INVESTIGATION OF TRANSCRIPTIONAL REGULATION OF *5'-NUCLEOTIDASE* IN *DICTYOSTELIUM DISCOIDEUM*

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ABSTRACT

A 5'AMP-degrading activity appears during the course of development in *Dictyostelium discoideum* between the prestalk and prespore zones. This enzyme is referred as 5'-Nucleotidase (5NT). Given the critical role of cyclic AMP in cell differentiation in this organism, 5NT is thought to be involved in cell positioning during development. Southern blot analysis showed a single form of the gene. The expression of the *5nt* gene is known to be developmentally regulated. The message appears first at about 5 hr of the *Dictyostelium* development and remains constant throughout the rest of the development. Primer extension indicated two potential transcriptional start sites (118 bp and 148 bp upstream of the ATG initiation codon) for the *5nt* expression. The *5nt* promoter region was cloned and analyzed to investigate the expression of *5nt*. Analysis of the cloned *5nt* promoter fused to *lacZ* enabled the localization of the *5nt* expression in pstAB cells during development. To identify *cis*-acting regulatory sequences, a series of 5' and internal promoter deletions were generated and fused to a *luciferase* reporter gene. The reporter activity driven by the 1,212 bp promoter started at the early aggregation stage, in agreement with temporal expression of the *5nt* gene. Also, the expression was

induced by exogenous cAMP. The reporter activity was high and relatively equivalent for all deletion constructs that contained 547 bp or more of the promoter region. No luciferase activity was detected using 365 bp or less of the promoter. A gradual decrease in activity was observed when three deletion constructs between –547 and –365 bp were tested suggesting the presence of at least two *cis*-regulatory elements within this region. Internal deletion analysis indicated another potential regulatory region located between –307 and –226 bp. To identify protein factor(s) that bind specifically to these regulatory sequences, gel shift assays were performed. Two bands, 0.33 R_f and 0.13 R_f, were detected in both cytoplasmic and nuclear extracts using radiolabeled DNA fragments located between –227 and -198 bp and -252 and -203 bp of the promoter region, respectively. Competition experiments confirmed the specificity of binding. The protein factors in these DNA binding activities were purified using various chromatography techniques. Mass spectrometry analysis of the purified 70 kDa protein corresponding to the 0.33 R_f band activity and a subsequent search in the *Dictyostelium* genomic database revealed that the purified protein was a putative formyltetrahydrofolate synthase.

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CHAPTER 1. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. The Life Cycle of *Dictyostelium discoideum*

Dictyostelium discoideum, a relatively simple eukaryotic slime mold, exists, in the presence of abundant food source, as a single cell and exhibits amoeboid movement. It feeds on bacteria and divides every 4-10 hr. Cells of *Dictyostelium discoideum* multiply by binary fission (equal mitotic division) as single-celled amoebae. Upon depletion of nutrients, however, the organism undergoes a relatively simple developmental program, which takes 24 hr to complete and generates a final fruiting body in which a ball of dormant spores is held on top of a cellular stalk (Raper, 1941; Bonner, 1952; Brown and Firtel, 1999; Yin *et al*., 1994a).

Upon starvation, up to 100,000 single cells migrate towards aggregation centers in response to a signal of extracellular cyclic AMP (cAMP) to form a discrete multicellular structure (Konijn *et al*., 1967; Gross, 1994; Firtel, 1995; Williams and Jermyn, 1991). At this point, cell differentiation occurs, generating a random pattern of two cell types, prestalk and prespore cells. These precursor cells ultimately differentiate into stalk cells and spores of the mature fruiting body (Fig. 1). The aggregate elongates and forms an upright structure called ‘first finger’. The anterior side of the aggregate is composed of prestalk cells. The anterior part of the prestalk region comprised of prestalk A (pstA) cells, a type of prestalk cells that express EcmA (an extracellular matrix protein). pstO cells are located in the posterior of the prestalk region. In the central portion of the prestalk region, the pstAB cells, which express EcmB (another extracellular matrix protein), are positioned. “Anterior-like cells” (ALCs), another type of prestalk cells, are dispersed throughout the prespore zone (Sternfeld and David, 1982).

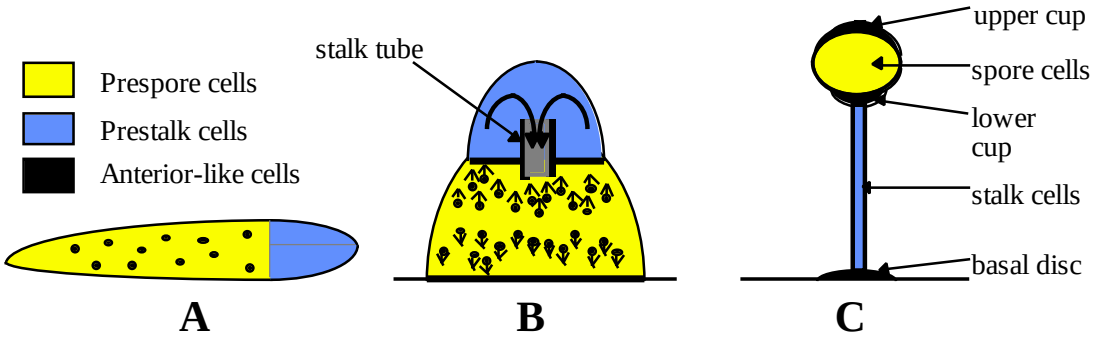

Figure 1. Cell sorting during development. A) Slug stage. Prestalk cells are located in the anterior of the slug and ALCs are dispersed throughout the prespore zone. **B)** Culmination. Slug sits on posterior and the apical prestalk cells synthesize a tube into which they migrate thus extending the tube through the underlying prespore cells. ALCs sorting occurs in two directions: ALCs fated to form the upper cup move upwards, while the cells that form the lower cup move to the posterior. **C)** Mature culminant. Terminal differentiation produces spore cells and stalk cells. Spore cells form a sorus that is raised on the stalk.

Later in development, some ALCs move upward to form the upper cup whereas some migrate downward to form the lower cup (Jermyn and Williams, 1991). ALCs are also involved in the formation of the basal disc that attaches the stalk to the substratum (Jermyn *et al.*, 1996). Finally, prespore cells that become spores at the end of development make up the posterior region of the finger structure (Fosnaugh and Loomis, 1991). The first finger structure chooses one of the two pathways: it either directly enters culmination stage at the aggregation site through a structure called ‘mexican hat’ or it elongates and then falls on the substratum forming a slug. Environmental conditions determine which of these two pathways will be chosen. In the migrating slug, precursors for stalk and spore cells become recognizable. Whether the slug is small or large, the

anterior 20 percent of the slug consists of prestalk cells and the posterior 80 percent consists of prespore cells (Kessin, 2001). If the slug is severed or cut, so that its prestalk and prespore regions are separated, each fragment will go on to form a fruiting body, but the fruiting body will be defective. The rear of the slug forms more normal fruiting bodies than the front, which is believed to be due to the presence of the prestalk-like cells (ALCs) in the prespore region, which can actually move to the front and form the prestalk region that is missing (Kessin, 2001). Slugs migrate towards heat and light in search of food and eventually arise and enter the culmination stage at a different location (Loomis, 1982).

Culmination involves a dramatic series of cell-sorting events with concurrent cell differentiation, processes believed to be regulated by extracellular signaling molecules (Kessin, 2001). The prestalk cells, on the top of the early culminant, move toward the bottom of the structure to form the stalk tube. This movement of prestalk cells requires involvement of cAMP. At the same time, ALCs in the prespore region either move up or move down to form the upper and lower cups of the mature organism. At the end of culmination, a mature organism or fruiting body is formed. The fruiting body consists of a spore head secured by ALC-derived upper and lower cups. The spore head is supported by a slender column of dead stalk cells, which is connected to the substratum by another ALC-derived structure, the basal disc. Each spore is surrounded by a firm spore coat, which is comprised of glycoproteins and polysaccharides secreted from spore cells. The spores are metabolically inactive and can tolerate dessication, ultraviolet light, and other severe treatments. Under favorable conditions, spores are dispersed to initiate another life cycle.

1.2. *Dictyostelium* as a Model System

Dictyostelium is similar to mammalian cells in many physiological functions such as directed amoeboid movement, cell-cell adhesion, tissue differentiation, proportioning and sorting (Loomis, 1996). It has similarity to plant cells in vacuolization and cellulose deposition during terminal stalk cell differentiation (Raper, 1935). Various molecular genetic techniques including gene disruption by homologous recombination, gene replacement, antisense methods, restriction enzyme-mediated integration (REMI) and expression of green fluorescent protein (GFP) fusion proteins have been successfully applied in this organism (Eichinger *et al*., 1999). *Dictyostelium* is used as a model system to study many different cellular processes including differentiation, signal transduction, phagocytosis, cytokinesis, chemotaxis and cell motility (Escalante and Vicente, 2000). In addition, this organism has been used as a host system to investigate the virulence factors of pathogenic strains of *Pseudomonas aeroginosa* (Cosson *et al*., 2002; Pukatzki *et al*., 2002).

Dictyostelium discoideum is a good model organism for a number of reasons. This organism has a small haploid genome of approximately 34 mega base pairs (Mb), which has been almost completely sequenced (Eichinger and Noegel, 2003). The genome has a small amount of non-coding sequences. The life cycle of this organism can be divided into vegetative growth and developmental phases. It is a good model system to study cell differentiation and cell sorting events during development because cellular differentiation produces two cell types and development takes only 24 hr to complete. Also, large (kilogram) quantities of cells can be obtained in a relatively short period of time. The haploid nature of the organism and the small amount of non-coding sequences simplify the genetic analysis of the mechanisms underlying differentiation and

morphogenesis. The genome can be saturated with mutations very easily and effectively to obtain developmental mutants and to identify the corresponding genes. In addition, *Dictyostelium* spores can be frozen and stored for many years and remain viable.

1.3. The Genome of *Dictyostelium discoideum*

Dictyostelium discoideum contains 6 chromosomes, the sizes of which range from 4 to 7 Mb (Loomis and Kuspa, 1997; Kuspa *et al*., 2001). Chromosome 2, the largest chromosome, is 8 Mb and represents ~25% of the genome. The genome has a relatively high average A+T content of 78%. Several interspersed repetitive elements make up about 10% of the *Dictyostelium* chromosomes (Glockner *et al*., 2001). Nuclei also contain approximately 100 identical copies of a 90 kb extrachromosomal element that carries the ribosomal RNA genes. The mitochondrial genome size in *Dictyostelium* is 54 kb and there are 200 copies per cell.

The 34 Mb genome of this organism has been almost entirely sequenced by an international consortium using a “whole chromosome shutgun” approach (Eichinger and Noegel, 2003). The availability and analysis of the genome sequence will greatly increase the attractiveness of this organism as a model to researchers. This will enhance the understanding the roles of the developmental genes during morphogenesis of multicellular organisms. In addition, a sequencing project in Japan contains thousands of cDNAs corresponding to genes expressed during growth and development.

It is estimated that *Dictyostelium* contains 8,000 to 10,000 genes (Loomis and Kuspa, 1997). It is relatively easy to recognize exons in this genome because translated regions have an average G+C content of 40% whereas introns and adjacent regions contain a G+C content of less than 15%. The genome size of this organism is

intermediate between the 6,000 genes of *Saccharomyces cerevisiae* and the 19,000 genes of *Caenorhabditis elegans*. It is estimated that there are several thousand of *Dictyostelium* genes that are homologous to genes in higher eukaryotes and that are not present in *S. cerevisiae*. Half of the *Dictyostelium* genome sequence is comprised of open reading frames (ORFs), which are found every 3 to 4 kb. Introns are rare and quite short with an average of about 100 bases.

1.4. cAMP and DIF

In *Dictyostelium*, two main extracellular signaling molecules have been characterized as morphogens, Differentiation Inducing Factor (DIF-1) and cAMP. These factors are present in different concentrations in the prestalk and prespore zones. The high level of DIF-1, a chlorinated hexaphenone, in the prespore region is due to its synthesis in this region (Loomis, 1993). The prestalk zone, on the other hand, has a low level of DIF-1. This concentration gradient is generated by a DIF-1 degrading activity present in the prestalk zone (Nayler *et al*., 1992). The lower concentration of DIF-1 in the prestalk zone stimulates prestalk-specific gene expression and represses prespore-specific gene expression, thereby maintaining the spatial pattern of the two major cell types.

cAMP is an important regulatory molecule in both prokaryotes and eukaryotes. In *Dictyostelium*, cAMP plays a role as both a morphogen and a regulator of cell differentiation. Prior to culmination, cAMP is essential for the stimulation of cell type-specific gene expression in both prestalk and prespore cells. However, during culmination, cAMP inhibits prestalk cell differentiation, whereas prespore differentiation is induced (Williams and Jermyn, 1991). During the later stages of development, the

expression of a cAMP-phosphodiesterase (cAMP-PDE) activity in the prestalk cells reduces cAMP concentration in this region, thereby allowing the continuation of stalk formation (Brown and Rutherford, 1980; Hall *et al*., 1993). Therefore, this cAMP gradient acts in the following way: high levels of cAMP in the prespore zone induce expression of prespore-specific genes, while lowered levels in the prestalk zone promote prestalk differentiation.

In *Dictyostelium discoideum* development, cAMP also acts as a chemoattractant and second messenger regulating chemotaxis, gene expression and cell differentiation (Parent and Devreotes, 1996; Ott *et al*., 2000). During aggregation, extracellular cAMP is detected by G protein-coupled cell surface receptors. There are four 7-span cell surface cAMP receptors (CAR1-4) produced by separate genes whose expression is regulated in a distinct temporal and cell-specific manner (Mu *et al*., 1998). These cAMP receptors activate different G proteins and intracellular second messengers such as inositol triphosphate, diacylglycerol or calcium. Cells precisely control the synthesis and degradation of cAMP. Adenylyl cyclase (ACA) synthesizes cAMP during aggregation. The hydrolysis of extracellular cAMP is achieved by a phosphodiesterase (PDE) that is either secreted or attached to the extracellular face of the plasma membrane (Gerisch, 1976). An intracellular PDE, RegA, has also been described (Shaulsky *et al*., 1996). In addition to its chemoattractant role, cAMP also acts as an intracellular second messenger activating protein kinase A (PKA), which functions in gene expression and cell differentiation in *Dictyostelium* development (Rutherford *et al*., 1984). PKA is activated when the regulatory subunit (PKA-R) interacts with cAMP and dissociates from its catalytic subunit (PKA-C). Extracellular cAMP is believed to be involved in post-

aggregative processes such as cell sorting, slug migration and terminal cell differentiation.

cAMP, synthesized by ACA, is degraded by cAMP-PDE to 5'AMP. The enzyme 5'-Nucleotidase (5NT) then degrades 5'AMP to adenosine and inorganic phosphate. Adenosine itself has been shown to have morphogenic properties (Schaap and Wang, 1986; Spek *et al*., 1988; Newell and Ross, 1982) and is believed to compete with cAMP for its cell surface receptor (Theibert and Devreotes, 1984; van Lookeren Campagne *et al*., 1986).

In addition to cAMP, DIF-1, and adenosine, cells also secrete peptide factors such as prestarvation factor (PSF), conditioned medium factor (CMF), and spore differentiation factor (SDF) that have important functions during several stages of development (Clarke *et al*., 1992; Clarke and Gomer, 1995; Anjard *et al*., 1998). Ammonia, a byproduct of protein and nucleic acid degradation, also acts as a developmental signal in *Dictyostelium*. It has been shown that this molecule facilitates prespore cell differentiation (Bradbury and Gross, 1989) and represses prestalk cell differentiation (Wang and Schaap, 1989). Furthermore, folic acid, another chemotactic signal in *Dictyostelium*, was shown to activate guanylyl cyclase through the stimulation of a G protein (van Haastert and Kuwayama, 1997).

The *Dictyostelium* morphogens, cAMP and DIF, have been shown to specifically induce expression of many genes including *7E, gp2* and *gp80* (Richards *et al*., 1990; Yin *et al*., 1994a; Desbarats *et al*., 1992). Deletion analysis of the *gp80* promoter led to the identification of a *cis*-acting regulatory element that was responsive to cAMP (CRE) and this element was specifically recognized by a nuclear protein (Desbarats *et al*., 1992).

Although several CREs have been identified in *Dictyostelium,* little sequence homology exists among these elements.

Morphogens have very important functions in the *Dictyostelium* development. Therefore it is imperative to understand how these morphogen gradients are established and maintained in order to better interpret the events of cell differentiation and cell sorting during development.

1.5. Gene Regulation

DNA microarray studies have suggested that approximately 2,000-3,000 of the *Dictyostelium* genes are developmentally regulated, indicating a vigorous cellular mechanism that controls development in this organism (Van Driessche *et al*., 2002). The analysis and characterization of the promoters of *Dictyostelium* genes aim to identify the *cis*-acting regulatory elements that are involved in the temporal and spatial expression of these genes. The goal of these studies is to understand the interactions between the developmental signals and signal transduction, between second messengers and transcription factors, and ultimately how transcription factors regulate specific gene expression.

During vegetative growth, the regulation of *Dictyostelium discoideum* genes appears to involve A/T rich elements (Bonfils *et al*., 1999; Hori and Firtel, 1994; Esch *et al*., 1992; Vauti *et al*., 1990; Maniak and Nellen, 1990; McPherson and Singleton, 1993). It has been shown that the A/T rich element of the actin 15 promoter can stimulate expression in vegetative cells when inserted in an otherwise developmentally-regulated promoter (Hori and Firtel, 1994). On the other hand, no similarity appears among these identified A/T rich sequences implying the complexity of gene regulation during

vegetative growth (Bonfils *et al*., 1999). In addition, an A/T rich element within the promoter of *celA* was shown to regulate spore germination-specific expression (Ramalingam *et al*., 1995).

Several transcription factors have been identified in *Dictyostelium discoideum*. GBF (G box regulatory element binding factor) stimulates the expression of post-aggregative and cell type-specific genes through the induction of cell surface cAMP receptors (Brown and Firtel, 2001). It has been shown that the central portion of GBF, which includes two predicted zinc finger motifs, is involved in DNA binding activity *in vitro*. However, only one zinc finger is required for *in vivo* function. The expression of GBF, which shows specificity for a number of C/G-rich regulatory elements, is induced by extracellular cAMP (Schnitzler *et al*., 1994). The transition from aggregation to postaggregative stages is mediated by increased concentrations of extracellular cAMP and other signaling molecules that inhibit the expression of genes essential for aggregation and induce genes that are needed for post-aggregative differentiation (Mann and Firtel, 1987; Schaap and van Driel, 1985). The disruption of *gbf* results in a developmental arrest at the loose-mound stage and *gbf*-null cells do not express postaggregative and cell-type specific genes (Schnitzler *et al*., 1994). This indicates that the GBF activity is essential in the transition from mound stage to the later stages in development by stimulating the expression of the genes involved in this transition.

Additional gene regulatory proteins include Dd-STATa, a member of the Signal Transducer and Activator of Transcription family. Dd-STATa has been shown to interact with an A/T-rich activator element in the prestalk gene *ecmB* and to regulate prestalk cell differentiation during culmination (Kawata *et al*., 1997; Araki *et al*., 1998). However, recent data shows that the *ecmB* expression is induced in Dd-STATa null cells calling

into question the role of Dd-STATa as an activator of *ecmB* expression (Mohanty *et al*., 1999). CMBF (the C-module-binding factor), another regulatory protein, is a nuclear DNA-binding protein involved in growth and development of *Dictyostelium* cells (Winckler *et al*., 2001). The transcription factor serum response factor A (srfA), an analog of mammalian serum response factor, is involved in slug migration, morphogenesis, and sporulation in *Dictyostelium* (Escalante *et al*., 2001). srfA shows a spatial and temporal expression pattern, which is achieved by 'alternative promoters'. Another regulatory protein, CAR1 transcription factor (CRTF), was shown to regulate the expression of the *cAMP receptor 1* (*CAR1*) gene at early developmental stages (Mu *et al*., 2001). CRTF is also involved in a number of other processes, including sporulation, throughout *Dictyostelium* development.

Myb proteins have been shown to regulate the expression of several genes in *Dictyostelium* including the *glucosamine-6-phosphate isomerase* (*gpi*) gene. *gpi* is expressed in a stage-specific and cell type-specific manner, mainly during vegetative growth and during mound and slug stages in prestalk cells (Matsuda *et al*., 1999; Tabata *et al*., 2001). GPI is involved in energy-generating processes (Tabata *et al*., 2001). GPI mutant cells can aggregate but not develop indicating the importance of this enzyme for development (Matsuda *et al*., 1999). It has been demonstrated that different protein factors recognize a Myb binding site on the *gpi* promoter and regulate the expression of the gene in a stage-specific and cell type-specific manner (Tabata *et al*., 2001). Three known Myb proteins (DdMyb1-3), nuclear factors that act as transcriptional regulators, have been reported in *Dictyostelium*. The function of DdMyb1, which recognizes the sequence 5'-TAACT/GG-3', is not known (Stober-Grasser *et al*., 1992). DdMyb2 is involved in inducing adenylyl cyclase (ACA) expression during early development

(Otsuka and van Haastert, 1998) while the prestalk-specific DdMyb3 functions in culmination (Guo *et al*., 1999).

The JAK/STAT signaling pathways play important roles in different organisms. In *Dictyostelium discoideum*, this pathway plays a role in cell proliferation, cell fate determination and cell migration (Hou *et al*., 2002). It has recently been shown that DIF inhibits the nuclear export of a STAT protein, Dd-STATc (Fukuzawa *et al*., 2003).

The control elements of several prespore-specific genes have been characterized. Most of these promoters have C/A-rich elements (CAEs), which play an important role in transcription of these genes (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al*., 1994; Haberstroh *et al*., 1991; Haberstroh and Firtel, 1990; Fosnaugh and Loomis, 1993; Stevens *et al*., 2001; Seager *et al*., 2001). Second control element, which is A/T rich, is also required and is located downstream of the CAEs (Powell-Coffman and Firtel, 1994; Powell-Coffman *et al*., 1994). Any of these two elements can drive prespore-specific gene expression when placed individually upstream of a basal promoter. However, gene expression is highly-induced, relative to expression by any of these two elements individually, when both together are fused to a basal promoter. The CAEs have been shown to interact with the transcription factor GBF (Schnitzler *et al*., 1994). Analysis of the transcriptional control of the developmentally-regulated *glycogen phosphorylase 2* (*gp2*) gene led to purification of a DNA-binding factor, TF2, which binds a C-rich sequence in the *gp2* promoter (Rutherford *et al*., 1997; Warner and Rutherford, 2000).

1.6. 5’-Nucleotidases

5’-Nucleotidase (5NT), a membrane-bound or soluble glycoprotein, is a ubiquitous enzyme found in a variety of species and in several different cellular locations

(Zimmermann, 1992). Although the physiological function of the enzyme differs in different organisms, these enzymes all convert nucleotides to nucleosides enabling transport across the cell membrane. The cell membrane provides a barrier to nucleotides but permits the passage of nucleosides in either direction. One group of nucleotidases (ecto-nucleotidases) are attached to the cell membrane via a glycosyl phosphoinositol anchor and through their action permit the uptake into cells of nucleosides derived by dephosphorylation of extracellular nucleotides (Zimmermann, 1992; Resta *et al*., 1998). Other nucleotidases are present in the cytosol. Through desphosphorylation of intracellular nucleotides these enzymes make possible the export of nucleosides into extracellular space (Gazziola *et al*., 1999).

5NTs in mammalian cells are found in cell membranes, in cytosol or in mitochondria and remove phosphate groups from ribo- and deoxyribonucleotides. The functions of mammalian 5NTs include preventing overproduction of deoxyribonucleotides, producing adenosine from AMP (Rampazzo *et al*., 2002). Human 5NT is involved in purine salvage process, which provides cells with nucleosides during nucleotide synthesis (Thompson, 1985). Because of the impermeability of cell membranes for 5'-mononucleotides, this molecule is hydrolyzed on the exterior of the cell membrane to inorganic phosphate and the respective nucleoside, e.g. adenosine, which is then taken up by the cells through adenosine channels.

5NTs generate adenosine and inorganic phosphate from extracellular AMP. Adenosine then reacts with a family of receptors that produce a wide range of physiological effects such as regulation of cardiovascular and cerebral blood flow, cytoprotection in myocardial ischemia, inhibition of certain aspects of immune function,

stimulation of angiogenesis or enhancement of cancer growth through increased expression of A1 adenosine receptors (Spychala *et al*., 1999).

Takayama *et al*. (2000) suggested that the enzyme may also play a role in sperm motility and male fertility. In some cell types the expression of this enzyme is variable and appears to be metabolically-regulated. In cells undergoing maturation, migration or growth, the expression of the enzyme is increased or decreased accordingly. The involvement of 5NT in cell-to-cell interaction in highly active cells and with certain membrane transport processes has also been demonstrated. Changes in the expression pattern of this enzyme at the apical surface of cells such as those of the uterine epithelium during the early stages of pregnancy may imply involvement of the enzyme in cellular recognition and adhesion activity (Bucci and Murphy, 1999).

1.7. 5'-Nucleotidase in *Dictyostelium*

More than 40 years ago researchers discovered a phosphatase activity during the course of cell differentiation that was localized in a narrow band of cells located between the prestalk and prespore cell types. The corresponding protein has been given a substantial attention throughout the years due to its possible involvement in the cAMP degradative pathway (Bonner *et al*., 1955; Krivanek, 1956; Krivanek and Krivanek, 1958; Gezelius and Wright, 1965; Armant and Rutherford, 1979; Das and Weeks, 1980; Das and Weeks, 1981; Susic *et al*., 1993). At the slug stage, cells containing this activity are dispersed throughout the multicellular aggregate (due perhaps to ALCs in prespore zone), but are enriched in the prestalk zone. At early culmination, activity becomes concentrated in the anterior prestalk zone and later in culmination becomes localized between prestalk and prespore zones.

Later work using ultramicrotechniques that were capable of quantitative analysis of the level of enzyme activity in each cellular region showed that the activity domain of the enzyme was on the extracellular surface of the plasma membrane (Armant *et al*., 1980; Armant and Rutherford, 1981; Armant and Rutherford, 1982). The phosphatase activity was specific for 5'AMP. No degradation occurred for any of the other nucleoside phosphates or sugar phosphates tested. This 5'AMP activity was shown to have an isoelectric point of 4.5-4.9, a pH optimum of pH 9.5 and a K_m for 5'AMP of 1.2 mM. The enzyme was also shown to be a zinc metalloprotein.

By utilizing microdissection of culminating individuals and enzymatic cycling of the products of 5'AMP degradation, the 5NT activity was found to be localized in the boundary between prestalk and prespore regions. Furthermore, electron microscopy studies revealed the presence of this enzymatic activity only in the prestalk cells (Armant *et al*., 1980). There is, in fact, no physical barrier between prestalk and prespore zones and the two cell types are in close contact, yet only prestalk cells showed 5NT activity. In addition, stalk cells show no activity, suggesting that the activity of the enzyme is down-regulated after prestalk cells enter the stalk tube (Armant and Rutherford, 1979).

This 5'AMP degrading activity is referred as 5'-Nucleotidase or 5NT. As mentioned earlier, the enzyme has received attention over the years due to the involvement of cAMP as a chemoattractant and morphogen in *Dictyostelium discoideum* (Schaap and Wang, 1986), in that this activity could remove the product of cyclic AMP degradation.

1.8. Previous Data on 5NT

5NT has been purified from culmination stage cells using Concanavalin A affinity, DEAE and 300 SW gel filtration chromatography (Chanchao *et al*., 1999). The relative molecular mass of the native protein was determined by gel filtration. The activity eluted at a position corresponding to approximately 90 kDa. On SDS-PAGE, three bands were present with apparent molecular masses of approximately 90, 120 and 140 kDa. A fourth band at 45 kDa was also detected occasionally. Subsequent enzymatic assays indicated that only the 90 kDa and 45 kDa peptides had enzymatic activity. The 90 kDa and 45 kDa bands were sequenced by Edman degradation. The N-terminal sequences for the 90 kDa and 45 kDa bands were QYSQTNEKIIVTGE and MLVVKTNVYNSPGCTGGVNK, respectively. In addition, three short sequences from the 90 kDa band were obtained from mass spectrometric analysis; GYSQTNEKIIVTGEC*K, GXDVNHDSNEFK and FKYEXPXNESXSVVDXK (C* designates carbamidomethyl modified C and X designates either I or L). Inspection of the 90 kDa N-terminal sequence and one of the sequences from mass spectrometric analysis showed 90% identity. Using these peptide sequences, no sequences with high similarity were found in the databanks available at the time. Degenerate PCR primers were designed from the DNA sequence of the 90 kDa peptide data because, as stated above, the size of the native protein determined by gel filtration was approximately 90 kDa. These primers were used to amplify several regions of *5nt* from *Dictyostelium* genomic DNA, which were cloned and sequenced (Chanchao *et al*., 1999). Using these nucleotide sequences, a *Dictyostelium* developmental cDNA project database search at the Institute of Biological Sciences, University of Tsukuba, Japan revealed three cDNA clones of the same gene, which when translated matched the peptide sequences obtained

for 5NT from mass spectrometric analysis and Edman degradation. This library also contained cDNA clones with high identity to "classical" alkaline phosphatase from other organisms. When all of these clones were sequenced in their entirety and compared to each other, no significant similarity was found between the *5nt* cDNA and that of the "classical" alkaline phosphatases. The translated amino acid sequence of the longest ORF of the *5nt* cDNA clone was used to search a protein database, revealing no close similarity between 5NT and any other protein. The closest similarity was between 5NT and the *Dictyostelium* Contact Site A (24% identity over 151 amino acids). Contact Site A, a membrane-bound glycoprotein, 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 *et al*., 1988; Bhanot and Weeks, 1989). The derived 5NT protein has a molecular mass of 63,423 Da, with 39 strongly basic amino acids, 49 strongly acidic amino acids, 165 hydrophobic amino acids, 262 polar amino acids, and an isoelectric point of 4.869. The estimated size of the 5NT from SDS-PAGE analysis is approximately 90,000 Da, a result considerably different from that estimated by translation of the cDNA clone. This difference may be due to the heavy glycosylation of the protein as observed during the analysis by mass spectrometry. The cDNA databank search also revealed another clone that matched the N-terminal sequence of the 45 kDa polypeptide. The protein product of this cDNA had a calculated molecular mass of 26,152 Da. The fact that sequences of the two cDNAs were not similar indicated that the 45 kDa peptide is not a degradation product of the 90 kDa peptide. Also, no other protein with high similarity to the 45 kDa sequence was found in GenBank, which suggested that 45 kDa peptide is a unique *Dictyostelium* protein with phosphatase activity.

Expression of *5nt* during *Dictyostelium* development was determined by northern blot analysis (Chanchao *et al*., 1999). This analysis showed that there was no expression in the vegetative cells. The message first appeared at about 5 hr of development and remained throughout the rest of development, indicating that *5nt* expression is developmentally regulated.

The *5nt* cDNA was overexpressed in *Escherichia coli* to produce a recombinant protein, which was then purified from bacterial cells and used to raise a polyclonal antibody (Ubeidat and Rutherford, 2002). Western blots were performed to determine the presence of endogenous 5NT during various stages of development. The antibody detected a protein of about 100 kDa in membrane fractions starting from the aggregation stage. The protein was also detected in slug and culmination stage extracts, however was absent in amoebae (vegetative cells) extracts. This result was consistent with the northern analysis that showed expression during development.

To test the functional significance of 5NT on development, the gene was disrupted by homologous recombination (Rutherford *et al*., 2003). Northern analysis of the *5nt* null clones indicated the absence of the *5nt* transcript in these cells. Moreover, western blot analysis showed that the gene disruption resulted in the loss of 5NT. In order to determine any phenotypic alterations in growth or development, the *5nt* null clones were subjected to various assays. It was found that cells from each of the five *5nt* null mutant clones were able to grow normally and aggregate at the same time as the wild type cells. However, before continuing to develop to the slug stage, they remained at the aggregation stage 3 to 5 hr longer than wild type cells. Furthermore, instead of forming the normal single tip for each aggregate, these null cells formed multiple tips. The tip is known to act as an ‘embryonic organizer’. Early grafting experiments by Raper (1940)

showed that if multiple tips were transplanted onto a single slug, a number of smaller slugs were formed as a result of each fresh tip recruiting cells from the host slug. All of these slugs then migrated and eventually formed normal mature organisms. It is now believed that the ability of the tip to regulate cAMP oscillations (adenylate cyclase and cAMP-PDE are synthesized in the tip) is the basis of this organizer property (Bretschneider *et al*., 1995; Schaap and Wang, 1986). The cAMP synthetic enzyme Adenylate cyclase A is synthesized in the tip (Verkerke-van Wijk *et al*., 2001). Furthermore, cAMP-PDE, the cAMP degradative enzyme, is localized in the anterior of the slug (Hall *et al*., 1993). The fact that ACA and cAMP-PDE are found in the tip and prestalk cells and that 5'AMP is the product of cAMP degradation, it was hypothesized that 5NT is involved in the establishment and maintenance of a cAMP gradient from the tip to the lower region of the prestalk cell zone. In short, knockout experiments suggested that 5NT is involved in the late aggregation stage, where it functions in the steps leading to tip formation, however the exact nature of this involvement is not known.

1.9. Proposed Function of 5NT in *Dictyostelium*

In *Dictyostelium*, 5NT is believed to be involved in the regulation of cAMP levels. cAMP is made from ATP by adenylate cyclase and converted to 5'AMP by cAMP-PDE (Hall *et al*., 1993). Further cAMP degradation can be blocked by feedback inhibition if 5'AMP accumulates in the environment. Therefore, it is believed that the function of 5NT is to clear 5'AMP from the environment so that cAMP degradation can continue. Furthermore, localization of 5NT at the interface of prestalk and prespore cells may have a key role in insulating the prestalk cells from the high concentrations of cAMP that exist in the prespore cells. During culmination, very low levels of cAMP are needed

for the proper progression of prestalk differentiation (Berks and Kay, 1988). For example, expression of *ecmB* (a late prestalk differentiation marker) is inhibited by high levels of cAMP (Harwood *et al*., 1993). This gene is expressed following the drop in cAMP levels in the anterior prestalk zone. The reduction in cAMP levels is likely achieved by cAMP-PDE, which is also induced late during prestalk differentiation (Hall *et al*., 1993). Thus, it is hypothesized that 5NT activity is also required in these cells to control cAMP levels in the prestalk zone. The activity is oriented towards the extracellular face of the plasma membrane so that 5'AMP resulting from cAMP degradation could be removed.

The accumulation of 5NT in specific cells may be the result of both temporal and cell-specific expression of the corresponding gene. Synthesis or modification of a transcription factor(s) in those cells can accomplish this specific expression.

1.10. Specific Research Objectives of the Current Study

The following specific objectives address transcriptional regulation of *5nt* in this study:

A. To obtain a genomic clone of *5nt* including upstream flanking sequences
B. To analyze the regulation of *5nt* expression by known morphogens
C. To define *cis*-acting regulatory element(s) of *5nt*
D. To identify *trans*-acting factors that bind specifically to the regulatory element(s)
E. To purify putative protein factor(s) that are involved in the developmental regulation of *5nt* expression
F. To localize the protein during development using the reporter β-galactosidase

G. To determine the transcriptional start site of *5nt* expression

1.11. Significance of the Current Investigation

As stated earlier, it is clear that extracellular signaling molecules direct morphogenesis in *Dictyostelium*. To better explain morphogenesis, the understanding of how these morphogen gradients are established and maintained is required. The working hypothesis is that cell-specific levels of cAMP are built and maintained as a result of a harmonious interaction of adenylate cyclase (synthesis of cAMP), cAMP-PDE (degradation of cAMP to 5'AMP) and 5NT (degradation of 5'AMP to adenosine). 5NT is expressed in cells that are specifically localized between the prespore and prestalk zones. This suggests that 5NT activity plays a key role in controlling morphogen levels and in positional differentiation of the two cell types. An understanding of how the expression of the corresponding gene is regulated is essential before an accurate model of the mechanisms that regulate morphogenesis can be constructed. Identification and characterization of the factors causing cell-specific expression of *5nt* has the potential to advance the understanding of morphogenesis in *Dictyostelium* as well as pattern formation and cellular differentiation in general.

CHAPTER 2. MATERIALS AND METHODS

2.1. Cell Growth and Development

Dictyostelium discoideum (strain AX3K) cells were grown in HL5 (5 g yeast extract, 10 g bacteriological peptone, 10 g glucose, 0.25 g Na_2HPO_4, 0.4 g KH_2PO_4, pH 6.5, per liter) supplemented with 50 μg/ml streptomycin sulfate and 100 μg/ml ampicillin as described previously (Rogers *et al*., 1992; Rutherford and Cloutier, 1986). The AX3K cells were kept at a density of less than 5.0 x 10^6 cells per ml. For initiation of multicellular development, the amoebae were washed free of nutrient media and plated on Gelman GN-6 cellulose filters supported by Gelman absorbent pads. Both the pads and membranes were presoaked in 1x MES-LPS buffer (7.7 mM N-morpholinoethanesulfonic acid (pH 6.5) containing 20 mM KCl and 5 mM $MgSO_4$). The plated cells were incubated at 21°C to allow development to the desired stage.

2.2. Genomic DNA Isolation

Five hundred milliliters of exponentially growing vegetative AX3K cells were pelleted by centrifugation at 1,400 x g for 5 min. The pellet was washed with 60 ml of TEN (10 mM Tris, pH 8.0, 10 mM EDTA, 10 mM NaCl) and resuspended in 8 ml of TEN. Two milliliters of 10% SDS was added and the mixture was incubated for 10 min at room temperature. Proteinase K (100 μg/ml final concentration) was then added and the mixture was incubated at 37°C for 1 hr. The sample was then centrifuged and the supernatant was transferred to a fresh tube. DNA was extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was then precipitated from the final aqueous phase with 1/10 volume of 3 M sodium acetate (pH 5.2) and equal

volume of isopropanol and washed with 70% ethanol. The pellet was resuspended in 1 ml TE buffer (pH 8.0). The sample was then incubated with RNase A (100 μg/ml final concentration) at 37°C for 1 hr. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. Genomic DNA was resuspended in 500 μl DI water using Milli Q apparatus (milliQ H_2O) and stored at –20°C.

2.3. Southern Blot Analysis

The *5nt* cDNA clone (Fig. 2) was a generous gift from Dr. Takahiro Morio of the University of Tsukuba, Japan and the *Dictyostelium* cDNA project group. The probe used in Southern blots was prepared as follows: 5 μg of pBluescript II KS+ vector carrying the *5nt* cDNA (1,921 bp) was digested with *Sal*I (10 U) and *Not*I (10 U) restriction enzymes at 37°C for 2 hr (the *5nt* cDNA was cloned between *Sal*I and *Not*I sites in pBluescript). The DNA was then fractionated on a 1% agarose/TAE gel. The insert bands were excised from the gel and the DNA was purified using a GeneClean III Kit (Bio 101) according to the manufacturer's instructions. The *5nt* cDNA was then labeled with [α-^{32}P] dATP (New England Nuclear) using the Random Priming method as described (Ausubel *et al*., 1995), using 100 ng of DNA and 10 U of Klenow enzyme. Labeled DNA was separated from the unincorporated [α-^{32}P] dATPs by gel filtration chromatography using a Micro Bio-Spin 6 column (Bio-Rad Laboratories). In some Southern blot experiments, a partial cDNA sequence containing the 5' 849 bp sequence was used as the probe. For this purpose, the cDNA was digested with *Eco*RV and the two fragments (1,051 bp and 849 bp) were separated on an agarose gel. The small fragment was excised from the gel, purified using a Quantum Prep™ Freeze 'N Squeeze Spin column (Bio-Rad Laboratories) and labeled as described above.

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CAATATTAATTTTTTTTTCACATAATTTTATTATTTGTATAATTTAATTATTCATAAATT  60
ATTCTATATTATAATTTTTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAATGAAATTA  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
TCATCATTATTTTTATTATTATTAATCTTTATTACTTTATCTTATTAAATCAAAACAAAT  1860
TACTTTTTCGTTTATAAAAATAAAATTAAATAAATAAATAAATATATATATACATTTTTA  1920
A                                                              1921
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Figure 2. Nucleotide sequence of the *5nt* cDNA. An open reading frame exists starting at position 112 (ATG, shown in shadow) through position 1845. A stop codon (TAA, shown in shadow) is at position 1846. Nucleotide numbering is on the right.

In order to prepare the DNA for Southern blotting, 1 μg of *Dictyostelium* genomic DNA was digested with 10 U of various restriction enzymes. Electrophoretic separation of digestion products was performed on a 1% agarose/TBE gel. Two micrograms of *Sty*I-digested λ DNA was used as standard size markers. The gel was prepared for DNA transfer to nylon membrane by gentle agitation in depurination solution (250 mM HCl) for 10 min at room temperature followed by denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 25 min at room temperature and finally by neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5) for 30 min at room temperature. DNA was transferred onto a nylon membrane (Hybond-N^+, Amersham) by vacuum blot at 68 mm Hg for 1 hr (PosiBlot 30-30 Pressure Blotter and Pressure Control Station, Stratagene). The transfer buffer contained 10X SSC (0.15 M $C_6H_5Na_3O_7$, 1.5 M NaCl) + 10X SSPE (3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 20 mM Na_2EDTA). Following transfer, the DNA was immobilized on the membrane using a Stratalinker UV crosslinker (Stratagene). The blot was incubated with prehybridization solution for 1 hr at 60°C. Hybridization was performed overnight at 60°C using 5x SSC, 5x Denhart solution, 1% SDS, 100 μg/ml herring sperm DNA and the radiolabeled cDNA probe. The blot was first washed with 6x SSC, then with 2x SSC + 0.1% SDS, and finally with 1x SSC + 0.1% SDS. All washes were performed twice at 60°C for 15 min. The bound probe was detected by either autoradiography or phospho imaging.

2.4. Sub-genomic Library Construction

2.4.1. Preparative DNA Digestion

Forty two micrograms of genomic DNA was digested with 200 U of *Taq*I at 65°C for 2 hr. The digested DNA was extracted with phenol:chloroform:isoamyl alcohol

(25:24:1), precipitated with ethanol and resuspended in 80 µl milliQ H_2O. The DNA was run on a 1% low-melting agarose (SeaPlaque GTG agarose, FMC BioProducts)/TAE gel at 50 V. Six 0.5 cm slices (fraction #1-6) were excised from the gel and the DNA was purified using GeneClean III Kit. PCR was performed to determine the fraction containing the fragment of interest (≈ 2.5 kb *Taq*I fragment). The two primers used were 5'-ATG GTG GAT TCT TGG TGT A-3' (primer *cc1*) and 5'-TGA GTG GTT TTA TTA TTA TTA TCT-3' (primer *nu1r*). The PCR conditions involved an initial denaturation step at 94°C for 2.5 min, followed by 34 cycles including denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec, extension at 72°C for 3 min, and a final extension step at 72°C for 10 min. Each PCR reaction contained 1.5 mM final $MgCl_2$ concentration. *Taq* DNA polymerase amplified the *5nt* coding region from 326 bp to 734 bp resulting in a 408 bp PCR product.

2.4.2. Ligation and Transformation of Bacteria

Three hundred nanograms of sub-genomic library fraction #4 (containing the fragment of interest) was ligated with 120 ng of pBluescript which was previously digested with *Cla*I and treated with calf intestinal alkaline phosphatase (CIAP, New England Biolabs). The ligation reaction was performed at 16°C for 16 hr. The ligation reaction was then precipitated with ethanol and resuspended in 10 µl milliQ H_2O. The ligation sample was added to 40 µl electro-competent cells (XL1-Blue, Stratagene) and the DNA/cell mixture was immediately transferred to an ice-cold 0.1 cm cuvette (Gene Pulser® Cuvettes, Bio-Rad Laboratories). The electroporation was performed at 25 µF (capacitor), 1.8 kV (voltage), and 200 Ω (controller) using a gene pulser apparatus (Gene Pulser™, Bio-Rad Laboratories). The electroporated cells were incubated in 1 ml of SOC

medium (0.5% yeast extract, 2% tryptone peptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) at 37°C for 1 hr, shaking at 225 rpm. The cell suspension (100 µl) was plated onto LB plates (0.5% yeast extract, 1% tryptone peptone, 170 mM NaCl and 1.5% bacto agar) containing 100 µg/ml ampicillin, 500 µg isopropyl-1-thio-β-*D*-galactoside (IPTG) and 1 mg 5-bromo-4-chloro-3-indolyl-β-*D*-galactoside (X-gal). The plates were incubated overnight at 37°C.

2.4.3. Screening the Sub-genomic Library

Colony-PCR method was used to identify the clone(s) containing the fragment of interest (Gussow and Clackson, 1989). The templates were prepared by placing a small amount of each colony in a microcentrifuge tube containing 50 µl colony lysis buffer (10 mM Tris, 1 mM EDTA, pH 8.0, 0.1% Tween-20). Five colonies were mixed in a given tube for preliminary screening. The tubes were incubated at 95°C for 10 min, then centrifuged at 14,000 x g for 2 min in a microcentrifuge. Two microliters of the supernatant was used for PCR analysis using gene-specific primers *cc1* and *nu1r*. The PCR conditions were the same as above (see 'preparative digestion'). As a positive control, the colony lysate prepared from the bacterial cells harboring the pBluescript II KS+ with the *5nt* cDNA was used as template.

2.5. Plasmid DNA Purification

For large scale plasmid isolation, a single colony was used to inoculate 3 ml LB (0.5% yeast extract, 1% tryptone peptone, 170 mM NaCl) containing 100 µg/ml ampicillin and incubated for 8 hr at 37°C, 200 rpm. One milliliter of the bacterial culture was transferred to a 500 ml fresh LB-amp and grown for 16 hr at 37°C, 250 rpm. The

plasmid DNA was purified using a plasmid maxiprep kit (Qiagen) according to manufacturer's instructions. The yield was determined by agarose gel electrophoresis using *Sty*I-digested λ DNA as standard size markers. Small scale plasmid DNA preparations were performed using Wizard Plus SV Minipreps DNA purification System (Promega).

2.6. DNA Sequencing

DNA sequencing was performed at the DNA Core Laboratory of the Virginia Bioinformatics Institute at Virginia Tech. The plasmid DNA isolated from the clone 'c167' (containing the fragment of interest) was used as the template with the *T3* and *T7* primers in separate sequencing reactions. For genomic sequencing, genomic DNA from vegetative Ax3K cells was amplified using *5nup1-5nup2* and *5nup3-gs2r* primer pairs. Table 1 shows various primers used in this study. Template for PCR reactions were prepared as described (Rivero *et al*., 1996). Cells were counted and about 1 million cells were harvested, washed twice in cold (4°C) milliQ H_2O and suspended in 99 µl PCR buffer (50 mM KCl, 10 mM Tris, pH 9.0, 0.1% Triton X-100). NP-40 (0.5% final concentration) and proteinase K (100 µg/ml final concentration) were added and the sample was incubated at 56°C for 45 min, then at 95°C for 10 min. The sample was centrifuged for 2 min at 14,000 x g in a microcentrifuge and 15 µl of the supernatant was used in 50 µl final PCR volume. The PCR conditions involved an initial denaturation step at 94°C for 2.5 min, followed by 34 cycles including denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 3 min, and a final extension step at 72°C for 10 min. Each PCR contained 2.5-3.0 mM final $MgCl_2$ concentration.

Table 1. Various primers used in this study. Some primers contain the *Hin*dIII restriction enzyme site, as indicated, for cloning purposes. All sequences are written in the 5' to 3' direction.

Primer	Sequence	Annealing region
cc1	ATGGTGGATTCTTGGTGTA	coding
nu1r	TGAGTGGTTTTATTATTATTATCT	coding
*5nup1 +Hin*dIII	TTTTAAGCTTTCCAAAAATAAATGCATTAAGTCT	promoter
*5nup2 +Hin*dIII	ACTTAAGCTTGCTGCAAAAATCAGACCTAAACTC	promoter
*5nup3 +Hin*dIII	GCATAAGCTTTTGGCTAATGGCTTTCCTCTTC	promoter
*gs1r +Hin*dIII	TCATAAGCTTGACTATAACCACATTTTGA	coding
*gs2r +Hin*dIII	TATTAAGCTTTGGTTTTATCAAATTCTCCAGTAA	coding
*gs3r +Hin*dIII	CTCAAAGCTTAATTATGTGAAAAAAAAAATTAATATTG	coding
*T7 +Hin*dIII	CCAAAGCTTGCGCGTAATACGACTCACTAT	pBluescript
gs1f	TCAAAATGTGGTTATAGTCAA	coding
gs3f	CAATATTAATTTTTTTTTCACATAATT	coding
pvtl2-1f	GGGCGAATTGGGTACCGG	pVTL2
luc1r	AATCCGTTTTAGAATCCAT	pVTL2
luc2r	GAACGTGTACATCGACTGAAATC	pVTL2

The PCR products were separated on a 1% agarose/TAE gel and the bands were removed and purified using a GeneClean III Kit. The purified DNA was then used as template for cycle sequencing. All sequences obtained were aligned using the DNAStar program.

2.7. Generation of 5' Promoter Deletions

Six micrograms of the 'c167' maxiprep plasmid DNA was digested with 20 U of *Sac*I in a 50 µl total volume for 2 hr, precipitated with ethanol and then digested with 40 U of *Eco*RI for 2 hr. The DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), then precipitated from the final aqueous phase using ethanol. The DNA pellet was dried and resuspended in 48 µl of Exonuclease III buffer. Two hundred and fifty units of Exonuclease III (New England Biolabs) was

added to the tube and incubated at 30°C for 5 min. A 10 µl aliquot (~1.2 µg DNA) was removed from the tube at 1 min intervals, placed in a chilled-tube containing 2 µl of 10x mung bean nuclease buffer and 6 µl milliQ H_2O, and frozen on dry ice. After all aliquots had been taken, the tubes were heated at 68°C for 15 min to inactivate the exonuclease. The tubes were placed on ice for 5 min, centrifuged briefly, then 3 U of mung bean nuclease (New England Biolabs) was added. The final reactions were incubated at 30°C for 30 min, the DNA was precipitated with ethanol, resuspended in milliQ H_2O and then run on an 1% agarose/TAE gel. The bands were excised and the DNA was purified from the gel using a GeneClean III Kit. The purified DNAs were self-ligated at 16°C for 16 hr and then used to transform XL1-Blue electro-competent cells (Stratagene). Deletion sizes were determined by PCR using the primers *T7* and *gs2r*.

Plasmid DNA was purified from the clones containing appropriate-sized deletions using the Wizard miniprep DNA purification system (Promega) and used as template for PCR primers *T7* and *gs1r*. Some promoter deletions were also amplified using *T7* and *gs3r* primers. All these primers contained engineered *Hin*dIII restriction enzyme sites. The PCR conditions involved an initial denaturation step at 94°C for 2.5 min, followed by 28 cycles including denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 2 min, and a final extension step at 72°C for 10 min. Each PCR reaction contained 3 mM final $MgCl_2$ concentration. The PCR-amplified DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). DNA from the aqueous phase was then precipitated with ethanol and resuspended in 30 µl milliQ H_2O. PCR products (approximately 6-8 µg) were then digested with 30 U of *Hin*dIII overnight at 37°C. The digests were separated on a 1% agarose/TAE gel and the bands were excised and purified using a GeneClean III Kit. The PCR products

containing longer than 547 bp promoter regions had to be partially digested due to an internal *Hin*dIII enzyme site located at –547 bp in the promoter. Partial digestion was carried out using 1 U of enzyme per 225 ng DNA for 90 min. The digests were separated on a 1% agarose/TAE gel and the bands were excised and purified.

2.8. Promoter-*luciferase* Fusions

The purified PCR products were ligated into the *Hin*dIII-cut and CIAP-treated pVTL2 reporter vector (Yin *et al*., 1994b) in a reaction volume of 20 µl. Each ligation reaction contained 150 ng total DNA with a 20:1 insert to vector molar ratio. The ligation was performed at 16°C for 16 hr. The DNA was precipitated with ethanol and used to transform XL1-Blue competent cells by electroporation. Cells were plated onto LB plates containing 100 µg/ml ampicillin and grown overnight at 37°C. Clones were initially screened for the presence of insert by PCR using *T7–gs1r* or *T7–gs3r* primer pairs. Once positive clones were determined, PCR was repeated in order to identify the clones with correctly oriented inserts. For this purpose, the following primers were used: *gs1f–luc1r*, *gs1f–luc2r*, *gs3f–luc1r*, *gs3f–luc2r* (*luc1r* and *luc2r* primers are pVTL2-vector-specific). The PCR conditions for these primers were the same as above except that the annealing temperature was 48°C instead of 54°C. The vector-specific *pvtl21f–luc1r* (or *pvtl21f–luc2r*) primers were also used to identify clones with one insert molecule. The plasmid DNA was isolated from positive clones using a Qiagen Maxiprep Kit. Glycerol stocks of bacteria were prepared by mixing 875 µl of exponentially growing cells with 125 µl of 80% glycerol, vortexing and freezing at –80°C.

2.9. Generation of Internal Promoter Deletions

Numerous internal promoter deletions were generated using the PCR-ligation-PCR method described previously (Ali and Steinkasserer, 1995). The strategy used to generate internal deletions is shown in Figure 3.

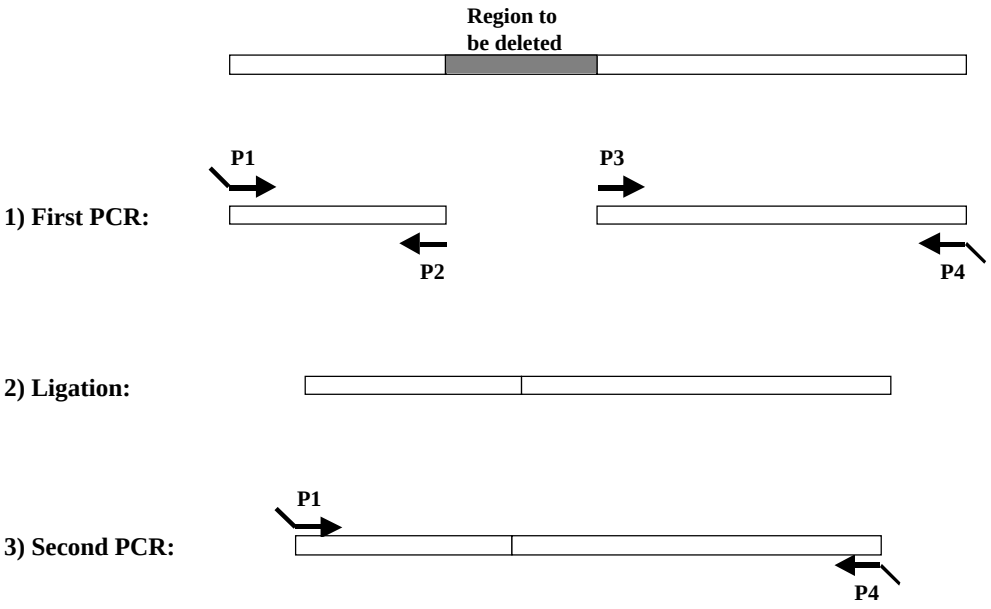


Figure 3. Strategy for generating internal promoter deletions. PCR-ligation-PCR method was used to generate internal deletions. In the first step, two separate PCR reactions amplified the two sides of the region to be deleted. In the second step, the two PCR products were ligated to produce the template for the second PCR. Finally, the second PCR amplified the entire sequence using the two flanking primers.

In the first step, two fragments that flank the region to be deleted were individually created by PCR. The upstream fragment was generated using the vector-specific *T7* primer and a reverse promoter-specific primer. The downstream fragment was generated using a forward promoter-specific primer and the gene-specific primer

gs2r. In the second step, these two PCR fragments were phosphorylated (T4 polynucleotide kinase, New England Biolabs) and ligated (T4 DNA ligase, New England Biolabs) at room temperature for 20 min to produce the template for the second PCR. Finally, 1 µl of this ligation sample was used as template in a second PCR reaction to amplify the entire sequence using two flanking primers (*5nup3* and *gs1r*) that contained engineered *Hin*dIII restriction enzyme sites at the 5' ends. The amplified DNA products were extracted with phenol, precipitated with ethanol and digested with *Hin*dIII. The digests were fractionated on a 1% agarose/TAE gel, the bands were excised and purified. These DNA fragments containing internal deletions were then fused in-frame to the *luciferase* (*luc*) in pVTL2 vector, which was used to transform XL1-Blue cells. Screening for the positive clones and for the correct insert orientation was performed as described above.

2.10. Transformation of *Dictyostelium*

Transformation of *Dictyostelium* was performed as described previously (Nellen *et al*., 1984) with minor modifications. AX3K amoebae cells (2-5 x 10^6 cells/ml) were chilled on ice for 15 min and then harvested at 2100 x g for 3 min in a CRU-5000 centrifuge (International Equipment Company). The cells were washed with ice-cold electroporation buffer (10 mM $NaPO_4$, pH 6.1, 50 mM sucrose). The cells (total 1.5 x 10^7 cells per transformation sample) were resuspended in 800 µl of ice-cold electroporation buffer. pVTL2 plasmid DNA (total 20 µg per sample) containing *5nt* promoter – luciferase reporter fusions was added to the cells and the DNA/cell mixture was transferred to an ice-cold 0.4 cm cuvette (Gene Pulser® Cuvettes, Bio-Rad Laboratories). The electroporation was performed at 3 µF (capacitor), 1.2 kV (voltage) using a gene

pulser apparatus (Gene Pulser™, Bio-Rad Laboratories). The electroporated cells were incubated on ice for 10 min and then transferred into a petri dish containing 20 ml HL5 media containing 100 µg/ml ampicillin and 50 µg/ml streptomycin sulfate. After incubation at 21°C for 18 hr, G418 (Alexis Biochemicals) was added to a final concentration of 10 µg/ml. The plates were incubated at 21°C for 3 days. After this incubation period, the medium was replaced with fresh HL5 and antibiotics and 30 µg/ml G418. The plates were incubated at 21°C for 3 more days. The cells were washed off the plate, pelleted, and then resuspended in 500 µl PBS (10 mM KCl, 10 mM NaCl, 16 mM Na_2HPO_4, 34 mM KH_2PO_4) and mixed with G418-resistant bacteria (*Escherichia coli* strain B/r $G418^R$). One hundred and twenty five microliters of this mixture was spread on DM agar plates (0.2% glucose, 1% oxoid peptone, 1.5% bacto agar, 3 mM Na_2HPO_4, 10 mM KH_2PO_4) containing 20 µg/ml G418. The plates were incubated until the amoebae plaques were approximately 1 cm in diameter (3-5 days or longer). Cells from individual plaques were transferred to HL5 containing 10-20 µg/ml G418. Cells were kept in liquid medium at a density of less than 5×10^6 cells per ml.

2.11. Luciferase Enzyme Activity Assays

2.11.1. Developmental Stage Study

Amoebae were harvested from liquid medium by centrifugation at 2,100 x g for 3 min in a CRU-5000 centrifuge (International Equipment Company). The pellet was washed twice with 1xMES-LPS, pH 6.5. The weighed pellet was resuspended in five cell pellet volumes of 1xMES-LPS. For initiation of multicellular development, 400 µl of cells/MES-LPS were plated on Gelman GN-6 membrane filters supported by Gelman pads. Both the pads and membranes were presoaked in 1xMES-LPS buffer. Pads were

incubated at 21°C until the desired stage. At different developmental stages, cells were harvested from the membranes with 1 ml of 30 mM glycyl-glycine (pH 8.3), then pelleted for 2 min at 2,500 x g in a microcentrifuge and stored at –20°C until assayed. Protein concentration was determined using the BCA protein assay kit (Pierce) according to manufacturer's protocol with BSA as a standard and a microplate reader (Bio-Rad Laboratories).

2.11.2. cAMP Induction Experiments

Amoebae were harvested from liquid media by centrifugation at 2,100 x g for 3 min in a CRU-5000 centrifuge in a (International Equipment Company). The pellet was washed once with 1xMES-LPS, pH 6.5. Cells were resuspended in a 125 ml flask in 1xMES-LPS to give 1 x 10^7 cells/ml final cell concentration. The cells were shaken at room temperature at 130 rpm for 10-15 hr to become aggregation competent and cAMP (Sigma) was then added to one flask to a final concentration of 1 mM. The cells were shaken with or without cAMP at the same speed and 1 ml samples of the cell suspension were removed at 1 hr time intervals for about 9 hr from both – and + cAMP cultures. The samples were centrifuged for 1 min at 2,500 x g in a microcentrifuge and the pellets were stored at –20°C until assayed.

2.11.3. Luciferase Assay

To assay luciferase activity, cells were thawed on ice and resuspended in 200 µl lysis buffer (25 mM glycyl-glycine, pH 8.3, 15 mM $MgCl_2$, 4 mM EGTA, 1 mM DTT, 20% glycerol, 1% Triton X-100, and the protease inhibitors 100 µg/ml PMSF, 0.5 µg/ml Leupeptin, 100 µg/ml TPCK, 50 µg/ml TLCK, 1 mg/ml Benzamidine). The luciferase

assay was performed by mixing 50 µl of 100-fold diluted extract (10 µl cell extract + 990 µl lysis buffer) with 50 µl of a reaction mixture containing 25 mM glycyl-glycine (pH 8.3), 15 mM K_2P04 (pH 8.3), 15 mM $MgCl_2$, 4 mM EGTA, 1 mM DTT and 2.8 mM ATP. After automatic injection of 100 µl of luciferin [0.6 mM luciferin (Promega) in 25 mM glycyl-glycine, pH 8.3 and 1 mM DTT], the light output was recorded as luciferase units in a luminometer (Lumat LB9501, Berthold). Specific activity was calculated as luciferase units per milligram of protein.

2.12. Gel Shift Assays

2.12.1. Labeling of DNA Fragments

Two complementary single-stranded oligonucleotides were first annealed in 20 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0, 50 mM NaCl. The annealing reaction was incubated at 70°C for 5 min and then cooled slowly to room temperature. The annealed DNA fragments were stored at –20°C. DNA fragments (100 ng) were then labeled with 30 µCi of [γ-^{32}P] ATP (New England Nuclear) in a 12 µl final reaction volume using 10 U of T4 polynucleotide kinase (New England Biolabs). The reaction was carried out at 37°C for 30 min. The reaction was heated at 65°C for 20 min to inactivate the enzyme and the reaction was cooled at room temperature. The unincorporated nucleotides were removed by passing the sample through a Micro Bio-Spin 6 column (Bio-Rad Laboratories). The radioactivity of the labeled fragment was measured in a scintillation counter (Beckman LS 6000SC). The amount of radioactivity used in each gel shift binding reaction was 30,000 cpm. PCR-amplified promoter fragments were also used as gel shift probes in some experiments. In this case, PCR products were extracted with phenol, precipitated with ethanol and fractionated on a 2% agarose gel. The desired

DNA band was excised from the gel and the DNA was purified using a MERmaid kit (Bio 101). Labeling of the DNA fragments was carried out as described above.

2.12.2. The Binding Assay

The gel shift binding assay was performed as described previously (Ausubel *et al*., 1995) with some modifications. The gel shift binding reaction contained 20 mM HEPES, pH 7.9, 12% glycerol, 2 µg BSA, 20 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM DTT, 2 mM $MgCl_2$, 0.04% NP-40, 0.5 µg non-specific carrier (polydAdT-polydAdT, polydA-polydT or polydIdC-polydIdC), 1.5 µl extract and 30,000 cpm probe DNA. The final binding reaction volume was 15 µl. The extract was always incubated on ice for approximately 10 min with the non-specific carrier DNA prior to the addition of the probe. In competition assays, the unlabeled excess DNA (100X excess in molar ratio) was added immediately after addition of the labeled probe. DNA-protein binding was allowed to take place at room temperature for 30 min. The reaction was then loaded on a 5% native polyacrylamide gel (34.5:1) in 0.5x TBE, which was pre-run at 150 V for 30 min. The electrophoresis was carried out in 0.5X TBE at 200 V for 70 to 100 min. After electrophoresis, the gel was dried at 80°C for 45 min, then placed on film (X-OMAT™ AR, Kodak) for autoradiography.

2.13. Preparation of Cytoplasmic and Nuclear Extracts

Protein extracts were prepared as described previously (Kawata *et al*., 1996) with minor modifications. Exponentially growing AX3K cells (8.5 L) were harvested from liquid media by centrifugation in an IEC clinical centrifuge (speed 5) [International Equipment Company]. The cells were washed once with 1xMES-LPS, pH 6.5. The

pellet was resuspended in two-cell pellet volumes of 1xMES-LPS. For initiation of multicellular development, 3.5 ml of cells/MES-LPS was plated on 2% agar/H_2O plates. Plates were incubated at room temperature until slugs were formed (approximately 19 hr). Slugs were removed from plates with 20 mM potassium phosphate buffer (pH 7.5), forced through a 21G needle to break up the slugs. The cells were pelleted at 2,600 x g for 5 min (Sorvall, GSA). Then five cell pellet volumes of lysis 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) were added to resuspend the cells. The mixture was forced through a 21G needle and then centrifuged at 10,000 x g for 10 min in 40 ml polycarbonate tubes (Sorvall, SA-600). The supernatant (cytoplasmic extract) was removed and frozen at –80°C. For the nuclear extract, the pellet was washed once with lysis buffer, then resuspended with two-nuclear pellet volumes of lysis buffer. While stirring, NaCl was slowly added to a final concentration of 0.4 M. This was placed on ice for 1 hr and then centrifuged at 11,000 x g for 10 min. The supernatant (nuclear fraction) was stored at –80°C.

2.14. Ammonium Sulfate Fractionation

The cytoplasmic extract was fractionated by adding solid ammonium sulfate (AS) to 20, 40, 60 and 80% saturation. Each AS pellet was resuspended in a buffer containing 50 mM Tris, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 20 µg/ml TLCK, 100 µg/ml PMSF. The samples were then dialyzed overnight at 4°C against 1 L of dialysis buffer (20 mM Tris, pH 7.5) with one change of the buffer during dialysis. The dialyzed samples were then centrifuged in 12 ml polycarbonate tubes for 15 min at 20,000 x g (Sorvall, SS34) to remove solids formed as a result of dialysis. The final supernatant was stored at –80°C.

2.15. Methods of Column Chromatography

High Performance Liquid Chromatography (HPLC) was performed using Waters model 650E Advanced Protein Purification System (Millipore). Extracts were either centrifuged or filtered before loading on different columns. All fractions were stored at 4°C.

2.15.1. DEAE Sephacel Column

The column (27 mm x 250 mm, Sigma) was equilibrated with Buffer D (20 mM Tris, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF. Thawed cytoplasmic extract was filtered (0.45 μm) and applied to the column using a peristaltic pump at 2.5 ml/min. The column was pumped with Buffer D at 3 ml/min. Bound proteins were eluted with a linear gradient to Buffer D containing 0.5 M NaCl at a flow rate of 5 ml/min (15 ml fractions). When not in use, the column was stored in 20% ethanol at 4°C.

2.15.2. Heparin Sepharose Column

Heparin can be used to purify DNA binding proteins because the nature of the polymer (sulfated glucosaminoglycan) resembles a nucleic acid molecule. The column (20 mm x 130 mm, Sigma) was equilibrated with Buffer H (10 mM potassium phosphate buffer, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.5 mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF). The DEAE column fractions containing DNA binding activity were pooled, concentrated to about 12 ml in a stirred filtration cell (Amicon YM10 membrane) and then the buffer was exchanged to Buffer H. The 12 ml sample was filtered and applied to the column at a flow rate of 1 ml/min. The column was pumped with Buffer H at 2

ml/min. Bound proteins were eluted with a linear gradient to Buffer H containing 0.8 M NaCl at a flow rate of 2 ml/min (4 ml fractions). When not in use, the column was stored in 20% ethanol at 4°C.

2.15.3. DNA Affinity Column

2.15.3.1. Preparing the DNA

Two complementary single-stranded oligonucleotides were first annealed using 20 nmol (approximately 330 μg) of each oligonucleotide. Phosphorylation was carried out in a 105 μl final volume containing 3 mM ATP and 100 U T4 polynucleotide kinase at 37°C for 2 hr. The DNA was ethanol precipitated and ligation was performed in 100 μl final volume with 2,000 U of T4 DNA ligase at 16°C for overnight. The DNA was extracted with phenol, precipitated with ethanol and resuspended in 100 μl milliQ H_2O.

2.15.3.2. Coupling of DNA to Sepharose

The DNA was coupled to cyanogen bromide (CNBr)-activated Sepharose-4B (Pharmacia) as described (Ausubel *et al*., 1995). One gram of CNBr-activated sepharose-4B (3.5 ml settled volume) was swelled in 100 ml of 1 mM HCl for 10 min, then transferred to a glass filter and washed with 200 ml of 1 mM HCl, with 300 ml milliQ H_2O and with 100 ml 10 mM potassium phosphate buffer (pH 8.0). The resin was transferred to a 15 ml screw-capped plastic tube and allowed to settle. The supernatant was removed and enough 10 mM potassium phosphate buffer (pH 8.0) (about 4 ml) was added to give a thick slurry. The oligonucleotide solution (100 μl in milliQ H_2O) was then added and the tube was incubated at room temperature overnight on a rotating wheel. The slurry was transferred to a glass filter and washed twice with 100 ml milliQ

H_2O and once with 100 ml of 1 M ethanolamine (pH 8.0). The resin was transferred to a 15 ml screw-capped tube and incubated at room temperature with 1 M ethanolamine to block excess active groups in the sepharose (final volume 14 ml). After 5 hr of rotation, the resin was packed in a column (13 mm x 25 mm, about 3 ml settled volume) and washed with 100 ml of 10 mM potassium phosphate buffer (pH 8.0), 1 M potassium phosphate buffer (pH 8.0), 1 M KCl, 100 ml milliQ H_2O and 'column storage buffer' (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 300 mM NaCl, 0.04% NaN_3), successively. The column was kept at 4°C in 'column storage buffer' (stable at least one year under these conditions).

2.15.3.3. Separation of Proteins

The column was equilibrated with 'binding reaction buffer' (20 mM HEPES, pH 7.9, 10% glycerol, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 0.04% NP-40). The active heparin column fractions were pooled, concentrated to about 2 ml using an Amicon YM10 membrane, and then the buffer was exchanged to 'binding reaction buffer'. The 2 ml sample was loaded on the column by gravity, then was recycled four times to obtain maximum binding to the column. The column was pumped with 'binding reaction buffer' at 1 ml/min to remove unbound proteins. Bound proteins were eluted with a linear gradient to 'binding reaction buffer' containing 1 M NaCl at a flow rate of 1 ml/min (1 ml fractions).

2.15.4. Gel Filtration Column

A gel filtration column (8 mm x 300 mm, 300SW, Waters) was equilibrated with Buffer H (10 mM potassium phosphate buffer, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.5

mM DTT, 20 μg/ml TLCK, 100 μg/ml PMSF). The active affinity column fractions were pooled, concentrated to about 600 μl using an Amicon YM10 membrane, and the buffer was exchanged to Buffer H. After the extract (600 μl) was applied, the column was pumped with the same buffer at a flow rate of 0.2 ml/min. When not in use, the column was stored at 4°C in 0.1% NaN_3 to prevent bacterial growth.

2.16. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were mixed with loading buffer and heated at 85°C for 5 min prior to loading on SDS-polyacrylamide gels (7.5 or 10%). Electrophoresis was carried out in 1X running buffer at 15 mA for approximately 30 min and at 25 mA for approximately 1 hr. The gels were stained either with a Coomassie Blue solution or Silver Stain (Merril *et al*., 1981).

2.17. Preparation of Samples for Mass Spectrometry

The active gel filtration fractions were pooled and concentrated to about 200 μl using a Centricon YM10 membrane (Millipore). The sample was washed twice with 50 mM ammonium bicarbonate buffer (pH 7.5). The final sample was frozen at –80°C. The protein sample was then shipped in dry ice to the Department of Chemistry and Pathology at the University of Virginia for mass spectrometric analysis.

2.18. *In situ* Staining of β-galactosidase and Assay of β-galactosidase Enzyme Activity

The *5nt* promoter region was amplified using primers with engineered *Xba*I restriction sites. The 1,361 bp PCR product was then digested with *Xba*I. The promoter

was then fused in-frame to super-labile *lacZ* (Rafols *et al*., 2001) within the *Xba*I site of the plasmid 63-iDQgal (the plasmid 63-iDQgal was kindly provided by Dr. Harry K. MacWilliams, Zoology Institute, Ludwig-Maximilians-University, Munich, Germany). Transformation and development of *Dictyostelium* cells were performed as described above. *In situ* staining of β-galactosidase activity was performed as described previously (Dingermann *et al*., 1989) with minor modifications. The filters containing cells at a desired developmental stage were transferred to a cellulose pad saturated with 1% glutaraldehyde in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) and incubated for 15 min. The filters were sprayed twice with 1% glutaraldehyde at 10 min intervals. The petri dishes were then filled with 1% glutaraldehyde and incubated for an additional 15 min to insure complete fixation, especially in the late developmental stages. Each filter was then transferred to a new petri dish and dried for 15 min. Z-buffer containing 0.1% NP-40 was added to the filters until the cells were rehydrated, then the developing individuals were covered with this same buffer and incubated for 15 min. The solution was removed and the filters were washed twice in Z-buffer. Sufficient β-galactosidase stain [5 mM $K_3(Fe(CN)_6)$, 5 mM $K_4(Fe(CN)_6)$, 1 mM X-gal and 1 mM EGTA in Z-buffer] was added to completely submerge the developing individuals and incubated 2-6 hr in the dark. The reaction was stopped with 50% methanol and the filters were stored under 50% methanol, or freeze-dried. For assaying β-galactosidase activity, cells at different developmental stages were scraped from the filters, centrifuged at 700 x g for 3 min, then washed twice in phosphate buffer (14.7 mM KH_2PO_4/2 mM Na_2HPO_4, pH 6.0). The cells were resuspended in 500 µl phosphate buffer, homogenized in a glass homogenizer, then frozen at –80°C until use. Immediately prior to the assay, the cell suspension was thawed at room temperature,

vortexed for 1 min and cleared at 20,000 x g for 10 min. A 100 µl sample of the supernatant was added to 500 µl reaction mix (300 µl Z-buffer containing 50 mM β-mercaptoethanol and 200 µl of O-nitrophenyl-β-D-galactoside (4 mg/ml) in 100 mM sodium phosphate buffer, pH 7.0). Reactions were incubated at room temperature then terminated by adding 400 µl of 1 M Na_2CO_3 and cleared for 2 min at 20,000 x g. The absorbance at 420 nm was determined. Enzyme activity was expressed as A_{420}/min/mg protein. Protein concentration was determined using the BCA protein assay kit (Pierce) as described above.

2.19. Primer Extension

Dictyostelium AX3K cells (1 x 10^7 cells/ml final cell concentration) were starved by shaking at room temperature at 130 rpm for 14 hr in 1xMES-LPS, pH 6.5. Then 1 mM final concentration of cAMP was added to the cells and continued shaking for 9 more hr. Total RNA was isolated from cAMP-induced cells by lysis in RNApure (GenHunter Corporation) guanidium thiocyanate solution according to manufacturer's protocol. Primers *gs1r* (located 179 bp downstream of the 5' end of the *5nt* cDNA) and *gs2r* (located 222 bp downstream of the 5' end of the *5nt* cDNA) were radioactively labeled as described above. The hybridization reaction included 10 µg total RNA, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, pH 8.0, 80% formamide and the primer (100,000 cpm) in a 20 µl total volume. The reaction was first incubated at 85°C for 2 min followed by an overnight incubation at 45°C. Then the mixture was ethanol precipitated and resuspended in a 20 µl volume including 1x RT buffer, 500 µM of each dNTP, 2 µg actinomycin D and 200 U of Moloney Murine Leukemia Virus reverse transcriptase (Promega) and DEPC water. The mixture was incubated at 42°C for 60

min. Then 4 μl formamide loading buffer was added and the sample was heated at 90°C for 10 min. The sample was cooled on ice for 2 min and then loaded on a 6% polyacrylamide/7 M urea gel. *Hin*fI digested φX174 DNA (Promega) were radioactively-labeled and used as standard size markers. The gel was performed at 150 V for approximately 6 hr. Detection was performed by autoradiography. A standard curve (generated based on the size versus migration distance data of the standard markers) was used to determine sizes of the primer extension products.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Southern Analysis of *5nt* Gene and Construction of a Sub-genomic Library

In order to clone the *5nt* promoter sequences, restriction enzyme sites in the genomic region in which the *5nt* gene is located were first determined by Southern analysis. Based on the genomic restriction map data, a sub-genomic library was generated in a plasmid vector. The library was screened by colony PCR and a colony carrying a restriction fragment containing the promoter was selected.

3.1.1. Southern Analysis of *5nt* Gene

In order to obtain a genomic restriction map of the region in which the *5nt* gene is located, several Southern blotting experiments were performed. Genomic DNA isolated from the *Dictyostelium* cells was digested to completion with a number of restriction endonucleases that were chosen based on the frequency of occurrence of the recognition sites in *5nt* cDNA. After transfer to nylon membrane the blots were hybridized with either the radiolabeled full-length *5nt* cDNA (Fig. 2) or an 849 bp fragment of the 5' region of the *5nt* cDNA. The overall banding patterns observed on the blots were consistent with the known restriction sites of the *5nt* cDNA (Fig. 4). *Kpn*I did not cut within the cDNA probe and produced a large band (>5kb) (Fig. 4A, lane 1). Lane 2 shows one band (5 kb) produced by *Bam*HI digestion. *Bam*HI cut within the cDNA probe once and the 5 kb band could contain either the upstream or downstream region. *Hinc*II did not cut within the cDNA probe and produced a 5 kb band (Fig. 4A, lane 3). *Eco*RV cut once within the full-length cDNA probe and yielded two bands (5 kb and 1.6 kb) on the blot (Fig. 4A, lane 4).

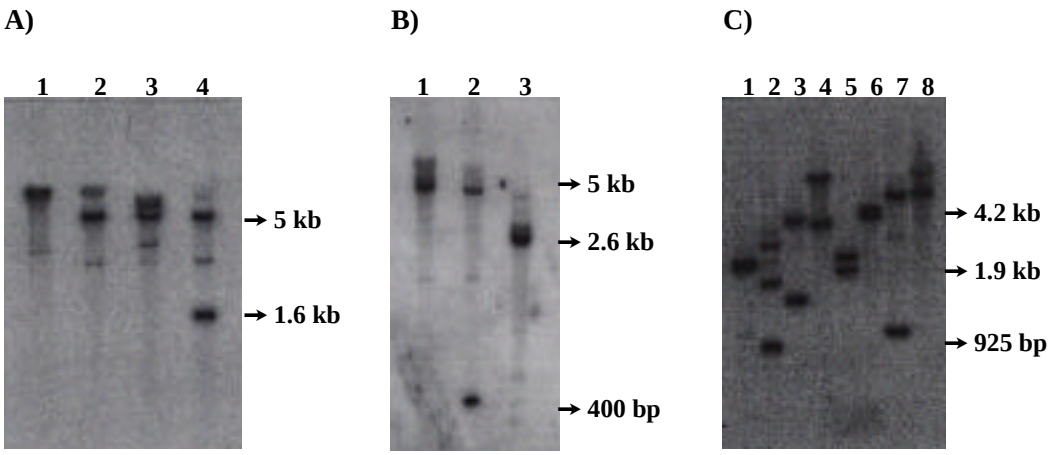


Figure 4. Southern blot hybridization of genomic DNA. Genomic DNA was digested to completion with various restriction endonucleases and resolved on 1% agarose/TBE gels. After transfer to nylon membrane the blot was hybridized with ^{32}P-labeled either the entire 1,921 bp *5nt* cDNA or the 5' 849 bp fragment of the *5nt* cDNA. **A)** *Kpn*I (lane 1), *Bam*HI (lane 2), *Hin*cII (lane 3) and *Eco*RV (lane 4). The blot was hybridized with the entire 1,921 bp *5nt* cDNA. **B)** *Eco*RV (lane 1), *Eco*RV plus *Bam*HI (lane 2) and *Taq*I (lane 3). The blot was hybridized with the 5' 849 bp fragment of the *5nt* cDNA. **C)** *Eco*RV plus *Taq*I (lane 1), *Bam*HI plus *Taq*I (lane 2), *Bam*HI plus *Bcl*I (lane 3), *Bam*HI plus *Bgl*II (lane 4), *Bam*HI plus *Acc*I (lane 5), *Bam*HI plus *Cla*I (lane 6), *Bam*HI plus *Hin*dIII (lane 7) and *Bam*HI plus *Sac*I (lane8). The blot was hybridized with the 5' 849 bp fragment of the *5nt* cDNA.

The genomic DNA was subjected to further restriction digestion and probed with a radiolabeled fragment of the *5nt* cDNA as shown in Fig. 4B. This fragment contained the 5' 849 bp of the *5nt* cDNA. Lanes 1, 2 and 3 show genomic DNA digested with *Eco*RV, *Eco*RV plus *Bam*HI and *Taq*I, respectively. A band of approximately 5 kb was observed when the genomic DNA was digested with *Eco*RV (Fig. 4B, lane 1). As stated earlier, *Eco*RV cut once within the cDNA, however, it did not cut within the cDNA probe used in this experiment. Therefore, this 5 kb band contained the upstream region. The 1.6 kb band observed previously (see Fig. 4A, lane 4) likely contained the downstream region. This 1.6 kb band was not present on this blot because the probe did not contain the corresponding region. When the genomic DNA was digested with *Eco*RV and *Bam*HI together, two bands (5 kb and 0.4 kb) were observed (Fig. 4B, lane 2). These two bands were produced because *Bam*HI cut within the cDNA probe once. Moreover, an approximately 2.6 kb band was detected when the genomic DNA was digested with *Taq*I (Fig. 4B, lane 3). Although there is one apparent *Taq*I recognition site located at 1,300 bp relative to the 5' end of the *5nt* cDNA, *Taq*I did not cut within the cDNA probe used in this experiment. This 2.6 kb *Taq*I fragment contained 1.3 kb coding region as well as approximately 1.3 kb of the 5' flanking sequence.

Southern analysis was used to determine if alternate forms of the 5NT-encoding gene existed. As shown in Figure 4C, *Bam*HI cut within the cDNA probe once and produced two bands of varying size depending on the second enzyme used. The hybridization pattern observed suggested that the 5NT protein is encoded by a single gene.

Based on the data obtained from the Southern blotting analyses, a genomic restriction map was generated (Fig. 5). The 2.6 kb *Taq*I fragment was chosen for cloning

into a plasmid vector in order to isolate the *5nt* promoter sequences. As mentioned above, this fragment contained approximately 1.3 kb of 5' flanking sequence of the *5nt* gene. This length was thought to be sufficient to control the *5nt* gene expression because the gene regulatory sequences in *Dictyostelium* generally reside within approximately 1 kb upstream of coding regions.

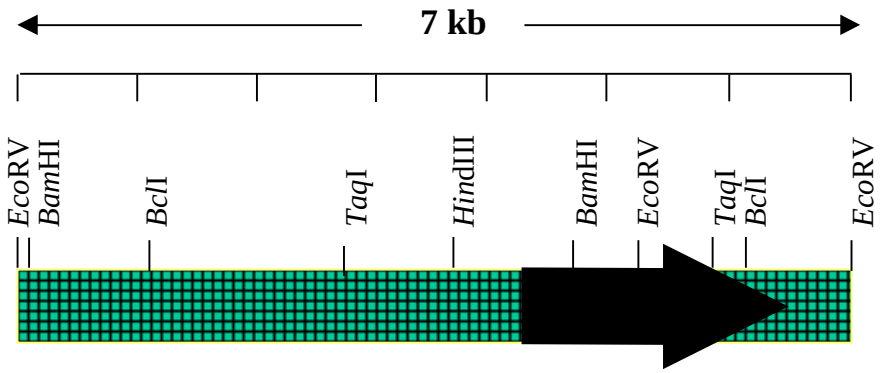


Figure 5. Genomic restriction map of *5nt*. Using the data obtained from Southern blotting analyses, a genomic restriction map of was generated. The large arrow shows the *5nt* coding region and direction of transcription. Approximately 4,200 bp upstream and 600 bp downstream (relative to the coding region) regions are shown.

3.1.2. Construction and Screening of Sub-genomic Library

In order to clone the 2.6 kb *Taq*I fragment containing the 1.3 kb of the coding region as well as approximately 1.3 kb of 5' flanking sequences, a sub-genomic library was generated. Approximately 40 μg of genomic DNA was digested with *Taq*I. The digested DNA was fractionated on an agarose gel (Fig. 6) and six gel fractions were excised. DNA was extracted from each fraction and PCR was performed (using gene-specific primers *cc1* and *nu1r*) to identify the fraction containing the fragment of interest. Amplification by *Taq* polymerase yielded a PCR product of 408 bp (see Table 1 for primers). The fragment was found in fraction 4, DNA from which was then ligated into *Cla*I-cut and CIAP-treated pBluescript KS vector.

After transformation of *E. coli* XL1-Blue cells, PCR was used to screen for positive clones. Colony lysates prepared from 1,083 white colonies were used as PCR templates for *cc1* and *nu1r* as primers. Five colonies were mixed in a given tube for preliminary screening. One PCR reaction (out of approximately 210) produced the 408 bp product indicating the presence of the *Taq*I fragment. Figure 7A shows the PCR results using templates prepared from the clones 147-221. The correct-sized PCR product was observed in the lane corresponding to pooled clones 167-171 (Fig. 7A, lane 5). To determine which of these five colonies contained the insert, lysates were prepared from these colonies individually and PCR was performed again. The template prepared from colony 167 resulted in a 408 bp-product (Fig. 7B, lane 1). This clone was designated as 'c167' and was used in subsequent experiments.

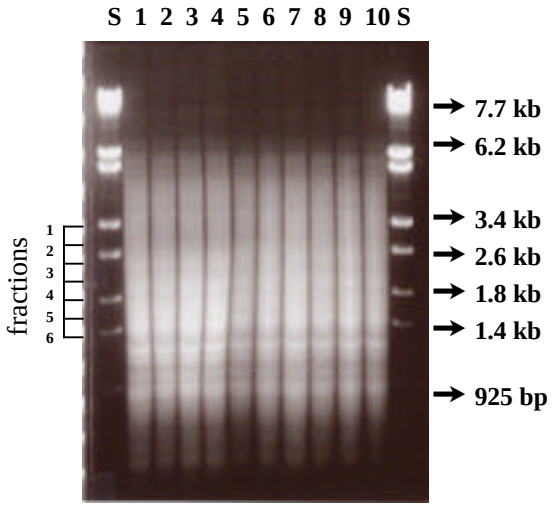


Figure 6. Agarose gel electrophoresis of genomic DNA digested with *Taq*I. Forty two micrograms of genomic DNA was digested with *Taq*I and resolved on a 1% low melt agarose/TAE gel. Lane S shows *Sty*I-digested λ DNA as standard size markers. Lanes 1 through 10 contain *Taq*I-digested genomic DNA (4.2 μg per lane). Six 0.5 cm slices were excised from the gel for subsequent DNA purification.

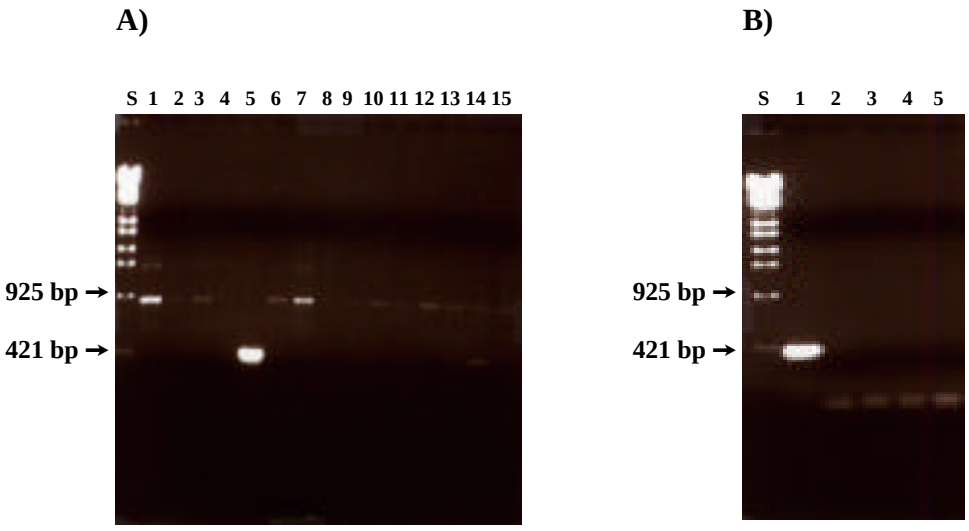


Figure 7. Agarose gel electrophoresis of PCR reactions used to screen *Taq*I sub-genomic library. PCR was performed to screen *Taq*I-subgenomic library for clones carrying the fragment of interest. Bacterial lysates prepared from colonies 147 to 221 were used as templates for primers *cc1* and *nu1r*. PCR samples were fractionated on a 1% agarose/TBE gel. **A)** PCR screening of colonies 147-221. Five pooled clones were simultaneously tested in each PCR reaction. Clones 147-151 (lane 1), 152-156 (lane 2), 157-161 (lane 3), 162-166 (lane 4), 167-171 (lane 5), 172-176 (lane 6), 177-181 (lane 7), 182-186 (lane 8), 187-191 (lane 9), 192-196 (lane 10), 197-201 (lane 11), 202-206 (lane 12), 207-211 (lane 13), 212-216 (lane 14) and 217-221 (lane 15). Lane S shows *Sty*I-digested λ DNA as standard size markers. **B)** PCR screening of colonies 167-171. Clone 167 (lane1), 168 (lane 2), 169 (lane 3), 170 (lane 4) and 171 (lane 5). Lane S shows *Sty*I-digested λ DNA as standard size markers.

3.2. The *5nt* Promoter is A/T Rich

The plasmid DNA isolated from clone 'c167' was used as template in sequencing reactions. DNA sequencing was performed at the Virginia Bioinformatics Institute's DNA Sequencing Facility at Virginia Tech. The *5nt* promoter sequence is typical of non-coding regions of *Dictyostelium*, being A/T rich and containing long sequences comprised of A's and T's (Fig. 8). The promoter region consists of 37.5% A's, 44.5% T's, 9.6% G's and 8.4% C's.

In order to confirm and obtain more accurate sequencing data, this newly obtained promoter sequence was used to design four PCR primers. Three of the primers (*5nup1*, *5nup2*, and *5nup3*) annealed the 5' flanking region and one (*gs2r*) annealed the coding region (Fig. 8, see also Table 1). Genomic DNA from vegetative cells was then amplified using *5nup1-5nup2* and *5nup3-gs2r* primer pairs, producing 466 bp and 989 bp PCR products, respectively. These PCR products were then used as template in sequencing reactions. The sequence was in agreement with that obtained from clone 'c167'. These sequences were subsequently verified in Blast searches of fragments obtained from the *Dictyostelium* Genome Sequencing Project. The nucleotide sequence of the upstream region of *5nt* was deposited in GenBank [Accession Number AF378371] (Appendix A).

The fact that *5nt* is developmentally regulated suggested the existence of regulatory element(s) in this promoter. However, searching the *5nt* promoter for consensus regulatory protein binding sites did not reveal any clear matches to known sites in other organisms.

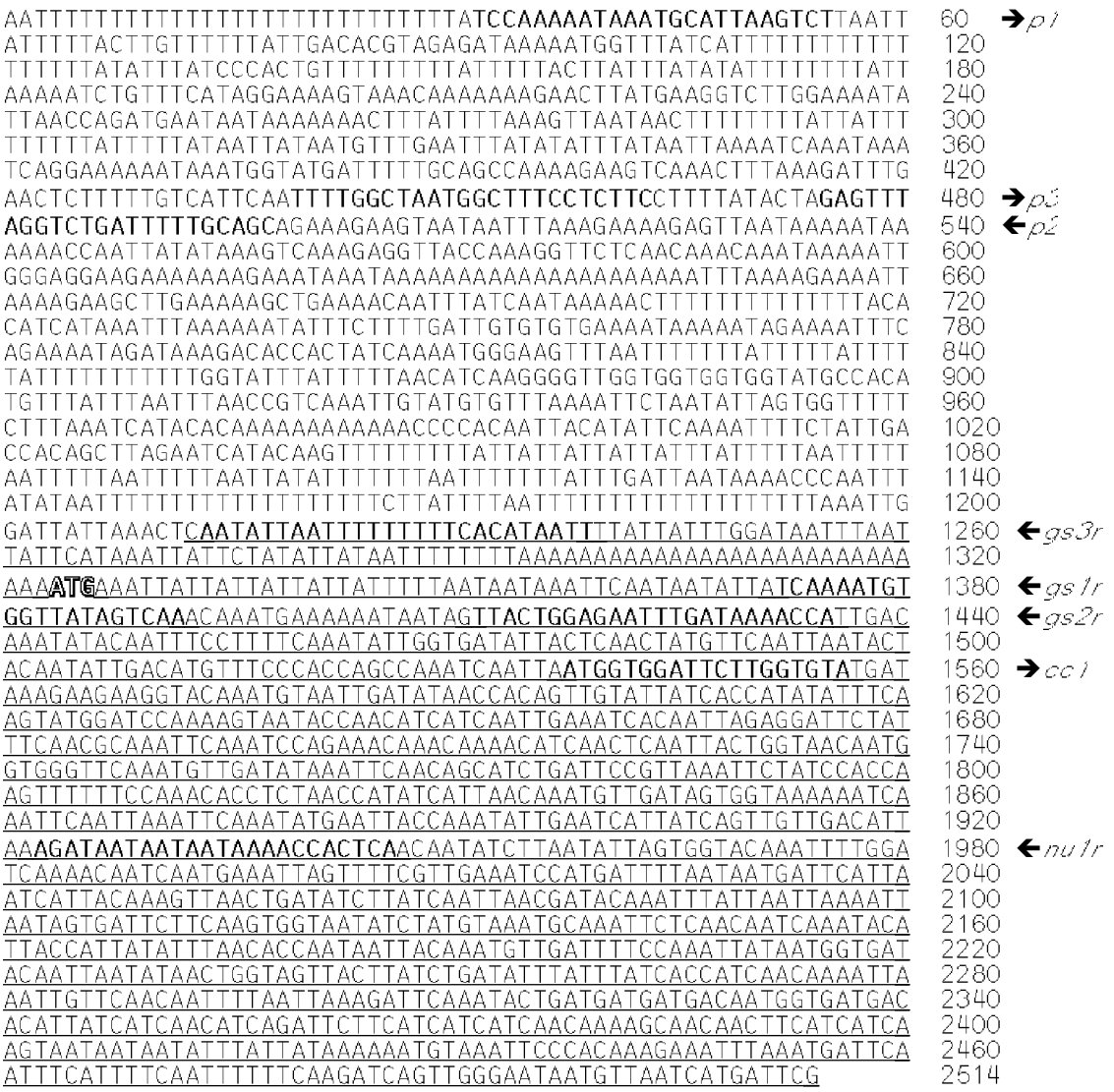


Figure 8. Nucleotide sequence of the 5' flanking and a part of the coding region of *5nt*. The underlined region (1213-2514) is the sequence consensus with the first 1301 bp of the *5nt* cDNA. The translational start site (ATG) is shown in shadow. Nucleotide numbering is on the right. Bold sequences indicate various primer annealing sites with respective primer names and their direction on the far right.

3.3. Construction of *5nt* Promoter-*luciferase* Fusions

To identify regulatory sequences within the *5nt* promoter through the use of a luciferase reporter gene system, a unidirectional nested series of 5' promoter deletions were generated using the exonuclease III/mung bean nuclease system (Henikoff, 1984). Approximately 65 different-sized promoter deletion clones were obtained (Fig. 9). Eleven deletion constructs as well as the 1,212 bp cloned promoter were initially chosen for fusion to the *luc* gene in the pVTL2 reporter gene vector. This 13 kb-extra-chromosomal reporter plasmid is maintained at 10-50 copies per cell and carries an Amp^R gene and a $G418^R$ gene (Yin *et al*., 1994b). The deletion constructs contained fragments of various lengths ranging from 1,117 bp to 27 bp. The strategy used to construct the 5*nt* promoter-*luc* fusions is shown in Figure 10. The entire (1,212 bp) and deleted promoter regions were PCR-amplified using the vector-specific *T7+Hin*dIII primer and the gene-specific *gs1r+Hin*dIII primer (Fig. 10A). The *5nt* ATG and 66 nucleotides downstream from the ATG were also amplified because the gene-specific *gs1r* primer is located downstream of the translational start site of the gene. Some deleted promoter regions were also amplified using *T7+Hin*dIII and the gene-specific *gs3r+Hin*dIII primer. These constructs did not contain the *5nt* ATG because the *gs3r* primer is located upstream of the translational start site.

The amplified promoter regions were then digested with *Hin*dIII to expose the restriction enzyme sites at both ends of the PCR products. Due to the presence of an internal *Hin*dIII site located at -547 bp within the promoter, amplified DNAs containing more than 547 bp of promoter region needed to be partially digested with the enzyme. Fragments were then selected in which the internal *Hin*dIII site had not been cleaved.

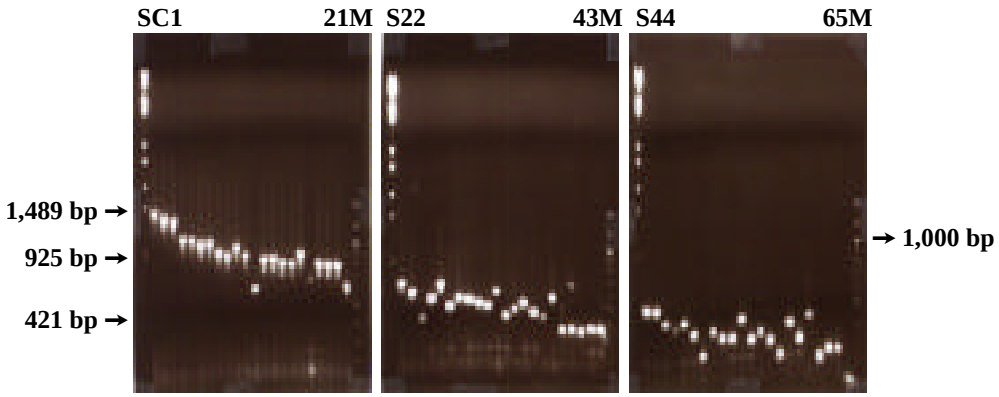
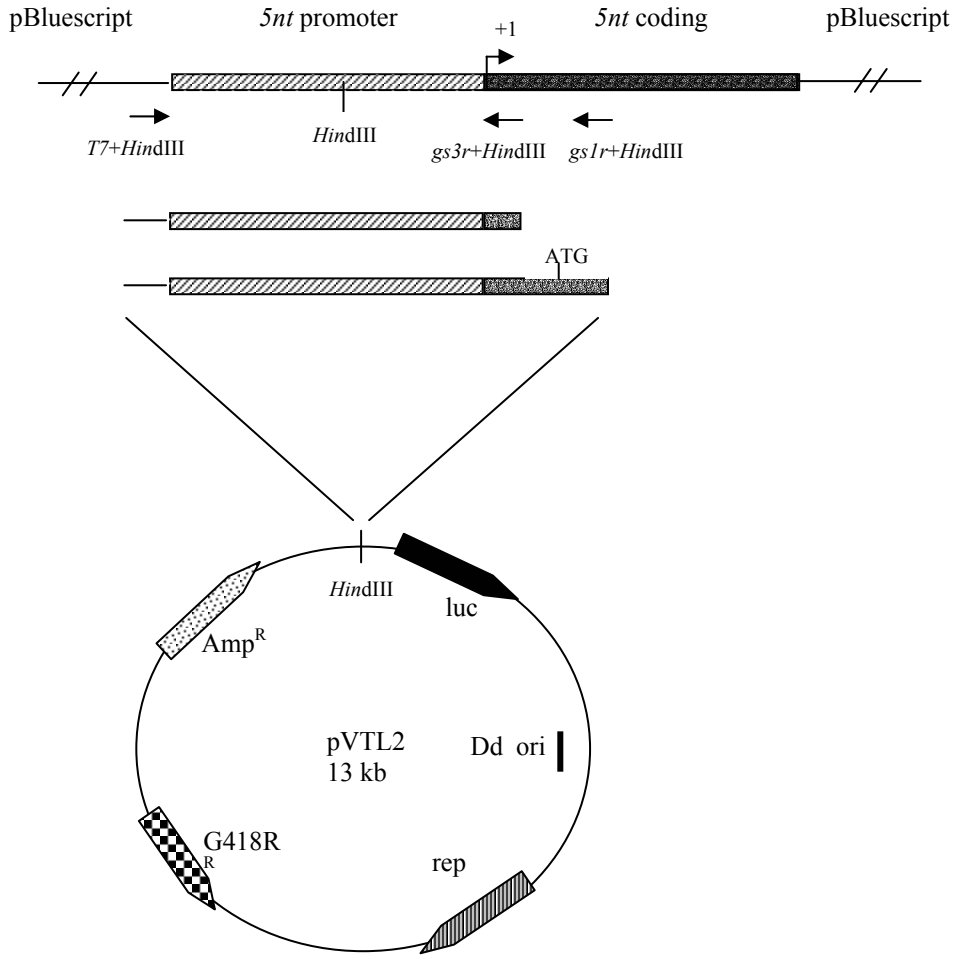


Figure 9. Agarose gel electrophoresis of PCR reactions to determine sizes of promoter deletions. Bacterial lysates prepared from 65 different clones were used as templates for primers *T7* and *gs2r*. PCR samples were fractionated on 1% agarose/TBE gels. Lane S shows *Sty*I-digested λ DNA as standard size markers. Lane M is 100 bp standard markers. Lane C is the control PCR, which included lysate prepared from clone 'c167' containing undeleted promoter. Lanes 1 through 65 are individual clones representing various promoter deletions. Not all lanes are numbered.

A)


pBluescript
5nt promoter
+1
5nt coding
pBluescript
T7+HindIII
HindIII
gs3r+HindIII
gs1r+HindIII
ATG
HindIII
luc
Amp^R
pVTL2
13 kb
Dd ori
G418R^R
rep

B)

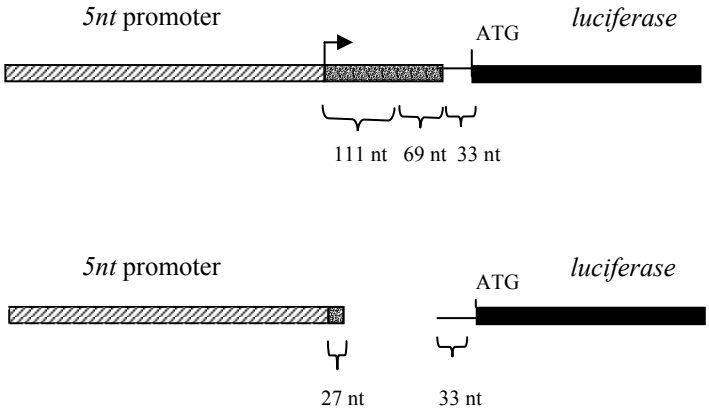

5nt promoter
ATG
luciferase
111 nt
69 nt
33 nt
5nt promoter
ATG
luciferase
27 nt
33 nt

Figure 10. Strategy for constructing promoter-*luciferase* fusions in pVTL2 vector. **A)** Cloning strategy. pBluescript vector carrying different promoter deletions were used as PCR template for primer pairs *T7-gs1r* and *T7-gs3r*. Resulting products were partially digested with *Hin*dIII followed by ligating into the *Hin*dIII site of the pVTL2 reporter vector. **B)** Resulting constructs. *gs1r*-constructs included the 111 nucleotide untranslated region and the first 69 nucleotide of the *5nt* coding region. *gs3r*-constructs included only 27 nucleotide of the untranslated region and none of the coding region. Therefore in the *gs3r* constructs, the translational start site was the *luc* ATG. Also, additional 33 nucleotides (encoding 11 amino acids) were present between the *Hin*dIII cloning site of the vector and the *luc* ATG.

Ligation into pVTL2 vector and transformation of bacteria were performed as described in the Materials and Methods. It was predicted that promoter regions amplified by *gs1r* would be in-frame with the luciferase coding sequence. As stated above, 69 nucleotides, coding for the first 23 amino acids of the 5NT as well as 111 nucleotides in the 5' leader sequence, were incorporated in the promoter-*luc* fusions. There are also 33 nucleotides (11 codons) between the *Hin*dIII cloning site and the translational start site of the *luc* gene in the vector. Therefore, in these constructs, the luciferase enzyme would have additional 34 amino acids at its N-terminus assuming that the *5nt* ATG is used as the start site for translation. The *gs3r*-amplified promoter regions, however, lacked the *5nt* ATG (27 nucleotides of the untranslated region were incorporated in these constructs). Thus, in these constructs, the luciferase enzyme would not have additional residues (Fig. 10B).

Dictyostelium amoebae cells were transformed with the pVTL2 vectors containing *5nt* promoter-*luc* fusions. In this vector, the promoter constructs drive expression of the *luc* reporter gene, which allows the quantification of (deleted) promoter activity.

3.4. *5nt* Expression is Developmentally Regulated

Luciferase assays were performed as described in the Materials and Methods. Crude cell extracts were used in assays because the activity measured in the supernatant (obtained after centrifugation of the crude extract) was 10-fold lower than that of crude extracts (data not shown).

Cells transformed with the empty vector as a negative control did not yield any luciferase activity as expected (data not shown). There was no difference in the level of

activity in any single construct with or without the *5nt* ATG (data not shown). This result suggested that 34 amino acid residues added to the N-terminus of the luciferase enzyme did not alter the enzyme activity. Figure 11 shows the luciferase activity as driven by the *5nt* promoter (1,212 bp) during the time course of development. There was very low background activity during early stages of development. Activity was first observed at 6 hr, then increased and remained constant during the remainder of development. This result was in agreement with northern analysis of the authentic *5nt* (Chanchao *et al.*, 1999). Similar results were obtained when the *5nt* promoter (1,212 bp) was fused in-frame to *lacZ* (see Fig. 36). Because of the similarity of the appearance of the authentic *5nt* and the reporter gene driven by the *5nt* promoter, it is likely that regulation of *5nt* occurs largely at the level of transcription.

3.5. *5nt* Expression is Induced by Extracellular cAMP

Because several *Dictyostelium* genes have been shown to be regulated by cAMP, the expression of *5nt* was tested in the presence and absence of this morphogen. Cells transformed with the cloned (1,212 bp) *5nt* promoter-*luc* fusion were starved and shaken in liquid culture in the absence and presence of 1 mM cAMP. A very low activity was detected in the absence of cAMP (Fig. 12). In the presence of cAMP, however, the enzyme activity appeared after 2 hr and continued to increase during the time course of the experiment (Fig. 12). This result indicated that the *5nt* expression is induced by cAMP in shaking cell suspension and very likely by cAMP during development on agar.

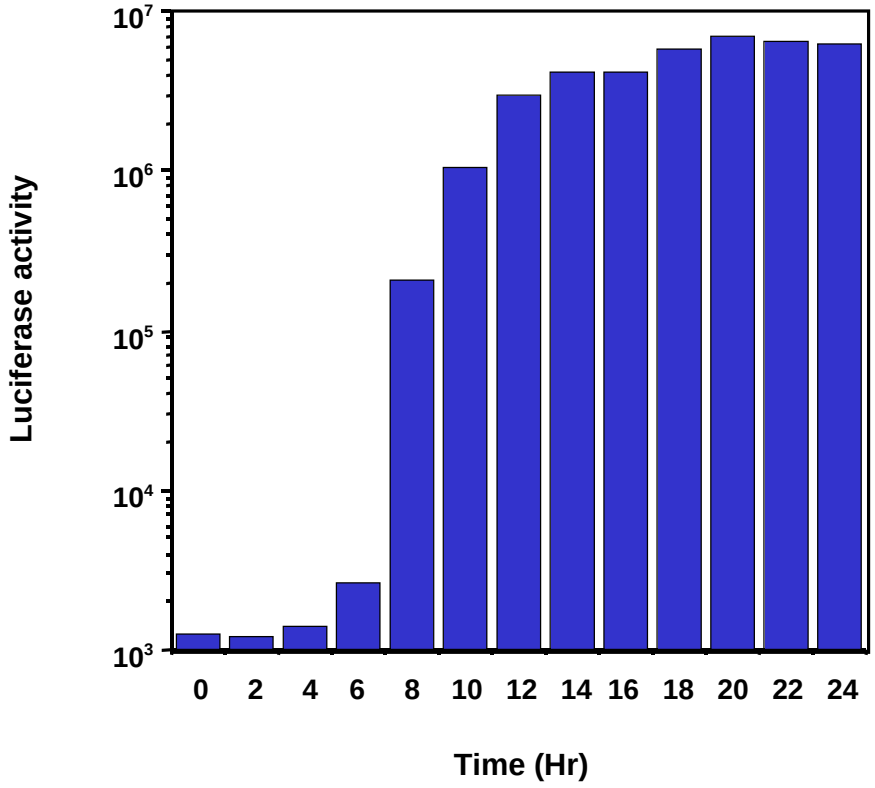


Figure 11. Luciferase reporter gene activity during development. Cells transformed with pVTL2 vector carrying the *5nt* promoter (1,212 bp)-*luc* fusion were developed on nitrocellulose filters for 24 hr. Cells were harvested every 2 hr and extracts were prepared for assay. The experiment was repeated several times with identical results.

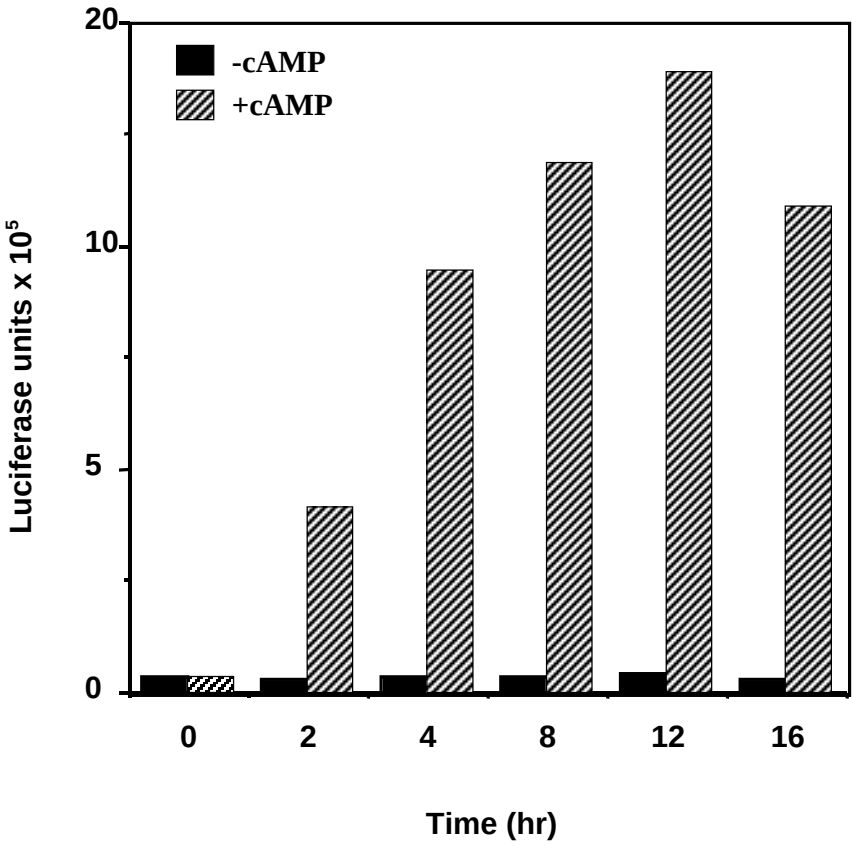


Figure 12. cAMP-induced expression of the *luciferase* reporter gene. Cells transformed with pVTL2 vector carrying the *5nt* promoter (1,212 bp)-*luc* fusion were shaken in non-nutrient buffer for 15 hr. To one flask of cells cAMP was added to a final concentration of 1 mM (+cAMP). An equal volume of buffer was added to a replicate sample of cells (-cAMP). Cells were then shaken for an additional 16 hr. Samples were taken at intervals and extracts were prepared for assay of luciferase activity. The experiment was repeated several times with identical results.

3.6. Identification of *cis*-acting Regulatory Elements in *5nt* Promoter

3.6.1. Gradual Decrease in Luciferase Activity in 5' Promoter Deletion Constructs

To locate regulatory sequence(s) in the promoter involved in the developmental/cAMP regulation of *5nt* expression, 5' promoter deletions were assayed for reporter activity. The level of reporter activity remained relatively constant when approximately 600 bp of the cloned promoter (1,212 bp) was deleted (Fig. 13). However in this region, the construct containing 884 bp of the promoter yielded higher activity than the 1,212 bp and the 1,117 bp promoter. This suggested a possible negative regulatory element between –1,117 bp and –884 bp region. However the –773 bp and –617 bp constructs yielded almost identical levels of activity to the –1,117 bp construct.

When additional sequences were deleted, the reporter activity decreased gradually (Fig. 13). No activity was observed with the –365 bp construct. This result showed that the –365 bp construct was not an effective promoter due to the lack of essential element(s) that drive the expression of the fusion gene. From the data obtained from the 5' deletion analysis, three potential promoter regions containing possible transcriptional regulatory sites were detected. These sites, contained in one contiguous region, are located between –547 and –480, between –480 and –392 and between –392 and –365 bp.

It should be noted that in the luciferase assays, the standard deviation was approximately 60% of the mean value. This high standard deviation could be due to the vector copy number, which was not determined in this study.

3.6.2. Luciferase Activity Resulting from Internal Promoter Deletion Constructs

To obtain additional data and to analyze interactions between regulatory elements within an otherwise intact promoter, numerous internal deletions were generated as

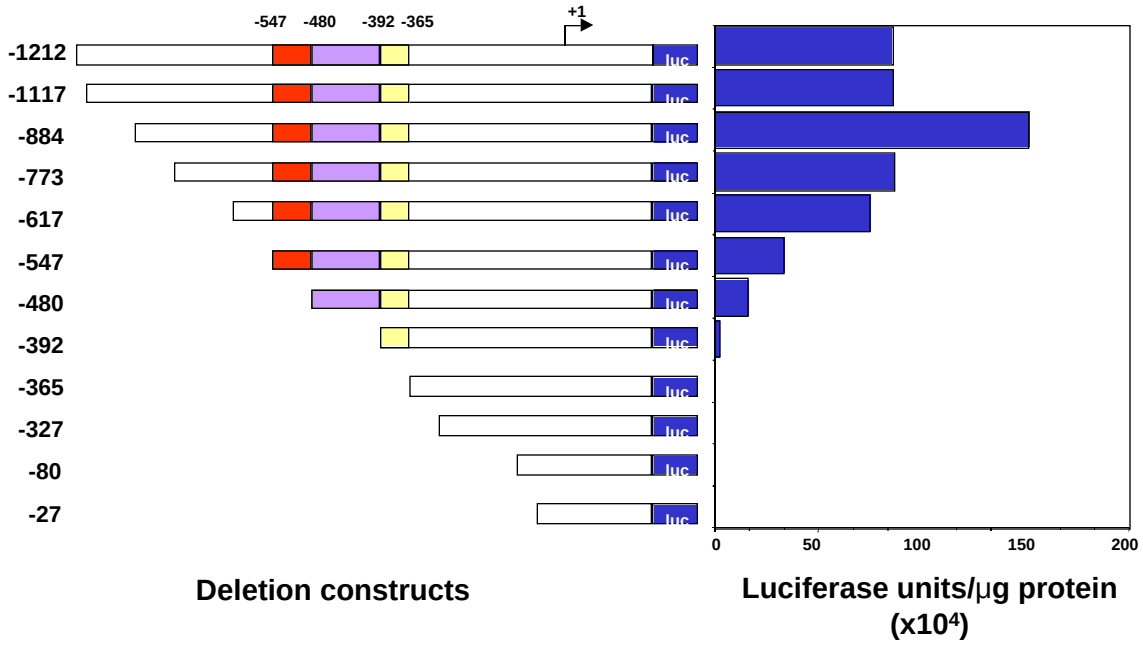


Figure 13. Luciferase activity in 5' promoter deletions. 5' promoter deletions were generated by exonuclease III and mung bean nuclease. In-frame *5nt* promoter-*luc* fusions were assembled in pVTL2. Luciferase assays were performed using crude extracts prepared from aggregation stage cells. Data shown are the average values of multiple clones assayed from each construct. The standard deviation was approximately 60% of the mean value. +1 is the 5' end of the *5nt* cDNA. Numbers on the left indicate the positions of the 5' ends of deletion constructs. Potential transcriptional regulatory sites affecting the reporter gene expression are shown.

described in Materials and Methods. Thirteen different internal deletions were generated for analysis. In addition, two constructs (the –773 and –547 constructs) containing promoter regions without any internal deletion were used as controls in these experiments.

The results obtained from internal deletion analysis were mostly in agreement with those of the 5' deletion analysis. Two controls yielded high level of reporter activity as expected (Fig. 14). When the region between –457 and –307 bp was deleted, the activity diminished dramatically (~96% decrease). Deleting the region between –457 and –421 bp did not have any effect on activity. However, the reporter activity decreased almost 100% when the region between –421 and –347 bp was deleted. The region between –307 and –226 bp also appeared to be critical for gene expression. When this part of the promoter was also deleted, the very low level of activity that was present in the -457Δ-307 construct was almost completely eliminated (~98% decrease). A similar result was observed for the -330Δ-192 construct (Fig. 14).

As stated earlier, 5' deletion analysis suggested no important regulatory elements within 365 bp promoter region because the –365 bp construct resulted in no luciferase activity (Fig. 13). The fact that a different result was obtained from this internal deletion analysis of the same region indicates that solely 5' deletion analysis may have misleading interpretations of the data and that it is important to perform additional analyses (i.e. internal deletions, site-directed mutagenesis) for accurate characterization of DNA elements.

The data obtained from 5' and internal deletion analysis indicated the presence of four potential regulatory regions within the *5nt* promoter. These regions were designated as a, b, c and d.

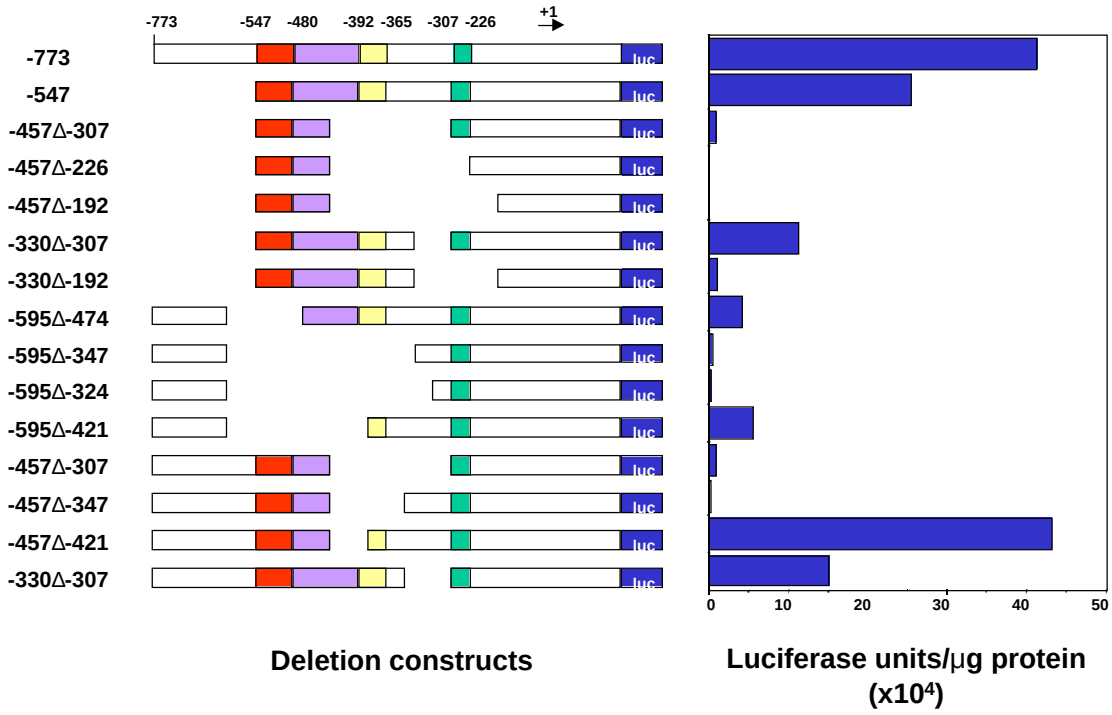


Figure 14. Luciferase activity in internal promoter deletions. Numerous internal deletions were generated by PCR-ligation-PCR method. Deletions were linked in-frame to the *luc* gene in pVTL2. Data shown are the average values of multiple clones assayed from each construct. The standard deviation was approximately 44% of the mean value. +1 is the 5' end of the *5nt* cDNA. Numbers on the left designate internal deletion constructs. The symbol Δ indicates deleted region. The first two constructs, –773 and –547, are controls. Potential transcriptional regulatory sites are shown.

3.7. Identification of Protein Factor(s) that Specifically Bind to Regulatory Elements in *5nt* Promoter

To identify *trans*-acting regulatory factor(s) that bind specifically to the regulatory sequences in the *5nt* promoter, gel mobility shift assays were performed. Cytoplasmic and nuclear proteins were extracted from *Dictyostelium* cells and incubated with ^{32}P-labeled DNA fragments as described in Materials and Methods. Numerous DNA fragments were used as probes in gel shift assays. Initially, the search for DNA binding factors was examined using the probes 1, 2, 3 and 6, which were produced by PCR and were 104, 144, 91 and 105 bp in size, respectively (Fig. 15). Smaller DNA fragments, 30 bp or 50 bp in size, were also used. Table 2 includes sequence information and locations on the promoter for individual DNA fragments. The relative locations of the gel shift probes are shown in Figure 15.

3.7.1. Detection and Characterization of a Gel Shift Band with 0.33 R_f Using Probe 19

In order to detect a gel shift band nuclear extracts were prepared from cells shaken in the presence of cAMP. The extract was applied to a heparin sepharose column, and the resulting fractions were assayed for DNA binding activity (Fig. 16). Three fractions were assayed, the flow through (F), fractions 3-27 eluting early in the salt gradient (E) and fractions 28-34 eluting late in the salt gradient (L).

In order to determine if DNA binding proteins were present in these fractions gel shift assays were performed. A 0.33 R_f band was observed in early fractions of the heparin column (Fig. 16). This band was present using probe 19 and was not present using any of the other probes (Fig. 16, lane 19E). Probe 19 represents the region between

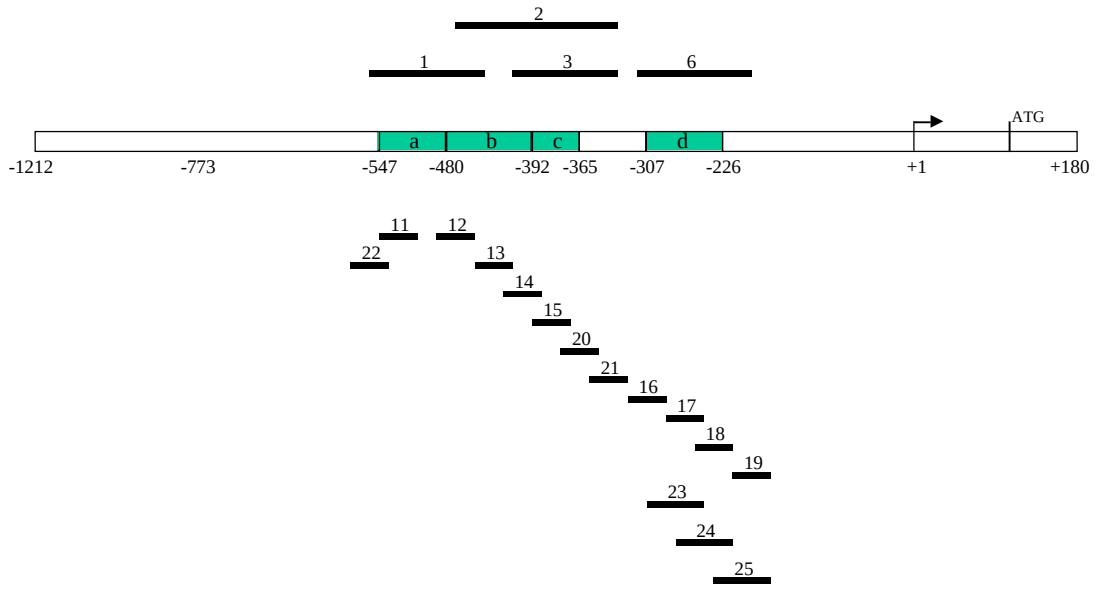


Figure 15. Probes used in gel shift assays. This figure shows relative locations of the probes used in gel shift assays. Several promoter regions containing potential transcription factor binding sites are indicated as a, b, c and d. Probes 1 (104 bp), 2 (144 bp), 3 (91 bp) and 6 (105 bp) were generated by PCR. Probes 11 through 22 were 30 bp oligonucleotides. Probes 23, 24 and 25 were 50 bp oligonucleotides.

Table 2. DNA fragments used as probes in gel shift assays. Probes 1, 2, 3 and 6 were generated by PCR using primer pairs F10-R1, F5-R2, F8-R2 and F1-R7, respectively. The sizes of these PCR products were 104 bp, 144 bp, 91 bp and 105 bp, respectively. Probes 11 to 25 were oligonucleotides (11-22 were 30 bp; 23-25 were 50 bp oligonucleotides). The sequence of the forward oligonucleotide is shown. All sequences are written in the 5' to 3' direction. The location refers to the promoter region represented by each DNA fragment (relative to the 5' end of the *5nt* cDNA). F, forward; R, reverse.

Probe	Sequence	Location
1		-561 to -458
F10	AAGAAAATTAAAAGAAG	-561 to -545
R1	CACAATCAAAAGAAATA	-458 to -474
2		-474 to -331
F5	TATTTCTTTTGATTGTG	-474 to -458
R2	CCAACCCCTTGATGTTA	-331 to -347
3		-421 to -331
F8	AAAGACACCACTATCAAAA	-421 to -441
R2	CCAACCCCTTGATGTTA	-331 to -347
6		-307 to -203
F1	ATTTAATTTAACCGTCAAA	-307 to -327
R7	ATTTTGAATATGTAATTGTGGGGT	-203 to -226
11	GCTTGAAAAAGCTGAAAACAATTTATCAAT	-545 to -516
12	TACACATCATAAATTTAAAAAATATTTCTT	-496 to -467
13	TTGATTGTGTGTGAAAATAAAAATAGAAAA	-466 to -437
14	TCAGAAAATAGATAAAGACACCACTATCAA	-434 to -405
15	AAAGACACCACTATCAAAATGGGAAGTTTA	-421 to -392
16	CAAGGGGTTGGTGGTGGTGGTATGCCACAT	-341 to -312
17	ACCGTCAAATTGTATGTGTTTAAAATTCTA	-297 to -268
18	TATTAGTGGTTTTTCTTTAAATCATACACA	-266 to -237
19	AACCCCACAATTACATATTCAAAATTTTCT	-227 to -198
20	GTTTAATTTTTTTATTTTTATTTTTATTTT	-396 to -367
21	TATTTTTTTTTTTGGTATTTATTTTTAACA	-372 to -343
22	ATTAAAAGAAGCTTGAAAAAGCTGAAAACA	-555 to -526
23	ATTTAATTTAACCGTCAAATTGTATGTGTTTAAAATTCTAATATTAGTGG	-307 to -258
24	GTTTAAAATTCTAATATTAGTGGTTTTTCTTTAAATCATACACAAAAAAA	-280 to -231
25	CTTTAAATCATACACAAAAAAAAAAAACCCCACAATTACATATTCAAAAT	-252 to -203

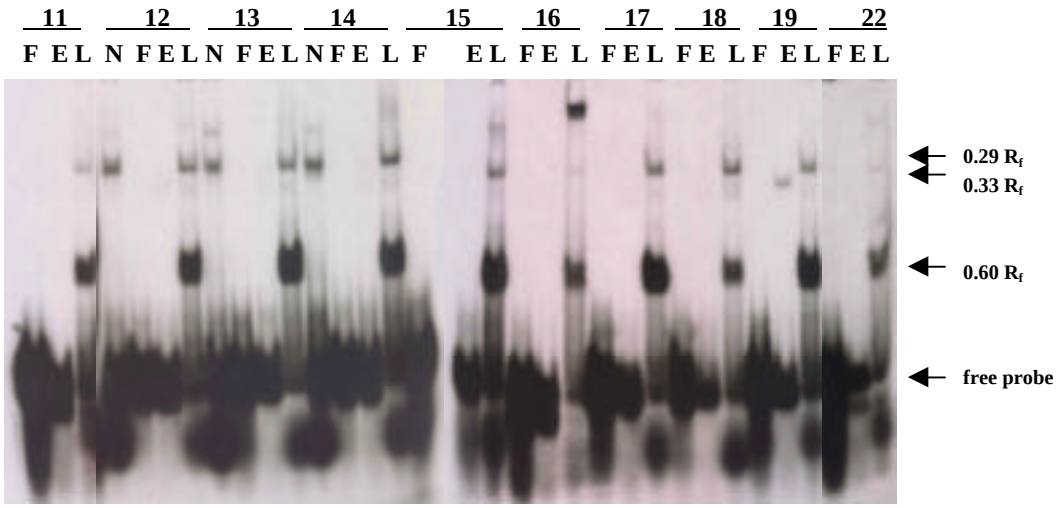
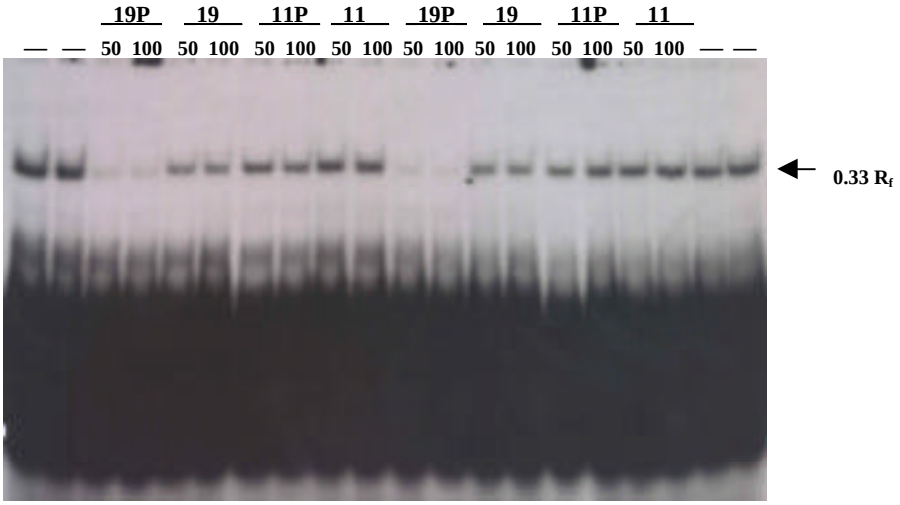


Figure 16. Gel shift assay of +cAMP nuclear extracts from heparin column using various probes. Nuclear extract was prepared from cells shaken in the presence (+) of cAMP. The extract was subjected to heparin sepharose chromatography. The flow through volume was collected then the bound proteins were eluted as described in Materials and Methods. Fractions 3 to 27 and fractions 28 to 34 of the eluted volume were separately pooled and concentrated. Gel shift assays were performed with ^{32}P-labeled probes 11 to 19 and 22. N, nuclear extract before heparin chromatography; F, flow through; E, early eluting fractions (3-27); L, late eluting fractions (28-34).

–227 bp and –198 bp of the 5nt promoter. All probes produced two additional bands, 0.29 R_f and 0.60 R_f, in the late fractions of the heparin column (Fig. 16). Therefore these bands were non-specific. The 0.29 R_f band was also visible in the nuclear extract before column fractionation (lanes labeled N in Fig. 16) when assayed with probes 12, 13 and 14. However the 0.60 R_f band was absent in the pre-column nuclear extract (Fig. 16). It should be noted that some additional bands, e.g. a low mobility band in the late fractions with probe 16, were also visible. However these were not observed consistently suggesting that artifacts can arise in the gel shift analysis.

The 0.33 R_f band was then characterized with respect to specificity, stability and ability to bind under a variety of reaction parameters. To determine the specificity of the binding, several different competition experiments were performed. Probe 19 was incubated with early heparin column fractions in the absence and presence of various unlabeled DNA fragments as competitors. As expected, the 0.33 R_f band was observed when no competitor was used. This band was reduced when unlabeled excess oligonucleotide 19 in the phosphorylated form was added to the binding reaction as the competitor (Fig. 17A, lanes labeled 19P). Phosphorylated form of the oligonucleotide was used as competitor in order to imitate the probe molecule in the reaction as much as possible. The non-phosphorylated form of the oligonucleotide 19 also competed with the binding to some extent but the level of competition was not as high as using the phosphorylated oligonucleotide. Therefore, the phosphorylated form of the oligonucleotide 19 was a better competitor than the non-phosphorylated form. The effect on the binding was nearly complete with both 50 fold and 100 fold molar excess competitor (Fig. 17A). Little or no competition was observed with oligonucleotide 11 in either the phosphorylated or non-phosphorylated form.

A)


19P 19 11P 11 19P 19 11P 11
— — 50 100 50 100 50 100 50 100 50 100 50 100 50 100 50 100 — —
0.33 R_f

B)

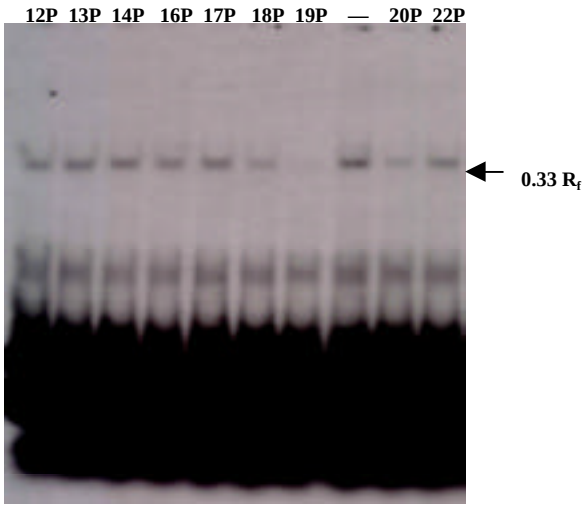

12P 13P 14P 16P 17P 18P 19P — 20P 22P
0.33 R_f

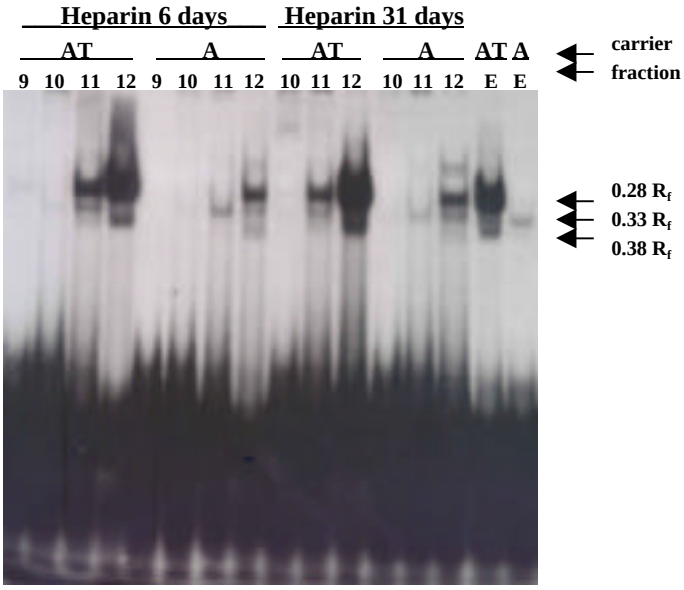
Figure 17. Competition experiments of the 0.33 R_f band by various unlabeled DNA fragments. Probe 19 was used in all reactions. The extract was the early heparin column fractions as described in Fig. 18. Lanes with the symbol '—' have no competitor. **A)** Competition with phosphorylated versus non-phosphorylated competitors. Unlabeled excess competitors (either non-phosphorylated or phosphorylated prior to use) are shown. P indicates the phosphorylated form of the competitors. The competitors were used at 50 and 100 fold molar excess. The first 10 and the last 10 lanes are the same except that in the first half the extract was added to the binding reaction last, whereas in the second half the extract was added followed by competitor and probe. **B)** Competition with various competitors. Numbers above gel lanes indicate the unlabeled excess competitor (all in phosphorylated form). The competitors were used at 70 fold molar excess. The extract was added to the binding reaction last.

Additional competition experiments were performed using several different unlabeled DNA fragments, all of which were phosphorylated prior to use. Again, the oligonucleotide 19 showed the highest degree of competition (Fig. 17B). These competition experiments as well as the binding results from Fig. 16 (see also Fig. 19B) suggest that the 0.33 R_f band has specificity for the nucleotide sequence contained in probe 19.

To determine the stability of the 0.33 R_f band during storage, heparin column fractions were held at 4°C for either 6 or 31 days (Fig. 18A). The 0.33 R_f band is visible in fractions 10, 11 and 12 with the highest visibility present in fraction 11. This band was present at the same intensity in both heparin column fractions showing that the binding protein(s) was quite stable at 4°C (Fig. 18A, compare the intensity of the bands from Heparin 6 days to Heparin 31 days).

Figure 18A also shows the effect of the non-specific carrier DNA molecules polydA.dT-polydA.dT or polydA-polydT. Fractions 9, 10, 11 and 12 from the early elution of the heparin column (Fig. 16) were incubated with probe 19 in the presence of the two non-specific competitors. When assayed in the presence of polydA.dT-polydA.dT as the non-specific competitor the 0.33 R_f band is almost non-detectable as it is obscured by the two non-specific bands at R_f 0.28 and 0.38 (Fig. 18A, see fractions 11 and 12). However, when assayed in the presence of polydA-polydT as the non-specific competitor the intensity of the 0.28 R_f and 0.38 R_f bands were severely reduced, whereas the intensity of the 0.33 R_f band is not changed. The effect of the non-specific competitor is even more evident when the early eluting heparin sepharose fractions were pooled and concentrated, then assayed for binding to probe 19 (Lanes labeled E in Fig. 18A). When assayed in the presence of polydA.dT-polydA.dT the 0.33 R_f band was partially obscured

A)


Heparin 6 days
Heparin 31 days
AT
A
AT
A
AT A
9 10 11 12 9 10 11 12 10 11 12 10 11 12 E E
carrier fraction
0.28 Rf
0.33 Rf
0.38 Rf

B)

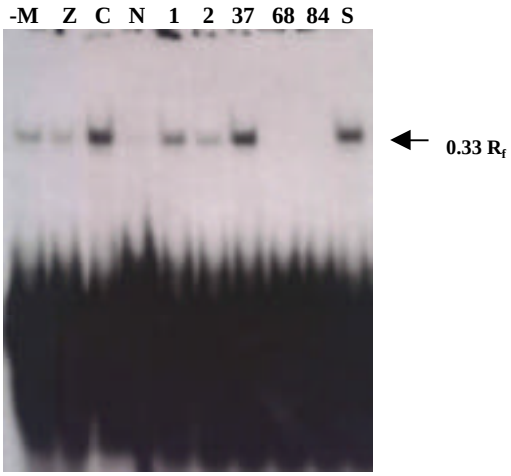

-M Z C N 1 2 37 68 84 S
0.33 Rf

Figure 18. Effects of various conditions on the 0.33 R_f band. Probe 19 was used in all reactions. **A)** Effects of stability and different non-specific carrier DNAs. Heparin 6 days, heparin column fractions (of +cAMP cytoplasmic extract) stored at 4°C for 6 days prior to assay. Heparin 31 days, heparin column fractions (of +cAMP cytoplasmic extract) stored at 4°C for 31 days prior to assay. Numbers 9-12 represent column fractions. E, pooled and concentrated early heparin fractions (of +cAMP nuclear extract); AT, non-specific carrier polydA.dT-polydA.dT; A, non-specific carrier polydA-polydT. **B)** Effects of various metal ions, salt concentration, different concentrations of non-specific carrier and temperature. The extract was the early heparin column fractions (of +cAMP nuclear extract). In reactions 37, 68 and 84, the extract was pre-heated at indicated temperatures for 10 min prior to assaying. S, standard binding reaction as described in Material and Methods (including magnesium, 20 mM NaCl, 0.5 μg polydA.polydT). –M, no magnesium; Z, 1 mM $ZnCl_2$; C, 1 mM $CaCl_2$; N, 100 mM NaCl; 1, 1 μg polydA.polydT; 2, 2 μg polydA.polydT; 37, 37°C; 68, 68°C and 84, 84°C.

by the 0.28 R_f band, while in the presence of polydA-polydT only the 0.33 R_f band was present. These results show the importance of the non-specific competitor in gel shift assays. In fact, the 0.33 R_f band would not have been detected at all if only polydA.dT-polydA.dT had been used in the initial screening for proteins binding to the *5nt* promoter. Therefore, polydA-polydT was the preferred non-specific DNA to detect the 0.33 R_f band in gel shift experiments.

The effects of various gel shift assay conditions on the binding of the 0.33 R_f band were also tested. Probe 19 was incubated with early heparin column fractions of +cAMP nuclear extract in the absence and presence of various metal ions, high salt, increased concentrations of non-specific carrier and pre-heated extract (Fig. 18B). Both magnesium and calcium at 1 mM concentrations induced the 0.33 R_f band whereas Zn had no effect. NaCl at a concentration of 100 mM inhibited the binding. Increased concentrations of polydA.polydT decreased the binding to some extent. Moreover, heating the extract at 37°C for 10 min prior to adding to the reaction had no effect indicating that the protein(s) survived this temperature for 10 min. However pre-heating the extract at 68°C and 84°C had inhibitory effects suggesting instability of the protein(s) at these temperatures (Fig. 18B).

In order to obtain information about the pI (isoelectric point) of the protein(s) that specifically bind(s) to probe 19 and, thereby, to determine the type of ion exchanger that could be used for purification of this protein, the protein's ionic characteristics were determined. The 0.33 R_f band was tested for its binding characteristics on an anion (DEAE sephacel) and a cation (CM cellulose) exchange resin at various pHs (pH 5, 6, 7, 8 and 9). Equal volumes of the extract (dialyzed at a specified pH) and the equilibrated resin were mixed in a total volume of 30 µl and incubated for 15 min at room

temperature. The tubes were then centrifuged and the supernatants were assayed in gel shift. The 0.33 R_f band was not present in DEAE supernatants at any pH. The band was not observed in the CM supernatant at pH 5 but was present in CM supernatants at pH 6 to 9 (data not shown). This indicated that binding to the resin did not occur in CM resin at pH 6-9 and that the protein did bind to DEAE resin over this pH range. To determine if the protein was binding to the resins, and if so, whether it could be recovered, proteins were eluted from each resin using 0.5 M NaCl and the supernatants containing eluted proteins were tested using probe 19. The 0.33 R_f band was not present in CM supernatants, in agreement with the previous assay (data not shown). The activity was recovered in DEAE eluted samples at pH 5-8 but less activity was recovered at pH 9. This showed that the binding to DEAE resin occurred, the protein could be eluted with 0.5 M NaCl, and that the protein was negatively charged at pH 5-9.

A cytoplasmic extract prepared from aggregation/slug cells was loaded on a DEAE sephacel column (pH 7.5) as described in Materials and Methods and fractions were tested with the gel shift assay using probe 19. The 0.33 R_f band activity was observed in the early eluting fractions of the salt gradient (Fig. 19A). When specificity of the binding was tested with different unlabeled fragments, only oligonucleotide 19 competed (Fig. 19B), again suggesting that the 0.33 R_f protein has binding specificity for the nucleotide sequence within oligonucleotide 19. The bands at 0.07 and 0.62 R_f (Fig. 19A) were shown to be non-specific.

The initial attempts to identify a factor that bound to the *5nt* promoter utilized aggregation/slug extracts because *5nt* is expressed only during cell differentiation. However, preparation of sufficient quantities of cell extracts from differentiated cells is time consuming and laborious. Undifferentiated amoebae, on the other hand, can be

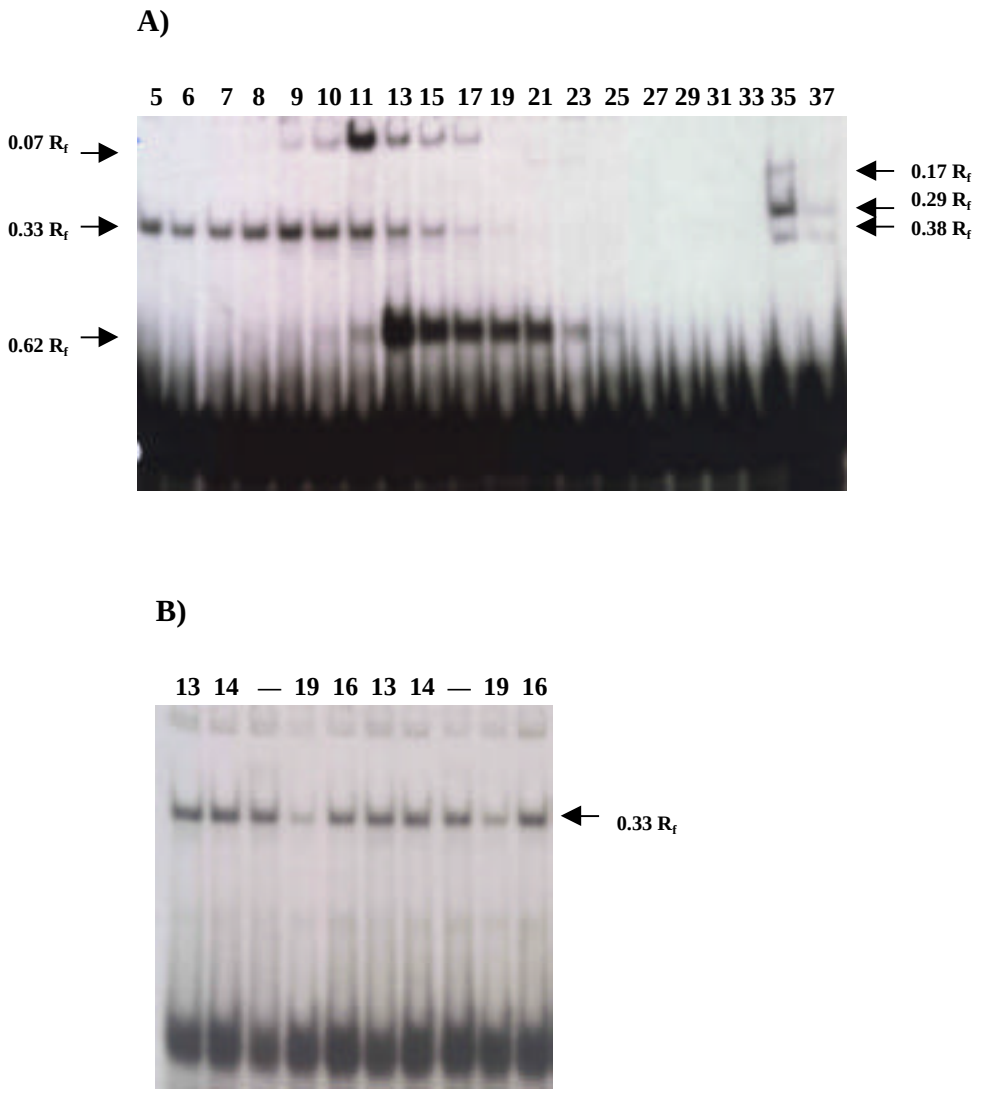


Figure 19. Gel shift assay of fractions eluted from DEAE sephacel column. A) Cytoplasmic extract prepared from aggregation/slug cells was loaded on a DEAE column and aliquots of fractions were tested for binding to probe 19. Numbers above gel lanes indicate fractions. **B)** Competition assay. The above DEAE fractions 5 to 10 were pooled, concentrated and assayed for competition. Numbers above gel lanes indicate unlabeled excess competitor. Lane with the symbol '—' has no competitor.

prepared in kilogram quantities quite readily. To determine if the 0.33 R_f binding activity was also present in amoebae cells, a cytoplasmic extract prepared from vegetative cells was applied to a heparin column and fractions were tested for binding to probe 19 and 15. The 0.33 R_f band was observed in fractions 9 to 15 using probe 19 but not probe 15 (Fig. 20). This indicated the presence of the protein(s) in amoebae cells. A non-specific band with 0.38 R_f was also observed with both probes (Fig. 20).

The cytoplasmic extract prepared from both vegetative and slug stage cells were fractionated using ammonium sulfate (AS) and each fraction was tested in gel shift assays. This fractionation should provide a convenient initial step in the purification procedure. The 0.33 R_f band was observed using probe 19 in the resuspended pellet of the 80% AS fraction of both vegetative and slug cell extracts (Fig. 21). Two additional bands, 0.26 R_f and 0.38 R_f, were also observed in the 60% AS fraction of slug extract. The two bands were diminished in the 80% fraction along with enhanced appearance of the 0.33 R_f band in both amoebae and slug extracts.

3.7.2. Detection of a Gel Shift Band with 0.13 R_f Using Probe 6 and 25

Probes that overlap oligonucleotide 19 were used to determine if additional factors were present in the extracts. A 0.13 R_f band was observed in both cytoplasmic and nuclear protein extracts with probe 6 (Fig. 22). Probe 6 was a PCR fragment of 105 bp. This band was not present with either probe 1 (104 bp) or 3 (91 bp) (Fig. 22). The band was also not detected using probe 2 (144 bp) (data not shown). The extracts used in this experiment were prepared from cells induced by cAMP (+cAMP). However, the same band was also detected using aggregation and slug stage cells.

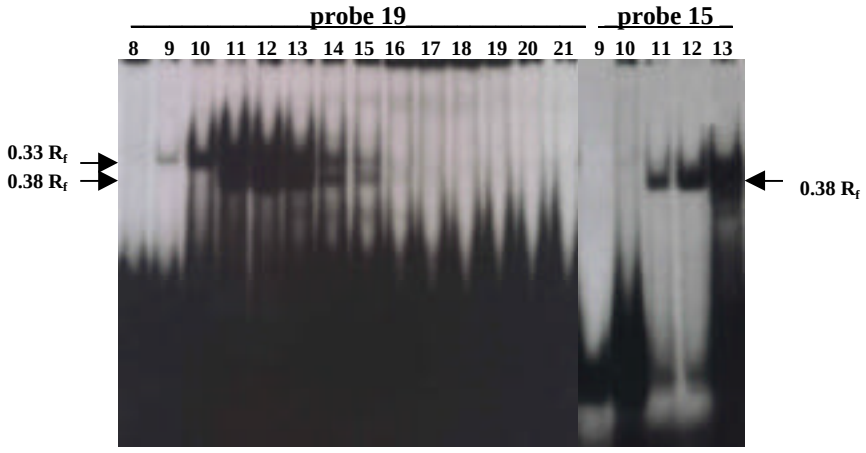


Figure 20. Gel shift assay of fractions of amoebae cytoplasmic extract eluted from heparin sepharose column. Cytoplasmic extract prepared from vegetative amoebae cells was subjected to a heparin sepharose chromatography and samples of fractions were tested for binding to probe 19 and 15. Numbers above gel lanes indicate fractions.

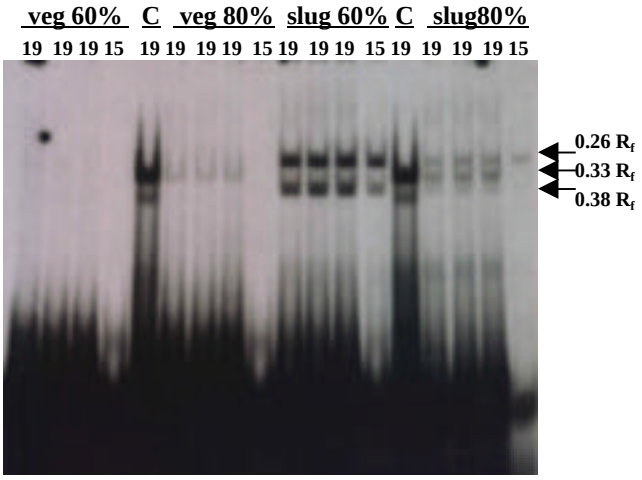


Figure 21. Ammonium sulfate fractionation of cytoplasmic extract from vegetative amoebae and slug cells. Cytoplasmic extract prepared from vegetative amoebae (veg) and slug stage cells were fractionated by ammonium sulfate (AS). The pellets of the 60% and 80% AS precipitation were resuspended and tested using probe 19 and 15 in gel shift assays. C, control extract [fraction 11 from Heparin column of amoebae cytoplasmic extract, (see Fig. 23)].

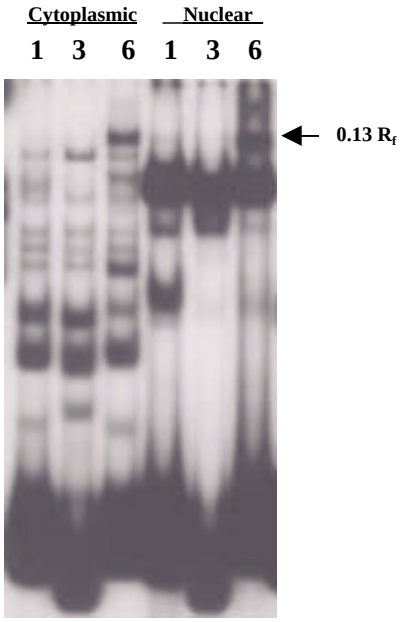


Figure 22. Gel shift assay of cytoplasmic and nuclear extracts using various probes. Cytoplasmic and nuclear extracts were prepared from cells shaken in the presence of cAMP. The gel shift assay was performed with ^{32}P-labeled probes 1, 3 and 6. Arrows indicate a band of 0.13 R_f present in both cytoplasmic and nuclear extracts using probe 6.

The 0.13 R_f band was also present in –cAMP extracts though with relatively less intensity as the +cAMP extracts (data not shown). As described earlier, even though no cAMP was added to –cAMP flasks during protein extraction, these cultures were grown in non-nutrient buffer for almost 24 hr. Cells in this condition of low nutrients produce and secrete cAMP, even if they are unable to aggregate and undergo development, which in turn stimulates signal transduction pathways that may result in the activation of certain promoters. Therefore, the presence of 0.13 R_f band in –cAMP extracts may not be unexpected. On the other hand, it should be noted that very low luciferase activity was detected in cells shaken in the absence of cAMP (see Fig. 12). Therefore, *in vivo* cAMP is required for full expression of the *5nt* gene.

To determine the specificity of the binding, competition experiments were performed. Probe 6 was incubated with +cAMP cytoplasmic protein extract in the absence and presence of various unlabeled DNA fragments as competitors. The competitors were used at 100 fold molar excess and were added to the binding reaction immediately after the addition of the labeled probe. As expected, the 0.13 R_f band was observed when no competitor was used and this band was not observed when unlabeled excess fragment 6 was added to the binding reaction as the competitor (Fig. 23A). If unlabeled fragment 3 was used as competitor, however, the 0.13 R_f band was detected at the same intensity compared to the no competitor reaction (Fig. 23A). These results indicated that the protein factor(s) present in the cytoplasmic extract bound to probe 6 specifically.

Four different 30 bp oligonucleotides were also used as competitors. Three of these oligonucleotides, 17, 18 and 19, partially overlapped with probe 6 whereas 16 did not. Oligonucleotide 16 did not compete with the binding as expected (Fig. 23A).

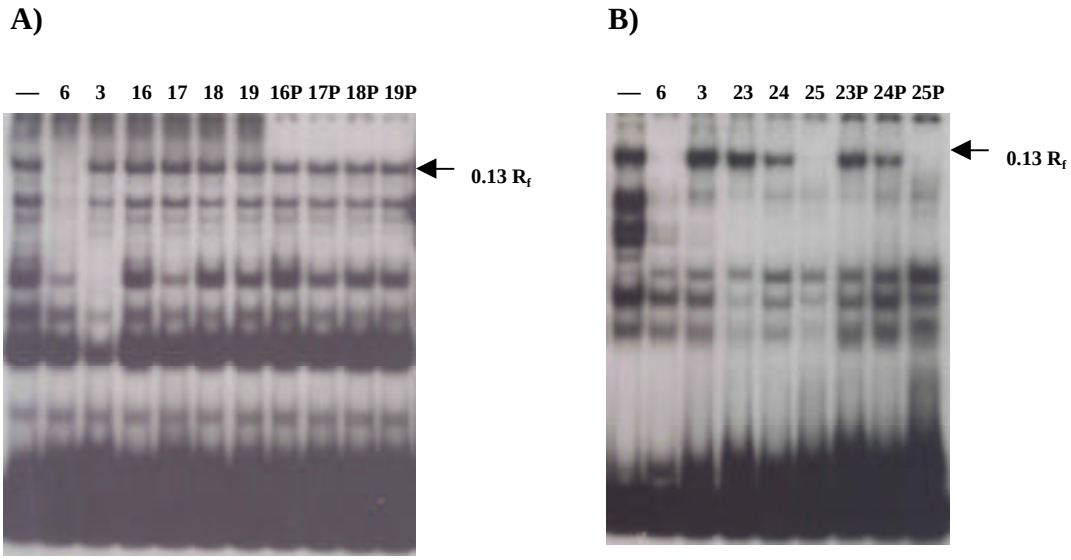


Figure 23. Competition experiment of the 0.13 R_f band by various unlabeled DNA fragments. Probe 6 was used in all reactions containing +cAMP cytoplasmic extract. Probe was added immediately after addition of the competitor DNA (100 fold molar excess). Numbers above gel lanes indicate competitors. Lanes with the symbol '—' have no competitor. Some competitor DNA fragments were phosphorylated prior to use. P indicates the phosphorylated form of competitors.

However, none of the other three oligonucleotides (17, 18 and 19) competed for binding (Fig. 23A). This result was not entirely unexpected because these oligonucleotides were relatively short, did not overlap each other and did not entirely cover the region represented by probe 6. Therefore, it is possible that the intact binding site was not represented by any of these oligonucleotides.

Moreover, in order to test the effect of phosphorylation, the 30 bp oligonucleotides were phosphorylated prior to the competition assays. As mentioned earlier, phosphorylated oligonucleotides were used as competitors to imitate the binding conditions as much as possible. However, no competition was observed using unlabeled excess phosphorylated oligonucleotides as competitors (Fig. 23A).

Additional competition experiments were performed using different unlabeled DNA fragments as described in 2.12.2. Again, fragment 6 competed with itself and fragment 3 did not compete with labeled probe (Fig. 23B). In addition, the 50 bp oligonucleotides, 23, 24 and 25, were also used as competitors. These oligonucleotides overlapped with probe 6. Oligonucleotide 23 did not compete, however inclusion of 25 lowered binding to labeled probe 6 considerably (Fig. 23B). This result suggested that the binding site was located within the region of probe 6 that overlapped with oligonucleotide 25. This sequence represents the region between –252 bp and –203 bp of the *5nt* promoter. Furthermore, there was an intermediate level of competition using oligonucleotide 24 (Fig. 23B). Oligonucleotides 24 and 25 overlap in a region of 22 bp, suggesting that this sequence may be involved in the binding. Identical results were obtained using phosphorylated 23, 24 and 25 as competitors (Fig. 23B).

In summary, gel shift assays indicated the presence of two bands (0.33 R_f and 0.13 R_f). The 0.33 R_f band was observed with probe 19 in both cytoplasmic and nuclear

extracts isolated from vegetative, aggregation and slug stage cells as well as from cAMP-induced cells. The 0.13 R_f band was observed with probe 6 and probe 25. This band was present in both cytoplasmic and nuclear extracts isolated from cAMP-induced and aggregation/slug stage cells. Competition assays showed the specificity of binding on both activities. These results led to the attempt to purify the protein factor(s) involved in these specific DNA-binding activities.

3.8. Purification of Probe 19-binding Protein(s)

In order to purify the protein factor(s) that specifically bind(s) to probe 19, a cytoplasmic protein extract prepared from slug stage cells was first fractionated by ammonium sulfate (AS) to obtain 80% cutoff fraction. The 80% $(NH_4)_2SO_4$ fraction was then subjected to ion exchange chromatography using a DEAE sephacel column. The 0.33 R_f band eluted at approximately 0.2 M NaCl. The highest activity was detected in the fraction 20 (Fig. 24). There was no activity detected in the flow through (F1, F2). In addition, a phosphatase activity was observed mainly in fractions 14 to 18 revealed from the release of the radioactive inorganic phosphate (Fig. 24). This phosphatase activity was also analyzed later in the purification steps because of the fact that the 0.33 R_f band and phosphatase activities eluted very closely in the salt gradient and that the two activities could be the same. Moreover, as stated above, the phosphorylated form of the oligonucleotide 19 was a better competitor than the non-phosphorylated form (see Fig. 17A), which suggests that the 0.33 R_f band activity may be the result of a phosphatase.

The DEAE fractions, 17 to 23, were pooled, concentrated and further purified by a heparin sepharose chromatography (Fig. 25). The 0.33 R_f band eluted at approximately 0.12 M NaCl. The highest activity was detected in fraction 17 (Fig. 25). Also, the

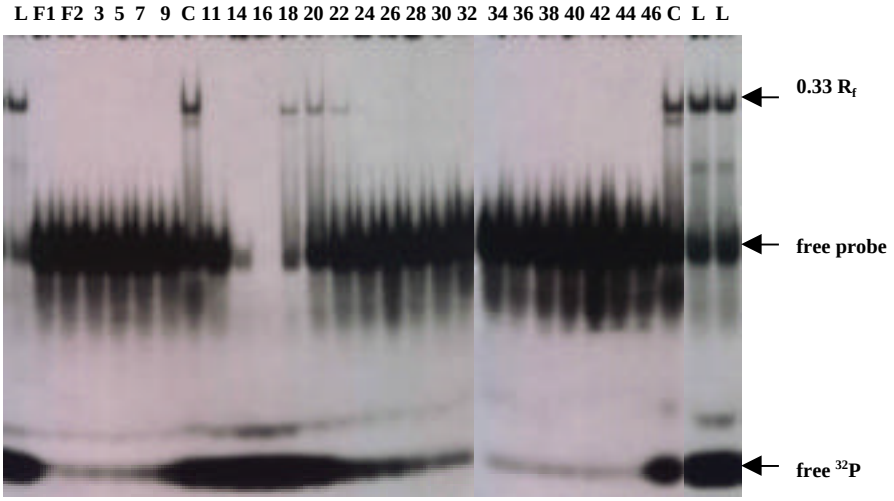


Figure 24. Gel shift assay of fractions eluted from DEAE sephacel column. The 80% ammonium sulfate cutoff sample from slug cytoplasmic extract was subjected to a DEAE sephacel chromatography and fractions were tested for binding to probe 19. L, load; F1, first flow through; F2, second flow through; 3-46, fraction number; C, control extract [fraction 11 from heparin column of amoebae cytoplasmic extract, (see Fig. 23)].

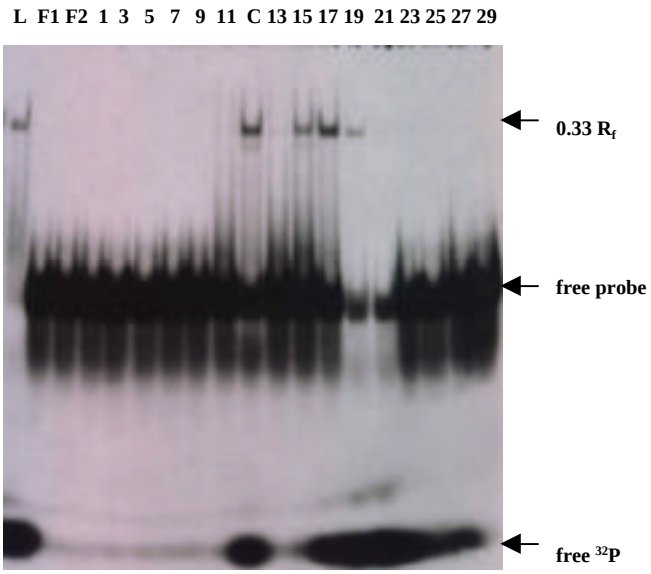


Figure 25. Gel shift assay of fractions eluted from heparin sepharose column. Active DEAE fractions were pooled and loaded on a heparin column and fractions were tested for binding to probe 19. Flowthrough (F1 and 2) and every other fraction from fraction 1 to 29 were assayed alongside with the sample that was loaded on the column (L). C, control extract [fraction 11 from heparin column of amoebae cytoplasmic extract, (see Fig. 23)].

phosphatase activity previously seen on the DEAE column (see Fig. 24) was located in fractions 19-21 of the heparin column (Fig. 25).

The heparin column fractions containing DNA binding activity (fractions 14 to 19) were pooled, concentrated and subjected to a DNA affinity chromatography using the oligonucleotide 19 ligand to separate the protein(s) of interest. The 0.33 R_f band eluted at approximately 0.05 M NaCl in fractions 13 to 15 (Fig. 26). However most of the activity was in the flow through. This may have been due to either overloading of the column or inefficient binding. In addition, it was noted that the phosphatase activity was mostly in the flow through and did not bind to the affinity column (Fig. 26). The flow through samples (because most of the 0.33 R_f band activity was in the flow through) were pooled, concentrated and then loaded with four passes back on the affinity column. The 0.33 R_f band eluted in fractions 15 to 19 (data not shown).

The active affinity column fractions were pooled, concentrated and then loaded on a gel filtration column. The DNA binding activity was in fraction starting from 14 and peaking in fractions 15 and 16 and continuing through fraction 26 (Fig. 27). It should be noted that the fractions were only 0.2 ml in volume. Thus, although the activity appears to be spread over a number of fractions the protein was recovered in only about 2 ml. Figure 27 also shows that the phosphatase activity eluted in fractions 17 and 18 (as determined by the loss of radioactivity from the unbound probe), different fractions than the 0.33 R_f band-containing fractions, which indicated that the 0.33 R_f band activity was not the result of a phosphatase.

Samples of the gel filtration fractions 12 to 20 were then run on a 10% SDS-polyacrylamide gel electrophoresis. A band of approximately 70 kDa was present starting from fraction 14 with highest intensity in fraction 16 (Fig. 28). This banding

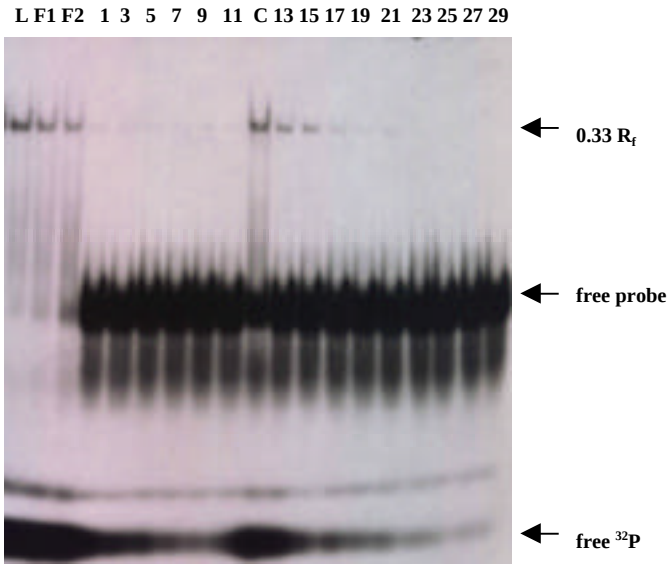


Figure 26. Gel shift assay of fractions eluted from DNA (oligonucleotide 19) affinity column. Active heparin column fractions were pooled and subjected to a DNA affinity chromatography and fractions were tested for binding to probe 19. Flow through (F1, F2) and every other fraction from fraction 1 to 29 were assayed alongside with the sample that was loaded on the column (L). C, control extract [fraction 11 from heparin column of amoebae cytoplasmic extract, (see Fig. 23)].

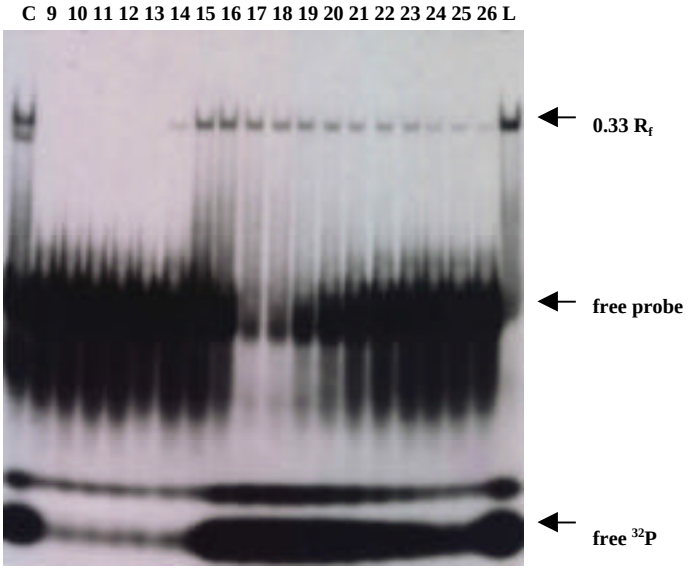


Figure 27. Gel shift assay of gel filtration column fractions. Active affinity column fractions were pooled and subjected to a gel filtration chromatography and fractions were tested for binding to probe 19. Fractions 9 to 26 are shown. C, control extract [fraction 11 from heparin column of amoebae cytoplasmic extract, (see Fig. 23)]; L, load.

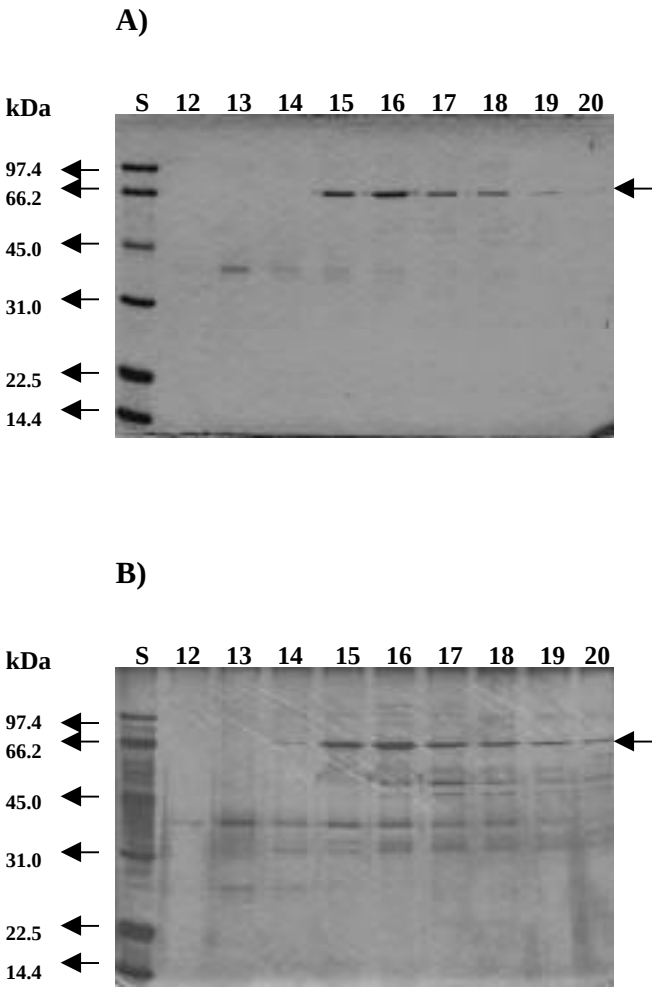


Figure 28. SDS-PAGE analysis of gel filtration column fractions. Samples of gel filtration fractions 12 to 20 were analyzed by 10% denaturing polyacrylamide gel electrophoresis. S, standard protein markers. The arrow points to an approximately 70 kDa band starting from fraction 14 with most intensity in fraction 16. **A)** Coomassie Blue stain. **B)** Silver stain.

pattern was consistent with the gel shift activity of the same fractions. The gel filtration fractions 15 to 17 were pooled, concentrated and estimated to contain about 4 μg total protein. The protein sample was then sent to the Mass Spectrometry Laboratory in the Department of Chemistry and Pathology at the University of Virginia for mass spectrometric analysis.

3.8.1. Formyltetrahydrofolate Synthase

Mass spectrometry analysis of the 0.33 R_f band yielded numerous peptide products (see Appendix B). Using these peptide sequences, a blast search in the *Dictyostelium* genomic database revealed that the purified protein was a putative formyltetrahydrofolate synthase (FTHFS).

FTHFS enzymes are involved in nucleotide transport and metabolism (Nour and Rabinowitz, 1992). The enzyme catalyzes the inter-conversion of reduced forms of folate to supply activated one carbon units for a variety of metabolic pathways. In eukaryotes the three functions of the enzyme (synthetase, cyclohydrolase and dehydrogenase) are found in a single polypeptide of approximately 100 kDa that exists as a homodimer. In bacteria the same reactions are usually catalyzed by separate mono (synthetase activity) and bifunctional (cyclohydrolase and dehydrogenase activities) polypeptides (Appling and Rabinowitz, 1985; Nour and Rabinowitz, 1992).

The putative *Dictyostelium* enzyme, which has not yet been studied, contains 638 amino acid residues (see Appendix C) and has very high homology to FTHFS enzymes in other organisms. The putative *Dictyostelium* FTHFS has 60%, 64% and 65% protein sequence identity to yeast, mouse and *Arabidopsis* enzymes, respectively. A 65% identity exists between *Dictyostelium* and human FTHFS enzymes.

Interestingly, DNA binding activity has been reported previously for FTHFS (Wahls *et al*., 1993). Studies on the *Schizosaccharomyces pombe* enzyme indicated a specific single-stranded DNA binding activity. The enzyme did not, however, bind to double-stranded DNA or RNA. The ssDNA binding was shown to be sequence-independent and not inhibited by the substrates or cofactors of the enzyme. Furthermore, the binding of the protein to the ssDNA occurred internally and required approximately 20-30 bases. Wahls *et al*. also showed that this single-strand binding activity was conserved among various species and was possibly carried out by the synthetase domain of the enzyme.

In order to determine if the 0.33 R_f band could be detected using a single-stranded probe, complementary single strands of oligonucleotide 19 were separately labeled and incubated with the purified sample. Interestingly, the forward probe (F19), but not the reverse probe (R19), resulted in the same shifted band as the double-stranded probe (Fig. 29A). This result indicated that the protein binds the single-stranded probe as well as double-stranded and that the binding is possibly sequence-dependent. However, the result of the competition experiments was the opposite. The reverse oligonucleotide but not the forward oligonucleotide competed with the double-stranded probe for binding of the protein(s) (Fig. 29B).

It was previously observed that ssDNA binding activity can be observed with a dsDNA probe if residual ssDNA is present in the probe preparation (Wahls *et al*., 1993). In our gel shift experiments the double-stranded probe was prepared by annealing two complementary single strands and then labeling the 5' ends with radioactive ATP using a polynucleotide kinase. It is possible that a residual amount of single-stranded DNA, which could not be detected on 5% polyacrylamide gels, could be present in the probe

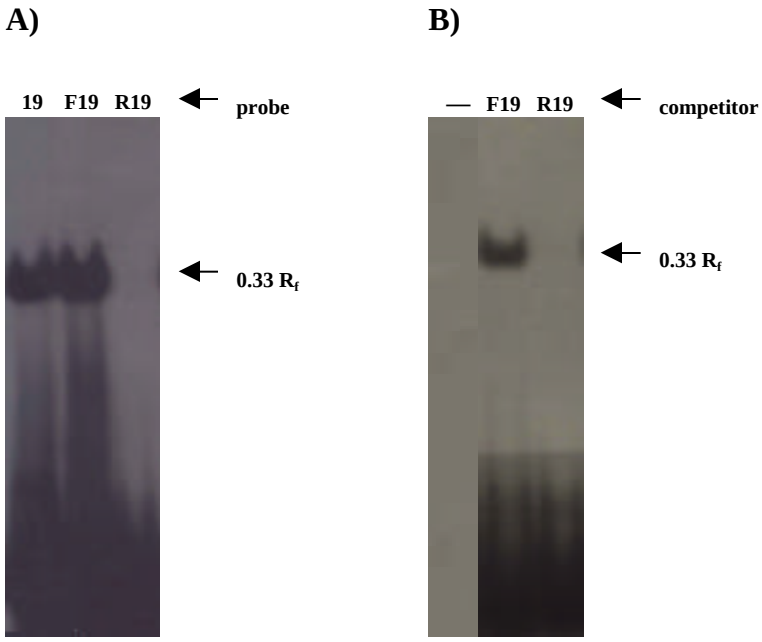


Figure 29. Gel shift assay using single-stranded probe 19. The extract was fraction 15 of gel filtration column (see Fig. 32). **A)** Single-stranded DNA as probe. Complementary strands (F19 and R19) of probe 19 were separately labeled and used as probes alongside with the double-stranded probe (19). F, forward; R, reverse. **B)** Competition assay using single-stranded DNA. Double-stranded probe 19 was used in all reactions. Unlabeled complementary strands of probe 19 were used as competitors (at 100 fold molar excess. Lane with the symbol '—' has no competitor.

preparation and therefore may result in the observed binding activity. However, the binding activity of the 0.33 R_f band occurred with approximately the same intensity with both ds and ss probes, suggesting that protein binds both DNAs in a similar manner. It is possible, however, that in the case of a limited amount of protein in the binding reaction the binding to two DNA species could not be distinguished. More experiments should be performed to obtain definitive answers to the question of whether the binding occurs to ds or ssDNA.

It is unclear whether the putative *Dictyostelium* FTHFS is involved in the regulation of the *5nt* expression. ssDNA binding proteins are involved in many significant biological processes, including recombination, DNA replication, DNA repair, transcription and translation. A biological role, if any, for the ssDNA binding activity of FTHFS remains to be determined.

As mentioned earlier, folic acid is known to be a chemoattractant and a regulator of gene expression in *Dictyostelium*. This molecule was shown to activate guanylyl cyclase through the stimulation of a G protein (van Haastert and Kuwayama, 1997), to regulate the expression of the *discoidin* genes (Blusch and Nellen, 1994) and to stimulate the activity of the *Dictyostelium* MAP kinase, ERK2 (Maeda and Firtel, 1997). The folate-activated ERK2 was shown to stimulate adenylyl cyclase activity (Maeda and Firtel, 1997). The involvement of folic acid in *Dictyostelium* development may suggest a role for FTHFS in the *5nt* expression.

3.9. Purification of Probe 25-binding Protein(s)

In order to purify the protein factor(s) that specifically binds to probe 25, cytoplasmic protein extract prepared from slug stage cells was first fractionated using

ammonium sulfate (AS) to obtain 20, 40, 60 and 80% cutoff fractions. Gel shift assays were performed using various probes to detect the DNA binding activity. The 0.13 R_f band was observed mostly in the 40% AS fraction using probes 6 and 25 (Fig. 30). As expected, other probes did not produce this band. The 40% $(NH_4)_2SO_4$ fraction was then subjected to ion exchange chromatography using a DEAE sephacel column. The 0.13 R_f band eluted at approximately 0.15 M NaCl. The highest activity was detected in fractions 16-20 (Fig. 31). There was very low activity detected in the flow through.

The DEAE fractions containing DNA binding activity (fractions 14 to 24) were pooled, concentrated and further purified by a heparin sepharose chromatography. The 0.13 R_f band eluted at approximately 0.22 M NaCl. The highest activity was detected in fractions 14-16 (Fig. 32).

The active heparin column fractions, 12 to 20, were pooled, concentrated and subjected to a DNA affinity chromatography using the oligonucleotide 25 ligand to bind the protein(s) of interest. The sample was loaded on the column by gravity, then was recycled 4 times to obtain maximum binding to the column. A 45 min linear gradient to binding reaction buffer containing 1 M NaCl was used to elute bound proteins. The 0.13 R_f band eluted at approximately 0.19 M NaCl. The highest activity was detected in fraction 20 (Fig. 33). There was little activity observed in the flow through. The active affinity column fractions, 17 to 24, were pooled, concentrated and then subjected to a gel filtration chromatography. The DNA binding activity was detected in fractions 36 to 48 with the highest activity in fractions 36-38 (Fig. 34).

In order to determine if the 0.13 R_f band could be detected using a single-stranded probe, complementary single strands of oligonucleotide 25 were separately labeled and incubated with the purified sample. The reverse oligonucleotide (R25) but not the

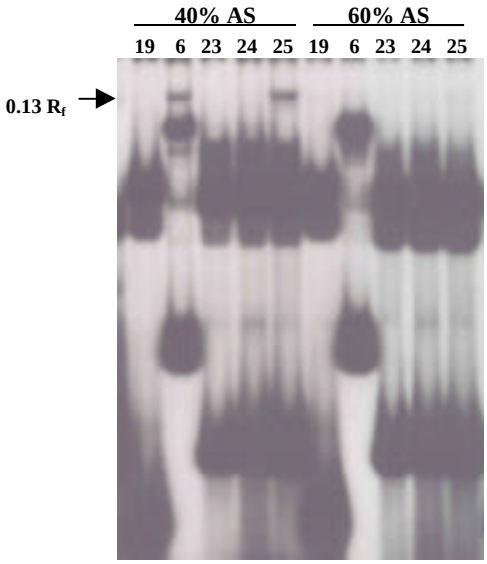


Figure 30. Ammonium sulfate fractionation of slug cytoplasmic proteins. Cytoplasmic proteins were extracted from slug stage cells and fractionated by ammonium sulfate (AS). The pellets of the 40% and 60% AS precipitation were resuspended and tested using different probes in gel shift assays.

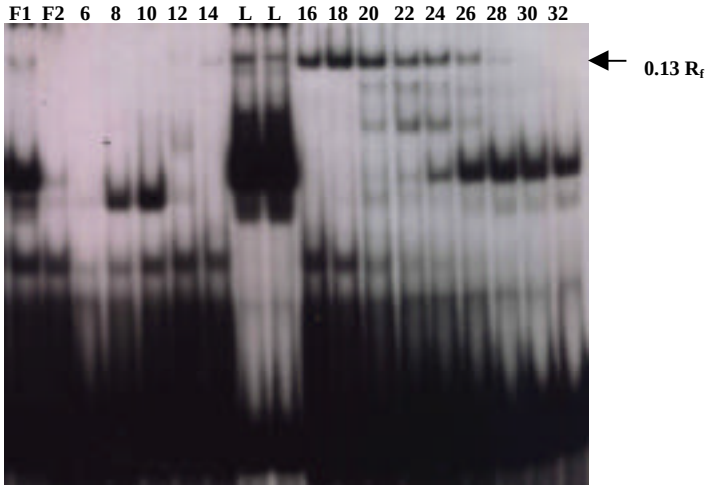


Figure 31. Gel shift assay of fractions eluted from DEAE sephacel column. Slug cytoplasmic 40% AS cutoff sample was subjected to a DEAE sephacel chromatography and fractions were tested for binding to probe 25. Flow through (F1 and 2) and every other fraction from fraction 6 to 32 were assayed alongside with the sample that was loaded on the column (L).

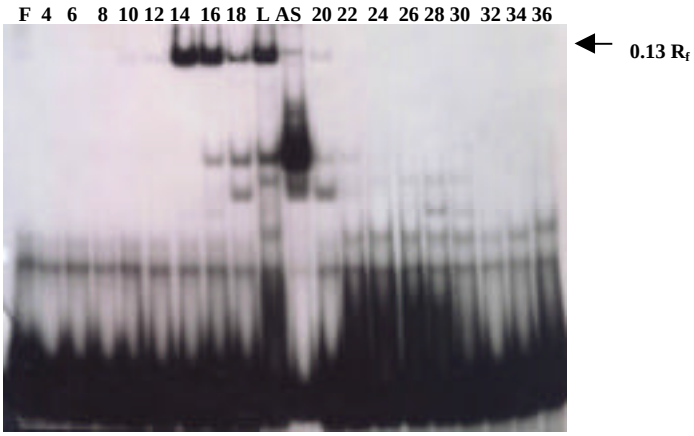


Figure 32. Gel shift assay of fractions eluted from heparin sepharose column. Active DEAE fractions were pooled and subjected to a heparin sepharose chromatography and fractions were tested for binding to probe 25. Flow through (F) and every other fraction from fraction 4 to 36 were assayed alongside with the sample that was loaded on the column (L) and slug cytoplasmic 40% AS cutoff (AS).

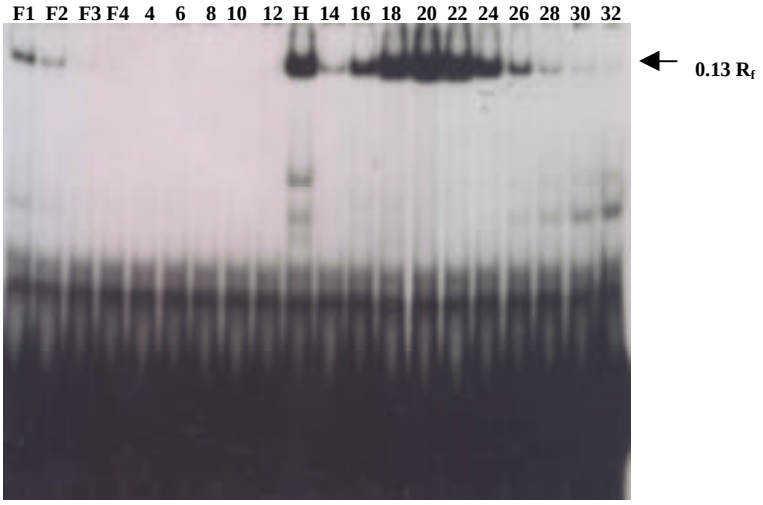


Figure 33. Gel shift assay of fractions eluted from DNA (oligonucleotide 25) affinity column. Active heparin column fractions were pooled and subjected to a DNA affinity chromatography and fractions were tested for binding to probe 25. Flow through (F1, 2, 3 and 4) and every other fraction from fraction 4 to 32 were assayed alongside with a sample of the heparin fraction 16 (H).

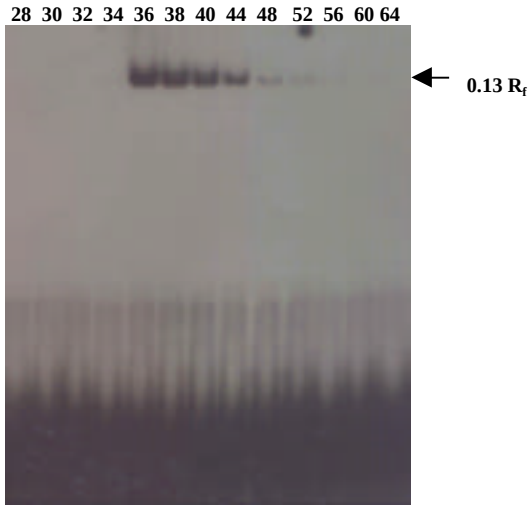


Figure 34. Gel shift assay of gel filtration column fractions. Active affinity column fractions were pooled and subjected to a gel filtration chromatography and fractions (28-64) were tested for binding to probe 25.

forward oligonucleotide (F25) resulted in the same shifted band as the double-stranded probe (data not shown). This result indicated that the protein(s) binds the single-stranded DNA as well as double-stranded and that the binding may be sequence dependent.

Aliquots of fractions 32 to 36 as well as fractions 38, 40, 44 and 48 were then fractionated on a 7.5% SDS-polyacrylamide gel electrophresis. After processing the gel with silver stain, no protein bands were observed in the lanes of the fractions 32, 33 and 34 (Fig. 35). Three bands, approximately 105 kDa, 100 kDa and 70 kDa in size, were visible in the fractions 35, 36 and 38. This banding pattern was correlated with the gel shift activity of the same fractions. The 70 kDa band was also visible in fraction 40, although at lower intensity. In addition, a low molecular weight band (33 kDa) was also detected in fractions 40 and 44 (Fig. 35).

The gel filtration fractions 35 to 38 were pooled, concentrated and estimated to contain about 30 ng total protein. Unfortunately, this amount of protein was not sufficient for analysis by mass spectrometry. Therefore, the protein purification procedure needs to be scaled up in order to obtain enough protein for an accurate protein sequence analysis. Also, it is possible that the three bands observed on the SDS-PAGE are the subunits of the same protein that might have been denatured during gel electrophoresis. Electrophoresis on native PAGE could be used to test this hypothesis.

3.10. Localization of Promoter Activity Using β-galactosidase

In order to determine the localization of *5nt* expression, the cloned *5nt* promoter (1,212 bp) was fused to *lacZ* in the reporter plasmid vector 63-iDQgal (Ubeidat *et al.*, 2002). The construct was fused in-frame to *lacZ* so that the construct contained the promoter region as well as the first 22 amino acids in the *5nt* coding sequence.

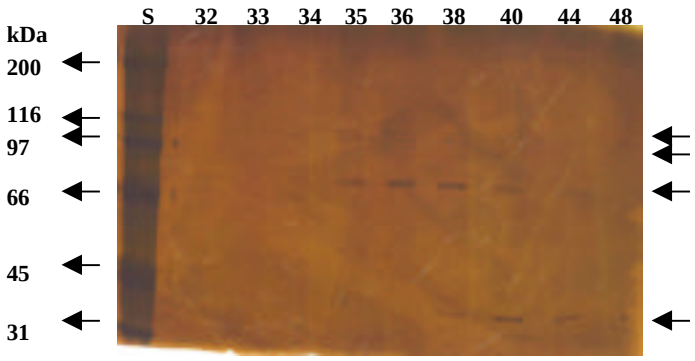


Figure 35. SDS-PAGE analysis of gel filtration column fractions. Samples of gel filtration fractions 32 to 48 were analyzed by a 7.5% denaturing polyacrylamide gel alongside with the standard protein markers (S). Protein bands were visualized with silver stain.

Dictyostelium cells transformed with 63-iDQgal carrying the *5nt* promoter-*lacZ* fusion were plated on nitrocellulose filters for development to the desired stage. Each stage of development was subjected to β-galactosidase staining for *in situ* localization of enzyme activity, as well as measurement of total enzyme activity in cell homogenates.

As shown in Figure 36, β-galactosidase activity was first detected during the aggregation stage, reached its highest level at the slug stage, and then remained constant during the late developmental stages. This pattern was identical to that of the endogenous *5nt* (Chanchao *et al*., 1999).

The localization of the *5nt* promoter activity during development is shown in Figure 37. The expression of *lacZ* appeared as soon as the cells start streaming toward the aggregation center (Fig. 37A). The aggregation center showed a higher proportion of cells stained for β-galactosidase activity than the cells in the streams. As stated earlier, the aggregation center is the site for the production of cAMP pulses, towards which cells start moving upon starvation. Thus, it is likely that the expression of *lacZ* in the early aggregates is the result of cAMP activation of the *5nt* promoter. In the tight aggregate, the cells expressing the fusion gene were concentrated in the upper 75% while the lower 25% were devoid of fusion gene activity (Fig. 37B). The origin of these cells in the upper 75% could not be determined because cells sort out during late aggregation and slug stages (Datta *et al*., 1986; Williams *et al*., 1989). The same pattern of expression has been previously shown for prestalk and prespore specific genes. Both the *D19* prespore-specific gene promoter and the *ecmA* prestalk-specific gene promoter was shown to drive *lacZ* expression only in the center of the tight aggregate (Williams *et al*., 1989; Kessin, 2001). However *5nt* expression is likely to be associated with the prestalk cells in the aggregate because there was no expression observed in spore cells in later stages.

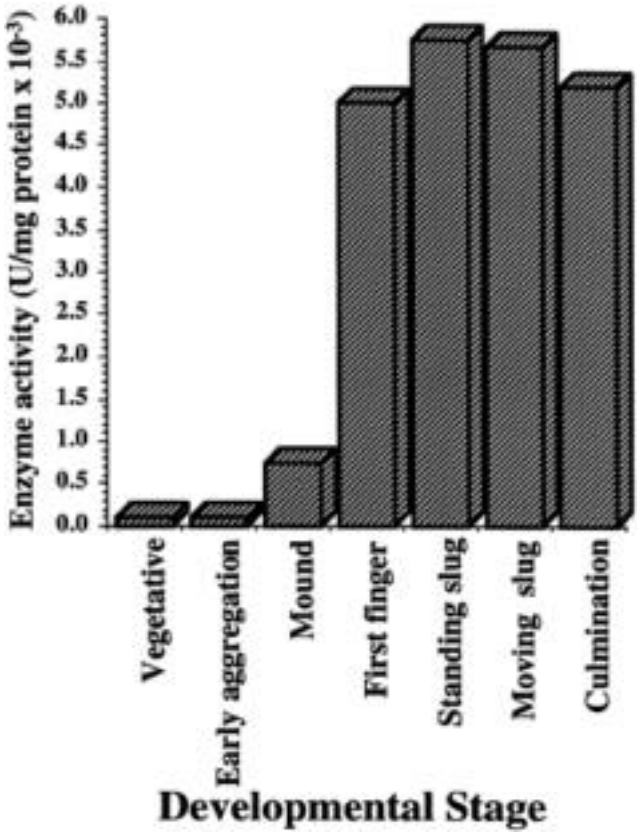


Figure 36. β-galactosidase activity during *Dictyostelium* development. *Dictyostelium* cells were transformed with 63-iDQgal vector carrying the *5nt* promoter(1,212 bp)-*lacZ* construct. Enzyme activity is expressed as the absorbance at 420 nm per min per mg protein (A_{420nm}/min/mg). The results are the average of triplicate determinations from different clones obtained from the same construct.

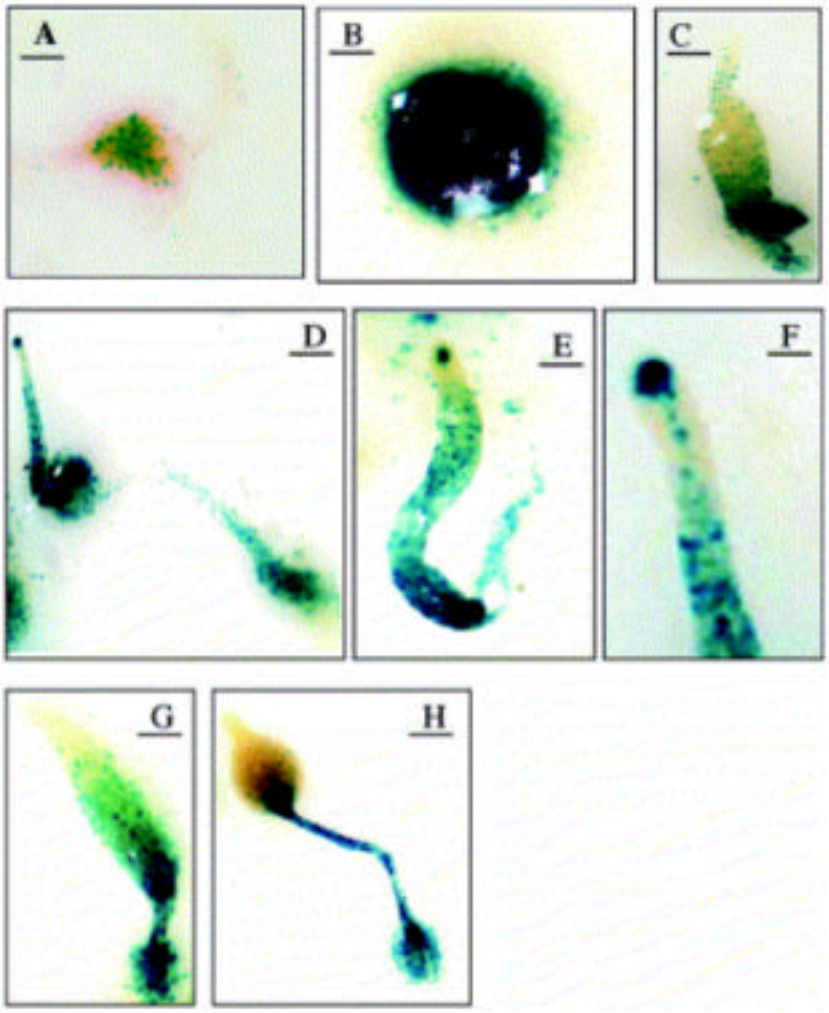


Figure 37. Localization of β-galactosidase activity in various developmental stages. Developmental stages shown are: A, streaming/early aggregation; B, tight aggregate; C, first finger; D, slug with relatively long slime trail; E, slug with short slime trail; F, magnification of the anterior region of the slug in D. In addition, D-F show the expression pattern of *5nt* in pstAB cells in the center of the anterior region of the slug as well as in scattered cells in the prespore region. At the culmination stage, gene expression is shown in G. H shows the expression in the complete fruiting body. The scale bars represent 1.2, 0.55, 0.6, 1.1, 0.7, 0.4, 0.75 and 0.65 mm for A, B, C, D, E, F, G and H, respectively.

In the first finger stage, the activity was localized in the base, with some expression in cells that are scattered throughout the structure (Fig. 37C). In migrating slugs, the expression was observed in a group of cells that form a funnel shaped core in the front of the slug (Fig. 37D,F). It is likely that the cells in this location are pstAB cells, which express the *ecmA* and *ecmB* genes (Jermyn *et al*., 1989; Sternfeld, 1992; Gaskell *et al*., 1992). Moreover, as the slug moved along the substratum, high activity was observed in cells that are left behind the migrating slug in the slime sheath (Fig. 37D,E). It was previously shown that pstAB cells migrate through the slug and are deposited in the slime trail (Sternfeld, 1992), which was another indication that the cells expressing *5nt* are likely the pstAB cells.

At the culmination stage, the expression was in the cells located in the region of the developing spore mass, and in cells along the newly formed stalk and at the basal disc (Fig. 37G). In the complete fruiting body, the activity was in the lower cup, in the slime sheath and the basal disc. No activity was observed in the upper cup, stalk tube or spore cells (Fig. 37H). It should be noted that in these experiments, non-transformed Ax3K cells were used as a control; no endogenous activity was observed at any developmental stage (data not shown).

3.11. Determination of Transcriptional Start Site of *5nt*

In order to determine the transcriptional start site of *5nt*, two primers, *gs1r* and *gs2r* were used in separate primer extension experiments. The annealing site for the primer *gs1r* was located 179 base pairs downstream of the 5' end of the *5nt* cDNA sequence (see Fig. 8). This primer includes 21 nucleotide annealing sequence as well as 8 nucleotide additional engineered sequence at the 5' end (see Table 1). This additional

sequence at the 5' end contained *Hin*dIII restriction site used for cloning purposes in other experiments as mentioned previously. The annealing site for the primer, *gs2r*, was located 222 base pairs downstream of the 5' end of the cDNA sequence (see Fig. 8). This primer contains 24 nt annealing sequence in addition to 10 nt engineered sequence at the 5' end (see Table 1), for cloning purposes as stated above.

Total RNA was isolated from cAMP-induced *Dictyostelium* cells, because *5nt* expression is stimulated by cAMP (see Fig. 12). Primers were allowed to separately hybridize to total RNA and primer extension was carried out as described in the Materials and Methods. Two bands were detected for each of the two reactions (Fig. 38). A 195 nt and a 225 nt bands were observed using the primer *gs1r*. This result suggested two transcriptional start sites for the *5nt* expression. These sites are located 7 bp upstream of the 5' end of the longest cDNA sequence reported by the Japanese cDNA project (118 bp upstream of the ATG initiation codon) and 37 bp upstream of the 5' end of the cDNA sequence (148 bp upstream of the ATG initiation codon).

Using *gs2r* primer, a 240 nt and a 270 nt bands were detected (Fig. 38). This result was in agreement with that of the *gs1r* experiment and indicated the same two locations for transcriptional start sites. The distance between the two primer annealing sites was 43 nt (see Fig. 8) and the additional nucleotides at the 5' end of these primers were 8 nt and 10 nt for *gs1r* and *gs2r*, respectively, as stated above. Therefore, the size of the primer extension product obtained with the *gs2r* primer would be 45 nt longer than that of the *gs1r* primer. As expected, the results indicated the 45 nt size difference between the two primer extension products.

These transcriptional start sites are preceded by a stretch of T residues, which is characteristic of transcription start sites of *Dictyostelium* genes (Kimmel and Firtel,

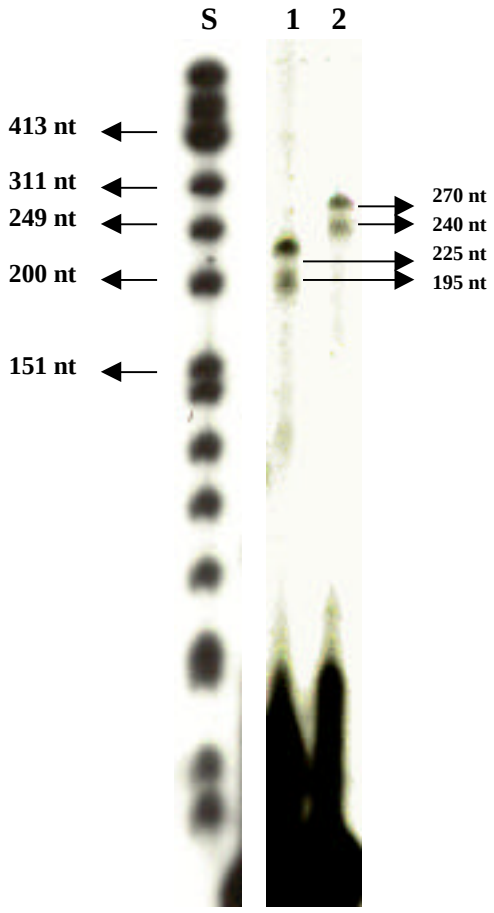


Figure 38. Primer extension for determining transcriptional start site of *5nt.* Total RNA isolated from cAMP-induced *Dictyostelium* cells was hybridized to primers *gs1r* (lane 1) and *gs2r* (lane 2) in separate reactions. Primer extension was carried out as described in Materials and Methods. S, *Hin*fI digested φX174 DNA standard size markers.

1983). Two start sites for transcription may suggest two alternate ways for mRNA production although it is also possible that the start site is located 148 bp upstream of the *5nt* ATG and that the message is modified post-transcriptionally.

CHAPTER 4. CONCLUSIONS

In order to investigate the regulation of *5nt* at the transcriptional level, the 5' flanking region of the gene was cloned. Southern blotting experiments indicated one form of the 5NT-encoding gene (Fig. 4C). Sequencing of the promoter revealed that the promoter sequence is typical of non-coding regions of *Dictyostelium*, being A/T rich and consisting of long sequences of A's and T's (Fig. 8).

The developmental expression pattern of *5nt* was investigated by fusing the *5nt* promoter in frame to the reporter genes *luc* and *lacZ*. The same results were obtained using both reporter genes (Figures 11 and 36). This result was in agreement with the expression pattern of *5nt* (Chanchao *et al*., 1999). It is likely that regulation of *5nt* occurs primarily at the level of transcription because of the similarity of the appearance of the authentic *5nt* and the reporter gene driven by the *5nt* promoter.

Whether cAMP could affect *5nt* expression was tested by the *5nt* promoter-*luc* fusion. Cells carrying *5nt* promoter–*luc* constructs were shaken in non-nutrient medium and then exposed to exogenous cAMP. Cells exposed to cAMP highly expressed the reporter gene while in the absence of cAMP there was no expression (Fig. 12). This result showed that cAMP stimulates *5nt* expression *in vivo*.

The 5' and internal promoter deletion analyses were performed to identify *cis*-regulatory sequences within the *5nt* promoter. The results suggested four potential regulatory regions within the *5nt* promoter (Figures 13 and 14). In order to identify *trans*-acting regulatory factor(s) that bind specifically to these regulatory sequences, gel shift assays were performed. Gel shift assays indicated the presence of two protein-DNA complexes (0.33 R_f and 0.13 R_f). The 0.33 R_f band was observed with probe 19 (-227 to

–198 bp) and the 0.13 R_f band was observed with probe 25 (-252 to –203 bp). The probe 25 sequence corresponded to the regulatory region 'd', which was from –307 bp to –226 bp (see Fig. 15). These activities were present in both cytoplasmic and nuclear extracts isolated from aggregation and slug stage cells as well as from cAMP-induced cells. Competition assays showed the specificity of the binding on both activities. Various chromatographic techniques were used to purify the protein factor(s) involved in these specific DNA-binding activities. Unfortunately, the purification of the protein factor(s) involved in the 0.13 R_f band activity did not yield sufficient protein for mass spectrometric analysis.

Mass spectrometry analysis of the purified protein generating the 0.33 R_f band activity yielded numerous peptide products. Using these peptide sequences, a search in the *Dictyostelium* genomic sequence database revealed that the purified protein was a putative formyltetrahydrofolate synthase (FTHFS). The *Dictyostelium* enzyme, which has not yet been studied, has very high homology to FTHFS enzymes in other organisms. It is unclear whether the putative *Dictyostelium* FTHFS is involved in the regulation of the *5nt* expression, although the involvement of folic acid in *Dictyostelium* development may suggest a role for the protein in the *5nt* expression. While DNA binding activity of FTHFS enzymes has previously been reported, a potential biological role, if any, for the DNA binding activity of these enzymes is yet to be determined.

The localization of *5nt* expresion during development was studied using a *5nt* promoter-*lacZ* fusion construct (Ubeidat *et al*., 2002). Assaying β-galactosidase activity during development indicated that the gene is expressed early in development (Fig. 36), which was in agreement with the expression of the authentic *5nt* (Chanchao *et al*., 1999). The expression of *lacZ* appeared as soon as the cells began streaming toward the

aggregation center (Fig. 37A). During the tight aggregation stage, only the cells in the upper 75% of the structure were stained (Fig. 37B). In slug stage, *5nt* is highly expressed in cells that form a funnel-shaped core in the front of the slug (Fig. 37D, F). These cells are likely pstAB cells. Moreover, as the slug moved along the substratum, high activity was observed in cells that were left behind the migrating slug in the slime sheath (Fig. 37D,E). In the complete fruiting body, the activity was in the lower cup, in the slime sheath and the basal disc (Fig. 37H).

In addition, primer extension was performed to determine the start site for transcription of *5nt*. The results indicated two potential transcriptional start sites for the *5nt* expression (Fig. 38). These sites were located 118 bp and 148 bp upstream of the ATG initiation codon. Two start sites for transcription may suggest two alternate ways for mRNA production or possibly post-transcriptional modification of the longer message.

Several more internal promoter deletions and site-directed mutagenesis studies are already underway to obtain additional insights into the transcriptional regulation of *5nt*. Ultimately, identification and characterization of protein factors through such methods as gene replacement, down regulation and overexpression will lead to the understanding of the mechanisms that regulate *5nt* expression. In addition, experiments that are aimed to rescue *5nt* null mutant cells with wild type and mutant forms of 5NT will provide further information on the role of this protein in *Dictyostelium* development.

REFERENCES

A**li, S. A., and Steinkasserer, A.** (1995). PCR-ligation-PCR mutagenesis: a protocol for creating gene fusions and mutations. *Biotechniques* **18**, 746-750.

Anjard, C., Chang, W. T., Gross, J., and Nellen, W. (1998). Production and activity of spore differentiation factors (SDFs) in *Dictyostelium*. *Development* **125**, 4067-4075.

Appling, D. R., and Rabinowitz, J. C. (1985). Evidence for overlapping active sites in a multifunctional enzyme: immunochemical and chemical modification studies on C1-tetrahydrofolate synthase from *Saccharomyces cerevisiae*. *Biochemistry* **24**, 3540-3547.

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., and Rutherford, C. L. (1979). 5'-AMP nucleotidase is localized in the area of the cell-cell contact of prespore and prestalk regions during culmination of *Dictyostelium discoideum*. *Mech. Ageing Devel.* **10**, 199-217.

Armant, D. R., and Rutherford, C. L. (1981). Copurification of alkaline phosphatase and 5'-AMP specific nucleotidase in *Dictyostelium discoideum*. *J. Biol. Chem.* **256**, 12710-12718.

Armant, D. R., and Rutherford, C. L. (1982). Properties of a 5'-AMP specific nucleotidase which accumulates in one cell type during development of *Dictyostelium discoideum*. *Arch. Biochem. Biophys.* **216**, 485-494.

Armant, D. R., Stetler, D. A., and Rutherford, C. L. (1980). Cell surface localization of 5'-AMP nucleotidase in prestalk cells of *Dictyostelium discoideum*. *J. Cell Sci.* **45**, 119-129.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995). Current Protocols in Molecular Biology. John Wiley & Sons, New York.

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

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

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

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. (1952). The pattern of differentiation in amoeboid slime molds. *Am. Naturalist* **86**, 79-89.

Bonner, J. T., Chiquoine, A. D., and Kolderie, M. Q. (1955). A histochemical study of differentiation in the cellular slime molds. *J. Exp. Zool.* **130**, 133-158.

Bradbury, J. M., and Gross, J. D. (1989). The effect of ammonia on cell-type-specific enzyme accumulation in *Dictyostelium discoideum*. *Cell Differ. Devel.* **27**, 121-128.

Bretschneider, T., Siegert, F., and Weijer, C. J. (1995). Three-dimensional scroll waves of cAMP could direct cell movement and gene expression in *Dictyostelium* slugs. *Proc. Natl. Acad. Sci. USA* **92**, 4387-4391.

Brown, J. M., and Firtel, R. A. (1999). Regulation of cell-fate determination in *Dictyostelium*. *Dev. Biol.* **216**, 426-441.

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

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

Bucci, M., and Murphy, C. R. (1999). Differential alterations in the distribution of three phophatase enzymes during the plasma membrane transformation of uterine epithelial cells in the rat. *Cell Biol. Int.* **23**, 21-30.

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.

Clarke, M., and Gomer, R. H. (1995). PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*. *Experientia* **51**, 1124-1134.

Cosson, P., Zulianello, L., Join-Lambert, O., Faurisson, F., Gebbie, L., Benghezal, M., van Delden, C., Curty, L. K., and Kohler, T. (2002). *Pseudomonas aeruginosa* virulence analyzed in a *Dictyostelium discoideum* host system. *J. Bact.* **184**, 3027-3033.

Das, D. V. M., and Weeks, G. (1980). Reversible heat activation of alkaline phosphatase of *Dictyostelium discoideum* and its developmental implication. *Nature* **288**, 166-168.

Das, D. V. M., and Weeks, G. (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.

Datta, S., Gomer, R. H., and Firtel, R. A. (1986). Spatial and temporal regulation of a foreign gene by a prestalk-specific promotor in transformed *Dictyostelium discoideum*. *Mol. Cell. Biol.* **6**, 811-820.

Desbarats, L., Lam, T. Y., Wong, L. M., and Siu, C. H. (1992). Identification of a unique cAMP-response element in the gene encoding the cell adhesion molecule gp80 in *Dictyostelium discoideum*. *J. Biol. Chem.* **267**, 19655-19664.

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

Eichinger, L., Lee, S. S., and Schleicher, M. (1999). *Dictyostelium* as model system for studies of the actin cytoskeleton by molecular genetics. *Microsc. Res. Techn.* **47**, 124-134.

Eichinger, L., and Noegel, A. A. (2003). Crawling into a new era-the *Dictyostelium* genome project. *EMBO J.* **22**, 1941-1946.

Escalante, R., and Vicente, J. J. (2000). *Dictyostelium discoideum*: a model system for differentiation and patterning. *Int. J. Dev. Biol.* **44**, 819- 835.

Escalante, R., Vicente, J. J., Moreno, N., and Sastre, L. (2001). The MADS-box gene *srfA* is expressed in a complex pattern under the control of alternative promoters and is essential for different aspects of *Dictyostelium* development. *Dev. Biol.* **235**, 314-329.

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.

Firtel, R. A. (1995). Integration of signaling information in controlling cell- fate decisions in *Dictyostelium*. *Genes Devel.* **9**, 1427-1444.

Fosnaugh, K. L., and Loomis, W. F. (1991). Coordinate regulation of the spore coat genes in *Dictyostelium discoideum*. *Dev. Genet.* **12**, 123-132.

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

Fukuzawa, M., Abe, T., and Williams, J. G. (2003). The *Dictyostelium* prestalk cell inducer DIF regulates nuclear accumulation of a STAT protein by controlling its rate of export from the nucleus. *Development* **130**, 797-804.

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.

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**, 474-482.

Gerisch, G. (1976). Extracellular cyclic-AMP phosphodiesterase regulation in agar plate cultures of *Dictyostelium discoideum*. *Cell Differ.* **5**, 21-25.

Gezelius, K., and Wright, B. E. (1965). Alkaline phosphatase in *Dictyostelium discoideum*. *J. Gen. Microbiol.* **38**, 309-327.

Glockner, G., Szafranski, K., Winckler, T., Dingermann, T., Quail, M. A., Cox, E., Eichinger, L., Noegel, A. A., and Rosenthal, A. (2001). The complex repeats of *Dictyostelium discoideum*. *Genome Res.* **11**, 585-594.

Gross, J. D. (1994). Developmental decisions in *Dictyostelium discoideum*. *Microbiol. Rev.* **58**, 330-351.

Gussow, D., and Clackson, T. (1989). Direct clone characterization from plaques and colonies by the polymerase chain reaction. *Nucl. Acids Res.* **17**, 4000.

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 Firtel, R. A. (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.

Harwood, A. J., Early, A., and Williams, J. G. (1993). A repressor controls the timing and spatial localisation of stalk cell-specific gene expression in *Dictyostelium*. *Development* **118**, 1041-1048.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351-359.

Hori, R., and Firtel, R. A. (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.

Hou, S. X., Zheng, Z. Y., Chen, X., and Perrimon, N. (2002). The JAK/STAT pathway in model organisms: emerging roles in cell movement. *Dev. Cell* **3**, 765-778.

Jermyn, K., Traynor, D., and Williams, J. (1996). The initiation of basal disc formation in *Dictyostelium discoideum* is an early event in culmination. *Development* **122**, 753-760.

Jermyn, K. A., Duffy, K. T., and Williams, J. G. (1989). A new anatomy of the prestalk zone in *Dictyostelium*. *Nature* **340**, 144-146.

Jermyn, K. A., and Williams, J. G. (1991). An analysis of culmination in *Dictyostelium* using prestalk and stalk-specific cell autonomous markers. *Development* **111**, 779-787.

Kawata, T., Early, A., and Williams, J. (1996). Evidence that a combined activator-repressor protein regulates *Dictyostelium* stalk cell differentiation. *EMBO J.* **15**, 3085-3092.

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.

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

Kimmel, A.R., and Firtel, R.A. (1983). Sequence organization in *Dictyostelium*: unique structure at the 5'-ends of protein coding genes. *Nucl. Acids Res*. **11**, 541-552.

Konijn, T. M., van de Meene, J. G. C., Bonner, J. T., and Barkley, D. S. (1967). The acrasin activity of adenosine-3',5'-cyclic phosphate. *Proc. Natl. Acad. Sci. USA* **58**, 1152-1154.

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

Krivanek, J. O., and Krivanek, R. C. (1958). The histochemical localization of certain biochemical intermediates and enzymes in the developing slime mold, *Dictyostelium discoideum* Raper. *J. Exp. Zool.* **137**, 89-115.

Kuspa, A., Sucgang, R., and Shaulsky, G. (2001). The promise of a protist: the *Dictyostelium* genome project. *Funct. Integr. Genomics* **1**, 279-293.

Loomis, W. F. (1982). "The development of *Dictyostelium discoideum*." Ac. Press, New York.

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

Loomis, W. F. (1996). Genetic networks that regulate development in *Dictyostelium* cells. *Microbiol. Rev.* **60**, 135-150.

Loomis, W. F., and Kuspa, A. (1997). The genome of *Dictyostelium discoideum*. *In* "*Dictyostelium* - A model system for cell and developmental biology." (Y. Maeda, K. Inouye, and I. Takeuchi, Eds.), pp. 15-30. Universal Academy Press, Tokyo, Japan.

Maeda, M., and Firtel, R. A. (1997). Activation of the mitogen-activated protein kinase ERK2 by the chemoattractant folic acid in *Dictyostelium*. *J. Biol. Chem.* **272**, 23690-23695.

Maniak, M., and Nellen, W. (1990). Two separable promoters control different aspects of expression of a *Dictyostelium* gene. *Nucl. Acids Res.* **18**, 3211-3217.

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

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

Merril, C. R., Goldman, D., Sedman, S. A., and Ebert, M. H. (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211**, 1437-1438.

Mohanty, S., Jermyn, K. A., Early, A., Kawata, T., Aubry, L., Ceccarelli, A., Schaap, P., Williams, J. G., and Firtel, R. A. (1999). Evidence that the *Dictyostelium* Dd-STATa protein is a repressor that regulates commitment to stalk cell differentiation and is also required for efficient chemotaxis. *Development* **126**, 3391-3405.

Mu, X. Q., Lee, B., Louis, J. M., and Kimmel, A. R. (1998). Sequence-specific protein interaction with a transcriptional enhancer involved in the autoregulated expression of cAMP receptor 1 in *Dictyostelium*. *Development* **125**, 3689-3698.

Mu, X. Q., Spanos, S. A., Shiloach, J., and Kimmel, A. (2001). CRTF is a novel transcription factor that regulates multiple stages of *Dictyostelium* development. *Development* **128**, 2569-2579.

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.

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

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

Nour, J. M., and Rabinowitz, J. C. (1992). Isolation and sequencing of the cDNA coding for spinach 10-formyltetrahydrofolate synthetase. *J. Biol. Chem.* **267**, 16292-16296.

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

Ott, A., Oehme, F., Keller, H., and Schuster, S. C. (2000). Osmotic stress response in *Dictyostelium* is mediated by cAMP. *EMBO J.* **19**, 5782-5792.

Parent, C. A., and Devreotes, P. N. (1996). Molecular genetics of signal transduction in *Dictyostelium*. *Annu. Rev. Biochem.* **65**, 411-440.

Powell-Coffman, J. A., and Firtel, R. A. (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.

Pukatzki, S., Kessin, R. H., and Mekalanos, J. J. (2002). The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA* **99**, 3159-3164.

Rafols, I., Amagai, A., Maeda, Y., MacWilliams, H. K., and Sawada, Y. (2001). Cell type proportioning in *Dictyostelium* slugs: lack of regulation within a 2.5-fold tolerance range. *Differentiation* **67**, 107-116.

Ramalingam, R., Blume, J. E., Ganguly, K., and Ennis, H. L. (1995). AT-rich upstream sequence elements regulate spore germination-specific expression of the *Dictyostelium discoideum celA* gene. *Nucl. Acids Res.* **23**, 3018-3025.

Rampazzo, C., Mazzon, C., Reichard, P., and Bianchi, V. (2002). 5'-Nucleotidases: specific assays for five different enzymes in cell extracts. *Biochem. Biophys. Res. Commun.* **293**, 258-263.

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. (1940). Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. *J. Elisha Mitchell Sci. Soc.* **56**, 241-282.

Raper, K. B. (1941). Developmental patterns in simple slime molds. *Growth* **5** (Suppl.), 41-76.

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

Richards, A. J., Corney, A. J., and Hames, B. D. (1990). Cell-type-specific genes expressed late in *Dictyostelium* development show markedly different responses to 3',5'-cyclic AMP. *Mol. Microbiol.* **4**, 1279-1291.

Rivero, F., Furukawa, R., Noegel, A. A., and Fechheimer, M. (1996). *Dictyostelium discoideum* cells lacking the 34,000-Dalton actin-binding protein can grow, locomote, and develop, but exhibit defects in regulation of cell structure and movement: A case of partial redundancy. *J. Cell Biol.* **135**, 965-980.

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

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

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., Selmin, O., and Peters-Weigel, S. (1997). Temporal regulation of the *Dictyostelium glycogen phosphorylase 2* gene. *Biochim. Biophys. Acta* **1351**, 111-125.

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

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

Schaap, P., and Wang, M. (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.

Shaulsky, G., Escalante, R., and Loomis, W. F. (1996). Developmental signal transduction pathways uncovered by genetic suppressors. *Proc. Natl. Acad. Sci. USA* **93**, 15260-15265.

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*. *In* "J. Cell Biol.", Vol. **103**, pp. 3a.

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.

Spek, W., van Drunen, K., van Eijk, R., and Schaap, P. (1988). Opposite effects of adenosine on two types of cAMP-induced gene expression in *Dictyostelium* indicate the involvement of at least two different intracellular pathways for the transduction of cAMP signals. *FEBS Lett.* **228**, 231-234.

Spychala, J., Zimmermann, A. G., and Mitchell, B. S. (1999). Tissue-specific regulation of the ecto-5'-nucleotidase promoter. *J. Biol. Chem.* **274**, 22705-22712.

Sternfeld, J. (1992). A study of pstB cells during *Dictyostelium* migration and culmination reveals a unidirectional cell type conversion process. *W.R. Arch. Dev. Biol.* **201**, 354-363.

Sternfeld, J., and David, C. N. (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.

Sucic, J. F., Selmin, O., and Rutherford, C. L. (1993). Regulation of the *Dictyostelium glycogen phosphorylase 2* gene by cyclic AMP. *Dev. Genet.* **14**, 313-322.

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.

Takayama, T., Matsubara, S., Shibahara, H., Minakami, H., Takizawa, T., and Sato, I. (2000). Ultracytochemical localization of 5'-nucleotidase activity in human ejaculated spermatozoa. *Int. J. Androl.* **23**, 106-108.

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

Thompson, L. F. (1985). Inosine 5'-monophosphate vs inosine and hypoxanthine as substrates for purine salvage in human lymphoid cells. *Proc. Soc. Exp. Biol. Med.* **179**, 432-436.

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

Ubeidat, M., and Rutherford, C. L. (2002). Expression and one-step purification of a developmentally regulated protein from *Dictyostelium discoideum*. *Prot. Expr. Purif.* **25**, 472-480.

Van Driessche, 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.

van Haastert, P. J. M., and Kuwayama, H. (1997). cGMP as second messenger during *Dictyostelium* chemotaxis. *FEBS Lett.* **410**, 25-28.

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

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., Fukuzawa, M., Devreotes, P. N., and Schaap, P. (2001). Adenylyl cyclase A expression is tip-specific in *Dictyostelium* slugs and directs StatA nuclear translocation and CudA gene expression. *Dev. Biol.* **234**, 151-160.

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**, 23792-23798.

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

Warner, N., and Rutherford, C. L. (2000). Purification and cloning of TF2: A novel protein that binds a regulatory site of the *gp2* promoter in *Dictyostelium*. *Arch. Biochem. Biophys* **373**, 462-470.

Williams, J. G., Duffy, K. T., Lane, D. P., McRobbie, S. J., Harwood, A. J., Traynor, D., Kay, R. R., and Jermyn, K. A. (1989). Origins of the prestalk-prespore pattern in *Dictyostelium* development. *Cell* **59**, 1157-1163.

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

Winckler, T., Trautwein, C., Tschepke, C., Neuhauser, C., Zundorf, I., Beck, P., Vogel, G., and Dingermann, T. (2001). Gene function analysis by amber stop codon suppression: CMBF is a nuclear protein that supports growth and development of *Dictyostelium* amoebae. *J. Mol. Biol.* **305**, 703-714.

Yin, Y. H., Rogers, P. V., and Rutherford, C. L. (1994a). Dual regulation of the *glycogen phosphorylase 2* gene of *Dictyostelium discoideum*: The effects of DIF-1, cAMP, NH3 and adenosine. *Development* **120**, 1169-1178.

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

Zimmermann, H. (1992). 5'-Nucleotidase: molecular structure and functional aspects. *Biochem. J.* **285**, 345-365.

APPENDIX A

AF378371. Dictyostelium dis...[gi:18253312]

```
LOCUS       AF378371               1435 bp    DNA     linear   INV 22-JAN-2002
DEFINITION  Dictyostelium discoideum 5'-nucleotidase gene, promoter region.
ACCESSION   AF378371
VERSION     AF378371.1  GI:18253312
KEYWORDS    .
SOURCE      Dictyostelium discoideum
  ORGANISM  Dictyostelium discoideum
            Eukaryota; Mycetozoa; Dictyosteliida; Dictyostelium.
REFERENCE   1  (bases 1 to 1435)
  AUTHORS   Ubeidat,M., Eristi,C.M. and Rutherford,C.L.
  TITLE     Expression pattern of 5'-nucleotidase in Dictyostelium
  JOURNAL   Mech. Dev. 110 (1-2), 237-239 (2002)
  MEDLINE   21612247
   PUBMED   11744390
REFERENCE   2  (bases 1 to 1435)
  AUTHORS   Eristi,C.M., Ubeidat,M. and Rutherford,C.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-MAY-2001) Biology, Virginia Polytechnic Institute and
            State University, 2119 Derring Hall, Blacksburg, VA 24061-0406, USA
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:44689"
     gene            1..>1435
                     /gene="5'-nucleotidase"
                     /note="5NT"
     promoter        1..1435
                     /gene="5'-nucleotidase"
BASE COUNT      564 a    112 c    129 g    630 t
ORIGIN
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       61 atttttactt gttttttatt gacacgtaga gataaaaatg gtttatcatt ttttttttt
      121 ttttttatat ttatcccact gttttttttt atttttactt atttatatat tttttttatt
      181 aaaaatctgt ttcataggaa aagtaaacaa aaaaagaact tatgaaggtc ttggaaaata
      241 ttaaccagat gaataataaa aaaactttat tttaaagtta ataacttttt tttattattt
      301 ttttttattt ttataattat aatgtttgaa tttatatatt tataattaaa atcaaataaa
      361 tcaggaaaaa ataaatggta tgatttttgc agccaaaaga agtcaaactt taaagatttg
      421 aactcttttt gtcattcaat tttggctaat ggctttcctc ttccttttat actagagttt
      481 aggtctgatt tttgcagcag aaagaagtaa taatttaaag aaaagagtta ataaaaataa
      541 aaaaccaatt atataaagtc aaagaggtta ccaaaggttc tcaacaaaca aataaaaatt
      601 gggaggaaga aaaaaagaaa taaataaaaa aaaaaaaaaa aaaaaattta aaagaaaatt
      661 aaaagaagct tgaaaaagct gaaaacaatt tatcaataaa aacttttttt ttttttaca
      721 catcataaat ttaaaaaata tttcttttga ttgtgtgtga aaataaaaat agaaaatttc
      781 agaaaataga taaagacacc actatcaaaa tgggaagttt aatttttta tttttatttt
      841 tatttttttt tttggtattt atttttaaca tcaaggggtt ggtggtggtg gtatgccaca
      901 tgtttattta atttaaccgt caaattgtat gtgtttaaaa ttctaatatt agtggttttt
      961 ctttaaatca tacacaaaaa aaaaaaaccc cacaattaca tattcaaaat tttctattga
     1021 ccacagctta gaatcataca agttttttt tattattatt attatttatt tttaattttt
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     1141 atataatttt ttttttttt tttttcttat tttaattttt ttttttttt ttttaaattg
     1201 gattattaaa ctcaatatta atttttttt cacataattt tattatttgg ataatttaat
     1261 tattcataaa ttattctata ttataatttt ttttaaaaaa aaaaaaaaaa aaaaaaaaaa
     1321 aaaatgaaat tattattatt attatttttа ataataaatt caataatatt atcaaaatgt
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```

APPENDIX B

Peptide Products Obtained from Mass Spectrometry Analysis.

K.MHGGGPK.V
K.FGVGVVVAINK.F
R.KGVPTGFILPIR.D
K.VVAGTPLDK.A
K.YLYPLDK.S
K.LPICMAK.T
K.THLSLSHDPER.K
K.LYTTQGFDK.L
K.GVPTGFILPIR.D
K.GVSNLAHHIK.N
K.EIYGADGVEYSPEADDKIK.L
K.THLSLSHDPERK.G
K.FGVGVVVAINK.F
K.LYTTQGFDKLPICMAK.T
K.GVPTGFILPIRDVR.A
K.THLSLSHDPERK.G
K.KFGVGVVVAINK.F
K.KTFACIR.Q
K.TFACIRQPSQGPTFGIK.G
R.KFAPIMLR.R
K.AKVSLEVIER.L
K.STTTIGLCQALGAHLGKK.T
K.SIGLLEEEIDFYGKYK.A
R.ILHEGTQTDAQLWK.R
R.LKETENGNYVVVTGINPTPLGEGK.S
K.LNLIKPVPSDIEIASSVEPLPIK.T

APPENDIX C

Sequence of the Putative *Dictyostelium discoideum* Formyltetrahydrofolate Synthase based on a blast search in the *Dictyostelium* database.

```
MENFNYPKLNLIKPVPSDIEIASSVEPLPIKTIAKSIGLLEEEIDFYGKYKAKVSLEVIE 60
RLKETENGNYVVVTGINPTPLGEGKSTTTIGLCQALGAHLGKKTFACIRQPSQGPTFGIK 120
GGAAGGGYSQVIPMEEFNLHMTGDIHAITAANNLLAAAIDTRILHEGTQTDAQLWKRLCP 180
VDSKDGSRKFAPIMLRRLKKLGIDKTDPNQLTEEEISKFVRLDIDPTRITWNRVLDTNDR 240
FLRGISVGQGKEEQRFERKTNFDISVASEIMAVLALCTSLSDMRERLGRMVVGPSRSGEP 300
ITADDLGVGGALTVLMKDAIMPTLMQTLEGTPVLVHAGPFANIAHGNSSIIADQIALKLA 360
GKDGYVVTEAVFGADIGAEKFFDIKCRSSGLKPNCAVIVATIRALKMHGGGPKVVAGTPL 420
DKAYTSENIELLKKGVSNLAHHIKNLKKFGVGVVVAINKFHTDSDAEVNLLVEASLTAGA 480
NDAVMSDHWAEGGNGALDLANAVEKACKETNKDNFKYLYPLDKSVKEKIETIAKEIYGAD 540
GVEYSPEADDKIKLYTTQGFDKLPICMAKTHLSLSHDPERKGVPTGFILPIRDVRASIGA 600
GFIYPLVGSMATIPGLPTRPCFYEIDIDTNTGKIIGLS                        638
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CURRICULUM VITAE

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Education

Ph.D. candidate, Molecular and Cellular Biology. 1998-present (expected August 2003), Virginia Poytechnic Institute and State University, Blacksburg, VA.

M.S., Biology. 1996-1997. University of Massachusetts at Boston, Boston, MA.

B.S., Biology. 1988-1992. Istanbul University, Istanbul, Turkey.

Publications

C.M. Eristi, and C.L. Rutherford. Investigation of transcriptional regulation of *5'-nucleotidase* in *Dictyostelium discoideum*. (In preparation)

M. Ubeidat, **C.M. Eristi**, and C.L. Rutherford. 2002. Expression pattern of 5'-nucleotidase in *Dictyostelium*. Mechanisms of Development, 110 (1-2): 237-239.

C. Chanchao, **C.M. Eristi**, R. Favis, and C.L. Rutherford. 1999. 5'-Nucleotidase in *Dictyostelium*: Protein Purification, Cloning, and Developmental Expression. Biochimica et Biophysica Acta, 1473: 376-390.

Presentations

C.M. Eristi. Investigation of 5'-Nucleotidase in *Dictyostelium.* Microbiology and Immunology Seminar series. Biology Department, Virginia Tech, Blacksburg, VA. December, 2002.

C.M. Eristi, M. Ubeidat, B.R. Joyce and C.L. Rutherford. Transcriptional Regulation of the 5'-Nucleotidase gene of *Dictyostelium discoideum*. Poster presentation. International Dictyostelium Conference, University of California-San Diego, San Diego, CA. July, 2001.

C.M. Eristi and C.L. Rutherford. Cloning and Analysis of Transcriptional Regulation of the 5'-Nucleotidase gene in *Dictyostelium.* Paper presentation. 78th Annual Meeting of the Virginia Academy of Science, Radford, VA. May, 2000.

C.M. Eristi. Analysis of compounds that inhibit colicin V toxin activity. Departmental seminar presentation. Biology Department, University of Massachusetts at Boston, Boston, MA. December,1997.

Selected Abstracts (National and International Meetings)

C.M. Eristi, M. Ubeidat, N.S. Wiles, B.R. Joyce and C.L. Rutherford. Analysis of regulatory elements within the promoter sequence of the developmentally expressed gene encoding 5'-Nucleotidase and subsequent identification of a potentially novel transcription factor in the model system *Dictyostelium discoideum*. Paper presentation. International Dictyostelium Conference, Lorne, Victoria, Australia. July, 2003.

B.R. Joyce, M. Ubeidat, **C.M. Eristi**, N.S. Wiles and C.L. Rutherford. Regulation and expression of alkaline phosphatase during *Dictyostelium* development. Poster presentation. International Dictyostelium Conference, Lorne, Victoria, Australia. July, 2003.

M. Ubeidat, **C.M. Eristi**, D.F. Overall, B.R. Joyce and C.L. Rutherford. 5'-Nucleotidase in *Dictyostelium*: Regulation, Promoter Analysis and Localization. Paper presentation. International Dictyostelium Conference, University of California-San Diego, San Diego, CA. July, 2001.

C.L. Rutherford, **C.M. Eristi**, M. Ubeidat, C. Chanchao, D.F. Overall, B.R. Joyce, and J.L. Goodin. Regulation of 5'-Nucleotidase (5NT) in *Dictyostelium discoideum.* Paper presentation. International Dictyostelium Conference, University of Dundee, Dundee, Scotland. August, 2000.

M. Ubeidat, **C.M. Eristi**, C. Chanchao, D.F. Overall, B.R. Joyce, and C.L. Rutherford. 5'-Nucleotidase in *Dictyostelium*: Cloning, Expression in *E. coli* and Immunological Studies. Poster presentation. International Dictyostelium Conference, University of Dundee, Dundee, Scotland. August, 2000.

C.M. Eristi and C.L. Rutherford. 2000. Cloning and Analysis of Transcriptional Regulation of the *5'-nucleotidase* gene in *Dictyostelium discoideum.* Abstract. Virginia Journal of Science, 51(2): 78.

S. Mullin, **C.M. Eristi**, J. Warner, and R.C. Skvirsky. Triazine dyes that inhibit the activity of the bacterial toxin colicin V. Paper presentation. 217th American Chemical Society National Meeting, Anaheim, CA. March, 1999.

Research Experience

Doctoral student. 1998-2003. Investigation of transcriptional regulation of *5'-nucleotidase* in *Dictyostelium discoideum*. Charles L. Rutherford (Advisor). Biology Department, Virginia Tech, Blacksburg, VA.

Master student. 1996-1997. Analysis of compounds that inhibit colicin V toxin activity. Rachel Skvirsky (Advisor). Biology Department, University of Massachusetts at Boston, Boston, MA.

Teaching Experience

Graduate Teaching Assistant. 2002-2003. Molecular Biology Laboratory (BIOL 4774), Virginia Tech, Blacksburg, VA.
Taught molecular biology techniques to both undergraduate and graduate students, graded lab reports, prepared and graded quizzes. Some of the molecular biology techniques used in the course included: restriction mapping, various forms of gel electrophoresis, cDNA library manipulation, plasmid cloning and subcloning, transformation of bacteria, PCR, DNA sequencing and computer analysis of DNA

sequences, recombinant protein expression, chromatography, western blotting, Southern blotting and use of various reporter genes to study gene expression.

Other Professional Activities

Graduate Research Assistant. Summers 2002, 2003. Biology Department, Virginia Tech, Blacksburg, VA.

Undergraduate Research Supervisor. 1999, 2000. Virginia Tech, Blacksburg, VA.

Undergraduate Research Supervisor. 1997. University of Massachusetts at Boston, Boston, MA.

Master of Science student. 1994. Biology Department, Uludag University, Bursa, Turkey.

Intern Research Assistant. Fall 1992. Experimental Medicine Research Institute (DETAE), Istanbul University, Istanbul, Turkey.

Grants Received

Virginia Academy of Science (VAS) Small Research Project Grant. 2001. $1,250 + $500 Biology Dept Matching Funds.

Graduate Student Assembly Travel Fund of Virginia Tech. 2001. $300.

Graduate Research Development Project (GRDP) Grant of Virginia Tech. 2000. $500 + $500 Biology Dept Matching Funds.

Sigma Xi Grants-in-Aid of Research. 1999. $600 + $500 Biology Dept Matching Funds.

Scholarships and Membership activities

Full scholarship by the Ministry of Education of Turkey to pursue master and doctoral studies in the United States. 1996-2001.

Virginia Academy of Science. 1999-present.