 kd protein (Naranan et al., 1988a; Naranan et al., 1988b; Rutherford et al., 1988). This, however, did not immediately imply that separate genes exist for gp-1 and gp-2, since in eukaryotic systems a single gene can produce two different proteins through differential splicing of mRNA molecules (Early et al., 1980; Young et al., 1981; Amara et al., 1982; Nabeshima et al., 1984; Breitbart et al., 1985). Indeed, alternate mRNA splicing has been reported in *Dictyostelium* (Podgorski et al., 1989; Grant et al., 1990).

To examine the relationship between, and the regulation of, gp-1 and gp-2, the molecular cloning of the gene or genes for these proteins was initiated. In this chapter, I present the results of this cloning and show that the gp-1 and gp-2 proteins are the products of distinct, but related, genes. I also show that these two glycogen phosphorylases are similar to glycogen phosphorylase enzymes from other organisms. Finally, the developmental regulation of gp-1 and gp-2 mRNA molecules is discussed.

Results

cDNA cloning of gp-1

A cDNA library was constructed from poly(A) enriched amoebae stage *Dictyostelium* RNA. Amoebae stage RNA was used because the activity of gp-1 is maximal in undifferentiated amoebae. The cDNA was ligated into the expression vector λgt11 and the library was then screened using polyclonal anti-gp-1 antiserum in plaque lift assays. Approximately 800,000 plaques were screened; 28 positives were identified in this screening. These positives were subjected to a secondary screening using a radiolabelled yeast glycogen phosphorylase fragment called gp-y (Hwang and Fletterick, 1986), which was a generous gift from Peter Hwang and Robert Fletterick of the

University of California, San Francisco. Twelve of the 28 positive clones detected by the antibodies hybridized to gp-y under conditions of relaxed stringency. Southern analysis and restriction mapping of the cDNA inserts from these 12 clones revealed that they were overlapping. These clones were further analyzed by subcloning into phage m13 and sequencing. Analysis of the deduced amino acid sequence from these cDNAs revealed a high degree of similarity between the cloned *Dictyostelium* DNA and glycogen phosphorylases from other organisms, proving that the inserts encoded a glycogen phosphorylase. Given the specificity of the anti-gp-1 antisera (Chapter 1, this dissertation; Naranan et al., 1988a) it seemed likely that the cloned phosphorylase was indeed the gene for gp-1; comparison of the deduced amino acid sequence from this cloned cDNA and the amino acid sequence of peptides derived from purified gp-1 protein confirmed that the inserts did code for gp-1 (Rogers et al., 1992).

The overlapping cDNA inserts represented 2327 bp of gp-1 DNA; this corresponds to an open reading frame of 767 amino acids. The characteristic *Dictyostelium* stop codon, TAA, and consensus polyadenylation signal, AATAAA (Nellen et al., 1987), were observed at the 3′ end of the cDNAs. Based on the observed 92 kd molecular weight for gp-1, and on sequence comparisons to other glycogen phosphorylases, the gp-1 cDNAs appeared to lack approximately 300 nucleotides (coding information for about 100 amino acids) at the 5′ end. To obtain the missing 5′ sequence, the technique of inverted Polymerase Chain Reaction (PCR) amplification was used.

Inverted PCR amplification of the gp-1 5′ region

The technique of inverted PCR allows amplification of an unknown region of DNA that flanks a region where sequence information is available (Triglia et al., 1988). A restriction fragment that contains the known and unknown regions is circularized by ligation. The circularized DNA is melted, and primers whose 3′ ends point away from each other are annealed; PCR is then carried out, amplifying the entire circular molecule, including the unknown sequence. Southern analysis of *Dictyostelium* genomic DNA showed that a 1.7 kilobase (kb) NdeI fragment contained the 5′

gp-1 sequence as well as some of the gp-1 sequence of the cloned cDNAs (Rogers et al., 1992). *Dictyostelium* genomic DNA was digested with NdeI, circularized, and amplified by two specific primers designed to anneal to the opposite ends of the known region of gp-1 found in the ligated, circular 1.7 kb fragment. The PCR reaction amplified a 990 bp fragment from the circularized template DNA. This fragment was sequenced directly and was found to contain 213 bp of 5′ non-coding DNA, followed (moving 3′) by a 117 bp ORF, a potential 139 bp intron flanked by consensus or near consensus *Dictyostelium* intron splice sites, and another ORF of 350 bp that overlapped the known gp-1 cDNA sequence. This 5′ sequence, combined with the cDNA sequences, yielded the complete gp-1 gene.

The sequence of the gp-1 gene

The complete coding sequence of gp-1, as well as the intron and some 5′ and 3′ flanking sequences, is shown in Figure 5. The transcriptional start site was mapped by primer extension of amoebae RNA, and PCR amplification of genomic and cDNAs was used to confirm the location and size of the intron (Rogers et al., 1992). Genomic cloning of the gp-1 gene (Rogers et al., 1992) confirmed that no other introns were located within the coding region. The gp-1 coding sequence of 2562 bp encodes 853 amino acids with a calculated molecular weight of 98.3 kd, a figure close to the observed molecular weight of 92 kd. The gp-1 gene was found to be a typical *Dictyostelium* gene in a number of respects: 1) the intron is short and is located at the 5′ region of the gene, similar to many other *Dictyostelium* genes; 2) like other *Dictyostelium* genes (Warrick and Spudich, 1988) the non-coding regions are extremely A + T rich (about 85%) and the coding region is about 64% A + T; 3) codon usage in gp-1 generally matches the codon preferences for *Dictyostelium* proteins expressed at medium levels (Warrick and Spudich, 1988; Sharp and Devine, 1989).

Sequence comparison of gp-1 to other glycogen phosphorylases

The complete deduced amino acid sequence of gp-1 was compared to the published sequences of a number of glycogen phosphorylases from several disparate organisms (yeast, potato, *E. coli*, rabbit muscle, and human muscle, brain, and liver isozymes). The sequence comparisons were performed using the Genetics Computer Group (GCG) program, version 5.0. The gp-1 enzyme was very similar at the amino acid level to all of these glycogen phosphorylases, showing an average of 48.8% identical amino acids and an average of 68.8% conserved amino acids with these other enzymes (Rogers et al., 1992). Much of the amino acid identity and conservation was seen at residues involved in the active site or in regulatory sites. Figure 6 shows a comparison of the amino acid sequence of gp-1 and glycogen phosporylases from rabbit muscle and yeast, two of the most extensively studied phosphorylases. Active site residues are clearly conserved among these phosphorylases; indeed, 16 out of 17 aligned residues involved in the active site are identical when comparing the rabbit muscle sequence to the *Dictyostelium* sequence. Phosphorylation sites also appear to be conserved, as are many of the sites involved in subunit dimerization. Curiously, many of the 5′ AMP binding sites of the rabbit muscle enzyme are not conserved in the *Dictyostelium* enzyme, even though gp-1 does exhibit allosteric activation by 5′AMP.

Developmental regulation of the gp-1 gene

To analyze the developmental expression of the gp-1 gene, total RNA from several developmental stages was extracted, resolved by denaturing agarose gel electrophoresis, transferred to nitrocellulose, and hybridized under stringent conditions to radiolabelled gp-1 cDNAs. The resultant Northern blots were used to generate the autoradiogram shown in Figure 7. The gp-1 mRNA exhibited developmental regulation; gp-1 mRNA levels were high in undifferentiated amoebae and in early developmental stages, decreased at about seven hours of development, then increased later in development. This pattern of expression is not consistent with the either the de-

crease in gp-1 activity during development or with the constant level of gp-1 protein observed during development. The fluctuation in gp-1 mRNA levels was highly repeatable in more than 10 experimental replications. In all of these replications total RNA was quantified by optical densities and by ethidium bromide staining of agarose gel pieces, and equivalent ammounts of RNA from every developmental stage were loaded onto the gel.

Molecular cloning of the gp-2 gene

Initial attempts to clone the gp-2 gene were with cDNA cloning techniques. A cDNA library was constructed from *Dictyostelium* slug stage RNA. Slug stage was selected since gp-2 activity is increasing at this developmental point. The cDNAs were ligated into λgt11 and the library was screened using the anti-gp-2 antibodies. A total of 41 positive clones were identified by the antibody screening. However, none of these 41 clones was found to encode a glycogen phosphorylase. Attempts to clone the gp-2 gene then shifted to PCR amplification. Amino acid sequence information for gp-2 was obtained by purifying and sequencing peptide fragments of the gp-2 protein (Rutherford et al., 1992). These sequences were compared to published amino acid sequences for other glycogen phosphorylases, and gp-2 amino acid sequences that corresponded to nucleotide sequences at the 5′ region of the gene, the middle of gene, and the 3′ region of the gene were identified. Oligonucleotides were then constructed based on the amino acid sequences, using codons that are preferentially utilized in *Dictyostelium.* The oligonucleotides were constructed to allow amplification of most of the gp-2 gene (Figure 8). Amplified PCR products were sequenced directly, and were found to encode a glycogen phosphorylase. These PCR products did not contain the most 5′ or 3′ regions of the gene; these regions were obtained by screening a partial EcoRI genomic library with PCR probes or by using inverted PCR on circularized genomic DNA fragments (Rutherford et al., 1992).

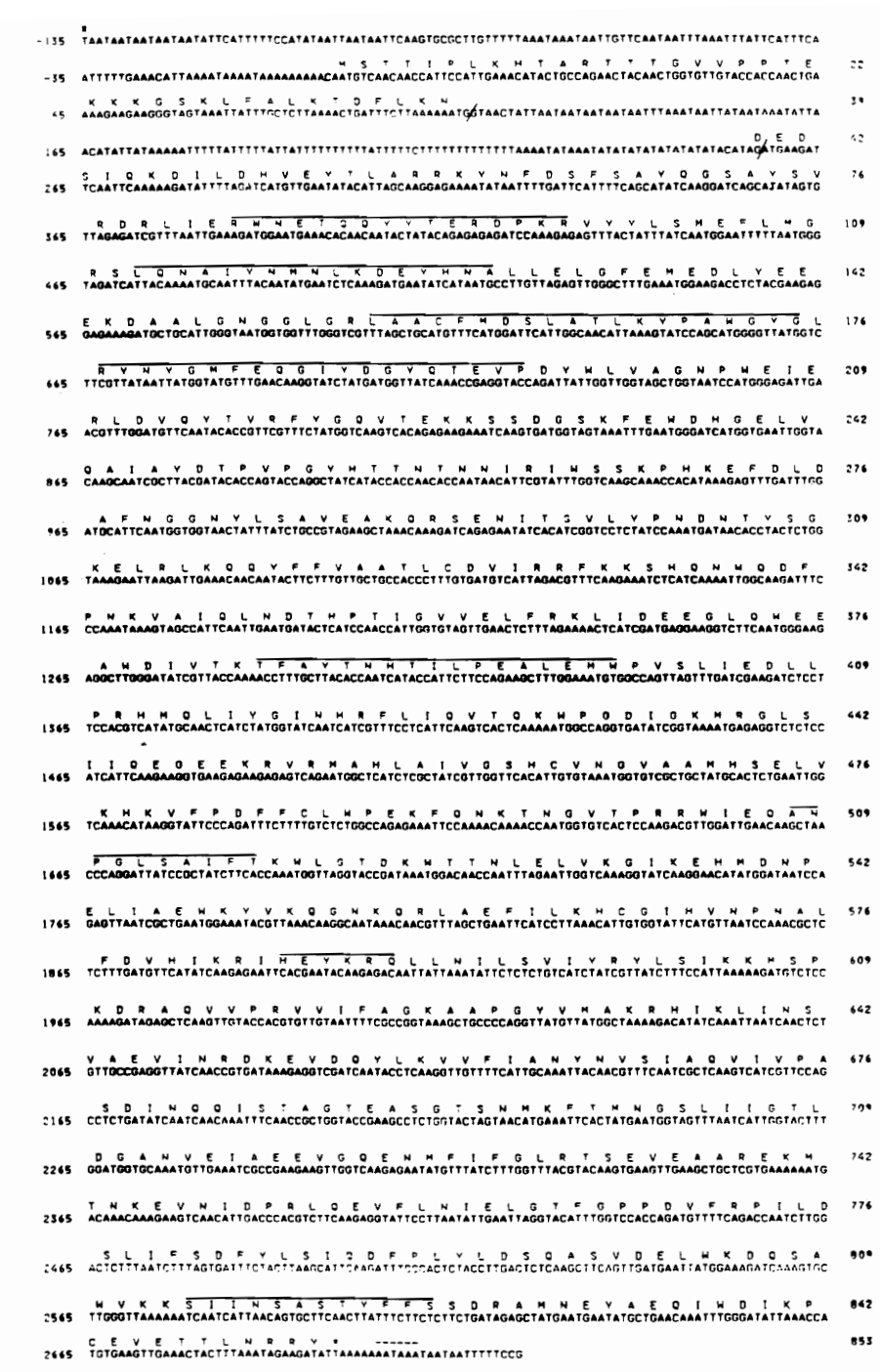

Figure 5. Nucleotide and deduced amino acid sequence of gp-1: The genomic sequence of gp-1 is numbered to the left of the figure. The amino acid sequences encoded by the exons are shown above the DNA sequences, and the numbering of these residues is to the right of the figure. Amino acid residues confirmed by protein sequencing are overlined. #, transcriptional start site; /, intron splice junctions; *, stop codon; ---, polyadenlyation signal.

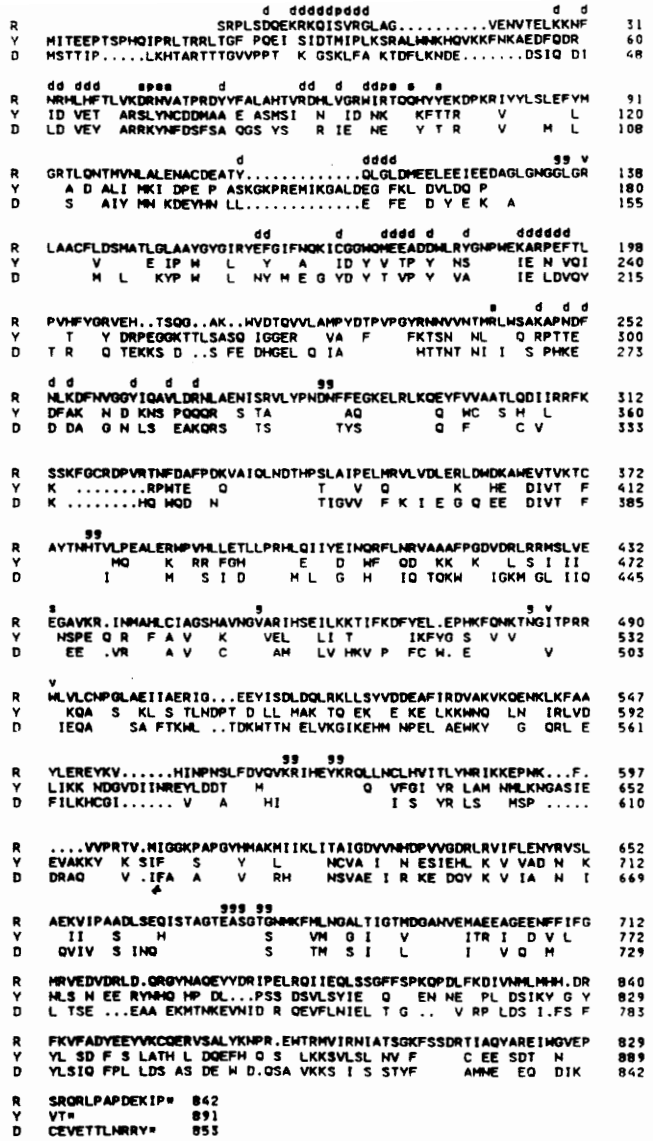

Figure 6. Comparison of the primary structure of gp-1 and other glycogen phosphorylases: The GCG "Gap" subprogram was used to prepare the multiple sequence alignment of rabbit muscle glycogen phosphorylase (R), yeast glycogen phosphorylase (Y), and gp-1 (D). Gaps to optimize amino acid sequence similarity are denoted by periods, and the stop codons are indicated by asterisks. Important sites of the rabbit muscle enzyme are indicated: a, AMP binding site; d, dimerization points; g, active sites; p, residues involved in phosphorylation.

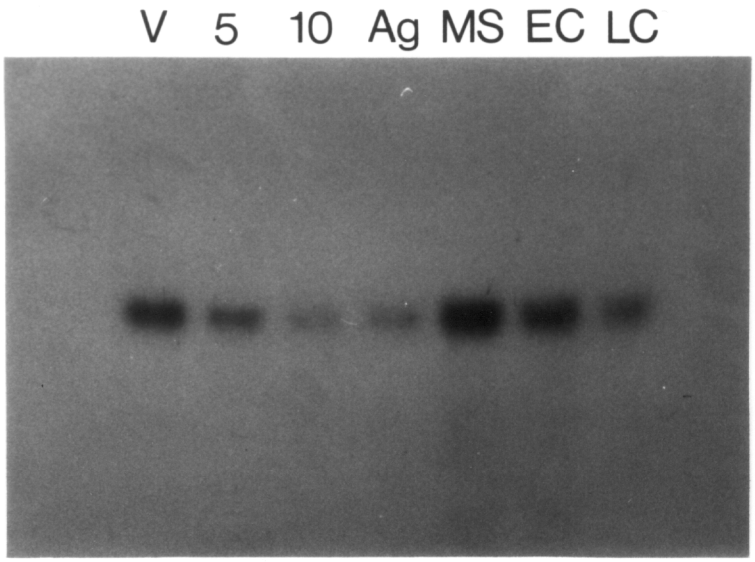

Figure 7. Northern blot showing developmental expression of gp-1 mRNA: Ten micrograms of total RNA were loaded from each developmental stage. The RNA was electrophoresed, transferred to nitrocellulose, and hybridized to radiolabelled gp-1 cDNA under stringent conditions. The developmental stages are: V, vegetative amoebae; 5 and 10, cells developed for 5h or 10h; Ag, late aggregation stage; MS, migrating slug stage; EC, early culmination stage; LC, late culmination stage. The gp-1 message is about 3.0 kb in size.

The sequence of the gp-2 gene

The complete sequence of the gp-2 structural gene, as well as some 5′ and 3′ flanking sequences, is shown in Figure 9. Two introns were observed in the gp-2 gene. Both introns are short (one of 109 bp and one of 105 bp) and are located at the 5′ end of the gene, typical of *Dictyostelium* introns. The coding sequence of the gene is 2976 bp, specifying 992 amino acids with a calculated molecular weight of 112.5 kd (Rutherford et al., 1992). The consensus stop codon and polyadenlyation signals were observed, and the transcriptional start site was determined by primer extension (Rutherford et al., 1992). An unusual sequence was observed from nucleotides 276 to 321. The nucleotide triplet CAA was repeated 11 times, giving 11 in frame glutamine residues out of 15 amino acids encoded by this region. Sequencing of cDNAs (amplified by PCR from slug stage RNA) confirmed that these repeats are transcribed (Rutherford et al., 1992). Nucleotide repeats are often observed in developmentally regulated genes in *Dictyostelium* (Rosen et al., 1983; Cohen et al., 1984; Kimmel and Firtel, 1985; Shaw et al., 1989) as well as in other organisms (Davidson and Posakony, 1982); these repeats are thought to play some regulatory function, although a precise role has not yet been been determined for such sequences.

Comparison of gp-2 to gp-1 and other glycogen phosphorylases

The nucleotide sequences of gp-1 and gp-2 were compared and differences were observed throughout the entire coding regions of these genes; the 5′ sequences were also different. Thus, it is clear that the gp-1 and gp-2 proteins are unique and are the products of two distinct genes, as previous data had suggested (Naranan et al., 1988a; Rutherford et al., 1988). Figure 10 shows an amino acid sequence comparison of gp-1 and gp-2. Regions of amino acid identity are seen, but there are also regions where no identity or similarity exists, showing, as did the nucleotide sequence comparisons, that these two proteins are unique. The deduced amino acid sequence of gp-2 was also compared to glycogen phosphorylases from other organisms (Rutherford et al., 1992). The

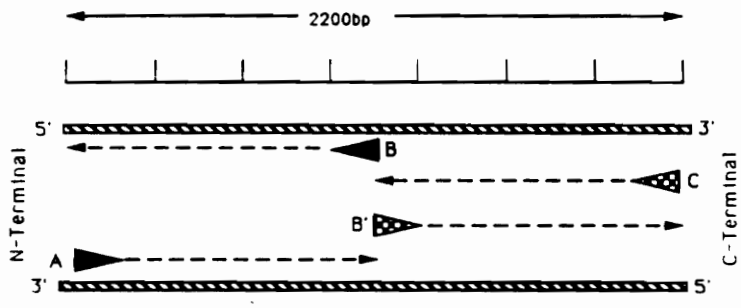

Figure 8. PCR amplification of gp-2 from genomic DNA: Oligonucleotide primers A, B, B′, and C were constructed from amino acid sequences of the gp-2 protein. Primers A and B were used to amplify the 5′ portion of the gene and primers A′ and B′ the 3′ portion. The striped lines represent the two strands of genomic DNA.

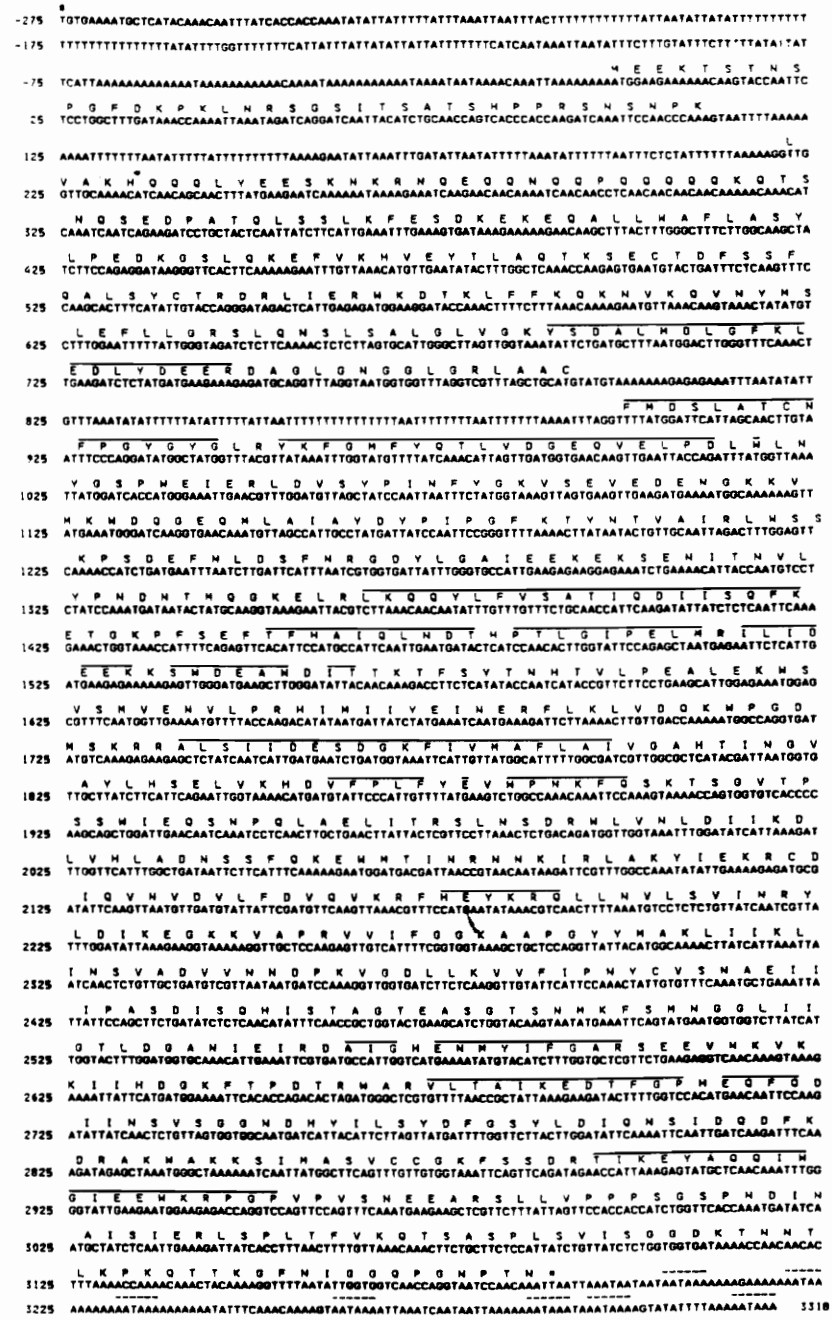

Figure 9. Sequence of the gp-2 gene: The nucleotide sequence of the gp-2 gene is shown as well as the deduced amino acid sequence. Overlined amino acid sequences were confirmed by peptide sequencing. Also shown are the start site for transcription (#), the stop codon (*), and consensus polyadenylation signals (---).

Dictyostelium enzyme was up to 50% identical to other glycogen phosphorylases, and active site residues were universally identical or conserved (Rutherford et al., 1992). However, when comparing gp-2 to rabbit muscle phosphorylase, no conservation was seen in AMP binding sites or phosphorylation sites (Rutherford et al., 1992). These results are consistent with the observed data on gp-2, which is not stimulated by 5′ AMP and does not appear to be regulated by phosphorylation.

Developmental expression of the gp-2 gene

Developmental changes in gp-2 mRNA levels were analyzed by Northern blotting in which RNA from different developmental stages was resolved by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to radiolabeled gp-2 PCR fragments. The hybridized filters were then used to expose x-ray film. The results of this experimentation are shown in Figure 11B. The gp-2 mRNA is not present in vegetative cells or in early developmental stages, but can be detected by slug stage; late in development, gp-2 mRNA levels decrease. Figure 11 also shows a Western blot of gp-2 protein levels during development, and a graph showing the developmental increase in gp-2 activity. Notice that, as shown earlier (Chapter 1), the anti-gp-2 antiserum detects a protein doublet. The upper member of the doublet is about 112 kd, close to the predicted 112.5 kd molecular weight of gp-2 based on the deduced gp-2 amino acid sequence. The lower member of the doublet is about 104 kd, which is the molecular weight of purified gp-2 protein. The appearance of gp-2 mRNA coincides with the appearance of the 112 kd upper band; however, the appearance of gp-2 activity coincides with the appearance of the 104 kd band. Since the gp-2 mRNA exhibits only one size, it is possible that the 112 kd molecule represents an inactive gp-2 precursor. Other circumstantial evidence also suggests that gp-2 may initially be synthesized as a larger, precursor molecule. For example, the *in vitro* translation experiments presented in Chapter 1 resulted in the immunoprecipitation of a 110-112 kd gp-2 molecule, rather than a 104 kd protein. If protein processsing of gp-2 does take place, it is unlikely that such processing would be seen in the *in vitro*

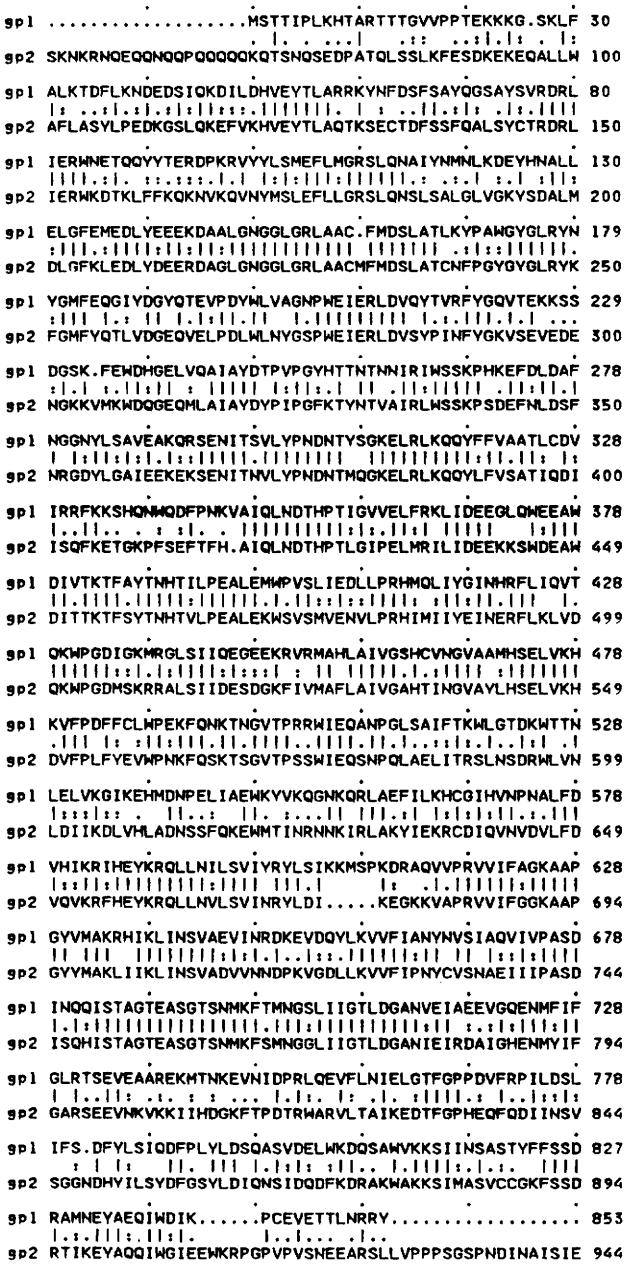

Figure 10. Comparison of the amino acid sequences of gp-1 and gp-2: Identical amino acids are indicated with lines. Conserved amino acids are indicated by two dots. Semi-conserved amino acids are indicated with one dot.

translation system; thus, the immunoprecipitation of the precursor would be expected. Also, Higgins and Dahmus (1982) also reported the immunoprecipitation of "precursor" forms of gp-2 from *in vitro* translation reactions when certain protease inhibitors were included in the reaction. Finally, during the sequencing of gp-2 peptides, which were derived from active, 104 kd gp-2, no amino acid sequence was ever observed from the extreme N-terminal region of the protein. This may suggest that the 112 kd precursor is cleaved to the active 104 kd form by the removal of N-terminal amino acids. All of these data, while certainly circumstantial, suggest that the gp-2 protein is initially synthesized as a larger, precursor molecule that is cleaved to yield active gp-2 enzyme.

Discussion

In this chapter I have outlined the molecular cloning of the gp-1 and gp-2 genes and described their developmental regulation. Molecular cloning of these genes provided conclusive evidence, in the form of direct nucleotide and amino acid sequence comparison, that the gp-1 and gp-2 activities are associated with two unique proteins that are the products of two distinct, but related genes. Both the gp-1 and gp-2 genes were observed to be typical of *Dictyostelium* genes in terms of A + T content, intron structure and location, and codon usage. Also, both of these genes were found to be similar in structure to glycogen phosphorylases from other organsims; the similarities were especially pronounced in residues involved in the active sites (Rogers et al., 1992; Rutherford, et al., 1992). The gp-1 protein was found to also possess structural similarities to other phosphorylases at residues involved in phosphorylation and in some residues involved in 5′ AMP activation. These findings are consistent with previous studies on gp-1, in which this enzyme was shown to be allosterically activated by 5′ AMP and covalently activated by reversible phosphorylation (Rutherford and Cloutier, 1986; Cloutier and Rutherford, 1987; Naranan et al., 1988b). The gp-2 enzyme lacked amino acid similarity to other phosphorylases at the 5′ AMP binding and phosphorylation sites, consistent with earlier data showing that gp-2 is not regulated by these mechanisms.

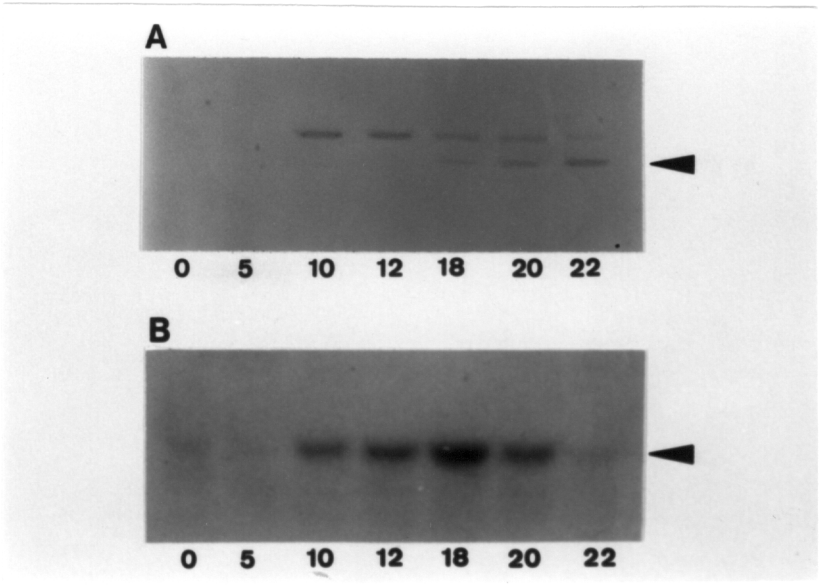

Figure 11. Analysis of gp-2 expression during *Dictyostelium* development: Figure 11A shows protein levels, while Figure 11B shows mRNA levels. The arrows indicate either 104 kd (Figure 11A) or 3.2 kb (Figure 11B). Figure 11C shows gp-2 activity during development. The numbers below each figure indicate time of development: 0, undifferentiated amoebae; 5 and 10, early aggregation stages; 12, aggregation stage; 18, slug stage; 20, early culmination stage; 22, late culmination.

Both glycogen phosphorylase genes were found to be developmentally regulated. The gp-1 mRNA levels were observed to be high early in development, decrease during the middle developmental stages, and then increase late in development. This fluctuation does not match the developmental decrease seen in gp-1 activity or the constant levels of gp-1 protein seen during development. Currently, these discrepancies have not been resolved. Certainly, protein stability could account for the constant levels of gp-1 protein, but this does not explain the loss in activity or the slug stage increase in mRNA levels that occur with no apparent increase in either gp-1 protein levels or activity. Sequestering gp-1 protein in pre-spore cells and spores, for use upon germination, also does not appear to be a viable explanation for these discrepancies (Chapter 5). It is also unclear at this point whether the changes in gp-1 mRNA levels seen during development are the result of transcriptional alterations or changes in mRNA stability, although reporter gene experiments (Chapter 4) suggest that the changes are the result of transcriptional events. The gp-2 gene is also developmentally regulated, with gp-2 mRNA levels peaking relatively late in development. It seems likely that the increase in gp-2 mRNA levels during development is due to activation of transcription. Clearly, the developmental increase in gp-2 activity is due to synthesis of gp-2 molecules as earlier researchers had suspected (Firtel and Bonner, 1972; Thomas and Wright, 1976; Higgins and Dahmus, 1982). Data presented in this chapter suggests that the gp-2 enzyme may be synthesized as a larger, inactive, precursor that is cleaved to produce active gp-2 enzyme. Some circumstantial evidence suggests that the cleavage occurs at the N-terminal end of the protein. If this is the case, it is possible that the glutamine repeats described above play a role in this cleavage. Extrapolating back from the termination codon, a 104 kd protein would begin just 3′ to the glutamine rich area. It may be that the glutamine repeats act as a signal for proteolytic processing; however, such a claim is purely speculative at this point and awaits the results of future experimentation.

Materials and Methods

Cell growth and development

Dictyostelium cells of the strain AX-3 were grown in HL5 media as previously described (Rutherford and Cloutier, 1986). Differentiated cells were obtained by washing vegetative amoebae free of nutrient media and plating on non-nutrient agar or onto Gelman GN-6 membrane filters supported by Gelman absorbent pads (both of which were soaked in 7 mM N-morpholinoethanesufonic acid [pH 6.5], 20 mM KCl, and 5 mM $MgSo_4$).

Isolation Of RNA and DNA

RNA was isolated from either vegetative amoebae or from cells at specific developmental stages as previously described (Nellen et al., 1987). Genomic DNA was isolated as previously described (Nellen et al., 1987).

cDNA and genomic cloning

Total RNA from *Dictyostelium* cells was passed over oligo(dT) cellulose columns to obtain poly(A) enriched RNA. cDNA was synthesized from this RNA using standard procedures. The cDNA was treated with mung bean nuclease and EcoRI methylase and ligated to phosphorylated EcoRI linkers and to dephosphorylated, EcoRI treated λgt11 arms. *In vitro* packaging was was performed, and the resultant library was screened with anti-gp-1 or anti-gp-2 antibodies according to the method of Young and Davis (1983). Positive plaques were subjected to at least three rounds of rescreening. Screening the amoebae cDNA library with radiolabelled gp-y was done as described by Rogers et al. (1992). Genomic cloning was done from a λ-Zap library obtained from Herb

Ennis at the Roche Institute of Molecular Biology in Nutley, New Jersey; this cloning was done following standard procedures (Rogers et al., 1992).

PCR amplification

Primers were constructed for PCR reactions based on amino acid or nucleotide sequence information. The primers were used to amplify specific regions of *Dictyostelium* genomic DNA in a Perkin-Elmer Cetus Instruments thermal cycler following procedures described by the manufacturer. Restriction enzyme sites were often engineered into the ends of the primers to facilitate subcloning. For inverted PCR, the procedure was the same except that specific partial digests of genomic DNA which were circularized by ligation were used as template DNA.

Nucleic acid sequencing

DNA to be sequenced was subcloned into restriction cut m13 or pBluescript (Stratagene) vectors as described (Rogers et al., 1992; Rutherford et al., 1992). Single or double stranded seqencing reactions were run, using the chain termination method (Sanger et al., 1977) according to the Sequenase (U.S. Biochemical) protocol; both vector and insert specific primers were used, depending upon the situation. Sequence analysis was done using the Genetics Computer Group program, version 5.0.

Western blots, Northern blots, and activity assays

Northern blotting was done by capillary action following gel electrophoresis of RNA in formaldehyde containing 1.2% Seakem (FMC) agarose; hybridizations to specific ^{32}P- labeled DNA probes were under stringent conditions of 50% formamide, 6 x SSPE, 1 x Denhardt's sol-

ution, 0.1% SDS, and 200 μg/ml salmon sperm DNA at 42° C. Washes were also carried out under stringent conditions (Rogers et al., 1992; Rutherford et al., 1992). DNA probes were labelled by random priming (Feinberg and Vogelstein, 1984). The hybridized filters were dried under vacuum and autoradiography was done by exposing the filters to Kodak XAR-5 film at -70° C. Western blotting and glycogen phosphorylase activity assays were done as described previously (Naranan et al., 1988a).

Protein purification and sequencing

The gp-1 and gp-2 proteins were purified to homogeneity as described previously (Rutherford and Cloutier, 1986; Cloutier and Rutherford, 1987). For peptide sequencing, purified protein was reduced and carboxymethylated and then digested with trypsin or protease V8. Proteolytic fragments were purified by microbore HPLC and sequenced using an Applied Biosystems Model 477A Sequencer with on-line identification of phenylthiohydantoins.

Chapter 3: Regulation of the gp-2 Gene by cAMP

Abstract

A crucial developmental event in the cellular slime mold, *Dictyostelium discoideum*, is glycogen degradation. The enzyme that catalyzes this degradation, glycogen phosphorylase 2 (gp-2), is developmentally regulated and cAMP appears to be involved in this regulation. I have examined several aspects of the cAMP regulation of gp-2. I show that the addition of exogenous cAMP to aggregation competent amoebae induced the appearance of gp-2 mRNA. The induction of gp-2 mRNA occured within 1h and 1.5h after the initial exposure to cAMP. Exposure to exogenous cAMP concentrations as low as 1.0 μM induced gp-2 mRNA. I also examined the molecular mechanism through which cAMP induction of gp-2 occurs. Induction of gp-2 appears to occur through a mechanism that does not require intracellular cAMP signalling, and may occur directly through a cAMP binding protein without the requirement of any intracellular signalling. I also examined *cis*-acting elements involved in the cAMP regulation of gp-2. Repeated TA rich sequences observed between -193 and -305 nucleotides from the transcriptional start site are required for maximal cAMP response. The involvement of TA rich sequences in cAMP responsiveness is similar to at least one other *Dictyostelium* gene that has been examined.

Introduction

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study eukaryotic cell differentiation and development. This organism proceeds through a well characterized developmental cycle in which a homogeneous population of vegetative cells will, under conditions of nutrient depletion, undergo differentiation to produce two distinct differentiated cell types (for review, see Loomis, 1982). Both differentiated cell types require the synthesis of complex structural polysaccharides such as cellulose. The glucose monomers used to synthesize these structural components are derived from glycogen stores (Wright et al., 1968; Marshall et al., 1970; Gustafson and Wright, 1972). Thus, a crucial event in cellular differentiation in *Dictyostelium* is glycogen degradation. Developmentally regulated glycogen degradation in *Dictyostelium* is catalyzed by the enzyme glycogen phosphorylase 2 (gp-2; 1,4-α-D-glucan:orthophosphate α-glucosyl transferase, EC 2.4.1.1). This enzyme is itself developmentally regulated; during development, gp-2 activity, protein, and mRNA levels all increase (Firtel and Bonner, 1972; Thomas and Wright, 1976; Higgins and Dahmus, 1982; Rutherford et al., 1992).

Cyclic adenosine monophosphate (cAMP) is an important regulatory molecule in both prokaryotes and eukaryotes, and cAMP plays several important roles in *Dictyostelium* development. Exogenous cAMP has been shown to be necessary for the expression of many developmentally regulated genes and stabilization of many developmentally controlled mRNA species (Landfear and Lodish, 1980; Landfear et al., 1982; Barklis and Lodish, 1983; Mangiarotti et al., 1983; Mehdy et al., 1983; Driscoll and Williams, 1987). Previous reports have shown that exogenous cAMP can induce gp-2 enzyme activity (Okamoto and Takeuchi, 1976; Takemoto et al., 1978; Okamoto et al., 1982; Schaap and Van Driel, 1985) as well as the appearance of the gp-2 protein (Brickey et al., 1990). The structural gene and promoter elements for gp-2 have recently been cloned and sequenced (Rutherford et al., 1992), and, in this chapter, I have carried these studies further to examine the role of exogenous cAMP in the regulation of the gp-2 gene. I show that exogenous cAMP induces the appearance of the gp-2 mRNA. I also examine some kinetic and physical pa-

rameters of this induction, and explore the intracellular molecular mechanism involved in the induction. Finally, I identify potential DNA sequence elements involved in the cAMP mediated regulation of this gene.

Results

Induction of gp-2 mRNA by exogenous cAMP

Undifferentiated, aggregation competent *Dictyostelium* amoebae were placed in MES-LPS buffer and were shaken at 180 rpm in the presence or absence of 1.0 mM exogenous cAMP, as detailed in Materials and Methods. In the presence of cAMP, gp-2 enzyme activity was induced as shown in Figure 12A; gp-2 enzyme activity was initially detected 2h after the initial exposure to cAMP, and continued to increase throughout the 8h experiment. Western blot analysis of these same extracts showed that the gp-2 protein accumulated in response to cAMP concomitant with the observed increase in gp-2 activity (Figure 12B). No gp-2 protein was observed in the absence of cAMP or in the extracts from aggregation competent cells. To examine the effect of cAMP on the expression of gp-2 mRNA levels, RNA was isolated from cells at 4h and 8h time points, resolved by electrophoresis in formaldehyde containing gels, and transferred to nitrocellulose filters. These filters were hybridized to radiolabeled PCR amplified fragments of gp-2 DNA. Figure 13 shows that no gp-2 mRNA was detected in vegetative cells (Lane V) or in aggregation competent (Lane AC) cells. A dramatic induction of gp-2 mRNA was seen in cells that were shaken in the presence of cAMP for either 4 or 8 hours. Cells shaken for these time periods in the absence of exogenous cAMP showed no induction of the gp-2 message. Induction of the gp-2 mRNA was not seen in the presence of several other exogenous compounds, such as adenosine, 5′adenosine monophosphate, or cyclic guanosine monophosphate (data not shown). I was also able to induce gp-2 mRNA accumulation with exogenous cAMP in cells that were not made aggregation compe-

tent (data not shown). Clearly, exogenous cAMP can regulate gp-2 enzyme activity, enzyme protein, and mRNA levels. Given previous studies on the regulation of the gp-2 enzyme and its synthesis (Firtel and Bonner, 1972; Thomas and Wright, 1976; Higgins and Dahmus, 1982), it is likely that the cAMP regulation is at the level of transcription, although confirmation will await the results of future experiments.

Timing of cAMP induction of the gp-2 mRNA and the effects of cAMP concentration on the induction of gp-2

Figure 13 shows that the gp-2 mRNA is induced within 4h of the addition of exogenous cAMP. In order to examine the kinetics of gp-2 mRNA accumulation in more detail, I extracted RNA from cells exposed to cAMP in shaking culture for 1h, 2h, 3h, and 4h. Figure 14A shows that by 2h after the addition of cAMP, maximal induction of gp-2 mRNA was observed; however, essentially no gp-2 mRNA was detected at 1h. In Figure 14B, the 0h to 2h time interval was examined in more detail by extracting RNA from cells exposed to cAMP for 0.5h, 1h, 1.5h, and 2h. The gp-2 mRNA was weakly detected at 1h, but was strongly induced by 1.5h after the addition of cAMP. Thus, the induction of gp-2 mRNA by cAMP occurs rapidly and markedly between 1h and 1.5h after the addition of cAMP, and is maximal by 2h.

I have also examined the effect of cAMP concentration on the induction of the gp-2 mRNA. Figure 15 shows that gp-2 mRNA was induced with the concentration of exogenous cAMP as low as 1 μM. However, the level of induced gp-2 mRNA increased with increasing cAMP concentrations. The induction of gp-2 mRNA by 1.0 μM cAMP occured with cAMP levels 100-1000 fold lower than those routinely used to induce *Dictyostelium* genes. These levels of cAMP are, nevertheless, several orders of magnitude higher than the endogenous concentration of cAMP (Pahlic and Rutherford, 1979). It is, of course, impossible to determine the effective concentration of cAMP *in vivo* due to the interaction of cAMP with cell surface receptors and the activities of adenylate cyclase and phosphodiesterase.

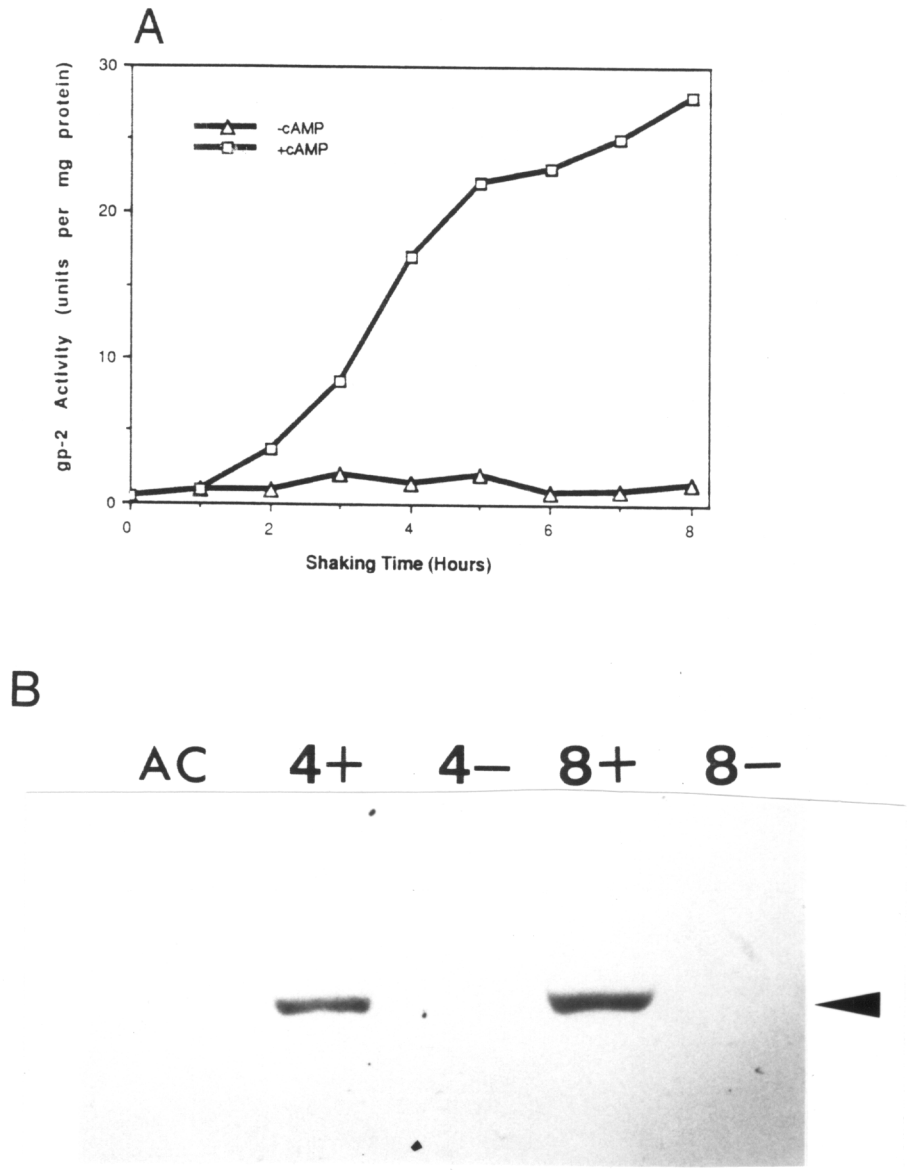

Figure 12. cAMP induction of gp-2 activity and gp-2 protein: (A) Aggregation competent cells of *Dictyostelium* were shaken in the presence (open squares) or absence (open triangles) of 1.0 mM cAMP. At the indicated times, samples of the cell suspension were removed and gp-2 enzyme activity was assayed as described in Methods and Materials. (B) Western blot of cell extracts after cAMP induction experiments. gp-2 protein detection was done by incubating the blot with anti-gp-2 antiserum. Lane AC indicates the extract from aggregation competent cells. The lanes marked 4 were cells cells shaken for 4h; those marked 8 were cells shaken for 8h. The +/- symbols indicate the presence (+) or absence (-) of 1.0 mM exogenous cAMP in the shaking culture. The arrow indicates 104 kd, which is the subunit molecular weight of the gp-2 protein.

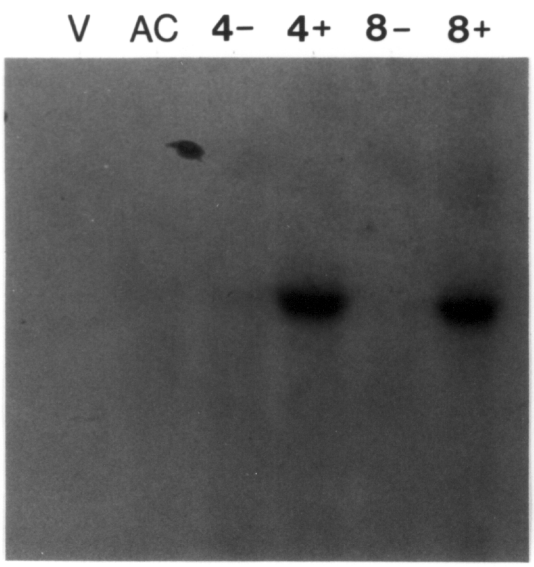

Figure 13. cAMP induction of the gp-2 mRNA: Aggregation competent amoebae were shaken in MES-LPS buffer either in the presence or absence of exogenous cAMP as described in Methods and Materials. Samples of the cell suspension were removed at different time points and RNA was extracted. The RNA was used for Northern blotting and was probed with a radiolabeled, 1098 bp fragment of gp-2 that was amplified by PCR. Lane V, RNA from vegetative cells; Lane AC, RNA from aggregation competent cells. Lanes labeled 4 indicate RNA from cells shaken for 4h in the absence (-) or presence (+) of exogenous cAMP. Lanes labeled 8 indicate RNA from cells shaken for 8h in the absence (-) or presence (+) of exogenous cAMP.

Molecular mechanism of cAMP activation of the gp-2 gene

Exogenous cAMP can modulate gene expression in *Dictyostelium* through at least two different intracellular signaling mechanisms (Europe-Finner and Newell, 1987; Gerisch, 1987; Janssens and Van Haastert, 1987; Kimmel, 1987). The two mechanisms appear to be mediated through unique cell surface cAMP receptors that activate different G-proteins and intracellular second messengers (Johnson et al., 1989; Saxe et al., 1991). One regulatory mechanism requires an intracellular increase in cAMP, suggesting a role for cAMP dependent protein kinases or other cAMP binding proteins. The second mechanism appears to be independent of intracellular increases in cAMP; in this mechanism, exogenous cAMP probably leads to the production of other intracellular second messengers such as inositol trisphosphate (IP_3), diacylglycerol (DAG), or calcium (Europe-Finner and Newell, 1986; Schaap et al., 1986; Janssens and Van Haastert, 1987; Europe-Finner and Newell, 1987; Ginsburg and Kimmel, 1989).

Two experimental approaches were used to examine the molecular mechanism of cAMP activation of the gp-2 gene. First, cAMP induction experiments were done in the presence of caffeine. Caffeine has been shown to inhibit adenylate cyclase activity, and therefore also inhibit intracellular increases in cAMP, in *Dictyostelium* (Brenner and Thoms, 1984). The second experimental approach involved the use of cAMP analogs that possess high affinity for either cell surface cAMP receptors or for the regulatory subunit of the cAMP dependent protein kinase. Figure 16 shows the results of cAMP induction experiments done in the presence of caffeine. Note that cAMP induction of the gp-2 mRNA occured even in the presence of caffeine (compare lanes 3 and 4), suggesting that cAMP regulation of this gene occurs via the cell surface receptors in a mechanism that is independent of intracellular cAMP. Similar cAMP induction experiments were done in which cells were suspended in a glucose albumin medium, a medium that is known to inhibit intracellular cAMP signalling in *Dictyostelium* (Oyama and Blumberg, 1986). Again, gp-2 mRNA was induced by cAMP in this medium, indicating that gp-2 mRNA induction can occur without intracellular increases in cAMP (data not shown). The effect of cAMP analogs on gp-2 mRNA levels also suggests that gp-2 induction occurs via cell surface receptors rather than through intra-

cellular cAMP signaling (Figure 17). The cAMP analogs used were 2′-deoxy-cAMP (2′d-cAMP) and dibutyryl-cAMP (Bt_2-cAMP). Oyama and Blumberg (1986) showed that 2′d-cAMP has a high affinity for *Dictyostelium* cell surface receptors, and a low affinity for the cAMP dependent protein kinase. Bt_2-cAMP is membrane permeable, possesses a high affinity for the regulatory subunit of the *Dictyostelium* cAMP dependent protein kinase (Oyama and Blumberg, 1986), and is widely used in eukaryotic systems to mimic the effects of intracellular cAMP. Figure 17 shows that inclusion of 2′d-cAMP in the cell suspension resulted in induction of gp-2 mRNA to levels essentially identical to those seen with authentic cAMP, while no induction was seen with Bt_2-cAMP. These results are consistent with the caffeine and glucose albumin results as described above and support the contention that cAMP regulation of the gp-2 gene occurs through a mechanism independent of intracellular cAMP. An alternate mechanism for intracellular signaling in the regulation of *Dictyostelium* genes involves IP_3 and DAG. I tested this possibility by bypassing the exogenous cAMP requirement for gp-2 induction with the use of IP_3 and DAG directly on cells in suspension. However, no gp-2 mRNA induction was observed under several sets of conditions using these compounds (data not shown).

Identification of DNA sequence elements involved in the cAMP regulation of the gp-2 gene

The data presented above clearly indicate that cAMP regulates the expression of the gp-2 mRNA, likely at the transcriptional stage. I have examined this regulation at the DNA sequence level to identify potential sequence elements involved in the cAMP-mediated regulation of this gene. This experimentation was carried out by studying the expression of a luciferase reporter gene that was driven by either the full length or deletions of the gp-2 promoter. The full length gp-2 promoter was isolated on a 1051 bp genomic fragment. I believe that this fragment represents the entire promoter because the coding sequence of another gene is observed just upstream of this fragment. Figure 18 shows a schematic diagram of this gp-2 promoter fragment (gp-2-d7) and the deletion

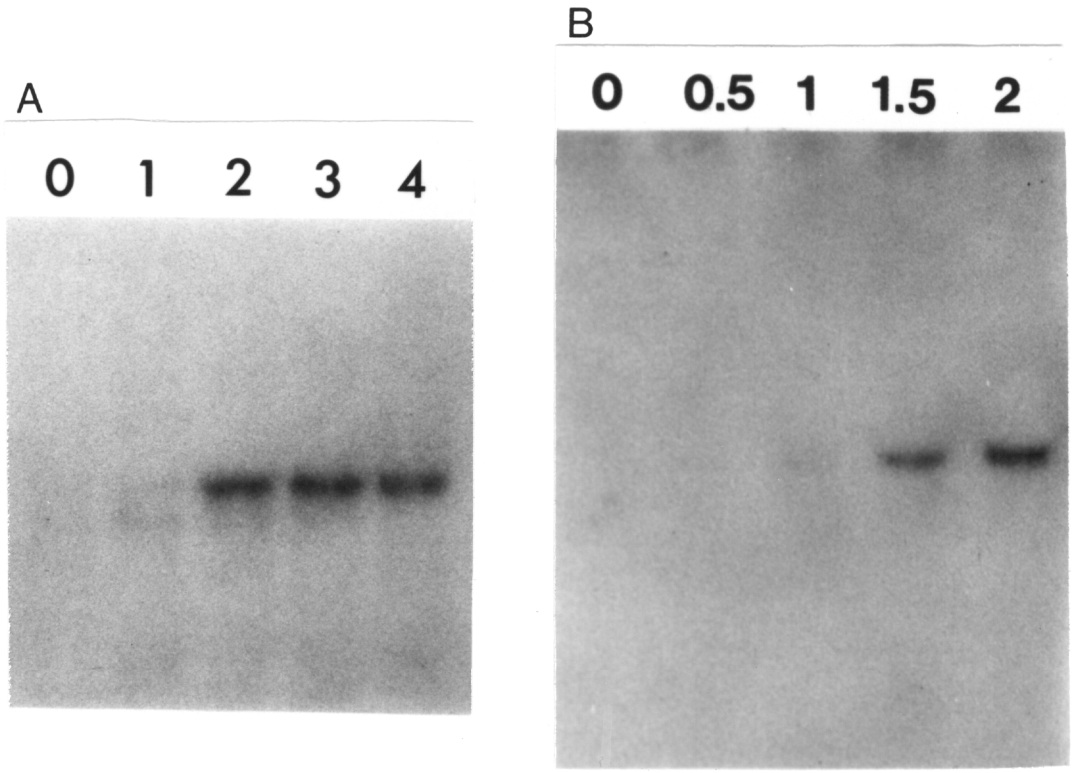

Figure 14. Timing of gp-2 induction by cAMP: (A) Northern blot showing RNA extracted from aggregation competent cells shaken in the presence of 1.0 mM cAMP for 1h, 2h, 3h, and 4h (Lanes 1-4, respectively). Lane 0 is RNA from aggregation competent cells. (B) Northern blot showing the levels of gp-2 mRNA in cells shaken in the presence of exogenous cAMP for 0.5h, 1h, 1.5h, and 2h. Lane 0 is RNA from aggregation competent cells. Radiolabeled PCR amplified gp-2 DNA was used to probe both blots.

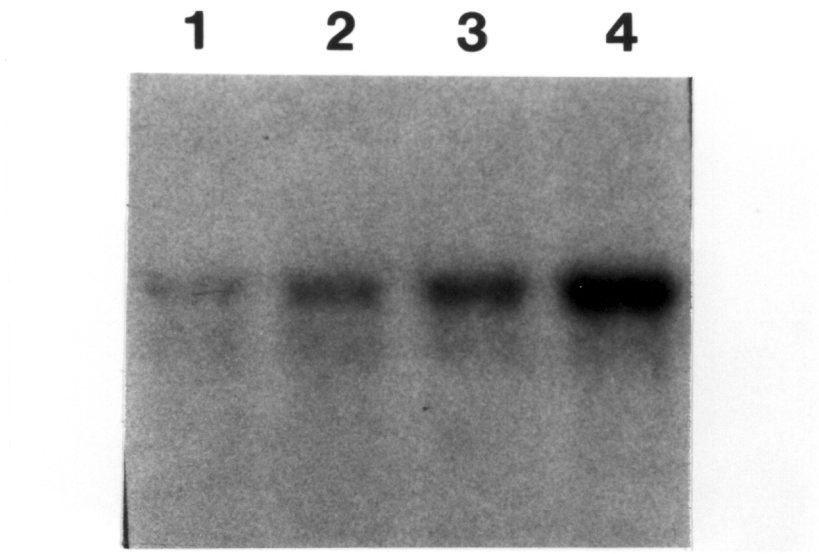

Figure 15. Effect of cAMP concentration on gp-2 mRNA induction: RNA was extracted from ***Dictyostelium*** cells shaken for 4h in the presence of varying concentrations of exogenous cAMP. The RNA was resolved by electrophoresis, transferred to nitrocellulose, and hybridized to a 1098 bp PCR amplified fragment of gp-2 that was labeled by random priming. The RNA in Lane 1 was extracted from cells shaken in the presence of 1.0 μM cAMP. The RNA in Lanes 2-4 was from cells shaken in 10 μM cAMP (Lane 2), 0.1 mM cAMP (Lane 3), and 1.0 mM cAMP (Lane 4).

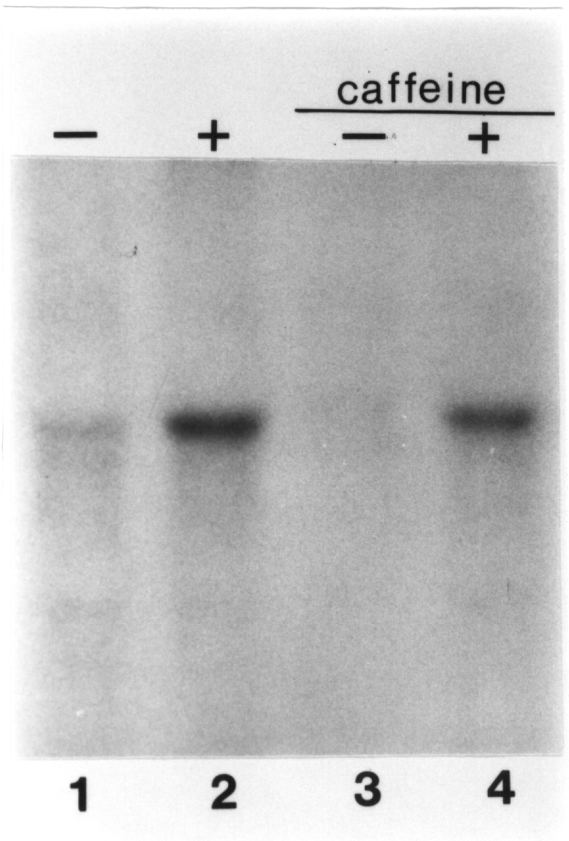

Figure 16. Induction of gp-2 mRNA in the presence of caffeine: Aggregation competent cells were shaken in MES-LPS buffer either in the presence of 2mM caffeine (Lanes 3 and 4; overlined by "caffeine") or in the absence of caffeine (Lanes 1 and 2). Cells were also incubated in the presence (+) or absence (-) of 1.0 mM cAMP. After 4h of these incubations, cells were harvested, RNA was extracted, and Northern blotting was done using labeled gp-2 DNA as a probe.

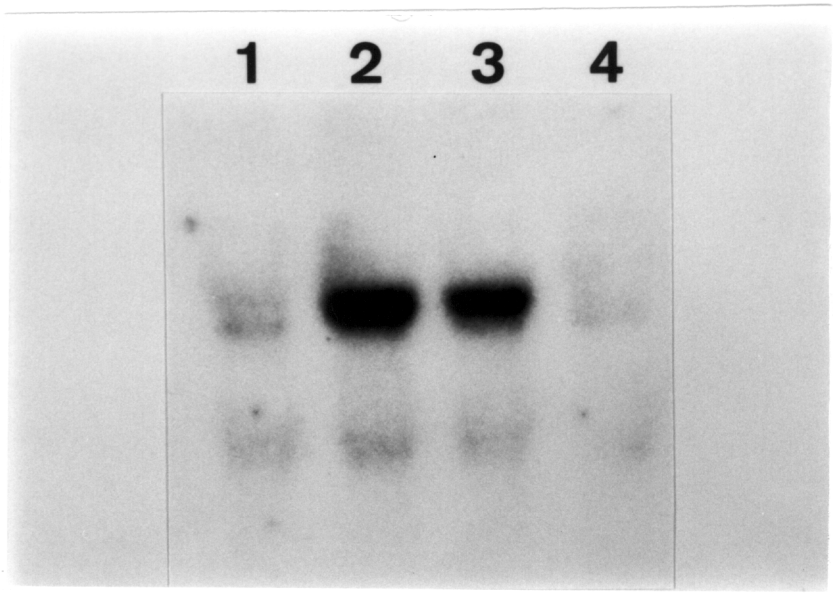

Figure 17. Test of cAMP analogs on the induction of gp-2 mRNA: Aggregation competent cells were shaken at 180 rpm in MES-LPS buffer alone and in MES-LPS containing either 1.0 mM cAMP, 1.0 mM 2′d-cAMP, or 1.0 mM Bt_2-cAMP. RNA was extracted after 4h of shaking in the respective conditions. The RNA was electrophoresed, transferred to nitrocellulose, and hybridized to labeled gp-2 DNA. Lane 1, RNA from cells shaken in MES-LPS buffer alone; Lane 2, RNA from cells shaken in the presence of 1.0 mM cAMP; Lane 3, RNA from cells shaken in the presence of 1.0 mM 2′d-cAMP; Lane 4, RNA from cells shaken in the presence of 1.0 mM Bt_2-cAMP.

constructs derived from this fragment. Examination of the sequence of the gp-2 promoter revealed several short, repeated sequences. The sequence 5′-ACCCACT-3′ is repeated twice. One of the repeats begins 42 nucleotides upstream of the transcriptional start site and the other begins at -79 (+1 being the transcriptional start site). I refer to these repeats as ″C boxes″ in Figure 18. The sequence 5′-TAAAAATGGA-3′ is also repeated twice, both repeats (″TAG boxes″) falling into a short region between -151 and -175. Further upstream, three repeats of the sequence 5′-TAATTATAA-3′ are observed (″TA boxes″); two of these repeats are adjacent, between -210 and -227 while the third begins 314 nucleotides upstream of the transcriptional start site. Since repeated sequences have been implicated as important *cis*-acting elements in the transcriptional regulation of some other eukaryotic genes (Minty and Kedes, 1986; Miwa and Kedes, 1987), I hypothesized that some or all of these repeats might be important in the cAMP regulation of the gp-2 gene. To test this hypothesis, the deletions indicated in Figure 18 were constructed and then ligated into a luciferase reporter gene vector as described in Materials and Methods. The ligations put the luciferase gene under the control of the gp-2 promoter or deletions thereof. These constructs were transfected into *Dictyostelium* cells, and transformants were then used in cAMP induction experiments. The two longest constructs, gp-2-d7 (full length promoter) and gp-2-d1.9, both showed a dramatic induction of luciferase activity in response to cAMP (about 30- and 60-fold stimulation over levels observed in the absence of cAMP, respectively [Figure 19]). Constructs gp-2-d11 and gp-2-d10 also showed cAMP responsiveness, although of only 10- and 5-fold, respectively. Note that gp-2-d11 lacks one of the TA repeats and gp-2-d10 lacks all three TA repeats. The lower cAMP responsiveness of these constructs may indicate that the TA repeats play a role in the cAMP regulation of the gp-2 gene. None of the shorter constructs (gp-2-d23, gp-2-d29, gp-2-d27, gp-2-d2.7, gp-2-d2) showed any responsiveness to cAMP. The lack of cAMP induction in these constructs provides evidence that the DNA sequence elements necessary for cAMP regulation are located upstream of gp-2-d23. The TAG repeats, therefore, may also be involved in the cAMP response of the gp-2 gene, since construct gp-2-d10 contains these elements and exhibits some cAMP responsiveness. The C-boxes are probably not involved in cAMP responsiveness,

since constructs gp-2-d23, gp-2-d29, and gp-2-d27 all contain these elements but show no response to cAMP.

Discussion

In this chapter I have examined several aspects of the cAMP regulation of the *Dictyostelium* gp-2 gene. I have shown that exogenous cAMP plays an important role in the regulation of the gp-2 gene in *Dictyostelium*. The presence of exogenous cAMP induces the gp-2 mRNA (Figure 13), a result that is consistent with the observed cAMP induction of gp-2 activity and the gp-2 protein (Figure 12). It seems likely that cAMP regulation occurs at the level of transcription, given previous studies on the regulation of gp-2 during *Dictyostelium* development (Firtel and Bonner, 1972; Thomas and Wright, 1976; Higgins and Dahmus, 1982). I have also shown that the induction of gp-2 mRNA by exogenous cAMP occurs rapidly between 1 and 1.5 hours after the initial exposure to cAMP, and that exogenous cAMP concentrations as low as 1.0 μM can induce the appearance of the gp-2 mRNA.

I have also examined several aspects of the molecular mechanism of cAMP induction of gp-2. Results showed that cAMP could induce the gp-2 mRNA under conditions in which intracellular cAMP signaling is inhibited in *Dictyostelium*; I also observed that 2'd-cAMP, but not Bt_2-cAMP, caused the appearance of the gp-2 message. These results suggest that cAMP regulation of gp-2 occurs independent of intracellular cAMP signalling. Several other *Dictyostelium* genes which are controlled by cAMP also appear to be regulated without intracellular alterations in cAMP levels (Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Kimmel, 1987; Mann and Firtel, 1987). Gene regulation without intracellular cAMP signaling may be accomplished through second messengers other than cAMP, such as IP_3 and DAG. However, my attempts to stimulate gp-2 induction with IP_3 and DAG did not result in increased gp-2 message (data not shown). Exogenous cAMP may also regulate gene expression in *Dictyostelium* directly through a cell surface binding protein like the CABP1 protein described by Kay et al. (1987); this protein has

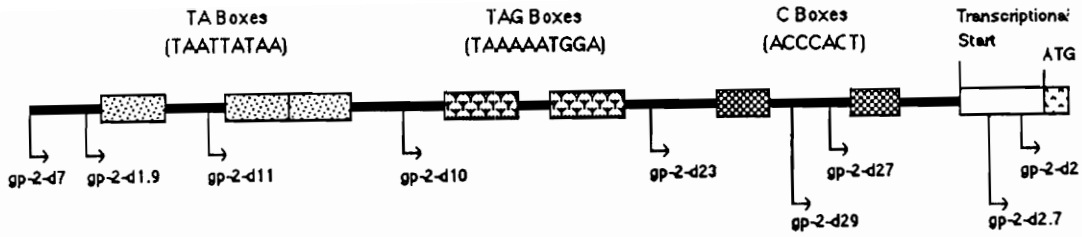

Figure 18. Diagram of the gp-2 promoter and the deletions of this promoter used to drive the expression of a luciferase reporter gene: Construct gp-2-d7 represents the full length gp-2 promoter. This fragment and the other constructs shown were ligated into the luciferase reporter gene vector as described in Materials and Methods. The ligated promoter fragments drive the expression of the luciferase gene. Potential regulatory sequences are shown. The diagram is not to scale. The C boxes begin at -42 and -79, respectively (the transcriptional start site is +1). The TAG boxes begin at -151; the TA boxes extend from -210 to -314.

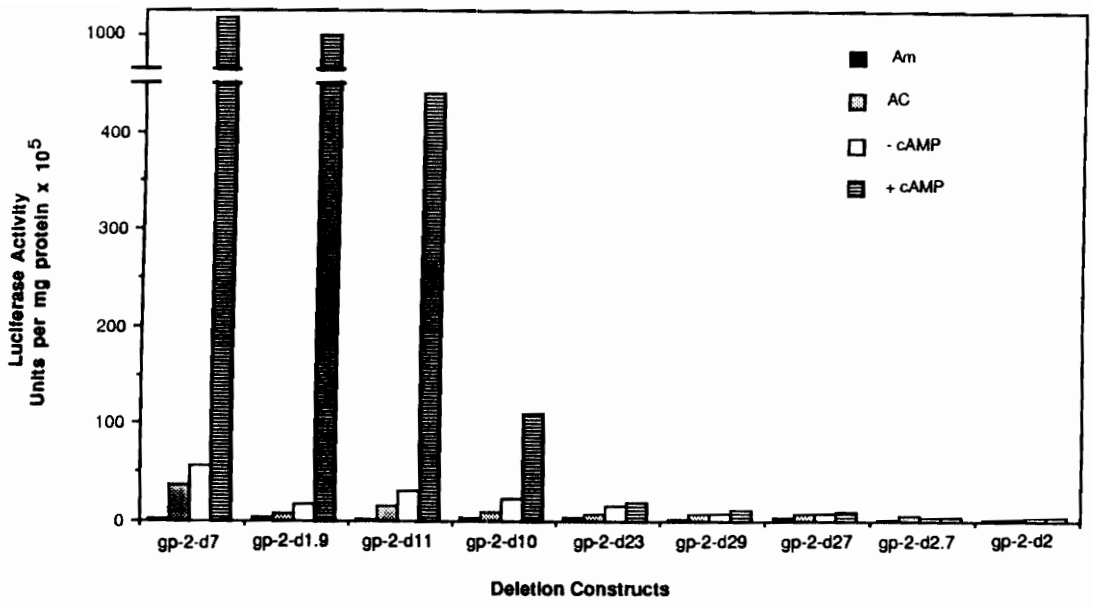

Figure 19. Results of luciferase assays on transformants carrying the gp-2 promoter/luciferase constructs: Specific activity of luciferase for each construct is shown for four conditions: undifferentiated amoebae (Am), aggregation competent cells (AC), and cells shaken for 8h in the absence or presence of 1.0 mM cAMP (-cAMP and + cAMP, respectively). The specific activites represent the average of between 4 and 7 experimental repeats.

been shown to migrate to the nucleus after binding cAMP at the cell surface, although its precise nuclear function remains unknown. Perhaps gp-2 expression is mediated through such a protein without the requirement of intracellular second messengers. Our laboratory is studying the gene for another glycogen phosphorylase, glycogen phosphorylase 1 (gp-1); gp-1 also shows cAMP responsiveness, and, interestingly, cAMP regulation of this gene does appear to require intracellular increases in cAMP (Chapter 4, this dissertation).

I have also examined the gp-2 promoter to identify potential *cis*-acting elements involved in the cAMP regulation of this gene. My results show that the gp-2 promoter exhibits maximal cAMP responsiveness when three repeats of a TA-rich element, located between -210 and -314, are present in the promoter. When one of these repeats is deleted, the cAMP response decreases at least three fold. When all three repeats are deleted, the cAMP response decreases at least six fold. However, even with all three of the TA repeats deleted, some cAMP induction of the luciferase reporter gene is seen. Further deletion, including the TAG repeats between -151 and -175, completely eliminates the cAMP induction of luciferase. These results suggest that both the TA repeats and the TAG repeats may play a role in the cAMP regulation of the gp-2 gene. Curiously, none of these elements appears to be required for temporal regulation of the gp-2 gene; constructs gp-2-d23, gp-2-d29, and gp-2-d27 all confer developmental regulation upon the reporter gene; that is, luciferase activity is observed only during the late developmental stages in transformants carrying these constructs. This suggests that cAMP and developmental regulation of the gp-2 gene occur through different *cis*-acting sites, and probably require multiple *trans*-acting factors.

The *cis*-acting elements involved in the cAMP regulation of several other *Dictyostelium* genes have been reported (Datta and Firtel, 1987; Pears and Williams, 1987; Datta and Firtel, 1988; Pears and Williams, 1988; Pavlovic et al., 1989; Haberstroh and Firtel, 1990). There does not appear to be any definitive consensus sequence for a *Dictyostelium* cAMP response element from these reports. Some of these reports implicate short, G-rich sequences as being necessary for cAMP responsiveness; others identify G/C rich regions and C/A rich sequences as being important in cAMP regulation. The sequences described in these reports are varying distances from the transcriptional start site. However, examination of the promoter of the *pst-cath* gene by Datta and

Firtel (1988) implicated a T/A rich sequence as being important in mediating the cAMP response of that gene, a result very similar to what I have reported here regarding the TA repeats that I observed. The T/A rich sequence of the *pst-cath* gene is located about 250 nucleotides upstream of the transcriptional start site, a location similar to the TA repeats of the gp-2 gene (Figure 18). Additionally, the T/A rich sequence of the *pst-cath* gene is observed to be just upstream of elements that are required for maximal developmental regulation, a result that is also similar to what is observed in the gp-2 gene. Indeed, the C boxes that I observe in the gp-2 gene are virtually identical to sequences shown to be important in the temporal and cell-type specific expression of at least one other *Dictyostelium* gene (Tasaka et al., 1992); similar or identical C boxes are seen in the promoters of several other *Dictyostelium* genes (Fosnaugh and Loomis, 1991). Unfortunately, there is little sequence homology between the T/A rich sequence seen in the *pst-cath* gene and the TA repeats that I observe in the gp-2 gene.

The results described in this report provide some information on a *Dictyostelium* gene that is regulated by cAMP, and provide some insight, in the form of DNA sequence information, into how this regulation occurs. Current experimentation involves analyzing the gp-2 promoter with gel shift assays and footprinting, from both cAMP induction experiments and developmental studies, to identify regions of the promoter that may be recognized by *trans*-acting factors. We are also beginning mutagenesis studies to further examine the importance of the TA repeats and TAG repeats in the cAMP regulation of gp-2. It will be interesting to see if the results from these experiments corroborate what I have reported here--that is, that the TA repeats and TAG repeats are important for cAMP regulation of the gp-2 gene. These studies will provide insight into the mechanism of regulation of a cAMP and developmentally regulated *Dictyostelium* gene.

Materials and Methods

Materials

Alpha labeled ^{32}P-dATP was obtained from New England Nuclear. Ultrapure phenol was from Bethesda Research Laboratories. Nitrocellulose (Nitro Plus 2000) was obtained from MSI. Unless otherwise indicated all other reagents were obtained at the highest purity available from Sigma Chemicals.

Cell Culture

Vegetative cells of *Dictyostelium discoideum* strain AX3 were cultured in HL5 as described previously (Naranan et al., 1988; Rogers et al., 1992). *Dictyostelium* stain AX3K was used for the reporter gene experiments. The AX3K cells were kept at a density of less than 5.0 x 10^6 per ml, and were used at a density of 1.0 x 10^6 per ml in the transformation experiments.

cAMP induction experiments

Dictyostelium amoebae were made aggregation competent by plating onto non-nutrient agar (1.5% Bacto agar buffered at pH 6.5 by either 30 mM potassium phosphate or 7 mM MES) and incubating overnight at 7° C. The aggregation competent amoebae were then harvested, washed once in MES-LPS (7 mM N-morpholinoethanesulfonic acid, pH 6.5, 5 mM $MgSO_4$, 20 mM KCl), and resuspended in 30 ml fresh MES-LPS at 0.5-1.0 x 10^6 cells per ml in 125 ml Erlenmeyer flasks. Cyclic AMP was added to a final concentration of 1.0 mM and the cells were shaken at 180 rpm on a Lab Line rotary shaker (Model 3520). Samples of the cell suspension were removed at 4h and/or 8h after cAMP addition for use in various assays. When 8h cells were used,

the cells were pelleted at the 4h time point and resuspended in fresh MES-LPS with or without cAMP. The experiments done testing the effect of caffeine were carried out as above except that 2.0 mM caffeine was added to the MES-LPS buffer prior to the addition of cAMP. In experiments that tested analogs of cAMP, the cAMP was replaced by either 2'd-cAMP or Bt_2-cAMP, added to a final concentration of 1.0 mM.

Glycogen phosphorylase activity assays and western blotting

Cell extracts from cAMP induction experiments were prepared by freeze-thaw lysis, and a 20 μl sample of each extract was added to 200 μl of a reaction mixture containing 50 mM imidazole (pH 6.8), 2.5 mg/ml glycogen, 2 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM NADP, 50 μg/ml glucose-1,6-bisphosphate, 0.3 unit glucose-6-phosphate dehydrogenase, and 0.4 unit phosphoglucomutase. The assay was carried out at 23° C. The amount of NADPH formed was followed on a strip chart recorder. A molar extinction coefficient of 6.2×10^3 was used to quantitate NADPH formation at 340 nm. One unit of activity is the amount of enzyme that catalyzes the synthesis of 1 μmol NADPH/min at 23° C.

For Western blotting, proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes using a Hoeffer transfer chamber and a power source (model TE51) set at 1.2 amps for 1h. The transfer buffer contained 192 mM glycine and 20% methanol in 25 mM Tris-HCl, pH 8.3. After transfer, the nitrocellulose was rinsed in 50 ml of TBS-Tween (50 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 0.1% Tween 20) for 30 min. The nitrocellulose was then incubated overnight in TBS-Tween containing anti-gp-2 antiserum. The antiserum was prepared as described previously (Naranan et al., 1988a). Following incubation with antiserum the filter was rinsed several times with fresh TBS-Tween and then incubated for 1h with shaking in TBS-Tween containing 1 μg/ml protein A-peroxidase. Following this incubation the filter was washed twice with TBS-Tween and twice with TBS alone. Colorimetric detection of

antigens was done with 48 ml of peroxidase reaction mixture containing 17% methanol, 24 mg of 4-chloro-1-napthol, and 0.008% hydrogen peroxide in TBS.

RNA isolation and Northern analysis

Total RNA was prepared from *Dictyostelium* cells by the simultaneous lysis and extraction in diethylpyrocarbonate (DEPC), SDS, and phenol/ chloroform as previously described (Nellen et al., 1987). Northern blotting was done by capillary action following gel electrophresis of the RNA in formaldehyde containing 1.2% Seakem (FMC) agarose. RNA was quantified by optical density readings at 260 nm, and 10 μg of total RNA were loaded per lane. Duplicate samples were run and stained with ethidium bromide to ensure that rRNA bands were of equal intensity in all lanes. Hybridizations were performed under stringent conditions of 50% formamide, 6 x SSPE, 1 x Denhardt's solution, 0.1% SDS, and 200 μg/ml salmon sperm DNA at 42° C. A 1098 bp fragment of gp-2, extending from nucleotides 691-1789, was amplified by PCR (Rutherford et al., 1992), labeled by random priming (Feinberg and Vogelstein, 1983) and used in the hybridizations. Hybridized filters were washed in 0.2 x SSC, 0.2% SDS at 42° C to 65° C. The hybridized filters were dried under vacuum and autoradiography was done by exposing the filters to Kodak XAR-5 film at -70° C.

Construction of gp-2 promoter deletions

A 1051 bp genomic fragment that extended from the HindIII site of the gp-2 gene (Rutherford et al., 1992) to 651 bp upstream of the translational start site was inserted into the SmaI site of the pBluescript II SK + cloning vector (Stratagene). Bacteria (XL1-Blue) were transformed with the ligation mixture and recombinant clones were sequenced. Deletions of the promoter region were prepared by digesting the vector at the HindIII site, filling with deoxythioderivatives using the Klenow fragment of *E. coli* DNA polymerase I, digesting the downstream EcoRI site of

pBluescript, and then treating with Exo III and mung bean nucleases for various time points. After ligation and transformation of bacteria, recombinant clones were selected and sequenced.

Ligation of the promoter deletions into the reporter gene vector

The promoter inserts were cut out of pBluescript with either ClaI/RsaI or BssHII/RsaI double digests and were blunt ends were formed by filling in overhangs with the Klenow enzyme. The vector SP60-luciferase-Δ20 (SP60-luc; see Haberstroh and Firtel, 1990) is shown in Figure 20 and was the generous gift of Richard A. Firtel from the University of California, San Diego. The SP60 promoter was excised from this vector with a NheI/BamHI double digest, and the overhangs were filled in. The gp-2 promoter inserts contain the translation initiation codon, so ligations were constructed to allow the in frame read through from the gp-2 initiation codon into the luciferase structural gene. Ligations were done with T4 DNA ligase (BMB) according to the supplier's instructions. The molar ratio of vector to insert was 1:10, as quantified by visualization of samples of vector and insert DNA in ethidium bromide stained agarose gels. Restriction digests and sequencing were used to confirm the orientation and in frame status of the inserts.

Transformation

The promoter constructs were transfected into *Dictyostelium* amoebae of the strain AX3K by using a calcium-phosphate precipitation/glycerol shock protocol (Nellen et al., 1984). Transformants were selected by incubation for 6 days in HL5 containing 20 μg/ml G418; resistance to G418 is encoded by the SP60-luc vector. After this selection, cells were harvested in PBS (10 mM KCl, 10 mM NaCl, 16 mM Na_2HPO_4, 34 mM KH_2PO_4, pH 6.5) and plated onto DM agar plates (2% glucose, 1% Oxoid peptone, 1.5% Bacto agar, 3 mM Na_2HPO_4, 10 mM KH_2PO_4) containing 40-50 μg/ml G418 and an overlay of neomycin resistant *E. coli* B/r cells (a generous gift from Dennis Welker of Utah State University). Plaques appeared in 2-5 days. Cells from individual

plaques were transferred to liquid HL5 + 5 μg/ml G418, and grown to a density of less than 1.0 x 10^6 cells per ml. These cells were then used in cAMP induction experiments.

Luciferase assays

Following the cAMP induction experiments (as described above), extracts of transformants carrying the promoter-luciferase constructs were prepared by freeze-thaw lysis of the cells. The extracts were centrifuged for 10 min at 12,000 x g and 5 μl of the supernatants were added to 100 μl of a reaction mix consisting of 0.54 M glycyl-glycine (pH 8.3), 4 mM $MgCl_2$, and 2.1 mM ATP. Luciferase units were quantified by a Berthold luminometer (Lumat LB9501) following the addition of 100 μl 0.6 mM luciferin. Specific activity is expressed as luciferase units per mg protein. Protein was measured by the method of Bradford (1976).

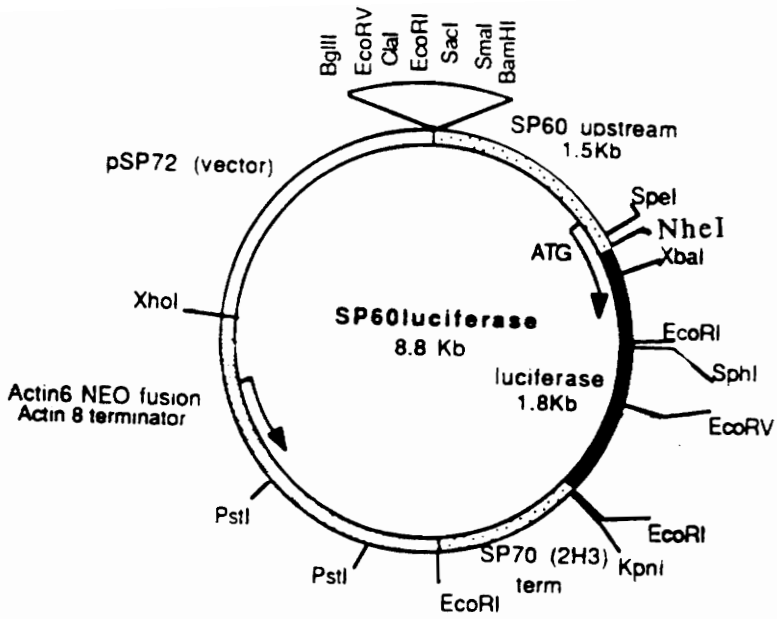

Figure 20. **The reporter gene vector SP60-luciferase-Δ20:** Important restriction sites are indicated. The SP60 promoter was excised and replaced by the gp-2 promoter or deletions thereof, thus placing the transcription of the luciferase gene under the control of the gp-2 promoter fragments.

Chapter 4: Developmental and cAMP Mediated Regulation of gp-1

Abstract

The *Dictyostelium* glycogen phosphorylase-1 (gp-1) exhibits a complex pattern of developmental expression in which differential temporal regulation of enzyme activity, protein levels, and mRNA levels is observed. This pattern of expression implies that gp-1 regulation occurs at multiple levels, probably involving both transcriptional and post-transcriptional events. In this chapter I have examined several facets of this regulation. I show that addition of exogenous cAMP to cells in suspension culture caused alterations in gp-1 enzyme activity and mRNA levels that are identical to those observed during normal development, suggesting that cAMP is involved in the regulation of gp-1. Exogenous cAMP regulated gp-1 mRNA expression at concentrations as low as 1.0 μM. cAMP regulation of gp-1 mRNA appeared to occur through a mechanism that required intracellular cAMP signalling. I also identified regions of the promoter necesssary for gp-1 expression by using gp-1 promoter deletions to drive the expression of a luciferase reporter gene. Results of these experiments suggested that developmental and cAMP mediated changes in gp-1 mRNA levels were the result of alterations in transcription. The promoter analysis also suggested that a DNA se-

quence element required for expression in vegetative cells is located between -690 and -2000 nucleotides from the transcriptional start site. Elements necessary for maximal developmental and cAMP mediated expression appear to be located between -1000 and -2000 nucleotides from the cap site. Sequence elements located between -230 and -1000 appear to be required for a basal level of late developmental expression.

Introduction

One of the central problems in biology is the elucidation of the molecular control mechanisms responsible for developmentally regulated gene expression. In recent years it has become increasingly clear that the expression of developmentally regulated genes can be controlled at many different levels; transcriptional control, mRNA processing and stability, translational control, and post translational protein modification have all been shown to play roles in regulating the developmental expression of various genes.

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study the molecular events associated with development and cell differentiation (for review, see Loomis, 1982). This organism proceeds through a short and well documented developmental cycle that is initiated by nutrient depletion. Upon nutrient depletion, vegetative cells, which are amoeboid in appearence, halt cell division, stream together, and ultimately form a multicellular fruiting structure composed of two distinct cell types. This developmental cycle not only possesses elegant simplicity, but also separates vegetative growth from differentiation (Sussman and Brackenbury, 1976). Additionally, *Dictyostelium* is readily amenable to the genetic manipulations required for modern molecular biology techniques.

Both differentiated cell types in *Dictyostelium* require the synthesis of complex structural polysaccharides such as cellulose, and the glucose monomers used to synthesize these structural components are derived from glycogen stores (Wright et al., 1968; Marshall et al., 1970; Gustafson and Wright, 1972). Thus, glycogen degradation is a crucial developmental event in *Dictyostelium.*

Glycogen degradation is catalyzed by glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyl transferase, EC 2.4.1.1), and I am examining the expression of two developmentally regulated glycogen phosphorylases in *Dictyostelium*. Glycogen phosphorylase-2 (gp-2) is expressed late in development, and the gp-2 mRNA and protein accumulate concomitant with increases in gp-2 activity (Naranan et al., 1988; Rutherford et al., 1992; Chapter 2, this dissertation). Glycogen phosphorylase-1 (gp-1) exhibits a more complex pattern of regulation. gp-1 enzyme activity is maximal in vegetative amoebae then decreases throughout the subsequent stages of development (Rutherford and Cloutier, 1986; Naranan et al., 1988). Although the gp-1 activity decreases, the level of gp-1 protein is constant during development (Naranan et al., 1988; Rogers et al., 1992; Chapter 2, this dissertation). The gp-1 mRNA also exhibits developmental regulation (Rogers et al., 1992). In vegetative amoebae and in very early developmental stages the gp-1 mRNA is highly expressed, but as development proceeds the level of gp-1 mRNA decreases; later in development, gp-1 mRNA levels increase again. The differential temporal expression of gp-1 enzyme activity, protein, and mRNA suggest that regulation of gp-1 occurs at multiple levels, possibly involving both transcriptional and post-transcriptional events. In this chapter I have examined several aspects of gp-1 regulation. I show that exogenous cAMP can mimic the developmental changes seen in gp-1 enzyme activity and mRNA levels. I also examine the molecular mechanism of cAMP regulation of gp-1 and examine the gp-1 promoter to identify regions necessary for developmental and cAMP mediated expression.

Results

Analysis of gp-1 during Dictyostelium development and in response to exogenous cAMP

In Figure 21 I show a comparison of gp-1 enzyme activity, protein levels, and mRNA expression during the developmental cycle of *Dictyostelium* (Rutherford and Cloutier, 1986; Naranan et al., 1988; Rogers et al., 1992). The gp-1 enzyme activity is maintained at high levels in undifferentiated amoebae, but slowly decreases during the time course of multicellular development (Figure 21A). Curiously, this decrease in gp-1 activity was not accompanied by a corresponding decrease in the level of gp-1 protein; gp-1 protein levels remained constant during the developmental stages (Figure 21B). In addition, Northern analysis showed that the gp-1 mRNA was highly expressed in vegetative cells and late in development, but decreased markedly during the initial stages of development (Figure 21C). Because the observed changes in gp-1 activity do not correlate with the observed levels of gp-1 protein and mRNA, it is probable that gp-1 is regulated at several different levels, possibly by both transcriptional and post transcriptional events.

It has been shown previously that exogenous cAMP causes a dramatic decrease in gp-1 enzyme activity (Brickey et al., 1990). Figure 22 shows that the decrease in gp-1 enzyme activity is not reflected in a corresponding loss of enzyme protein, but instead, gp-1 protein levels remain essentially constant in the presence or absence of exogenous cAMP (Figure 22B). In Figure 22C, we have examined the effect of exogenous cAMP on gp-1 mRNA levels. Aggregation competent *Dictyostelium* cells were suspended in MES-LPS buffer in either the presence or absence of 1.0 mM cAMP. At 4h and/or 8h after cAMP addition cells were removed and RNA was extracted, resolved by electrophoresis, transferred to nitrocellulose filters, and hybridized to radiolabeled gp-1 cDNA. Essentially no gp-1 mRNA was seen in aggregation competent amoebae (Lane 1). Cells shaken for 4h or 8h in the presence of cAMP (Lanes 3 and 5, repectively) showed a marked increase in the level of gp-1 mRNA when compared to cells shaken in the absence of cAMP (Lanes 2 and 4).

These results are consistent with the hypothesis that regulation of gp-1 occurs at several levels; the changes in gp-1 activity seen in response to exogenous cAMP are not a direct consequence of regulation of the levels of gp-1 protein or gp-1 mRNA. Also, these data clearly show that the effect of exogenous cAMP on gp-1 enzyme activity, protein levels, and mRNA expression is identical to that observed during normal development, suggesting that cAMP is involved in the regulation of gp-1 *in vivo*.

Timing of cAMP enhancement and the effect of cAMP concentration on gp-1 mRNA levels

As shown in Figure 22C, the cAMP enhancement of gp-1 mRNA levels appears to be maximal by 4h after the addition of exogenous cAMP. I examined the kinetics of this enhancement in more detail by extracting RNA from aggregation competent cells that had been exposed to exogenous cAMP for 1h, 2h, 3h, or 4h (Figure 23A). The gp-1 mRNA levels increased linearly from 1h to 4h. Cells shaken in the absence of exogenous cAMP showed markedly less enhancement of gp-1 mRNA levels after 4h of shaking (data not shown).

We also examined the effect of cAMP concentration on the enhancement of gp-1 mRNA levels (Figure 23B). Aggregation competent *Dictyostelium* cells were shaken in the presence of various concentrations of exogenous cAMP for 4h or 8h. After the incubations RNA was extracted, resolved by electrophoresis in formaldehyde-containing agarose gels, and transferred to nitrocellulose filters. These filters were hybridized to a 836 bp fragment of radiolabeled gp-1 cDNA. After 4h of shaking, only cAMP concentrations of 1.0 mM and 0.1 mM were effective in enhancing gp-1 mRNA levels. However, after 8h of shaking, cAMP concentrations as low as 1.0 μM resulted in elevated gp-1 mRNA levels. The enhancement of gp-1 mRNA expression with 1.0 μM occured with cAMP concentrations 100-1000 times lower than those routinely used to examine the regulation of *Dictyostelium* genes, but these concentrations are still several orders of magnitude higher than the endogenous cAMP concentration (Pahlic and Rutherford, 1979).

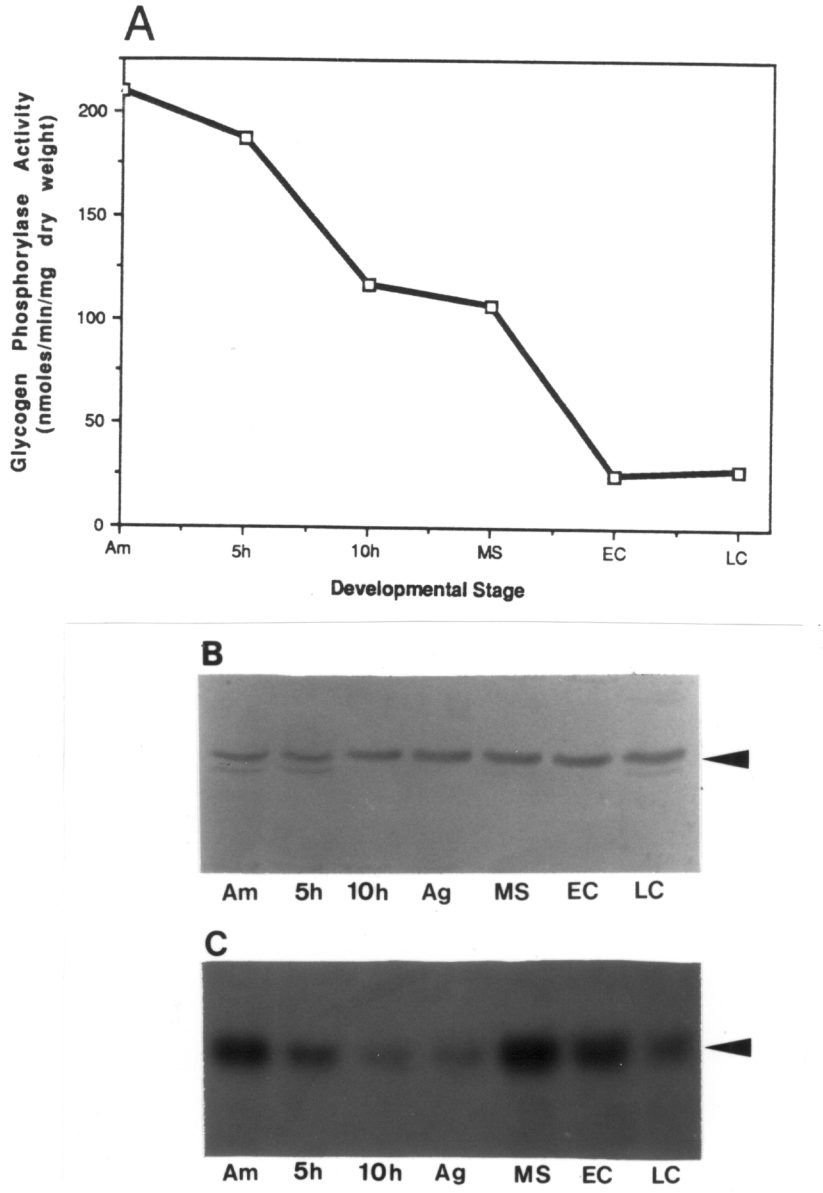

Figure 21. Developmental expression of gp-1 during *Dictyostelium* development: (A) gp-1 activity during the development of *Dictyostelium*. Cells at different developmental stages were harvested, lysed, and the extracts were assayed for gp-1 activity as described in Materials and Methods. Activity is expressed as nanomoles NADPH produced/min/mg dry weight. (B) Western analysis showing gp-1 protein levels during *Dictyostelium* development. Cells at the indicated developmental stages were harvested and lysed. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and exposed to anti-gp-1 antiserum. The arrow indicates 92 kd. (C) Northern analysis of gp-1 mRNA levels during the development of *Dictyostelium* RNA was extracted from cells at the indicated developmental stages, resolved by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to radiolabeled gp-1 cDNA. The arrow indicates 3.0 kb. The abbreviations used in (A), (B), and (C) are: Am, vegetative amoebae; 5h and 10h, cells plated for 5h and 10h; Ag, late aggregation stage; MS, migrating slugs; EC, early culmination stage; LC, late culmination stage.

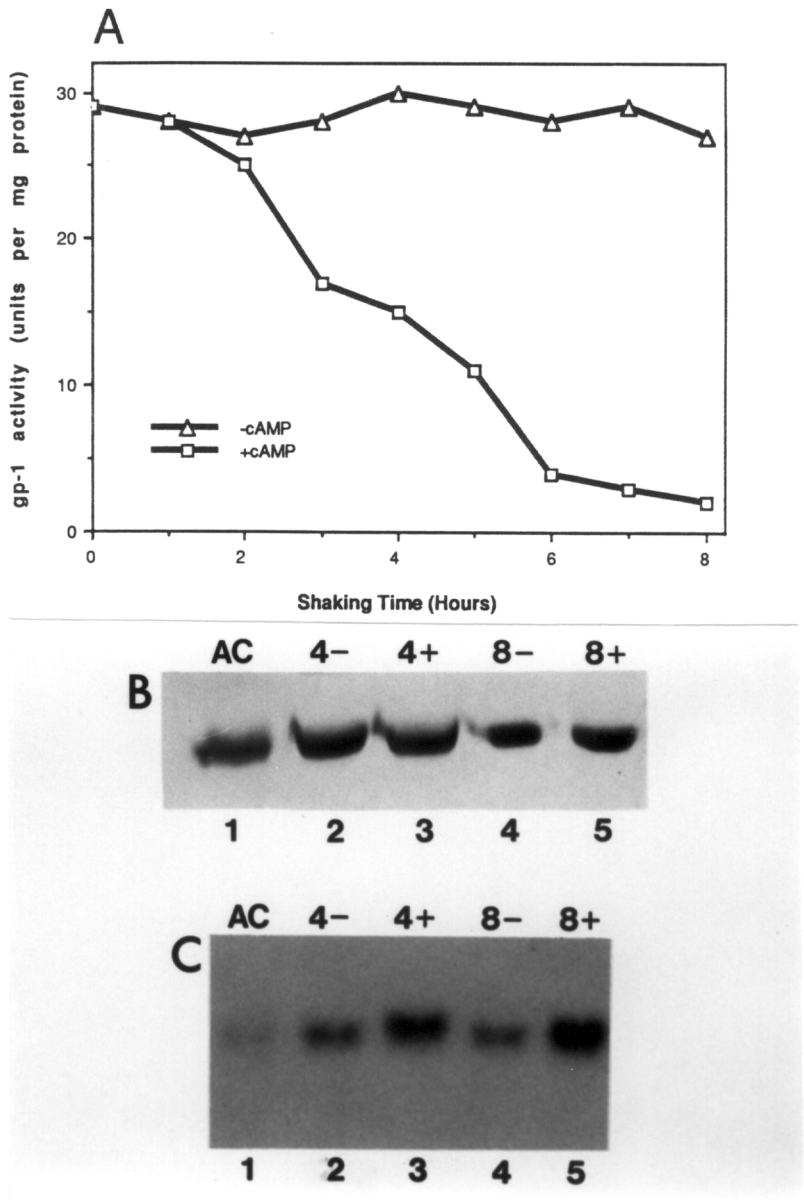

Figure 22. The effects of exogenous cAMP on gp-1 enzyme activity, protein levels, and mRNA levels: Aggregation competent amoebae were resuspended in MES-LPS and were shaken at 160 rpm in either the presence or absence of 1 mM cAMP. At various time points cells were removed from the shaking cultures, lysed, and the resultant extracts were assayed for gp-1 activity or were used for Western and Northern blotting. (A) gp-1 activity in cells shaken in the presence and absence of exogenous cAMP. gp-1 activity is expressed as units per mg protein. (B) gp-1 protein levels in cells shaken for 4h (Lanes 2 and 3; also marked 4h) or 8h (Lanes 4 and 5; also marked 8h) in either the absence (Lanes 2 and 4; also marked -) or presence (Lanes 3 and 5; also marked +) of exogenous cAMP. Lane 1 (marked AC) represents protein from aggregation competent cells. (C) gp-1 mRNA levels in cells shaken for 4h (Lanes 2 and 3; also marked 4h) or 8h (Lanes 4 and 5; also marked 8h) in either the absence (Lanes 2 and 4; marked -) or presence (Lanes 3 and 5; marked +) or exogenous cAMP. Lane 1 (also marked AC) represents RNA from aggregation competent cells.

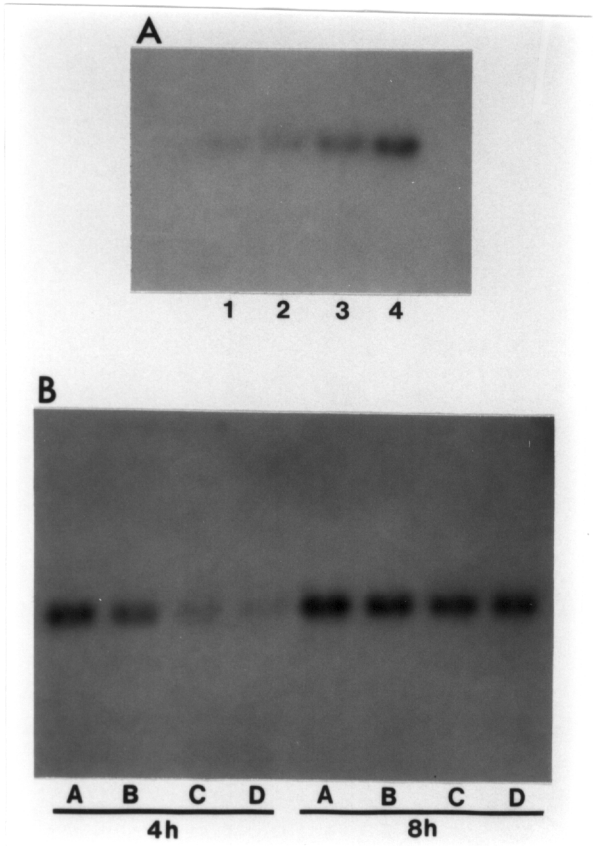

Figure 23. Timing of cAMP enhancement and the effect of cAMP concentration on gp-1 mRNA levels: (A) Timing of cAMP enhancement of gp-1 mRNA levels. The northern blot shows RNA from aggregation competent cells that were resuspended in MES-LPS buffer containing 1.0 mM cAMP and shaken at 160 rpm for 1h, 2h, 3h, or 4h (Lanes 1-4, respectively). (B) The effect of cAMP concentration on gp-1 mRNA levels. The RNA for this blot was extracted from aggregation competent cells that were shaken in the presence of cAMP at concentrations of 1.0 mM (Lanes A), 0.1 mM (Lanes B), 10 μM (Lanes C), or 1.0 μM (Lanes D) for either 4h (Lanes underlined by 4h) or 8h (Lanes underlined by 8h).

Molecular mechanism of cAMP enhancement of gp-1 expression

The above results provide evidence that cAMP is directly involved in the regulation of gp-1 *in vivo*, in that exposure of cells to exogenous cAMP results in changes in the level of gp-1 enzyme activity and mRNA levels which mimic the changes that occur in normal developmental. In *Dictyostelium*, exogenous cAMP can lead to alterations in intracellular processes through at least two different molecular mechanisms (Europe-Finner and Newell, 1987; Gerisch, 1987; Janssens and Van Haastert, 1987; Kimmel, 1987). One mechanism involves intracellular cAMP signaling and leads to intracellular manifestations through the action of cAMP-dependent protein kinases or other cAMP binding proteins. The second mechanism is independent of intracellular cAMP signaling and appears to act through second messengers such as inositol trisphosphate (IP_3), diacylglycerol, or calcium (Europe-Finner and Newell, 1986; Schaap et al., 1986; Janssens and Van Haastert, 1987; Europe-Finner and Newell, 1987; Ginsburg and Kimmel, 1989).

To examine the molecular mechanism of cAMP regulation of gp-1, aggregation competent cells were resuspended in MES-LPS buffer in the absence or presence of cAMP as well as in MES-LPS buffer plus 2.0 mM caffeine containing either the cAMP analog 2′-deoxy-cAMP (2′d-cAMP) or the cAMP analog dibutyryl-cAMP (Bt_2-cAMP). These analogs were selected based on their membrane permeability and their affinity for either the *Dictyostelium* cAMP dependent protein kinase or for cell surface cAMP receptors. Oyama and Blumberg (1986) demonstrated that 2′d-cAMP has a high affinity for cell surface cAMP receptors and a low affinity for the cAMP dependent kinase, while Bt_2-cAMP possesses a high affinity for the regulatory subunit of the *Dictyostelium* cAMP dependent protein kinase. Also, Bt_2-cAMP is membrane permeable and is often used to mimic the effects of intracellular cAMP. Caffeine has been shown to inhibit intracellular cAMP signaling in *Dictyostelium* (Brenner and Thoms, 1984). If enhancement of gp-1 mRNA expression occurs independent of intracellular cAMP signaling, under these experimental conditions only the addition of 2′d-cAMP should result in increased gp-1 mRNA levels. If, however, this enhancement is regulated via intracellular cAMP signaling, increased gp-1 mRNA levels should be observed with only Bt_2-cAMP. After 4h or 8h incubation in the absence or presence

of cAMP or the analogs, RNA was extracted and Northern analyses were performed. Figure 24 shows that Bt_2-cAMP (Lane 4) was able to enhance the expression of gp-1 mRNA to the levels seen with authentic cAMP (Lane 2); essentially no enhancement was observed with 2'd-cAMP (Lane 3). These results suggest that the cAMP regulation of gp-1 occurs through a mechanism that requires intracellular cAMP signaling.

Analysis of the gp-1 promoter: identification of elements necessary for gp-1 expression

The complete gp-1 promoter was isolated on a 2.2 kb RsaI genomic fragment. Presumably the entire promoter is contained in this fragment because the coding sequence of another gene overlaps this fragment. Deletion constructs of the gp-1 promoter were prepared as described in Materials and Methods, then were ligated to a luciferase reporter gene such that the gp-1 promoter directs luciferase expression (Figure 25). The luciferase reporter gene was derived from the vector SP60-luciferase-Δ20, which was the generous gift of R. A. Firtel of the University of California, San Diego. The promoter deletion-luciferase constructs were then transfected into *Dictyostelium* amoebae. In order to analyze regions of the promoter required for gp-1 expression, transformants were assayed for luciferase activity at various stages of development and after being shaken in liquid culture in the presence of exogenous cAMP.

To analyze regions of the gp-1 promoter that are required for gp-1 expression during development, transformants carrying the different promoter deletion-reporter gene constructs were removed from nutrient medium and spread onto filters buffered with MES-LPS. Removal from nutrient medium triggers development; cells were harvested from the filters at various developmental stages, lysed, and the extracts were assayed for luciferase activity. Figure 26 shows that construct gp-1-d7 (extending to approximately -2000, with + 1 being the transcriptional start site) directed the expression of luciferase in a pattern that is qualitatively the same as the developmental expression pattern observed for gp-1 mRNA (Figure 21C); that is, high activity was observed in

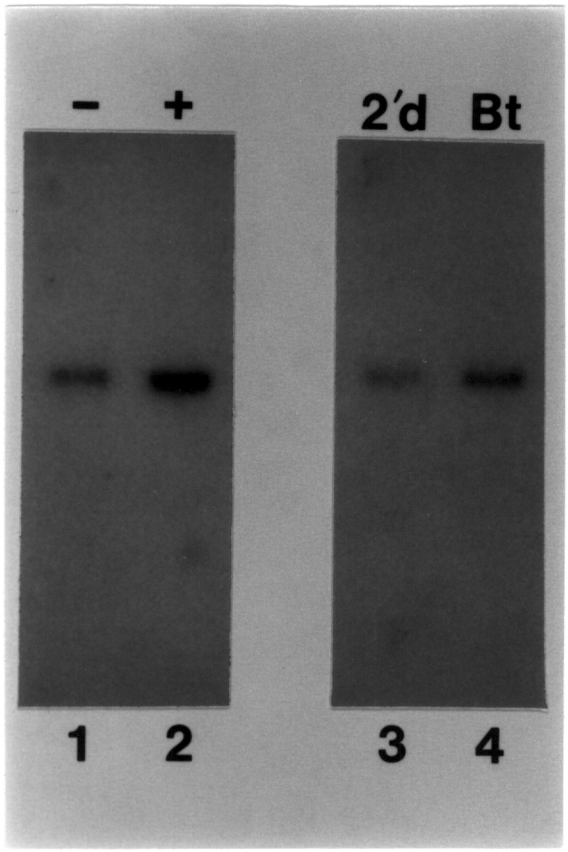

Figure 24. Test of cAMP analogs on the enhancement of gp-1 mRNA levels: Aggregation competent cells were shaken at 160 rpm in either MES-LPS (Lanes 1 and 2) or MES-LPS containing 2.0 mM caffeine (Lanes 3 and 4). Cells received either no additional treatment (Lane 1; marked -), or were exposed to 1.0 mM concentrations of cAMP (Lane 2; marked +), 2'd-cAMP (Lane 3; marked 2'd), or Bt_2-cAMP (Lane 4; marked Bt). RNA was extracted after 4h or 8h of shaking, resolved by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to a radiolabeled gp-1 cDNA fragment.

amoebae, followed by a decrease at the initiation of development and an increase in the late stages. Construct gp-1-d94 (deleted to approximately -1000) also showed high luciferase activity, but the pattern of expression differed from gp-1-d7 in that the late increase in activity was not seen. Constructs gp-1-d81 (-690) and gp-1-d105 (-540) showed greatly reduced luciferase activity at all stages of development, particularly in vegetative amoebae where 50 to 70 fold less activity was observed as compared to the longer constructs. However, these constructs did appear to show a basal level of activity in the later developmental stages. Construct gp-1-d89 (deleted to -230) showed essentially no reporter gene activity either in vegetative cells or in cells at later developmental stages. These results suggest to us that the alterations in gp-1 mRNA levels during development occur as a result of transcriptional regulation, since our promoter deletions alter both the level and pattern of reporter gene expression. If gp-1 mRNA levels were controlled at the level of message stability, we would expect that promoter deletions could alter the level of expression, but the pattern of expression would remain the same for all constructs. The data presented in Figure 26 also suggest to us that at least three regulatory sites exist in the gp-1 promoter. A regulatory site required for vegetative expression appears to be located upstream of construct gp-1-d81 (-690). Luciferase expression in later developmental stages is maximal only in deletion gp-1-d7, suggesting the presence of a regulatory element necessary for late expression over 1000 nucleotides upstream of the transcription initiation site. There also may be elements able to act as a minimal promoter for late developmental activity in constructs gp-1-d105, gp-1-d81, and elevated, but not normal, late activity in construct gp-1-d94. Note that these constructs extend from -230 to -1000.

To analyze elements of the gp-1 promoter required for regulation by exogenous cAMP, the transformants carrying the promoter deletion-luciferase constructs were examined for cAMP responsiveness. Figure 27 shows that construct gp-1-d7 (-2000) directed a pattern of luciferase expression similar to that observed for gp-1 mRNA expression in cAMP response experiments. In both cases, the presence of exogenous cAMP enhanced the expression of luciferase or mRNA (Figure 27; compare to Figure 22C) over the levels of cells shaken in the absence of cAMP. Interestingly, the specific activities of luciferase for this construct were essentially identical in these cAMP response experiments and in the experiments examining developmental expression (compare

Figures 26 and 27). Construct gp-1-d94 (-1000) showed high luciferase activity but, unlike construct gp-1-d7, no enhancement of luciferase activity was seen in the presence of cAMP. Constructs gp-1-d81 and gp-1-d105 (-540 and -690, respectively) showed greatly reduced luciferase activity, while deletion gp-1-d89 (-230) showed no reporter activity. These results suggest that the cAMP enhancement of gp-1 mRNA levels is a transcriptional event, since, again, promoter deletions altered both the level and pattern of expression. These results also suggest that the sequence elements necessary for cAMP regulation are seen only in construct gp-1-d7, and, therefore, are located at least 1000 nucleotides from the transcriptional start site.

Discussion

In this report I have examined the expression of gp-1 during the development of *Dictyostelium* and have found evidence that the system is regulated at both the transcriptional and post-transcriptional levels. During development, gp-1 enzyme activity decreases while gp-1 protein levels remain constant (Rutherford and Cloutier, 1986; Naranan et al., 1988; Figure 21). Interestingly, the gp-1 mRNA shows a pattern of expression in which high levels are observed in vegetative cells and late in development, with little or no gp-1 message seen during the intervening developmental stages (Rogers et al., 1992; Figure 21C). While these results clearly imply that regulation of gp-1 occurs at several levels, many specific facets of this regulation remain unknown. For example, it is not clear at this point what mechanism is involved in the apparent inactivation of the gp-1 enzyme during development. Presumably, some developmentally regulated modification(s) mediate this process. It is also unclear if there is any functional role for the inactive gp-1 protein that remains present throughout the late developmental stages. Preliminary information on the involvement of gp-1 enzyme in the late stages of development comes from studies of transformed mutants which lack **gp-2**. In these transformed cells there is no developmental decrease in gp-1 activity. That is, both gp-1 activity and gp-1 protein levels remain high throughout development. It is possible that the normal expression of gp-2 may be involved in regulating the activity

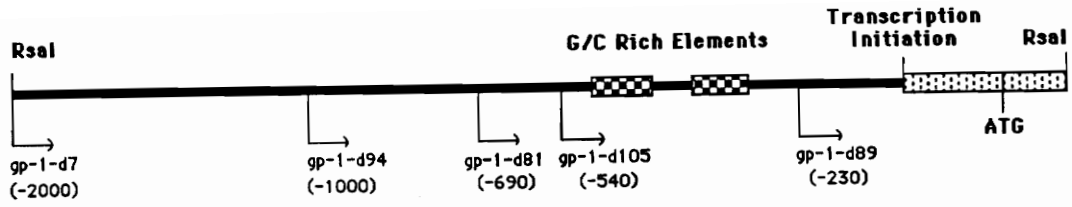

Figure 25. Diagram of the gp-1 promoter deletions used to drive the expression of a luciferase reporter gene: Construct gp-1-d7 represents the full length gp-1 promoter, and extends to approximately -2000 (+1 is transcription initiation). The other constructs are shown along with the distance of the deletion from the transcriptional start site. These promoter fragments were ligated into the luciferase reporter gene vector as described in Materials and Methods. The promoter fragments drive the expression of the luciferase gene. The positions of two relatively G/C rich sequence elements are indicated. The diagram is not to scale.

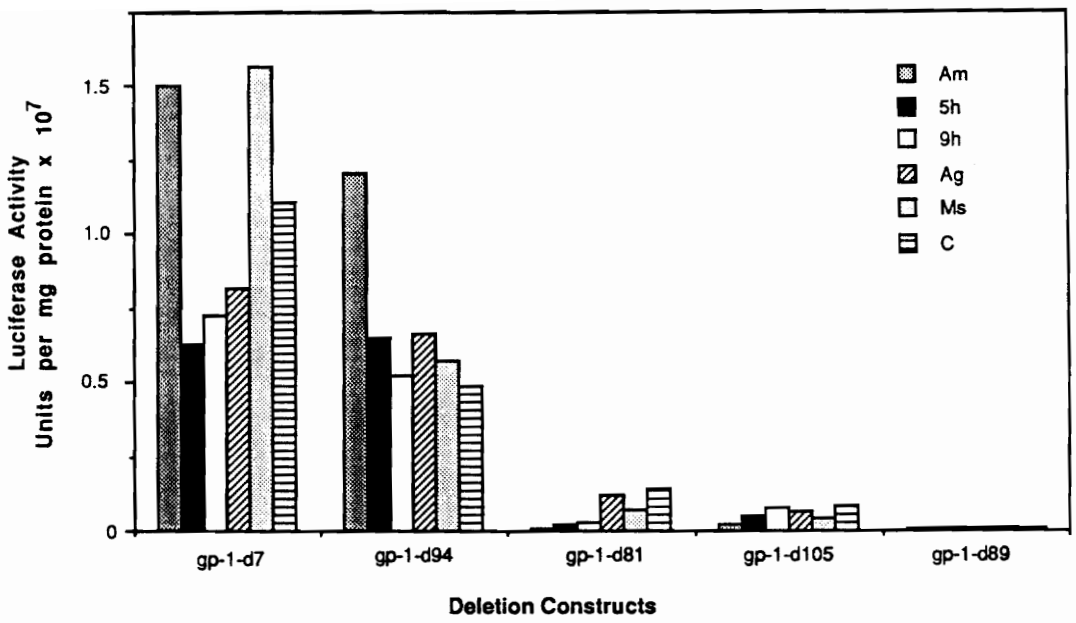

Figure 26. Developmentally regulated luciferase activity in transformants carrying the promoter deletion-luciferase constructs: Specific activity of luciferase for each construct is shown for six developmental stages (Am, vegetative amoebae; 5h and 9h, cells developed on filters for 5h or 9h; Ag, late aggregation stage; MS, migrating slug stage; C, mid-culmination stage). Specific activity is expressed as units per mg protein.

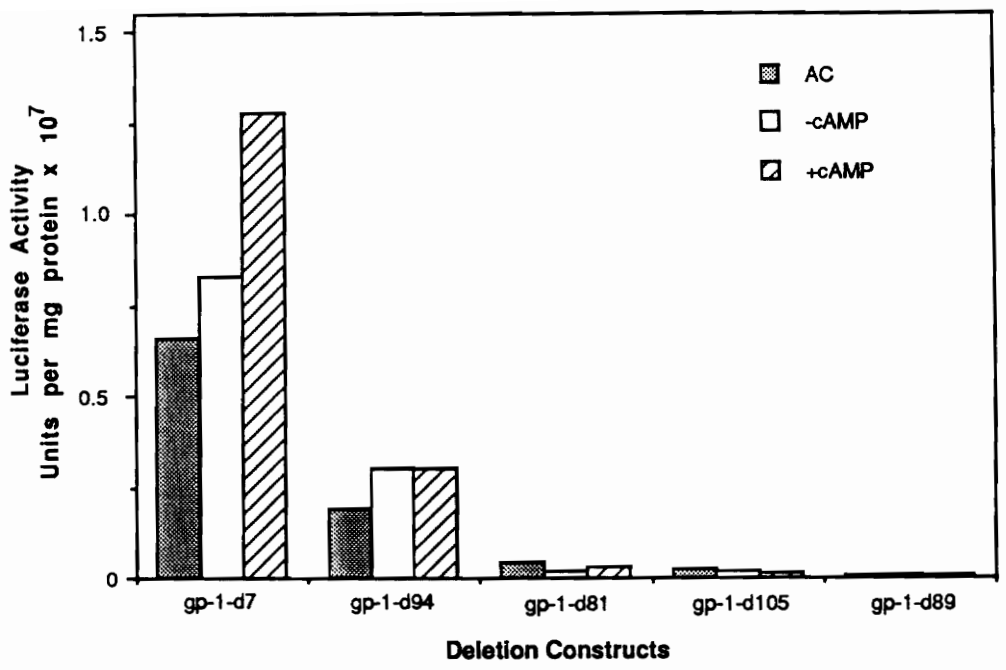

Figure 27. Effect of exogenous cAMP on luciferase activity in transformants carrying the promoter deletion-luciferase constructs: Luciferase activity is shown for three experimental conditions: aggregation competent amoebae (AC), cells shaken for 8h in the absence of exogenous cAMP (-cAMP), and cells shaken for 8h in the presence of 1.0 mM cAMP (+cAMP). Luciferase activity is expressed as units per mg protein.

of gp-1 (manuscript in preparation). In addition, I have observed that the inactive gp-1 protein is highly localized in pre-stalk cells in *Dictyostelium* slugs and culminates that express gp-2, but in the gp-2 transformants the active gp-1 protein is observed in both cell types (Chapter 5, this dissertation). This result suggests, again, that coordinate regulation between gp-1 and gp-2 may occur. The functional significance of an increase in gp-1 mRNA levels during the terminal stages of development is also unclear. One possibility is that this mRNA is sequestered in spores for use upon germination, but this hypothesis has not yet been directly tested.

I have also shown that addition of exogenous cAMP can result in changes in gp-1 enzyme activity and mRNA levels that mimic those occuring during normal development, a result that suggests that cAMP is involved in the regulation of gp-1. I have shown that the cAMP enhancement of gp-1 mRNA levels occurs in a linear fashion over 4h after the initial exposure to exogenous cAMP and that exogenous cAMP concentrations as low as 1.0 μM can enhance gp-1 mRNA levels after 8h of exposure. I also examined the molecular mechanism through which cAMP regulates gp-1 expression, and found evidence to support the involvement of intracellular cAMP signaling. Since cAMP has unique effects on both gp-1 activity and gp-1 mRNA levels, it seems likely that intracellular cAMP signaling triggers both post translational regulation of gp-1 as well as alterations in gp-1 transcription. Most of the other cAMP regulated *Dictyostelium* genes that have been examined, including the gp-2 gene (Chapter 4, this dissertation), are regulated without intracellular cAMP signaling (Oyama and Blumberg, 1986; Haribabu and Dottin, 1986; Kimmel, 1987; Mann and Firtel, 1987).

I have also examined the gp-1 promoter and identified regions which are necessary for gp-1 expression. The results of these experiments suggest that at least three regulatory sites may be present in the gp-1 promoter. Maximal vegetative expression was observed only when the region from between -690 to -2000 is not deleted from the promoter, suggesting the presence of a vegetative cell-specific element in this region. Late developmental expression of gp-1 was not observed if the region between -1000 and -2000 was deleted. This region also appears to be required for cAMP enhancement, suggesting that the *cis*-acting elements necessary for these aspects of gp-1 expression are located in this region of the promoter. A basal, late developmental activity was detected in

constructs that extended from -230 to -690, and elevated, but not normal, late activity was observed when the region between -690 and -1000 was included in the constructs. These results suggest the presence of elements in these regions that may be required, but are not alone sufficient, for normal late gp-1 expression.

Based on my deletion analysis, it appears that the gp-1 promoter is very different from most of the other *Dictyostelium* promoters in several respects. The presence of putative *cis*-acting elements over 1000 nucleotides from the transcriptional start site is very unusual, especially for developmental and cAMP response elements, which have usually been identified between -200 and -700 nucleotides of the transcriptional start site (Pears and Williams, 1987; Datta and Firtel, 1988; Early and Williams, 1989; Pavlovic et al., 1989; Fosnaugh and Loomis, 1991; Haberstroh et al., 1991; Faix et al., 1992; Tasaka et al., 1992). The distance between the transcriptional start and these potential gp-1 regulatory elements is characteristic of enhancer sequences seen in higher eukaryotes (Khoury and Gruss, 1983; Schaffner et al., 1985). Sequence analysis of the region between + 1 and -500 showed that two relatively G/C rich "boxes" are located in an otherwise extremely A/T rich region between -230 and -400 (data not shown); recall that constructs containing only this region exhibited a basal level of late developmental activity. G/C rich or G rich sequences have been implicated in mediating the expression of other *Dictyostelium* genes that exhibit developmental and cAMP mediated regulation (Pears and Williams, 1987; Datta and Firtel, 1988; Pears and Williams, 1988; Pavlovic et al., 1989; Faix et al., 1992). Other workers in our laboratory are currently beginning gel shift and mutagenesis studies to examine the potential role of these G/C rich sequences in the regulation of gp-1. Others are also currently sequencing the remainder of the gp-1 promoter, and will shortly begin to expand the gel shift studies to other regions of the gp-1 5′ sequence. It will be interesting to see if specific proteins bind to gp-1 promoter sequences located 1000 bp or more from the transcriptional start site.

The results of promoter deletion experiments also suggest that the changes in gp-1 mRNA levels that are observed during development occur as a result in transcriptional changes, rather than as result of changes in mRNA stability, because the promoter deletions are able to alter both the level and pattern of reporter gene expression. If mRNA stability controlled gp-1 mRNA levels, I

would have expected that the promoter deletions would alter the level of expression, but not the pattern of expression, in different constructs. The observation that only the longest construct directs the expression of the reporter gene in a pattern that matches the developmental pattern of gp-1 mRNA also suggests that the information required for this expression is located in the gp-1 promoter sequence contained in that construct. If nucleotide sequences in the gp-1 mRNA 5′ untranslated region or 3′ untranslated region regulated mRNA levels, I would not have expected this result, since all of the constructs contained the complete gp-1 5′ untranslated mRNA sequence and all of them lacked any 3′ untranslated sequence from the gp-1 mRNA.

Materials and Methods

Materials

Radiolabeled ^{32}P-dATP was obtained from New England Nuclear. Ultrapure phenol was from Bethesda Research Laboratories. Nitrocellulose (Nitro Plus 2000) was obtained from MSI. Unless otherwise indicated all other reagents were obtained at the highest purity available from Sigma Chemicals.

Cell Culture

Vegetative cells of *Dictyostelium discoideum* strain AX3 were cultured in HL5 as described previously (Naranan et al., 1988; Rogers et al., 1992). *Dictyostelium* strain AX3K was used for the reporter gene experiments. The AX3K cells were kept at a density of less than 5.0 x 10^6 per ml, and were used at a density of 1.0 x 10^6 per ml in the transformation protocol. To obtain cells at various developmental stages, vegetative amoebae were washed free of nutrient media and plated

onto Gelman GN-6 cellulose acetate filters supported by Gelman absorbent pads saturated with MES-LPS buffer (7 mM N-morpholinoethanesulfonic acid, pH 6.5, 5 mM $MgSO_4$, 20 mM KCl).

cAMP response experiments

Dictyostelium amoebae were made aggregation competent by plating onto non-nutrient agar (1.5% Bacto agar buffered at pH 6.5 by 7 mM MES) and incubating for 12h at 7° C. The aggregation competent cells were then harvested, washed once in MES-LPS, and resuspended in 30 ml fresh MES-LPS at 0.5-1.0 x 10^6 cells per ml in 125 ml Erlenmeyer flasks. Cyclic AMP was added to a final concentration of 1.0 mM and the cells were shaken at 180 rpm on a Lab Line rotary shaker (Model 3250). Samples of the cell suspension were removed at 4h and/or 8h after cAMP addition for use in various assays. When 8h cells were used, the cells were pelleted by centrifugation after 4h shaking and resuspended in fresh MES-LPS with or without added cAMP. The analog experiments were carried out as above except that 2.0 mM caffeine was added to the MES-LPS prior to the addition of either 1.0 mM 2'd-cAMP or 1.0 mM Bt_2-cAMP

Glycogen phosphorylase activity assays and western analysis

Extracts from cells exposed to exogenous cAMP or isolated at different developmental stages were prepared by freeze-thaw lysis, and a 20 μl sample of each extract was added to 200 μl of a reaction mixture containing 50 mM imidazole (pH 6.8), 2.5 mg/ml glycogen, 2 mM K_2HPO_4, 5 mM $MgCl_2$, 0.5 mM NADP, 50 μg/ml glucose-1,6-bisphosphate, 0.3 units glucose-6-phosphate dehydrogenase, and 0.4 units phosphoglucomutase. The assay was carried out at 23° C. The amount of NADPH formed was followed on a strip chart recorder. A molar extinction coefficient of 6.2 x 10^3 was used to quantitate NADPH formation at 340 nm. One unit of activity is the amount of enzyme that catalyzes the synthesis of 1 μmol NADPH/min at 23° C.

For Western analysis, proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes using a Hoeffer transfer chamber and a power source (model TE51) set at 1.2 amps for 1h. The transfer buffer contained 192 mM glycine and 20% methanol in 25 mM Tris-HCl, pH 8.3. After transfer, the visualization of gp-1 protein was done as previously described (Naranan et al., 1988), using polyclonal anti-gp-1 antibodies, a protein A-peroxidase conjugate, and colorimetric antigen detection with 4-chloro-1-napthol and hydrogen peroxide.

RNA isolation and Northern analysis

Total RNA was prepared from *Dictyostelium* cells by the simultaneous lysis and extraction in diethylpyrocarbonate (DEPC), SDS, and phenol/chloroform as described previously (Nellen et al., 1987). Northern blotting was done by capillary action following gel electrophoresis of the RNA in formaldehyde containing 1.2% Seakem (FMC) agarose. RNA was quantified by optical density reading at 260 nm, and 10 μg of total RNA were loaded per lane. Duplicate samples were run and stained with ethidium bromide to ensure that rRNA bands were of equal intensity in all lanes. Hybridizations were performed under stringent conditions of 50% formamide, 6 x SSPE, 1 x Denhardt's solution, 0.1% SDS, and 200 μg salmon sperm DNA at 42° C. Purified cDNA fragments of either 1421 bp or 836 bp (Rogers et al., 1992) were labeled by random priming (Feinberg and Vogelstein, 1983) and used in the hybridizations. Hybridized filters were washed in 0.2 x SSC, 0.2% SDS at 42° C to 65° C. The hybridized filters were dried under vacuum and exposed to Kodak XAR-5 film at -70° C for autoradiography.

Construction of the gp-1 promoter deletions

A 2.2 kb RsaI genomic fragment containing approximately 200 nucleotides of transcribed sequence and 2.0 kb of 5′ sequence was isolated and inserted into the the vector pBluescript II

SK+. Deletions of the promoter region were prepared by digesting the vector at the SacI site, filling with deoxythioderivatives using the Klenow fragment of *E. coli* DNA polymerase I, and treating with Exo III and mung bean nucleases for various times. After ligation and transformation of bacteria, recombinant clones were selected and sequenced.

Ligation of the promoter deletions into the reporter gene vector

The promoter inserts were cut out of pBluescript with ClaI/BssHII double digests and were blunt ended by filling in overhangs with the Klenow enzyme. The vector SP60-luciferase-Δ20 (SP60-luc; see Haberstroh and Firtel, 1990; also Figure 20, Chapter 3, this dissertation) was the generous gift of Richard A. Firtel from the University of California, San Diego. The SP60 promoter was excised from this vector with a NheI/ClaI double digest, and the overhangs were filled in. The gp-1 promoter inserts contain the translation initiation codon, so ligations were constructed to allow the in-frame read through from the gp-1 initiation codon, through a small portion of pBluescript sequence, and into the luciferase structural gene. Ligations were done with T4 DNA ligase (BMB) according to the supplier's instructions. The molar ratio of vector to insert was 1:10, as quantified by visualization of samples of vector and insert DNA in ethidium bromide stained agarose gels. Restriction digests and sequencing were done to confirm the orientation and in frame status of the inserts.

Transformation

The promoter-reporter gene constructs were transfected into *Dictyostelium* amoebae of the strain AX3K by using a calcium-phosphate precipitation/glyceral shock protocol (Nellen et al., 1984). Transformants were selected by incubation for 6 days in HL5 containing 20 μg G418, resistance to which is encoded by the SP60-luc vector. After this selection, cells were harvested in PBS (10 mM KCl, 10 mM NaCl, 16 mM Na_2HPO_4, 34 mM KH_2PO_4, pH 6.5) and plated onto

DM agar plates (2% glucose, 1% Oxoid peptone, 1.5% Bacto agar, 3 mM Na_2HPO_4, 10 mM KH_2PO_4) containing 40-50 μg/ml G418 and an overlay of neomycin resistant *E. coli* B/r cells (a generous gift from Dennis Welker of Utah State University). Plaques appeared in 2-5 days. Cells from individual plaques were transferred to liquid HL5 + 5 μg/ml G418, and grown to a density of less than 1.0 x 10^6 cells per ml. These cells were then used in stage studies or in cAMP shaking experiments.

Luciferase assays

Following the cAMP response experiments or experiments analyzing developmental expression, extracts of transformants carrying the promoter-luciferase constructs were prepared by freeze-thaw lysis of the cells. The extracts were centrifuged for 10 min at 12,000 x g and 5 μl of the supernatant fluid was added to 100 μl of a reaction mix consisting of 0.54 M glycyl-glycine (pH 8.3), 4 mM $MgCl_2$, and 2.1 mM ATP. Luciferase units were quantified by a Berthold luminometer (Lumat LB9501) following the addition of 100 μl 0.6 mM luciferin. Specific activity is expressed as luciferase units per mg protein. Protein was measured by the method of Bradford (1976).

Chapter 5: Expression of the gp-1 and gp-2 Proteins in Pre-stalk and Pre-spore Cells

Abstract

In this chapter, I discuss the expression of the gp-1 and gp-2 proteins in the two differentiating cell types during *Dictyostelium* development. Microdissection experiments showed that the gp-1 protein was expressed primarily in pre-stalk cells of slugs and culminates. The gp-2 protein was expressed in both cell types when derived from microdissections of slugs and culminates. Experiments with a β-galactosidase reporter gene suggested that the expression of gp-2 in the two cell types is temporally regulated; in slugs and early culminates, the gp-2 promoter directed the pre-spore expression of the reporter gene, but the expression switched to pre-stalk cells in later culminates. I have also shown that it is possible to detect the gp-1 protein in tissue samples from a single *Dictyostelium* slug or culminate. In addition, I examined the expression of the gp-1 protein in the two cell types in mutants that lack the expression of gp-2. Results of this experimentation showed that in mutants in which gp-2 was not expressed, normal developmental regulation of gp-1 activity and cell-type expression was altered. These results suggest that normal expression of gp-2 may be

involved in the regulation of gp-1 activity as well as the pre-stalk enrichment of the gp-1 protein seen during *Dictyostelium* development.

Introduction

The mechanisms involved in regulating developmentally expressed and tissue specific genes comprise some of the most important molecular circuitry in eukaryotic systems. Understanding these regulatory mechanisms will shed light not only on how multicellular organisms develop from a single fertilized egg, but also on developmental anomalies such as cancer, birth defects, and premature aging.

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study the molecular events involved in mediating developmentally regulated and tissue specific gene expression (Loomis, 1982). This organism proceeds through a short, well documented developmental cycle in which one initial cell type (vegetatively growing, amoeboid cells) differentiates into two cell types (stalk and spore cells, derived from pre-stalk and pre-spore cells). Thus, in *Dictyostelium*, vegetative growth and cellular differentiation are separated (Sussman and Brackenbury, 1976), which greatly simplifies the analysis of developmentally significant events. Additionally, this organism is readily amenable to the genetic manipulations required for modern molecular biology procedures. Both differentiated cell types in *Dictyostelium* require the synthesis of complex structural polycaccharrides such as cellulose. The glucose monomers required for cellulose synthesis are derived from glycogen degradation. Thus, regulation of glycogen degradation is likely to be of enormous developmental significance in this organism. Glycogen degradation is catalyzed by glycogen phosphorylase, and two glycogen phosphorylase activities have been identified in *Dictyostelium*; these two activities have been shown to result from two related but distinct glycogen phosphorylase genes (Rogers et al., 1992; Rutherford et al., 1992; Chapter 2, this dissertation). These two genes, called gp-1 and gp-2, are developmentally regulated and are regulated by cAMP. Since glycogen degradation is normally required for both pre-stalk and pre-spore differentiation, the

cell-type-specific expression of these genes is also likely to be of considerable developmental importance. In this chapter, I examine the cell-type localization of the gp-1 and gp-2 proteins. The information in this chapter is, at this point, neither conclusive enough nor comprehensive enough to be submitted for publication, but is intended to provide a useful framework for future investigation.

Results

Analysis of the expression of gp-1 and gp-2 in pre-stalk and pre-spore cells

A number of techniques are currently available to isolate pre-stalk and pre-spore cells from *Dictyostelium* slugs and culminates and analyze cell-type-specific gene expression. Perhaps the most widely used technique involves density gradient centrifugation. The two cell types have been shown to exhibit different densities under specific conditions and can often be separated with isopycnic Percoll gradients (Tsang and Bradbury, 1981; Ratner and Borth, 1983). My attempts to isolate the two cell types from dissociated multicellular individuals using this approach were unsuccessful. Attempts to separate pre-stalk and pre-spore cells by using automated flow cytometry were also unsuccessful. I then turned to microdissection of lyophilized slugs and culminates. During the later stages of *Dictyostelium* development, a spatial separation of pre-stalk and pre-spore cells is observed (Figure 28). This separation makes it possible to isolate relatively pure samples of pre-stalk and pre-spore tissue by simply removing a specific area of any given individual. There are at least three obvious drawbacks to this approach. First, microdissection is time consuming and tedious. Second, only small quantities of tissue can be obtained. Finally, lyophilization makes it impossible to analyze the cell types for the presence of specific mRNAs. Nevertheless, this approach does allow the examination of proteins in the two cell types, and I was able to show that the dissection of

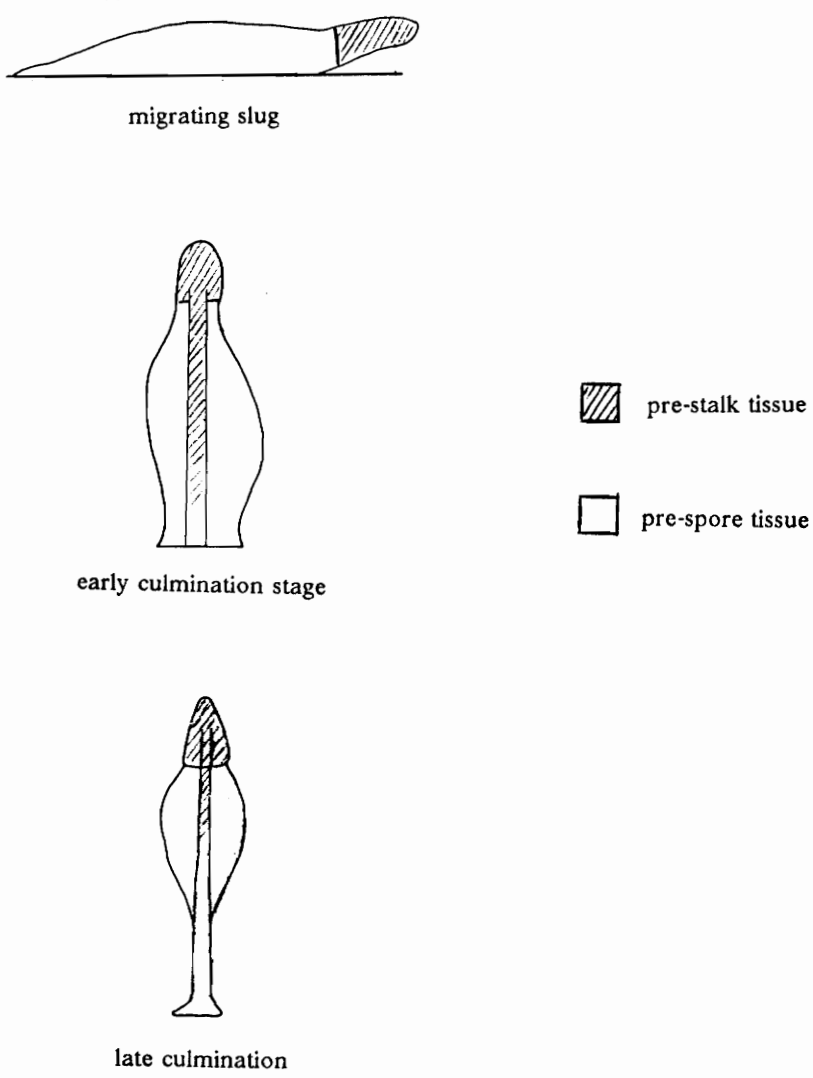

Figure 28. Spatial localization of pre-stalk and pre-spore cells during *Dictyostelium* development: The localization of the two cell types allows the preparation of relatively pure pre-stalk and pre-spore samples by microdissection. The diagrams are approximately 100x actual size.

approximately 200 individuals would give enough protein to be detected on Western blots using conventional colorimetric antigen detection.

In an attempt to increase the sensitivity of antigen detection in my Western blotting experiments, I utilized Amersham's Enhanced Chemiluminescence (ECL) system and found that it was indeed more sensitive than colorimetric detection, as claimed by the manufacturer. In the ECL system, the electron donor for the coupled peroxidase luminesces when oxidized; the resultant light is used to expose X-ray film, much like radioactive emissions expose film during autoradiography.

Results of microdissections and ECL antigen detection using 50 slugs and culminates are shown in Figure 29. The lyophilized pre-stalk and pre-spore tissue was resuspended directly in SDS-PAGE buffer, and electrophoresis, Western transfer, and detection were carried out as described in Materials and Methods. Figure 29A shows that the gp-1 protein is highly enriched in pre-stalk cells (compare Lanes St and Sp). Figure 29B shows that the gp-1 protein is present in both cell types, and is perhaps expressed at a higher level in pre-spore cells (Lanes St and Sp).

Analysis of gp-1 protein localization by microdissection of single multicellular individuals

While the results shown in Figure 29 were obtained with ECL antigen detection, the protocol steps preceeding detection were carried out by performing standard Western blotting techniques. That is, antibody incubations, etc., were done in 50-100 ml volumes of buffer. I hypothesized that if the volumes in these steps could be decreased, the antibody concentration could be increased (without requiring large volumes of antiserum), and the sensitivity of the system could be even further increased, perhaps to the point of detecting protein in tissue samples from only one or a few multicellular individuals. I was able to greatly decrease the buffer volume by carrying out the antibody incubation in small plastic bags. I constructed the small bags from larger plastic bags, that we normally use for Southern and Northern hybridizations, by simply cutting the plastic to the appropriate size (usually about 5-7 cm^2) and using a heat sealer to melt the loose ends

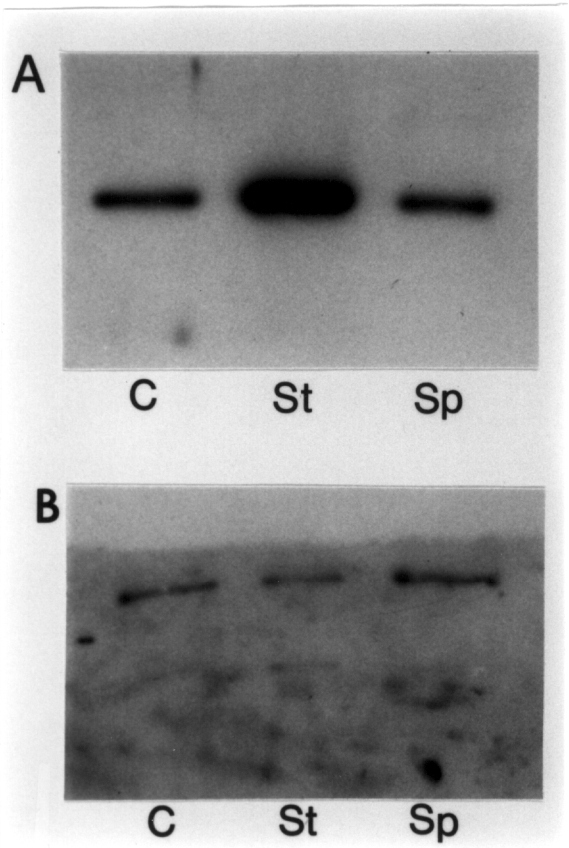

Figure 29. Expression of the gp-1 and gp-2 proteins in pre-stalk and pre-spore cells: Approximately 50 slugs and culminates were microdissected into pre-stalk and pre-spore tissue samples. The samples were resuspended in SDS-PAGE buffer, carried through SDS-PAGE, and transferred to nitrocellulose. Western blotting was carried out as described in Materials and Methods. Antigen detection was done using ECL. Figure 29A represents anti-gp-1 antibody staining, while Figure 29A represents anti-gp-2 staining.

together. Bags of this size allowed antibody incubation in volumes of 5 ml or less. Thus, without increasing the volume of antiserum needed in each experiment I could increase the effective antibody concentration by at least 10 to 20 fold over the levels that I had been using. In order to use these small bags, and what I refer to as "mini Westerns", I included Bio Rad prestained molecular weight markers in the SDS-PAGE and, after the transfer, cut out a small area (less than 5 cm^2) of nitrocellulose containing the glycogen phosphorylase molecular weight range.

To examine cell type expression of phosphorylase in single individuals with the "mini Westerns" described above, samples of pre-stalk and pre-spore tissue were removed from a slug or culminate. The tissue from each cell type was quantified by using a quartz fiber microbalance, and was directly resuspended in SDS-PAGE buffer. Figure 30 shows the results of two replications of this experimentation in which the expression of gp-1 was examined. Lane C is a control; in this lane, an entire individual was resuspended and run on the gel. Lane St represents a sample of pre-stalk tissue, while Lane Sp represents an equivalent amount of pre-spore tissue. The "mini Western" technique is clearly sensitive enough to detect protein from samples of a single individual. These blots also showed that the gp-1 protein is highly enriched in pre-stalk cells, corroborating the results obtained in Figure 29A. In many other replications (not shown) I always observed enrichment of gp-1 protein in pre-stalk cells. Attempts to analyze the expression of gp-2 in single individuals were not successful. The problem in these experiments appeared to be the anti-gp-2 antiserum, which in the "mini Western" procedure consistently produced high backgrounds that made it impossible to observe any protein bands (note that even in Figure 29B, the anti-gp-2 blot shows much higher background than the anti-gp-1 blot). Attempts to lower the background by altering the concentration of the blocker, the substance used as the blocker, and the concentration of Tween 20 (see Materials and Methods) were all unsuccessful.

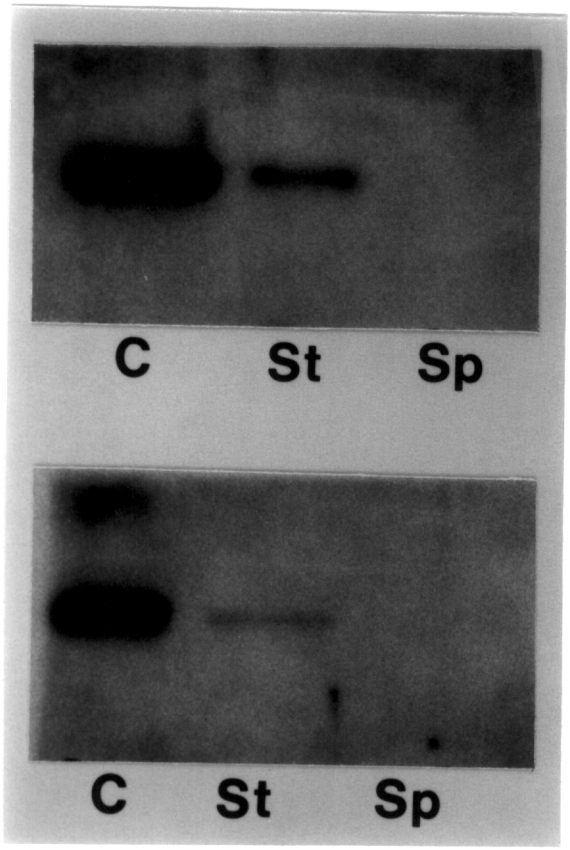

Figure 30. Analysis of gp-1 protein expression in pre-stalk and pre-spore tissue derived from a single *Dictyostelium* slug or culminate: Prestalk and pre-spore tissue was excised from a single lyophilized slug or culminate, quantified by using a quartz fiber microbalance, and resuspended in SDS-PAGE buffer. After SDS-PAGE and transfer to nitrocellulose, a small piece of nitrocellulose containing the gp-1 molecular weight range was cut and used in the "mini Western" procedure to visualize the gp-1 protein. The two blots represent tissue from two different microdissected individuals. Abbreviations are: C, control lane containing an entire slug or culminate; St, pre-stalk tissue; Sp, pre-spore tissue.

Analysis of gp-2 expression in pre-stalk and pre-spore cells using a β-galactosidase reporter gene

I attempted to analyze the cell-type-specific expression of gp-2 in more detail using a reporter gene. The reporter gene in these experiments was the *E. coli* lac-z gene, encoding β-galactosidase. This gene has been engineered into a vector called pDdGal-16, just downstream of a multiple cloning site. The vector pDdGal-16 was designed and constructed by Adrian Harwood of the Imperial Cancer Research Fund in England, and was a generous gift from Richard Kessin of Columbia University. I ligated the gp-2 promoter fragment gp-2-d1.9 (Chapter 3, Figure 18) into this vector such that the expression of the B-galactosidase gene was driven by the gp-2 promoter. Thus, any cell-type-specific expression directed by the gp-2 promoter should be reflected in the expression of the reporter gene. Expression of the reporter gene was assayed by using X-Gal staining as detailed in Materials and Methods. Results of this experimentation showed that prior to the mid-culmination stage, β-galactosidase was expressed preferentially in pre-spore cells; however, after this stage, expression was observed in pre-stalk cells and in the stalk tube (data not shown). Identical results were also obtained by others in our laboratory using different gp-2 promoter fragments and lac-z constructs (Yizhong Yin, personal communication). This apparent temporally regulated switch in cell-type specific expression has not been reported for any other *Dictyostelium* genes. However, considering the importance of glycogen degradation in *Dictyostelium* development, and the fact that glycogen-derived end products are required in both cell types, it is not inconceivable that such regulation would be needed. These reporter gene results are consistent with the Western blot results shown in Figure 29B, which showed gp-2 protein in both cell types; recall that microdissected slugs and culminates were used to generate that Western blot.

Alterations in the cell-type-specific expression of gp-1 in mutants which do not express gp-2

A central issue regarding gp-1 regulation and expression has always been the functional role, if any exists, for the presence of inactive gp-1 protein during the late developmental stages. One hypothesis, that the gp-1 protein was sequestered in spores for use upon germination, is apparently ruled out by the cell-type studies described above. In an effort to better understand the role of gp-1 in *Dictyostelium* development, transformed mutants were constructed such that the expression of gp-1 or gp-2 was abolished. These mutants were generated by transforming amoebae with plasmids carrying fragments of the gp-1 or gp-2 gene; disruption and inactivation of the native genes was via homologous recombination or gene conversion (Rogers et al., manuscript in preparation).

Figure 31 shows glycogen phosphorylase activity in extracts prepared from these transformants at different developmental stages. Figure 31A shows control extracts from cells transformed only with vector DNA (no gp-1 or gp-2 inserts). The developmental activities of gp-1 and gp-2 exhibit the normal regulatory pattern in these cells. That is, gp-1 activity is predominant early in development and then decreases at later developmental stages as gp-2 activity peaks. Northern blots and Western blots showed that the gp-1 and gp-2 mRNAs and proteins also exhibited the pattern of regulation seen in normal, non-transformed cells (data not shown). Figure 31B shows developmental phosphorylase activities in cells transformed with plasmids carrying a gp-1 gene fragment. No gp-1 activity was detected at any developmental stage in these transformants; however, the usual, late developmental increase in gp-2 activity was observed and these cells developed normally. Northern analysis showed that gp-1 mRNA expression in these cells was also disrupted and no gp-1 protein could be detected on Western blots (data not shown). Figure 31C shows developmental phosphorylase activities in cells transformed with plasmids carrying a gp-2 gene fragment. In these cells, no gp-2 activity was detected at any developmental stage; additionally, expression of gp-2 protein and mRNA was disrupted. Interestingly, these transformants showed high gp-1 activity throughout development and did develop normally. These

results suggest that the expression of gp-2 may be required for normal regulation of gp-1 activity during development.

Figure 32 shows the expression of gp-1 protein in pre-stalk and pre-spore cells of the control, gp-1$^-$, and gp-2$^-$ transformants. The westerns in this figure were all prepared from microdissections of single slugs or early culminates. Figure 32A shows gp-1 expression in control cells transfected with vector DNA only. In these cells gp-1 is expressed preferentially in pre-stalk tissue, a result consistent with the results described earlier in this chapter. Figure 32B shows tissue from gp-1$^-$ cells; no gp-1 protein was detected in either pre-stalk or pre-spore cells from these transformants. Figures 32C and 32D show tissue samples from gp-2$^-$ transformants. Note that in these individuals the expression of the gp-1 protein was altered: the gp-1 protein was detected in both pre-stalk and pre-spore cells. This result suggests that the expression of gp-2 may not only play a role in regulating gp-1 activity, but may also be involved in the pre-stalk enrichment of the gp-1 protein in normal development.

Discussion

In this chapter I have examined the expression of the gp-1 and gp-2 proteins in pre-stalk and pre-spore cells of *Dictyostelium*. I showed that during normal development the gp-1 protein is highly enriched in pre-stalk cells; microdissections of slugs and culminates suggested that the gp-2 protein is present in both cell types. I was also able to show that a "mini Western" technique increased the sensitivity of gp-1 antigen detection and allowed the examination of gp-1 protein expression in pre-stalk and pre-spore tissue derived from a single slug or culminate. Unfortunately, this technique could not be successfully used to examine gp-2 expression due to background problems observed when using the anti-gp-2 antiserum. Examination of gp-2 expression with a β-galactosidase reporter gene showed that the gp-2 promoter directed the pre-spore specific expression of the reporter gene early in development, and then switched to pre-stalk enriched expression at about mid-culmination stage.

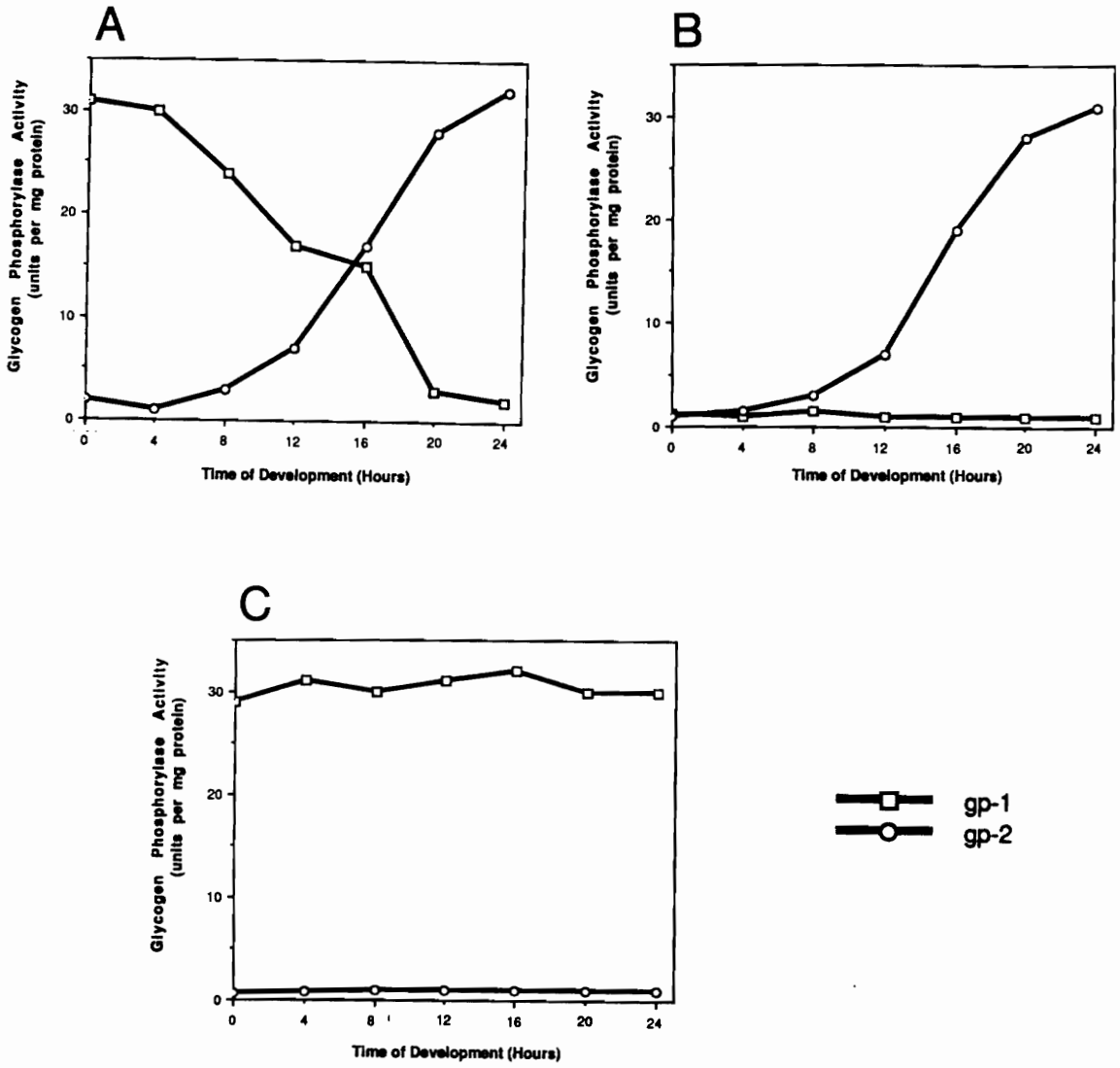

Figure 31. Glycogen phosphorylase activity during the development of transformants lacking gp-1 or gp-2: Mutants lacking gp-1 or gp-2 expression were constructed by transfecting amoebae with plasmids carrying fragments of either the gp-1 or gp-2 genes (Rogers et al., manuscript in preparation). Homologous recombination or gene conversion eliminated the expression of the native genes. The mutants were removed from nutrient medium, plated onto buffered filters, and allowed to develop. At the indicated stages, cells were harvested, lysed, and the extracts were assayed for gp-1 and gp-2 activity. (A) Assays of extracts from cells transfected with vector DNA only. (B) Assays of extracts from cells transformed with a plasmid carrying a fragment of the gp-1 gene. (C) Assays of extracts from cells transformed with a plasmid carrying a fragment of the gp-2 gene.

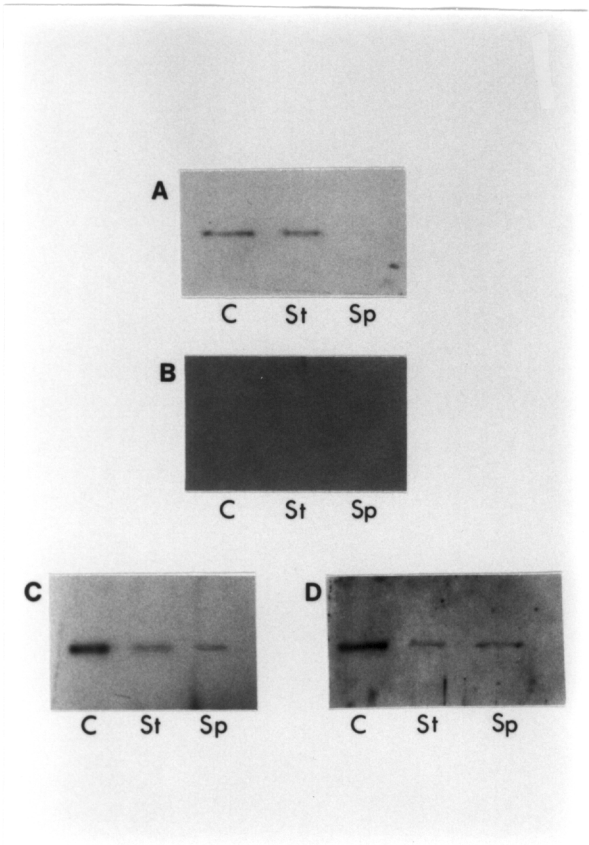

Figure 32. Expression of the gp-1 protein in pre-stalk and pre-spore cells of transformants: Mutants lacking the expression of gp-1 or gp-2 were constructed through homologous recombination or gene conversion. These transformants were removed from nutrient culture and plated onto filters buffered by MES-LPS. Cells were allowed to develop to the migrating slug or early culmination stage, and were then lyophilized. Samples of pre-stalk and pre-spore tissue were prepared by microdissection and analyzed for gp-1 or gp-2 protein expression by Western blotting and ECL detection. (A) Pre-stalk and pre-spore tissue from transformants carrying vector DNA only. (B) Tissue from transformants lacking gp-1 expression. (C) and (D) represent pre-stalk and pre-spore tissue samples from transformants lacking gp-2 expression. In (A) through (D) the abbreviations are: C, control lane with an intact slug or culminate; St, pre-stalk tissue; Sp, prespore tissue.

These results answer some questions about the cell-type-specific expression of the gp-1 and gp-2 proteins, but many aspects of this expression remain unresolved. For example, the functional significance of the pre-stalk enrichment of the inactive gp-1 protein is not clear. The mechanism through which this enrichment occurs is also unknown at the present time. The late developmental increase in the gp-1 mRNA (Chapter 2 and Chapter 4, this dissertation; Rogers et al., 1992) is also currently unexplained, and, unfortunately, could not be analyzed with the techniques that I used to examine the expression of the gp-1 protein. It may be that the gp-1 mRNA expressed late in development is sequestered in spores for immediate translation upon germination, but this hypothesis has not been directly tested. Examining this possibility would require isolation of the two cell types. Cell separation by Percoll gradients or by flow cytometry have proven to be unsuccessful. The apparent temporally-regulated switch in the cell-type-specific expression of gp-2 is novel. This phenomenon has not been reported for any other *Dictyoselium* gene that has been examined. Since this switch in cell-type expression was observed with several different promoter-reporter gene constructs by two different researchers, it likely represents the *in vivo* pattern of expression. The switch may be regulated at the transcriptional level or by sequences present in the 5′ untranslated region of the gp-2 mRNA, since all of the constructs used in these experiments contained the complete gp-2 5′ untranslated mRNA region.

I also examined the developmental regulation of gp-1 enzyme activity and the cell-type-specific expression of the gp-1 protein in transformed mutants which did not express gp-2. In these mutants, no developmental decrease in gp-1 activity was observed, and the apparently active gp-1 protein was observed in both pre-stalk and pre-spore cells. Some coordinate regulation may be involved in the normal expression of the two glycogen phosphorylases in *Dictyostelium.* For example, in the gp-2$^-$ transformants, gp-1 activity does not decrease during development, suggesting that the normal expression of gp-2 may regulate the activity of gp-1. Also, the normal expression of gp-2 may be involved in the pre-stalk enrichment of the inactive gp-1 protein. The mechanism(s) of this coordinate regulation are currently unknown. The regulation of gp-1 activity is almost certainly a post-translational event, but how gp-2 expression may mediate this process is not known. The alterations in the cell-type-specific expression of the gp-1 protein in the gp-2 transformants

may be transcriptional or post-transcriptional. If this is a post-transcriptional event, perhaps the inactivation of gp-1 during normal development, which appears to involve gp-2 expression, somehow leads to the pre-stalk specific sorting of the inactive gp-1 protein; in the gp-2$^-$cells this obviously could not occur. Clearly, there is still much to learn about regulation of the two glycogen phosphorylases that are expressed during the developmental cycle of *Dictyostelium.*

Materials and Methods

Materials

The ECL detection system was obtained from Amersham. Pre-stained molecular weight markers for SDS-PAGE were from Bio Rad. Nitrocellulose was obtained from MSI. Unless otherwise indicated, all other reagents were obtained at the highest purity available from Sigma Chemicals.

Cell culture and development

Cells of *Dictyostelium* strain AX3 were grown in HL5 medium as previously described (Naranan et al., 1988; Rogers et al., 1992). To obtain cells at different developmental stages, vegetative amoebae were washed removed from HL5, washed, and plated onto Gelman GN6 cellulose acetate filters supported by Gelman absorbent pads saturated with MES-LPS buffer (7 mM N-morpholinoethanesulfonic acid, pH 6.5, 5 mM $MgSO_4$, 20 mM KCl). *Dictyostelium* strain AX3K was used for transformations.

Glycogen phosphorylase activity assays and Western blotting

Glycogen phosphorylase activity was assayed as previously described (Rutherford and Cloutier, 1986; Naranan et al., 1988; Chapters 2, 3, and 4, this dissertation). For Western blotting, proteins were resolved by SDS-PAGE and were electrophoretically transferred to nitrocellulose. Conventional colorimetric antigen detection was done as described previously (Naranan et al., 1988). For ECL antigen detection, the nitrocellulose was blocked by incubating for 1h at room temperature in TBS (50 mM Tris-HCl, pH 7.6, 200 mM NaCl) containing 5% dried milk and 0.1% Tween 20. The filter was then washed a total of five times in TBS containing 0.1% Tween 20 (2 x 2 min, 1 x 15 min, 2 x 5 min). Antibody incubation was overnight at room temperature in TBS containing 5% dried milk and 0.1% Tween 20; 100 μl of antiserum were used per 50-100 ml of buffer. After the antibody incubation, the filter was washed five times as described above, except that the concentration of Tween 20 was increased to 0.3%. The filter was then incubated for 1h at room temperature in TBS containing 5% milk, 0.1% Tween 20, and 1 μg/ml of a protein-A-peroxidase conjugate. Following this incubation the filter was washed five times again, as described above, except that the concentration of Tween 20 was increased to 0.5%. Detection was done as described by the manufacturer, using reagents provided in the ECL kit. Film (Kodak XAR-5) was exposed at room temperature for 5 sec to 30 min. The film was developed using Kodak GBX developer and fixer.

For "mini Westerns", Bio Rad prestained molecular weight markers were separated along with the tissue samples during SDS-PAGE, and the area of nitrocellulose that contained the phosphorylase molecular weight range was excised from the nitrocellulose filter after the Western transfer. The blotting procedure for the "mini Westerns" was essentially identical to that described above, except that the antibody incubation was done in small plastic bags in buffer volumes of 5 ml or less.

Microdissections

Gelman filters containing developing slugs and culminates were removed from the supporting pads and placed into lyophilizing jars. The jars were placed at -80° C for at least 1h and were then transferred, while packed in dry ice, to the lyphilizer. Lyophilization was carried out overnight. Samples of prestalk or prespore tissue were excised from individual lyophilized slugs or culminates using a microscalpel. The tissue samples were quantified by placing onto a quartz fiber microbalance and measuring the degree of deflection with an occular micrometer. Samples of lyophilized tissue were resuspended directly in SDS-PAGE run buffer.

Construction of mutants lacking the expression of gp-1 or gp-2

Fragments of the gp-1 or gp-2 gene were cloned into the vector pDdNeoII. These plasmids were transfected into amoebae of *Dictyostelium* strain AX3K using a calcium-phosphate/glycerol shock protocol (Nellen et al., 1984). Transformants were selected by incubating in G418, resistance to which is encoded by pDdNeo. After selection, transformants were plated as described (Chapters 3 and 4, this dissertation) and individual clones were selected. Southern analysis was used to confirm the presence of the inserts and the disruption of the native gp-1 or gp-2 genes (Rogers et al., manuscript in preparation).

ß-galactosidase reporter gene experiments

The gp-2 promoter fragment gp-2-d1.9 was blunt ended and ligated into blunt ended restriction digested pDdGal-16 such that the lac-z gene was under the transcriptional control of the gp-2 promoter. This construct was transfected into amoebae of *Dictyostelium* strain AX3K using the transformation protocol described above (Nellen et al., 1984). Transformants were selected and grown in HL5 containing 5 μg/ml G418, then were washed free of HL5 and plated onto filters.

Filters containing cells at various developmental stages were stained for β-galactosidase activity as follows. The cells were permeabilized in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) containing 0.1% NP40 for 20 min at room temperature. Cells were then washed twice with Z buffer and fixed by incubating for 10 min at room temperature in Z buffer containing 0.5% glutaraldehyde. The filters were washed two more times with Z buffer and then stained with an X-Gal solution containing 7.5 ml Z buffer, 0.5 ml 100 mM $K_3[Fe(CN)_6]$, 0.5 ml 100 mM $K_4[Fe(CN)_6]$, 0.5 ml 20 mM X-Gal (in N,N-dimethylformamide), and 1.0 ml 10 mM EGTA. Staining was usually visible within 10 min and was usually allowed to continue for approximately 30 min.

Chapter 6: Summary

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study eukaryotic development and cell differentiation. A crucial developmental event in *Dictyostelium* is glycogen degradation; the degradation of glycogen provides glucose precursors used to synthesize components of differentiated cells. Glycogen degradation is catalyzed by glycogen phosphorylase and two developmentally regulated glycogen phosphorylase activities have been identified in this organism. The activity of gp-1 is dependent upon 5′ AMP as a positive allosteric modifier, is predominant in vegetative amoebae and in the very early stages of development, and then decreases as development progresses. gp-1 activity is associated with a 92 kd protein. The activity of gp-2 is associated with a 104 kd protein. gp-2 activity is independent of 5′ AMP, is undetectable early in development, but is the predominant form during the late developmental stages. I have presented the results of experimentation examining the regulation of these two glycogen phosphorylases.

I have presented conclusive evidence, in the form of direct nucleotide and amino acid sequences, that the gp-1 and gp-2 activities are associated with two unique proteins which are the products of two distinct, but related, genes (Chapter 2). This finding came was surprising, since in virtually every other eukaryotic system where 5′ AMP dependent and 5′ AMP independent glycogen phosphorylase activities are observed, the activities reside in the same protein molecule and are regulated by reversible phosphorylation. However, the discovery that two genes exist for

gp-1 and gp-2 was consistent with earlier work (presented in Chapter 1) which showed that these two glycogen phosphorylases were not immunologically cross-reactive, showed different peptide maps, and appeared to be synthesized from distinct mRNA molecules. Both gp-1 and gp-2 were similar in several respects to glycogen phosphorylases from other organisms.

I also examined the developmental expression of the gp-1 and gp-2 proteins and mRNAs. The gp-2 protein and mRNA accumulated during development, and closely matched the increase in gp-2 activity observed during development. The increase in the gp-2 mRNA is probably regulated at the transcriptional level, although this has not been confirmed by experimentation. The gp-2 protein may be initially synthesized as an inactive precursor that is proteolytically cleaved to yield active gp-2 enzyme. The immunoprecipitation of a 110-112 kd gp-2 protein from *in vitro* translation reactions lends supports this hypothesis, as does the observation of a 110-112 kd gp-2 protein in extracts from developmental stages which show gp-2 mRNA but do not exhibit gp-2 enzyme activity; indeed, a 104 kd gp-2 protein was only detected on Western blots when gp-2 activity could to be detected. The presence of an unusual number of glutamine residues near the N terminus of the gp-2 protein molecule may serve as a signal for proteolytic cleavage, although this is speculative. The developmental regulation of gp-1 is considerably more complex than the regulation of gp-2. During development, gp-1 protein levels remain essentially constant while gp-1 activity decreases. The gp-1 mRNA exhibits a developmental pattern in which high levels are observed in vegetative cells and late in development with markedly lower levels observed in the intervening developmental stages. The lack of direct correspondence between gp-1 enzyme activity, gp-1 protein levels, and gp-1 mRNA levels suggests that regulation of gp-1 occurs at multiple levels, probably at both the transcriptional and post-transcriptional steps. However, the specific details of the developmental regulation of gp-1 have yet to be elucidated.

I have also examined the regulation of gp-1 and gp-2 by cAMP (Chapters 3 and 4). Exogenous cAMP caused induction of the gp-2 enzyme activity, protein, and mRNA. This induction is probably regulated at the level of transcription, and appears to occur independent of intracellular cAMP signaling. Repeated T/A rich DNA sequence elements located in the gp-2 promoter appear to be required for cAMP regulation. The involvement of T/A rich sequences in

cAMP responsiveness is unlike most cAMP regulated *Dictyostelium* genes, although the *pst-cath* gene, as described by Datta and Firtel (1988), also appears to require T/A rich sequence elements for normal cAMP responsiveness The T/A rich sequences of the gp-2 promoter are also very dissimilar to cAMP response elements identified in other eukaryotic genes (Wynshaw-Boris et al., 1984; Nagamine and Reich, 1985; Deutsch et al., 1988). It will be interesting to see if specific DNA binding proteins can be shown to bind to these sequences in a cAMP dependent fashion. Curiously, these sequences do not appear to be required for developmental expression of gp-2. Exogenous cAMP can also regulate gp-1; cAMP causes a decrease in gp-1 enzyme activity and enhances the level of gp-1 mRNA, results which reflect the normal developmental changes in these parameters. The cAMP regulation of gp-1 appears to require intracellular signalling. Results from reporter gene experiments suggested that the alterations in gp-1 levels during development and in response to cAMP are mediated through changes in transcription. Reporter gene studies also suggested that DNA sequence elements at least 1000 nucleotides away from the transcriptional start site are involved in the cAMP responsiveness of the gp-1 gene. It will be interesting to see if sequence elements that far from the cap site show any sequence similarity to other sequences believed to be involved in cAMP mediated gene regulation in *Dictyostelium.* It will also be interesting to see if any sequence similarity exists between elements of the gp-1 promoter and elements in other *Dictyostelium* genes expressed in vegetative cells and/or early in development (Knecht et al., 1986; Cohen et al., 1986).

I also examined the cell-type-specific expression of the gp-1 and gp-2 proteins by carrying out microdissections of slugs or culminates and examining the resultant tissue samples for the presence of the gp-1 and gp-2 proteins with Western blots. I showed that the gp-1 protein was highly enriched in pre-stalk cells, while the gp-2 protein appeared to be expressed in both cell types. Reporter gene experiments showed that the cell type specific expression of the gp-2 protein was temporally regulated; at earlier developmental stages, the gp-2 promoter directed pre-spore expression of the reporter gene, but later in development pre-stalk expression was observed. Using a "mini Western" procedure I showed that the gp-1 protein could be detected in samples of tissue from a single slug or culminate. I also presented results which showed that in transformed mutants

which lack gp-2 expression, the normal developmental regulation of gp-1 is altered; in these cells, gp-1 activity remains high during development and the active gp-1 protein is observed in both cell types. These results suggest that some coordinate regulation of gp-1 and gp-2 takes place during normal development. Much remains to be discovered about this regulation, however.

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Vita

CURRICULUM VITA

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PERSONAL DATA

Birth Date: September 1, 1962
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EDUCATIONAL BACKGROUND

B.A. Biology, 1984
Thiel College, Greenville, PA

M.S. Microbiology, 1988
Virginia Polytechnic Institute and State University
Blacksburg, VA

Candidate--Ph.D. Program, Microbiology (from May, 1988)
Virginia Polytechnic Institute and State University

ACADEMIC HONORS AND PROFESSIONAL SOCIETIES

Dean's List for 8 Semesters at Thiel College
Lambda Sigma Honor Society
Alpha Chi National Honor Society
Beta Beta Beta Biological Honor Society
Member, American Association for the Advancement of Science
Member, Virginia Academy of Science

RESEARCH EXPERIENCE

September 1983 to May 1984: Independent Research Project
Biology Department, Thiel College, Greenville, PA
Research Interest: The effects of elevated phosphate levels on the photosynthetic activity of *Scenedesmus* sp. algae.

September 1985 to present: Thesis and Dissertation Research
Research Interests: Molecular biology of cell differentiation in *Dictyostelium discoideum*; developmental regulation of glycogen phosphorylase genes in *D. discoideum.*

TEACHING EXPERIENCE

Laboratory Assistant at Thiel College in:
Principles of Biology Laboratory (freshman science majors)
Cell Biology Laboratory
Genetics Laboratory
Microbiology Laboratory

Graduate Teaching Assistant at Virginia Tech in:
General Biology Laboratory (freshman non-science majors)
Principles of Biology Laboratory (freshman science majors)
Embryology Laboratory
General Microbiology Laboratory

PUBLICATIONS

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Sucic, J.F., S. Luo, B.D. Williamson, and C.L. Rutherford.
"Developmental and Cyclic AMP Mediated Regulation of Glycogen Phosphorylase-1 in *Dictyostelium*"

Rogers, P.V., J.F. Sucic, Y. Yin, and C.L. Rutheford.
"Analysis of *Dictyostelium* Glycogen Phosphorylase Mutants:
Evidence for Coordinated Regulation of the gp-1 and gp-2 Gene Products"

ABSTRACTS

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Sucic, J.F., V. Naranan, D.A. Brickey, and C.L. Rutherford. 1987.
Graduate Research Symposium, Virginia Polytechnic Institute and State University.
"The Relationship Between Two Forms of Glycogen Phosphorylase in *Dictyostelium discoideum*"

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Y. Yin, R.B. Peery, and C.L. Rutherford. 1991.
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Rutherford, C.L., S. Luo, J.F. Sucic, Y. Yin,
P.V. Rogers, O. Selmin, and R.B. Peery. 1991.
Proceedings of International *Dictyostelium* Conference.
"Glycogen Phosphorylase 1 and 2 Gene Expression in *Dictyostelium discoideum*"

PRESENTATIONS

Brickey, D.A., J.F. Sucic, V. Naranan, and C.L. Rutherford.
American Society of Biological Chemists National Meeting.
June, 1987--Philadelphia, PA; Poster Session
"The Relationship Between Two Forms of Glycogen Phosphorylase in *Dictyostelium discoideum*"
Presented by D.A. Brickey and J.F. Sucic.

Sucic, J.F., V. Naranan, D.A. Brickey, and C.L. Rutherford.
Graduate Research Symposium, Virginia Polytechnic Institute and State University.
November, 1987--Blacksburg, VA; Poster Session
"The Relationship Between Two Forms of Glycogen Phosphorylase in *Dictyostelium discoideum*" Presented by J.F. Sucic.

Rutherford, C.L., V. Naranan, D.A. Brickey, J.F. Sucic, P.V. Rogers, and O. Selmin.
International Symposium; Molecular Biology of *Dictyostelium* Development.
November, 1987--Airlie, VA; Poster Session
"Glycogen Phosphorylase in *Dictyostelium*"
Presented by J.F. Sucic and O. Selmin.

Rogers, P.V., J.F. Sucic, S. Luo, and C.L. Rutherford.
American Society of Biological Chemistry and Molecular Biology National Meeting.
January, 1989--San Francisco, CA; Poster Session
"Glycogen Phosphorylase in *Dictyostelium discoideum*"
Presented by P.V. Rogers and J.F. Sucic.

Sucic, J.F., S. Luo, P.V. Rogers, O. Selmin, Y. Yin,
R.B. Peery, K.P. Lindgren, and C.L. Rutherford.
Virginia Academy of Science Annual Meeting.
May, 1991--Blacksburg, VA
"Regulation of Glycogen Phosphorylase Genes in *Dictyostelium discoideum*"
Talk presented by J.F. Sucic.

Sucic, J.F., K.P. Lindgren, S. Luo, P.V. Rogers,
O. Selmin, Y. Yin, R.B. Peery, and C.L. Rutherford.
Chinese Agriculture Association of Students and Scholars; Third Conference.
June, 1991--Blacksburg, VA
"Glycogen Phosphorylase Genes in *Dictyostelium discoideum* Are Regulated by Cyclic-AMP"
Talk presented by J.F. Sucic.

Sucic, J.F., S. Luo, P.V. Rogers, Y. Yin, K.P. Lindgren, and C.L. Rutherford.
International *Dictyostelium* Conference.
August, 1991--Vancouver, British Columbia, Canada; Poster Session
"The Regulation of Glycogen Phosphorylase Genes in *Dictyostelium discoideum*"
Presented by J.F. Sucic and S. Luo.

GRANTS RECEIVED

Sigma Xi Research Grant, 1988
"The Relationship Between The Two Forms of Glycogen Phosphorylase in *Dictyostelium discoideum*" Award (with departmental matching funds): $700.00

Graduate Research Development Project Award, 1989
"Cyclic AMP Mediated Regulation Of Glycogen Phosphorylase 2 in *Dictyostelium discoideum*" Award (with departmental matching funds): $1000.00