Identification of Regulatory Binding Sites and Corresponding Transcription Factors Involved in the Developmental Control of *5'-nucleotidase* Expression in *Dictyostelium discoideum*

by

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Abstract

Gene regulation is a critical aspect of normal development, energy conservation, metabolic control, and responses to environmental cues, diseases and pathogens in eukaryotic organisms. In order to appropriately respond to environmental changes and advance through the life cycle, an organism must manage the expression levels of a large number of genes by utilizing available gene regulation mechanisms. The developmental control of *5'-nucleotidase* (*5nt*) expression in the model system *Dictyostelium discoideum* has provided a focal point for studies of gene regulation at the level of transcription.

In order to identify temporally-regulated control elements within the promoter of the *5nt* gene, 5' and internal promoter deletions were designed and fused to the *luciferase* and *lacZ* reporter genes, and reporter enzyme activity was measured in cells from the slug stage of development. The results from these experiments enabled the identification of a 250 bp region of the promoter, which was used as a template for subsequent site-directed mutagenesis experiments. These experiments involved altering 6-12 bp regions of the promoter by substitution. Twelve mutagenized promoters were fused to the *luciferase* and *lacZ* reporter genes, and activity was measured at the slug stage of development to more precisely locate *cis*-acting temporally-regulated control elements. In addition, cAMP induction experiments were performed on amoebae transformed with the mutagenized

promoters to identify control elements within the promoter influenced by the presence of cAMP. The regions between -530 and -560 bp and -440 and -460 bp from the ATG translation start site.

In order to evaluate the functions of the *cis*-acting promoter control elements, electromobility gel shift assays were performed to identify specific DNA-protein interactions on the *5nt* promoter. These assays enabled the detection of a 0.13 Rf and 0.33 Rf binding activity to specific sites of the promoter. After characterization of these binding activities, both proteins were purified by a series of column chromatography techniques and characterized after mass spectrometry. The proteins purified were identified as formyltetrahydrofolate synthase and hydroxymethylpterin pyrophosphokinase. These enzymes function in the biosynthetic pathway of tetrahydrofolate and the production of folate coenzymes. The specific interactions of these enzymes with the *5nt* promoter suggest these proteins may also function in regulating *5nt* expression.

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List of Abbreviations

5NT	5'-nucleotidase
ALC	Anterior-like cells
ß-gal	ß-galactosidase
cAMP	Cyclic adenosine 3', 5'-monophosphate
CAE	CA rich elements
dDSTAT	Dictyostelium signal transducer and activator of transcription
DIF	Differentiation inducing factor
EMSA	Electromobility gel shift assay
FTHFS	Formyltetrahydrofolate synthase
GBF	G-box binding factor
HPPK	Hydroxymethylpterin pyrophosphokinase
LINE	Longer interspersed nuclear elements
LTR	Long terminal repeats
LUC	Luciferase
Myb	Myeloblastosis proteins
ONPG	Orthonitrophenyl ß-D-galactoside
PCR	Polymerase chain reaction
PKA	Protein kinase A
PNK	Polynucleotide kinase
PSF	Prestarvation factor
Rf	Retention factor
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel eletrophoresis
THF	Tetrahydrofolate

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Chapter 1

General Introduction and Literature Review

1.1. *Dictyostelium* as a Model Organism.

Dictyostelium discoidium is a simple eukaryotic soil-living amoeba that has been used as a model system in molecular studies of developmental gene expression, cell-type differentiation, cell motility, cell sorting, signal transduction, chemotaxis, and cohesive cell contacts (Raper and Thom 1932; Raper 1935; Bonner 1944; Bonner 1947; Kessin 2001). The organism serves as a useful model organism because in many ways these functions are analogous to mammalian systems. The manageable genome, the short life cycle of the organism, the differentiation of two major cells types, and the ease of maintaining the organism under laboratory conditions are a few characteristics of *Dictyostelium* that make it a good model system.

Molecular phylogenetics has shown that *Dictyostelium* is not as closely related to fungi as previously thought, though the precise phylogenetic position of the organism remains a point of debate (Kessin 2001). Ribosome analysis suggests that *Dictyostelium* diverged before the evolution of plants, animals and fungi. Based upon the sequenced mitochondrial genome of *Dictyostelium*, it has been proposed that the organism is more closely related to green plants than to animals and fungi. The similarity to plant cells extends to cellulose deposition during terminal stalk cell differentiation and vacuolization. However, a closer relationship with animals has been suggested based upon similarities between protein sequences encoded by nuclear genes. Molecular similarities do exist between *Dictyostelium* and mammals, and the relative simplicity of the genome makes the model organism suitable for manipulative processes.

A consortium including Baylor College of Medicine, the Wellcome Trust Sanger Institute, the Institut Pasteur and the Genome Sequencing Center at Jena, in collaboration with the Institute of Biochemistry in Cologne, has completed the *Dictyostelium* genome sequencing project. The haploid genome of *Dictyostelium* is comprised of 3.4×10^7 bp, and resides on a total of 6 chromosomes (Kessin 2001). The genome is very AT rich, with an overall composition of 77% AT. Coding sequences are approximately 65% AT, and introns and promoters are typically 90% AT. An estimated 10,000 genes are coded by the organism, with an average bp length of a single gene being 3,000, including regulatory sequences and introns. Introns do reside within the genome, though they are relatively short, an average 100 bp in length. Approximately 3% of the genome is comprised of transposable elements. These have been characterized as long terminal repeats (LTRs) and longer interspersed nuclear elements (LINEs). Outside of the chromosomal genome and within the nucleus, ribosomal rRNA genes reside on a 90 kilobase linear extrachromosomal palindrome, and several families of replicating plasmids exist. The mitochondrial genome is comprised of 5.55×10^4 bp, and is AT rich, as well, with an overall composition of 70.8% (Kessin 2001).

Mutagenesis of the genome has been achieved using high efficiency extrachromosomal vectors, homologous recombination, and restriction enzyme-mediated integration (REMI) (Kessin 2001). In addition, transformation is sufficient to enable complentation of mutants after chemical mutagenesis. The functions of target genes can be easily evaluated due to the haploid nature of the genome. In addition, the consequences of gene disruption can be monitored throughout the 24 hr life cycle of the organism.

1.2. The Life Cycle of *Dictyostelium*

The life cycle of *Dictyostelium* consists of distinct developmental stages characterized by both unicellular and multicellular forms (Fig. 1.1) (Raper 1935). The transition between the single-celled amoebae and the multicellular form of the organism is dependent upon nutrient availability (Raper 1937). When bacterial nutrients are plentiful, the organism exists as single-celled amoebae and grows vegetatively. The initial developmental change occurs upon depletion of the bacterial food source.

In response to the absence of nutrients, some individual cells begin to emit pulses of cyclic adenosine 3', 5'-monophosphate (cAMP) (Bonner et al. 1970). Neighboring cells respond to the presence of cAMP by releasing their own cAMP and aggregating towards the original source of cAMP by chemotaxis. The cAR1 and cAR3 cell surface receptors are involved in the chemotactic cellular response to the presence of extracellular cAMP (Kimmel 1987; Kimmel and Firtel 1991; Milne and Devreotes 1993). As a result of the chemotactic response, a mound of single-celled amoebae form a multicellular aggregate, consisting of approximately 10^5 cells. In addition to the requirement of cAMP as a morphogen to regulate differentiation of the cell types, DIF (differentiation inducing factor), a small chlorinated organic molecule, is also required (Kay et al. 1979; Kay 1997).

During the aggregate stage of development, differentiation of the precursors of two distinct cell types occurs. The precursors to the prespore cells comprise approximately 70% of the total cells, whereas the precursors to the prestalk cells comprise the rest. A distinct spatial pattern of the cell types is generated within the

mound, which includes the presence of prestalk cells at the top of the aggregate (Raper and Fennell 1952).

The tip of the aggregate elongates to form a structure, referred to as the first finger stage. Additional cell sub-types have been described based upon the expression of two specific genes that encode for extracellular matrix proteins: *ecmA* and *ecmB* (Ceccarelli et al. 1991; Gaskell et al. 1992). Within the anterior region of the prestalk cells, there are prestalk A (pstA) cells, which express *ecmA*. Within the posterior region of the prestalk cells, there are prestalk O (pstO) cells, which express *ecmA* at very low levels (Gomer and Ammann 1996). In addition, there are prestalk AB (pstAB) cells, which express *ecmA* and *ecmB* genes at high levels. Another type of prestalk cells, anterior-like cells (ALCs), are dispersed throughout the posterior prespore zone (Sternfeld and David 1982; Devine and Loomis 1985). As the life cycle progresses, these sub-types of prestalk cells are involved in sorting events, which are summarized in Figure 1.2 (Mohanty and Firtel 1999).

The tip of the aggregate elongates and falls over to form the pseudoplasmodium, or slug. The prestalk cells encompass 30% of the slug in the anterior region, whereas the prespore cells encompass 70% of the slug in the posterior region. The sensitivity and ability of the slug to migrate through the slime sheath towards heat and light enable the organism to occupy an appropriate site for culmination and eventual development of the fruiting body.

After slug migration is complete, the differentiated cells reorient within the organism. The slug positions itself in a vertical formation, with the prespore region of the organism in contact with the substratum. The prestalk cells, located in the anterior

region, synthesize a central stalk tube through which they migrate to the base of the structure, often referred to as the "Mexican hat" conformation (Sussman and Sussman 1967). This "reverse fountain" movement of cells results in the basal tubular stalk structure. The ALCs, once scattered throughout the prespore region of the organism, migrate to form the upper and lower cups of the spore head (Fig. 1.2). Maturation of the spores occurs when cAMP-dependent protein kinase is activated. The spore cells produce spores, surrounded by a glycoprotein and polysaccharide coating that are released when favorable conditions exist. Following spore dispersal, germination occurs. The single-celled amoebae that are released continue the life cycle.

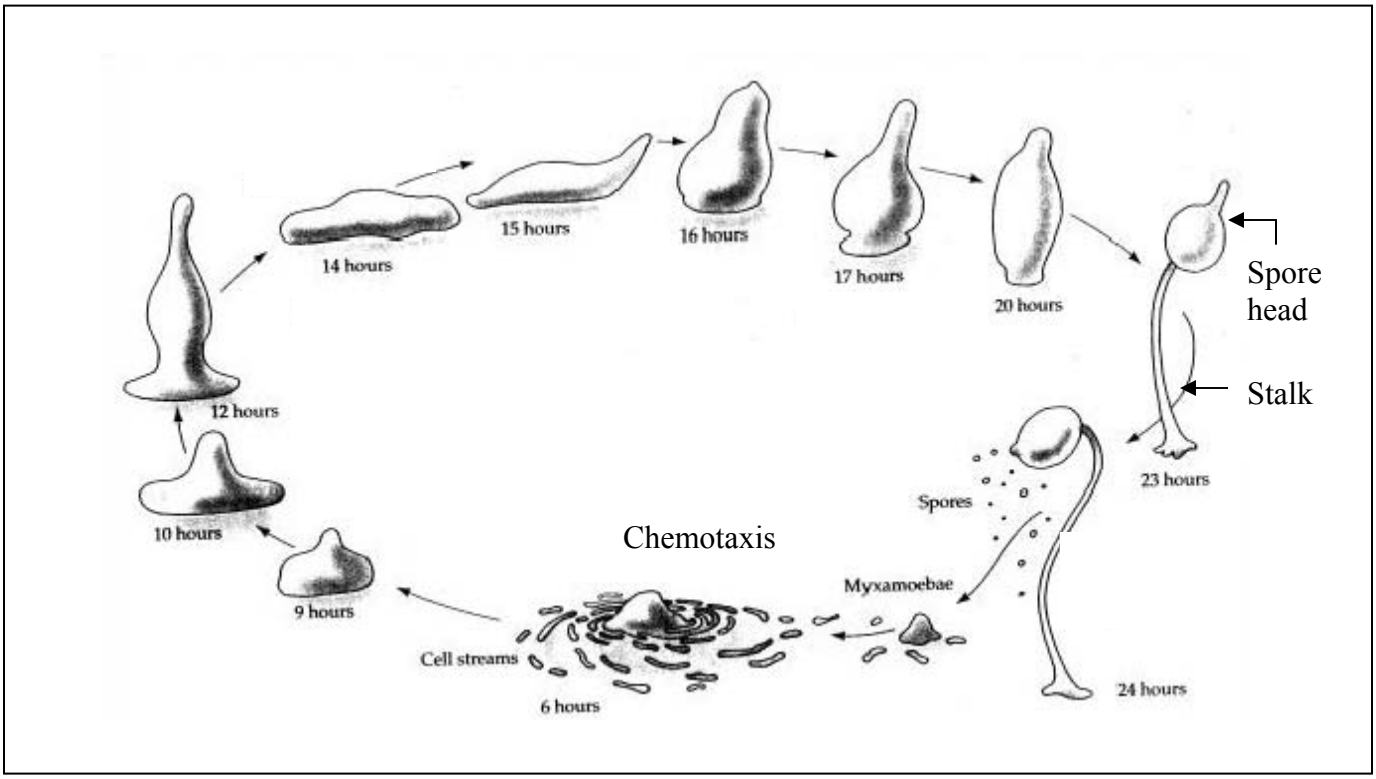

Figure 1.1. The Life Cycle of *Dictyostelium* (modified from Raper 1935)**.** The 24 h life cycle of *Dictyostelium* includes unicellular and multicellular stages. When nutrients become depleted, amoebae emit oscillatory pulses of the chemoattractant cAMP and aggregate into a multicellular mound formation approximately 6 h into the developmental process. Between 9 h and 12 h of development, the tip of the mound extends and elongates. The pseudoplasmodium, or slug, forms at 14 h after the tip of the mound falls over, and migrates towards a culmination site. During the culmination stage of development a cell sorting process of prestalk and prespore cells generates a stalk tube. Terminal differentiation of the two major cell types occur in the mature culminant. The spore cells generate a sorus, present at the top of the stalk that releases individual spores.

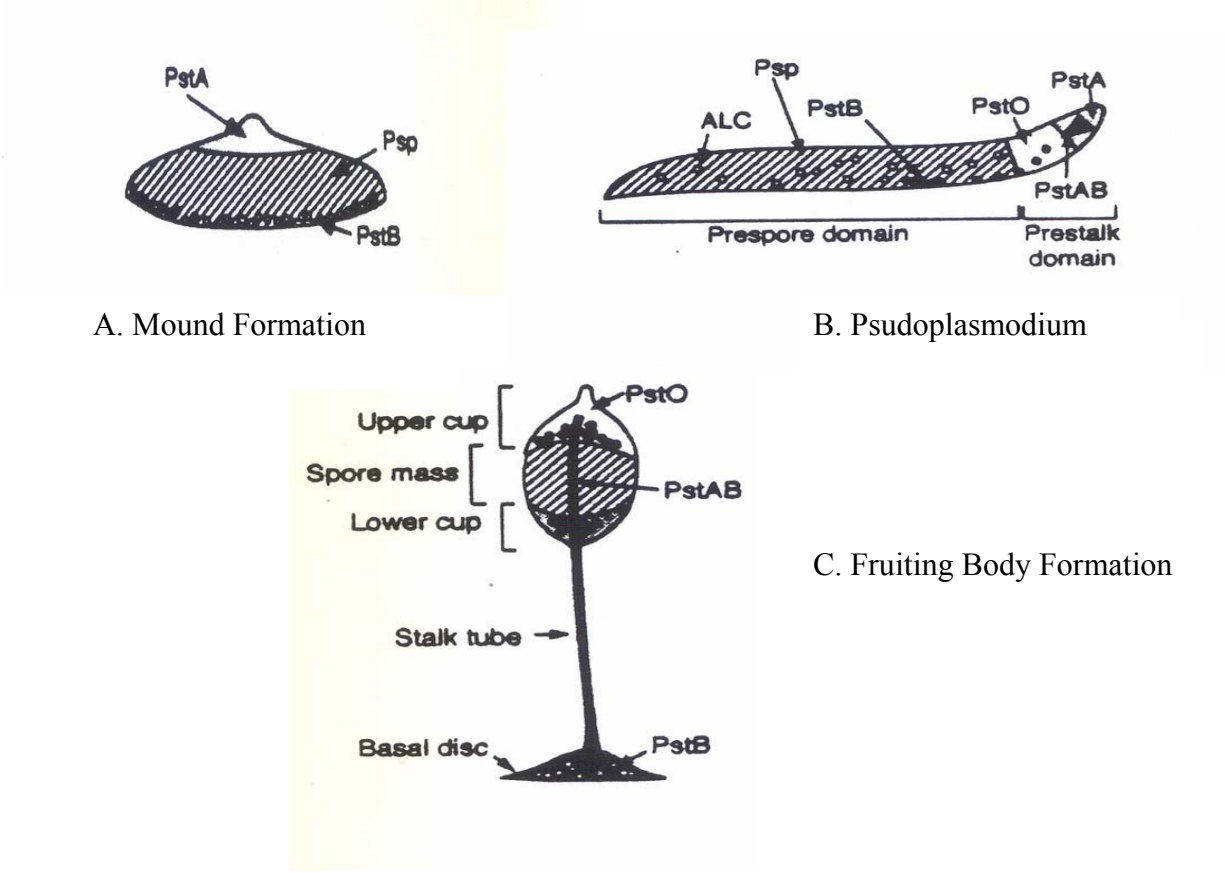

Figure 1.2. Cell Types and Sorting Events of the Mound, Slug and Fruiting Body Developmental Stages (modified from Mohanty and Firtel 1999). **A.** After the aggregation of amoebae, differentiation between prestalk and prespore becomes apparent. Prestalk cells comprise 30% of the mound formation, and include PstA cells and PstB cells. These cells express the extracellular matrix proteins EcmA and EcmB, respectively. Prespore cells comprise the remaining 70% of the mound structure. **B.** The slug forms after the tip of the mound elongates and falls over. While the prestalk cells generally comprise the anterior of the organism and the prespore cells generally comprise the posterior of the organism, there are prestalk cell subtypes scattered in the prespore domain. In addition to the PstA and PstB cell subtypes, there are ALCs, PstAB cells, and PstO cells also present. **C.** Cell sorting events occur between the slug and fruiting body stages, resulting in considerable redistribution of cell types within the organism. The ALCs form the upper and lower cup of the organism. PstO cells are found in the anterior region of the structure, while PstB cells are found at the base of the structure. PstAB cells are found in the upper region of the stalk tube, surrounded by the spore mass.

1.3. The Effects of cAMP and DIF during *Dictyostelium* Development

Cyclic AMP is an important regulatory molecule within prokaryotic and eukaryotic systems (Fig. 1.3). The important role of this chemoattractant to induce the switch between a unicellular and multicellular existence is evident from the response of cells to extracellular cAMP, and the production of cAMP by cells during the aggregation stage of *Dictyostelium* development. The initial detection of reduced amounts of nutrients (bacteria) by individual cells involves a density-sensing mechanism. One of these mechanisms is controlled by a protein called the prestarvation factor (PSF), whose expression increases when nutrients become low (Clarke et al. 1992; Rathi and Clarke 1992). In response to the increase in PSF, there is activation of a number of genes involved in the initial stages of development, many of which are involved in the chemotactic relay system directed by cAMP.

The basis of secretion of cAMP is not known, though several of the major cell surface receptors, including cAR1, cAR2, cAR3 and cAR4, have been the focus of many studies. These transmembrane protein receptors are coupled to GTP-binding proteins, activating various intracellular second messengers and their corresponding signal transduction pathways. One of the responses to extracellular cAMP detection is intracellular synthesis and secretion of cAMP. The quantity of cAMP produced by adenylyl cyclase (AC) is proportional to the levels of the extracellular cAMP stimulus (Dinauer et al. 1980). Neighboring cells respond to levels of cAMP by migrating towards the most concentrated source of the acrasin. The release of oscillatory cAMP pulses and

the subsequent migration of cells towards high levels of the chemoattractant result in the formation of the multicellular aggregate.

While there is a peak of cAMP present during the aggregation stage, significant levels persist throughout development (Merkle et al. 1984). In addition to its role as a chemoattractant and secondary messenger, cAMP is also critical for cell differentiation and morphogenesis due to its role in regulating gene expression. In both prestalk and prespore cells, cell-type specific gene expression is dependent upon cAMP before the culmination stage of development. Once the culmination stage is reached, cAMP stimulates prespore cell differentiation, but inhibits prestalk cell differentiation (Williams and Jermyn 1991). In order to maintain prestalk cell differentiation, a cAMP phosphodiesterase (cAMP PDE) hydrolyzes cAMP, reducing the levels present within this cell type (Brown and Rutherford 1980; Hall et al. 1993). One of the specific genes activated by cAMP during development encodes protein kinase A (PKA) (Rutherford et al. 1982). The regulatory subunit of PKA directly interacts with cAMP, for which it has a high affinity. This process releases and activates the catalytic subunit. The active PKA plays crucial roles at all stages of *Dictyostelium* development, including aggregation, induction of prespore gene expression, and multicellular development.

In addition to cAMP, DIF is also involved in achieving cell-type specific gene expression in prestalk and prespore cells (Fig. 1.4). DIF is synthesized in the prespore region of the organism, where it is maintained at high levels (Loomis 1993). DIF is degraded in the prestalk zone, resulting in low levels of the morphogen (Nayler et al. 1992). As a result of these activities, a concentration gradient of DIF is established. Prestalk-specific gene expression is stimulated by low levels of DIF and prespore-

specific gene expression is stimulated by high levels of DIF. There is evidence that increased levels of calcium are required for the DIF induction that occurs in prestalk cells (Schaap et al. 1996; Verkerke-van Wijk et al. 1998). The combined effects of DIF and cAMP are not consistent for all types of prestalk cells; in some prestalk cells gene expression is induced, whereas in other cells gene expression is repressed. In prespore cells, transcription of genes are repressed by DIF and induced by cAMP (Kay et al. 1999).

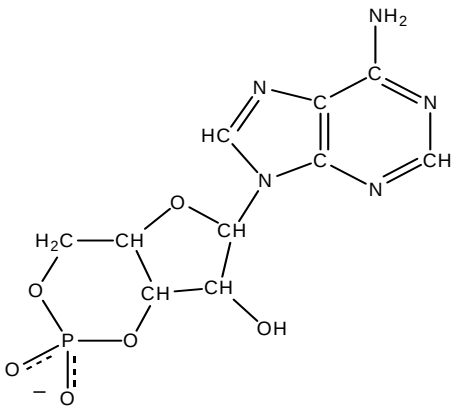

Figure 1.3. The Chemical Structure of cAMP

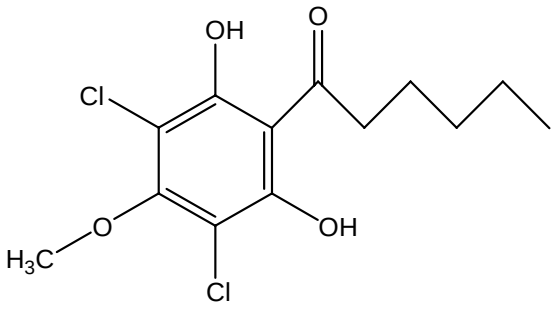

Figure 1.4. The Chemical Structure of the Differentiation Inducing Factor (DIF)

1.4. General Mechanisms of Gene Regulation

By the time the transcription machinery is recruited to a gene to the production of an active protein product, there are several points at which expression can be regulated (Carey and Smale, 2000). Within the nucleus of the eukaryotic cell, chromatin state and architecture can modulate the initiation of transcription, a process influenced by chemical modifications of the DNA. When access to a specific gene is available by the transcription machinery, then the interaction of the transcription complex with the gene promoter sequence may initiate the production of mRNA, depending upon the presence or absence of transcription activators, repressors or co-repressors.

Eukaryotic DNA is organized in a compact fashion within the nucleus of the cell. Interaction of the DNA double helix with histone proteins to produce nucleosomes, and the coiling of adjacent nucleosomes to form a chromatin fiber with a diameter of approximately 30 nm, enables the extensive amount of DNA in the nucleus to be compact and organized. Further looping patterns formed by the chromatin fiber result in the characteristic chromosome. The architecture of chromosomes within the eukaryotic cell changes as progression through the cell cycle occurs. Mitotic chromosomes are highly condensed in a form referred to as heterochromatin. This highly condensed form of the DNA prevents access of transcription and replication machinery, and therefore, these cellular processes are less likely to occur. In contrast, the euchromatic form of DNA is more loosely organized. This form of DNA is predominant during the replication phase of the cell cycle. In this architectural state, the components of the transcription machinery usually have better access to gene promoters, so transcription is more likely to occur.

In addition to the architectural state of the chromatin, chemical modification of the DNA may also influence accessibility of protein machinery, and therefore the occurrence of transcription (Carey and Smale, 2000). Hyperacetylation of histones has been associated with chromatin that is transcriptionally active, while regions of chromatin that are hypoacetylated are typically inactive with respect to transcription. Chemical modifications involving methyl groups can also influence gene regulation. Methylation of cytosine bases is often associated with low levels of transcription, while genes present in regions of DNA containing demethylated cytosine bases are usually transcriptionally active.

When chromatin architecture and chemical modifications are conducive for the association of the transcription machinery with gene promoters, then transcription initiation may ensue (Carey and Smale, 2000). The frequency of transcription initiation is typically dependent upon a complex network of specific protein-protein and protein-DNA interactions involving the association of the enhanceosome with the general transcription machinery at the core promoter to form the pre-initiation complex.

The promoter core is generally located between -40 and +50 bp from the transcription start site and usually contains a TATA motif, an initiator element, a downstream core promoter element and a TFIIB recognition element (Carey and Smale, 2000). The TATA box, represented by the sequence "TATAAA," is the location where the TATA-Binding Protein (TBP) of the TFIID general transcription machinery component binds. The Inr, or initiator element, serves a similar function as the TATA box in those promoters without this feature. It determines the start site location, and physically interacts with the TFIID complex to direct the formation of the pre-initiation

complex. In many promoter sequences that are "TATA-less," including many found in *Drosophila*, there is an additional downstream core promoter element (DPE) that acts in conjunction with the Inr element to initiate transcription. This DPE is usually located approximately 30 bp downstream of the transcription start site. The TFIIB recognition element (BRE) was discovered and reported by Lagrange et al in 1989. It is a GC rich element found immediately upstream from the TATA box, is found in many eukaryotic promoters as the site of specific TFIIB interaction.

Transcription activators are modular proteins with specifically structured DNA-binding domains (Carey and Smale, 2000). Common structural motifs found in eukaryotic activators that enable DNA-binding include zinc fingers, leucine zippers, and helix-turn-helix motifs. Some transcription activators are required to directly interact with the general transcription machinery or other co-activators in order for initiation to occur. The requirement for the presence of transcription activators in order for gene expression to occur provides yet another level for regulation. For example, the presence of the activator protein may be regulated by its own transcription or by required posttranslational modifications.

In addition to activation mechanisms, repressor and co-repressor activities may also influence whether or not a gene is transcribed (Carey and Smale, 2000). There are three general categories for this process, the first of which involves inactivating a transcription activator. This may involve posttranslational modification of an activator that would render it non-functional. Dimerization of the activator could also cause inactivation. In a third scenario, a repressor protein would prevent the binding of the activator by physically blocking its recognition site on the promoter sequence. The

second category for repressive activity targets the preinitiation complex. Some repressors, for example, form tight physical associations with general transcription factors, preventing assembly of the pre-initiation complex. The final broad category of repression mechanisms involves the interaction of specific DNA-binding protein with a specific DNA element, which may interfere with either enhanced or basal levels of transcription.

Gene expression is commonly regulated at the level of transcription initiation, but many points of regulation exist after the production of an mRNA transcript (Carey and Smale, 2000). Temporal control may be achieved if the transcript is maintained in the nucleus, where translation may not proceed if cap and tail processing does not occur immediately. After export to the cytoplasm, the time period before mRNA degradation occurs influences how often the transcript can be translated. Interaction of the mature mRNA with ribosomes may produce a poylpeptide, but this polypeptide may not be functionally active until it is properly cleaved, chemically modified, folded, and transported to its appropriate cellular destination. Once these steps have been completed, degradation processes further influence the effectiveness of the active protein.

Regulation of gene activity involves complex interactions and interconnections of numerous cellular components. Through the mechanisms previously described, the expression of a particular gene can be precisely controlled to fit the specific demands and needs of an organism.

1.5. Mechanisms of Gene Regulation in *Dictyostelium*

During the life cycle of *Dictyostelium*, an estimated 2,500 genes are developmentally regulated, as shown from microarray analysis (VanDriessche et al. 2002). In order to temporally and spatially regulate expression of genes throughout the course of development, specific control mechanisms must be employed. As described in the section General Mechanisms of Gene Regulation, gene expression is often controlled at the level of transcription by *cis*-acting regulatory proteins that interact with gene promoter and / or upstream activator sequences (UAS). Cell differentiation and morphological changes that occur during development rely upon these regulatory mechanisms. In order to acquire a complete understanding of normal developmental processes, it is necessary to put the components of cell signaling, signal transduction and the effects on gene expression in context with one another. Several regulatory elements in promoter sequences and transcription factors have been identified and characterized in *Dictyostelium*.

The AT bias of the *Dictyostelium* genome extends to gene promoters, which are typically comprised of 90% AT. *Plasmodia falciparum* is another organism whose genome is exceptionally AT rich, and it is not known what led to this characteristic (Kessin 2001). Research has shown that throughout vegetative growth, gene expression is controlled by AT rich promoter control elements (Maniak and Nellen 1990; Vauti et al. 1990; Esch et al. 1992; McPherson and Singleton 1993; Hori and Firtel 1994; Bonfils et al. 1999). As an example, an AT rich element within the actin 15 promoter has been identified. The insertion of this control element into a developmentally-regulated promoter induces expression in the vegetative stage (Hori and Firtel 1994).

Several prespore-specific genes have been identified in *Dictyostelium*, and many of the control elements that regulate the expression of these genes are CA rich. These CA rich elements (CAEs) are critical sites involved in the stimulation of prespore-specific gene expression (Haberstroh and Firtel 1990; Haberstroh et al. 1991; Fosnaugh and Loomis 1993; Powell-Coffman and Firtel 1994; Powell-Coffman et al. 1994; Seager et al. 2001; Stevens et al. 2001). In addition to the CAE, there resides an AT rich control element, located further downstream, in many of these gene promoters. Either of these elements are capable of inducing gene expression when they are integrated upstream of a basal promoter. Several proteins that interact with *Dictyostelium* gene promoter control elements have been identified.

Some of the transcription factors that have been identified in *Dictyostelium* include the G-box regulatory binding factor (GBF), the signal transducer and activator of transcription protein family (DdSTATs), some myeloblastosis proteins (Myb), the C-module-binding factor (CMBF), the serum response factor (srfA), the CAR1 transcription factor (CRTF), and transcription factor 2 (TF2). Research has shown that the GBF transcription factor interacts with a CAE (Schnitzler et al. 1994). The induction of cell surface cAMP receptors causes the GBF transcription factor to interact with recognized control elements and stimulate the expression of cell-type specific genes (Brown and Firtel 2001). The interaction of GBF with DNA relies upon zinc finger motifs. When the gene encoding GBF is disrupted, development stops at the loose mound stage, and mutants do not express cell-type specific genes, thereby indicating that this regulatory protein plays a critical function for transition into the first finger stage of development (Schnitzler et al. 1994).

Dictyostelium STATs, which localize to the nucleus during aggregation, are produced throughout growth and development. A member of the DdSTAT family, DdSTATa, is a transcription factor that is produced in pstA cells (Araki et al. 1998). The transcription factor becomes localized to the nucleus of cells in the prestalk zone when cAMP levels are high. DdSTATa interacts with an AT rich control element in the prestalk gene *ecmB* to regulate prestalk cell differentiation during the culmination stage of development (Kawata et al. 1997; Araki et al. 1998). When the DdSTATa gene is disrupted, there is wider expression of the *ecmB* gene in the pstA zone, and the organism has a defective culmination.

Several genes in the *Dictyostelium* genome are regulated by Myb proteins. The glucosamine-6-phosphate isomerase gene (*gpi*), is expressed during vegetative growth, and in the prestalk cells of the mound and slug stages (Matsuda et al. 1999; Tabata et al. 2001). A Myb binding site, which is involved in the regulatory control of expression, resides in the promoter of *gpi*. A total of three Myb proteins have been identified in *Dictyostelium*, although the precise function of each has not been completely determined. DdMyb2 has been shown to participate in the activation of the *adenylyl cyclase* gene during the early stages of development (Otsuka and van Haastert 1998). DdMyb3, which is expressed in prestalk cells, functions during the culmination stage of development (Guo et al. 1999). While it is known that DdMyb1 recognizes and binds to the sequence 5' TAACT/GG 3', the functional significance of the protein has not been determined (Stober-Grasser et al. 1992).

1.6. The Functions of 5’-nucleotidases

5'-nucleotidase is a membrane-bound glycoprotein ubiquitous to numerous species and cell types. Functioning similarly to other ectonucleotidases, 5'-nucleotidase exports nucleosides, derived from dephosphorylated nucleotides present in the extracellular matrix, into the cell (Zimmerman and Weijer 1993). Although also capable of allowing nucleoside passage in the intracellular to extracellular direction, the enzyme prevents passage of nucleotides across the membrane barrier (Gazziola et al, 1999).

5'-nucleotidase has also been shown to be involved in other membrane transport processes and cell-cell interactions (Uusitalo 1981; Codogno et al. 1988). Research has shown the involvement of 5'-nucleotidase in adhesion and cellular recognition activities. Alterations in the distribution of this enzyme at the apical surface of uterine epithelial cells have been shown during early stages of pregnancy in humans (Bucci and Murphy, 1999). Other processes in which the enzyme has been implicated include sperm motility and male fertility, lymphocyte-endothelial cell interactions, signal transduction in the immune system and T cell activation (Takayama et al. 2000).

The involvement of 5'-nucleotidase in the cAMP degradation pathway has made understanding the regulation of this enzyme particularly important. Cyclic AMP is degraded by cAMP phosphodiesterase, producing 5’AMP. 5NT removes 5’AMP, producing inorganic phosphate and adenosine. 5’AMP levels would accumulate if 5NT were not present, and may inhibit further cAMP degradation by feedback inhibition.

1.7. Previous Studies of *5'-nucleotidase* in *Dictyostelium*

The gene encoding 5'-nucleotidase (5NT) is located on chromosome 4 of the *Dictyostelium* genome. Protein functions of genes located in the proximity of *5nt* on chromosome 4 include cap binding activity, ubiquitin carrier activity, electron transport activity and oxidoreductase activity (http://dictybase.org/). The next upstream gene on chromosome 4, which is involved in ubiquitin conjugation, is located approximately 3,300 bp away, and oriented in the opposite direction. TheAT-rich sequence between the translation start site of *5nt* and the start of makes analysis a challenge, though the possibility exists that sequences controlling the regulation of *5nt* may also be involved in the regulation of this gene. The regulation of this neighboring gene has not been characterized in *Dictyostelium*. Bidirectional gene pairs have been identified in many genomes, including the human genome (Trinklein et al. 2004). Their analysis showed that many promoter segments between two bidirectional gene pairs included shared elements that regulated both genes and initiated transcription in both directions.

The biochemical characterization of 5NT has provided insight into many of the functions of the enzyme. From *Dictyostelium* culminant cells, 5NT was purified by Concanavalin A affinity, DEAE and 300 SW gel filtration chromatography (Chanchao et al. 1999). The alkaline phosphatase activity of 5NT, which has a pH optimum of 9, was shown to be specific for the artificial substrate para-nitrophenyl phosphate (PNPP) and 5'AMP (Armant 1980). Activity from the gel filtration fractions and by enzymatic assays corresponded to a 90 kDa band and a 45 kDa band detected by SDS-PAGE. Peptide sequences were obtained by mass spectrometry. At the time, the sequence did not show significant similarity to any known sequence in the database. The highest similarity was

with a *Dictyostelium* contact site A protein (24% identity, 151 amino acids), a membrane-bound glycoprotein that is expressed at the aggregation stage under cAMP regulation and is involved in cell adhesion and cell-cell interactions (Das and Weeks 1981; Siu et al. 1986; Siu et al. 1987; Siu and Lam 1988; Siu et al. 1988; Bhanot and Weeks 1989).

To amplify several regions of *5nt* from the genomic DNA, degenerate primers were used, and the products obtained were cloned, sequenced, and utilized to search the *Dictyostelium* developmental cDNA project at the Intitute of Biological Sciences at the University of Tsukuba in Japan (Chanchao et al. 1999). Three cDNA clones from the same gene were found from the database. The predicted amino acid sequence for these clones matched the 5NT sequences that were previously obtained by Edman degradation and mass spectrometry (Fig.1.5).

The *5nt* gene is developmentally regulated. Northern analysis has shown that levels of mRNA transcript increase as development proceeds. During the vegetative stage of development, *5nt* transcript is not present (Chanchao et al. 1999). However, expression of the gene increases during the culmination stage of development. Western blot analysis was also performed to detect the gene product at various stages of development. A polyclonal antibody to 5NT was raised and used to detect the presence of endogenous protein from membrane extracts (Ubeidat et al. 2002). The protein was not present in membrane extracts from amoebae, but was detected in extracts from slugs and culminants. Results from the Northern and Western analyses suggest a similar pattern for the accumulation of mRNA and protein throughout development.

Fusion of the *5nt* promoter to the *gfp* reporter gene has enabled the localization of expression to be determined at each stage of development through confocal microscopy

(Ubeidat et al. 2002). Activity was initially seen in scattered cells as they began to migrate towards the aggregation centers. The highest levels of activity were observed in the central portion of the tight aggregate. In the first finger stage of development, activity was present in a gradient, with highest levels detected at the base of the structure. During the slug stage of development, activity was apparent in the slime sheath through which the slug migrates towards an appropriate culmination site. In the early culminant, activity was observed at the boundary between prestalk and prespore cells. In the late culminant, expression of the reporter gene was detectable in a cell layer surrounding the prespore mass. In the final fruiting body stage of development, activity was present in the lower cup, stalk and basal disk.

Insertional mutagenesis of the 1.9 kb *5nt* gene using the blastocidin resistance cassette in homologous recombination experiments have shown developmental consequences (Rutherford 2003). The effect occurs after the tight aggregation stage, and instead of a single finger protruding from the aggregate, small fingers form on several parts of the mound. These fingers continue to extend throughout the remainder of the lifecycle and result in the development of multiple fruiting bodies as opposed to one. These experiments suggest that 5NT functions in the late aggregation stage when tip formation occurs. The tip of the finger structure serves as the "embryonic organizer" of the organism at this stage of development, presumably by regulating cAMP oscillations (Schaap and Wang 1986).

CAATATTAATTTTTTTTTCACATAATTTTATTATTTGTATAATTTAATTATTCATAAATT 60
ATTCTATATTATAATTTTTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA**ATG**AAATTA 120
TTATTATTATTATTTTTAATAATAAATTCAATAATATTATCAAAATGTGGTTATAGTCAA 180
ACAAATGAAAAAATAATAGTTACTGGAGAATTTGATAAAACCATTGACAAATATACAATT 240
TCCTTTTCAAATATTGGTGATATTACTCAACTATGTTCAATTAATACTACAATATTGACA 300
TGTTTCCCACCAGCCAAATCAATTAATGGTGGATTCTTGGTGTATGATAAAGAAGAAGGT 360
ACAAATGTAATTGATATAACCACAGTTGTATTATCACCATATATTTCAAGTATGGATCCA 420
AAAGTAATACCAACATCATCAATTGAAATCACAATTAGAGGATTCTATTTCAACGCAAAT 480
TCAAATCCAGAAACAAACAAAACATCAACTCAATTACTGGTAACAATGGTGGGTTCAAAT 540
GTTGATATAAATTCAACAGCATCTGATTCCGTTAAATTCTATCCACCAAGTTTTTTCCAA 600
ACACCTCTAACCATATCATTAACAAATGTTGATAGTGGTAAAAAATCAAATTCAATTAAA 660
TTCAAATATGAATTACCAAATATTGAATCATTATCAGTTGTTGACATTAAAGATAATAAT 720
AATAAAACCACTCAACAATATCTTAATATTAGTGGTACAAATTTTGGATCAAAACAATCA 780
ATGAAATTAGTTTTCGTTGAAATCCATGATTTTAATAATGATTCATTAATCATTACAAAG 840
TTAACTGATATCTTATCAATTAACGATACAAATTTATTAATTAAAATTAATAGTGATTCT 900
TCAAGTGGTAATATCTATGTAAATGCAAATTCTCAACAATCAAATACATTACCATTATAT 960
TTAACACCAATAATTACAAATGTTGATTTTCCAAATTATAATGGTGATACAATTAATATA 1020
ACTGGTAGTTACTTATCTGATATTTATTTATCACCATCAACAAAATTAAATTGTTCAACA 1080
ATTTTAATTAAAGATTCAAATACTGATGATGATGACAATGGTGATGACACATTATCATCA 1140
ACATCAGATTCTTCATCATCATCAACAAAAGCAACAACTTCATCATCAAGTAATAATAAT 1200
ATTTATTATAAAAAATGTAAATTCCCACAAAGAAATTTAAATGATTCAATTTCATTTTCA 1260
ATTTTTTCAAGATCAGTTGGGAATAATGTTAATCATGATTCGAATGAATTTAAATCACAT 1320
TATCAAAAACCAATTATTGATGCAGTTGTTCCAAATGGATTTTATGTTAATAATAAATTG 1380
AATTTCACATTTTATGGTACAAATTGGGCAAATTATACAAATACTACAATTACAATCGCT 1440
GATAAACCATGTAAAGTATTAGAAATTACAAGTTCAACAATTGTATGTTACTATGAAGCT 1500
GGTGTTGAAATTTTACAAAATCCAATCTCTTATGTTATAACTGTTGATGGTCAAAGAAAT 1560
AATATAGCACCAGATAGTGATACTTCAACAATTTCATTCTATTCACTTTGCCCAGGTCAA 1620
TCATTTTCCAATGGTACCACTTCTCCAACTTCTTCAACCAATACAACAACACAAGGTTGT 1680
AGTAATAGTGGTACTTGTAATCCTGTCACTGGTCTATGCCAATGTTTACCAACCAAAACT 1740
GGTAAAATTTGTGATCAAGATAAATATTCAAGTAATTCAACTTCAAAATTATTATCAACA 1800
TCATCATTATTTTTATTATTATTAATCTTTATTACTTTATCTTAT**TAA**ATCAAAACAAAT 1860
TACTTTTTCGTTTATAAAAATAAAATTAAATAAATAAATAAATATATATATACATTTTTA 1920
A 1921

Figure 1.5. The *5nt* cDNA Nucleotide Sequence. The start codon ATG, underlined at position 112, represents the beginning of an open reading frame. A stretch of A's immediately precedes the translation start site, which is characteristic of many *Dictyostelium* genes. The stop codon TAA is underlined at position 1845.

1.8. The Use of *Luciferase* and *ß-galactosidase* as Reporters of *in vivo* Processes

Reporter genes that encode for ß-glucoronidase (GUS), green fluorescent protein (GFP), chloramphenicol-acetyltransferase (CAT), orotidine 5'-phosphate decarboxylase (URA3), luciferase (LUC), and ß-gal, have been successfully employed in *Dictyostelium* to evaluate various cellular phenomena. The identification of regulatory binding sites in the promoter of *5nt* involved the use of two reporter genes, LUC and ß-gal.

The luciferase reporter vector, pVTL2 (Fig. 1.6), was derived from the nuclear-associated plasmid Ddp2 (Yin et al. 1994). The presence of a Dd *ori* in the vector enables autonomous propagation. This characteristic is useful because it prevents random integration into the genome, which could potentially alter reporter gene activity if the site were near a highly active or inactive region. The vector is 13 kb in size, has two multiple cloning sites, T3 and T7 primer binding sites to test for insert presence, and genes that confer ampicillin and G418 resistance for selective purposes. The vector has a relatively low copy number of 10-50 per cell. The luciferase enzyme produced from the reporter gene oxidizes the decarboxylation of the luciferin substrate in an ATP-dependent manner. The energy that is produced during this reaction is released as bioluminescence. The light emission occurs at a wavelength of 562 nm, and can be quantitatively measured using a luminometer.

The ß-gal reporter vector, 63-iDQgal, is 12 kb in size, with two multiple cloning sites. The ecm promoter of the vector was replaced with the *5nt* promoter in this study. The Tn5 gene was replaced with the G418 gene, so the vector contains genes that confer ampicillin and G418 resistance for positive transformant selection (Fig. 1.7). The product of the *lacZ* reporter gene is ß-gal, the first gene in the lac operon, which converts lactose to galactose and glucose. To assay for ß-galactosidase activity, an analog of lactose,

orthonitrophenyl ß-D-galactoside (ONPG), is used. When ß-galactosidase hydrolyzes this substrate a bright yellow nitrophenolate ion is produced. Quantification can be achieved by measuring absorbance at 415 nm.

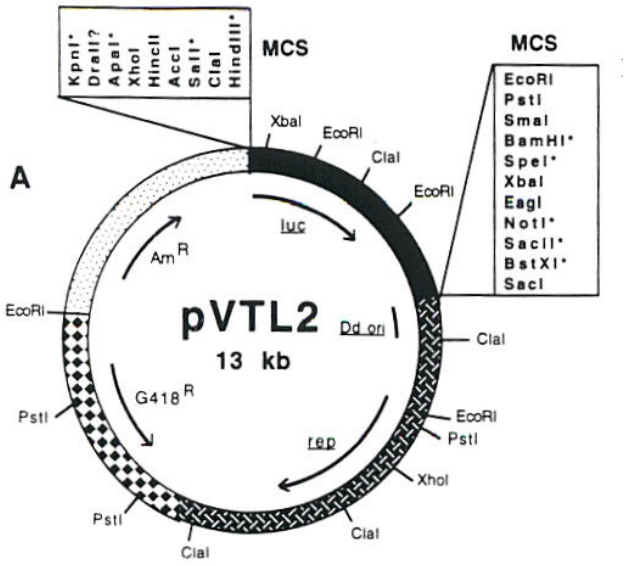

Figure 1.6. Features of the pVTL2 Reporter Vector. This 13 kb autonomously propagating, low copy vector contains the reporter gene, *luciferase*. In this study, the upstream multiple cloning site was used to incorporate various mutagenized forms of a particular promoter. Selection of cells successfully transformed with the vector was based upon resistance to ampicillin and G418. The ability of the promoter to drive expression was quantified by measuring the bioluminescence produced from the reaction of luciferase with the substrate luciferin.

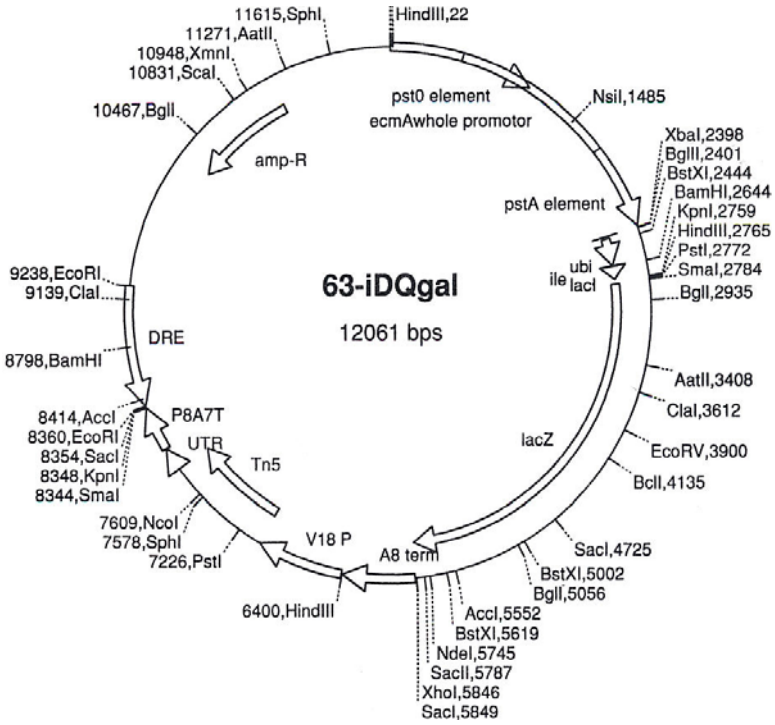

Figure 1.7. Features of the 63-iDQgal Reporter Vector. This 12 kb vector contains the reporter gene, *lac Z*. In this study, a cloning site upstream from the reporter gene was used to incorporate various mutagenized forms of a particular promoter. Cells successfully transformed with the vectors were selected by their ability to survive in the presence of ampicillin and G418. The ability of the promoter to drive expression was quantified by measuring absorbance at 415 nm after extract was incubated with the artificial substrate ONPG.

1.9. Significance of Research and Research Objectives

The regulation of gene expression throughout the development of multicellular organisms is critical to the process of morphogenesis. In response to specific environmental cues, a complex network of control mechanisms work together to stimulate or inhibit certain genes as appropriate to the cell type and life cycle stage. During morphogenesis in *Dictyostelium*, levels of cAMP induce the expression of genes in some cell types and repress genes in other cells types, a process critical for proper cell differentiation to be achieved.

5nt is a developmentally-regulated gene whose expression is also induced in the presence of cAMP. The product of *5nt* has been implicated in the cAMP degradation pathway. By elucidating the transcriptional regulatory mechanisms that control *5nt* expression throughout development, a more detailed model of morphogenesis can be made. The objectives of this research were to: 1) delineate *cis*-acting elements within the promoter of *5nt* involved in temporal regulation, 2) define *cis*-acting elements within the promoter of *5nt* whose regulatory activities are influenced by the presence of cAMP, 3) identify *trans*-acting proteins that associate with specific nucleotide cis-acting elements, and 4) purify and characterize the *trans*-acting proteins.

The four research objectives were achieved as a result of a collaborative effort within our research group. To identify elements in the *5nt* promoter involved in the temporal regulation of gene expression, 5' and internal promoter deletions were created and fused to the *luciferase* and *lacZ* reporter genes. After the cloning step of this type of experiment is achieved, the subsequent steps of *Dictyostelium* transformation, growth and development, and enzyme activity assays require a considerable period of time. By

working with a former graduate student, Can Eristi and a former undergraduate researcher, Lauren Sangenario, a large number of promoter deletions were generated and tested. Lauren Sangenario also contributed to site-directed mutagenesis experiments. The electromobility gel shift assays to identify *trans*-acting proteins interacting with the *5nt* promoter were performed by Can Eristi and Dr. Rutherford. The purification of these proteins involved chromatographic techniques followed by the analysis of fractions by electromobility shift assay. My participation in the chromatographic processes of the two proteins purified in these studies involved the preparation of ammonium sulfate fractions from cytoplasmic slug extract, preparing and setting up DEAE Sephacel and Heparin Sepharose Columns, and testing fractions collected by EMSA.

References

Araki, T., Gamper, M., Early, A., Fukuzawa, M., Abe, T., Kawata, T., Kim, E., Firtel, R. A., and Williams, J. G. (1998). "Developmentally and spatially regulated activation of a Dictyostelium STAT protein by a serpentine receptor." EMBO J. **17**: 4018-4028.

Armant, D. R. (1980). Purification, characterization and localization of 5'AMP nucleotidase during pattern formation in Dictyostelium discoideum. Blacksburg, VA, Virginia Polytechnic Institute and State University**:** 108.

Bhanot, P. and G. Weeks (1989). "The activation of Dictyostelium discoideum alkaline phosphatase by carbohydrate binding proteins." Biochem. Cell Biol. **67**: 246-249.

Bonfils, C., Gaudet, P., and Tsang, A. (1999). "Identification of cis-regulating elements and trans-acting factors regulating the expression of the gene encoding the small subunit of ribonucleotide reductase in Dictyostelium discoideum." J. Biol. Chem. **274**: 20384-20390.

Bonner, J. T. (1944). "A descriptive study of the development of the slime mold Dictyostelium discoideum." Am. J. Bot. **31**: 175-182.

Bonner, J. T. (1947). "Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold Dictyostelium discoideum." J. Exp. Zool. **106**: 1-26.

Bonner, J. T., Hall, E. M., Sachsenmaier, W., and Walker, B. K. (1970). "Evidence for a second chemotactic system in the cellular slime mold, Dictyostelium discoideum." J. Bacteriol. **102**: 682-687.

Brown, J. M. and R. A. Firtel (2001). "Functional and regulatory analysis of the Dictyostelium G-box binding factor." Dev. Biol. **234**: 521-534.

Brown, S. S. and C. L. Rutherford (1980). "Localization of cyclic nucleotide phosphodiesterase in the multicellular stages of Dictyostelium discoideum." Differentiation **16**: 173-183.

Carey, M. and S.T. Smale (2000). Transcriptional Regulation in Eukaryotes: Concepts, Strategies and Techniques. Cold Spring Harbor Laboratory Press.

Ceccarelli, A., Mahbubani, H., and Williams, J. G. (1991). "Positively and negatively acting signals regulating stalk cell and anterior-like cell differentiation in Dictyostelium." Cell **65**: 983-989.

Chanchao, C., Eristi, C. M., Favis, R., and Rutherford, C. L. (1999). "5'-Nucleotidase in Dictyostelium: protein purification, cloning, and developmental expression." Biochim. Biophys. Acta **1473**: 376-390.

Clarke, M., Dominguez, N., Yuen, I. S., and Gomer, R. H. (1992). "Growing and starving Dictyostelium cells produce distinct density-sensing factors." Dev. Biol. **152**: 403-406.

Codogno, P., M. A. Doyennette-Moyne, Aubery M, Dieckhoff J, Lietzke R, and Mannherz HG. (1988). "Polyclonal and monoclonal antibodies against chicken gizzard 5'-nucleotidase inhibit the spreading process of chicken embryonic fibroblasts on laminin substratum." Exp Cell Res **174**(2): 344-54.

Das, D. V. M. and G. Weeks (1981). "The inhibition of Dictyostelium discoideum alkaline phosphatase by a low molecular weight factor and its implication for the developmental regulation of the enzyme." FEBS Lett. **130**: 249-252.

Devine, K. M. and W. F. Loomis (1985). "Molecular characterization of anterior-like cells in Dictyostelium discoideum." Dev. Biol. **107**: 364-372.

Dinauer, M. C., MacKay, S. A., and Devreotes, P. N. (1980). "Cyclic 3',5'-AMP relay in Dictyostelium discoideum III. The relationship of cAMP synthesis and secretion during the cAMP signaling response." J. Cell Biol. **86**: 537-544.

Esch, R. K., Howard, P. K., and Firtel, R. A. (1992). "Regulation of the Dictyostelium cAMP-induced, prestalk-specific DdrasD gene - Identification of cis-acting elements." Nucl. Acids Res. **20**: 1325-1332.

Fosnaugh, K. L. and W. F. Loomis (1993). "Enhancer regions responsible for temporal and cell-type-specific expression of a spore coat gene in Dictyostelium." Dev. Biol. **157**: 38-48.

Gaskell, M. J., Jermyn, K. A., Watts, D. J., Treffry, T., and Williams, J. G. (1992). "Immunolocalization and separation of multiple prestalk cell types in Dictyostelium." Differentiation **51**: 171-176.

Gomer, R. H. and R. R. Ammann (1996). "A cell-cycle phase-associated cell-type choice mechanism monitors the cell cycle rather than using an independent timer." Dev. Biol. **174**: 82-91.

Guo, K., Anjard, C., Harwood, A., Kim, H. J., Newell, P. C., and Gross, J. D. (1999). "A myb-related protein required for culmination in Dictyostelium." Development **126**: 2813-2822.

Haberstroh, L. and R. A. Firtel (1990). "A spatial gradient of expresssion of a cAMP-regulated prespore cell type-specific gene in Dictyostelium." Genes Devel. **4**: 596-612.

Haberstroh, L., Galindo, J., and Firtel, R. A. (1991). "Developmental and spatial regulation of a Dictyostelium prespore gene - cis-acting elements and a cAMP-induced, developmentally regulated DNA binding activity." Development **113**: 947-958.

Hall, A. L., Franke, J., Faure, M., and Kessin, R. H. (1993). "The role of the cyclic nucleotide phosphodiesterase of Dictyostelium discoideum during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the pde." Dev. Biol. **157**: 73-84.

Hori, R. and R. A. Firtel (1994). "Identification and characterization of multiple A/T-rich cis-acting elements that control expression from Dictyostelium actin promoters: The Dictyostelium actin upstream activating sequence confers growth phase expression and has enhancer-like properties." Nucl. Acids Res. **22**: 5099-5111.

Kawata, T., Shevchenko, A., Fukuzawa, M., Jermyn, K. A., Totty, N. F., Zhukovskaya, N. V., Sterling, A. E., Mann, M., and Williams, J. G. (1997). "SH2 signaling in a lower eukaryote: A STAT protein that regulates stalk cell differentiation in Dictyostelium." Cell **89**: 909-916.

Kay, R. R. (1997). DIF signalling. Dictyostelium - A model system for cell and developmental biology. Y. Maeda, K. Inouye and I. Takeuchi. Tokyo, Japan, Universal Academy Press**:** 279-292.

Kay, R. R., Flatman, P., and Thompson, C. R. L. (1999). "DIF signalling and cell fate." Sem. Cell Dev. Biol. **10**: 577-585.

Kay, R. R., Town, C. D., and Gross, J. D. (1979). "Cell differentiation in Dictyostelium doiscoideum." Differentiation **13**: 7-14.

Kessin, R. H. (2001). Dictyostelium - Evolution, cell biology, and the development of multicellularity. Cambridge, UK, Cambridge Univ. Press.

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

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

Loomis, W. F. (1993). "Lateral inhibition and pattern formation in Dictyostelium." Curr. Topics Dev. Biol. **28**: 1-46.

Maniak, M. and W. Nellen (1990). "Evidence for a feedback regulated back-up promoter which controls permanent expression of a Dictyostelium gene." Nucl. Acids Res. **18**: 5375-5380.

Matsuda, Y., Masamune, Y., Kodaira, K., and Yasukawa, H. (1999). "Analysis of the disruption mutant of the oscillin homolog gene of Dictyostelium discoideum." Biol. Pharmaceut. Bull. **22**: 915-919.

McPherson, C. E. and C. K. Singleton (1993). "Nutrient-responsive promoter elements of the V4 gene of Dictyostelium discoideum." J. Mol. Biol. **232**: 386-396.

Merkle, R. K., Cooper, K. K., and Rutherford, C. L. (1984). "Localization and levels of cyclic AMP during development of Dictyostelium discoideum." Cell. Differ. **14**: 257-266.

Milne, J. L. and P. N. Devreotes (1993). "The surface cyclic AMP receptors, cAR1, cAR2, and cAR3, promote Ca2+ influx in Dictyostelium discoideum by a Galpha2-independent mechanism." Mol. Biol. Cell **4**: 283-292.

Mohanty S and Firtel RA (1999). Control of spatial patterning and cell-type proportioning in Dictyostelium. Semin Cell Dev Biol.10(6):597-607. Review.

Nayler, O., Insall, R., and Kay, R. R. (1992). "Differentiation-inducing-factor dechlorinase, a novel cytosolic dechlorinating enzyme from Dictyostelium discoideum." Eur. J. Biochem. **208**: 531-536.

Otsuka, H. and P. J. M. van Haastert (1998). "A novel Myb homolog initiates Dictyostelium development by induction of adenylyl cyclase expression." Genes Devel. **12**: 1738-1748.

Powell-Coffman, J. A. and R. A. Firtel (1994). "Characterization of a novel Dictyostelium discoideum prespore-specific gene, PspB, reveals conserved regulatory sequences." Development **120**: 1601-1611.

Powell-Coffman, J. A., Schnitzler, G. R., and Firtel, R. A. (1994). "A GBF-binding site and a novel AT element define the minimal sequences sufficient to direct prespore-specific expression in Dictyostelium discoideum." Mol. Cell. Biol. **14**: 5840-5849.

Raper, K. B. (1935). "Dictyostelium discoideum, a new species of slime mold from decaying forest leaves." J. Agr. Res. **50**: 135-147.

Raper, K. B. (1937). "Growth and development of Dictyostelium discoideum with different bacterial associates." J. Agr. Res. **55**: 289-316.

Raper, K. B. and D. I. Fennell (1952). "Stalk formation in Dictyostelium." Bull. Torrey Bot. Club **79**: 25-51.

Raper, K. B. and C. Thom (1932). "The distribution of Dictyostelium and other slime molds in soil." J. Wash. Acad. Sci. **22**: 93-96.

Rathi, A. and M. Clarke (1992). "Expression of early developmental genes in Dictyostelium discoideum is initiated during exponential growth by an autocrine-dependent mechanism." Mech. Devel. **36**: 173-182.

Rutherford, C. L., Overall, D. F., Ubeidat, M., and Joyce, B. R. (2003). "Analysis of 5' nucleotidase and alkaline phosphatase by gene disruption in Dictyostelium." Genesis **35**: 202-213.

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

Schaap, P., Tang, Y. H., and Othmer, H. G. (1996). "A model for pattern formation in Dictyostelium discoideum." Differentiation **60**: 1-16.

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

Schnitzler, G. R., Fischer, W. H., and Firtel, R. A. (1994). "Cloning and characterization of the G-box binding factor, an essential component of the developmental switch between early and late development in Dictyostelium." Genes Devel. **8**: 502-514.

Seager, J. H. J., Stevens, B. A., McCaffery, I., and Hames, B. D. (2001). "Identification of novel elements which regulate the cell-type specificity of Dictyostelium 7E gene expression." Differentiation **68**: 22-30.

Siu, C.-H., Cho, A., and Choi, H. C. (1987). "The contact site a glycoprotein mediates cell-cell adhesion by homophilic binding in Dictyostelium discoideum." J. Cell Biol. **105**: 2523-2533.

Siu, C.-H., Cho, A. S., and Choi, A. (1986). Mechanism of action of the contact site A glycoprotein during development of Dictyostelium discoideum. J. Cell Biol.

Siu, C.-H. and T. Y. Lam (1988). "Mediation of cell-cell adhesion by the altered contact site A glycoprotein expressed in modB mutants of Dictyostelium discoideum." Exp. Cell Res. **177**: 338-346.

Siu, C.-H., Lam, T. Y., and Wong, L. M. (1988). "Expression of the contact site A glycoprotein in Dictyostelium discoideum: quantitation and developmental regulation." Biochim. Biophys. Acta **968**: 283-290.

Sternfeld, J. and C. N. David (1982). "Fate and regulation of anterior-like cells in Dictyostelium slugs." Dev. Biol. **93**: 111-118.

Stevens, B. A., Flynn, P. J., Wilson, G. A., and Hames, B. D. (2001). "Control elements of Dictyostelium discoideum prespore specific gene 3B." Differentiation **68**: 92-105.

Stober-Grasser, U., Brydolf, B., Bin, X., Grasser, F., Firtel, R. A., and Lipsick, J. S. (1992). "The myb DNA-binding domain is highly conserved in Dictyostelium discoideum." Oncogene **7**: 589-596.

Sussman, R. and M. Sussman (1967). "Cultivation of Dictyostelium discoideum in axenic culture." Biochem. Biophys. Res. Commun. **29**: 53-55.

Tabata, K., Matsuda, Y., Viller, E., Masamune, Y., Katayama, T., and Yasukawa, H. (2001). "Myb-binding site regulates the expression of glucosamine-6-phosphate isomerase in Dictyostelium discoideum." Devel. Growth Differ. **43**: 583-589.

Trinklein ND, Aldred SF, Hartman SJ, Schroeder DI, Otillar RP, Myers RM. An abundance of bidirectional promoters in the human genome. Genome Res. 2004 Jan;14(1):62-6.

Ubeidat, M., Eristi, C. M., and Rutherford, C. L. (2002). "Expression pattern of 5'-nucleotidase in Dictyostelium." Mech. Devel. **110**: 237-239.

Uusitalo, R. J. (1981). "5'-Nucleotidase activity in lymphocytes." Histochem J **13**(4): 525-34.

VanDriessche, N., Shaw, C., Katoh, M., Morio, T., Sucgang, R., Ibarra, M., Kuwayama, H., Saito, T., Urushihara, H., Maeda, M., Takeuchi, I., Ochiai, H., Eaton, W., Tollett, J., Halter, J., Kuspa, A., Tanaka, Y., and Shaulsky, G. (2002). "A transcriptional profile of multicellular development in Dictyostelium discoideum." Development **129**: 1543-1552.

Vauti, F., Morandini, P., Blusch, J., Sachse, A., and Nellen, W. (1990). "Regulation of the discoidin-Igamma-gene in Dictyostelium discoideum - identification of individual promoter elements mediating induction of transcription and repression by cyclic AMP." Mol. Cell. Biol. **10**: 4080-4088.

Verkerke-van Wijk, I., Brandt, R., Bosman, L., and Schaap, P. (1998). "Two distinct signaling pathways mediate DIF induction of prestalk gene expression in Dictyostelium." Exp. Cell Res. **245**: 179-185.

Williams, J. G. and K. A. Jermyn (1991). Cell sorting and positional differentiation during Dictyostelium morphogenesis. Cell-Cell Interactions in Early Development. J. Gerhart. New York, Wiley-Liss**:** 261-272.

Yin, Y. Z., Williamson, B. D., and Rutherford, C. L. (1994). "An autonomously propagating luciferase-encoding vector for Dictyostelium discoideum." Gene **150**: 293-298.

Zimmerman, W. and C. J. Weijer (1993). "Analysis of cell cycle progression during the development of Dictyostelium and its relationship to differentiation." Dev. Biol. **160**: 178-185.

Chapter 2

Identification of Temporal Sites Involved in the Transcriptional Regulation of 5'-*nucleotidase* in *Dictyostelium discoideum* by Deletion and Mutagenesis Analysis

Abstract

In order to evaluate control mechanisms of transcription in a developmentally regulated gene, an extensive analysis of the *Dictyostelium discoideum 5'-nucleotidase* (*5nt*) promoter was performed. Assessment of the promoter involved the construction and fusion of 5' and internal promoter deletions to the *luciferase* and *lacZ* reporter genes, followed by measurements of reporter enzyme activity in cells from the slug stage of development. Regions of potential interest in the promoter were subsequently examined by creating point mutations. These sites were then evaluated by comparing the enzyme activity levels in slugs after fusion of the promoters to the *luciferase* and *lacZ* reporter genes. The data obtained from these studies provided a useful resource as electromobility gel shift assays were performed to identify sites on the promoter to which proteins were bound. As a result of the electromobility gel shift assays, two DNA- binding activities have been identified. The binding sites have been evaluated, and their specific interactions with the proteins confirmed. The function of these binding activities as transcription regulators has not been established, although their recognition and interaction with specific sequences in the *5nt promoter* suggest this.

Introduction

Gene regulation is a critical aspect of normal development, energy conservation, metabolic control, and responses to environmental cues, disease and pathogens in eukaryotic organisms. In order to appropriately respond to environmental changes and progress through the life cycle, an organism must control the expression levels of a large number of genes by utilizing available gene regulation mechanisms. While mechanisms of regulation can range from the architecture of nuclear chromatin to RNA splicing and protein modification, one of the most common control points exists at the level of gene transcription. The transcription machinery required for the production of an RNA transcript must have access to specific regions of the gene. The interaction of proteins with specific nucleotide sequences within the gene promoter requires accessibility. To elucidate some of the mechanisms involved in the regulation of gene expression at the level of transcription, this study has involved an extensive analysis of the *Dictyostelium discoideum 5nt* promoter to identify potential control elements necessary for normal levels of expression.

The protein product of *5nt* is present in a great variety of species, where it is found in many cell types, and in many locations, including the cell membrane, the cytosol and mitochondria. The enzyme's residence on the exterior surface of plasma membranes enables it to carry out one of its major functions of converting nucleotides to nucleosides, thereby permitting their entry into the interior of cells (Resta et al. 1998; Gazziola et al. 1999). While this function is common to most ecto-nucleotidases, 5NT has been implicated with additional activities as well. Other functions of the membrane-bound

protein include the involvement in cell-cell interactions as well as other general transport processes (Uusitalo 1981; Codogno et al. 1988). In mammals, 5NT has been associated with the prevention of deoxyribonucleotide accumulation by generating adenosine from AMP, suggesting the involvement of the enzyme in regulating intracellular cAMP levels. Adenylate cyclase is responsible for the production of cAMP, while cAMP phosphodiesterase degrades the product, generating 5'AMP during the process. Because the product of *5nt* removes 5'AMP by degrading it to produce adenosine and inorganic phosphate, it is thought to prevent accumulation of 5'AMP that could otherwise inhibit further cAMP degradation (Krivanek 1956; Bhanot and Weeks 1985; Chanchao et al. 1999). In humans, abnormal levels of *5nt* expression have been associated with autism, and changes in the pattern of 5NT localization have been observed on the apical surface of cells, suggesting that the enzyme may function in adhesion activities and cellular recognition (Yoneyama et al. 2000).

5nt is a developmentally regulated gene that is temporally expressed during the life cycle of *Dictyostelium.* The 24-hr life cycle of this model organism has enabled a progressive analysis of *5nt* activity at each of the distinct stages of development. Previous research has shown that expression of the gene is detected 5 hr after the initiation of multicellular development, when amoebae cells first begin to form the loose aggregate (Chanchao et al. 1999). Localization of activity was determined to be scattered at this stage by observing in situ staining of *ß-galactosidase* activity after fusion of the *5nt* promoter to the *lacZ* reporter gene (Ubeidat et al. 2002). Levels of *5nt* activity increase during the formation of the first finger, and that activity was mainly localized to the base of the structure. As activity levels remained high through the slug stage and culmination

stage of development, the majority of activity was localized to the pstAB cells and slime sheath of the slug. In the late culminant, activity levels were detected in the lower cup, stalk and basal disk. Due to the nature of *5nt* expression throughout development, the gene provides an ideal platform from which transcriptional regulation can be analyzed and better understood. The initial approach to these studies involved creating a series of 5' and internal promoter deletions and fusing them to the *luciferase* and *lacZ* reporter genes.

A thorough analysis of the *5nt* promoter sequence was conducted in these studies in order to identify potential control elements critical for normal levels of gene expression during development. While increasingly sizeable 5' promoter deletions enabled a general region of interest to be identified for use in further experiments, internal promoter deletions fused to both the *luciferase* and *lacZ* reporter genes offered insight into more precise potential regulatory sites. To account for the potential requirement of spatial integrity for normal levels of gene expression, a 250 bp region of the promoter, selected due to the results obtained from the deletion studies, was targeted for site directed mutagenesis experiments. Taking into account the data produced from the deletion experiments and site-directed mutagenesis assays, approximately 4 defined elements were identified as potential control points on the *5nt* promoter. Probes designed to span a large region of the *5nt* promoter were used in subsequent gel shift assays, and two specific DNA-protein binding activities were identified as potential transcription regulators of *5nt*.

Materials and Methods

Exonuclease-Mung Bean System for 5' Deletions

The template used to generate deletions was c167, the pBluescript vector containing the full *5nt* promoter and coding region. The initial digestion reaction utilized 6 µg of plasmid DNA, purified by a Wizard Plus SV Maxiprep DNA Purification kit (Qiagen), with 20U of the *Sac*I restriction enzyme in a total reaction mix volume of 50 µl. After a 2 h digestion at room temperature, ethanol was added to precipitate the DNA, which was then incubated with 40U of the *Eco*RI restriction enzyme for an additional 2 h. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was then added to the reaction to extract the DNA, which was subsequently precipitated using ethanol from the final aqueous phase. After resuspending the dried DNA pellet in 48 µl Exonuclease III buffer, 250U of the Exonuclease III enzyme were added and the reaction mix was incubated for 5 min in a 30°C water bath. Tubes chilled on dry ice containing 2 µl of 10X Mung Bean nuclease buffer and 6 µl of milliQH_2O were placed beside the water bath, and 10 µl aliquots from the reaction mix were transferred to these tubes at 1 min intervals.

To inactivate the exonuclease enzyme, the tubes were incubated at 68°C for 15 min, and then incubated on ice for 5 min. After the incubation periods, 3U of Mung Bean nuclease were added to each tube, and the reactions were incubated at 30°C for 30 min. After the incubation period, ethanol was used to precipitate the DNA, which was then resuspended in milliQH_2O and separated on a 1% TAE agarose gel. The Exonuclease III

enzyme and buffer, and the Mung Bean nuclease enzyme and buffer used in this experiment were from New England BioLabs (NEB). The bands observed on the gel were excised, and a GeneClean III Kit (BIO101) was used to purify the DNA samples. To re-circularize the plasmid DNA that had been digested and exposed to Exonuclease III, the purified samples were incubated in the presence of T4 DNA ligase at 16°C for 16 hr. Electrocompetent XL1-Blue cells (Stratagene) were transformed with the plasmid DNA, a Wizard Plus SV Miniprep DNA Purification kit (Qiagen) was used to isolate the plasmid, and the sizes of the 5' promoter deletions were determined by PCR.

The plasmid DNA was then used as template in a PCR reaction to amplify the 5'deleted promoter. Primers T7 and GS3R with engineered *Hind*III sites were used for amplification using the following thermocycler program: 2.5 min initial denaturation at 94°C, 28 cycles including 30 sec at 94°C, 30 sec at 54°C, and 2 min at 72°C, followed by a final 10 min extension at 72°C. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was used to extract the DNA from the PCR reactions, and the DNA was subsequently precipitated using ethanol. The PCR products were then incubated with 30U *Hind*III overnight at 37°C. There is an endogenous *Hind*III site -663 bp from ATG translation start site. Promoter constructs that extended past this site were subjected to a "partial" digestion using 1U *Hind*III / 225ng DNA for 90 min. The digested PCR products were visualized on a 1% TAE agarose gel, and bands were excised and purified using the GeneClean III Kit (BIO101).

Fragment Ligation Approach for Internal Deletions

Generation of internal promoter deletions by fragment-ligation entailed designing pairs of PCR reactions that would amplify two sections of the full promoter. The nucleotide sequence between the fragments produced represented the region of the promoter to be deleted. A variety of forward and reverse primers were designed to be used for this approach. Using the pBluescript T7 primer in combination with various reverse primers enabled the generation of multiple "upstream fragments." Similarly, using the GSIR-*Hind*III primer in combination with various forward primers enabled the generation of multiple "downstream fragments." The generation of this pool of upstream and downstream fragments involved 50 µl PCR reactions, which used 10 ng of the C167 template previously described. The reaction mix included 1 µM each of the forward and reverse primer, 80 µM dNTP, 1.2 mM $MgCl_2$, 0.01% Tween 20, and 2.5 U Taq polymerase. These components were thoroughly mixed with PCR buffer, comprised of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, and 50 mM KCl. The thermocycler program used included an initial 2.5 min denaturation at 94°C. The next 30 cycles included: 30 sec at 94°C, 1 min at 56°C, and 2 min at 72°C. The final extension step was 10 min at 72°C.

The upstream and downstream fragments produced were phosphorylated by combining 2.5 µl of the PCR product with 1 mM ATP in T4 polynucleotide kinase buffer in a reaction volume of 48 µl. The reaction mix was incubated at 70°C for 5 min, followed by incubation on ice for 5 min. After the reaction had cooled, 20 U T4 PNK were added and the reaction mix was incubated at 37°C for 30 min. After phosphorylation, individual upstream and downstream fragments were combined in a

ligation reaction, which included 5 µl of each fragment and 400 U T4 DNA ligase. The ligation reactions were incubated at room temperature for 20 min.

The products of the ligation reactions were used as templates for a second PCR reaction that amplified the internally deleted promoter sequence. The reaction mix included 1 µM of the promoter-specific F22-*Hind*III primer, 1 µM of the gene-specific GSIR-*Hind*III primer, 80 µM dNTP, 1.5 mM $MgCl_2$, 0.01% Tween 20, and 2.5 U Taq polymerase in PCR buffer. The same thermocycler program previously described was used, and the amplified products were visualized on a 1%TBE gel to confirm promoter size. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was used to extract the DNA from the PCR reactions, and the DNA was subsequently precipitated using ethanol. The PCR products were then incubated with 10.6 U *Hind*III overnight at 37°C. The digested PCR products were visualized on a 1% TAE agarose gel, and bands were excised and purified using the GeneClean III Kit (BIO101).

Vector Amplification Approach for Internal Deletions

The generation of internal promoter deletions by vector amplification involved designing various combinations of forward and reverse promoter-specific primers that directed away from each other on the C167 vector template. With the primers directed away from each other within the promoter, a PCR reaction with a long extension time enabled the amplification of the entire vector, except for the nucleotides in the promoter between the two primers. Various combinations of forward and reverse primers were made to specifically remove internal portions of the promoter. The amplification of the

vector to precisely remove internal promoter sequence involved 50 µl PCR reactions, which used 10 ng of the C167 template previously described. The reaction mix included 1 µM each of the forward and reverse primer, 80 µM dNTP, 1.5 mM $MgCl_2$, 0.01% Tween 20, and 5 U Taq polymerase in PCR buffer. The thermocycler program used included an initial 2.5 min denaturation at 94°C. The next 25 cycles included: 30 sec at 94°C, 30 sec at 50°C, and 10 min at 72°C. The final extension step was 10 min at 72°C.

To digest overhangs remaining after PCR, 135 µl of the PCR reaction was combined with 2.5 U Klenow and 30 µM dNTP and incubated at room temperature for 30 min. DNA was purified from the reaction using a GeneClean III kit (BIO101) and resuspended in 20 µl of water. To phosphorylate the purified PCR products, 0.8 mM ATP and T4 Polynucleotide Kinase (PNK) buffer were added, the reaction mixture was incubated at 70°C for 5 min, and then incubated on ice for an additional 5 min. After incubation, 8 U T4 PNK were added, and the reaction mix was incubated at 37°C for 30 min. The phosphorylated products were visualized on a 1% TAE agarose gel, and the band were excised and purified using the GeneClean III kit (BIO101). In order to re-circularize the vectors containing precise internal promoter deletions, 5 µl of the phosphorylated product were combined with 400 U T4 DNA ligase in T4 DNA ligase buffer in a total reaction volume of 10 µl.

The products of the ligation reactions were used as templates for a second PCR reaction that amplified the internally deleted promoter sequence. The reaction mix included 1 µM of the promoter-specific F22-*Hind*III primer, 1 µM of the gene-specific GSIR-*Hind*III primer, 80 µM dNTP, 1.5 mM $MgCl_2$, 0.01% Tween 20, and 2.5 U Taq polymerase in PCR buffer. While the aforementioned reaction prepared the DNA for

insertion into a *Hind*III cut pVTL2 reporter vector, F22-*Xba*I and GSIR-*Xba*I primers were used to prepare the DNA for insertion into the *Xba*I cut ß-gal reporter vector. The same thermocycler program described above was used, and the amplified products were visualized on a 1% TBE gel to confirm promoter size. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was used to extract the DNA from the PCR reactions, and the DNA was subsequently precipitated using ethanol. The PCR products were then incubated with 10.6 U *Hind*III overnight at 37°C. The digested PCR products were visualized on a 1% TAE agarose gel, and bands were excised and purified using the GeneClean III Kit (BIO101).

Site-Directed Mutagenesis

Forward and reverse primers, ranging between 38 and 44 bp in length were designed to contain specific point mutations that would be transferred to the *5nt* promoter template at the completion of the procedure. The mutations were designed to be in the middle of the primer. Primers were designed to anneal to regions of the promoter that were relatively GC rich. The mutagenized sites were 6 or 12 nucleotides in length, with an internal portion replaced with sequence containing the *Sty*I restriction enzyme recognition site: 5' CCAAGG 3'.

The mutant strand synthesis reaction using thermal cycling followed the protocol described in a QuikChange Site-Directed Mutagenesis Kit (Stratagene). In the first thermal cycling reaction, each reaction contained a single primer. The reaction components included the C167 template, 80 μM dNTP, 1.5 mM $MgCl_2$, 0.01% Tween

20, and 2.5 U Taq polymerase in PCR buffer. The program used consisted of a 95°C initial denaturation for 30 sec, followed by 5 cycles consisting of: 30 sec at 95°C, 1 min at 50°C and 5 min at 68°C. At the completion of cycling, the forward primer reactions were combined with the corresponding reverse primer reaction, and additional Taq polymerase was added for a second program. The second program consisted of a 95°C initial denaturation for 30 sec, followed by 18 cycles consisting of: 95°C, 1 min at 50°C and 5 min at 68°C. Nicked, circular strands containing the mutagenic sites were the result of these reactions. To remove the methylated, non-mutated parental strands from the reaction, 10 U / µl of *Dpn*I endonuclease was added and the reactions were incubated at 37°C for 1 hr. XL1-Blue supercompetent cells (Stratagene) were then transformed by heat shock with the nicked vector containing the specified mutation.

Heat shock bacterial transformation entailed first cooling polypropylene culture tubes on ice. A tube containing NZY broth (described in QuikChange Stratagene kit) was incubated at 42°C, and XL1-Blue supercompetent cells were thawed on ice. Into the chilled culture tubes, 50 µl of competent cells were added followed by the addition of 5 µl of the *Dpn*I reaction mix. The contents were gently swirled and then incubated on ice for 30 min. The reactions were heat-pulsed at 42°C for 45 sec, and placed back on ice for 2 min. To each tube, 500 µl of NZY broth was added and the reactions were shaken 250 rpm at 37°C for 1 hr. After the incubation period, 250 µl of each reaction were plated on a warm LB amp plate and incubated overnight at 37°C.

Colonies present on plates were re-streaked and then used to start liquid cultures of the cells in LB amp medium. Cultures were grown in 3 ml of medium overnight, and a Wizard Plus SV Miniprep DNA Purification kit (Qiagen) was used to isolate the

plasmids. Plasmids were then digested overnight with *Sty*I to identify those in which the mutagenized site had been successfully incorporated. Those that were successfully digested were then sequenced at the VBI Core Lab at Virginia Tech to confirm that the mutation was intact and that the sequence was correct. The plasmids were then used as template in a PCR reaction to prepare the insert for incorporation into the ß-gal and pVTL2 reporter vectors. T7 and GSIR-*Hind*III primers were used to amplify the mutagenized promoter for incorporation into the pVTL2 vector, while T7 and GSIR-*Xba*I primers were used in preparation for insertion into the ß-gal vector. The program used for these reactions included an initial denaturation step at 94°C for 2 min and 30 sec, followed by 29 cycles consisting of: 30 sec at 94°C, 30 sec at 50°C, and 2 min at 72°C. There was a final extension period of 10 min at 72°C. Five reactions for each construct were prepared for PCR, and then pooled. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was used to extract the DNA from the PCR reactions, and the DNA was precipitated using ethanol. The products were incubated overnight at 37°C with either 20 U / µl of *Hind*III or *Xba*I. Digested DNA was then visualized on a 1% TAE agarose gel, and bands were excised and purified using the Gene Clean III kit (BIO101).

Cloning into Reporter Vectors and Transformation into E.coli SURE cells

The final products of the 5' and internally mutagenized promoters generated by the Exonuclease Mung Bean system, Fragment Ligation approach and Vector Amplification approach were gel-purified products, containing either *Hind*III restriction

sites or *Xba*I restriction sites on both ends of the mutagenized promoter fragment. The method of incorporating the mutagenized promoter into the appropriate reporter vector was the same regardless of the initial mutagenesis step.

Mutagenized promoters with 5' *Hind*III overhangs were incubated with a CIP-treated pVTL2 reporter vector. The vector, containing a single *Hind*III site within the multiple cloning region, had been digested with the enzyme before the incubation period. The ligation reaction consisted of a 20:1 insert: vector ratio and 320U T4 DNA ligase in the T4 ligase buffer. Mutagenized promoters with 3' *Xba*I overhangs were incubated with a CIP-treated ß-gal reporter vector. The vector, containing a single *Xba*I site in the multiple cloning region, had been digested with the enzyme before the incubation period. The ligation reaction consisted of a 2:1 insert: vector ratio and 320U T4 DNA ligase in the T4 ligase buffer. All ligation reactions were incubated at 16°C for 16 hr. Ethanol was used to precipitate the product, which was then resuspended in 10µl of water. The product was then transformed into *E.coli* SURE cells by electroporation.

The 10 µl ligation reaction was added to electrocompetent SURE cells and transferred to a chilled cuvette, where electroporation conditions of the BioRad Gene Pulser included 25 µF (gene pulser), 200 Ω, 960 µF (capacitance) at 1.8 kV. After electroporation, cells were immediately transferred to warm SOC medium in culture tubes and shaken at 250 rpm for 1 hr. After an hr, 250 µl from the culture tubes were spread on warm LB amp plates and incubated overnight at 37°C. Colonies formed on the plates were tested by PCR. Colony lysates were prepared by transferring bacteria cells from the colony into colony lysis buffer, followed by a 10 min incubation period at 95°C. To detect the presence of the mutagenized promoter insert and its orientation in the ß-gal

vector, GalIR, a vector-specific primer, and F19, a promoter-specific primer were utilized. To detect the presence of the mutagenized promoter insert and its orientation in the pVTL2 vector, Luc2R, a vector-specific primer, and GSIF, a promoter-specific primer, were utilized. The PCR conditions for both types of reactions included an initial denaturation step for 2 min and 30 sec at 94°C, followed by 29 cycles of: 30 sec at 94°C, 30 sec at 50°C, and 2 min at 72°C. The final extension step was 10 min at 72°C. Colonies containing cells determined to include the vector with insert were grown in liquid LB amp medium overnight and a midiprep kit (Qiagen) was used for plasmid isolation. The reporter vectors containing mutagenized promoter inserts were then used to transform *Dictyostelium* AX3K cells.

Dictyostelium Transformation by Electroporation and Calcium / Glycerol Shock

Transformation of reporter vectors into *Dictyostelium* AX3K cells was performed by either electroporation or a calcium/glycerol shock approach. AX3K cells used for transformation experiments were shaken in HL5 media with antibiotics. Cells were grown from freeze-dried spore stock. Transformation by electroporation entailed incubating cells (2×10^6 cells/ml) on ice for 15 min, followed by centrifugation at 2,100 x g for 3 min in a CRU-5000 centrifuge (International Equipment Company). Cells were subsequently washed with electroporation buffer (10mM $NaPO_4$, 50mM sucrose, pH 6.1), then resuspended in 800 µl of chilled electroporation buffer. To the cells, 20 µg of the reporter vector containing the mutagenized promoter were added, and then the entire contents were transferred to 0.4 cm cuvettes that had been chilled on ice. Electroporation

conditions of the BioRad Gene Pulser included 3 μF (capacitor) at 1.2 kV. After electroporation, cuvettes were incubated on ice for 10 min, and then 400 μl of the contents were transferred into Petri plates containing 10 ml HL5 media with antibiotics. Cells were incubated under these conditions for at least 18 hr, G418 was then added to the plates at a final concentration of 10 μg/ml, and the plates were incubated at 21°C for 3 days. The HL5 medium was changed after this time period, the final G418 concentration was increased to 40 μg/ml, and the plates were incubated at 21°C for 3 additional days.

For transformation by calcium / glycerol shock, AX3K cells were first grown in shaking flasks as previously described for electroporation. When a concentration of 4 x 10^6 cells / ml was reached, the cells were harvested by centrifugation at 2,100 x g for 4 min, resuspended in MOPS-HL5 media containing antibiotics, and shaken in flasks at 150 rpm overnight. For each transformation, 10 ml from the flasks were transferred to a Petri plate. Cells adhered to the Petri dish during a 2 hr incubation period at room temperature. An hr before completion of this incubation period, DNA precipitates were prepared by adding 600 μl HEPES-buffered saline (HBS) and 36 μl 2M $CaCl_2$ to approximately 10 μg of the reporter vector containing the mutagenized promoter. After the mixture was gently vortexed, the DNA precipitates were incubated at room temperature for 30 min. The MOPS-HL5 media was aspirated from the Petri plates, and the DNA precipitates were added drop-wise to the center of the plates, which were then incubated at room temperature for 30 min. After the incubation period, 10 ml MOPS-HL5 with antibiotics were then added to the plates, which were then incubated at room temperature for 4 hr. The media was aspirated from the plates and 2 ml 18% glycerol in

HBS was added to the plate, which was slowly tilted to enable complete coverage. The plates were incubated at room temperature for 5 min, the glycerol was aspirated, and 10 ml HL5 with antibiotics were added to the plates, which were then incubated overnight at 21°C. The following day, the media was replaced with HL5 media containing antibiotics and a final G418 concentration of 20 µg/ml, and the cells were incubated at 21°C for 1 week. During this period of time, the media was periodically changed to prevent bacterial contamination.

The final stage of transformation by electroporation or calcium / glycerol shock entailed harvesting the cells and plating them on DM agar plates for plaque formation. When cells had reached an optimal density in the plates under these conditions, the cells were scraped off, centrifuged at 2,100 x g for 3 min and resuspended in 500 µl of PBS (34 mM KH_2PO_4, 16 mM Na_2HPO_4, 10 mM KCl, 10 mM NaCl) with G418-resistant *E.coli* cells (strain B/r $G418^R$). To DM agar plates containing 20 µg/ml G418, 125 µl of the resuspended cells were spread, and the plates were incubated at 21°C approximately 5 days until sizeable plaques formed. Cells from the plaques were transferred to 24 well culture plates containing HL5 medium with antibiotics and a final G418 concentration of 5 µg/ml. Cells were tested by colony PCR to confirm that the individual clones contained the vector with the insert in the proper orientation. Positive clones were then transferred to shaking (150 rpm) 125 ml flasks.

Dictyostelium Growth and Development.

Prior to enzyme activity assays and protein extractions from cells at various stages of multicellular development, ameobae cells from shaking flasks were plated on nitrocellulose filters or 2% water agar plates to induce development. Cells from shaking flasks were first harvested by centrifugation at 2,100 x g for 3 min in a CRU-5000 centrifuge, washed with MES-LPS, then resuspended in a volume of MES-LPS that was 5 times the weight of the cell pellet and placed on ice. Gelman GN-6 membrane filters, supported by Gelman pads (previously soaked in MES-LPS buffer), were placed into small Petri dishes, and 400 µl of the resuspended cells were applied to the filters using a plastic spatula. Cells on pads were incubated at room temperature until the desired stage of development was observed. For most experiments, the cells were harvested from the slug stage of development, which was reached in approximately 8 hr. In preparation for luciferase enzyme activity assays, cells were scraped from the plate with 1ml 30 mM glycyl-glycine (pH 8.3), centrifuged at 4,000 x g for 2 min, and stored at -80°C until the enzyme assay was performed. In preparation for ß-galactosidase enzyme activity assays, cells were scraped from the plate with ß-gal lysis buffer (14.7 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.0), centrifuged at 4,000 x g for 2 min, and stored at -80°C until the enzyme assay was performed. Prior to slug protein extraction for use in electromobility gel shift assays (EMSA), cells were harvested and resuspended in MES-LPS as previously described, though from a larger initial volume. Water agar plates (2%) were used because of the quantity required and the ease of slug removal from the surface.

Enzyme Activity Assays

Luciferase enzyme activity assays were performed on cells transformed with the pVTL2 reporter gene from the slug stage of development. ß -galactosidase enzyme activity assays were performed on cells transformed with the ß -gal reporter vector from the slug stage of development. Slug pellets stored at -80°C were thawed on ice and resuspended in 200 µl of lysis buffer (25 mM glycyl-glycine, pH 8.3, 15 mM $MgCl_2$, 4 mM EGTA, 1% Triton X-100, 20% glycerol, 1 mM DTT, 100 µg/ml PMSF, 0.5 µg/ml leupeptin, 100 µg/ml TPCK, 50 µg/ml TLCK, and 1 mg/ml benzamidine). To acquire linear kinetics of the particular assay, sometimes an additional dilution was required. From the cell extract, 50 µl were added to 50 µl of the luciferase reaction mix (25 mM glycyl-glycine, pH 8.3, 15 mM $MgCl_2$, 4 mM EGTA, 1 mM DTT and 2.8 mM ATP) in a luminometer tube, and then placed into the chamber of a Berthold Lumat LB 9501. The luciferin substrate (0.6 mM luciferin in 25 mM glycyl-glycine, pH 8.3, with 1 mM DTT) was automatically injected at a volume of 100 µl and the light output was displayed on the machine in luciferase units. Specific activity was calculated as luciferase units / mg protein. Protein amount was measured using a BCA protein assay kit (Pierce) and the protocol described in the accompanying manual.

For ß -galactosidase enzyme activity assays, slug pellets stored at -80°C were thawed on ice and resuspended in 100 µl ß -gal phosphate lysis buffer (14.7 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.0). The samples were then centrifuged for 5 min at 2,500 x g in a microcentrifuge (Eppendorf Centrifuge 5147R), and 35 µl from the supernatant was transferred to a 96-well microtiter plate containing 165 µl of reaction mix [100 µl Z-

buffer (40 mM KH_2PO_4, 60 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and 65 µl of O-nitrophenyl-B-D-galactoside (4 mg/ml)]. Absorbance was measured at 415 nm in a BioRad Benchmark Microplate Reader. Specific activity was calculated as A_{415} / min / mg protein. Protein amount was measured using a BCA protein assay kit (Pierce).

For the promoter deletion analysis, the average specific activity (luciferase or ß-galactosidase) was calculated for all clones tested. The average number of clones tested for a single construct was 7. The average variation of activity between clones of a single construct ranged from 40-50%. When extract from a single clone was tested in consecutive assays, the variation in activity was 3%.

Cyclic AMP Induction Prior to Protein Extraction

Some of the protein extracts used in the electromobility gel shift assays described were from amoebae cells incubated in the presence or absence of cAMP (+ / - cAMP). AX3K amoebae shaking at 150 rpm were harvested by centrifugation for 3 min at 2,100 x g in a CRU-5000 centrifuge. After washing the pellet in 1 x MES-LPS buffer (pH 6.5), the cells were resuspended in a volume of 1 x MES LPS that gave a final concentration of 1 x 10^7 cells / ml. The volume of cells obtained was divided into two separate flasks, which were shaken at 150 rpm overnight at room temperature. In one shaking flask, cAMP was added to a final concentration of 1 mM, and the other shaking flask received no cAMP. After 8 hr, cells from both flasks were centrifuged at 10,000 x g for 10 min (Sorvall, GSA). The supernatant was aspirated and the cells were resuspended in 5 times the pellet weight of lysis buffer A as described in the section below.

Cytoplasmic and Nuclear Protein Fractionation

Cytoplasmic and nuclear protein extraction from + / - cAMP amoebae was initiated by resuspending the harvested cells described above in 5 times the pellet weight of lysis buffer A (buffer (50 mM Tris, pH 7.5, 10% glycerol, 2 mM $MgCl_2$, 1% NP40, 1 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF). The lysed cells were centrifuged at 10,000 x g for 10 min. The supernatant, containing cytoplasmic proteins was removed and stored at -80°C. The remaining pellet was suspended in 4 ml of lysis buffer A, and then centrifuged at 10,000 x g for 10 min. The resulting supernatant, or "1st Wash," was removed and stored at -80°C. The remaining pellet was weighed, resuspended in 2X volume of lysis buffer A, vortexed, and then NaCl was added to a final concentration of 0.42 M. After incubation on ice for 1 hr, the sample was centrifuged at 10,000 x g for 10 min. The supernatant, containing nuclear proteins, was removed and stored at -80°C.

Cytoplasmic and nuclear protein extraction from slugs was performed in a similar way to that described above for amoebae. Cells were induced to undergo multicellular development on 2% water agar plates as described in the section on *Dictyostelium* Growth and Development. A 20 mM potassium phosphate buffer, pH 7.5, was used to remove the slugs from the agar plates. The slugs were passed through a 21 G needle syringe to disaggregate the slugs into single cells, and then centrifuged at 2,600 x g for 5 min. After aspirating the supernatant, the pellets were weighed and resuspended in 5 times the pellet weight of lysis buffer A. Cytoplasmic, 1st wash and nuclear protein extracts were obtained as described in the previous paragraph.

Preparing and Labeling DNA Probes

Some probes were prepared by PCR while others were comprised of 2 complementary single-stranded oligonucleotides that were annealed together. The annealing reaction, which included 20 mM Tris-Cl, pH 8.0, 1 mM EDTA (pH 8.0), and 50 mM NaCl, was incubated at 70°C for 5 min, and then slowly cooled back to room temperature. PCR products to be used as probes were extracted from the PCR reaction mixture with phenol and precipitated with ethanol. The resulting precipitates were resuspended and applied to a 2% TAE agarose gel and the DNA was purified using a MERmaid kit (BIO101). PCR products and annealed oligonucleotides were stored at –20°C until the probes were labeled for EMSA. In order to label the DNA probes, 100ng of the PCR or oligonucleotide probe was combined with 30 μCi of [γ-^{32}P] ATP, T4 PNK buffer and water in a total reaction volume of 11 μl. The reaction was incubated at 70°C for 5 min and then chilled on ice, after which 10 U/μl T4 PNK was added. The reaction was incubated at 37°C for 30 min, then incubated at 65°C for 20 min, and chilled on ice. Nucleotides that were not incorporated during the labeling reaction were removed using a Micro Bio-Spin 6 column (BioRad), and the resulting radioactivity of the labeled probe was measured in a scintillation counter (Beckman LS 6000SC).

Binding Reactions for Electromobility Gel Shift Assays

The cocktail used for the gel shift binding reactions included 20 mM HEPES, pH 7.9, 12% glycerol, 20 mM NaCl, 1 mM EDTA, pH 8.0, 2 mM $MgCl_2$, 2 μg BSA, 1 mM DTT, 0.04% NP40 and 0.5 μg of a non-specific carrier. Three non-specific carriers were consistently used in these assays: polydIdC-polydIdC, polydA-polydT, polydAdT-polydAdT. The cocktail was combined with 1.5 μl of protein extract (cytoplasmic or nuclear), and 30,000 cpm of labeled DNA probe were added after a 10 min incubation period on ice. In binding reactions that involved competition, unlabeled DNA was added in 100X excess molar ratio and added after the labeled probe. The reaction was then run on a 5% native polyacrylamide gel in 0.5X TBE, which had previously run for 30 min at 150 V. After the samples were loaded, the voltage was increased to 200 V for approximately 2 hr. The gel was then transferred to chromatography paper and placed into a gel dryer at 80°C for 45 min. Once dry, the gel was placed on X-OMAT AR Kodak film and exposed at -80°C for a variable period of time prior to film development.

Results

Validation of Luciferase and LacZ Reporter Genes

In order to identify regions of the promoter sequence that may be implicated in the developmental regulation of *5nt* gene expression, 5' and internal *5nt* promoter deletions were generated and fused to the *luciferase* and *lacZ* reporter genes. *5nt* is a developmentally regulated gene, with very low levels of expression at the amoebae stage of development (Chanchao et al. 1999). As development progresses, *5nt* expression increases to higher levels in the aggregate and standing slug stage. High levels of expression are persistent throughout the remainder of the life cycle when the culminant stage is reached. In order to determine if a reporter gene driven by the *5nt* promoter followed the same expression pattern as the authentic gene, 1,212 bp of the *5nt* promoter were fused to the *luciferase* reporter gene and incorporated into the pVTL2 vector. After *Dictyostelium* transformation, initiation of aggregation was induced by plating the cells on nitrocellulose filters. As development progressed, extracts were tested in the luminometer every two hr after plating until the completion of the life cycle was reached at 24 hr (Fig. 2.1). The reporter gene activity observed at each stage of development, and the overall pattern of expression observed were identical to that of the endogenous *5nt*, thus validating its use as an indicator for *5nt* promoter analyses.

Similarly, in order to assess *lacZ* as a valid indicator of *5nt* expression in promoter deletion assays, 1,212 bp upstream of the translation start site of the *5nt* promoter were fused to the reporter gene and expression levels were analyzed throughout

development (Fig. 2.2). The temporal expression of *lacZ* activity was similar to that of the endogenous *5nt* gene and the *luciferase* reporter enzyme activity.

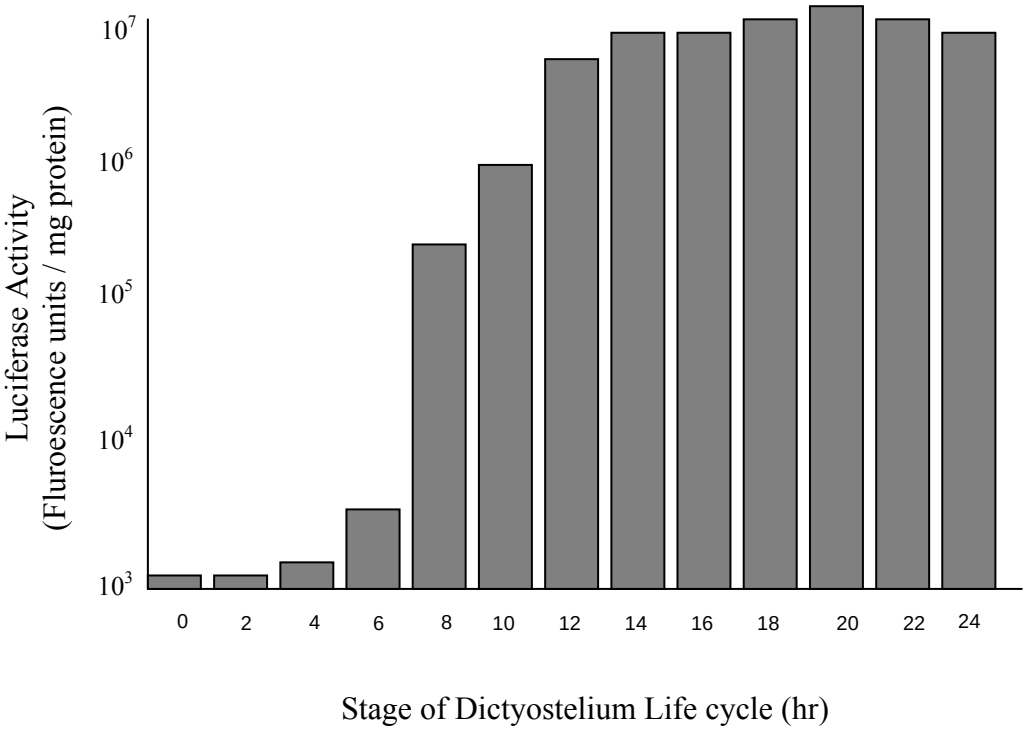

Figure 2.1. Luciferase Activity Measured from the *5nt* Promoter. The *5nt* promoter was fused to the *luciferase* reporter gene in the pVTL2 vector. After transformation of *Dictyostelium* cells, multicellular development was induced by plating in non-nutrient media on nitrocellulose filters (= time 0). At each stage of development, reporter gene activity was measured using a luminometer. While *luciferase* activity was very low during initial development, an increase in activity was detected as individual amoebae began to aggregate into the mound formation at 4 hr into development. There was a considerable increase in activity during the standing slug and migrating slug stage of development between 6 and 12 hr, and *luciferase* activity levels remained high throughout the remainder of the 24 hr life cycle. The pattern of activity level observed coincides with the activity observed from *5nt* gene expression, thereby confirming that the *luciferase* reporter gene is a valid indicator of *5nt* gene regulation.

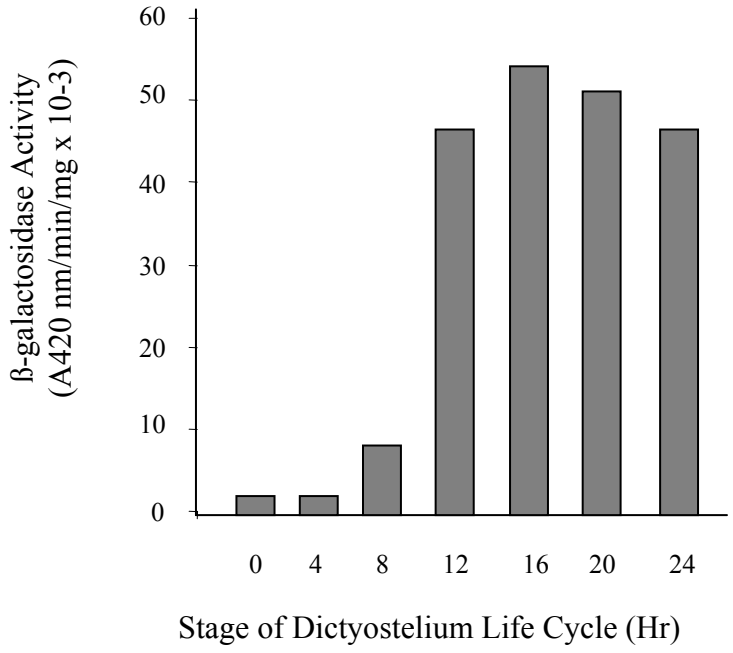

Figure 2.2. ß-galactosidase Activity Driven By the Full *5nt* Promoter at Defined Stages of *Dictyostelium* Development. After fusion of the *5nt* full promoter to the *lacZ* gene of the reporter vector, *Dictyostelium* cells were transformed and multicellular development was induced by plating on nitrocellulose filters. At each defined stage of development, reporter gene activity was measured and determined in units per milligram of protein. Initial activity was evident during late aggregation as individual cells reached the aggregation center and began mound formation. Levels of reporter gene activity increased dramatically as the formation of the first finger began (12 hr), and levels remained high throughout the remainder of the life cycle. The expression pattern correlates to that observed of 5nt activity; therefore, the reporter gene is a valid indicator of *5nt* gene expression.

Identification of the Limits of the Upstream Region of the 5nt Promoter Required for Temporal Regulation

Initial analysis of the *5nt* promoter involved the creation of internal and 5' promoter deletions and the fusion to the *lacZ* or *luciferase* reporter genes. The activities of a series of 5' promoter deletions were assayed in *Dictyostelium* slugs after transformation in the amoebae stage. The *luciferase* activity levels obtained from these experiments are shown in Fig 2.3. Reporter gene activity was not significantly reduced with 5' deletions between -1325 bp and -730 bp from the ATG translational start site, thereby indicating that this region of the promoter does not contain regulatory sites essential for the normal temporal regulation of the gene. The increase in activity evident when the -997 bp promoter drives the reporter gene may indicate the presence of a negative control element between -1230 base and -997 bp from the ATG. As the size of the 5' deletions increased to the region of the promoter downstream of -730 bp, *luciferase* activity levels progressively decreased. The data obtained from the 5' promoter deletion experiment defined a region of the promoter in which essential regulatory elements may exist. This region of potential interest was delineated between -505 and -730 bp from the ATG.

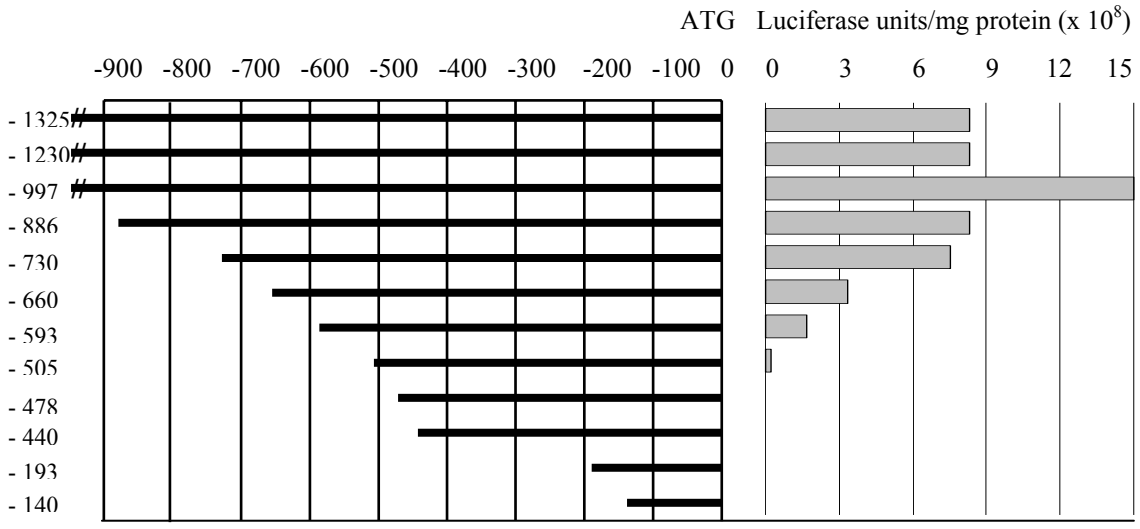

Figure 2.3. Luciferase Activity Measured from a Series of 5' Deleted *5nt* Promoters. Using the exonuclease III / mung bean nuclease system, a series of 5' *5nt* promoter deletions were created and fused in-frame to the *luciferase* reporter gene in the pVTL2 reporter vector. *Luciferase* activity was measured from extracts obtained from the slug stage of development and are reported as the average obtained from multiple clones assayed. Numbers in the left column represent the extent of the 5' deletion in bp from the ATG translational start site. An average of 7 clones were tested for each construct. The average variation between clones of a single construction was 45%. The average variation between repeat measurements of a single clone was 3%.

Analysis of the Internal 5nt Promoter Deletions Enabled the Identification of Potential Transcriptional Control Elements

Because the absence of large 5' portions of the promoter in the deletion analysis shown in Fig 2.3 may confound the ability to identify precise regulatory sites in the promoter, an internal promoter deletion analysis was performed on shorter segments in the nucleotide sequence. Internal promoter deletions in the *5nt* promoter were generated by a PCR-based approach or a vector amplification approach, as described in the Materials and Methods. The mutant promoters were then fused to the *luciferase* reporter gene. The reporter activity levels achieved from these promoters were assessed in *Dictyostelium* slugs, where endogenous *5nt* activity is known to be at its highest level. Twenty internal promoter deletions were analyzed and the luciferase reporter activity levels were compared to that of the control promoter (Fig. 2.4). In this experiment, the control was a segment 889 bp upstream of the translation start site. Previous experiments had determined that deletions upstream from -889 did not significantly reduce activity levels. Control promoters 1 and 2 show that the reporter activity obtained from the -663 promoter is not significantly reduced relative to the control. Therefore, the region between -889 and -663 was eliminated for regulatory element identification. The large internal deletions in constructs 3, 4, 5, and 7 showed dramatically reduced *luciferase* activity levels, while the smaller deletion is promoter 6 maintained a higher level of activity. Deletions 8-15 included more upstream nucleotide sequence than 3-7 or 16-22. The activity level achieved by promoter 8 and promoter 11 suggest that a regulatory site may be present between -537 and -663. Activity levels measured from promoters 9, 10,

12 and 13 may indicate that additional regulatory sites extend into the nucleotide sequence between -537 and -423. Deletion of the nucleotide sequence between -446 and -423 in promoter 15 resulted in somewhat reduced *luciferase* activity, but not to the extent of the other deletions, and was therefore not judged to be a potential regulatory site. Similarly, deletion of the nucleotide sequence between -537 and -573 (construct 14) resulted in activity levels very similar to the control promoter 1. The activity levels produced by a deletion of a similar region (-579 to -534) in the shorter promoter 17 agreed with those of promoter 14, and also supported the notion that the nucleotide sequence between -663 and -889 is not critical to maintain normal levels of gene activity. In contrast, compared to the activity levels observed from control promoter 2, internal deletions represented in promoters 16, 17, 18, 19, 20 and 21 all resulted in reduced *luciferase* activity, and therefore represent a region of the promoter where potential regulatory elements may reside.

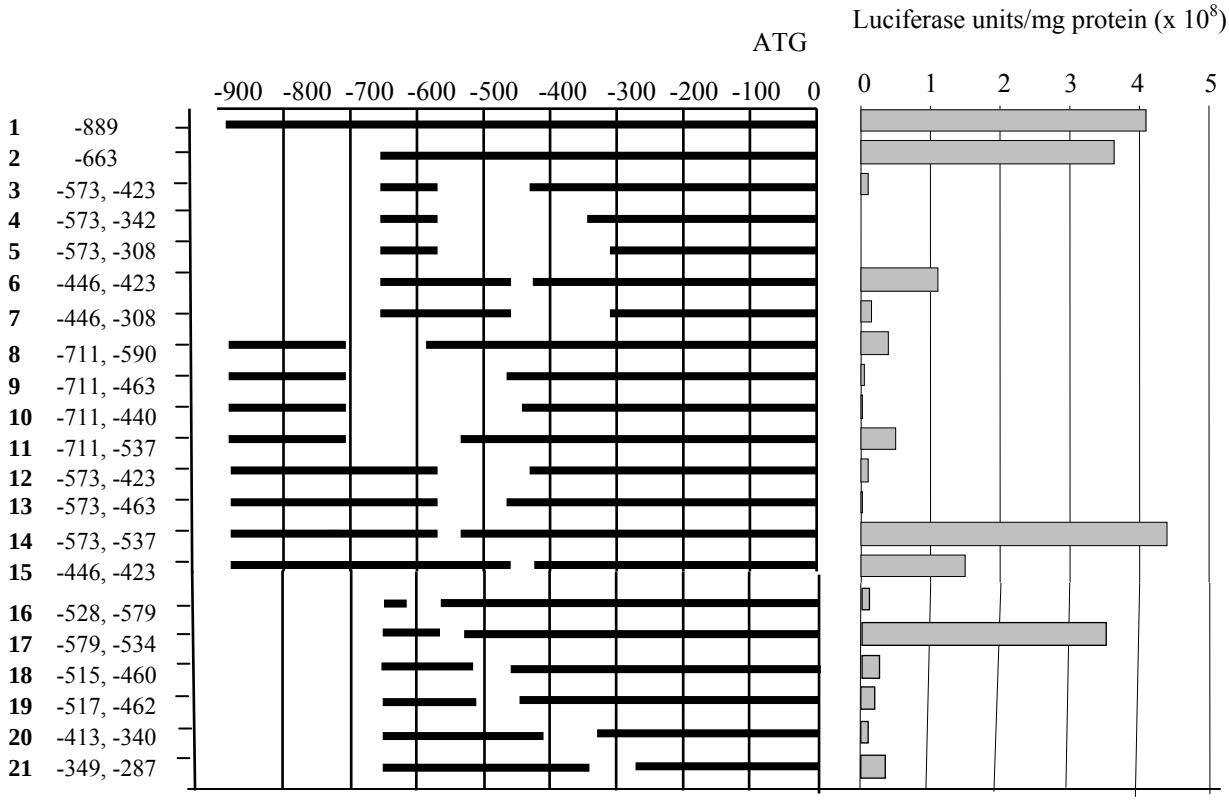

Figure 2.4. Luciferase Activity Measured From a Series of Internally Deleted *5nt* Promoters. Using a PCR-based approach or a fragment ligation approach, a series of internal *5nt* promoter deletions were created and fused in-frame to the *luciferase* reporter gene in the pVTL2 reporter vector. Luciferase activity was measured from extracts obtained from the slug stage of development and are reported as the average obtained from multiple clones assayed. Numbers in the left column represent the internal deletion created as distance from the start site of translation. An average of 7 clones was tested for each construct. The average variation between clones of a single construct was 45%. The average variation between repeat measurements of a single clone was 3%.

5nt internal promoter deletion constructs were also created in which the *luciferase* reporter gene was replaced with the *lacZ* reporter gene. The *lacZ* reporter gene activity levels produced were quantified in *Dictyostelium* slugs (Fig. 2.5). Promoter 1 represents the ß-galactosidase activity level obtained from 1,290 bp of the *5nt* promoter. The internal deletions of promoters 2, 3 and 4 prevented any ß-galactosidase activity, suggesting that the nucleotides between 509 and 628 may be critical to achieve normal expression levels. Similarly, the absence of reporter gene activity in promoters 6, 7, 8, 9 and 10 suggest that the region between 410 and 486 may represent sequence containing control elements. The higher levels of activity observed in promoters 11 and 12 suggest that the region between 340 and 410 are not required for normal levels of gene expression. The dramatic increase in reporter activity observed when the nucleotides between 486 and 521 are removed, may suggest that the region contains a negative control element, which when removed enables high gene expression levels. This region is proximal to and downstream from the potential negative control element described from the *luciferase* internal deletion experiments.

The 5' and internal *5nt* promoter deletion experiments identified a 250 bp region of the promoter that appears to be required for normal levels of gene expression. This region comprises the nucleotide sequence between -413 and -663 from the ATG translational start site.

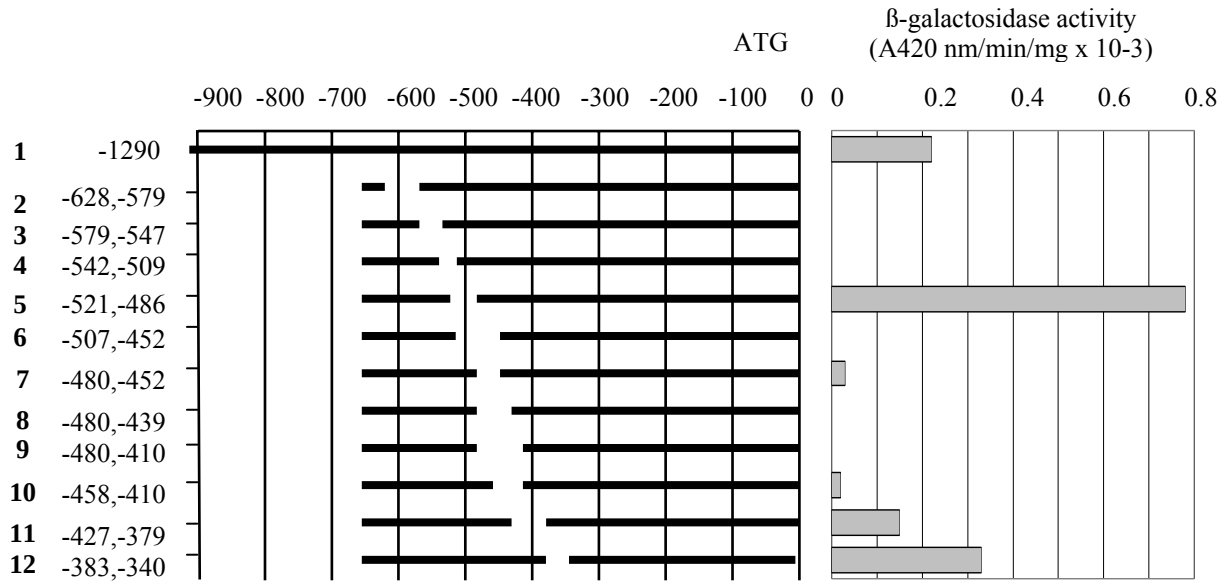

Figure 2.5. ß-galactosidase Activity Measured From a Series of Internally Deleted *5nt* Promoters. Using a PCR-based approach or a fragment ligation approach, a series of internal *5nt* promoter deletions were created and fused in-frame to the *lacZ* reporter gene. Reporter gene activity was measured from extracts obtained from the slug stage of development and are reported as the average obtained from multiple clones assayed. Numbers in the left column represent the internal deletion created as distance from the start site of translation. An average of 7 clones were tested for each construct. The average variation between clones of a single construct was 45%. The average variation between repeat measurements of a single clone was 3%.

Identification of Control Elements on the 5nt Promoter by Site-Directed Mutagenesis.

In order to further investigate the 250 bp region of the promoter and define more precise locations for potential control elements, site-directed mutagenesis experiments were performed using the QuickChange Mutagenesis Kit (Stratagene). Because maintaining the spatial integrity of the *5nt* promoter may be critical to its normal function, the site-directed mutagenesis experiments enabled the maintenance of this factor, which is not taken into account by promoter deletion analysis.

The promoter sequence targeted for site-directed mutagenesis is shown in relation to the entire *5nt* promoter in Fig 2.6. The sequence begins with an endogenous *Hind*III restriction site, centrally located within the promoter sequence. Fourteen sites within the sequence were mutagenized, and are represented as black rectangles in the figure. The mutagenized sites of some promoters, such as promoters 2 and 5, were as short as 6 bp, while the mutagenized sites of the other promoters comprised a 12 bp sequence. The mutagenized promoters were fused to the *luciferase* and *lacZ* reporter genes and transformed into *Dictyostelium* cells. Reporter gene activity was then measured in slugs after the induction of multicellular development.

Ten of the twelve mutagenized promoters were successfully cloned into the pVTL2 *Luciferase* reporter vector and transformed into *Dictyostelium* cells (Fig. 2.7). In most cases, multiple clones for each promoter construct were tested from the slug stage of multicellular development. The reporter activity for each clone is presented for each clone rather than an average of the activity from each construct because in several cases there was substantial variation among clones. This presentation of the data is particularly

important for constructs in which all clones show no activity except for one clone with exceptionally high activity. It is likely that these single clones have deletion constructs that have integrated into the genomic DNA and are under the control of a strong promoter or enhancer. In some cases, a single clone was tested more than once, as indicated by columns immediately adjacent to one another. A high level of *luciferase* activity was obtained from multiple clones with promoters 6, 10, 12, 13 and 14, suggesting that the sites targeted for mutagenesis in this study are not included within regulatory control elements. In contrast, the target sites of promoters 7, 8 and 11 may represent control elements, since mutagenized, reporter gene activity becomes significantly reduced. Few clones were obtained after *Dictyostelium* transformation for constructs 1 and 2, and although *luciferase* reporter gene activity levels are low for these cases, the sites cannot be conclusively eliminated as potential regulatory elements.

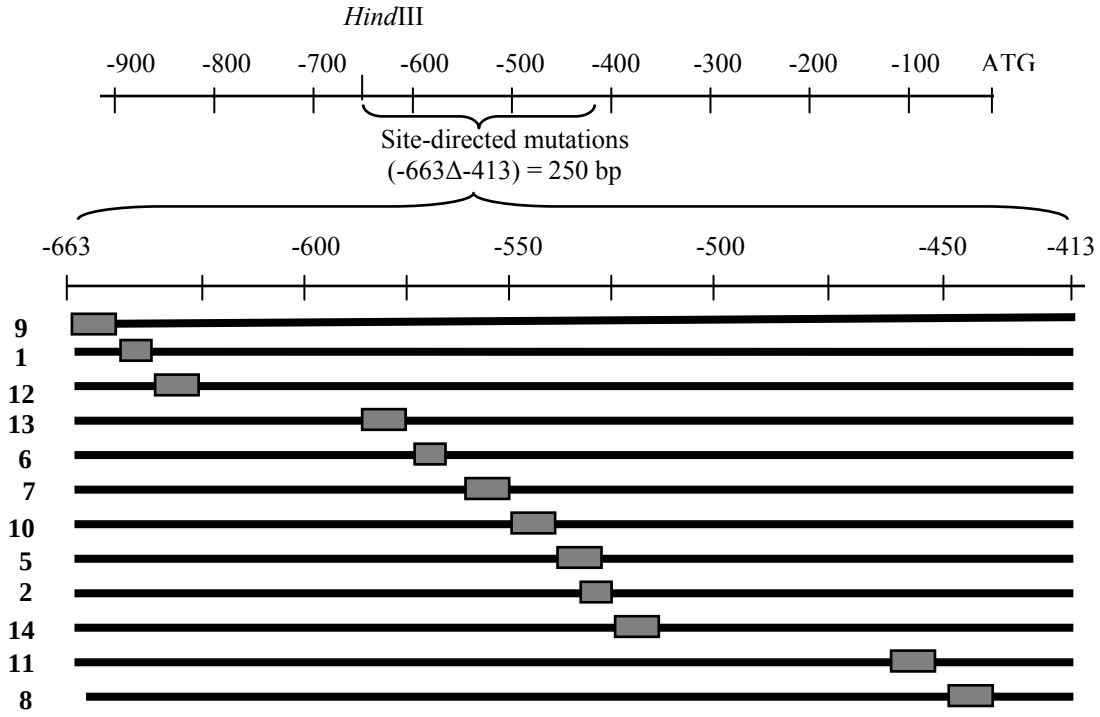

Figure 2.6. Target Points for Site-Directed Mutagenesis of the *5nt* Promoter. The 250 bp region of the *5nt* promoter identified by promoter deletion studies is shown. Within this 250 bp sequence, a total of 12 specific sites within the sequence were targeted for mutagenesis. Sequences mutagenized were between 6 and 12 bp in length. The numbers in the left column represent the reference number of each mutagenized promoter that was subsequently fused to the *luciferase* or *lacZ* reporter genes.

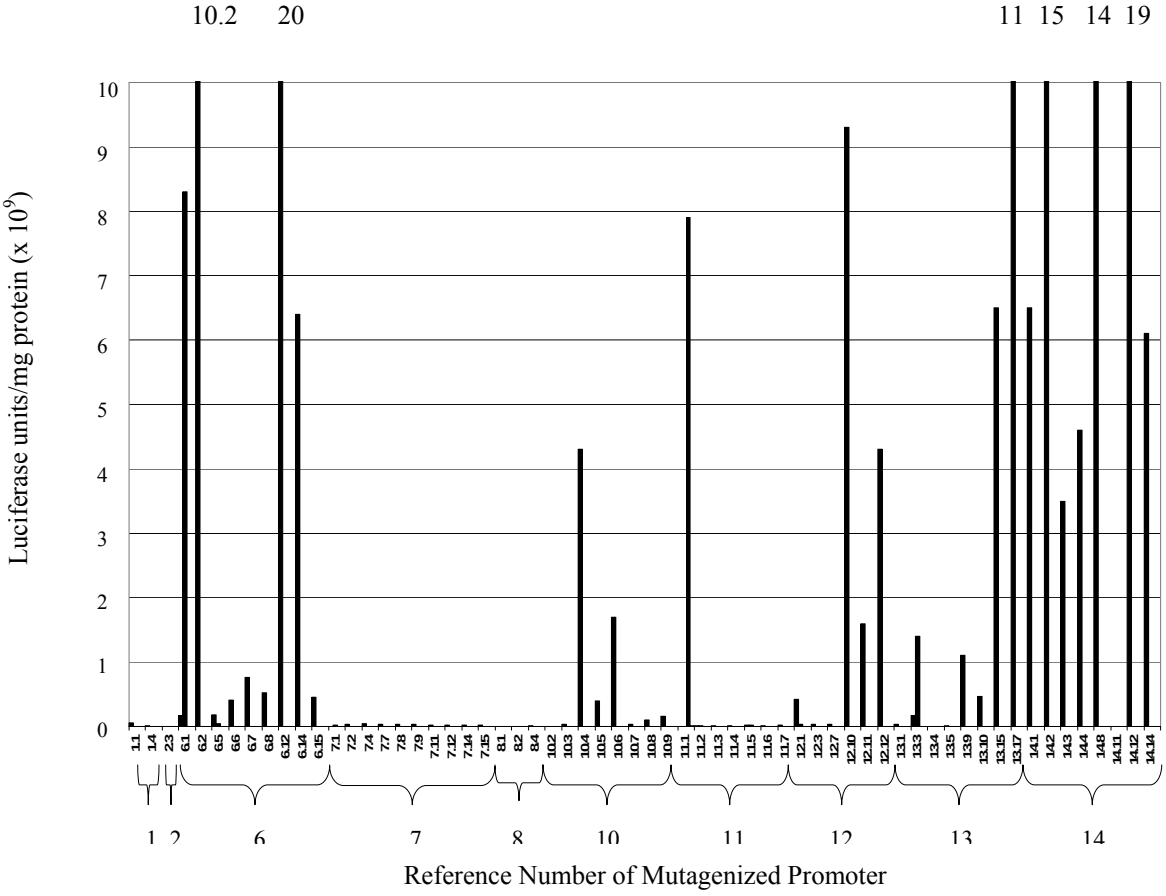

Figure 2.7. Luciferase Activity From Site-Directed Mutagenized *5nt* Promoters. Mutagenized 5nt promoters were fused to the *lacZ* reporter gene. Transformed cells were induced to undergo multicellular development, and reporter gene activity was measured at the slug stage of development. For each mutagenized promoter, multiple clones were assayed. In some cases, a single clone was assayed more than once. These cases are represented by columns immediately adjacent to one another. Activity levels exceeding the boundaries of the graph are enumerated at the top of the figure.

Twelve mutagenized promoters were successfully fused to the *lacZ* reporter gene. *Dictyostelium* cells were transformed with the reporter vector, and multiple clones for each promoter construct were grown and tested at the slug stage of development (Fig. 2.8). Multiple clones from promoters 2, 6, 9, 10 and 14 showed high levels of *ß-galactosidase* activity, suggesting that the regulatory elements do not reside in the target sites of these

promoters. Alternatively, the target sites of promoters 1, 7, 8, 11, 12, and 13 may represent control sequences involved in regulated gene expression. By comparing the results of the site-directed mutagenesis slug stage study with the *luciferase* and *lacZ* reporter genes, the most potential sites involved in temporal regulation may be 5, 7, 8 11, and possibly 13 (Fig. 2.6).

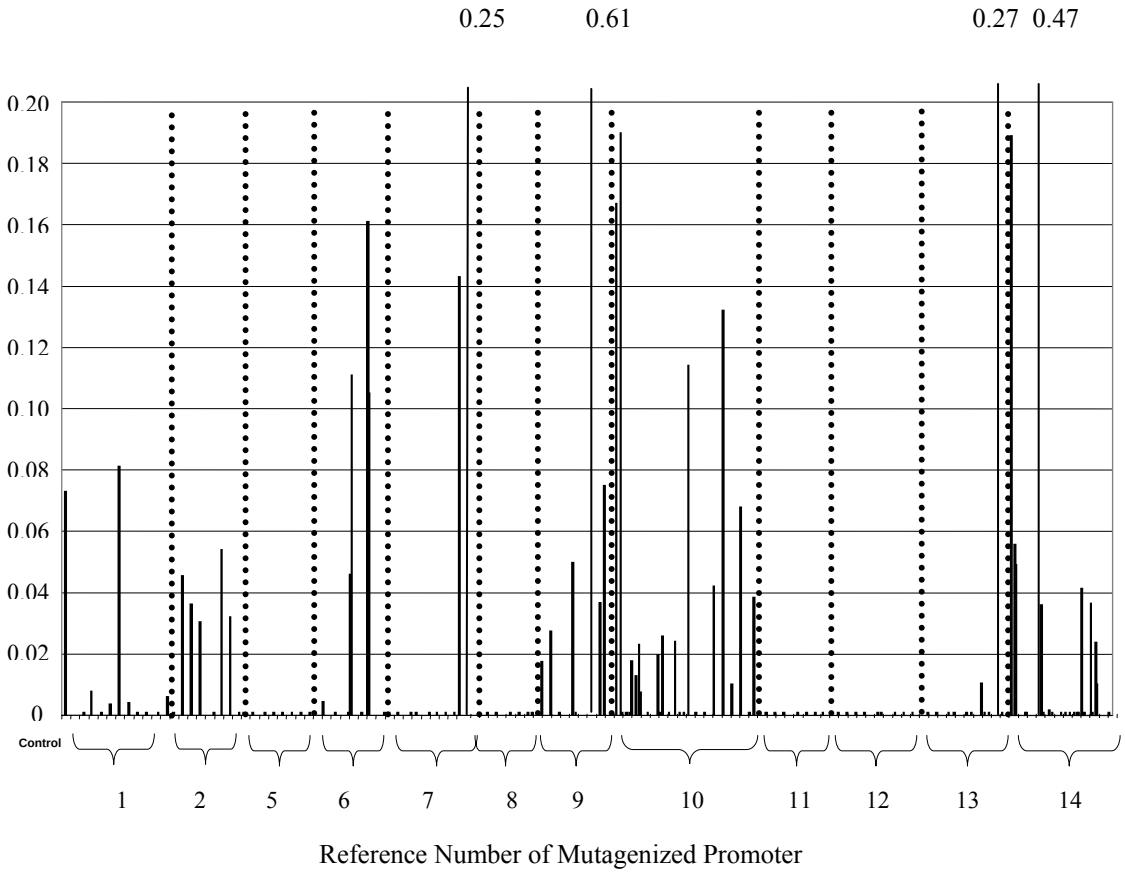

Figure 2.8. ß-galactosidase Activity From Site-Directed Mutagenized *5nt* Promoters. Mutagenized 5nt promoters were fused to the lacZ reporter gene. Transformed cells were induced to undergo multicellular development, and reporter gene activity was measured at the slug stage of development. For each mutagenized promoter, multiple clones were assayed. Clones for each construct are separated by dotted vertical lines. In some cases, a single clone was assayed more than once. These cases are represented by columns immediately adjacent to one another as a single clone number. Activity levels exceeding the boundaries of the graph, are enumerated at the top of the figure.

The Identification of Two Proteins That Bind to Specific Sequences of the 5nt Promoter

In order to assess the possibility of proteins binding to the DNA sequences identified by promoter deletion and site-directed mutagenesis studies, EMSA experiments were performed. Some of the probes designed for use at this stage in the promoter analysis are depicted in Fig 2.9, which shows the location of the probe relative to the ATG translation start site. Initial binding reactions utilizing these probes with various protein extract were performed to identify binding activities that showed specificity, or preference for certain nucleotide sequences. A binding activity of interest (Rf = 0.13) was detected using probe 3, nuclear or cytoplasmic protein extracts from cells in the aggregate and slug stages on development, and the polyIC non-specific carrier (data not shown). In addition to being present with protein extracts from slugs, the binding activity was also evident in protein extracts obtained from amoebae grown in the presence of cAMP. In order to evaluate the specificity of the 0.13 Rf binding activity, competition experiments were performed (Fig. 2.10). Lane 1 shows the intensity of the 0.13 Rf band produced when the binding reaction includes probe 3 and no additional competitor. As expected, competition was observed when unlabeled excess probe 3 was included in the binding reaction (lane 2). The faster migrating bands that were not diminished represent non-specific DNA-protein interactions. Competition was also apparent when unlabeled excess probe 11 (unphosphorylated or phosphorylated) was present (lanes 6 and 9, respectively), and slight competition occurred in the presence of unphosphorylated or phosphorylated

unlabeled excess probe 10 (lanes 5 and 8, respectively). Because probes 10 and 11 contain nucleotide sequence within probe 3, these results were not unexpected. Specificity of the 0.13 Rf binding activity was further shown by the inability of probe 2 (lane 3) to compete for the activity. In addition, no competition occurred in the presence of unphosphorylated or phosphorylated unlabeled excess probe 9 (lanes 4 and 7, respectively). While the protein extracts tested in Fig 2.10 were from cells induced by cAMP, it should be noted that the 0.13 Rf band was also detected, though not as intense, from cells in which cAMP was not added (data not shown.). In conclusion, a DNA-protein interaction with an Rf = 0.13 was identified and shown to be specific to the nucleotide sequence present in probe 3 and probe 11.

Electromobility gel shift assays enabled the identification of an additional discrete DNA-protein binding activity. This binding activity (Rf = 0.33) was detected using probe 8, a short oligonucleotide probe whose sequence partially overlaps probe 3. The 0.33 Rf binding activity was detected only in binding reaction condition that included the non-specific carrier polydApolydT. Binding reaction conditions that included the polydIdC-polydIdC or the polydAdT-polydAdT did not enable the visualization of the 0.33 Rf band. Fig 2.11 shows the specificity of this binding activity with probe 8. The protein extract used in the experiment were pooled and concentrated fractions collected from a DEAE column onto which cytoplasmic slug extract had been applied. The intensity level of the 0.33 Rf band in lanes 3 and 8 represent that observed when no competitor was added to the reaction. As expected, the binding activity was diminished under conditions in which the excess unlabeled probe 8 was present (lanes 4 and 9). In the other lanes, no competition occurred, so the intensity of the 0.33 Rf band was maintained. In conclusion,

a DNA-protein interaction with an Rf = 0.33 was identified and shown to be specific to the nucleotide sequence present in probe 8.

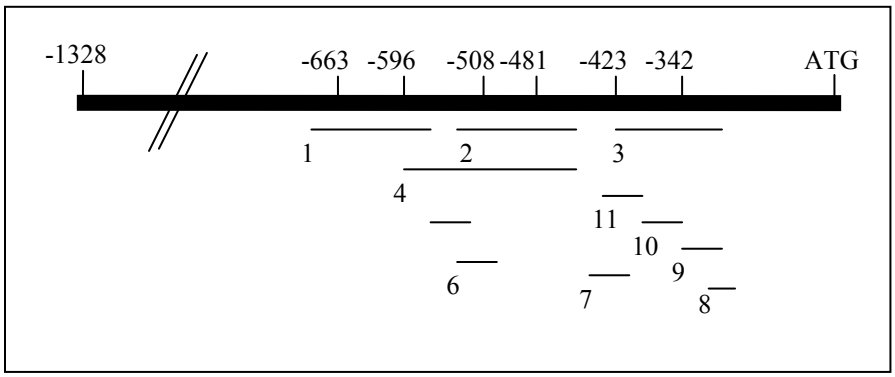

Figure 2.9. Probes Used to Test the Specificity of the 0.13 Rf and 0.33 Rf Binding Activities. Probes 1, 2, 3 and 4 were generated by PCR. Probe 1 is 104 base pairs, probe 2 is 144 base pairs, probe 3 is 105 base pairs, and probe 4 is 91 base pairs. Probes 9, 10 and 11 are 50 base pair oligonucleotides, and probes 5, 6, 7 and 8 are 30 base pair oligonucleotides. The precise location of the probes relative to the ATG site are as follows: 1 (677-574), 2 (537-447), 3 (423-319), 4 (590-477), 5 (582-553), 6 (550-521), 7 (457-428), 8 (343-314), 9 (423-374), 10 (396-347), 11 (368-319).

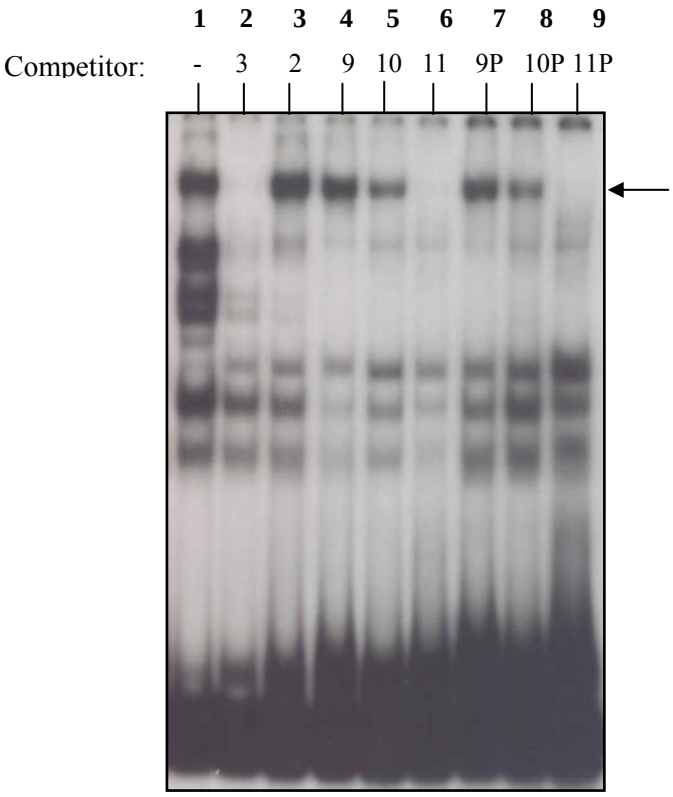

Figure 2.10. DNA-Protein Binding Activity (Rf = 0.13) is Specific to Probe 3 and Probe 11. Cytoplasmic extract from +cAMP induced cells were tested with various probes for competition analysis. Labeled probe 3 was used in all lanes. In lane 1, no competitor was used, while 100 fold molar excess unlabeled competitors were used in the subsequent lanes. In lane 2, unlabeled excess probe 6 was used, and competition was evident for the 0.13 Rf band. Competition was also observed in lanes 6 and 9, where probe 11, nonphosphorylated and phosphorylated respectively, were used.

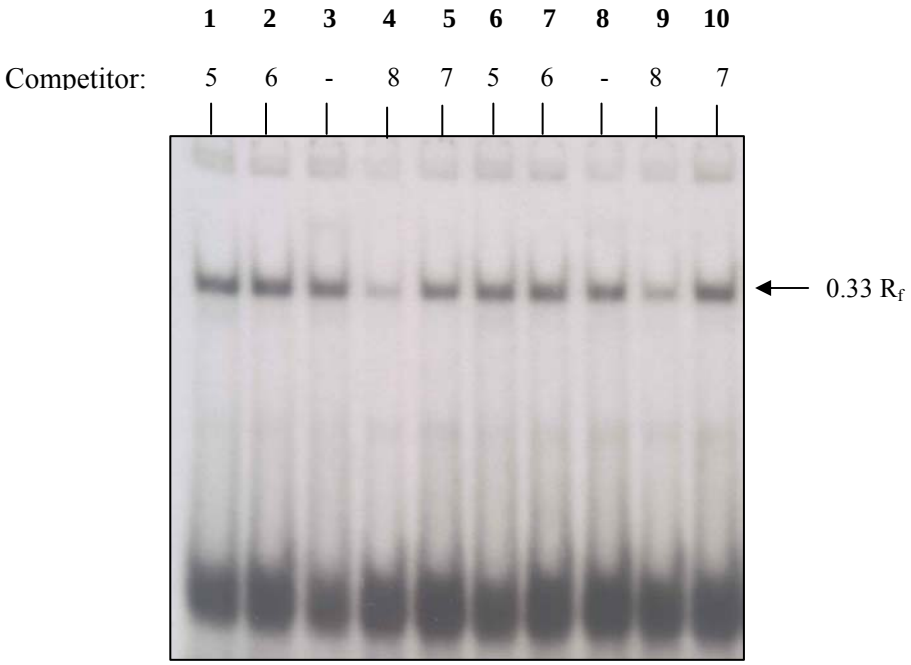

Figure 2.11. DNA-Protein Binding Activity (Rf = 0.33) is Specific to Probe 8. Slug cytoplasmic extracts were applied to a DEAE column, and eluted fractions containing the 0.33 Rf binding activity were pooled and concentrated. Competition assays on this extract was then performed to test the specificity of binding. Labeled probe 8 was used in all lanes and the effects of various 100 fold molar excess unlabeled competitors were assessed. In lanes 3 and 8, no competitor was added to the binding reaction. Competition was only apparent in lanes 4 and 9 where excess unlabeled probe 8 was added to the reaction.

Discussion

Because *5nt* is a developmentally regulated gene that is temporally expressed, its upstream promoter served as an appropriate platform for studies of transcriptional regulation. The temporal expression pattern of the endogenous gene throughout the 24-hr life cycle of *Dictyostelium* was accurately represented by the expression of the *luciferase* and *lacZ* reporter gene upon fusion with the full *5nt* promoter. Therefore, reduced expression of the reporter genes when fused to promoter with 5' or internal deletions would suggest that a critical element of the promoter had been removed.

Data obtained from the 5' deletions fused to the *luciferase* reporter gene suggested that the deletion of promoter sequence between -663 bp and -1325 bp did not significantly affect activity levels, and therefore was not critical to maintain normal levels of gene expression during development. Data from the internal promoter deletions fused to the *luciferase* and *lacZ* reporter genes supported this notion. Therefore, the search for control elements could be considerably narrowed down to a 663 bp sequence. Many of the internal promoter deletions tested focused upon sites within a 663 promoter construct (Fig. 2.4, constructs 2-7 and 17-22; Fig. 2.5).

Comparing the deletion sites and their corresponding effect on reporter gene activity between the two reporter genes was a useful tool during data analysis. For some deleted promoters, the effects on the reporter activity were similar for luciferase and ß-galactosidase. Construct 16 in Fig. 2.4 and construct 2 in Fig. 2.5, for example, were in agreement; the deletion of sequence between -579 and -628 caused a significant decrease in both reporter activities, and therefore represented a potential control element. The

absence of activity from construct 2 in Fig. 2.5 coincided with some of the results of constructs 3, 4, 5, 12 and 13 in Fig. 2.4. These constructs from Fig. 2.4 all had internal deletions that included the region that had been deleted in promoter 2 (Fig. 2.5), and they showed a dramatic decrease in reporter gene activity as well. In contrast, construct 14 and 17 (Fig. 2.4) showed no loss of luciferase activity compared to the control upon deletion of sequence between -534 and -579. Although the precise deletion was not identical between constructs 14 and 18 relative to construct 2 (Fig. 2.5), the similarity between the activity levels obtained were not expected. Although some divergence was observed when comparing the results from the ß-galactosidase and luciferase assays of the reporter deletions, the data obtained enabled the identification of a 250 bp region that potentially contained control elements, located between -663 and -413 bp from the ATG translation start site. This sequence was used as the template for site-directed mutagenesis experiments.

Because the site-directed mutagenized promoters were fused to both the *luciferase* and *lacZ* reporter genes, the effect on the two separate reporters could be compared between assays. Constructs 6, 10 and 14 showed relatively consistent high levels of luciferase and ß-galactosidase activity for most of the clones, while the activity levels for both reporter genes were consistently low for most clones from constructs 7 and 11. Some inconsistencies were observed with constructs 12 and 13. While relatively high luciferase activity levels were observed for many clones from 12 and 13, ß-galactosidase activity levels were very low. The single clone of construct 2 showed no *luciferase* activity, while a relatively high level of ß-galactosidase activity was consistent among the clones with mutagenized promoter 2. *Dictyostelium* cells transformed with the *luciferase*

reporter vector pVTL2 containing mutagenized promoters 5 and 9 did not grow to high densities in 24-well plates, and were unable to grow in 125 ml shaking flasks. Therefore, activity levels between the reporter genes could not be compared. However, the results from the ß-galactosidase assays (Fig. 2.8) suggested that site 9 was not a candidate for a control element within the promoter, while site 5 may be required for normal levels of gene expression. With both assays taken into account, the most likely sites representing regulatory elements were 5, 7, 8 and 11. While sites 5 and 7 are located in the middle of the target 250 bp sequence, 8 and 11 are located close together in the more downstream region. Sites 8 and 11 represent nucleotides approximately between -440 and -460, while sites 5 and 7 represent nucleotides approximately between -530 and -560.

By combining the location of the sites identified by site-directed mutagenesis experiments with the results of the deletion assays, some interesting comparisons can be made. In most cases, deletions including nucleotides between -440 and -460 showed low reporter gene activity levels, therefore supporting the results observed. Large 5' and internal deletions that included nucleotides between -530 and -560 also generally showed low levels of luciferase or ß-galactosidase activity. In contrast, constructs 14 and 18 in Fig. 2.4 showed no effect on luciferase activity compared to the controls when nucleotides between -530 and -580 were removed. It is possible that the inclusion of the 20 nucleotides between -560 and -580 results in normal activity that would otherwise not be expected. Another possibility is that maintenance of spatial integrity is critical, and inconsistencies may arise when comparing results observed when portions of the promoter are deleted relative to when point mutations are made in short segments.

EMSA enabled the identification of two specific DNA-protein binding activities. Multiple probes spanning over 300 bp of the *5nt* promoter were used to initialize these experiments. The 0.13 Rf binding activity identified was determined to specifically associate with probe 3, located between -319 and -423. The 0.13 Rf binding activity was also specific to probe 11, enabling the precise region of binding to be reduced to the 51 bp between -319 and -368. Its additional specific association with the overlapping probe 11 narrowed the area of association to the 51 bp between -319 and -368. Most of the 5' and internal deletions made were upstream from this region, except for constructs 20 and 21 (Fig. 2.4). Deletion of the nucleotides between -287 and -413 in these constructs resulted in over 90% reduction of the *luciferase* activity as compared to the control promoter. Construct 12 (Fig. 2.5), in contrast, showed no loss of ß-galactosidase activity when nucleotides between -340 and -383 were deleted. It is possible that the presence of nucleotides between -368 and -383 were sufficient to retain full activity. The results of the mutagenesis experiments suggest that sites 5, 7, 8 and 11 may represent potential control elements to which regulatory protein may bind. However, the 0.13 Rf binding activities identified by EMSA were found to specifically associate with nucleotide sequences located further downstream. It is possible that the sites identified from the mutagenesis assay may be additional protein binding control elements whose associations were not detected with the specific conditions of the binding experiments performed in this study.

The 0.33 Rf binding activity identified from gel shift assays was found to associate specifically with probes 3 and 8. Probe 8 is a short sequence that partially overlaps with the sequence of probe 3 between nucleotides -314 and -343. The DNA-

protein binding activity can therefore be focused to a 30 bp site on the promoter sequence. Again, the activity resides within a region of the promoter more downstream from most of the deletion and site-directed mutagenesis sites examined. Construct 21 (Fig. 2.4) contains a deletion that includes this site, and does show reduced luciferase activity levels, thus supporting the notion that a control element could reside within this region. As previously suggested, the potential elements identified from the site-directed mutagenesis assays may be intimately involved in the successful binding activities of 0.13 Rf, 0.33 Rf, or possibly both. They may represent sites whose spatial arrangement or nucleotide sequence enables the binding of transcription factors to other regions of the promoter.

Regulated gene expression can involve numerous mechanisms and components, whose roles must be played in a precise order or arrangement for activity levels to change. The interconnectedness of these components can be critical for normal levels of expression, and therefore represents challenges in the interpretations of promoter deletion analysis. The DNA-protein binding activities identified by the studies presented here represent potential components involved in the transcriptional regulation of the *5nt* gene. While their activities have been determined to be sequence specific to specific regions of the promoter, their roles in regulating *5nt* expression will be the focus of future investigations. An immediate goal in the analysis of *5nt* gene regulation is the purification of the 0.13 Rf and 0.33 Rf proteins identified from the electromobility gel shift assays.

References

Bhanot, P. and G. Weeks (1985). "The membrane-bound alkaline phosphatase and 5'-nucleotidase activities of vegetative cells of Dictyostelium discoideum." Arch. Biophys. Biochem. **236**: 497-505.

Chanchao, C., Eristi, C. M., Favis, R., and Rutherford, C. L. (1999). "5'-Nucleotidase in Dictyostelium: protein purification, cloning, and developmental expression." Biochim. Biophys. Acta **1473**: 376-390.

Codogno P, Doyennette-Moyne MA, Aubery M, Dieckhoff J, Lietzke R, and Mannherz HG. (1988). "Polyclonal and monoclonal antibodies against chicken gizzard 5'-nucleotidase inhibit the spreading process of chicken embryonic fibroblasts on laminin substratum." Exp Cell Res **174**(2): 344-54.

Gazziola C, Moras M, Ferraro P, Gallinaro L, Verin R, Rampazzo C, Reichard P, and Bianchi V. (1999). "Induction of human high K(M) 5'-nucleotidase in cultured 293 cells." Exp Cell Res **253**(2): 474-82.

Krivanek, J. O. (1956). "Alkaline phosphatase activity in the developing slime mold Dictyostelium discoideum Raper." J. Exp. Zool. **133**: 459-479.

Resta R, Yamashita Y, and Thompson LF. (1998). "Ecto-enzyme and signaling functions of lymphocyte CD73." Immunol Rev **161**: 95-109.

Ubeidat, M., Eristi, C. M., and Rutherford, C. L. (2002). "Expression pattern of 5'-nucleotidase in Dictyostelium." Mech. Devel. **110**: 237-239.

Uusitalo, R. J. (1981). "5'-Nucleotidase activity in lymphocytes." Histochem J **13**(4): 525-34.

Yoneyama Y, Suzuki S, Sawa R, Otsubo Y, Power GG, and Araki T. (2000). "Plasma adenosine levels increase in women with normal pregnancies." Am J Obstet Gynecol **182**(5): 1200-3.

Chapter 3

Site-Directed Mutagenesis of the *5'-nucleotidase* Promoter to Identify Control Elements Influenced by cAMP that Participate in the Transcriptional Regulation of *5nt* in *Dictyostelium discoideum*

Abstract

A 250 bp region of the *5'-nucleotidase* (*5nt)* promoter was subjected to site-directed mutagenesis, generating 12 mutagenized promoter sequences. Each of the mutagenized promoters was fused to the *luciferase* reporter gene and the *lacZ* reporter gene. In order to identify control elements within the promoter influenced by cAMP, *Dictyostelium* AX3K cells transformed with these constructs were incubated in the absence and presence of cAMP. Probes designed to span specific sites of the promoter were utilized in electromobility gel shift assays (EMSA) with protein extracts from cells incubated in the absence and presence of cAMP. A binding activity, detected only in protein fractions obtained from cells treated with cAMP, was identified with an Rf = 0.47.

Results and Discussion

Within the life cycle of *Dictyostelium discoideum*, multicellular development is initiated when nutrient levels become diminished. Individual amoebae cells emit pulses of cAMP, and neighboring cells respond by migrating towards the highest concentration of the chemoattractant (Bonner 1947). The signaling network that occurs among the single-celled population involves two cAMP receptors, cAR1 and cAR3, while two other cAMP receptors, cAR2 and cAR4, are additionally utilized by cells during the later multicellular stages of development (Kimmel 1987; Kimmel and Firtel 1991; Milne and Devreotes 1993). As a result of this migration, the individual cells aggregate together into

a mound formation, and the remainder of the life cycle ensues. The role of cAMP as a chemoattractant in the life cycle is clearly established, though its role in gene regulation remains to be described at the molecular level. This study has focused upon cAMP as it influences gene expression levels.

5NT is an enzyme prevalent in a variety of species. Found in cellular locations including the cytoplasm, the mitochondria and the membrane, it is most ubiquitous on membrane exteriors, where it is attached by glycosyl phosphoinositol anchors. The enzyme has been implicated in many cellular activities, although one of its main functions as a membrane-bound ecto-nucleotidase is to produce nucleosides from nucleotides. This conversion enables nucleosides to cross the membrane barrier and enter the cell. 5NT has also been associated with activities including cell interactions, signal transduction, T-cell activation, cell motility, and membrane transport processes, and the cAMP degradation pathway.

5nt is developmentally regulated throughout the life cycle of *Dictyostelium*. Gene expression is initially evident as individual cells begin to assemble into the tight aggregate formation (Chanchao et al. 1999). As the first finger stage is reached, mRNA levels increase, and these levels remain high throughout the remainder of development. The expression of *5nt* at a stage of development when cAMP is present at increased levels marks the gene as a candidate for its regulation by cAMP. Transcriptional regulation of the *5nt* promoter was therefore analyzed in this study to identify regulatory binding sites whose protein associations were dependent upon the presence of cAMP.

To identify control elements in the *5nt* promoter that may be influenced by the presence of cAMP, a series of mutagenized promoters were created within a 250 bp

region of the promoter. This region of the promoter, chosen from the results of an extensive promoter deletion study, is located between -413 bp and -663 bp from the ATG translational start site. Within this target sequence of the promoter, precise point mutations were generated at twelve distinct sites (Fig. 3.1). Sites for point mutations were chosen to collectively cover most of the 250 bp target sequence. The *Dictyostelium* genome is very AT rich, and this characteristic extends to gene promoters. Past research has identified relatively GC rich control elements, and therefore some of the sites chosen for mutagensis had this quality (Gollap and Kimmel 1997). Mutation sequences were designed to contain a *Sty*I site for selection purposes during the cloning process.

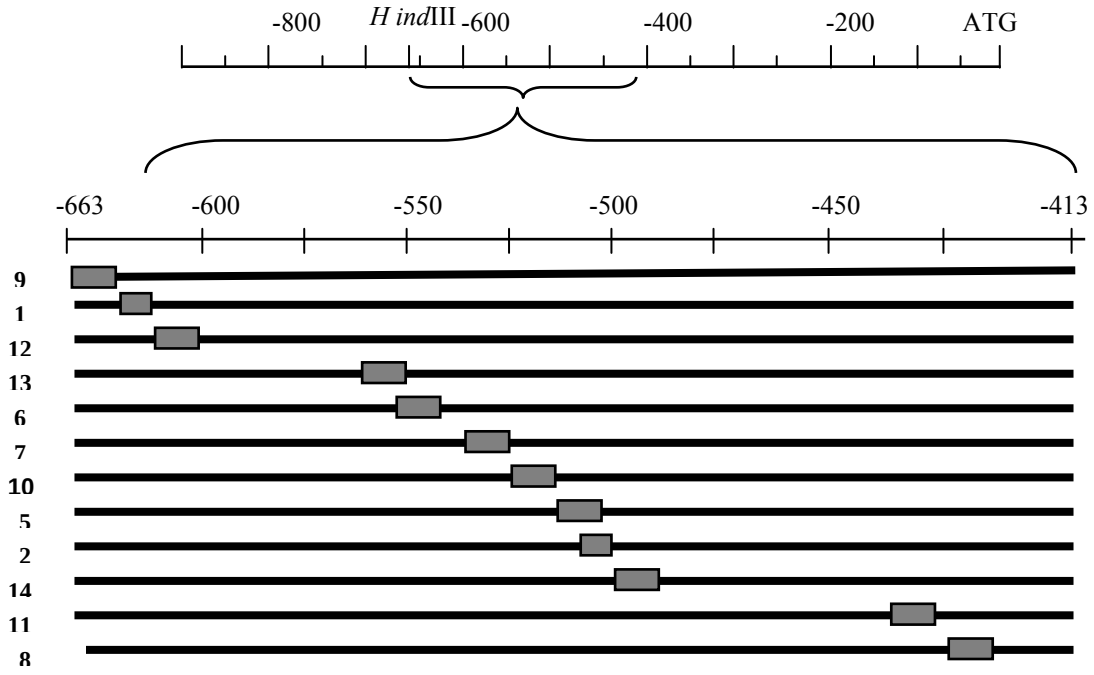

Figure 3.1. Targeted Sites for Site-Directed Mutagenesis of the *5nt* Promoter. A 250 bp region, beginning with an endogenous *Hind*III restriction site, of the *5nt* promoter was selected for site-directed mutagenesis analysis. By utilizing various mutagenic primers to introduce altered sites into the 5nt promoter contained within the pBluescript vector, 12 different mutagenized promoter sequences were generated using the protocol of a QuikChange Site-Directed Mutagenesis Kit (Stratagene). The mutagenized sequence, introduced using strand synthesis by thermal cycling, was either 6 or 12 bp in length and contained a *Sty*I restriction site. XL1-Blue supercompetent cells were transformed by heat shock, and colonies produced after overnight incubation at 37°C on LBamp plates were tested by PCR. Plasmids were isolated using a Wizard Plus SV Miniprep DNA Purification kit (Qiagen) and sequenced at the VBI Core Lab of Virginia Tech.

Previous studies with the *luciferase* and *lacZ* reporter genes have revealed that after fusion of the genes to the *5nt* promoter, the expression levels observed throughout *Dictyostelium* development are consistent with the expression pattern observed with the endogenous *5nt* gene. Because both reporter genes are reliable indicators of *5nt* activity, both were used in this study, allowing activity from each mutagenized promoter to be evaluated using both enzyme assays. Normally, *5nt* is not expressed in amoebae cells, although its activity is induced in the presence of cAMP. Therefore, the lack of cAMP induction from a mutagenized promoter in this study would mark the site of mutation as a potential cAMP regulatory element.

Transformation of *Dictyostelium* cells with mutagenized promoters fused to the *luciferase* gene of the pVTL2 reporter vector resulted in the successful development of 8 different transformants. Cells transformed with promoters 1, 2, 5 and 9 fused to *luciferase* did not grow to an optimal density for further experimentation, and were therefore not considered in this portion of the study. Stable clones were obtained for 8 constructs, and the results of the *luciferase* reporter gene expression from these cells shaken in the presence or absence of cAMP is shown in Fig 3.2. Seven clones from construct 7, 2 clones from construct 8 and 8 clones from construct 10 were tested for an effect of cAMP on reporter gene expression. None of these clones were induced by cAMP. This suggests that these sites may be potential cAMP regulatory elements, required for the detection of cAMP and appropriate genetic response to cAMP. Based upon the fact that cAMP induction was observed in at least one of the clones from constructs 6, 11, 12, 13 and 14, these sites were not considered potential cAMP regulatory elements. To further validate

and investigate the response of these promoter sites to cAMP, the same mutagenized promoters were tested with an additional reporter gene.

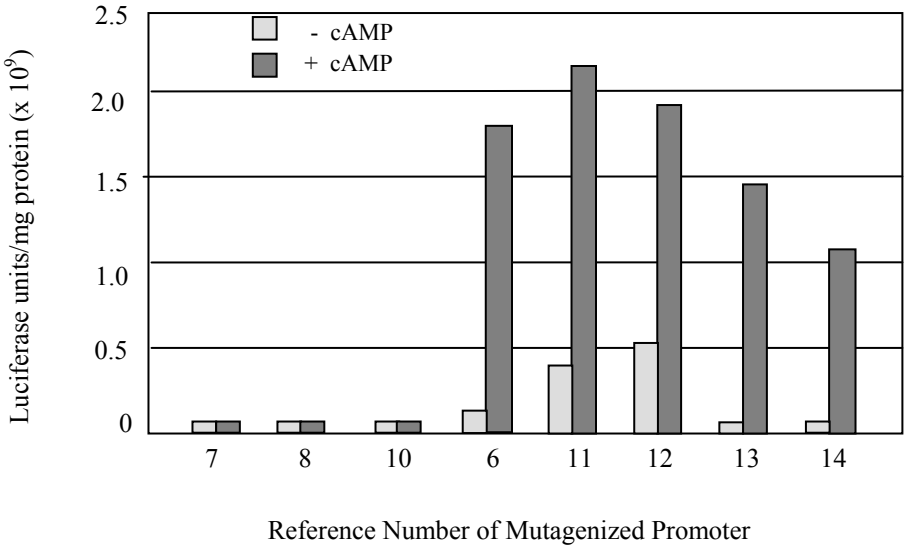

Figure 3.2. Luciferase Activity From Site-Directed Mutagenized Promoters in the Absence and Presence of cAMP. Mutagenized *5nt* promoters were fused to the *luciferase* reporter gene of the pVTL2 reporter vector using a 20:1 insert:vector ligation ratio. After transformation of *Dictyostelium* cells by calcium / glycerol shock, multiple clones for each type of mutagenized promoter construct were grown to a density of 4 x 10^6 cells / ml in shaking flasks. Each clone was grown in two separate 125 ml flasks. Approximately 24 hr after the cells were transferred to non-nutrient media (MES-LPS), a final concentration of 1mM cAMP was added to one flask for each clone (+ cAMP), while the other flask remained (- cAMP). Cells were collected 8 hr later, resuspended in 30mM glycyl-glycine (pH 8.3), and *luciferase* enzyme activity assays were performed as previously described. To determine specific activity, protein amounts were measured using a BCA protein assay kit (Pierce). In many cases, induction of reporter gene activity was observed. A single clone representative of the activity levels measured for each mutagenized promoter construct is shown. The activity levels measured for constructs 7, 8, and 10 were similar to activity levels measured for negative control samples without extract. No induction was observed with any of the clones from these 3 constructs.

Mutagenized promoters were also fused to the *lacZ* reporter gene of the ß-gal vector, resulting in the growth and development of 11 different transformants. Cells transformed with mutagenized promoter 5 did not thrive to an appropriate cell density for reporter gene analysis. The effect of cAMP on expression of *lacZ* and resulting ß-galactosidase enzyme activity is shown in Fig 3.3. Eight clones from construct 2, 9 clones from construct 8, 8 clones from construct 10, and 10 clones from construct 11 were tested, and ß-galactosidase activity was not induced by cAMP for any of these clones, suggesting that these sites may be potential cAMP regulatory elements. Based upon the fact that cAMP induction was observed in at least one of the clones from constructs 1, 6, 7, 9, 12, 13 and 14, these sites were not considered potential cAMP regulatory elements.

Comparing the results obtained from the *luciferase* cAMP induction study and the *lacZ* cAMP induction study, it was determined that the two sites most likely to be cAMP regulatory elements were sites 8 and 10. None of the clones obtained for these constructs showed cAMP induction in either enzyme assay. Site 8 is located -438 and -450 bp from the ATG translation start site. Site 10 is located -538 and -550 bp from the ATG translation start site (Fig. 3.1).

Analyses of protein binding with regions of the promoter were enabled using electromobility gel shift assays. Three probes generated by PCR were utilized in this study. Probe A represented the promoter sequence between -543 and -663 bp from the ATG site, probe B represented the promoter sequence between -428 and -538 bp from the ATG site, and probe C represented the promoter sequence between -423 and -293 bp from the ATG site. With reference to the site-directed mutagenesis experiments, site 10 was present in probe A, while site 8 was present in probe B. Probe C was located further

downstream, and included promoter sequence that was not analyzed in the site-directed mutagenesis experiments.

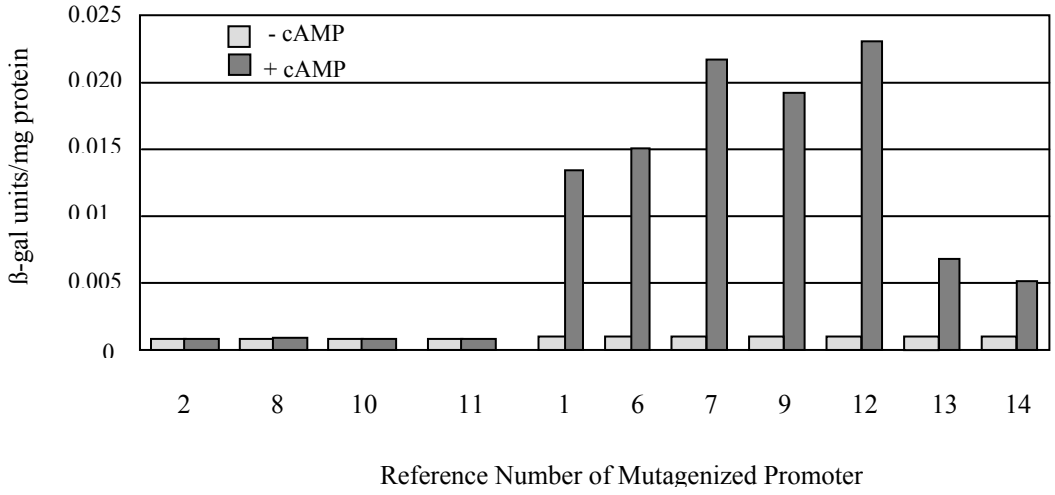

Figure 3.3. ß-galactosidase Activity From Site-Directed Mutagenized Promoters in the Absence and Presence of cAMP. Mutagenized *5nt* promoters were fused to the *lacZ* reporter gene in the ß-gal reporter vector using a 2:1 insert:vector ligation ratio. After transformation of *Dictyostelium* cells by calcium / glycerol shock, multiple clones for each type of mutagenized promoter construct were grown in shaking flasks. Each clone was grown in two separate flasks. Approximately 24 hr after the cells were transferred to non-nutrient media, a final concentration of 1mM cAMP was added to one flask for each clone (+ cAMP), while the other flask remained (- cAMP). Cells were collected 8 hr later, resuspended in a phosphate lysis buffer buffer (14.7 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.0) and *ß-galactosidase* enzyme activity assays were performed were performed as previously described. To determine specific activity, protein amounts were measured using a BCA protein assay kit (Pierce). A single clone representative of the activity levels measured for each mutagenized promoter construct is shown. The activity levels measured for constructs 2, 8, 10 and 11 were similar to activity levels measured for negative controls samples without extract. No induction was observed with any of the clones from these 4 constructs.

Cytoplasmic and nuclear protein extracts used in gel shift assays were obtained from amoebae incubated in the presence and absence of 1 mM cAMP. Cytoplasmic and nuclear fractions from each of these conditions were then subjected to ammonium sulfate precipitation to yield 20%, 40%, 60% and 80% fractions. Each of the three probes was tested with the cytoplasmic (+ / - cAMP) ammonium sulfate fractions and the nuclear (+ / -cAMP) ammonium sulfate fractions with three different non-specific carriers: polydIdC-polydIdC, polydA-polydT, polydAdT-polydAdT. Under the conditions tested, a DNA-protein binding activity with an Rf = 0.47 was detected using probe A and the non-specific polydIdC-polydIdC carrier (Fig. 3.4). The band was present in the cytoplasmic (+cAMP) protein fraction, but not present in the cytoplasmic (-cAMP) protein fraction. The band was not detected using any of the conditions tested with probes B or C (data not shown).

The analysis of protein binding activity using gel shift assays has enabled the identification of a band of potential interest, whose activity appears to be influenced by the presence of cAMP. Further tests substantiating the specificity and stability of the 0.47 Rf binding activity will further elucidate the potential control region identified by this study. Site 10, identified as candidate cAMP control elements in the mutagenesis study, may be involved in this activity. The sequence of site 10 that was mutagenized in the aforementioned experiments contains a near perfect palindrome: 5' CAGAAAATAGATAAAGAC 3', a motif that is frequently associated with transcription factor binding. When this sequence was changed to 5' CAttcccaaggcttAGAC 3', no cAMP induced expression was observed for either *luciferase* or *lacZ* reporter genes.

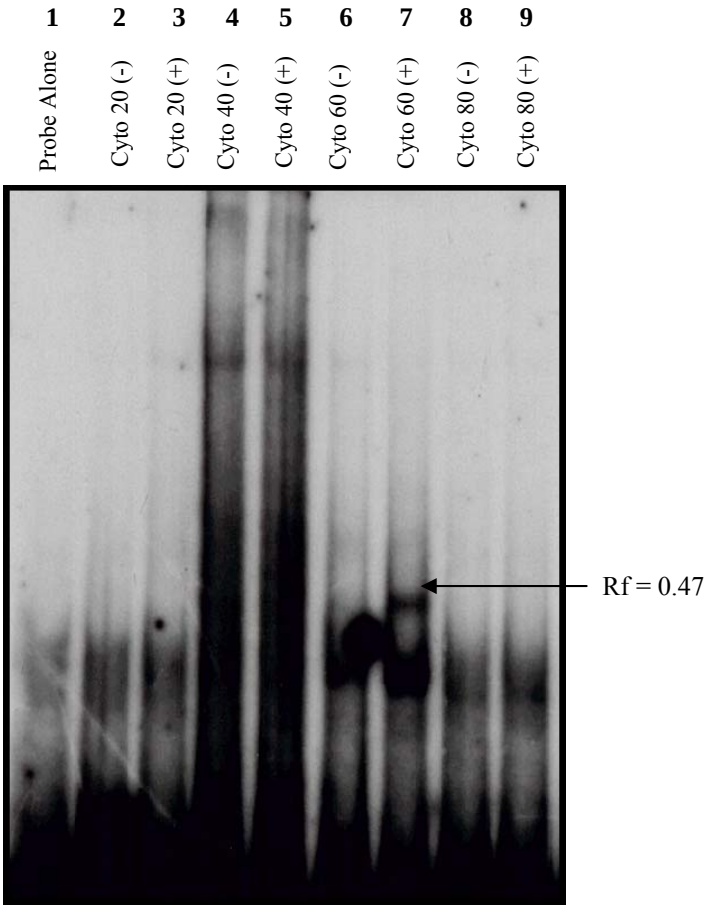

Figure 3.4. A 0.47 Rf DNA-Protein Binding Activity Detected Specifically with Probe A. Cytoplasmic protein extracts were prepared from amoebae cells in the absence (-) and presence (+) of 1 mM cAMP as previously described. Following ammonium sulfate precipitation of the cytoplasmic extract, electromobility gel shift assays were performed. The cocktail used for the gel shift binding reactions included 20mM HEPES, pH 7.9, 12% glycerol, 20 mM NaCl, 1 mM EDTA, pH 8.0, 2 mM $MgCl_2$, 2 μg BSA, 1 mM DTT, 0.04% NP40 and 0.5 μg of a non-specific carrier (poly IC shown here) combined with the protein extract. Labeled probe A (30,000 cpm) generated by PCR was added and the reaction incubated at room temperature for 25 min. The reaction was then run for 2 hr on a 5% native polyacrylamide gel in 0.5X TBE, which had previously run for 30 min at 150 V. Gels were dried at 80°C for 50 min then exposed on X-OMAT AR Kodak film at -80°C before development. An activity with Rf = 0.47 was found that specifically interacted with probe A. This activity was not present with probes B or C (data not shown), and therefore may represent a control element within the 5nt promoter that is regulated by cAMP.

References

Bonner, J. T. (1947). "Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold Dictyostelium discoideum." J. Exp. Zool. **106**: 1-26.

Chanchao, C., Eristi, C. M., Favis, R., and Rutherford, C. L. (1999). "5'-Nucleotidase in Dictyostelium: protein purification, cloning, and developmental expression." Biochim. Biophys. Acta **1473**: 376-390.

Gollap, R. and A.R. Kimmel (1997). "Control of cell-type specific gene expression in Dictyostelium by the general transcription factor GBF," Development. **124** (17) 33905-405

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

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

Milne, J. L. and P. N. Devreotes (1993). "The surface cyclic AMP receptors, cAR1, cAR2, and cAR3, promote Ca2+ influx in Dictyostelium discoideum by a Galpha2-independent mechanism." Mol. Biol. Cell **4**: 283-292.

Zimmerman, W. and C. J. Weijer (1993). "Analysis of cell cycle progression during the development of Dictyostelium and its relationship to differentiation." Dev. Biol. **160**: 178-185.

Chapter 4

Identification and Purification of a DNA-Binding Protein Interacting with the Promoter of *5-nucleotidase* in *Dictyostelium discoideum*

Abstract

The developmental management of *5'-nucleotidase* (*5nt*) expression in the model system *Dictyostelium discoideum* has provided a focal point for studies of gene regulation at the level of transcription (Armant 1980; Chanchao et al. 1999; Ubeidat et al. 2002; Rutherford et al. 2003). In efforts to identify DNA-protein binding activities that may be critical for the regulation of *5nt*, electromobility gel shift assays were performed. Numerous short oligonucleotides, whose sequences were designed to analyze a region of the promoter approximately 450 bp in length, were used in combination with various protein extracts. Initial assays revealed a binding activity (Rf = 0.33), which through competition assays with several unlabeled probes, appeared to be specific to the promoter sequence located between -342 and -313 from the ATG translation start site. Characterization of the binding activity, including the effects of parameters such as salts and temperature provided insight into the nature and stability of the DNA- protein interaction. The protein responsible for the 0.33 Rf binding activity was purified in a series of chromatographic stages, which included DEAE sephacel chromatography, Heparin Sepharose chromatography, DNA affinity chromatography and gel filtration chromatography. SDS-PAGE analysis of the gel filtration fractions revealed a polypeptide with an approximate molecular weight of 70 kDa. Peptide sequences obtained after analysis of the isolated protein by mass spectrometry revealed that the protein purified in this study was a putative formyltetrahydrofolate synthase (FTHFS). The specificity of the protein's association with the *5nt* promoter suggests the potential role of the protein as a regulatory transcription factor.

Introduction

5nt is an enzyme that is ubiquitous to numerous species and resides in a variety of cellular locations, including the cell membrane, the mitochondria, and the cytosol. The functions of the enzyme as it resides on the extracellular surface of the membrane include the conversion of nucleotides to nucleosides to enable transmembrane trafficking, cell adhesion activities, cellular recognition events and cell/cell interactions. 5NT is also involved in the cyclic AMP (cAMP) degradation pathway. 5NT removes 5'AMP by breaking it down into adenosine and inorganic phosphate. This activity precludes the accumulation of 5'AMP, which could otherwise prevent the additional degradation of cAMP by feedback inhibition. The role of cAMP as a chemoattractant and morphogen during the growth and development of many organisms has been established, and extends to the model organism utilized in this study.

Initiation of the multicellular stages of the *Dictyostelium* life cycle is dependent upon the chemoattractant cAMP, which is emitted as pulses by individual amoebae when nutrients become diminished (Bonner 1947). As a result of neighboring cells responding to the chemoattractant by migrating towards its most concentrated source, a multicellular aggregate structure is formed. Throughout the remainder of development, the differentiation of two major cell types occurs, a process that is dependent upon two morphogens: cAMP and DIF-1 (differentiation-inducing factor) (Kessin 2001).

It has been established that the concentrations of cyclic AMP and DIF-1 are different in prestalk and prespore cells (Kay et al. 1999). Before the culmination stage of the life cycle is reached, cAMP induces cell-type specific gene expression in both cell

types. After the culmination stage, however, prestalk cell differentiation is inhibited by cAMP, while prespore cell differentiation is induced (Williams and Jermyn 1991). In prestalk cells, the expression of a cAMP-phosphosdiesterase causes a reduction in cAMP concentration, thereby resulting in the inhibitory effects observed (Brown and Rutherford 1980; Hall et al. 1993). Another morphogen involved in differentiation, DIF-1, is present in high concentrations in the prespore region, where it is thought to be synthesized (Loomis 1993). The presence of an enzyme in prestalk cells that degrades DIF-1 results in low levels of the morphogen in this zone. These low concentrations stimulate prestalk-specific gene expression and repress prespore-specific gene expression.

In *Dictyostelium, 5nt* is expressed only during multicellular stages of development (Chanchao et al. 1999). Expression of the gene is first apparent approximately 5 hr after the initiation of multicellular development when nutrients are depleted. At this stage, individual amoebae have created a loose aggregate formation. The expression of the gene increases as the organism enters the first finger and slug stages of development, and high levels of expression are maintained throughout the remainder of the life cycle (Chanchao et al. 1999).

Fusion of the *5nt* promoter to the *lacZ* reporter gene enabled the cellular localization of enzyme activity to be determined within the organism at each developmental stage (Ubeidat et al. 2002). During the formation of the aggregate, activity was determined to be highest in cells residing in the aggregate center relative to cells still streaming. In the tight aggregate, activity resided mainly in cells from the upper portion of the structure. While in the first finger stage of development, activity was highest in cells at the base of the structure, activity was prevalent in anterior-like cells (ALCs) and

slime sheath cells at the slug stage. Activity in early culminants was concentrated to the boundary between the prespore and prestalk cells, while in the more mature culminants activity was found in the lower section of the pre-spore mass. Upon fruiting body formation, activity was localized to the basal disc, lower stalk and upper cup (Ubeidat et al. 2002).

The changes of *5nt* expression levels and localization during the course of development in *Dictyostelium* require the employment of mechanisms to regulate activity in a temporal and spatial context. The transcriptional regulation of *5nt* was investigated in our lab through a thorough analysis of the gene's promoter (Wiles et al. in preparation). A study of numerous internal and 5' promoter deletions enabled the identification of a general region of the promoter in which regulatory elements may reside. Further evaluation of this promoter region by site-directed mutagenesis provided additional insight into potential sequences in the promoter critical to the achievement of normal levels of expression. In this study, electromobility gel shift assays (EMSA) were performed to identify DNA-protein binding activities involved in the temporal regulation of *5nt* gene expression. By designing and utilizing numerous short oligonucleotide probes that spanned a chosen region of the promoter, DNA-protein binding activities specific to particular nucleotide sequences in the *5nt* promoter could be identified.

We report here the identification of a DNA-protein interaction specific to a 30 bp sequence of the promoter. We describe the characterization of this binding activity with respect to parameters including temperature, salt and the presence of different non-specific competitors. We explain the purification processes utilized to isolate the DNA-binding protein, describe the mass spectrometry analysis of the characterized protein, and

discuss the potential role of the presumed formyltetrahydrofolate synthase (FTHFS) as a regulator of *5nt* expression.

Materials and Methods

Extraction of Cytoplasmic and Nuclear Proteins from Dictyostelium Amoebae and Slugs

Cytoplasmic and nuclear protein extracts used for EMSA were derived from two sources: AX3K *Dictyostelium* amoebae induced with cAMP in shaking flasks, and AX3K *Dictyostelium* slugs grown on 2% water agar plates. Preparation of amoebae extracts were obtained by first enabling cells to grow to a density of about 3 x 10^6 cells / ml in shaking flasks at 150 rpm. Cells were harvested by centrifugation at 2,100 x g in a CRU-5000 centrifuge (International Equipment Company) for 3 min, and the pellets obtained were washed in a 1X MES-LPS buffer (pH 6.5). Resuspension of the washed pellet in 1X MES-LPS was in an amount required to obtain a final concentration of 1 x 10^7 cells / ml. Cells were returned to shaking flasks and grown under non-nutrient conditions overnight, after which cAMP was added to a final concentration of 1mM. Cells were shaken in the presence or absence of cAMP for 8 hr, and then harvested by centrifugation at 10,000 x g in a Sorvall GSA for 10 min. After aspiration of the supernatant, the pellets obtained were resuspended in 5 times the pellet weight in lysis A buffer, which contained 50 mM Tris (pH 7.5), 10% glycerol, 2 mM $MgCl_2$, 1% NP40, 1 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF, and then harvested by centrifugation at 10,000 x g for 10 min. The supernatant, containing cytoplasmic proteins, was stored at -80°C until EMSA were performed. The pellet was thoroughly washed and resuspended with 4ml of lysis buffer A, and then centrifuged at 10,000 x g for 10 min. The supernatant was removed and

stored at -80°C. The pellet was weighed and resuspended thoroughly by vortexing in 2X volume lysis buffer A. NaCl was then added to a final concentration of 0.42 M, and the samples were incubated on ice for 1 hr. After centrifugation at 10,000 x g for 10 min, the supernatant, containing nuclear proteins, was stored at -80°C.

Preparation of cytoplasmic and nuclear protein extracts from slugs also began with growing amoebae in shaking flasks to a density of approximately 3 x 10^6 cells / ml. Cells were harvested by centrifugation at 2,100 x g in a CRU-5000 centrifuge, and the pellets obtained were washed in a 1X MES-LPS buffer (pH 6.5). Resuspension of the washed pellet in 1X MES-LPS was in an amount required to obtain a final concentration of 1 x 10^7 cells / ml. To induce multicellular development, the samples were placed on ice, and then plated on the surface of 2% water agar Petri plates. Plates were incubated at room temperature until the slug stage of development was reached, approximately 18 hr later. To remove the slugs from the plate, a 20 mM potassium phosphate buffer (pH 7.5) was used. To dissagregate the slugs into single cells, the samples were passed through a 21 G syringe, and then centrifuged at 2,600 x g for 5 min. The supernatant was removed and the pellets were resuspended in 5 times the pellet weight of lysis buffer A. Cytoplasmic and nuclear protein fractions were then obtained in the same manner as described for the amoebae samples.

Ammonium Sulfate Fractionation of Cytoplasmic and Nuclear Protein Extracts

Cytoplasmic and nuclear protein extracts obtained from amoebae and slugs were subjected to further fractionation by ammonium sulfate precipitation for some

experiments. The cytoplasmic or nuclear extract was kept in a beaker on ice while stirring, as solid ammonium sulfate was slowly added to 20% saturation over a period of 5 min. The sample was stirred on the plate for an additional 30 min, after which the extract solution was poured into a 40 ml polycarbonate tube and centrifuged at 10,000 x g in a SS34 rotor for 20 min. The supernatants were poured back into the beaker while the pellets were resuspended in buffer containing 50 mM Tris (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH8.0), 0.5 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF, and then placed on ice. Meanwhile, solid ammonium sulfate was slowly added to 40% saturation over a period of 5 min to the supernatants in the beaker. The sample was stirred for an additional 30 min and then harvested by centrifugation. This process was repeated in the same manner for 60% and 80% saturation samples. At the end of the experiment, the pellets that had been resuspended in buffer and incubated on ice were dialyzed overnight in 1 L of dialysis buffer (20 mM Tris, pH 7.5) at 4°C. In order to remove any solids formed during the dialysis process, the samples were transferred to 12 ml polycarbonate tubes and centrifuged at 20,000 x g for 15 min. The supernatants were then stored at –80°C until used for subsequent EMSA.

Generating and Labeling Probes for EMSA

The probes prepared and used in this study were short double-stranded oligonucleotides. Annealing of the complementary single strands entailed the addition of a buffer containing 20 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), and 50 mM NaCl. The sample was incubated for 5 min at 70°C, and then cooled to room temperature. For

the labeling reaction, 100 ng of the double-stranded oligonucleotide was combined with T4 Polynucleotide Kinase (PNK) buffer, and 30 μCi of [γ-^{32}P] ATP in a total reaction volume of 11 μl. The reaction mix was incubated for 5 min at 70°C, and then chilled on ice before the addition of 10U/μl T4 PNK. Subsequently, the reaction mix was incubated for 1 hr at 37°C, and then incubated for 20 min at 65°C. To remove unincorporated nucleotides, the labeling reaction was subjected to a Micro Bio-Spin 6 column (BioRad). The radioactivity of the labeled probes was then measured using a scintillation counter (Beckman LS 6000SC).

Binding Reactions and EMSA Conditions

The standard binding reaction utilized a cocktail that included 20 mM HEPES (pH 7.9), 12% glycerol, 20 mM NaCl, 1 mM EDTA (pH 8.0), 2 mM $MgCl_2$, 2 μg BSA, 1 mM DTT, 0.04% NP40 and 0.5 μg of a non-specific carrier (polydIdC-polydIdC, polydA-polydT, or polydAdT-polydAdT). In eppendorf tubes, the cocktail was combined with 1.5 μl of the protein extract chosen for analysis. After adding 30,000 cpm of the labeled DNA probe, the reactions were incubated on ice for 10 min. If the reaction included the addition of a competitor, then it was added after the addition of the labeled probe. After pre-running the gel at 150 V for 30 min, the samples were loaded on a 5% native polyacrylamide gel in 0.5X TBE. The voltage was increased to 200 V for approximately 2 hr. Subsequently, the gels were then transferred to chromatography paper and dehydrated for 45 min at 80°C in a gel dryer. After the gel had dried onto the chromatography paper, the paper was placed on X-OMAT AR Kodak film in a cassette

and exposed at -80°C. Exposure time prior to development was generally between 24 and 72 hr.

DEAE Sephacel Column Chromatography

A Waters Advanced Protein Purification System (Model 650E) was used to perform all of the High Performance Liquid Chromatography (HPLC) experiments in this study. Prior to chromatography, the DEAE Sephacel (Sigma) column (27 mm x 250 mm) was equilibrated with Buffer D, comprised of 20 mM Tris (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH8.0), 0.5 mM DTT, 20 μg/ml TLCK, and 100 μg/ml PMSF. The first steps of protein purification utilized the 80% ammonium sulfate cytoplasmic protein fraction. The protein extract was slowly thawed on ice and loaded onto the column with a syringe. Buffer D was run through the column using a peristaltic pump at a rate of 3 ml / min. Flow-through fractions were collected until a baseline was reached as indicated by an absorbance monitor. Once the baseline was reached, negatively charged proteins bound to the DEAE Sephacel resin were eluted using a linear gradient to 0.5 M NaCl in Buffer D at a flow rate of 5 ml / min. As fractions were collected, their contents were assessed by an abosorbance monitor, and then analyzed by EMSA. The column was stored at 4°C in a 20% ethanol solution.

Heparin Sepharose Column Chromatography

Prior to chromatography, the Heparin Sepharose (Sigma) column (20 mm x 130 mm) was equilibrated with Buffer H, comprised of 10 mM potassium phosphate buffer (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF. This chromatography was used as the second stage of protein purification, and therefore, active fractions obtained from DEAE Sephacel chromatography were pooled and concentrated to approximately 12 ml (in Buffer H) using an Amicon YM10 membrane. The protein extract was loaded onto the column using a syringe. Flow-through fractions were collected until a baseline was reached. Once the baseline was reached, potential DNA-binding proteins bound to the sulfated glucosaminoglycan polymer were eluted using a linear gradient to 0.5 M NaCl in Buffer H at a flow rate of 2 ml / min. As fractions were collected, their contents were assessed by an absorbance reader, and then analyzed by EMSA. The column was stored at 4°C in a 20% ethanol solution.

Preparation of the DNA Affinity Column

In preparation for DNA affinity chromatography, double-stranded oligonucleotides (same sequence as probe 12) were prepared, phosphorylated, and then coupled to CNBr- activated Sepharose-4B resin (Pharmacia). Phosphorylation of the double-stranded oligonucleotides was achieved by adding 3 mM ATP and 100 U of T4 PNK in a total reaction volume of 100 μl with T4 PNK buffer. After incubating the

phosphorylation reaction for 2 hr at 37°C, the DNA was precipitated using ethanol. The DNA was then subjected to ligation by adding 2,000 U T4 DNA ligase in a total reaction volume of 100 µl. The sample was incubated overnight at 16°C, after which the DNA was extracted from the ligation reaction with phenol, precipitated with ethanol, and resuspended in 100 µl milliQH_2O.

To couple the phosphorylated double-stranded oligonucleotides to the activated resin, 1g of the resin was first added to 100 ml 1 mM HCl for 10 min. After the resin had swelled, it was transferred to a glass filter, where it was first washed with 200 ml of 1 mM HCl, then with 300 ml milliQH_2O, and lastly with 100 ml 10mM potassium phosphate buffer (pH 8.0). After transferring the resin to a 15 ml polypropylene tube to settle, the supernatant was removed and 10 mM potassium phosphate buffer (pH 8.0) was added to a volume sufficient to create a thick slurry solution. The oligonucleotide sample and thick slurry were then combined in a rotating wheel, where they incubated overnight at room temperature.

After the overnight incubation, the sample was again transferred to a glass filter, where it was washed two times with 100 ml milliQH_2O, and then washed with 100 ml 1 M ethanolamine (pH 8.0). The washed sample was then transferred to a 15 ml polypropylene tube, and 1M ethanolamine was added to a final volume of 14 ml in order to block excess active groups. After rotation of the tube for 5 hr at room temperature, the resin was packed into the column (13 mm x 25 mm) and washed first with 100 ml of 10 mM potassium phosphate buffer (pH 8.0), then with 100 ml 1 M potassium phosphate buffer (pH 8.0), then with 100 ml 1M KCl, then with 100 ml milliQH_2O and finally with

100 ml of storage buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 0.04% NaN_3). The column was stored at 4°C in this solution.

DNA Affinity Chromatography

Prior to chromatography, the DNA affinity column was equilibrated with Buffer D, comprised of 20 mM HEPES (pH 7.9), 10% glycerol, 1 mM EDTA (pH 8.0), 2 mM $MgCl_2$, 1 mM DTT, and 0.04% NP-40. The active fractions obtained from Heparin Sepharose chromatography were pooled and concentrated to approximately 2 ml (in buffer D) using an Amicon YM10 membrane. The protein extract was loaded onto the column using a syringe, and Buffer D was pumped through the column at a rate of 1ml / min. The same sample was passed through the column four times in order to maximize the binding of proteins to the resin. Proteins binding to the DNA affinity resin were eluted using a linear gradient to 1 M NaCl in buffer D at a flow rate of 1 ml / min. As fractions were collected, their contents were assessed by an absorbance monitor, and then analyzed by EMSA.

Gel Filtration Column Chromatography

Prior to chromatography, the 300SW gel filtration column (Waters, 8 mm x 300 mm) was equilibrated with Buffer H, comprised of 10mM potassium phosphate buffer (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF. The active fractions obtained from DNA affinity chromatography were

pooled and concentrated to approximately 600 µl (in buffer H) using an Amicon YM10 membrane, and then loaded onto the column at a flow rate of 0.2 ml/ min. One min fractions were collected, then analyzed by EMSA. The column was stored at 4°C in 0.1% NaN_3.

Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

A 10% SDS-polyacrylamide gel was used to analyze the gel filtration fractions obtained from the purification process. The protein samples analyzed were initially combined with a loading buffer and incubated for 5 min at 85°C. After the samples were loaded on the gel, the conditions were set to 15 mA for 30 min using a running buffer (2.5 mM Tris, 19 mM glycine, 1% SDS), and then increased to 25 mA for one hr. Gels were fixed by incubating them overnight in a solution comprised of 50% ethanol in 10% acetic acid. After the overnight incubation, the gel was washed for 30 min in a solution comprised of 50% methanol in 5% acetic acid. A second wash with the same solution was performed for an additional 45 min. In this study, the gel was analyzed by staining with Coomassie Blue and silver, though only results of the Coomassie Blue stain are shown. After the gel was fixed, protein bands were visualized by Coomassie stain by incubation of the gel with a solution of 0.1% Coomassie Blue, 45% methanol in 10% acetic acid for 5 hr. Destaining of the gel consisted of an incubation period with 50% methanol in 5% acetic acid solution. The peptide bands were then removed and analyzed by mass spectrometry in the W.M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia in Charlottesville.

Results

Identification of a Specific DNA-Protein Binding Activity by EMSA

Analysis of the *5nt* promoter focused upon the upstream region of the sequence beginning at -663 bp from the ATG translation start site, where an endogenous *Hind*III restriction site resides (Fig. 4.1). This fragment has been shown to contain all sequences necessary for proper temporal regulation of *5nt* expression (Wiles et al. in prep). Short oligonucleotides, approximately 30 bp in length, were designed to span this region of interest that had previously been selected for study by an extensive promoter deletion analysis and site-directed mutagenesis experiments. For probe 1, the upstream limit was -670 bp from the ATG start site, and the sequence extended downstream to -641. For probes 2-12, the upstream limits were -660, -611, -581, -549, -536, -511, -487, -456, -412, -381, and -342, respectively. The use of these labeled probes in EMSA enabled a thorough analysis of protein binding activity on this region of the *5nt* promoter.

Probes 1-6 and 9-12 were used in an initial quest to identify protein binding activities that may be specific to some of the sequences of the probes generated (Fig. 4.2). Nuclear protein extract obtained from *Dictyostelium* amoebae, grown in the presence of 1 mM cAMP for 8 hr, was subjected to heparin sepharose chromatography. Fractions collected were divided into early (fractions 3-27) and late (fractions 28-34) eluting groups, pooled, concentrated, and then tested with various radiolabeled probes. Two binding activities, with Rf values of 0.29 and 0.60, were consistently seen in the late-eluting fractions with all of the probes tested. An additional band in the late-eluting

fractions was observed exclusively with probe 9. Though not evident with the other probes tested, this low mobility band was not consistently observed with probe 9, suggesting that it may be an artifact. A specific band was also detected in the early-eluting fractions with probe 12. This binding activity was not observed with other probes, and its consistent appearance in the presence of probe 12 made further analysis of the specific association a successive goal.

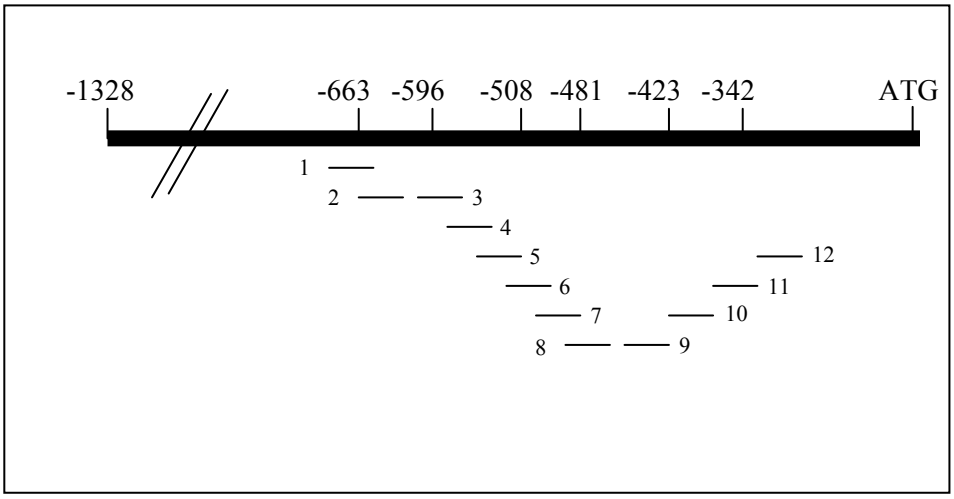

Figure 4.1. Oligonucleotide Probes Designed to Analyze the 663 Bp Upstream Region of the *5nt* Promoter. Double-stranded oligonucleotide probes of 30 bp lengths were designed to span the region of the *5nt* promoter between the endogenous HindIII restriction site (-663 bp) and the ATG translation start site. Probes were utilized in electromobility gel shift assays to locate specific sites on the promoter with which regulatory proteins may be interacting.

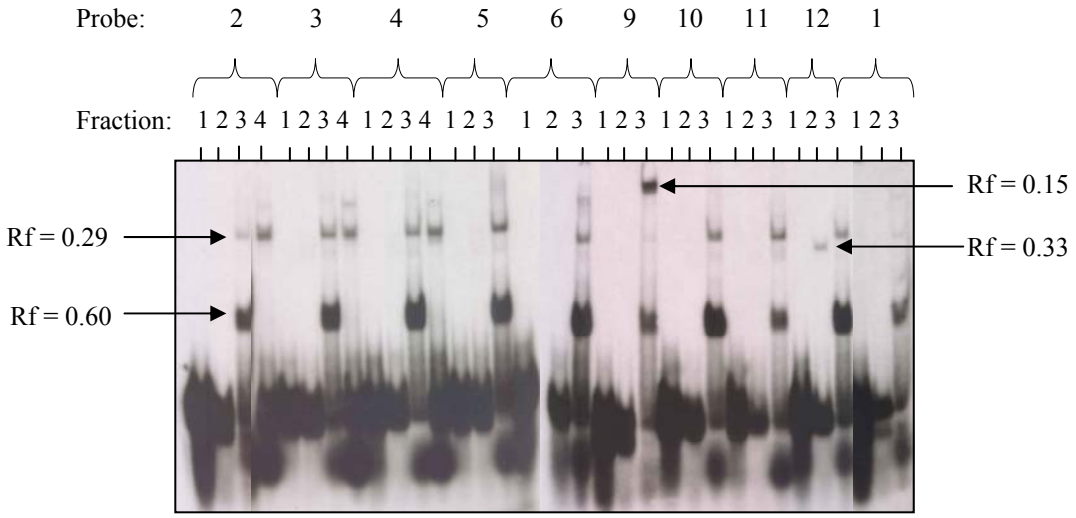

Figure 4.2. EMSA Reveals a Specific 0.33 Rf DNA-Protein Binding Activity. Nuclear protein extract obtained from *Dictyostelium* cells grown in the presence of 1mM cAMP was subjected to heparin sepharose chromatography. The flow through extract collected before the addition of salt was tested with various probes (1). Fractions collected from the separation were divided and concentrated into early eluting (2) and late eluting (3) fractions. In some lanes, nuclear extract (4) of the protein sample before chromatography was assayed. Ten different probes were tested with the protein fractions described to reveal a unique band with an Rf = 0.33 that was specific to probe 12 was detected in the concentrated early eluting fraction.

To further assess the specificity of the 0.33 Rf binding activity, gel shift competition assays were performed with a variety of probes (Fig. 4.3). In this study, unlabeled probes used as competitors were phosphorylated to simulate the labeled probe as much as possible in the binding reactions, and added to the binding reactions in a 70 molar excess. As expected, competition was the most pronounced in the presence of phosphorylated unlabeled probe 12 (lane 7). Competition was also observed, though not as prominently, when the unlabeled competitor was in an unphosphorylated form, suggesting that the phosphorylation of competitors may provide an optimal platform from which to analyze specificity (data not shown). Results from the assay reveal that the 0.33 binding activity is specific to the 30 bp nucleotide sequence that comprises probe 12. This sequence is located between -342 and -313 bp from the ATG translation start site.

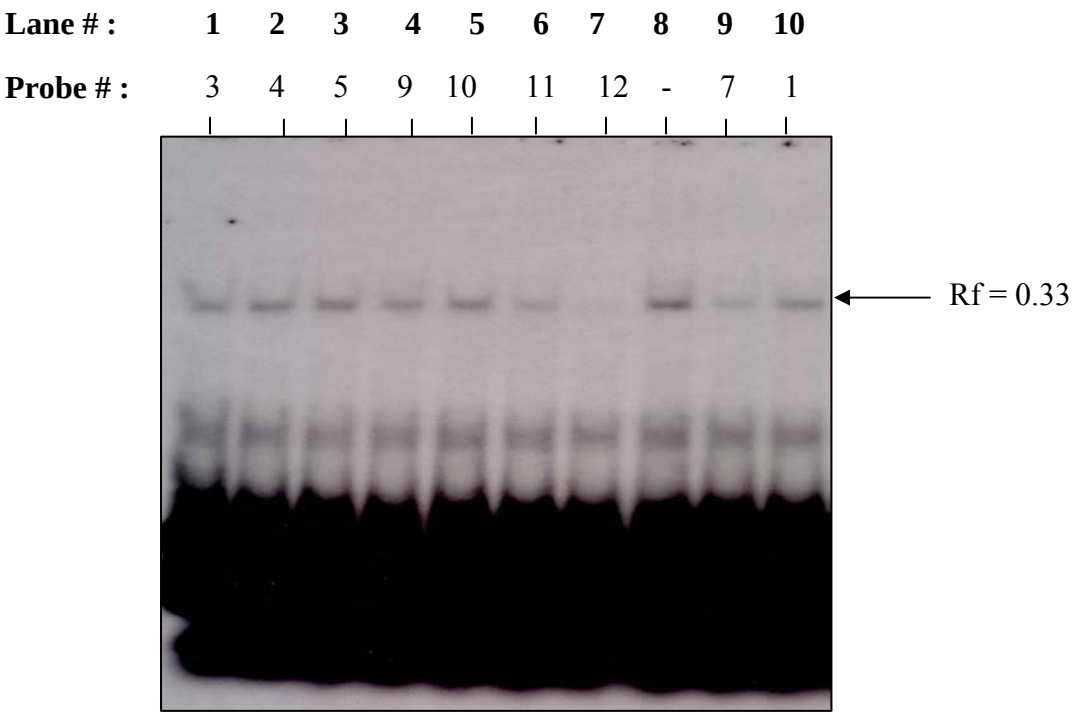

Figure 4.3. Competition Assays Validate the Specificity of the 0.33 Rf DNA-Protein Binding Activity with Probe 12. The early eluting fractions collected and concentrated after heparin sepharose chromatography were further analyzed by testing the effects of different unlabeled phosphorylated competitors. In lane 8 there was no unlabeled competitor tested. In the other lanes, unlabeled probes were added to the reaction mix in a 70 fold molar excess as the final reaction component. As expected, the addition of excess unlabeled probe 12 competed for the binding activity, as is evident by the diminished band intensity in lane 7. The extent of competition observed in lane 7 was not observed using any other excess unlabeled competitors. The slightly diminished band intensities observed in lanes 6 and 9 suggest that some competition did occur with unlabeled probes 11 and 7.

Characterization of the Specific 0.33 Rf DNA-Protein Binding Activity by EMSA

Characterization of the 0.33 Rf binding activity was addressed by an experiment in which the effects of various parameters on the binding activity were assessed (Fig. 4). The protein extract analyzed in this experiment was the pooled and concentrated early-eluting heparin sepharose fractions used and described in the experiments shown in Fig 4.2 and 4.3. The removal of magnesium from the binding reaction caused a decrease in the 0.33 Rf binding activity, suggesting that its presence enhances the DNA-protein interaction (Fig. 4.4, compare lanes 1 and 10). The substitution of 1mM calcium for the magnesium in the binding reaction in lane 3 resulted in a 0.33 Rf band of similar intensity as compared to lane 10. The substitution of 1 mM $ZnCl_2$ in lane 2 slightly reduced the 0.33 Rf binding activity, as is evident by band of lessened intensity. The addition of 100 mM NaCl, in place of 10 mM NaCl, clearly inhibited the DNA-protein interaction, as is seen in lane 4.

Visualization of the 0.33 Rf binding activity by EMSA was largely dependent upon the type of non-specific competitor used in the binding reaction. When the polydAdT-polydAdT non-specific competitor was used, the 0.33 Rf band was not detected due to the presence of two non-specific bands (Rf = 0.28 and 0.38) that masked its presence (data not shown). However, when the polydA-polydT non-specific competitor was used in the binding reaction, the 0.28 Rf and 0.38 Rf bands became significantly diminished, enabling the visualization of the 0.33 Rf band. The amount of non-specific competitor used in the binding reaction was tested, as shown in Fig 4.4. In lane 5, 1μg of polydA-polydT was used in the reaction, while in lane 6, 2 μg of the non-

specific competitor was used. Increasing amounts of the competitor does appear to slightly reduce the DNA-protein interaction, as is evident by comparing the intensities of the bands present in lanes 5, 6, and 10.

The effect of temperature on the 0.33 Rf binding activity was also assessed in Fig 4.4. Three experiments were performed in which the protein extract tested was incubated at 37°C, 68°C, or 84°C for 10 min before its addition to the other components of the binding reaction. While the 10 min incubation at 37°C (lane 7) had no effect on the 0.33 Rf binding activity, incubations at the higher temperatures significantly reduced the activity, as is shown in lanes 8 and 9.

Prior to protein purification, cytoplasmic extract obtained from *Dictyostelium* slugs was subjected to analysis with DEAE Sephacel resin and CM cellulose resin in order to assess the ionic characteristics of the protein of interest at a pH range of 5-9. After dialysis of the protein extract at the designated pH points and incubation with the specified resin type, the contents were centrifuged, and the supernatants were tested by EMSA with probe 12. The results of the experiment revealed that the protein was bound to the DEAE resin over the pH range 6-9. It was not bound to the CM cellulose resin, except at pH 5. Therefore, the protein is negatively charged in a pH range of approximately 6-9. To assess the ability to elute the bound protein from the DEAE resin, 0.5 M NaCl was added. The supernatants tested by EMSA showed that the protein represented by the 0.33 Rf could be eluted under these conditions.

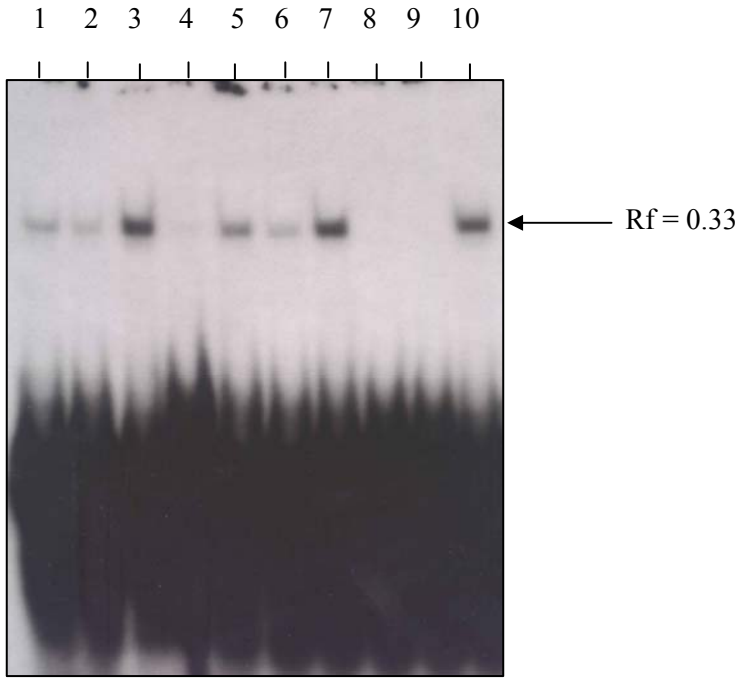

Figure 4.4. The Effects of Changing Binding Reaction Parameters such as Temperature, Salt, Metal Ions, and Non-Specific Carrier Concentration on 0.33 Rf Binding Activity. The early eluting fractions collected and concentrated after heparin sepharose chromatography were further tested with probe 19 by altering binding reaction conditions and assessing the effects of these changes on the 0.33 binding activity. The binding activity observed under standardized reaction conditions included 20 mM HEPES, 12% glycerol, 2 µg BSA, 2 mM $MgCl_2$, 20 mM NaCl, 0.5 µg polydA-polydT, 1 mM EDTA (pH 8.0), 1 mM DTT, and 0.04% NP40, and is represented in lane 10. In lane 1, magnesium was removed from the binding reaction, while in lanes 2 and 4 1 mM $ZnCl_2$ or 100 mM NaCl was included in the binding reaction. Lane 3 represents the effects seen when 1 mM $CaCl_2$ was included in the binding reaction. The binding reaction in lane 5 included 1 µg polydA-polydT non-specific carrier, while the binding reaction in lane 6 included 2 µg of the carrier. Protein extract was incubated at 37°C, 68°C and 84°C for 10 min prior to its addition into the binding reaction, and these effects are shown in lanes 7, 8 and 9 respectively.

Purification of the 0.33 Rf DNA-Binding Protein by Column Chromatography

Based upon the ionic characteristics of the protein involved in the 0.33 binding activity, cytoplasmic protein extract from slugs were subjected to DEAE Sephacel column chromatography at pH 7.5. Fractions collected after the application of a linear gradient to 0.5 M NaCl gradient were tested by EMSA with probe 12 (Fig. 4.5). The 0.33 Rf binding activity was present in the early eluting fractions, and therefore, the fractions (5-10) represented in lanes 1-6 were pooled and concentrated. These pooled and concentrated fractions were then subjected to additional competition assays with excess unlabeled probes 12, 13, 14 and 16. These results confirmed the identity of the 0.33 Rf band in the pooled and concentrated fractions from the DEAE chromatography, and substantiated the specificity of the protein's association with the sequence of probe 12 (data not shown).

While the 0.33 Rf binding activity is present in the nuclear amoebae extract, protein extraction of sufficient quantity for chromatographic isolation was considerably faster and simpler from the cytoplasmic fraction. Therefore, to ease the efforts of the purification process, tests were performed to determine if the 0.33 Rf binding activity was found in cytoplasmic amoebae extract. The cytoplasmic fractions obtained from *Dictyostelium* amoebae were further separated by ammonium sulfate fractionation, and then tested with probe 12 (Fig. 4.6). Lanes 5 and 14 contained a purified sample of the 0.33 Rf DNA binding protein that served as a marker of the 0.33 Rf band. The 0.33 Rf binding activity was detected in the 80% ammonium sulfate fraction from cytoplasmic slug protein extract and cytoplasmic amoebae protein extract. Fig 4.6 also demonstrates

the specificity of binding for the 0.33 Rf band. Although both probes bind to the non-specific bands at 0.26 Rf and 0.38 Rf, only probe 12 binds to the 0.33 Rf band.

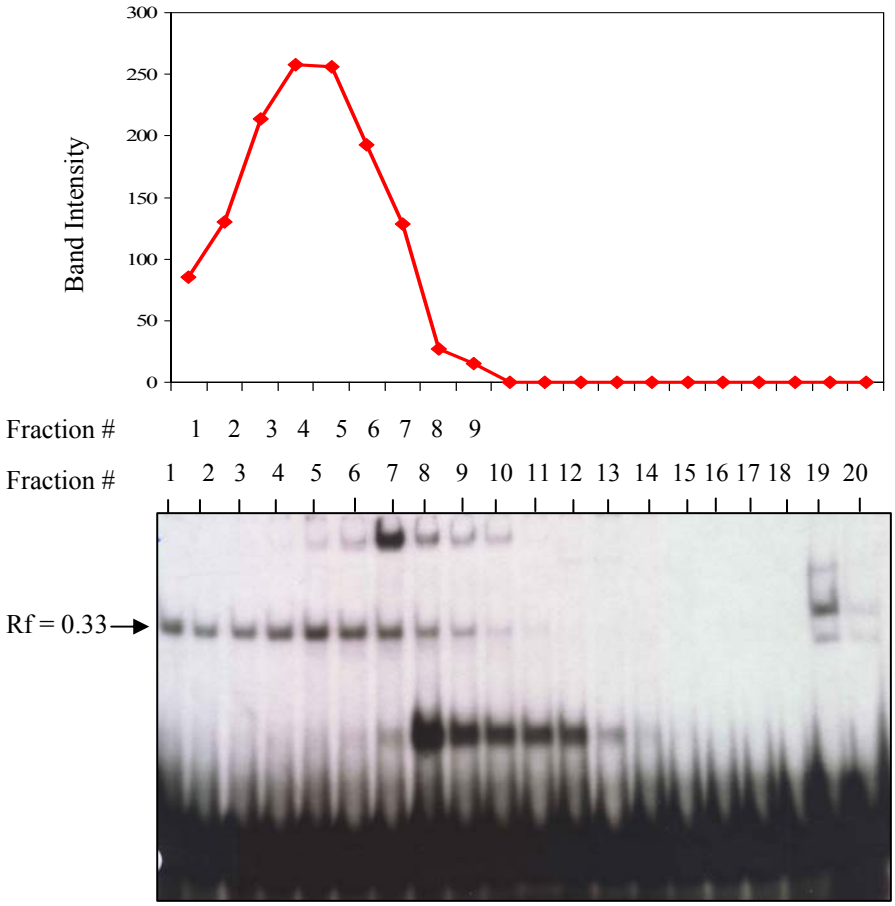

Figure 4.5. Fractions Collected After DEAE Column Chromatography Were Tested with Probe 12, Revealing the 0.33 Rf Binding Activity in the Early Eluting Fractions. Cytoplasmic protein extract obtained from *Dictyostelium* slug cells were subjected to DEAE chromatography and fractions were assessed by EMSA using labeled Probe 12. The 0.33 DNA-protein binding activity was detected as shown in lanes 1-10, corresponding to fractions 5-17 collected from the column. Fractions represented in lanes 1-6 were pooled and concentrated for further analysis. The 0.33 Rf binding activity detected in lanes 1-10 were quantified using NIH Image 1.54, and is shown in the graph above.

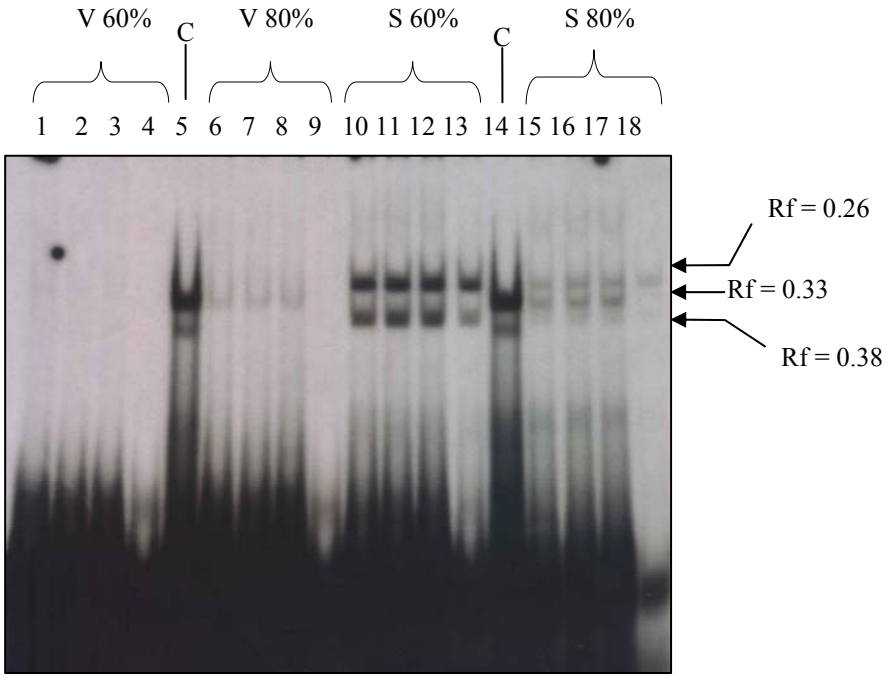

Figure 4.6. Testing Ammonium Sulfate Protein Fractions from Amoebae and Slug Cells for 0.33 Rf Binding Activity with Probes 6 and 12. Cytoplasmic protein fractions obtained from amoebae cells and from slug cells were further separated by ammonium sulfate fractionation into 20%, 40%, 60% and 80% fractions. Lanes 5 and 14 are control lanes in which the cytoplasmic protein extract used was from a previously obtained heparin fraction with labeled probe 12. The AS 60% cytoplasmic extract from vegetative cells was tested with labeled probe 12 in lanes 1-3, and tested with labeled probe 6 in lane 4. The AS 80% cytoplasmic extract from vegetative cells was tested with labeled probe 12 in lanes 6-8, and tested with labeled probe 6 in lane 9. The AS 60% cytoplasmic extract from slugs was tested with labeled probe 12 in lanes 10-12, and tested with probe 6 in lane 13. The AS 80% cytoplasmic extract from slugs was tested with labeled probe 12 in lanes 15-17, and tested with probe 6 in lane 18.

I. DEAE Chromatography

For the first steps of protein purification, the 80% ammonium sulfate fraction from amoebae was subjected to DEAE Sephacel column chromatography (Fig. 4.7). The total protein elution profile revealed two major peaks. The first peak represented the flow –through fractions and the second appeared after application of the linear gradient to 0.5 M NaCl. Elution of the 0.33 Rf protein occurred in the second major elution peak at about 0.2 M NaCl. EMSA of the fractions collected during chromatography with probe 12 showed that the 0.33 Rf band was not present in either of the flow-through fractions. The 0.33 Rf band was present in fractions 18-24, with the highest level of DNA-protein binding activity in fraction 20.

II. Heparin Sepharose Chromatography

Active DEAE fractions (18-24) were pooled, concentrated, and subjected to Heparin Sepharose column chromatography (Fig. 4.8). The total protein elution profile revealed three defined peaks. The first peak represented the flow-through fractions, and the other peaks appeared after application of the linear gradient to 0.5 M NaCl linear gradient. The 0.33 Rf protein eluted in the middle peak at about 0.12 M NaCl. EMSA of the collected fractions with probe 12 revealed the 0.33 Rf band was not found in either of the flow-through fractions. While the 0.33 Rf band was present in fractions 13-19, the highest level of DNA-protein binding activity resided in fraction 17.

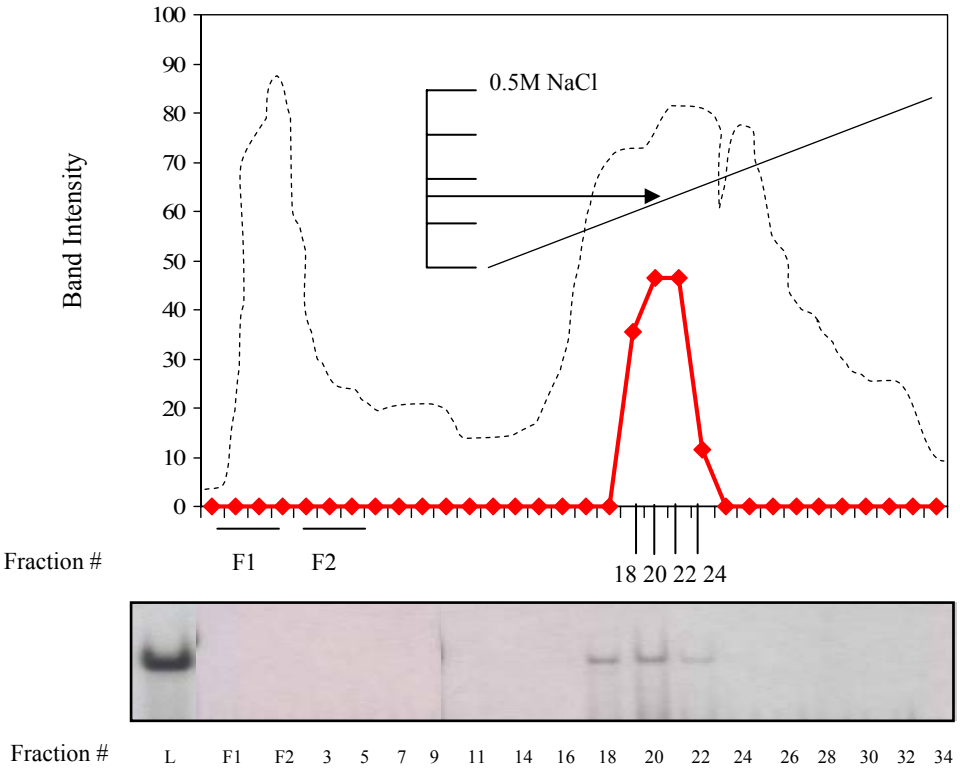

Figure 4.7. DEAE Column Chromatography of Cytoplasmic 80% Ammonium Sulfate Protein Extract. Cytoplasmic protein extract from amoebae collected from ammonium sulfate precipitation at 80% saturation was then further separated by DEAE sephacel chromatography. Bound proteins were eluted with a 2 hr linear gradient to 0.5 M NaCl, and the 3 ml fractions collected were subjected to EMSA with probe 12 (box below elution profile). Assays of protein loaded onto the column (L), flow through 1 and 2 fractions (F1 / F2), and every other fraction between 3 and 34 are shown. Binding activity was quantified using NIH Image 1.54, and is represented by the solid line. The total protein elution profile is shown as the dashed line. The 0.33 Rf band was evident in fractions 18-24. The protein eluted at 0.2 M NaCl.

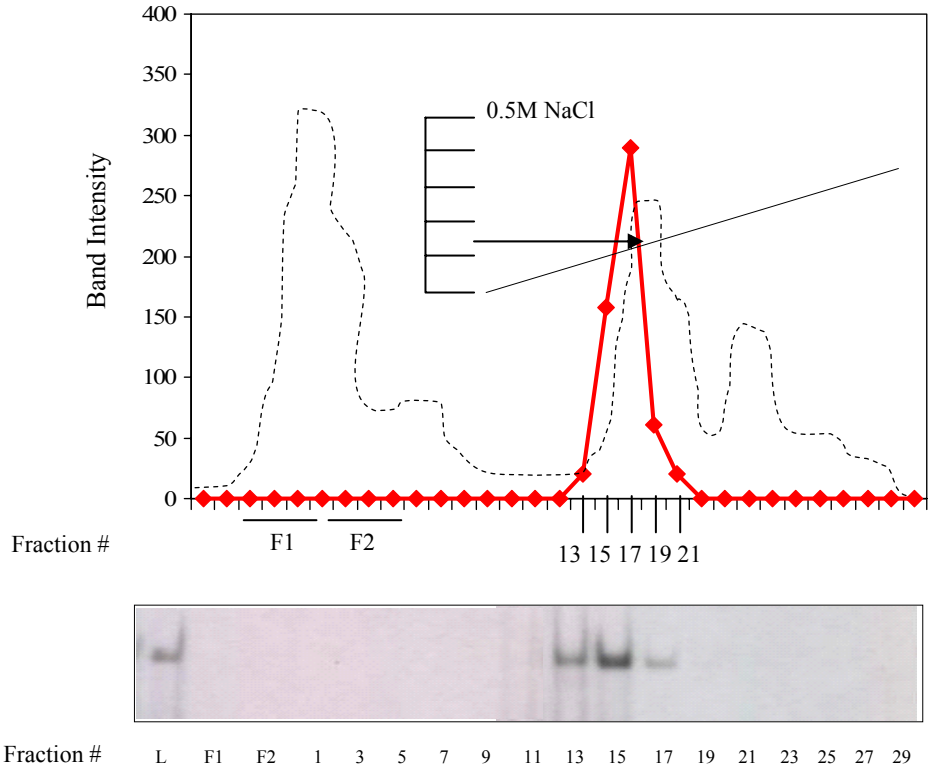

Figure 4.8. Heparin Sepharose Column Chromatography of Active DEAE Fractions. Active DEAE fractions (shown in Fig 4.7) were pooled and concentrated and loaded onto a Heparin Sepharose column. Bound proteins were eluted with a linear gradient to 0.5 M NaCl, and the 2 ml fractions collected were subjected to EMSA with probe 12 (box below elution profile). Assays of protein loaded onto the column (L), flow through 1 and 2 fractions (F1 / F2), and every other fraction between 1 and 29 are shown. Binding activity was quantified using NIH Image 1.54, and is represented by the solid line. The total protein elution profile is shown as the dashed line. The 0.33 Rf band was evident in fractions 13-19. The protein eluted with 0.12 M NaCl.

III. DNA Affinity Chromatography

Active heparin sepharose fractions (13-19) were pooled, concentrated, and subjected to DNA affinity column chromatography (Fig. 4.9). The total protein elution profile revealed three peaks. The first peak represented the flow-through fractions, and the other two peaks appeared after the application of a linear gradient to 1 M NaCl. The 0.33 Rf protein eluted in the third peak at about 0.2 M NaCl. EMSA of the collected fractions with probe 12 revealed the 0.33 Rf band was present in both of the flow-through fractions collected. Therefore, the flow through samples were pooled, concentrated and passed through the column four more times. The 0.33 Rf band was also present in fractions 13-15, with the highest level of DNA-protein binding activity resided in fraction 13.

IV. Gel Filtration Chromatography and SDS PAGE Analysis

Active DNA affinity fractions (13-15) were pooled, concentrated and subjected to gel filtration column chromatography (Fig. 4.10). Analysis of the fractions collected by EMSA with probe 12 showed that the 0.33 Rf binding activity was present in fractions 14-20, though the highest level of activity resided in fraction 15. Samples from each of the gel filtration fractions 14-20 were applied to a 10% SDS-polyacrylamide gel (Fig. 4. 11). With reference to the standard protein sample, a band estimated at about 70kDa was detected in lanes 5-10, corresponding in levels of intensity to the level of DNA binding activity as detected by EMSA shown in Fig 4.10. Quantification of the 0.33 Rf gel

filtration DNA binding activity and the 70 kDa polypeptide bands observed from the SDS gel using NIH Image 1.54 enabled analysis correlations to be made between the amount of protein present and the activity levels observed from each fraction. The quantification curves had similar patterns, with dark bands in the SDS gel coinciding with high levels of activity from the gel filtration fractions. Gel filtration fractions 15-17 were then pooled and concentrated to achieve a final protein amount of approximately 4 μg. Mass spectrometry was performed on the sample in the W.M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia in Charlottesville, and the peptide sequences obtained aligned 100% with the *Dictyostelium* Formyltetrahydrofolate synthase (FTHFS; EC 6.3.4.3) (Fig. 4.12).

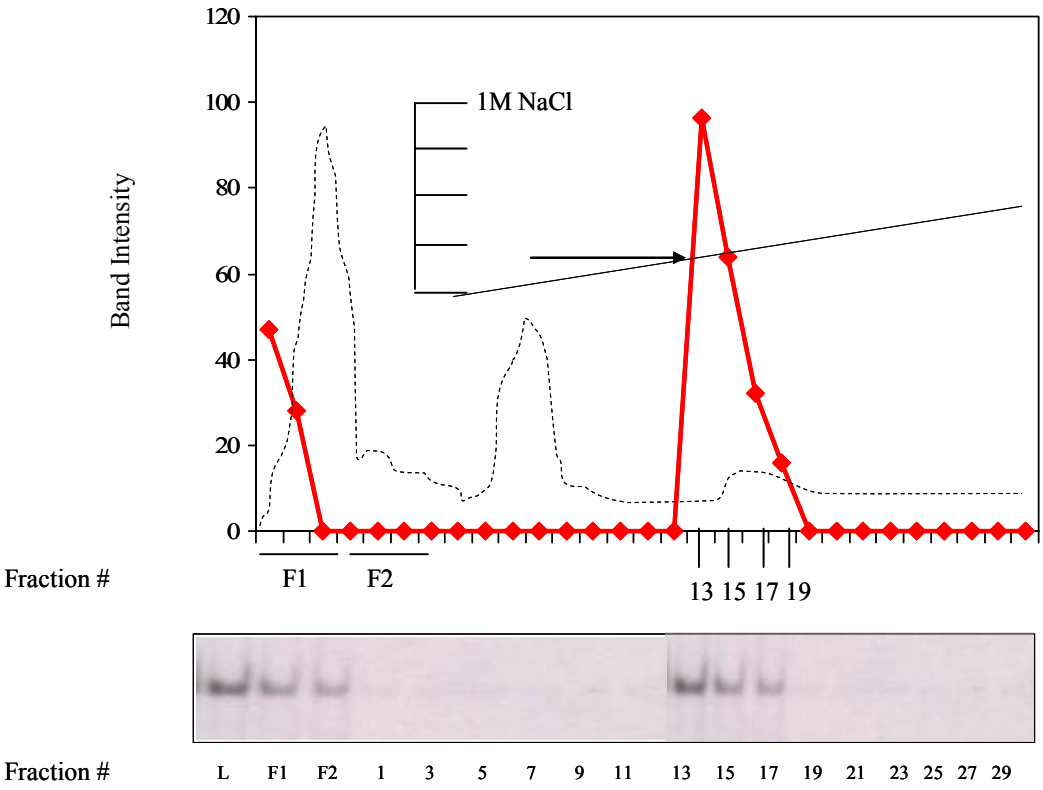

Figure 4.9. DNA Affinity Column Chromatography of Active Heparin Sepharose Fractions. Active heparin fractions (shown in Figure 4.8) were pooled and loaded onto a DNA affinity column. Bound proteins were eluted with a linear gradient to 1 M NaCl, and the 1 ml fractions collected were subjected to EMSA with probe 12 (box below elution profile). Assays of protein loaded onto the column (L), flow through 1 and 2 fractions (F1 / F2), and every other fraction between 1 and 29 are shown. Binding activity was quantified, and is represented by the solid line. The total protein elution profile is shown as the dashed line. The 0.33 Rf band was evident in fractions 13-19. The protein eluted with 0.05 M NaCl.

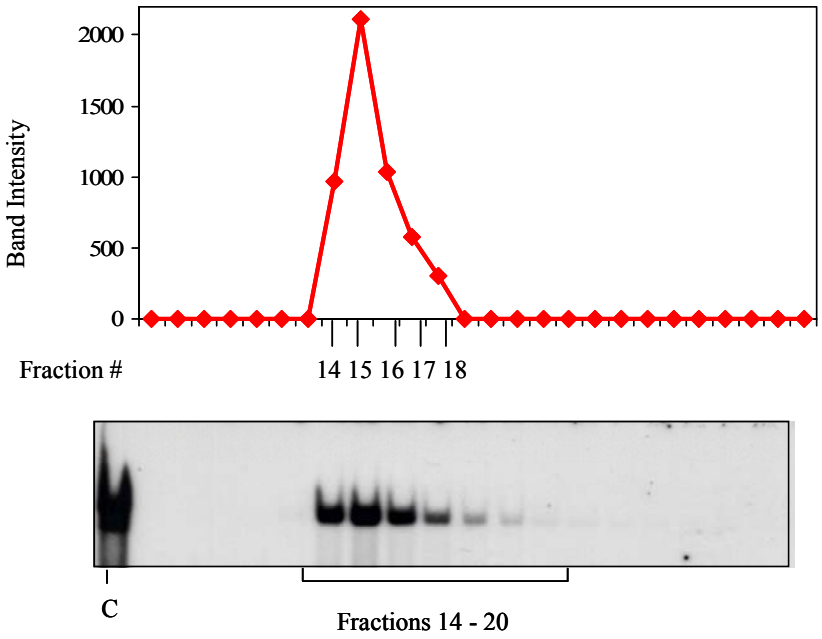

Figure 4.10. Gel Filtration Column Chromatography of Active DNA Affinity Fractions. Active DNA affinity fractions (shown in Fig 4.9) were pooled and loaded onto gel filtration column. Lane 1 is a control in which the protein extract used was from a previously obtained heparin fraction with labeled probe 12. EMSA activity was quantified for each band using NIH Image 1.54 and is shown in the graph above the gel image.

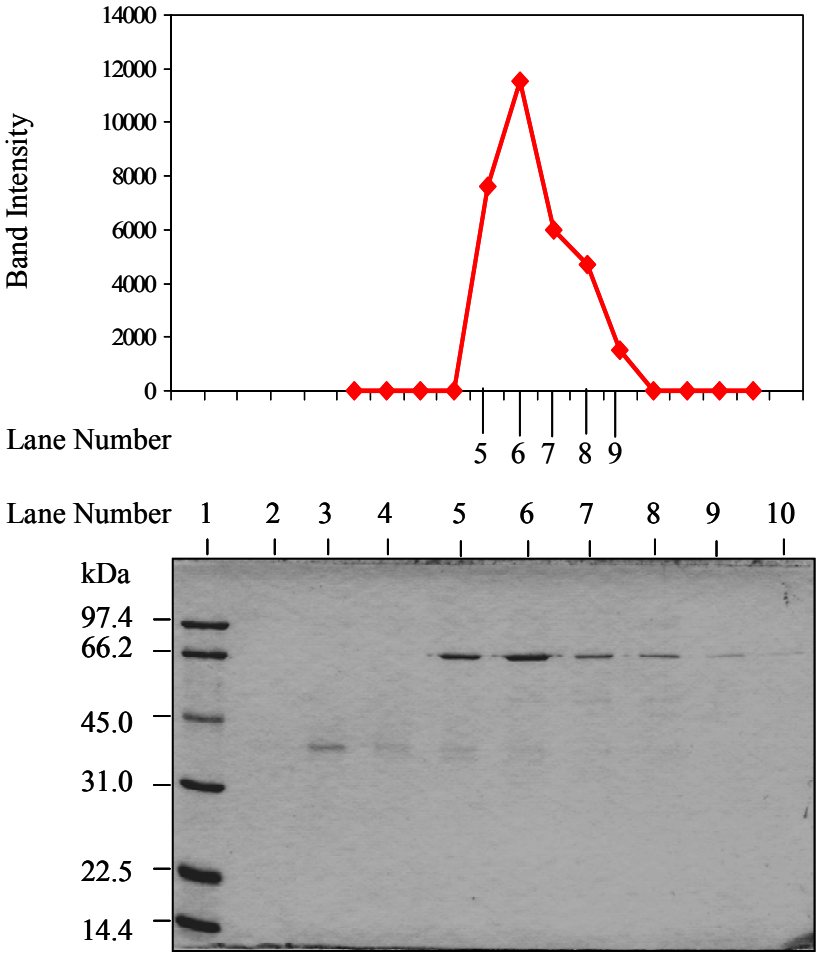

Figure 4.11. SDS PAGE of Gel Filtration Fractions Stained with Coomassie Blue. Active gel filtration fractions were separated on a 10% denaturing gel. Bands from the standard in lane 1 are listed in kDa. Bands of ~ 70 kDa are detected in lanes 5-10, which correspond to gel filtration fractions 14-19. EMSA activity was quantified using NIH Image 1.54 and is shown in the graph above the gel image

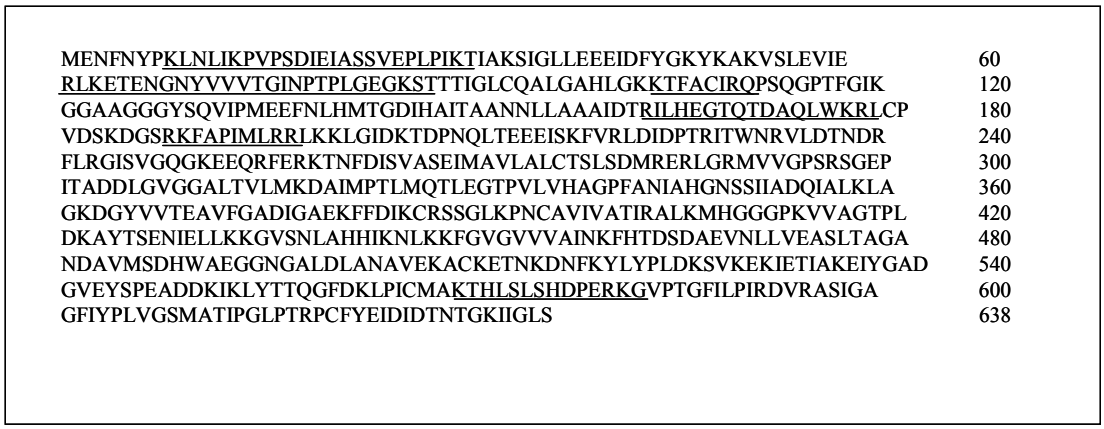

Figure 4.12. The Amino Acid Sequence of Formyltetrahydrofolate synthase (FTHFS). The 638 amino acid sequence of the *Dictyostelium* Formate Tetrahydrofolate Ligase is shown. Underlined portions of the sequence aligned identically to the peptide sequences obtained from protein analysis.

Discussion

Throughout the life cycle of *Dictyostelium*, *5nt* expression is regulated both temporally and spatially (Chanchao et al. 1999; Ubeidat et al. 2002; Rutherford et al. 2003). To elucidate some of the mechanisms that may contribute to this regulation at the level of transcription, EMSA were performed to identify *trans*-acting protein factors that may recognize and bind specific sequences of the *5nt* promoter. Initial EMSA enabled the identification of several DNA-protein binding activities (Fig. 4.2). Two binding activities, with Rf values of 0.29 and 0.60 appeared with all of the probes tested. The non-specificity of these interactions eliminated them as potential regulatory elements. Some bands were detected in these assays that appeared to be specific to the nucleotide sequence of certain probes. For example, the low mobility band detected in the presence of probe 9 was absent when other probes were examined (Fig. 4.2). Although this suggested specificity, additional assays did not consistently reproduce this band with probe 9. This example emphasizes the importance of thoroughly testing binding specificity through competition assays to minimize artifacts. However, the 0.33 Rf band detected with probe 12 showed high specificity and reproducibility.

The competition assays performed to evaluate the 0.33 Rf binding activity identified in Fig 4.2 supported the notion that the protein was specifically recognizing and interacting with the nucleotide sequence of probe 12. The binding activity was most clearly diminished when excess unlabeled probe 12 was added to the reaction as a competitor. In the presence of competitor probe 7 and probe 11 there was some competition for binding, although not to the extent seen with competitor probe 12. It is

possible that the protein has some affinity for probe 7 and 11 due to the partial presence of a recognition sequence or other characteristic, although its detection and binding affinity with probe 12 is much greater. In other cases, the binding activity was slightly reduced in the presence of other unlabeled competitors with respect to the control. The effects of adding the competitor before or after the labeled probe were examined and no changes in competition were noted.

Like much of the *Dictyostelium* genome, the nucleotide sequence of the *5nt* promoter is AT rich. The sequence of probe 12 is 73% AT rich. Within the 30 bp sequence, there are no previously characterized recognition sites present, such as a TA-box, a TAG-box or C-box. In order to analyze the nature and stability of the protein interaction with this sequence, a variety of parameters were examined.

The presence of magnesium and calcium in the binding reaction appeared to enhance the 0.33 Rf DNA-protein binding activity. Some proteins require the presence of these positively charged ions in order to participate in sequence specific binding activities. While the 0.33 Rf protein maintains the ability to specifically bind to the sequence of probe 12 in the absence of these ions, their presence does increase the affinity of the interaction. The chemical nature of the physical associations between protein molecule and DNA sequences are variable, though ionic interactions typically play a crucial role. Therefore it was not surprising that the addition of increasingly high concentrations of salt to the binding reactions decreased the affinity of the interaction.

A variable of considerable importance in the identification of the 0.33 Rf binding activity was the type of non-specific competitor utilized in the binding reaction. Three competitors were compared in many of the initial assays: polydA-polydT, polydAdT-

polydAdT, and polydIC-polydIC. The 0.33 Rf binding activity was not detected using either the polydAdT- polydAdT or polydIC-polydIC non-specific competitors due to the presence of additional bands that masked it. If assays had not been performed with polydA-polydT, then the 0.33 Rf band may not have been discovered. As the nature of this circumstance demonstrates, it is important to use a variety of non-specific competitors in initial assays to enable the visualization of as many binding activities as possible.

The initial assays that identified the 0.33 Rf binding activity used nuclear protein extract obtained from amoebae in the presence of 1 mM cAMP. The analysis of nuclear and cytoplasmic protein extracts from amoebae and slugs showed that the 0.33 Rf binding activity was present in all extracts. While the binding activity did not appear to be inducible by cAMP, the fact that the protein was present in both amoebae and slugs suggest that it may be present in an inactive state or sequestered *in vivo* early in development before *5nt* activity levels are first detected. In order to maximize the amount of protein extract for purification, cytoplasmic extracts were fractionated by ammonium sulfate fractionation prior to column chromatography.

Sequential purification of the 0.33 Rf protein by DEAE, Heparin Sepharose, DNA Affinity and Gel Filtration chromatography enabled the identification of a 70 kDa band in a 10% SDS. The banding pattern present on the gel was consistent with the pattern of activity observed from the gel filtration fractions. Mass spectrometry of the band resulted in numerous peptide products, whose sequences, when BLAST searched with the *Dictyostelium* genome database, showed that the protein was a formyltetrahydrofolate synthase (FTHFS), also known as formate-tetrahydrofolate ligase. Sequence homology

between the *Dictyostelium* FTHFS and the FTHFS found in yeast, mouse, *Arabidopsis,* and humans was 60%, 64%, 65%, and 65%, respectively. In *Dictyostelium*, the protein is comprised of 638 amino acid residues.

While FTHFS has not been studied in *Dictyostelium*, its presence and functions have been described in a variety of other organisms. While some general functions of FTHFS enzymes include nucleotide transport and metabolism, a major activity of the enzyme is in the biosynthetic pathway of tetrahydrofolate (Martinasevic et al. 1999). Tetrahydrofolate provides C1 carrier units to the 1-carbon pool, which are then utilized in various biosynthetic pathways, including nucleotide and purine metabolism (Nagy et al. 1995; Jabrin et al. 2003). The biosynthetic pathway of tetrahydrofolate includes DHNA (dihydroneopterin aldolase), HPPK (7,8-dihydro-6-hydroxymethylpterin-pyrophosphokinase), and DHPS (7,8-dihydropteroate synthase) activities. In most eukaryotic organisms, a single polypeptide enzyme that exists as a homodimer is responsible for these three activities, while in prokaryotic organisms there are separate enzymes involved (Appling and Rabinowitz 1985). After its production, tetrahydrofolate combines with formate in the presence of ATP to produce N (10)-formyltetrahydrofolate. This reaction requires the enzyme FTHFS, and the product of this reaction is an active coenzyme that functions as a carrier of the formyl group for a number of enzymatic reactions.

While the function of this enzyme has been clearly established within the context of N (10)-formyltetrahydrofolate biosynthesis, its role as a DNA-binding transcription factor has not been confirmed. The nature of a FTHFS interaction with DNA in *Schizosaccharomyces pombe* has been analyzed (Wahls et al. 1993). In this study, the

interaction was described to be specific to single-stranded DNA in a sequence-independent manner. While ssDNA binding proteins generally play roles in physiological processes, including transcription, translation, DNA repair and recombination, the function of FTHFS when bound to ssDNA in the study was not completely established. It was, however, suggested that the enzyme may be involved in autoregulating its synthesis.

Recently, an additional protein that specifically binds to a different region of the *5nt* promoter has been isolated and purified in our lab (Wiles et al. in prep). Mass spectrometric analysis of the protein has revealed that it is also a component of the tetrahydrofolate biosynthetic pathway. The specific interaction of two components of this pathway with the *5nt* promoter suggests that these metabolic enzymes may be involved in gene regulatory activity worthy of further investigation. Along with this investigation, the *5nt* gene will be closely evaluated. While the characteristics of *5nt* and its promoter have been thoroughly analyzed in our laboratory, the amino acid sequence of the purified 5nt revealed no strong similarity between it and any other protein, raising the possibility that the protein purified may function in activities other than those known to be associated with ecto-nucleotidases.

References

Appling, D. R. and J. C. Rabinowitz (1985). "Evidence for overlapping active sites in a multifunctional enzyme: immunochemical and chemical modification studies on C1-tetrahydrofolate synthase from Saccharomyces cerevisiae." Biochemistry **24**(14): 3540-7.

Armant, D. R. (1980). Purification, characterization and localization of 5'AMP nucleotidase during pattern formation in Dictyostelium discoideum. Blacksburg, VA, Virginia Polytechnic Institute and State University**:** 108.

Bonner, J. T. (1947). "Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold Dictyostelium discoideum." J. Exp. Zool. **106**: 1-26.

Brown, S. S. and C. L. Rutherford (1980). "Localization of cyclic nucleotide phosphodiesterase in the multicellular stages of Dictyostelium discoideum." Differentiation **16**: 173-183.

Chanchao, C., Eristi, C. M., Favis, R., and Rutherford, C. L. (1999). "5'-Nucleotidase in Dictyostelium: protein purification, cloning, and developmental expression." Biochim. Biophys. Acta **1473**: 376-390.

Hall, A. L., Franke, J., Faure, M., and Kessin, R. H. (1993). "The role of the cyclic nucleotide phosphodiesterase of Dictyostelium discoideum during growth, aggregation, and morphogenesis: overexpression and localization studies with the separate promoters of the pde." Dev. Biol. **157**: 73-84.

Jabrin, S., Ravanel, S., Gambonnet, B., Douce, R., and Rebeille, F. (2003). "One-carbon metabolism in plants. Regulation of tetrahydrofolate synthesis during germination and seedling development." Plant Physiol **131**(3): 1431-9.

Kay, R. R., Flatman, P., and Thompson, C. R. L. (1999). "DIF signalling and cell fate." Sem. Cell Dev. Biol. **10**: 577-585.

Kessin, R. H. (2001). Dictyostelium - Evolution, cell biology, and the development of multicellularity. Cambridge, UK, Cambridge Univ. Press.

Loomis, W. F. (1993). "Lateral inhibition and pattern formation in Dictyostelium." Curr. Topics Dev. Biol. **28**: 1-46.

Martinasevic, M. K., Rios, G. R., Miller, M. W., and Tephly, T. R. (1999). "Folate and folate-dependent enzymes associated with rat CNS development." Dev Neurosci **21**(1): 29-35.

Nagy, P. L., Marolewski, A., Benkovic, S. J., and Zalkin, H. (1995). "Formyltetrahydrofolate hydrolase, a regulatory enzyme that functions to balance pools of tetrahydrofolate and one-carbon tetrahydrofolate adducts in Escherichia coli." J Bacteriol **177**(5): 1292-8.

Rutherford, C. L., Overall, D. F., Ubeidat, M., and Joyce, B. R. (2003). "Analysis of 5' nucleotidase and alkaline phosphatase by gene disruption in Dictyostelium." Genesis **35**: 202-213.

Ubeidat, M., Eristi, C. M., and Rutherford, C. L. (2002). "Expression pattern of 5'-nucleotidase in Dictyostelium." Mech. Devel. **110**: 237-239.

Wahls, W. P., Song, J. M., and Smith, G. R. (1993). "Single-stranded DNA binding activity of C1-tetrahydrofolate synthase enzymes." J Biol Chem **268**(32): 23792-8.

Williams, J. G. and K. A. Jermyn (1991). Cell sorting and positional differentiation during Dictyostelium morphogenesis. Cell-Cell Interactions in Early Development. J. Gerhart. New York, Wiley-Liss**:** 261-272.

Chapter 5

Identification and Purification of a DNA-Binding Protein in *Dictyostelium discoideum* Identified as a Dihydro-6-hydroxymethylpterin Pyrophosphokinase

Abstract

Analysis of the transcriptional regulation of a developmentally regulated gene in *Dictyostelium* has involved a thorough study of protein binding activities on the promoter sequence of the gene by electromobility gel shift assays (EMSA). Three probes, approximately 100 bp in length, were designed to span a downstream region of the *5'-nucleotidase* (*5nt*) promoter, and tested with nuclear and cytoplasmic protein extracts from ameobae, aggregates and slugs by EMSA. A unique binding activity with an Rf = 0.13 was observed exclusively with probe 3. To more precisely ascertain the site of protein interaction, short oligonucleotide probes were generated to span the sequence represented by probe 3, and EMSA with these probes enabled the binding activity to be localized to a 50 bp region of the promoter located between -367 and -318 bp from the ATG translation start site. Competition assays revealed the inability of many unlabeled probes to compete for the binding activity, thereby supporting the notion that the protein associates with the specific 50 bp sequence. The protein involved in this binding activity was purified using DEAE, Heparin Sepharose, DNA Affinity and Gel Filtration column chromatography. Gel filtration fractions were analyzed by SDS-PAGE, revealing a polypeptide with an approximate molecular weight of 70 kDa. Peptide sequences obtained after analysis of the isolated protein by mass spectrometry showed 100% identity to the *Dictyostelium* protein dihydro-6-hydroxymethylpterin pyrophosphokinase. The specificity of the protein's binding activity to the *5nt* promoter suggests the potential role of the protein as a regulatory transcription factor.

Introduction

Dictyostelium discoideum is a simple eukaryotic soil-dwelling amoeba that has been used as a model system in molecular studies of developmental gene expression, cell-type differentiation, cell motility, signal transduction, chemotaxis, and cohesive cell contacts (Raper and Thom 1932; Raper 1935; Bonner 1944; Bonner 1947; Kessin 2001). The 24-hr life cycle of *Dictyostelium* makes the organism ideal for molecular studies (Loomis 1975). The life cycle is comprised of distinct developmental stages that include both unicellular and multicellular forms.

The transition from the unicellular form of the organism to the aggregation mound is elicited when nutrients become diminished and the cells begin to emit pulses of cyclic adenosine 3',5'-monophosphate (cAMP) (Raper 1937; Bonner et al. 1970). After the multicellular mound is formed, comprised of approximately 10^5 cells, cell-specific gene expression causes the initial distinctions between the precursors of the prestalk and prespore cells to become apparent (Raper and Fennell 1952). The tip of the mound structure elongates and falls over to form the pseudoplasmodium, which then migrates through a slime sheath towards heat and light, indicators of an appropriate culmination site. Throughout the lifecycle of *Dictyostelium*, two morphogens, cAMP and DIF (differentiation-inducing factor), are critical for the process of normal cell differentiation (Kay 1979; Kay 1997). For example, low concentrations of DIF stimulate prestalk-specific gene expression and repress prespore-specific gene expression in prestalk cells (Nayler et al. 1992). After the culmination stage, prestalk cell differentiation is inhibited by cAMP, while prespore cell differentiation is induced (Williams and Jermyn 1991).

Regulation of gene expression throughout the life cycle of any organism is critical for normal development. While the expression of cell-specific genes is critical for the process of differentiation, gene regulation through signal transduction also provides a critical means by which organisms can appropriately respond to environmental changes. The regulation of gene expression at the level of transcription is a common means to achieve cellular changes, such as the activation or inactivation of metabolic pathways, suitable under specific conditions. To maintain precise control over the magnitude of proteins involved in the numerous biosynthetic pathways and other activities of the cell, there must be control mechanisms that specifically recognize, and activate or inhibit the expression of certain genes. When components of these control mechanisms become dysfunctional, the consequences to the organism can be detrimental or fatal.

In order to identify components involved in the transcriptional control of the developmentally regulated gene, *5nt*, a thorough analysis of the promoter was previously performed in our laboratory (Wiles et al. in prep). The generation of numerous 5' and internal promoter deletions, and the fusion of these deletions to the *luciferase* and *lacZ* reporter genes enabled the identification of a 250 bp region of the promoter apparently required for normal levels of gene expression. This region of the promoter was further evaluated by site-directed mutagenesis experiments that resulted in the identification of precise positions on the promoter that may serve as control elements during transcriptional regulation.

The primary focus of this study was the identification of specific protein interactions with the *5nt* promoter. We report here the identification of a DNA-protein interaction determined to be specific to a 50 bp sequence of the promoter through EMSA

competition experiments. In addition, we describe the purification processes employed to isolate the DNA-binding protein. The results of mass spectrometry of the isolated protein identify the molecule as a dihydro-6-hydroxymethylpterin pyrophosphokinase, and we discuss the potential role of this protein as a transcriptional regulator. Furthermore, the putative association of this protein with another protein previously identified in our laboratory is addressed.

Materials and Methods

Cytoplasmic and Nuclear Protein Extraction from Dictyostelium Amoebae and Slugs

EMSA binding reactions utilized two sources of protein extract: AX3K *Dictyostelium* amoebae induced with cAMP in shaking flasks, and AX3K *Dictyostelium* slugs grown on 2% water agar plates. Protein extracts from amoebae were acquired by first enabling the cells to grow to a density of 3 x 10^6 cells / ml in shaking flasks at 150 rpm. Cells were harvested by centrifugation in a CRU-5000 centrifuge (International Equipment Company) at 2,100 x g for 3 min. Subsequently, the pellets obtained were washed in a MES-LPS buffer (pH 6.5), and final resuspension of the washed pellet was in a volume of buffer required to obtain a final concentration of 1 x 10^7 cells / ml. The resuspended cells were returned to shaking flasks and incubated in MES-LPS (pH 6.5) buffer overnight. Cyclic AMP was then added to a final concentration of 1 mM, and flasks were shaken for 8 hr. The cells were then harvested by centrifugation in a Sorvall GSA at 10,000 x g for 10 min. After the supernatant was aspirated, the pellets were resuspended in 5 times the pellet weight in lysis A buffer, which contained 50 mM Tris (pH 7.5), 10% glycerol, 2 mM $MgCl_2$, 1% NP40, 1 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF. The resuspended cells were then harvested by centrifugation at 10,000 x g for 10 min, and the supernatant, containing cytoplasmic proteins, was stored at -80°C until EMSA were performed. The remaining pellet was thoroughly washed and resuspended with 4 ml of lysis buffer A, and centrifuged at 10,000 x g for 10 min. The resulting supernatant was removed and stored at -80°C. The pellet was weighed and

resuspended thoroughly by vortexing in 2X volume lysis buffer A. NaCl was then added to a final concentration of 0.42 M. The samples were incubated on ice for 1 hr, and then harvested by centrifugation at 10,000 x g for 10 min. The supernatant, containing nuclear proteins, was stored at -80°C.

The preparation of nuclear and cytoplasmic protein extracts from slugs also entailed first growing the amoebae in shaking flasks to a density of 3 x 10^6 cells / ml. Once the appropriate cell density was reached, cells were harvested by centrifugation in a CRU-5000 centrifuge at 2,100 x g. The resulting pellets were washed in a 1X MES-LPS buffer (pH 6.5), and then resuspended in a volume of buffer required to obtain a final concentration of 1 x 10^7 cells / ml. Cells were kept on ice until they were spread on the surface of 2% water agar Petri plates. The plates were kept at room temperature until the slug developmental stage became apparent, approximately 18 hr later. The slugs were then removed in 20 mM potassium phosphate buffer (pH 7.5), and passed through a 21 G syringe to disaggregate the slugs into single cells. The samples were then centrifuged at 2,600 x g for 5 min, and the supernatant was removed. The pellets were resuspended in 5 times the pellet weight of lysis buffer A. Nuclear and cytoplasmic protein fractions were then acquired using the same procedure described for the amoebae samples.

Ammonium Sulfate Fractionation of Cytoplasmic and Nuclear Protein Extracts

Ammonium sulfate precipitation was used to further fractionate the cytoplasmic and nuclear protein extracts obtained from amoebae and slugs. Protein extracts were maintained on ice while the contents were stirred during this procedure. First, ammonium

sulfate was slowly added to 20% saturation over a period of 5 min for the protein extract being fractionated. After stirring the sample for an additional 30 min, the solution was transferred to a 40 ml polycarbonate tube and centrifuged in a SS34 rotor at 10,000 x g for 20 min. The supernatants were then transferred back into the original beaker, and the pellets were resuspended in a buffer containing 50 mM Tris (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH8.0), 0.5 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF, and placed on ice. Solid ammonium sulfate was then slowly added to 40% saturation over a period of 5 min to the supernatants in the beaker on the stir plate. After stirring the sample for an additional 30 min, the samples were separated by centrifugation. The same procedures were repeated for the 60% and 80% saturation samples. All of the pellets that had been resuspended in buffer and were incubating on ice at the end of the experiment were transferred to dialysis bags, which were then placed in 1L of dialysis buffer (20 mM Tris, pH 7.5) at 4°C overnight. After the overnight incubation, the samples were transferred to 12 ml polycarbonate tubes and centrifuged at 20,000 x g for 15 min. The supernatants were then stored at –80°C until used in subsequent EMSA.

Generating and Labeling Probes for EMSA

The probes prepared and used in this study were either PCR products (probes 1, 2 and 3) or short double-stranded oligonucleotides (all others). To anneal the complementary single strands for the oligonucleotide probes, a buffer containing 20 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0), and 50 mM NaCl was used. The oligonucleotide sample in buffer was incubated for 5 min at 70°C, and then cooled to room temperature.

To radioactively label the probe, 100 ng of the double-stranded oligonucleotide was added to T4 Polynucleotide Kinase (PNK) buffer, water, and 30 μCi of [γ-^{32}P] ATP in a total reaction volume of 11 μl. After an incubation period of 5 min at 70°C, the reactions were chilled on ice and then 10 U/μl T4 PNK were added. The reaction mix was incubated for 1 hr at 37°C, and then incubated for 20 min at 65°C. Unincorporated nucleotides were removed from the labeling reaction by passing the sample through a Micro Bio-Spin 6 column (BioRad). A scintillation counter (Beckman LS 6000SC) was used to measure the radioactivity of the labeled probes.

Binding Reactions and EMSA Conditions

Although binding reactions varied from experiment to experiment depending upon the protein extract, probe, and specific conditions chosen, the standard reaction included a cocktail comprised of 20 mM HEPES (pH 7.9), 12% glycerol, 20 mM NaCl, 1 mM EDTA (pH 8.0), 2 mM $MgCl_2$, 2 μg BSA, 1 mM DTT, 0.04% NP40 and 0.5 μg of a non-specific carrier (polydIdC-polydIdC, polydA-polydT, or polydAdT-polydAdT). The binding reaction was prepared by first combining the cocktail with 1.5 μl of the protein extract chosen for analysis. Then, 30,000 cpm of the labeled DNA probe was added. If a competitor was used, it was added immediately thereafter. The reactions were incubated on ice for 10 min before the samples were applied to the gel. The 5% native polyacrylamide gel in 0.5X TBE gel was subjected to 150 V for 30 min before the samples were loaded. After the samples had been loaded, the voltage was increased to 200 V for 2 hr. The gels were transferred to chromatography paper, and then incubated

for 45 min at 80°C in a gel dryer. Once the gel had dried onto the chromatography paper, the paper was positioned in a cassette with X-OMAT AR Kodak film and exposed at -80°C between 24 and 72 hr before development.

DEAE Sephacel Column Chromatography

All of the High Performance Liquid Chromatography (HPLC) experiments in this study were performed with a Waters Advanced Protein Purification System (Model 650E). For the first purification stage, Buffer D, comprised of 20 mM Tris (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH8.0), 0.5 mM DTT, 20 μg/ml TLCK, and 100 μg/ml PMSF, was used to equilibrate a DEAE Sephacel (Sigma) column (27 mm x 250 mm). The first steps of protein purification used the 40% ammonium sulfate cytoplasmic protein fraction from *Dictyostelium* slugs. Before being loaded onto the column with a syringe, the cytoplasmic extract was slowly thawed on ice. A peristaltic pump was used to run Buffer D through the column at a rate of 3 ml / min. An absorbance monitor was used to analyze the flow-through fractions collected. Once a baseline was reached during the collection of flow-through fractions, negatively charged proteins bound to the DEAE anionic Sephacel resin were eluted using a 60 min linear gradient to 0.5M NaCl in Buffer D at a flow rate of 5 ml / min. An absorbance monitor was used to assess the contents of the 3 min fractions as they were being collected in order to create an elution profile. Samples from the fractions were analyzed by EMSA with probe 10 and the intensity of the bands produced was quantified using NIH Image 1.54. The column was stored at 4°C in a 20% ethanol solution.

Heparin Sepharose Column Chromatography

Prior to the second stage of protein purification a Heparin Sepharose (Sigma) column (20 mm x 130 mm) was equilibrated with Buffer H, comprised of 10 mM potassium phosphate buffer (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF. Active fractions collected from DEAE sephacel chromatography were pooled and concentrated in Buffer H using an Amicon YM10 membrane to approximately 12 ml, and then loaded onto the column using a syringe. Once the majority of the unbound proteins passed through the column, and the absorbance monitor baseline was reached, potential DNA-binding proteins bound to the sulfated glucosaminoglycan polymer were eluted using a linear 100 min gradient to 0.8 M NaCl in buffer H. The flow rate of the gradient was 2 ml / min, and 2 min fractions were collected. Samples from the fractions were analyzed by EMSA with probe 10 and the intensity of the bands produced was quantified using NIH Image 1.54. The column was stored at 4°C in a 20% ethanol solution.

Preparation of the DNA Affinity Column

To prepare the resin of the DNA affinity column, double-stranded oligonucleotides (same sequence as probe 10) were produced, phosphorylated, and then coupled to CNBr- activated Sepharose-4B resin (Pharmacia). Double-stranded oligonucleotides were phosphorylated by adding 3 mM ATP and 100 U of T4 PNK in a

total reaction volume of 100 µl with T4 PNK buffer. The reaction was incubated at 37°C for 2 hr, after which the DNA was precipitated using ethanol. T4 DNA ligase (2,000 U) was then added to a total reaction volume of 100 µl, and the sample was incubated at 16°C overnight. Phenol was used to extract the DNA from the ligation reaction, and ethanol was used to precipitate the DNA, which was resuspended in 100 µl milliQH_2O.

To activate the CNBr-Sepharose-4B resin, 1 g was added to 100 ml 1mM HCl and incubated for 10 min. After the resin had swelled during this incubation period, it was transferred to a glass filter. On this filter, the resin was washed with 200 ml of 1 mM HCl, then with 300 ml milliQH_2O, and lastly with 100 ml 10 mM potassium phosphate buffer (pH 8.0). The resin was then transferred to a plastic tube where it was allowed to settle, and the supernatant was removed. A volume of 10 mM potassium phosphate buffer adequate to create a thick slurry solution was added to the washed resin. The oligonucleotide sample was then added to the activated resin, and placed into a rotating wheel for an overnight incubation at room temperature.

The sample was transferred to a glass filter after the overnight incubation on the rotating wheel, where it was washed two times with 100 ml milliQH_2O, then washed with 100 ml 1 M ethanolamine (pH 8.0), and then transferred to a 15 ml polypropylene tube. To block excess active groups, 1 M ethanolamine was added to a final volume of 14 ml. The tube was placed on a rotator for a 5 hr incubation period at room temperature, and then the resin was packed into the column (13 mm x 25 mm). The resin was washed first with 100 ml of 10 mM potassium phosphate buffer (pH 8.0), then with 100 ml 1 M potassium phosphate buffer (pH 8.0), 100 ml 1 M KCl, 100 ml milliQH_2O and finally

with 100 ml of storage buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 0.04% NaN_3). The column was stored at 4°C in this solution.

DNA Affinity Chromatography

Prior to the third stage of protein purification a DNA affinity column was equilibrated with Buffer D, comprised of 20 mM HEPES (pH 7.9), 10% glycerol, 1 mM EDTA (pH 8.0), 2 mM $MgCl_2$, 1 mM DTT, and 0.04% NP-40. Active fractions obtained from heparin sepharose chromatography were pooled and concentrated in buffer D using an Amicon YM10 membrane to approximately 2 ml. The protein extract was loaded onto the affinity column using a syringe, and Buffer D was pumped through the column at a rate of 1 ml / min. Proteins binding to the DNA affinity resin were eluted using a linear 45 min gradient to 1 M NaCl in Buffer D at a flow rate of 1 ml / min. As 2 min fractions were collected, their contents were assessed by an absorbance monitor to generate an elution profile, and then analyzed with probe 10 by EMSA.

Gel Filtration Column Chromatography

Prior to the fourth stage of protein purification a 300SW gel filtration column (Waters, 8 mm x 300 mm) was equilibrated with Buffer H, comprised of 10 mM potassium phosphate buffer (pH 7.5), 10% glycerol, 0.5 mM EDTA (pH 8.0), 0.5 mM DTT, 20 μg/ml TLCK, and 100 μg/ml PMSF. Active fractions collected from DNA affinity chromatography were pooled and concentrated in buffer H using an Amicon

YM10 membrane to approximately 600 μl, then loaded onto the column at a flow rate of 0.2 ml / min. One min fractions were collected and then analyzed by EMSA. The column was stored at 4°C in 0.1% NaN_3.

Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

A 7.5% SDS-polyacrylamide gel was used to analyze the gel filtration fractions. First, the protein samples analyzed were combined with a loading buffer and incubated for 5 min at 85°C. The samples were loaded on the gel, and the conditions were set to 15 mA for 30 min using a running buffer (2.5 mM Tris, 19 mM glycine, 1% SDS), and then increased to 25 mA for one hr. The gel was fixed in a solution comprised of 50% ethanol in 10% acetic acid, after which the gel was washed for 30 min in a solution comprised of 50% methanol in 5% acetic acid. A 45 min wash with the same solution followed. In this study, the gel was analyzed by staining with both Coomassie Blue and silver, though only results of the silver stain are shown. For silver staining, the gel was first placed in a solution comprised of 50% methanol and 12% acetic acid and incubated with shaking for 15 min. The gel was then washed and incubated for 4 min two times with a solution comprised of 10% ethanol and 5% acetic acid. The third solution, comprised of 3.4 mM potassium dichromate and 3.2 mM nitric acid, was then added to the gel, and incubated under shaking conditions in the dark for 5 min. Subsequently, the gel was washed twice in mQH_2O, and then 12 mM silver nitrate was added and the gel incubated under shaking conditions in the light for 5 min. The gel was rinsed twice with a solution comprised of 0.28 M sodium bicarbonate and 0.0185% formaldehyde, then incubated in the solution on

a shaker until protein bands began to appear. Development was stopped by adding 1% acetic acid solution to the gel. The gel was taken to the Virginia Bioinformatics Institute at Virginia Polytechnic Institute and State University, where the 70 kDa band was analyzed by mass spectrometry.

Results

EMSA Using Probes Designed from the 5nt Promoter Reveal a Band of Interest

Analysis of protein binding activity on the *5nt* promoter was initialized by EMSA using probes 1, 2 and 3. These double-stranded probes, generated by PCR, were designed to assess a 400 bp region of the promoter (Fig. 5.1). For probe 1, the upstream limit was -676 bp from the ATG translation start site, and the sequence extended downstream to -573. The upstream limit for probe 2 was -536 bp from the ATG site, and its sequence extended to -446. Probe 3 was designed to span the region between -422 bp and -318 bp from the ATG site. The use of these labeled probes in EMSA enabled a thorough analysis of protein binding activity on this region of the *5nt* promoter.

Cytoplasmic and nuclear extract obtained from amoebae cells were analyzed with the three probes by EMSA (Fig. 5.2). Numerous bands common to the three probes, interpreted as non-specific binding, were detected in both cytoplasmic and nuclear protein extracts. However, a band with an Rf = 0.13 was repeatedly observed with probe 3 (lanes 3 and 6) that was not present with probe 1 (lanes 1 and 4) or probe 2 (lanes 2 and 5). The 0.13 Rf binding activity with probe 3 was present when the reaction included either cytoplasmic (lanes 1-3) or nuclear (lanes 4-6) protein extract from amoebae cells.

The binding activity was further characterized by assessing its presence or absence in various protein extracts by EMSA (data not shown). For example, cytoplasmic and nuclear protein extracts obtained from amoebae cells in shaking flasks without 1mM cAMP were tested with probe 3. The 0.13 Rf binding activity was present under these

conditions as well. In addition, nuclear and cytoplasmic protein extracts obtained from cells at the aggregate and slug stages of development also produced the 0.13 activity in the presence of probe 3.

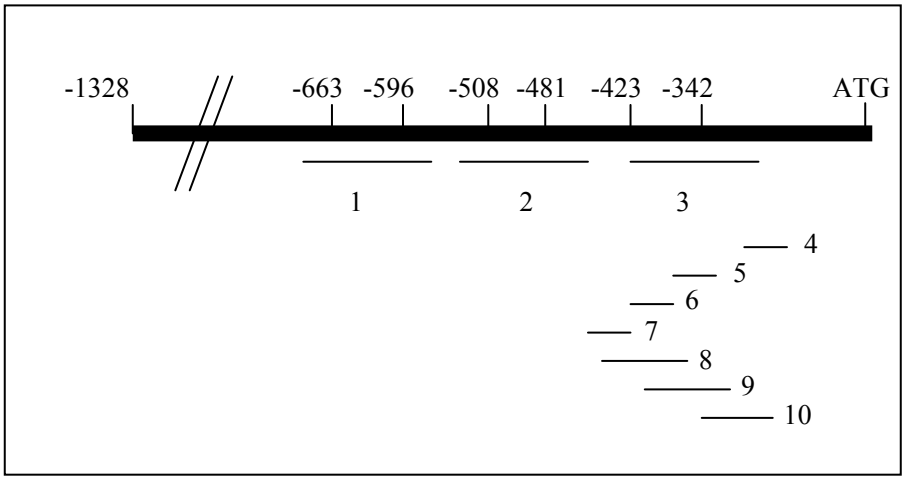

Figure 5.1. Probes Designed to Analyze the 663 Bp Upstream Region of the *5nt* Promoter. Probes 1-3 were designed by PCR and were 104, 91 and 105 bp in length, respectively. Probes 4-7 were double-stranded oligonucleotides and were 30 bp in length, while probes 8-10 were double-stranded oligonucleotides and were 50 bp in length. Probes were utilized in electromobility gel shift assays to locate specific sites on the promoter with which regulatory proteins may be interacting.

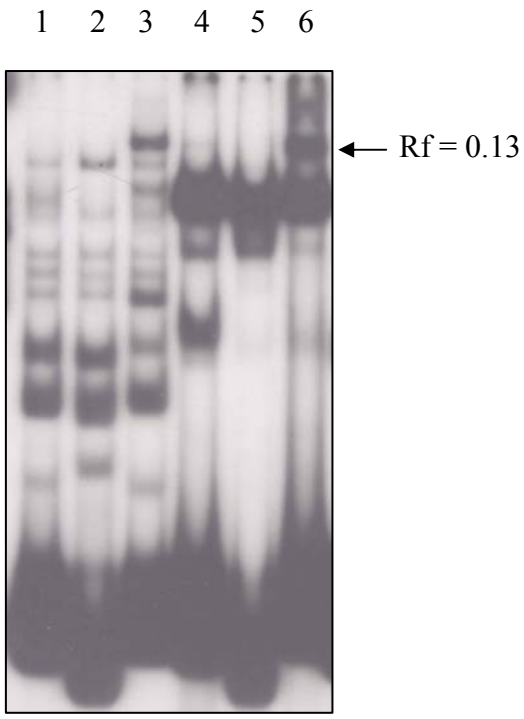

Figure 5.2. EMSA Reveals a 0.13 Rf Binding Activity In Cytoplasmic and Nuclear Protein Extract With Probe 3. Cytoplasmic and nuclear protein extract obtained from amoebae cells in shaking flasks with 1mM cAMP were tested by EMSA with three different labeled probes. Binding reactions including cytoplasmic extract and probes 1, 2 or 3 were analyzed in lanes 1-3, respectively. Binding reactions including nuclear extract and probes 1, 2 or 3 were analyzed in lanes 4-6, respectively. A binding activity (Rf = 0.13), present in both cytoplasmic and nuclear protein extracts, was detected with probe 3.

The 0.13 Rf DNA-Binding Activity is Specific to the Sequence of Probe 3

To determine further the degree of specificity of the 0.13 Rf binding activity to probe 3, competition assays were performed with unlabeled probes (Fig. 5.3). Cytoplasmic protein extract from cAMP induced amoebae cells was utilized in these studies. All of the reactions included labeled probe 3. In lane 1, the binding reaction was prepared without the addition of an unlabeled competitor to provide a standard of comparison. When probe 3 was added in 100 fold molar excess to the binding reaction (lane 2), the band was no longer discernable. However, when probe 2 was added in 100 fold molar excess to the binding reaction (lane 3), the binding activity was maintained.

To further examine the precise region of binding activity, additional shorter probes were generated to span the sequence of probe 3 (Fig. 5.1). Four 30 bp oligonucleotide probes were created. For probe 4, the upstream limit was -342 bp from the ATG translation start site, and the sequence extended downstream to -313. The sequence of probe 5 extended from -381 bp to -352 bp, the sequence of probe 6 extended from -412 bp to 383 bp, and the sequence of probe 7 extended from -456 bp to -427 bp. In addition to these short probes, 50 bp oligonucleotide probes were created. For probe 8, the upstream limit was -422 bp from the ATG translation start site, and the sequence extended downstream to -373. The sequence of probe 9 extended from -395 bp to -346 bp, while the sequence of probe 10 extended from -367 bp to -318 bp. EMSA with these probes enabled a more precise examination of the 0.13 Rf binding activity.

The results of the competition assays with the shorter probes showed that the addition of excess unlabeled probes 4, 5, 6, 7, 8 or 9 to the standard binding reaction did

not reduce the 0.13 Rf DNA-protein binding activity (Fig. 5.3; lanes 7, 6, 5, 4, 12 and 13). In contrast, the addition of excess unlabeled probe 10 to the binding reaction resulted in the disappearance of the 0.13 Rf band. Probe 10 acted as a competitor for the 0.13 Rf band whether or not probe 10 was phosphorylated (lanes 14 and 17). The nucleotide sequence of probe 10 is shown in Fig 5.4. Probes 4, 5 and 9, which have overlapping nucleotide sequence with probe 10, but do not compete for 0.13 Rf binding, are shown as well.

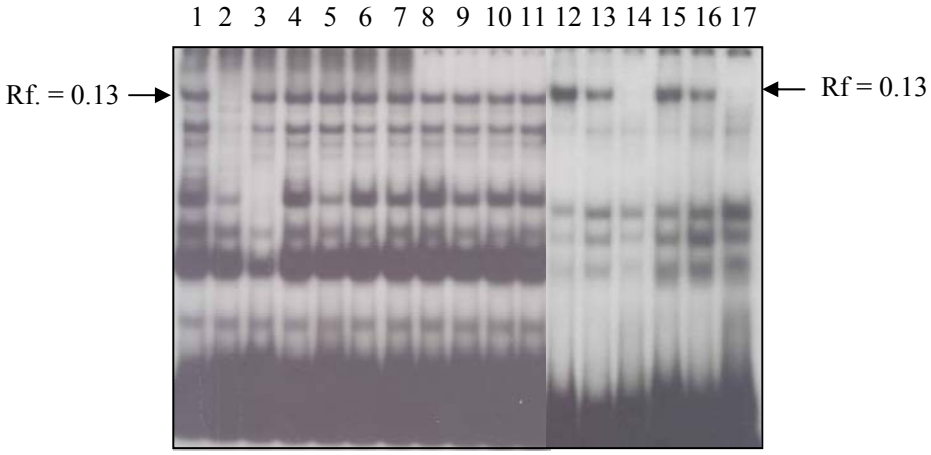

Figure 5.3. EMSA with Unlabeled Competitors Reveals that the 0.13 Rf Binding Activity is Specific to Probe 3 and Probe 10. Cytoplasmic extract from cAMP induced amoebae cells was analyzed in competition assays with various unlabeled probes to assess the specificity of the 0.13 Rf binding activity. Labeled probe 3 was used in all of the EMSA reactions. No competitor was added to the binding reaction in lane 1. In lanes 2, 3, 4, 5, 6 and 7, unlabeled probes 3, 2, 7, 6, 5 and 4 were added, respectively, to the binding reaction in a 100 fold molar excess. In lanes 8, 9, 10 and 11, phosphorylated unlabeled probes 7, 6, 5 and 4 were added, respectively, in 100 fold molar excess. In lanes 12, 13, and 14, unlabeled probes 8, 9 and 10 were added, respectively, to the binding reaction in a 100 fold molar excess. In lanes 15, 16 and 17, phosphorylated unlabeled probes 8, 9 and 10 were used, respectively.

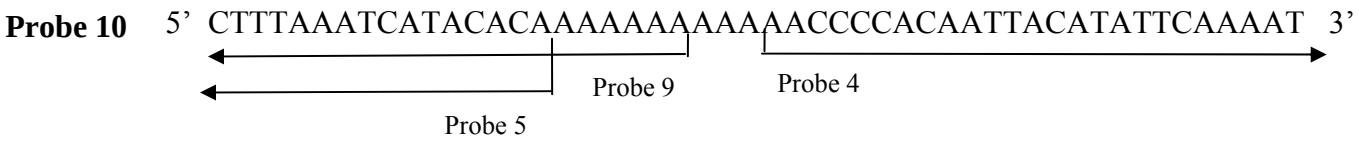

Figure 5.4. Nucleotide Sequence of Probe 10 and Regions of Overlap with Probes 4, 5 and 9. The 50 bp nucleotide sequence of probe 10 is shown. The 30 bp probe 4 overlaps the downstream sequence of probe 10 and extends slightly beyond the 3’ end of probe 10. Probes 5 and 9 also have regions of overlap with probe 10 but extend beyond the 5’ end of probe 10.

Purification of the 0.13 Rf DNA-Binding Protein by Column Chromatography

I. DEAE Chromatography

For the first step of protein purification, the 40% ammonium sulfate fraction of cytoplasmic protein extract from slugs was subjected to DEAE Sephacel column chromatography (Fig. 5.5). The total protein elution profile revealed two major peaks. The first peak represented the flow –through fractions and the second appeared after application of the linear gradient to 0.5 M NaCl. Elution of the 0.13 Rf protein occurred in the second major elution peak at about 0.15 M NaCl. EMSA of the fractions collected during chromatography with probe 10 showed that the 0.13 Rf binding activity was minimally discernable in the first flow through fraction, and undetectable in the second flow through fraction. While the 0.13 Rf band was present in fractions 16-26, the highest level of DNA-protein binding activity resided in fraction 18.

II. Heparin Sepharose Chromatography

Active DEAE fractions (16-26) were pooled, concentrated, and subjected to heparin sepharose column chromatography (Fig. 5.6). The total protein elution profile revealed 2 major peaks, one before and one after the 0.8 M NaCl linear gradient was applied to the column. Within the second major peak, there were two smaller peaks. The 0.13 Rf protein eluted in the second higher peak at about 0.22 M NaCl. EMSA of the collected fractions with probe 10 revealed the 0.13 Rf band was not found in the flow-

through fraction. While the 0.13 Rf band was present in fractions 16-20, the highest level of DNA-protein binding activity resided in fraction 16.

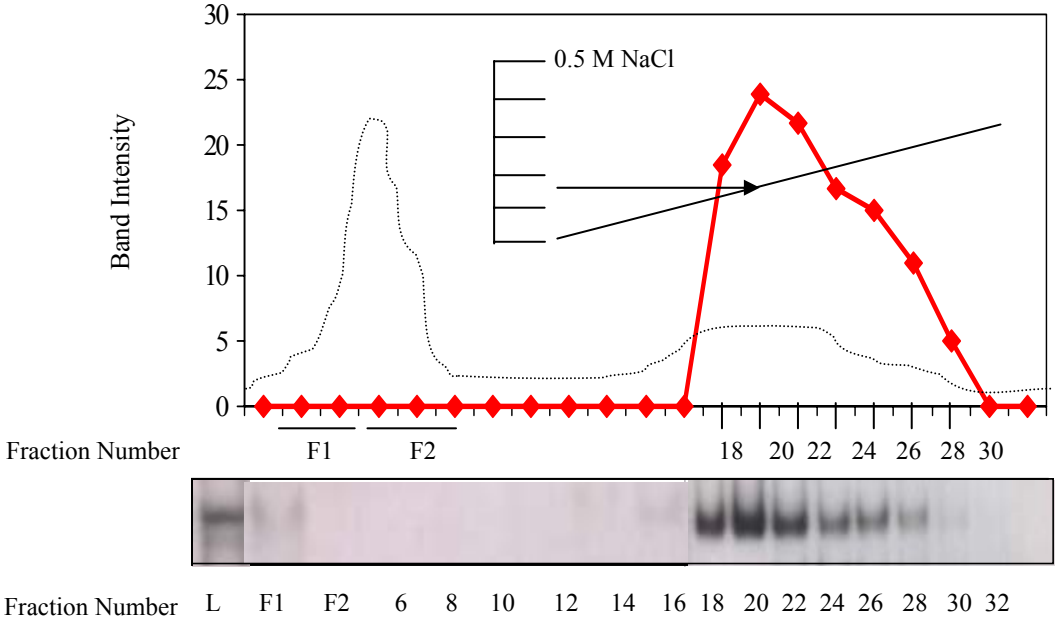

Figure 5.5. Analysis of Fractions Collected after Cytoplasmic 40% Ammonium Sulfate Extract was Subjected to DEAE Column Chromatography. Cytoplasmic protein extract collected from ammonium sulfate precipitation at 40% saturation was further separated into fractions collected from a DEAE sephacel column. Bound proteins were eluted with a linear gradient to 0.5 M NaCl over 120 min, and the 3 min fractions collected were subjected to EMSA with probe 10 (box below elution profile). Assays of protein loaded onto the column (L), flow through 1 and 2 fractions (F1 / F2), and every other fraction between 6 and 32 are shown. Binding activity was quantified using NIH Image 1.54, and is represented by the thick solid line in the graph. The elution of total protein profile is shown as the dashed line. The 0.13 Rf band was evident in fractions 16-26. The protein eluted with 0.15 M NaCl.

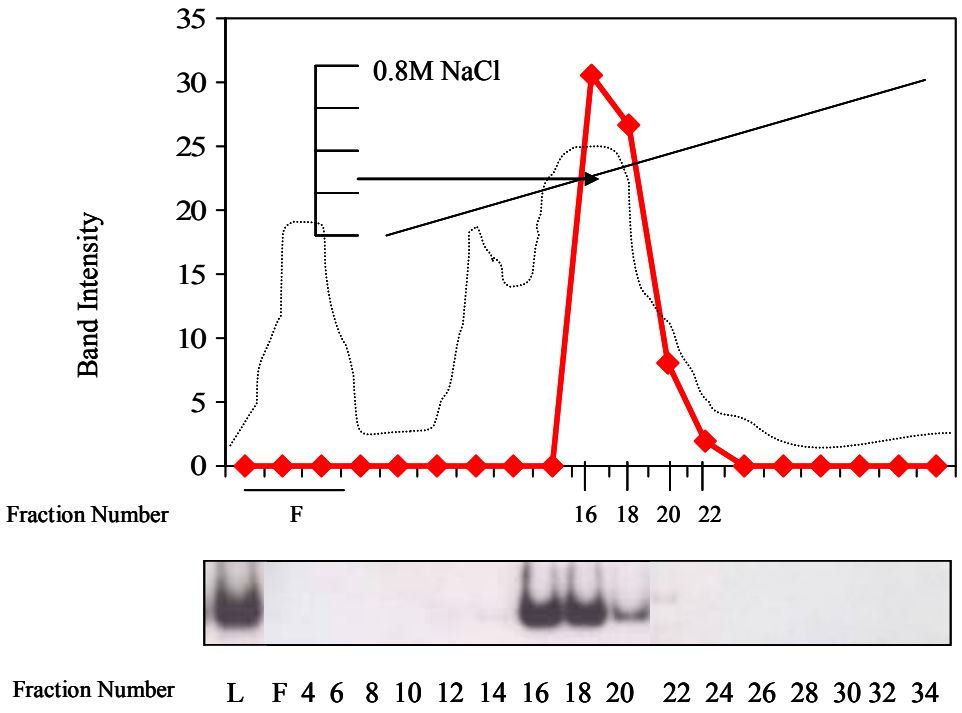

Figure 5.6. Heparin Sepharose Column Chromatography of Active DEAE Fractions. Active DEAE fractions (Fig. 5.5) were pooled and loaded onto a heparin sepharose column. Bound proteins were eluted with a linear gradient to 0.8 M NaCl over 100 min, and the 2 min fractions collected were subjected to EMSA with probe 10 (box below elution profile). Assays of protein loaded onto the column (L), flow through (F), and every other fraction between 4 and 34 are shown. Binding activity was quantified using NIH Image 1.54, and is represented by the thick solid line in the graph. The elution of total protein profile is shown as the dashed line. The 0.13 Rf band was evident in fractions 16-20. The protein eluted with 0.22 M NaCl.

III. DNA Affinity Chromatography

Active heparin sepharose fractions (16-20) were pooled, concentrated, and subjected to DNA affinity column chromatography (Fig. 5.7). The total protein elution profile revealed 2 peaks. The first peak represented the flow–through fractions and the second appeared after application of the linear gradient to 1 M NaCl. The 0.13 Rf protein eluted in the second peak at about 0.19 M NaCl. EMSA of the collected fractions with probe 10 revealed the 0.13 binding activity was detectable in the first two of four flow-through fractions collected, though its presence was much more prominent in fractions 14-28, with the highest level of DNA-protein binding activity resided in fraction 20.

IV. Gel Filtration Chromatography and SDS PAGE Analysis

Active DNA affinity fractions (17-24) were pooled, concentrated and subjected to gel filtration column chromatography (Fig. 5.8). Analysis of the fractions collected by EMSA with probe 10 showed that the 0.13 Rf binding activity was present in fractions 36-48, though the highest level of activity resided in fraction 36. Gel filtration fractions 35, 36, 38, 40 and 44 were subsequently analyzed on a 7.5% SDS-polyacrylamide gel (Fig. 5.9).

Three distinguishable bands were detected in fractions 35, 36, 38 and 40. The approximate sizes of these bands were 105 kDa, 100 kDa, and 70 kDa. Quantification of the 0.13 gel filtration bands and the bands observed from the SDS gel using NIH Image 1.54 enabled analysis correlations to be made between the amount of protein present and

the activity levels observed from each fraction. The quantification curves had similar patterns, with dark bands in the SDS gel coinciding with high levels of activity from the gel filtration fractions. The polypeptide was analyzed at the Virginia Bioinformatics Institute at Virginia Polytechnic Institute and State University, where mass spectrometry was performed to characterize and identify the isolated protein. Six peptides were identified as shown underlined in Fig 5.10. Blast searches with these peptides showed 100% identity to the *Dictyostelium* dihydro-6-hydroxymethylpterin pyrophosphokinase (HPPK; EC2.5.1.15).

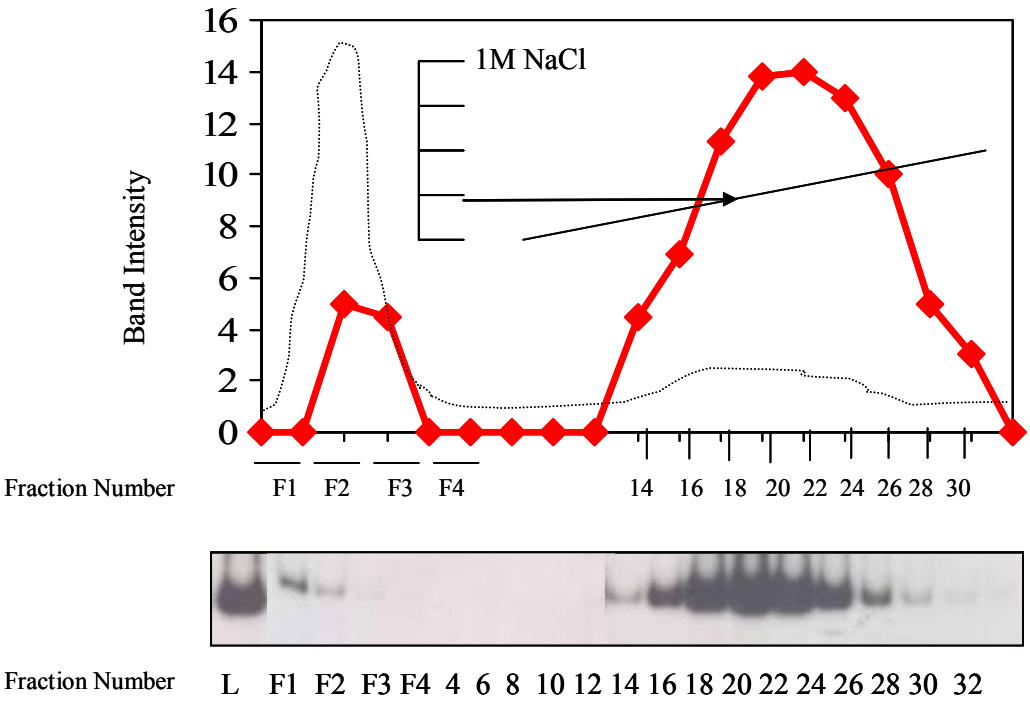

Figure 5.7. DNA Affinity Column Chromatography of Active Heparin Sepharose Fractions. Active heparin fractions (Fig. 5.6) were pooled and loaded onto a DNA affinity column. Bound proteins were eluted with 1 M NaCl over 45 min, and 1 min fractions collected were subjected to EMSA with probe 10 (box below elution profile). Assays of protein loaded onto the column (L), flow through 1-4 fractions (F1-F4), and every other fraction between 4 and 32 are shown. Binding activity was quantified, and is represented by the solid line in the graph. The elution of total protein profile is shown as the dashed line. The 0.13 Rf band was evident in fractions 14-28. The protein eluted with 0.19 M NaCl.

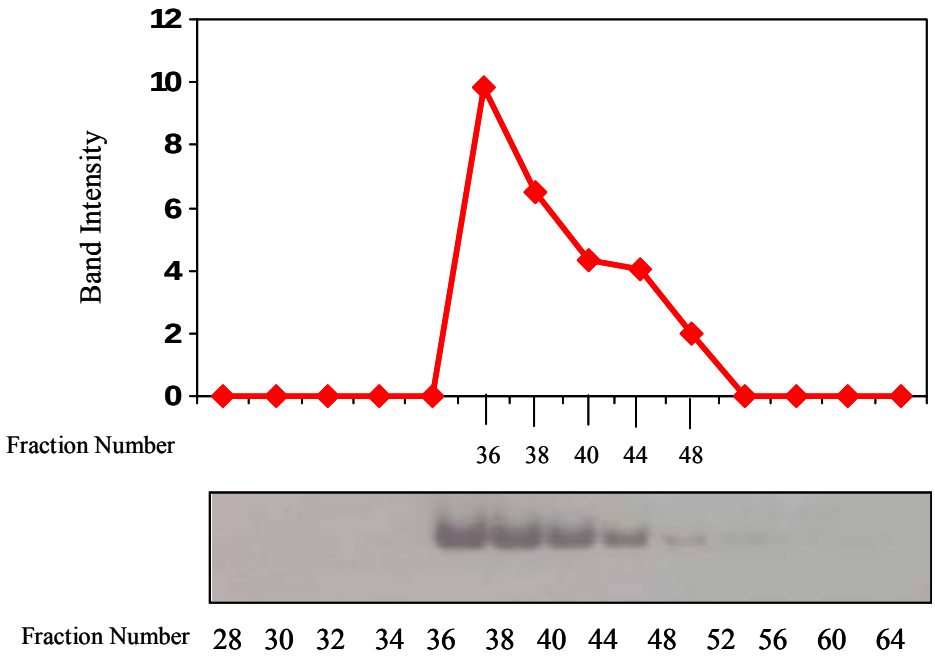

Figure 5.8. Gel Filtration Column Chromatography of Active DNA Affinity Fractions. Active DNA affinity fractions (Fig. 5.7) were pooled and loaded onto a gel filtration column. Every other fraction between 28 and 40 in addition to 44, 48, 52, 56, 60 and 64 were tested with probe 10 by EMSA. EMSA activity was quantified for each band using NIH Image 1.54 and is shown in the graph above the gel image. The 0.13 Rf band was evident in fractions 36-44.

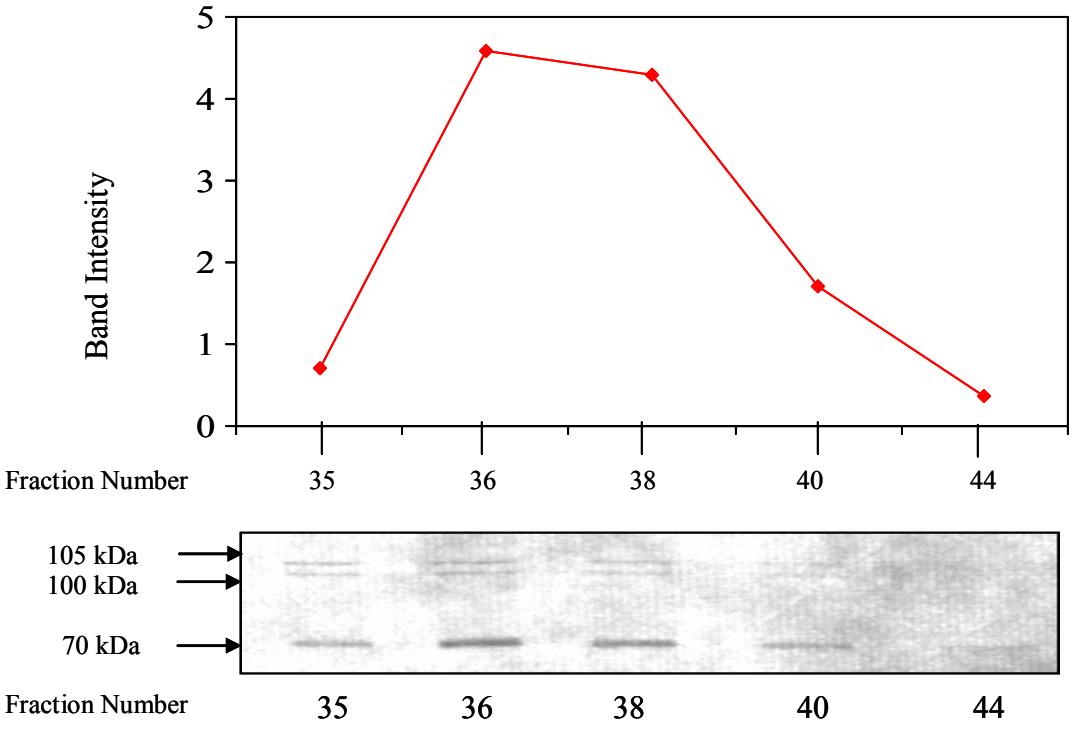

Figure 5.9. SDS PAGE of Gel Filtration Fractions (Silver Stained). Active gel filtration fractions were separated on a 7.5% denaturing gel. Each of the fractions shown here revealed three distinct bands, with an approximate molecular weight of 105, 100 and 70 kDa. The banding pattern of the 70 kDa band corresponds to the pattern observed from gel filtration fractions 36-44. Fractions 40 and 44 revealed an additional band with an approximate molecular weight of 30 kDa (not shown). Band intensity was quantified using NIH Image 1.54 and is shown in the graph above.

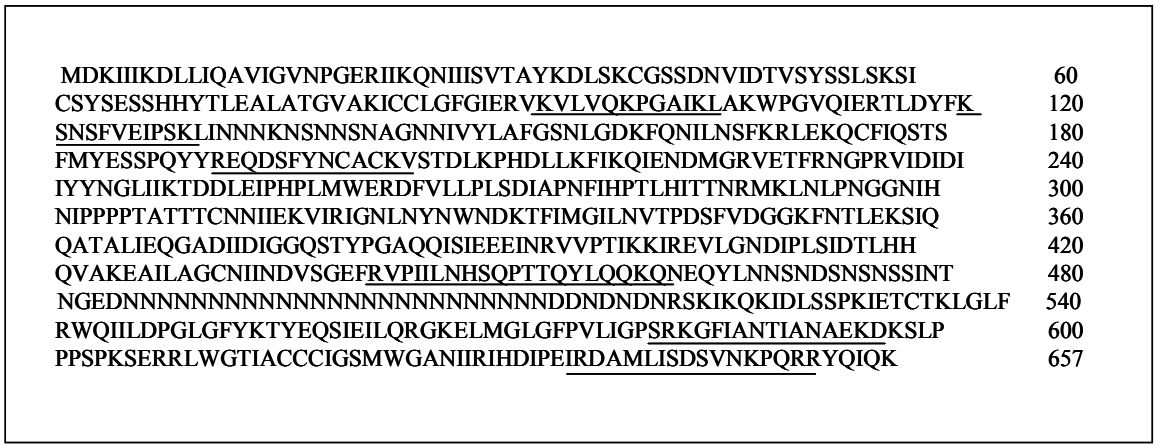

Figure 5.10. The Amino Acid Sequence of Dihydro-6-hydroxymethylpterin Pyrophosphokinase. The 657 amino acid sequence of the *Dictyostelium* Dihydro-6-hydroxymethylpterin Pyrophosphokinase is shown. Underlined portions of the sequence aligned identically to the peptide sequences obtained from protein analysis at the Virginia Bioinformatics Institute of Virginia Polytechnic Institute and State University.

Discussion

Throughout the life cycle of *Dictyostelium*, there exist control mechanisms that temporally and spatially regulate gene expression. These control mechanisms, often critical for the normal development of the organism and appropriate responses of the organism to environmental stimuli, can function at many levels within the cell. To elucidate the mechanisms required for the regulation of *5nt* at the level of transcription, the presence of protein binding activity on the *5nt* promoter sequence was analyzed by EMSA. Initial assays utilized three probes, designed to span an approximate 400 bp region of the promoter (Fig. 5.1). These assays revealed a 0.13 Rf binding activity that appeared exclusively with probe 3.

The binding activity was absent in the presence of probe 1 or probe 2, suggesting that the protein was specifically interacting with the nucleotide sequence represented by probe 3, a 105 bp probe (Fig. 5.2). The location of this sequence within the 5nt promoter is between -422 and -318 bp from the ATG translation start site. By utilizing oligonucleotide probes, the region of the promoter bound by the 0.13 Rf band was narrowed even further. It was found that a 50 bp fragment (probe 10, -367 to -318) that was contained within probe 3 comprised sufficient sequence to bind the 0.13 Rf band (Fig. 5.4-5.7). Likewise, probe 10 acted as a specific competitor for labeled probe 3 (Fig. 5.3; lane 14). Probes 4, 5, and 9 have overlapping nucleotide sequence with probe 10, but did not compete for the 0.13 Rf binding activity (Fig. 5.3 and Fig. 5.4). It is possible that in order for the 0.13 Rf protein to interact with the 50 bp nucleotide sequence of probe 10, it must associate with two sites. For example, the protein may bind to the “CAAAA”

site that is present in the overlapping region between probes 10 and 4 and to the "CAAAA" site that is present in the overlapping region between probes 10 and 9. If both sites must be intact for the protein to bind, then individual probes 4 and 9 would be unable to compete for the 0.13 Rf activity, supporting the results obtained from the competition experiments.

Although previous studies have shown that endogenous *5nt* expression is absent in amoebae, it has been shown that expression in these cells can be induced in the presence of cAMP (Chanchao et al. 1999). Therefore, DNA-protein interactions that normally activate *5nt* expression should be present in amoebae cells that are in shaking flasks with 1 mM cAMP. The protein responsible for the 0.13 Rf binding activity was first detected in cytoplasmic protein extract from cAMP induced amoebae cells. To determine if the protein involved in the 0.13 Rf activity was a cAMP-response element-binding protein (CREB) transcription factor, cytoplasmic protein extract from amoebae grown in the absence of cAMP was analyzed as well (data not shown). Because the 0.13 Rf binding activity was detected with this extract, this suggests that the protein is not a CREB transcription factor. However, cells growing in non-nutrient media for 24 hr do secrete cAMP at levels that may be sufficient for the activation of some signal transduction pathways.

The 0.13 Rf binding activity was detected with nuclear and cytoplasmic protein extract from both aggregates and slugs. If the 0.13 Rf activity is not induced by low levels of cAMP secreted by amoebae in non-nutrient media, then this compilation of data suggests that the interaction persists at several stages of development. If this interaction does persist throughout the entire life cycle, then it is possible that an additional

regulatory protein component may interact to form a complex at this site to achieve activation of expression. The conditions of these binding assays may not be conducive to the interaction of additional components at this site.

The specificity of the 0.13 Rf binding activity with the sequence of probe 3 was further evaluated through competition assays (Fig. 5.3). As expected, the addition of excess unlabeled probe 3 to the binding reaction did compete for the 0.13 Rf binding activity. The only other probe able to compete with the activity was probe 10. In some cases, competitor probes were phosphorylated in order to more resemble the radioactive labeled probe in the reaction. In its phosphorylated and unphosphorylated form, probe 10 successfully competed for the 0.13 Rf binding activity. This result was not unexpected because there is a region of overlap between probe 3 and probe 10 (Fig. 5.1). The sequence of probe 10 is included in the downstream portion of probe 3, between -367 and -318 bp from the ATG translation start site. Therefore, these results enabled the site of the DNA-protein interaction to be further specified to a 50 bp sequence on the *5nt* promoter. Examination of this 50 bp sequence did not reveal any known binding elements, such as a TA-box, a TAG-box or a C-box.

The protein interacting at this site was isolated through a series of chromatographic techniques, and the silver-stained SDS gel was subjected to peptide sequencing. Peptide sequences obtained from the analysis showed that the protein is a dihydro-6-hydroxymethylpterin pyrophosphokinase. The molecular weight of the protein is 73,720 Da, and it is comprised of 657 amino acids. The gene that encodes the protein is located on chromosome 3 of the *Dictyostelium* genome. In several other eukaryotes, this

enzyme is a trifunctional component of the tetrahydrolfolate biosynthetic pathway (Appling and Rabinowitz 1985).

In the biosynthetic pathway of tetrahydrofolate, successive reactions are catalyzed by the enzymes GTPCH (GTP-cyclohydrolase), DHNA (dihydroneopterin aldolase), HPPK (7,8-dihydro-6-hydroxymethylpterin-phyrophosphokinase), DHPS (7,8-dihydropteroate synthase), DHFS (dihydrofolate synthase), and DHFR (dihydrofolate reductase) (Martinasevic et al. 1999). In most bacteria, these activities reside on individual enzymes, resulting in 6 separate enzymes involved in the pathway. In contrast, in many eukaryotes, the DHNA, HPPK and DHPS activities are found on a single enzyme (Appling and Rabinowitz 1985).

In prokaryotes and simple eukaryotes, folate is synthesized rather than acquired through the diet, as in mammals. The synthesis of folate requires the trifunctional dihydro-6-hydroxymethylpterin pyrophosphokinase. Previously in our lab, a separate protein was isolated that specifically interacted with the *5nt* promoter sequence between -342 and -313 from the ATG translation start site. This protein, a formate tetrahydrofolate ligase, is also involved in the tetrahydrofolate pathway. In an ATP-catalyzed reaction with formate and folate, formate tetrahydrofolate ligase produces 10-formyltetrahydrofolate, an active coenzyme that functions as a carrier of the formyl group for a variety of enzymatic reactions. While the functions of HPPK and FTHFS in this biosynthetic pathway are clearly established, their functions during interactions with the *5nt* promoter are less clear.

These proteins may be involved in “moonlighting,” a phenomenon that describes metabolic proteins with additional functional activities. For example, phosphoglucose

isomerase (PGI) is an enzyme involved in the glycolytic pathway, a potent cytokine in the development of the central nervous system, an autocrine motility factor, and a mediator of the differentiation of leukemia cells (Petsko 2001). As a consequence of some metabolic proteins having more then one functional activity, the issue of organelle localization becomes an important consideration. Direct involvement in gene regulatory processes requires a nuclear localization, and involvement in many biosynthetic pathways requires localization to the mitochondria.

Chromatin immunoprecipitation experiments have shown that Arg5,6 is localized to the mitochondria and the nucleus, and associates with specific sequences of different genes in both locations (Hall et al. 2004). The enzyme's localization to the mitochondria and involvement in arginine biosynthesis has been established, though its function in the nucleus was previously unknown. The role of Arg5,6 in gene expression was evaluated by measuring mRNA transcript of several target genes from both organelles using RT-PCR in *arg5,6* Δ yeast cells grown in rich or limited medium. Results from the study suggested that the Arg5,6 is directly involved in regulating the transcript levels of specific nuclear and mitochondrial genes in cells grown in medium lacking amino acids or limited for nitrogen. Further, the interaction of Arg5,6 with sequences located in the intronic regions of these genes suggest that the enzyme may be involved in RNA processing.

Similar to Arg5,6, it is possible that HPPK and FTHFS are also multifunctional enzymes involved in biosynthetic activities as well as gene regulatory functions. The proposed link between Arg5,6 and nitrogen metabolism in yeast and some hypotheses and general models proposing a link between HPPK and FTHFS and folic acid detection /

metabolism during the development of *Dictyostelium*, are proposed in the general conclusions.

References

Bonner, J. T. (1944). "A descriptive study of the development of the slime mold Dictyostelium discoideum." Am. J. Bot. 31: 175-182.

Bonner, J. T. (1947). "Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold Dictyostelium discoideum." J. Exp. Zool. 106: 1-26.

Bonner, J. T., Hall, E. M., Sachsenmaier, W., and Walker, B. K. (1970). "Evidence for a second chemotactic system in the cellular slime mold, Dictyostelium discoideum." J. Bacteriol. 102: 682-687.

Hall DA, Zhu H, Zhu X, Royce T, Gerstein M, and Snyder M. (2004). "Regulation of gene expression by a metabolic enzyme." Science 306: 482-484.

Kay, R. R. (1979). "Gene expression in Dictyostelium discoideum: mutually antagonistic roles of cyclic-AMP and ammonia." J. Embryol. Exp. Morphol. 52: 171-182.

Kay, R. R. (1997). DIF signalling. Dictyostelium - A model system for cell and developmental biology. Y. Maeda, K. Inouye and I. Takeuchi. Tokyo, Japan, Universal Academy Press: 279-292.

Kessin, R. H. (2001). Dictyostelium - Evolution, cell biology, and the development of multicellularity. Cambridge, UK, Cambridge Univ. Press.

Loomis, W. F. (1975). Dictyostelium discoideum. A developmental system. New York, Ac. Press.

Nayler, O., Insall, R., and Kay, R. R. (1992). "Differentiation-inducing-factor dechlorinase, a novel cytosolic dechlorinating enzyme from Dictyostelium discoideum." Eur. J. Biochem. 208: 531-536.

Petsko, G.M. (2001). "Size Doesn't Matter." Genome Biol. 2001;2 (3).

Raper, K. B. (1935). "Dictyostelium discoideum, a new species of slime mold from decaying forest leaves." J. Agr. Res. 50: 135-147.

Raper, K. B. (1937). "Growth and development of Dictyostelium discoideum with different bacterial associates." J. Agr. Res. 55: 289-316.

Raper, K. B. and D. I. Fennell (1952). "Stalk formation in Dictyostelium." Bull. Torrey Bot. Club 79: 25-51.

Raper, K. B. and C. Thom (1932). "The distribution of Dictyostelium and other slime molds in soil." J. Wash. Acad. Sci. 22: 93-96.

Williams, J. G. and K. A. Jermyn (1991). Cell sorting and positional differentiation during Dictyostelium morphogenesis. Cell-Cell Interactions in Early Development. J. Gerhart. New York, Wiley-Liss: 261-272.

Chapter 6

General Conclusions

General Conclusions

In this dissertation, a more complete model of transcriptional regulation of the *Dictyostelium 5nt* gene was established by 1) delineating cis-acting elements within the promoter of *5nt* involved in temporal regulation, 2) defining cis-acting elements within the promoter of *5nt* whose regulatory activities are influenced by the presence of cAMP, 3) identifying trans-acting proteins that associate with the specific nucleotide sequences of defined cis-acting elements, and 4) purifying and characterizing the trans-acting proteins. In the process of characterizing the two DNA-binding proteins, a connection was established between the *5nt* gene and the folate biosynthetic and folate coenzyme pathways. A proposed link between *5nt* gene regulation and the enzymes involved in these pathways may exist and is described in a model.

The information acquired from the promoter deletion experiments and the site-directed mutagenesis experiments revealed the presence of several potential promoter control elements. Promoter deletion studies revealed that 663 bp of the promoter were sufficient to drive normal levels of gene expression. Comparisons of reporter activity among many promoter deletions enabled the identification of a site, located between -579 and -628 bp from the ATG translation start site, whose presence appeared to be required for normal levels of gene expression to be achieved (Fig. 6.1). These studies also enabled the identification of a 250 bp region of the promoter that was the focus of subsequent site-directed mutagenesis experiments.

Site-directed mutagenesis experiments in a developmental stage study revealed the presence of two potential regions that may be involved in temporal regulation of gene

expression. These regions were located between -530 and -560 bp and -440 and -460 bp from the ATG translation start site (Fig. 6.1). Deletion of these promoter regions resulted in low levels of reporter gene activity. Site-directed mutagenesis experiments were also performed in a cAMP induction study to determine if any of the sites tested were cAMP regulatory elements. As a result of these experiments, two sites were located between -438 and -450 bp and -550 and -562 bp from the start site. Therefore, the regions between -530 and -560 bp and -440 and -460 bp may be control elements that are regulated temporally and by the presence of cAMP that work cooperatively. Alternatively, there may be individual temporal sites and cAMP sites that function independently within each of these regions. Although the precise functions of these three cis-acting regulatory elements are unknown, their presence is necessary to achieve normal levels of gene expression. Electromobility gel shift assays did not reveal any protein binding activity with these regions of the promoters under the specific conditions used. It is possible that the binding reaction conditions were not optimal for the DNA-protein interactions at these sites to occur. It is also possible that these sites are required to fulfill a spatial requirement for other DNA-protein interactions in the vicinity.

Electromobility gel shift assays did reveal two individual DNA-protein interactions further downstream on the promoter sequence than the sites aforementioned. The 0.33 Rf protein specifically bound to the nucleotide sequence between -314 and -343 bp, and the 0.13 Rf protein specifically bound to the nucleotide sequence between -318 and -367 bp from the ATG translation start site (Fig. 6.1). The binding sites are in close proximity and may share sequence in common, which would be conducive for any required protein-protein interactions to occur.

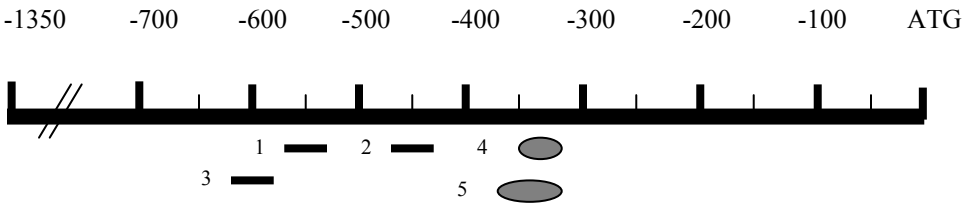

Figure 6.1. Summary Depiction of *Cis*-Acting and *Trans*-Acting *5nt* Promoter Elements. Promoter mutagenesis experiments revealed the presence of three potential regulatory elements (1, 2 and 3). Sites 1 and 2 were identified through site-directed mutagenesis experiments, and are located between -530 and -560 bp and -440 and -460 bp from the ATG translation start site. Promoter deletions that included these sites had low levels of reporter activity. Site 3 was identified by promoter deletion experiments. Located further upstream, this sequence was not included in the site-directed mutagenesis experiments. Cyclic AMP experiments with mutagenized promoters revealed two potential cAMP regulated elements, located between -438 and -450 bp and -550 to -562 bp from the ATG translation start site. The sequences of these sites are located within 1 and 2. The DNA-binding proteins identified associated with the promoter sequence further downstream. Factor 4 (0.33 Rf) bound specifically to the promoter between -314 and -343 bp from the ATG translation start site, and factor 5 (0.13 Rf) bound specifically to the promoter between -318 and -367 bp from the start site.

In addition to having binding sites in close proximity, the two DNA-binding proteins isolated in these studies share other similarities. The 0.33 Rf protein is a formyltetrahydrofolate synthase (FTHFS; EC 6.3.4.3), and the 0.13 Rf protein is a dihydro-6-hydroxymethylpterin pyrophosphokinase (HPPK; EC 2.5.1.15). Tetrahydrofolate (THF) is a central cofactor for one-carbon transfer reactions in all living organisms and is required for the synthesis of compounds including methionine and purines. Most plants, microbial eukaryotes, and prokaryotes are capable of synthesizing their own folate, while other organisms must acquire folate through their diet. The biosynthesis of THF in eukaryotes requires a trifunctional enzyme. This enzyme catalyzes three reactions in the pathway, and their functions are as a dihydroneopterin aldolase (DHPS), hydroxymethylpterin pyrophosphokinase (HPPK), and dihydropteroate synthase (DHPS) (Fig. 6.2) (http://www.chem.qmul.ac.uk/iubmb/). The nature of this enzyme has not been characterized in *Dictyostelium*, though it is presumed that it is tri-functional in this simple eukaryote. FTHFS is responsible for the recruitment of single carbon units from the formate pool into various folate dependent biosynthetic pathways (Fig. 6.3) (http://www.jbc.org/cgi/content/full/278/44/43178). In an ATP-catalyzed reaction with formate and folate, formate tetrahydrofolate ligase produces 10-formyltetrahydrofolate, an active coenzyme that functions as a carrier of the formyl group for a variety of enzymatic reactions. In *Saccharomyces cerevisiae*, all of the enzymes involved in the THF biosynthetic pathway are encoded by nuclear genes, although the pathway resides in mitochondria, and the trifunctional enzyme involved in the biosynthesis of THF has been localized to mitochondria (Guldener et al. 2004).

Late Stages of Folate Biosynthesis

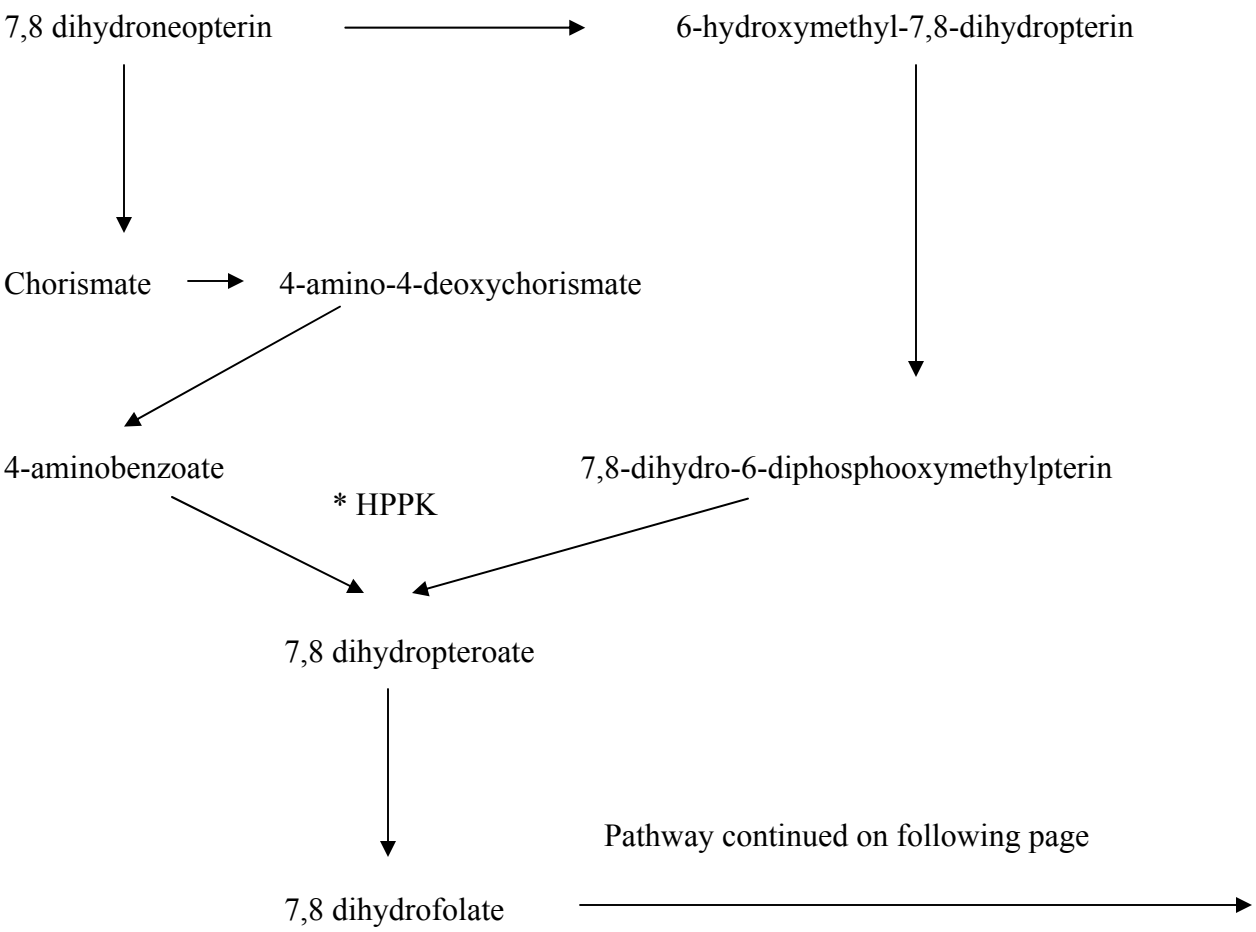

Figure 6.2. Function of HPPK in the Tetrahydrofolate Biosynthetic Pathway. The HPPK (EC2.5.1.15) enzyme is trifunctional in the biosynthetic pathway of tetrahydrofolate, producing 7,8-dihydropteroate from 7,8-dihydro-6-diphosphooxymethylpterin. After synthesis of the dihydrofolate, tetrahydrofolate is produced in a reduction reaction, as shown during the first stages shown in Fig 6.3.

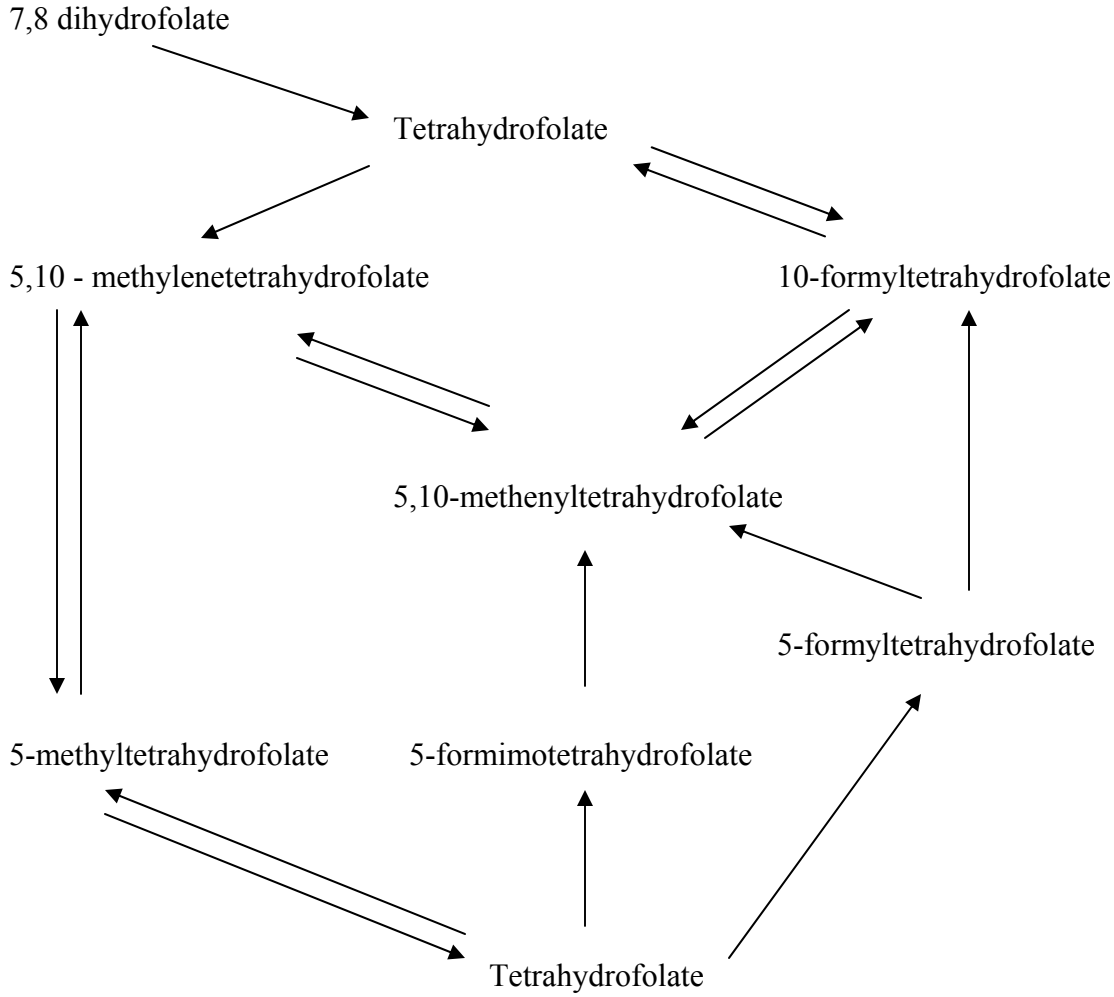

Figure 6.3. Function of FTHFS in the Synthesis of 10-formyltetrahydrofolate. After folate biosynthesis takes place, as shown in Fig 2, FTHFS (EC 6.3.4.3) catalyzes an ATP-requiring reaction that combines tetrahydrofolate with formate to produce 10-formyltetrahydrofolate. 10-formyltetrahydrofolate is an active coenzyme that functions as a carrier of the formyl group for a variety of enzymatic reactions.

The identification of FTHFS and HPPK in *Dictyostelium* is not surprising, and suggests that the organism is capable of synthesizing its own tetrahydrofolate, as is common in many prokaryotes, plants, and simple eukaryotes. However, the specific DNA interactions of these proteins with the *5nt* promoter suggest that these enzymes may be involved in an additional regulatory activity.

FTHFS and HPPK may be involved in "moonlighting," a phenomenon that describes metabolic proteins with additional functional activities and may compensate For example, phosphoglucose isomerase (PGI) is an enzyme involved in the glycolytic pathway, a potent cytokine in the development of the central nervous system, an autocrine motility factor, and a mediator of the differentiation of leukemia cells (Petsko 2001). Described as a "super multi-functional protein," the eukaryotic polypeptide elongation factor, EF-1, is involved in translation, signal transduction, cytoskeletal organization, apoptosis, oncogenic transformation, RNA synthesis and mitosis (Ejiri 2002). In *C.elegans,* the phosphatase Cdc14 prevents re-entry into the cell cycle during periods of quiescence, in addition to participating in the development of the central mitotic spindle during anaphase (Kipreos 2004). In adition, the ability of metabolic enzymes to directly regulate eukaryotic gene expression has been shown (Hall et al 2004). In this study, a yeast proteome microarray was screened with DNA probes, and a Arg5,6, a arginine biosynthetic enzyme, was identified. Subsequent chromatin immunoprecipitation experiments have shown that Arg5,6 is localized to the mitochondria and the nucleus, and associates with specific sequences of different genes in both locations. The enzyme's localization to the mitochondria and involvement in arginine biosynthesis has been established, though its function in the nucleus was

previously unknown. The role of Arg5,6 in gene expression was evaluated by measuring mRNA transcript of several target genes from both organelles using RT-PCR in *arg5,6* Δ yeast cells grown in rich or limited medium. Results from the study suggested that the Arg5,6 is directly involved in regulating the transcript levels of specific nuclear and mitochondrial genes in cells grown in medium lacking amino acids or limited for nitrogen. Further, the interaction of Arg5,6 with sequences located in the intronic regions of these genes suggest that the enzyme may be involved in RNA processing. In yeast, nitrogen metabolism metabolism occurs in the mitochondria. The proposed link between Arg5,6 in ornithine biosynthesis, nitrogen metabolism and mitochondrial gene expression may explain the link to mitochondrial function. In the case of HPPK and FTHFS, a similar link may exist with the folate biosynthesis pathway.

Folic acid and pteridine derivatives have been described as chemoattractants during the vegetative stages of many cellular slime molds, including *Dictyostelium* (Pan et al. 1972; Pan et al. 1975). During aggregation of *Dictyostelium*, the oscillation period of cAMP waves is 8 min. Temperature changes and the addition of small doses of cAMP or folic acid can cause a shift in these oscillatory patterns (Gerisch and Hess 1974; Wurster 1976; Gerisch et al. 1977; Malchow et al. 1978). It has been shown that in AX3K cells, levels of cAMP secretion in response to folate or xanthopterin are higher than levels achieved in response to cAMP itself (Devreotes 1983).

It has been suggested that the ability of amoebae to detect folate gradients, and move toward high concentrations of the chemoattractant, may serve as food detection mechanisms for these organisms (Blusch and Nellen 1994). In aggregation competent cells, the MAP kinase, ERK2, is activated by extracellular cAMP through G-coupled

cAMP receptors. Later in development, folate serves an additional function involving adenylyl cyclase activation. Folic acid receptors are present at their highest levels at the tipped aggregate stage of development, as are the Gα and Gα4 subunits of a G-protein that is required for activation of ERK2. ERK2 becomes phosphorylated on tyrosine residues and activates adenylyl cyclase. As a result of this activation, cAMP protein kinase becomes activated, and this activation is required at several stages of the life cycle. The G-protein pathway resulting in ERK2 activation by folic acid is unique from the pathway used by cAMP to activate ERK2 (Maeda 1997). Previous research has proposed that all chemotactic activity in *Dictyostelium* are mediated through G-protein –dependent signal transduction pathways (Kesbeke et al. 1990).

Based upon general information regarding 1) folic acid as a chemoattractant 2) the requirement for low levels of cAMP during prestalk differentiation 3) the localization of 5NT and several components of the folic acid receptor to the plasma membrane 4) the specific binding of two enzymes of folic acid metabolism to the *5nt* promoter and 5) the phenotype of 5nt null mutants, possible interactions of the components are suggested in Fig 6.4. For example, it has been shown previously that when *5nt* is disrupted, multiple tips form on the tight aggregate, suggesting that 5NT is a required component of tip formation in prestalk cells. It is also during tip formation that folate receptors and the Gα4 are maximally expressed. If 5NT is required for normal folate reception and / or signal transduction, then the disruption of the *5nt* gene may interfere with the ability of folate receptors to detect folic acid and activate ERK2. Previous research has shown that a folic acid deaminase, residing on the cell surface, is involved in the inactivation of folic acid (Kakebeeke 1980). Folate receptors, folic acid deaminase and 5nt are all present in

the cell membrane. If FTHFS and HPPK are regulating the expression of *5nt*, then it may be reasonable to suggest that membrane-bound 5NT interacts with folate receptors, and / or folic acid deaminase, and / or the G-proteins involved in the signal transduction events (Fig. 6.4).

During the culminant stage of development, 5NT is localized to the membranes of prestalk AB cells in the extreme tip of the slug and culmination stages (Fig. 6.4). These tip cells have been shown to act as the "organizer" of prestalk migration into the correct location for stalk construction. It is thought that cAMP is produced in these tip cells and that prestalk cells respond by migrating up the cAMP gradient. At this stage, there are major cell sorting events taking place to produce the stalk structure, and prespore cell differentiation is influenced by high levels of cAMP, while prestalk cell differentiation is inhibited by cAMP. It is possible that as development proceeds, two (or more) enzymes of folic acid metabolism enter the nucleus and specifically bind to the *5nt* promoter, localizing expression of the *5nt* gene to PstAB cells. If one role of 5NT at this stage is to act as a component of folic acid reception, for example to down-regulate folic acid deaminase, then the interaction of folic acid to cell surface receptors would be enabled. Likewise, pathways activating ERK2 and its signal transduction pathways would then occur. The positive feedback on the *5nt* gene by enzymes of folic acid metabolism would thus ensure that folic acid reception and signal transduction could be retained in the PstAB cells of the tip organizer. In addition, if 5NT is also involved in the cAMP degradation pathway, as shown in Fig 6.4, then its presence may participate in preventing the signal transduction events caused by cAMP, and contribute toward lowering the levels of cAMP in prestalk cells, a requirement for their differentiation.

While the characteristics of *5nt* and its promoter have been thoroughly analyzed in our laboratory, the amino acid sequence of the purified 5NT revealed no similarity between it and any other protein, raising the possibility that the protein purified may function in activities other than those known to be associated with ecto-nucleotidases. BLAST searches of the *5nt* sequence do not result in any matches with folic acid deaminase or folic acid receptors.

Future research endeavors could involve a functional analysis of FTHFS and HPPK. Using homologous recombination to disrupt the genes that encode these enzymes, over-expressing the genes, and down-regulating the genes, additional functions of these proteins may be elucidated. Due to the critical role of these enzymes in the biosynthesis of 10-formyltetrahydrofolate, mutants may be lethal. Potential interactions between FTHFS and HPPK as they interact with the *5nt* promoter could be analyzed by a yeast 2-hybrid system.

Technological advances have enabled the identification of DNA-protein interactions on a more global scale (Mukherjee et al 2004). Microarray analysis of chromatin precipitation (ChIP-chip) is a high-throughput method by which DNA-protein interactions can be identified, which requires the use of specific antibodies. Protein binding microarrays (PBMs) have emerged as a technique that enables the site-sequence specificities of transcription factors to be identified in one day, and does not require the availability of a specific antibody. In this process, a double-stranded DNA microarray is created to contain numerous potential DNA binding sites. The epitope tagged protein of interest is bound to the microarray and detected using a fluorophore-conjugated antibody. Such advances will enable the identification of potential transcription factors and their

respective binding sites to be identified efficiently on a comprehensive scale, and facilitate the identification of other genes that may be regulated by a specific transcription factor.

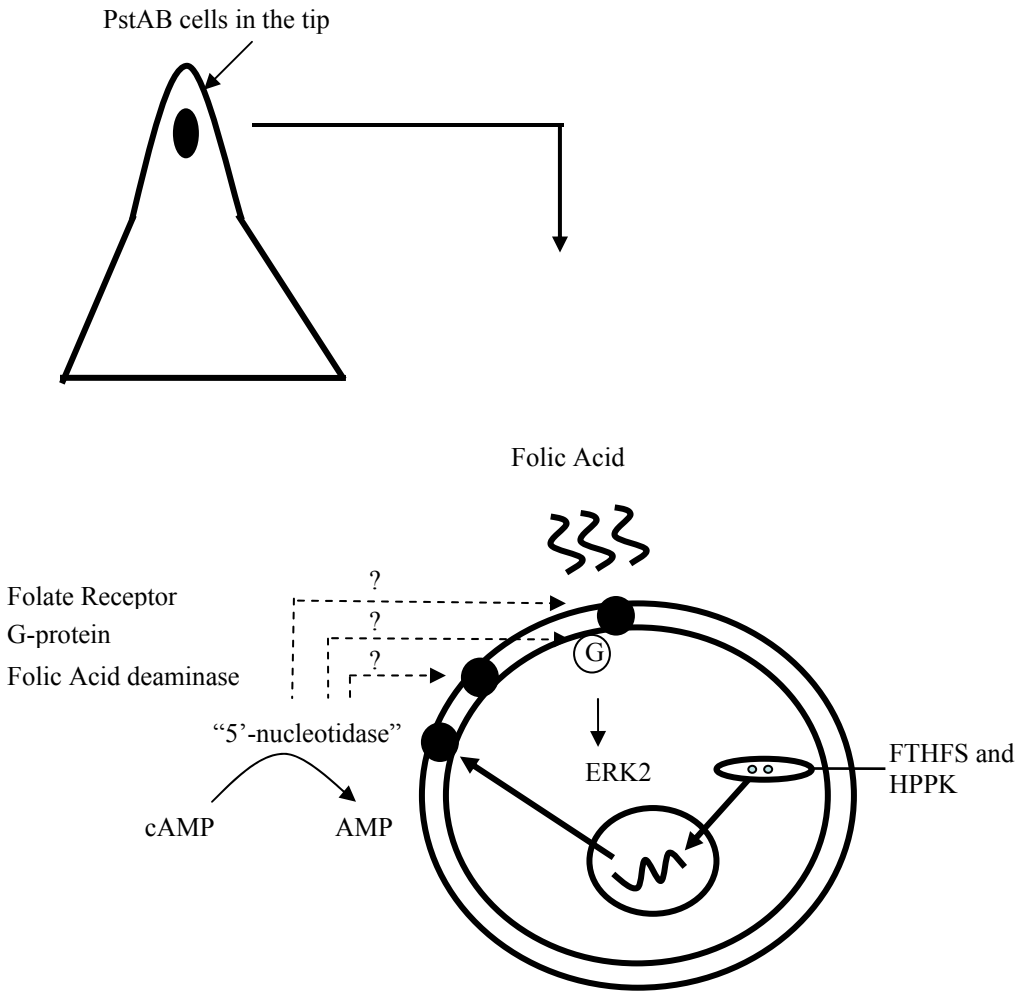

Figure 6.4. Possible Interactions of 5NT and Components of Folic Acid Reception and Signal Transduction. FTHFS and HPPK enter the nucleus and regulate the expression of *5nt* at the first finger and slug stages of development. The 5NT protein product resides on the extracellular surface of the cells. In the mid-culminant, this activity resides in the membranes of pstAB cells in the tip of the culminant. Along with 5NT, folic acid deaminase and folate receptors are present in the cell membrane. 5NT may interact with folic acid deaminase and / or folate receptors, and / or the G-proteins involved in the signal transduction of ERK2 activation. In addition to its interaction with the folic acid pathway, the catalytic function of 5NT would provide enhanced degradation capacity of cAMP in PstAB cells, where low levels of cAMP are required for the progression of prestalk differentiation.

References

Blusch, J. H. and W. Nellen (1994). "Folate responsiveness during growth and development of Dictyostelium - separate but related pathways control chemotaxis and gene regulation." Mol. Microbiol. 11: 331-335.

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

Ejiri, S. Moonlighting functions of polypeptide elongation factor 1: from actin bundling to zinc finger protein R1-associated nuclear localization. Biosci Biotechnol Biochem. 2002 Jan;66(1):1-21. Review.

Gerisch, G. and B. Hess (1974). "Cyclic-AMP-controlled oscillations in suspended Dictyostelium cells: Their relation to morphogenetic cell interactions." Proc. Natl. Acad. Sci. USA 71: 2118-2122.

Gerisch, G., Malchow, D., Roos, W., Wick, U., and Wurster, B. (1977). Periodic cyclic-AMP signals and membrane differentiation in Dictyostelium. Cell Interactions in Differentiation (Sigrid Juselius Symposium, Helsinki). N. Y. Karkinen-Jaaskelainen, L. Saxen and L. Weiss. New York, Ac. Press: 377-388.

Guldener U, Koehler GJ, Haussmann C, Bacher A, Kricke J, Becher D, and Hegemann JH. (2004). "Characterization of the Saccharomyces cerevisiae Fol1 protein: starvation for C1 carrier induces pseudohyphal growth." Mol Biol Cell 15(8): 3811-28.

Hall DA, Zhu H, Zhu X, Royce T, Gerstein M, and Snyder M. (2004). "Regulation of gene expression by a metabolic enzyme." Science 306: 482-484.

Kakebeeke, P. I. J. (1980). Chemotaxis signals during differentiation in the cellular slime molds. Leiden, RUL.

Kesbeke, F., van Haastert, P. J. M., de Wit, R. J. W., and Snaar-Jagalska, B. E. (1990). "Chemotaxis to cyclic AMP and folic acid is mediated by different G-proteins in Dictyostelium discoideum." J. Cell Sci. 96: 669-673.

Kipreos, ET. Developmental quiescence: Cdc14 moonlighting in G1. Nat Cell Biol. 2004 Aug;6(8):693-5.

Maeda, M. (1997). Aggregation-related protein kinases in Dictyostelium, MAP-kinase ERK2 and cAMP-dependent protein kinase PKA. Dictyostelium - A model system for cell and developmental biology. Y. Maeda, K. Inouye and I. Takeuchi. Tokyo, Japan, Universal Academy Press: 193-203.

Malchow, D., Nanjundiah, V., Wurster, B., Eckstein, F., and Gerisch, G. (1978). "Cyclic AMP-induced pH changes in Dictyostelium discoideum and their control by calcium." Biochim. Biophys. Acta 538: 473-480.

Mukherjee S, Berger MF, Jona G, Wang XS, Muzzey D, Snyder M, Young RA, and Bulyk ML. (2004). "Rapid analysis of the DNA-binding specificities of transcription factors with DNA microarrays." Nat Genet. 2004 Dec;36(12):1331-9.

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

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

Wurster, B. (1976). Responses of amoebae of the cellular slime mould Polysphondylium violaceum to their specific chemoattractant. Hoppe-Seyler's Z. Physiol. Chemie.

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EDUCATION

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Selected coursework: Molecular Biology Lab, Biochemistry, Molecular Biology of the Life Sciences, Microbial Physiology, Eukaryotic Gene Expression, Topics in Molecular Cell Biotechnology, and Seminar in Molecular Cell Biotechnology.

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Bachelor of Arts, College of Arts and Science August 1994- December 1998
Major: Psychology
Minors: Biology and Animal Behavior
Cumulative GPA: 3.42
Honors and Awards: Academic Honors, January 1995-August 1997
High Scholastic Achievement Award, August 1997-March 1998

RESEARCH EXPERIENCE

9/00 – present Dissertation research at Virginia Tech has involved the analysis of transcriptional regulation of the *5'-nucleotidase* gene in *Dictyostelium discoideum* by the production of a series of promoter deletions in the upstream regulatory sequence. Fusion of these deletions with the luciferase and ß-galactosidase reporter genes enabled the identification of a region in the promoter, which was subsequently further analyzed by site-directed mutagenesis experiments. Implementation of electromobility gel shift assays has enabled the identification of specific DNA-protein interactions in the region of the promoter. Further characterization of this specific activity is being evaluated for isolation of potential transcription factor(s) responsible for the regulation of *5'-nucleotidase* expression during development.

PRESENTATIONS, PUBLICATIONS, AWARDS

5/03 Oral presentation of dissertation research at the 81st annual Virginia Academy of Sciences meeting at the University of Virginia in Charlottesville, Virginia. Received "Best Student Paper Award" in the Biology section.

6/03 Identification of Regulatory Binding Sites and Corresponding Transcription Factors Involved in the Developmental Control of 5'-nucleotidase Expression in *Dictyostelium discoideum*. N.S. Wiles, C.M. Eristi, M.Ubeidat, B.R Joyce and C.L Rutherford. Virginia Journal of Science, Summer 2003, Vol.54, No.2, pg.61.

6/03	Expression and Regulation of Alkaline Phosphatase During Development of *Dictyosteium discoideum*. B.R. Joyce, M.Ubeidat, C.M. Eristi, N.S.Wiles and C.L. Rutherford.Virginia Journal of Science, Summer 2003, Vol.54,No.2, pg.61.
7/03	Oral presentation of dissertation research and laboratory group research at the annual international *Dictyostelium* conference in Lorne, Victoria, Australia.
1/05	Selected by the Biology Department at Virginia Tech to receive Biology Department Teaching Award
1/05	Selected as Biology Department nominee for the Univeristy Graduate Teaching Excellence Award

GRANTS

4/02	Applied for a Graduate Research Development Project Grant for outstanding graduate work. Received $500
9/03	Applied for a Graduate Research Development Project Grant for outstanding graduate work.
10/03	Applied for a travel fund grant from the Graduate Student Assembly of Virginia Tech. Received $300
11/03	Applied for a $20,000 grant from the American Association for University Women.
4/04	Applied for a Graduate Research Development Project Grant for outstanding graduate work.
9/04	Applied for a Graduate Research Development Project Grant for outstanding graduate work.

MEMBERSHIPS

Virginia Academy of Science
Phi Sigma National National Biological Honors Society

TEACHING EXPERIENCE

9/00-5/00	**Virginia Tech, Blacksburg, Virginia** **Graduate Teaching Assistant-** Instructor for General Biology and Principles of Biology laboratories for the first and second semesters. Responsibilities included designing coursework to embellish various laboratories, teaching laboratory techniques and basic biological principles to non-biology majors
9/01-5/02 9/02-5/03 9/03-5/04 9/04-current	**Graduate Teaching Assistant-** Instructor for the General Biology laboratories for majors. Responsibilities included designing coursework to complement and reiterate laboratories performed in class, teaching laboratory techniques and basic biological principles, with an additional focus on how to research topics in biology and write effective laboratory and research papers.
5/02-7/02 5/03-7/03	**Instructor for the General Biology Lecture Course-** Responsible for designing and implementing a six-week biology summer course for non-major students. Broad areas of focus included evolution, ecology, molecular biology, biochemistry, plant biology and cell biology.
5/04-7/04	**Instructor for the General Biology Lecture Course for Majors-** Responsible for designing and implementing a six-week biology summer course for science majors and biology majors. Broad areas of focus included detailed studies of principles important to cell biology, biochemistry, molecular biology,

plant biology, genetics, developmental biology, ecology and evolution. Coursework included research of current biological topics present in the news and group discussions of ethical considerations surrounding these topics.

8/02- 6/04 **Mentor of Undergraduate Researcher and Honors Thesis Project-** Organized projects, provided guidance throughout a two year period, and offered assistance to the production of an honors thesis manuscript for an undergraduate researcher in the Biology Department Honors Program.

REFERENCES

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