Cloning and Characterization of Glycogen Synthase From *Dictyostelium discoideum*

By

Brian Williamson

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Approved:

Charles L. Rutherford, Chair

Joseph O. Falkinham

Brenda W. Shirley

Patricia V. Rogers

Eric A. Wong

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(ABSTRACT)

In *Dictyostelium*, glycogen metabolism plays a major role in development. Undifferentiated cells contain stores of glycogen that are broken down and converted to structural components in differentiated cells. The enzyme that synthesizes this developmentally important pool of glycogen is glycogen synthase. I have cloned the entire coding and 1.3 kb of upstream noncoding region, of glycogen synthase, using PCR amplification and genomic library screening. In order to clone the 3' portion of the gene it was necessary to develop a new technique, enrichment-PCR, that relies on the base composition of the *Dictyostelium* genome. Due to the high A+T content of the *Dictyostelium* genome, a polyT primer and a gene specific primer were used to amplify an unknown DNA fragment, flanking a known sequence. Analysis of the complete coding region showed that glycogen synthase possesses three introns that contain the consensus splice sites for *Dictyostelium.*

The luciferase reporter gene was used to study the transcriptional regulation of glycogen synthase. I defined the *cis*-acting elements that are required for proper transcriptional regulation of glycogen synthase by using promoter/luciferase fusions of varying sizes. Using the luciferase reporter system a putative promoter element was identified. Additional luciferase constructs were made to identify the specific nucleotides

involved in transcription of the glycogen synthase gene. Small defined deletions are often necessary for reporter gene analysis. We have developed a deletion cassette that can expand the functionality of any commonly-used vector. The deletion cassette confers the ability to make small specific sequential deletions of the DNA flanking the cassette. We have shown that this altered vector (pNBL) now has the ability to create 2, 4, 5 or 9 bp deletions.

A number of experimental approaches were taken to study the regulation of glycogen synthase. Homologous recombination was used to try to generate a glycogen synthase (-) cell line. In addition, I have constructed a green fluorescent protein (GFP) vector (pNV) based on the pVTL2 vector. This reporter gene is useful for monitoring the expression of a particular gene *in vivo*.

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I would like to thank Dr. Rutherford and my committee; Dr. Falkinham, Dr. Shirley, Dr. Rogers and Dr. Wong, for their invaluable training and advice. I would like to thank all my labmates over the years for friendship and support.

I wish to thank my family who instilled in me a desire to learn.

And lastly, I give my warmest thanks to my wife, Suzanne, thanks for waiting all of these years. Thanks also for being patient and understanding, maybe now we can get on with our life together.

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Chapter 1: Introduction

Dictyostelium discoideum as a Model System

The development of a multicellular organism from a single nondifferentiated cell requires a highly specific and complex series of biochemical processes. Because all cells in an organism start with the same genetic information, the differential expression of genes is critical for development. There are several cellular regulatory mechanisms that control how different proteins are expressed. The major control points occur at the level of transcription, translation, protein stability, mRNA stability and localization. Control of translation occurs on two different levels, on a general level of protein synthesis and the translational control of individual gene products. Examples of global translational control include mRNAs that are sequestered and unavailable for translation in the unfertilized egg. In embryos there is a large store of mRNA that is quiescent until fertilization when the cell undergoes a tremendous pulse of protein synthesis (Bracht et al. 1963; Craig and Piatigorsky 1971; Rogers and Gross 1978). When an organism is exposed to elevated temperatures heat shock genes are expressed. One of the mechanisms for this control is at the level of translation. During heat shock the amount of protein translation is reduced while the amount of heat shock mRNA translation increases (Storti et al. 1980; Manrow and Jacobson 1987).

There are examples of specific genes being regulated at the level of translation. In yeast the enzyme that controls sterol biosynthesis, HMG-CoA reductase 1, is controlled at the level of translation (Demster-Dink et al. 1994). One of the suggested mechanisms of translational control is secondary structure prior to the initiation codon. A poly (A) tract has been implicated in heat shock protein control (Darnell et al. 1990). Interestingly, it is not unusual to find a long stretch of (A)s directly upstream of *Dictyostelium* initiation

codons (Steel and Jacobson 1991). *Dictyostelium* glycogen synthase has a run of twenty-one (A)s directly upstream of the ATG (see **Chapter 2**). Because of the large number of *Dictyostelium* genes that have (A)s prior to their initiation codons, one might predict a large number of *Dictyostelium* genes to be regulated at the level of translation. Although only a small percentage of *Dictyostelium* genes have been examined at the level of translational regulation, to date no strong correlation has been established (Steel and Jacobson 1991).

Cells can control protein activity through degradation or post-translational modification. Glycogen synthase is controlled by phosphorylation in other organisms (See **Chapter 2**). The HMG-CoA reductase 2 enzyme in yeast is controlled by degradation; when the end products of the sterol pathway are plentiful, HMG-CoA reductase 2 becomes unstable (Hampton and Rine 1994).

One of the most important control mechanisms available to a cell is the initiation of transcription. Our laboratory has shown that glycogen phosphorylase-1 and 2 are regulated at the transcriptional level during development (Rogers et al. 1992; Rutherford et al. 1992). The glycogen phosphorylase 2 gene is expressed in response to exogenous cAMP, a *Dictyostelium* morphogen (Sucic et al. 1993). The use of reporter genes is one way to study the transcriptional regulation of a gene. The use of the luciferase reporter system to study transcriptional regulation of glycogen synthase is discussed in **Chapter 2**. The development of a extrachromosomal reporter vector is discussed in **Chapter 4**. The development of glycogen synthase knock-out mutants should facilitate the study of the levels of control other than transcription (See **Chapter 6**).

Recent research has shown that the signals that control a cell's fate may rely on a relatively small number of overlapping pathways and that signals are generally shared by different organisms (Wolpert 1994). This developmental control system is further complicated in that the temporal exposure to these signals may play a role in the cell's response to a signal. Previous signals may prime some genes to respond when exposed to

a second signal (Katz and Sternberg 1992; Maruta and Burgess 1994). Neighboring cells may send additional signals so that the final developmental fate of a cell is a complex interaction of a limited number of signals.

Analysis of developmental regulation is simplified by the use of model systems. The knowledge learned from studying simpler organisms can be extrapolated to higher systems. *Dictyostelium* has become a model system for studying the molecular events associated with cellular differentiation and development because of its relative simplicity.

Dictyostelium has two alternative life cycles, an asexual and sexual life cycle. The sexual life cycle is difficult to produce under laboratory conditions. The asexual life cycle is readily induced and has many advantages that make *Dictyostelium* an ideal system to study development. During asexual development one initial cell type, vegetative amoebae, differentiates into two distinct cell types, spore and stalk cells. An important feature of the asexual life cycle is the division of growth and development into two separate phases. The asexual amoebae feed and multiply as separate organisms and then develop as a social multicellular organism. This separation of growth and development is a marked difference from most other organisms in which growth is tightly linked to morphogenesis (Sussman and Brackenbury 1976). An understanding of the signals regulating development in a model system such as *Dictyostelium* will help facilitate the understanding of other developmental systems.

The Life Cycle of *Dictyostelium discoideum*

Cellular slime molds were discovered in 1869 by Brefeld (Brefeld 1869). *Dictyostelium discoideum* is a cellular slime mold normally found in the leaf mulch of temperate forests (Raper 1935). *Dictyostelium* exists as haploid, single-celled, vegetative amoebae which are genetically, morphologically and biochemically identical (Raper 1940;

Bonner 1952). The amoebae can be readily triggered to undergo differentiation by transferring them to a minimal medium (Fig. 1-1). One cell type undergoes aging and programmed death while the other cell type starts the life cycle over again. Following nutrient depletion, the amoebae begin to emit cAMP which triggers a chemotactic response and migration of the cells to aggregation centers. Approximately 12 hours after nutrient depletion, the amoebae form a multicellular cell mass or slug. For the first time in the developmental cycle, two distinct cell types are detectable (Bonner 1952; MacWilliams and Bonner 1979). The cells in the anterior portion of the slug eventually give rise to the stalk of a mature fruiting body. For this reason these anterior cells are called prestalk cells. The cells in the posterior portion of the slug eventually form the spore cells and, therefore, are called prespore cells. In the final stage the stalk cells die while the spore cells germinate and continue the cycle. *Dictyostelium* provides one of the simplest models of cellular differentiation. The easily-triggered, well-characterized, and rapid differentiation of *Dictyostelium* make it an ideal system to study gene expression during differentiation.

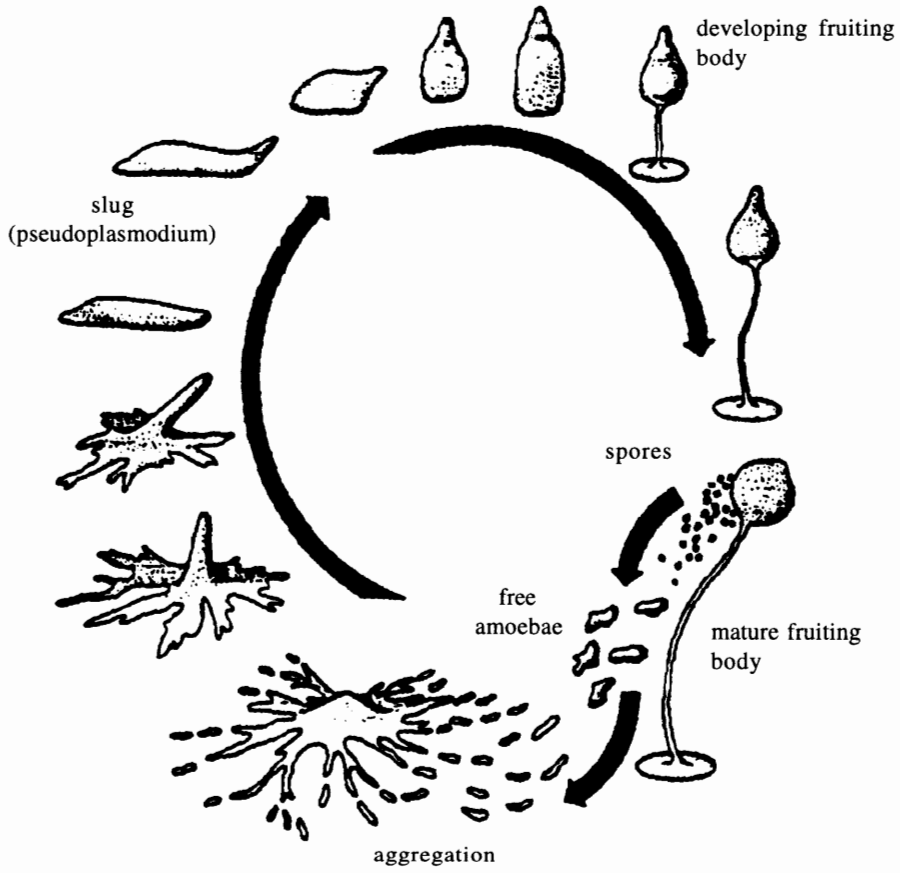


Fig. 1-1: Asexual Reproduction in *Dictyostelium discoideum*

Chapter 2: Cloning of Glycogen Synthase and Promoter Analysis Using The Luciferase Reporter System

Abstract

The glycogen synthase gene was cloned, by library screening, in order to study its regulation. A *Dictyostelium* glycogen synthase specific probe was created using PCR amplification. This 600 bp fragment of *Dictyostelium* glycogen synthase was amplified using degenerate primers designed to conserved regions of glycogen synthases from other organisms. A *Dictyostelium Eco*RI genomic library was constructed and screened, using the 600 bp PCR product, for the glycogen synthase gene. Eight clones containing 3.5 kb of genomic DNA were obtained containing 1.3 kb of upstream noncoding region and 2.2 kb of coding region. Sequencing of genomic clones revealed the presence of three introns that contain *Dictyostelium* consensus splice sites. The results from rtPCR confirmed the existence of the three introns.

The transcriptional regulation of glycogen synthase was tested using various promoter deletions fused to the luciferase gene. Using the luciferase system a putative promoter element has been identified. Additional luciferase constructs were made to identify the specific nucleotides involved in transcription of the glycogen synthase gene.

Introduction

The utilization of glycogen is one of the key events that occurs during the development of *Dictyostelium*. Experiments have shown that the amount of glycogen in a *Dictyostelium* amoeba prior to development correlates with its final developmental fate. Those cells that contain higher amounts of glycogen tend to form spore cells, while cells that have lower amounts of glycogen tend to form stalk cells (Inouye and Takeuchi 1982). During the early stages of development, glycogen is the only polysaccharide present (White and Sussman 1963; Ashworth and Watts 1970). In *Dictyostelium,* protein degradation, rather than glycogen metabolism, provides energy for differentiation, while glycogen breakdown provides glucose units that are required for the synthesis of mucopolysaccharide in spores and for the synthesis of cellulose cell walls in both spore and stalk cells (Fig. 2-1) (Wright et al. 1968; Marshall et al. 1970; Gustafson and Wright 1972; Loomis 1975). As multicellular development commences, glycogen is degraded and the breakdown products serve as precursors for cellulose, mucopolysaccharide, and trehalose, all of which accumulate and together account for about 13% of the dry weight of the fruiting body (Sussman and Sussman 1969).

The enzyme that initiates glycogen degradation, glycogen phosphorylase, has been well defined in *Dictyostelium*. Glycogen phosphorylase has been shown to be due to the activity of two separate gene products, the genes for which have been cloned by this laboratory (Rogers et al. 1992; Rutherford et al. 1992). One form of this enzyme, GP1, is expressed early in development and decreases as development proceeds (Rutherford and Cloutier 1986; Cloutier and Rutherford 1987; Brickey et al. 1990). The second enzyme,

GP2, is expressed in later stages of development and persists through terminal differentiation (Fig. 2-2) (Rutherford et al. 1992).

The enzymatic reaction that controls the synthesis of glycogen, and therefore a great majority of the other carbohydrates synthesized during development, is glycogen synthase. Glycogen synthase (EC 2.4.1.11) catalyzes the formation of α-1,4 glycosidic bonds adding glucose monomers to the growing chain of glycogen. In *Dictyostelium*, as in some other organisms, there are two forms of glycogen synthase, glucose 6-phosphate dependent (D) and glucose 6-phosphate independent (I) (Wright and Dahlberg 1967; Hames et al. 1972). While the total I+D activity remains constant, the D form is prevalent in aggregation and early slug stages and the I form is predominant in later stages (Fig. 2-2) (Wright et al. 1968; Wright et al. 1973). It is unknown if the shift from one form of glycogen synthase to the other is the result of differential expression of multiple gene products or the modification of a single gene product. There are examples of both types of control in other organisms. In mammals, glycogen synthase is a single gene product and is converted by kinase and phosphatase between the D and I forms depending on the metabolic needs of the organism (Cohen 1986). In yeast, the two different forms of glycogen synthase are the products of two different genes (Farkas et al. 1990; Peng et al. 1990). In *Dictyostelium* the method of regulation, differential expression to multiple gene products or the covalent modification of a single gene product, has not been ascertained. Attempts to convert one form of *Dictyostelium* glycogen synthase to the other through phosphorylation have failed; this failure could indicate the presence of two different gene products (Brickey 1988). However, low stringency hybridization of Southern blots argues against the presence of a second gene. Previous attempts to study the regulation of glycogen synthase have centered on the purification and characterization of either the D or I activities. During purification the proteins became increasingly unstable, preventing

purification to homogeneity (Saunders and Wright 1977; Brickey 1988). I took an alternative approach and studied the gene by molecular cloning. The gene encoding glycogen synthase was cloned from genomic DNA using library screening (see Results) and enrichment PCR (**Chapter 3**). We have studied the transcriptional regulation of the glycogen synthase gene through the use of the luciferase reporter system. I report in this chapter the analysis of a series of promoter deletion constructs that identified a putative regulatory sequence within the promoter of glycogen synthase. The construction of the reporter vector is detailed in **Chapter 4**.

Methods

Probe Construction

A 600 bp probe was amplified from genomic DNA using the polymerase chain reaction (PCR) and degenerate primers designed to conserved regions of yeast glycogen synthase. Amplification was performed in a 100 µl reaction mixture containing 1 µg of *Dictyostelium* genomic DNA, 200 µM each of dATP, dCTP, dGTP and dTTP, 50 pmoles of Primer 1 (5'GATTT(GT)GT(CT)(AC)G(AT)GGTCA(CT)TT 3'), 50 pmoles of Primer 2 (5'(AG)TAACCCCATGGTT(CG)AT(AG)ATA 3'), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, and 2.5 Units *Taq* DNA Polymerase (Boehringer Mannheim, Indianapolis, IN) with a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). The PCR cocktail was overlaid with 100 µl mineral oil, then denatured at 94°C for 2 min.. Thirty amplification cycles were carried out as follows: 94°C for 45 sec, 37°C for 45 sec, and 72°C for 2 min with a 10 min final extension step at 72°C. The PCR fragment was subcloned into the SmaI site of pBluescript II SK^+ (Stratagene, La Jolla, CA) and sequenced.

To construct a probe, the 600 bp PCR fragment was agarose gel-purified using the Geneclean system (Bio101, Vista, CA) and labeled with [α^{32}P] dATP (3000 Ci/mmole; DuPont New England Nuclear) using the Random Primed DNA Labeling Kit (Feinberg and Vogelstein 1983) (Boehringer Mannheim).

Genomic Library Construction

Genomic DNA (1 µg) from *Dictyostelium* AX3K cells was partially digested with 1 unit *Eco*RI for 10 min at 21°C. The degree of digestion was quantified by gel electrophoresis. The partially-digested genomic DNA was ligated to 100 ng of *Eco*RI-digested and Calf Intestinal Alkaline Phosphatase (CIAP)-treated vector pBluescript II SK^+ using T4 DNA Ligase at 12°C for 16 hours. Transformation of *E. coli* XL1-Blue cells (Stratagene) was performed using the calcium chloride transformation procedure (Sambrook et al. 1989).

To screen for glycogen synthase clones, a total of 50,000 colonies were transferred onto BA-S NC supported-nitrocellulose membranes (Schleicher and Schuell, Keene, NH) according to the manufacturer's recommendations. The membranes were prehybridized (50% deionized formamide, 5X SSPE, 2X Denhardt's solution, 0.25% SDS, and 150 µg/ml denatured salmon sperm DNA) at 42°C for 2 hours, and hybridized with the 600 bp PCR derived probe described above, for 16 hours at 42°C in (50% deionized formamide, 5X SSPE, 2X Denhardt's solution, 0.25% SDS, and 150 µg/ml denatured salmon sperm DNA) . The membranes were washed 2 times in 2X SSC/0.2% SDS at 42°C for 30 min each, then twice in 1X SSC/0.1% SDS at 60°C for 30 min. The membranes were then exposed to XAR-5 Scientific Imaging Film (Eastman Kodak Company; Rochester, NY) and the positive clones selected.

Deletion Construction

Exonuclease III/mung bean nuclease deletions (Stratagene) were created according to the manufacturer's protocol. Clones 1-7 and 7-1 were chosen because they contain

genomic fragments in opposite orientations relative to the multiple cloning site (MCS) of pBluescript II SK$^+$. A total of 35 µg of each clone was digested with *Cla*I. The *Cla*I overhang was protected with deoxy-thioderivatives then digested with *Hind*III. The DNA was then digested with exonuclease III for various time points. Room temperature digestions were used causing exonuclease III to digest ~ 125 bp/min (Fig 2-5). Six time points were chosen for each orientation 2, 4, 6, 8, 10 and 12 min. This should have created a 600 bp overlap. A total of 10 colonies were selected from each time point and 2-6 different clones/time point were sequenced (nucleic acid sequencing/methods). The regions of the glycogen synthase gene that remained beyond the range of vector-specific primers were sequenced by using gene specific primers.

Luciferase Constructs

All promoter constructs were made from the glycogen synthase genomic clone described above. Deletions D1, D5, D6 and D7 were constructed using an exonuclease-III/mung bean deletion kit (Stratagene). All of the exonuclease-III/mung bean deletions were amplified by PCR from pBluescript II SK+ using a 5' primer (T7 primer) and an internal primer LGS2(5'CCCCAAGCTTATGTTGAATTTGGTGTTCC3'), located downstream of the ATG and including 28 nucleotides of the glycogen synthase coding region. Deletions D2, D3 and D4 were constructed by using primer LGS2 and promoter-specific primers. After ligation of the promoter fragments into the *Hind*III site of the pVTL2 vector (**Chapter 4**; Yin et al. 1994), the constructs were in frame with the luciferase gene. The orientation and frame of all promoter constructs were verified by sequence analysis.

Luciferase assay

Transformed cells were grown in HL5 +5 µg/ml G418 to a density between 10^5-10^6 cells/ml. The transformed cells ($1X10^6$) were centrifuged, washed in 10 ml water, then resuspended in 3 vols. of suspension buffer (30 mM glycyl-glycine pH 8.3; containing 5 µg/ml each of PMSF, pepstatin, and leupeptin). Cell extracts were prepared by freezing the cells at -80°C for 15 min, then thawing them at room temperature. After centrifugation at 14,000 X g for 15 min, 5 µl of supernatant and 100 µl reaction mix (0.54 M glycyl-glycine pH 8.3, 4 mM $MgCl_2$, 2.1 mM ATP) were placed in a Berthold luminometer (Lumat LB9501) for quantification. The luminometer was programmed to inject 100 µl of 0.6 mM luciferin into the reaction cocktail and to record units of luciferase activity. Protein was measured by the Bradford method (Bradford 1976). The specific activity of luciferase is expressed as units of activity per mg of protein.

Transformation

For the genomic library and the subcloning of the 600 bp probe, the transformation of *E. coli* XL1-Blue cells (Stratagene) was performed using the calcium chloride transformation procedure (Sambrook et al. 1989). All other *E. coli* transformations were electroporated using a Gene Pulser (Bio Rad Laboratories, Inc., Hercules, CA) in 0.1 cm cuvettes (960 µFD Capacitance Extender, 200 Ω Pulse Controller, 25 µFD Pulser at 1.8 Kvolts).

Transformation of DNA into *Dictyostelium discoideum* cells was achieved by calcium chloride precipitation as previously described (Nellen et al. 1984) or by electroporation. Cells were harvested at densities ranging from 10^5 to 10^6 cells/ml. A total of 1-3x10^7 cells, in HL5, were chilled on ice for 15 min with periodic swirling, pelleted

and washed in chilled electroporation buffer (10 mM Na_2HPO4-NaH_2PO_4 pH 6.1 containing, 50 mM sucrose). The washed cells were resuspended in 0.8 ml electroporation buffer along with 30 µg transforming DNA. Electroporation was performed in 0.4 cm cuvettes (3 µFD capacitance, 1.0 Kvolts and the pulse controller set at 200 Ω). Ten min after electroporation, the cells were plated in a culture dish containing HL5 media. Following overnight incubation in HL5, 20 µg G418/ml was added; G418 resistance was conferred by the pVTL2 vector (**Chapter 4**). The cells were grown in the presence of G418 for two days before they were plated along with G418 resistant *E. coli* cells (B/R cells) on DM plates (2% glucose, 1% Oxoid peptone, 1.5% Bacto agar, 3 mM Na_2HPO_4, 10 mM KH_2PO_4) containing 70 µg G418/ml (Podgorski and Deering 1980; Hughes et al. 1992). The appearance of plaques on the bacterial lawn indicated the presence of transformed cells. The transformed cells were then assayed for luciferase activity.

Cloning of the 3' Fragment of Glycogen Synthase:

The Enrichment-PCR cloning of the 3' end of the *Dictyostelium* glycogen synthase (DGS) gene is described in detail in **Chapter 3.**

Nucleic Acid Sequencing:

Single- or double-stranded DNA was prepared by standard methods (Sambrook et al. 1989). All DNA preparations were sequenced by the chain termination method (Sanger et al. 1977) according to the sequenase protocol (Sequenase Version 2.0 Sequencing Kit; United States Biochemical, Cleveland, OH) using either vector primers (-20, -40, T3 or T7 promoter) or DGS-specific primers.

Sequencing Kit; United States Biochemical, Cleveland, OH) using either vector primers (-20, -40, T3 or T7 promoter) or DGS-specific primers.

RNA Extraction

RNA was extracted from vegetative amoebae using a modification of the phenol-chloroform technique (Spudich 1987). Cells (2 X 10^7) were frozen and thawed in 50 mM Tris (pH 8.4) and 2% SDS. The cell suspension was extracted 3 times with an equal volume of phenol-chloroform. Sodium acetate (pH 4.7) was added to the last extraction to a final volume of 0.4 M to exclude DNA from the aqueous phase. The resulting aqueous phase was mixed with 2.5 volumes of ethanol for 2-3 hours. The precipitated RNA was collected by centrifugation and resuspended in 10 mM HEPES, 1 mM EDTA.

rtPCR

An aliquot of total RNA was suspended at 100 ng/µl for use in rtPCR (Brenner et al. 1989). Three hundred ng of total RNA were added to each reverse transcriptase reaction (10 mMoles 3' Primer, 2.5 x PCR buffer, 2.5 mM dNTP, 2.5 U AMV RT, 20 U RNasin) at 42°C for 1 hour. The reactions were boiled for 5 minutes to inactivate the reverse transcriptase and held at 4°C. The cDNA was synthesized using the same PCR conditions described in **Chapter 2**/Methods.

Results

Probe construction

A *Dictyostelium* glycogen synthase specific probe was created using PCR amplification. Degenerate primers were designed to match conserved amino acid regions of other glycogen synthases. These primers were used in a Polymerase Chain Reaction (PCR) to amplify a 600 base pair fragment from *Dictyostelium* genomic DNA (Methods) (Fig. 2-3). The PCR fragment was subcloned into the SmaI site of pBluescript II SK$^+$ and sequenced completely. The deduced amino acid sequence indicated that the fragment encoded glycogen synthase (data not shown). Southern analysis showed that the 600 bp fragment gave a specific signal when used as a probe against *Dictyostelium* genomic DNA (Fig. 2-4). A restriction map was created using restriction sites found on the PCR fragment. The restriction mapping showed that there was an *Eco*RI site upstream of the PCR fragment. The *Eco*RI sites were used to clone the glycogen synthase gene.

Molecular cloning of Glycogen Synthase

The DGS gene was cloned using genomic library screening. An *Eco*RI genomic *Dictyostelium* library was constructed using pBluescript II SK$^+$ as the cloning vector. In order to obtain a representative sample of the *Dictyostelium* genome, a total of 50,000 colonies were probed with the PCR amplified glycogen synthase fragment (Methods). Eight positive clones were purified from the partial library and verified by sequencing. The orientation of the inserts was determined by restriction digestion and sequencing (data not shown) (Methods). All inserts yielded a 3.5 kb genomic fragment that included 1.3 kb of

upstream non-coding sequence and 2.2 kb of coding sequence. The remaining coding region was cloned using enrichment PCR (**Chapter 3**). The isolated clones produced a restriction map that matched the pattern of hybridizing bands observed on a genomic DNA Southern blot. In order to facilitate the sequencing of the genomic clones, various nested deletions were created using the exonuclease III/mung bean nuclease system (Fig 2-5; Methods). To determine copy number of the glycogen synthase gene, genomic DNA was digested with various restriction enzymes and then analyzed by Southern blotting using the 600 bp PCR probe. Each digest produced the expected bands, indicating that glycogen synthase is present as by a single-copy gene (Fig. 2-4). Genomic Southern blots were probed at low stringency, with increasingly stringent washes. The blots all showed high background hybridization, however, in no case did the probe hybridize with an alternative glycogen synthase gene (data not shown).

Identifying the start site of transcription can help identify the upstream noncoding regions that are important in the transcriptional regulation of a gene. In order to determine the start site of transcription, hot primer extension was used (Sambrook et al. 1989). Hot primer extension shows that the start site of transcription was about 300 bp upstream of the ATG codon. Due to the extremely A-T rich nature of the noncoding regions of *Dictyostelium* genomic DNA it was impossible to design a primer that would identify the precise sequence involved in transcriptional initiation.

The genomic clones encoding *Dictyostelium* glycogen synthase were sequenced (Methods), and analyzed. The genomic sequence and deduced primary amino acid sequence are shown in Fig. 2-6. The coding region is split by three introns forming four open reading frames that encode a protein of 775 amino acids, with a predicted molecular mass of 87 kD. The introns are flanked by consensus or near-consensus *Dictyostelium* intron splice sites (Datta and Firtel 1987; Grant et al. 1990; Loomis and Fuller 1990). The

presence of the three introns was confirmed by rtPCR (Fig. 2-7, Methods). Fig. 2-8 shows the degree of similarity and identity between the deduced amino acid sequences of glycogen synthase from various organisms. *Dictyostelium* glycogen synthase is 41.8 percent identical to human, 40.3 percent identical to rat, 40.4 percent identical to yeast YGS1, and 40.1 percent identical to yeast YGS2, with a large amount of divergence at the amino and carboxyl termini (Browner et al. 1989; Bai et al. 1990; Farkas et al. 1990; Farkas et al. 1991).

Glycogen Synthase Promoter Analysis

The transcriptional activity of the glycogen synthase promoter was studied through the use of reporter genes. The nucleotide sequence of the glycogen synthase promoter is shown in Fig. 2-9A. In order to study the promoter activity of the glycogen synthase gene the 5' non-coding region of glycogen synthase and various deletions were placed upstream of a luciferase reporter gene (Fig. 2-9A). The promoter deletions were fused in-frame with the luciferase gene. The full-length glycogen synthase promoter/luciferase fusion extends from 1314 upstream to 28 bp downstream of the ATG (construct DFL in Fig. 2-9). This is assumed to be the full length promoter because of the presence of a short reading frame ending prior to deletion D1. The luciferase activity resulting from constructs DFL and D1 showed constant activity during development (data not shown). This constant level of luciferase activity agrees with the constitutive mRNA levels (data not shown). It should be added, however, that the *in vivo* stability of the luciferase protein has not been well established in *Dictyostelium*. As seen in Fig. 2-9B, deletion of 485 bp (D1) and 654 bp (D2), from the 5' end resulted in a reduction to 89% and 60% of the full length promoter luciferase activity, respectively. An additional deletion of 67 bp, (deletion D3), abolishes luciferase activity. Further deletions, D4-D7, failed to restore luciferase activity. These

results suggest that a putative regulatory element lies within the 67 bp between deletion D2 and deletion D3. Within this 67 bp fragment there is a G-C box (CCCAACCAACCAGTG). This type of G-C rich sequence has been implicated in the regulation of other *Dictyostelium* promoters (Datta and Firtel 1988; Pears and Williams 1988).

Discussion

The comparison of functionally similar proteins can help to elucidate regions that are important for regulation. Regions of high similarity can represent active sites or important regulatory functions. Regions of low similarity can be either nonessential or sites of unique regulatory function. The predicted molecular weight of *Dictyostelium* glycogen synthase (87 kD) is larger than that reported in other organisms; the added size of the *Dictyostelium* enzyme is due primarily to unique sequences near the amino and carboxy termini. It has been suggested that the conserved central region is responsible for catalysis and allosteric regulation while the amino and carboxyl termini are involved in covalent regulation of the protein (Farkas et al. 1990).

The UDPG binding site appears to be conserved in *Dictyostelium*; Lys-318 and the sequence surrounding Lys-61 (KVGG) correspond to sequences that have been implicated in UDPG binding in other organisms (Raper 1935; Furukawa et al. 1990; Roach 1990). Other sites are conserved at the amino acid level, in *Dictyostelium* glycogen synthase, including numerous phosphorylation sites. Extensive studies of rabbit muscle glycogen synthase have shown its conversion from the G6P dependent form to G6P independent form of the enzyme is catalyzed by cAMP-dependent protein kinase (cAMPdPK) (Soderling et al. 1970; Soderling et al. 1977), as well as other protein kinases including casein kinases and phosphorylase kinase (DePaoli-Roach et al. 1979; Huang et al. 1983).

Seven potential cAMPdPK sites (R/KXXS) are present in *Dictyostelium* glycogen synthase, Ser-16, Ser-403, Ser-433, Ser-457, Ser-578, Ser-642, Ser-646. Based on the recognition sequence for glycogen synthase kinase-3 (GSK-3) (SXXXS(P)) (Fiol et al. 1988), 8 putative GSK-3 sites can be located in the *Dictyostelium* sequence, six of which are clustered between amino acids 640 and 660. Of these six glycogen synthase kinase sites, four are arranged in tandem so that the phosphorylation of one creates a new GSK-3 site. This type of hierachal protein phosphorylation has been observed in glycogen synthases in other organisms (Roach 1990).

The physiological relevance of these putative phosphorylation sites is unknown. Attempts have been made to force the conversion of glycogen synthase D to glycogen synthase I with phosphatases and kinases (Brickey 1988). None of the treatments employed resulted in loss of glycogen synthase I activity that could be relieved by the addition of G6P. However, this lack of conversion of the two forms does not rule out phosphorylation as a method of regulating glycogen synthase activity but only shows that it does not occur under the conditions of the assay. Initial investigations of rabbit muscle glycogen synthase indicated that several factors must be included in conversion reaction mixtures (Friedman and Larner 1963); these factors may differ for the *Dictyostelium* enzyme and may explain the inability to convert the enzyme.

The glycogen synthase 3' coding region contains numerous CAA repeats varying from seven to ten repeats, a total of 40 repeats within 300 bp. The CAA repeats are translated in all three reading frames in glycogen synthase producing the amino acids asparagine, glutamine or threonine (Fig. 2-6). Similar CAA repeats have been found in other *Dictyostelium* genes, including the enzymatically complementary protein glycogen phosphorylase-2 (Rutherford et al. 1992). CAA repeats are found in both coding and non-coding regions of *Dictyostelium* genes and transposons (Kimmel and Firtel 1985; Andre et

al. 1988; Shaw et al. 1989; Grant and Tsang 1990). There are examples of CAA repeats being associated with proteins in other organisms (Davidson and Posakony 1982) and various regulatory functions have been proposed, yet no definitive function has been assigned to these CAA repeats. We are currently attempting to disrupt the DGS gene through double homologous recombination, and if successful, we should be able to employ various modified enzyme constructs to ascertain the function of these CAA repeats (**Chapter 6**).

Control of a gene expression at the level of transcription is one way that cells control the overall activity of the enzyme. The presence of a G-C rich element between D2 and D3 provides an obvious candidate for a transcriptional regulatory element. *Dictyostelium* genomic DNA is highly A-T rich. The protein coding regions of genes are approximately 30-40% G-C (Kimmel and Firtel 1982), while the noncoding flanking regions are only 5-20% G-C (Jacobsen et al. 1974). A protein has been identified that binds to regions of high G-C content called the G-box binding factor (GBF), and has been implicated in transcriptional regulation of a large number of coordinately regulated genes in *Dictyostelium* (Schnitzler et al. 1994). The GBF gene is transcribed around 4 hours into development. The effect of deleting the first G-C element in glycogen synthase results in total loss of luciferase activity in vegetative amoebae, that is, at a stage of development in which GBF is not present. Thus, GBF is not involved in the basal transcription of the glycogen synthase gene. These findings suggest an additional G-C binding factor or other factor is expressed in vegetative amoebae. Two palindromic G-C boxes downstream of D3 have been identified (Fig. 2-9). The role of these two palindromic G-C boxes in the transcriptional regulation of DGS has not been ascertained.

We are using the pVTL2 vector to further characterize the glycogen synthase promoter (See Fig. 2-10). The G-C box located at the 5' end of promoter deletion D2 has

been studied further by mutation of specific sites in this G-C box. Preliminary screening of some of these mutants has not shown an effect on promoter activity (data not shown). Other *Dictyostelium* transcription factors, such as GBF, have highly degenerate specificity and this might account for the lack of a dramatic effect of point mutations on luciferase activity (Schnitzler et al. 1994). Constructs GF1-2 and GF1-3 are more promising; in these constructs this G-C box was placed on the 5' end of the deletions D3 and D4 to determine whether the G-C box is necessary and sufficient to restore activity to these inactive constructs (Fig. 2-10). Preliminary data indicate that the addition of the G-C box to D4 is sufficient to restore activity (Data not shown). These results, suggest that the G-C box is important to the transcriptional regulation of the glycogen synthase promoter.

The results that are presented in this chapter indicate that a single gene encodes *Dictyostelium* glycogen synthase. Southern blotting of genomic DNA showed single bands of hybridization. Low stringency hybridization did not reveal the presence of a second gene that possessed similarity to DGS, but instead led to an increase in the overall background binding to the probe. In addition, partial purification of the protein did not result in separation of the I and D forms of the enzyme, although the instability of the activity precluded purification to homogeneity. Finally, "enrichment PCR" (Williamson and Rutherford 1994), a procedure that utilizes a gene specific primer and a non-specific poly T primer that binds to non-coding regions resulted in amplification of a single fragment of DNA.

Although these results are compelling with regard to the presence of a single DGS gene, they must be viewed in the context of similar studies of the *Dictyostelium* glycogen phosphorylase enzyme (Rutherford and Cloutier 1986; Rogers et al. 1992; Rutherford et al. 1992). The early literature on glycogen synthase and glycogen phosphorylase described analogous properties of the enzymes. Both glycogen synthase and glycogen

phosphorylase had been shown to exist as two forms of enzyme activity; the former dependent on G6P, and the latter dependent on 5'AMP at certain stages of development. The 5'AMP dependent (D) form of phosphorylase predominated in the early stages of development, then shifted to the (I) form during cellular differentiation. Because the total activity of the I and D phosphorylase was constant at all stages of *Dictyostelium* development, and because conversion of I to D forms of the enzyme was known to occur in a wide variety of organisms and tissue types (Krebs et al. 1964; Yunis and Assaf 1970; Gold et al. 1974; Bai et al. 1988; Van Marrewijk et al. 1988), it was thought that the D to I conversion of phosphorylase in *Dictyostelium* was the result of a phosphorylation/dephosphorylation of a single gene product. Cloning of the phosphorylase D form provided probes that yielded results similar to those described above for glycogen synthase. That is, (1) only one species of mRNA was evident on northern analysis, (2) hybridization of genomic DNA showed no evidence of a second gene, and (3) western blotting or screening of expression libraries did not reveal the presence of a second gene product. Yet, repeated attempts to interconvert the two forms *in vitro* were unsuccessful, a result identical to that described for glycogen synthase (**Chapter 2**/Brickey 1988). Finally, upon purification of the phosphorylase enzyme activity from stages of development that contained both I and D forms, the two forms could be separated into different fractions on ion-exchange chromatography, and eventually were purified to homogeneity. Sequencing of the proteins and cloning of both forms showed unequivocally that the two forms were encoded by separate genes.

Based on the evidence available I conclude that glycogen synthase in *Dictyostelium* is the product of a single gene. It needs to be added however, that a similar conclusion was reached with regard to the glycogen degradative enzyme, and that eventual purification of

glycogen synthase protein (if possible) will be required before a definitive statement can be made about the regulation of glycogen synthase during development.

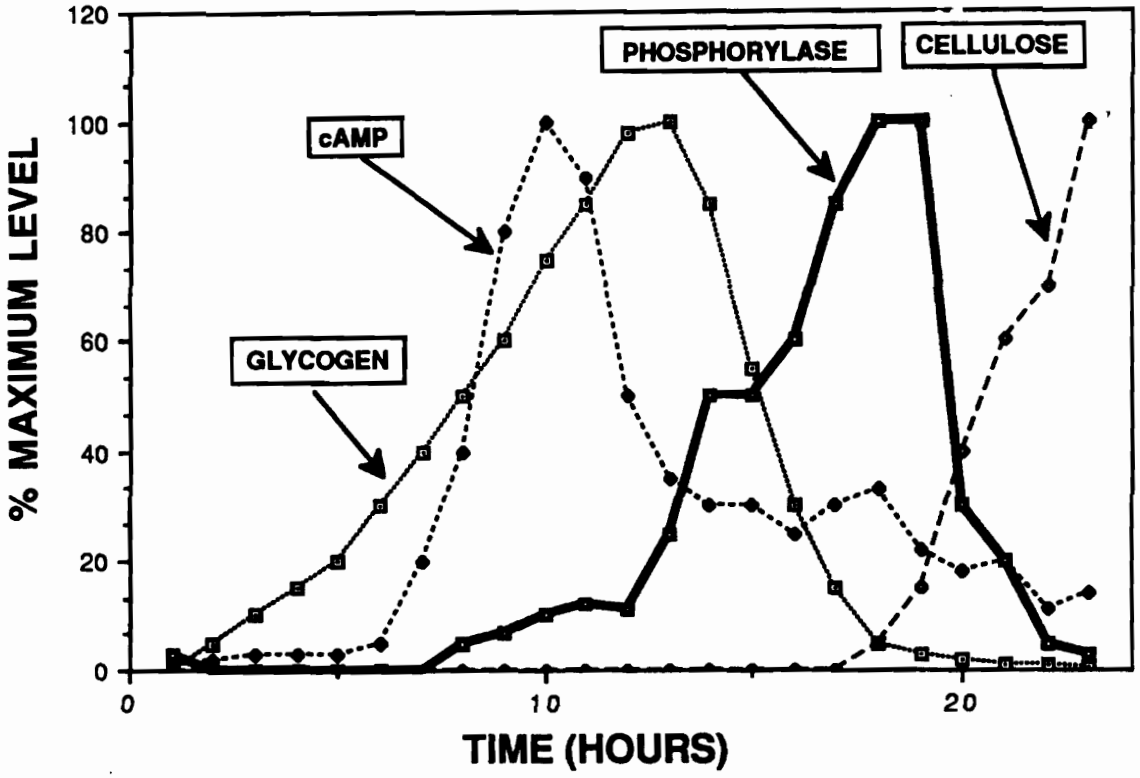


Fig. 2-1: Glycogen Metabolism During Development
All percentages are shown relative to their maximum activity. Time of development is shown on the X-axis with 24 hours representing culmination. Glycogen accumulates to a maximum point at around the aggregation stage. The glycogen accumulated is then rapidly degraded and converted into cellulose and other structural end products of development.

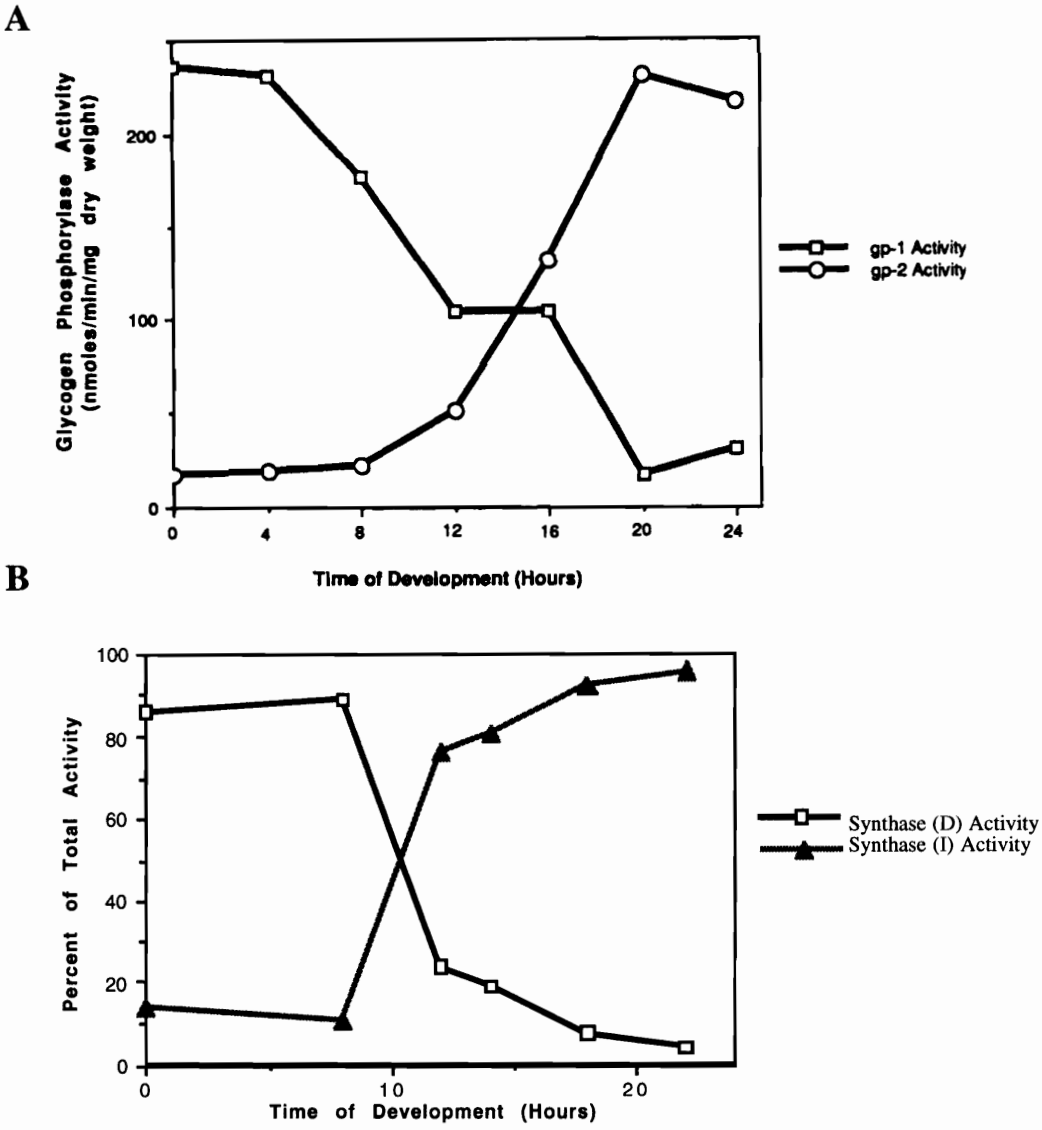


Fig. 2-2: Glycogen Phosphorylase and Glycogen Synthase Regulation Throughout Development.

(**A**) Cells were allowed to grow on pads with activity being measured in the presence and absence of 5' AMP. Glycogen phosphorylase-1 (5' AMP independent) is on early in development and decreases around aggregation. Glycogen phosphorylase-2 (5' AMP dependent) turns on around aggregation and remains on throughout development (Sucic 1992). (**B**) The total glycogen synthase activity remains constant while the enzyme switches from a glucose-6 phosphate dependent to a glucose 6 phosphate independent form of the enzyme (Brickey 1988).

A.

#1 5' ATTTATCATAGATATTGT 3'
C C CC T C

#2 5' GATTTTGTTAGAGGTCATTT 3'
C CC T C

#3 5' ATAACCCCATGGTTCATAATA 3'
G G G

#4 5' ATCAACAATATAAATACCATA
G G G G G

B.

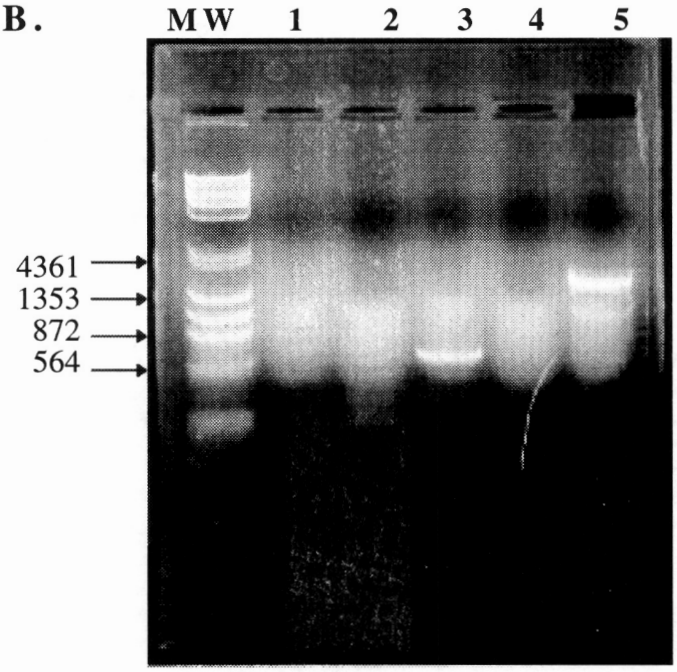


Fig. 2-3: Amplification of Glycogen Synthase using degenerate primers.
(**A**) Degenerate oligonucleotides generated to conserved regions of glycogen synthase. Oligonucleotides 1 and 2 extend towards the 3' end of glycogen synthase and oligonucleotides 3 and 4 extend towards the 5' end of glycogen synthase (**B**) Ethidium bromide stained 1% TBE agarose gel of PCR products. All PCR products were amplified as described in Materials and Methods. Lane 1: PCR amplification using primers 1 and 3. Lane 2: PCR amplification using primers 1 and 4. Lane 3: PCR amplification using primers 2 and 3, Lane 4: PCR amplification using primers 3 and 4, Lane 5: positive control using glycogen phosphorylase-1 primers.

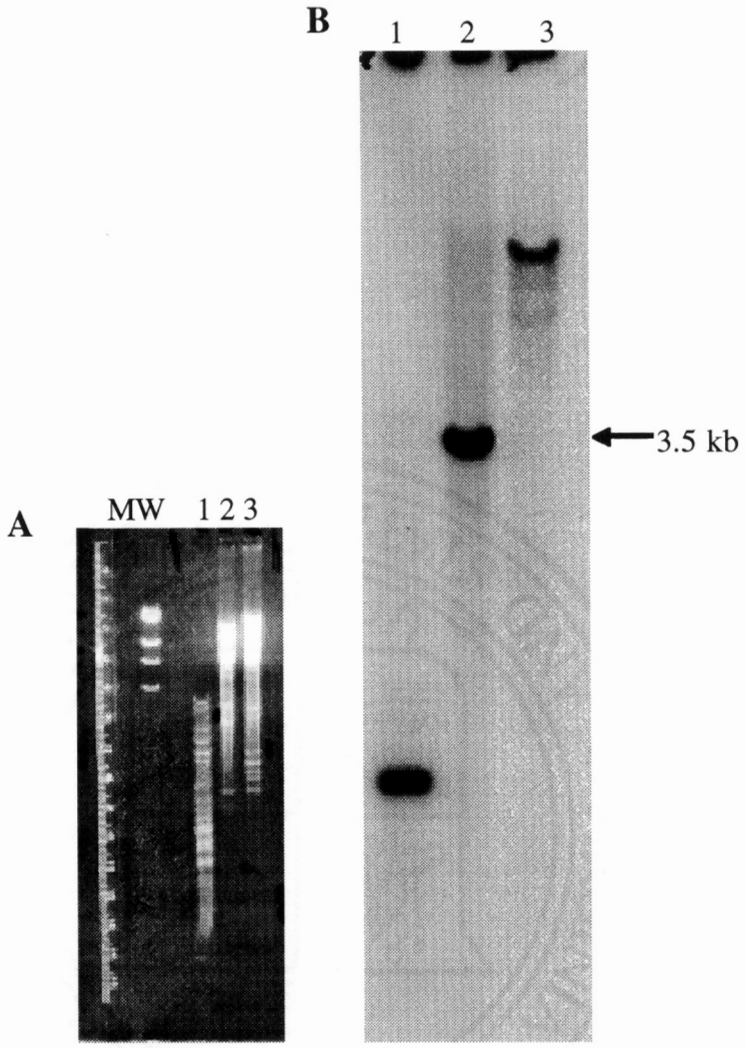


Fig. 2-4: Genomic Southern Blot Using 600 bp Glycogen Synthase Specific Probe.

For both **A** and **B**: 2.5 µg Genomic DNA/Lane. Lane 1 *Dra*I cut genomic DNA. Lane 2 *Eco*RI cut genomic DNA. Lane 3 *Hin*DIII cut genomic DNA. (**A**) Ethidium bromide stained agarose gel. Molecular weight standards are Lambda *Hin*D III. (**B**) The corresponding autoradiograph of the genomic DNA transferred onto supported Nitrocellulose and probed with the 600 bp fragment of glycogen synthase (described above) under high stringency and exposed for 4 hours.

A

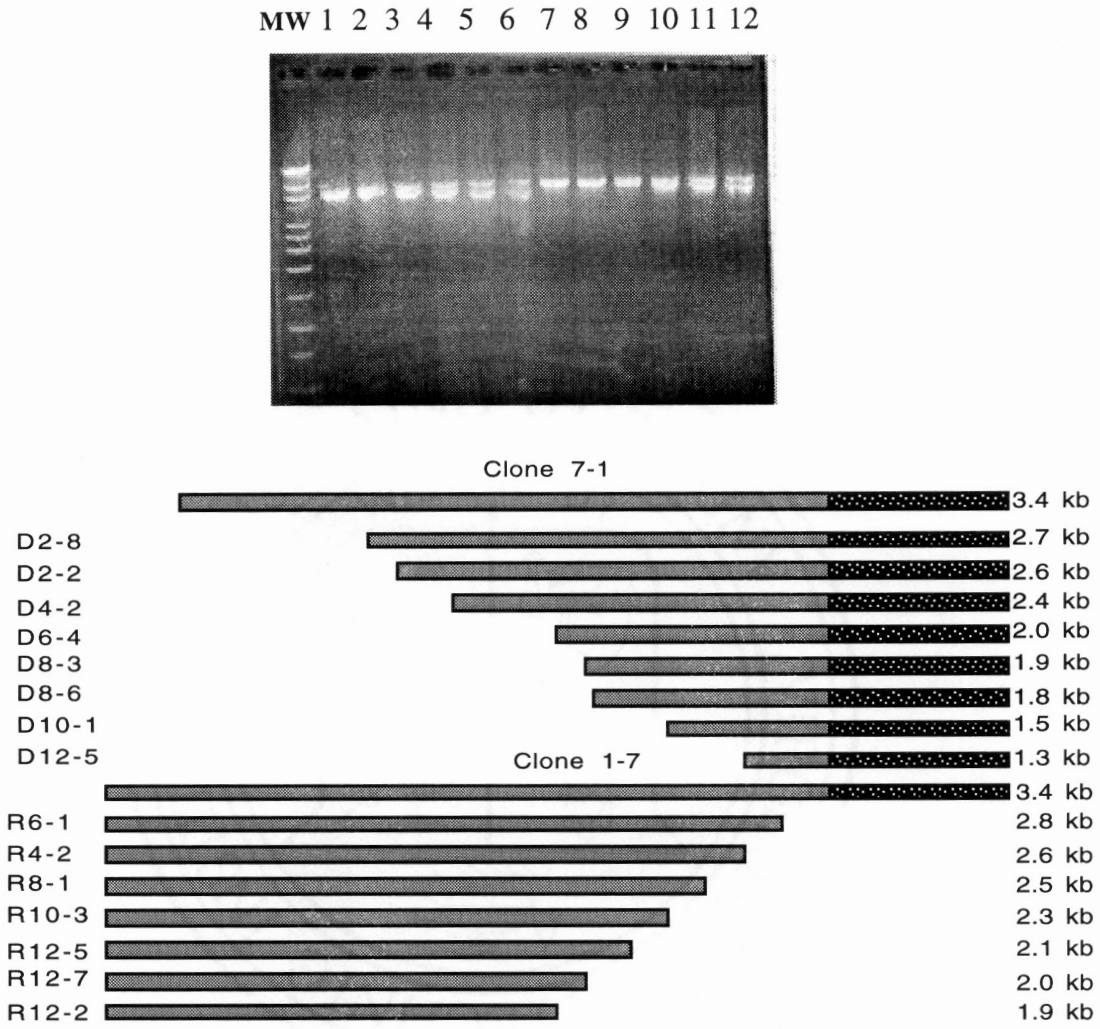


Fig. 2-5: Deletion Constructs of Glycogen Synthase.

(**A**) Ethidium bromide stained 1% TBE agarose gel showing exonuclease III/mung bean treated DNA (Methods). **Lanes 1-6** show clone 7-1. **Lanes 7-12** show clone 1-7 the two clones are in opposite orientation. (**B**) Schematic diagram of selected deletion clones (not to scale). The size of the genomic clone is shown to the right of the deletion and the clone number to the left. The black box refers to the location of the PCR probe (Methods). The number designation refers to the time point the clone was obtained from and the number of that clone from that time point respectively. Deletions progressed much more rapidly through repetitive sequences *i.e.* promoter (see **Chapter 6**).

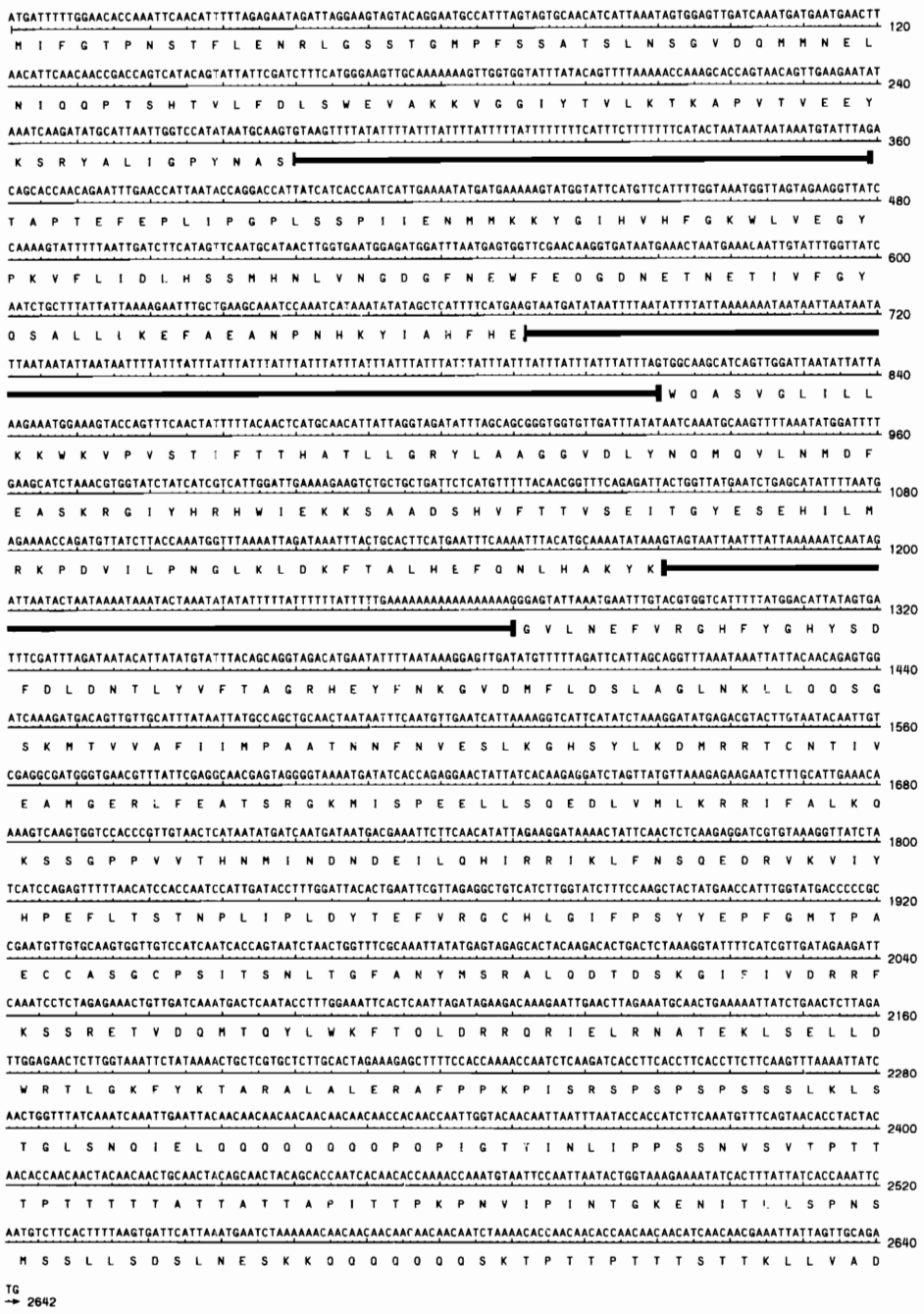
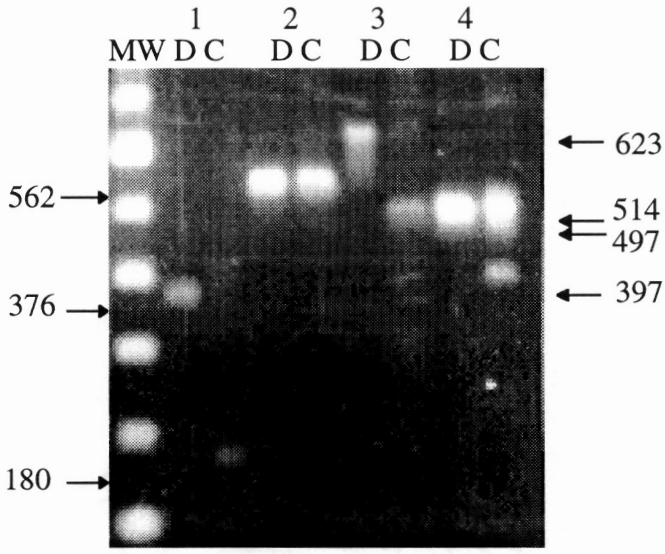


Fig. 2-6: Coding Region of *Dictyostelium* Glycogen Synthase.
The genomic nucleotide sequence of the *Dictyostelium* glycogen synthase gene is shown together with the deduced amino acid sequence of *Dictyostelium* glycogen synthase. The three introns are shown as solid bars, beginning at the splice site and ending just before the next open reading frame.



+1 Primer I1
ATGATTTTTGGAACACCAAATTCAACATTTTTAGAAGAATA....230bp....AATGCAAGTGTAAGTTTTATATTTTATTTAT

TTTATTTTTATTTTTTTTCATTTCTTTTTTTCATACTAATAATAATAAATGTATTTAGACAGCACCAACAGAAATTTGAA

CCATTAATAC + 387 Primer X11

+350 Primer I2
ATGTATTTAGACAGCACCAACAGAATTTGAACCATTAATAC....280bp....AGTAATGATATAATTTTAATATTTTATTAA

AAAAATAATAATTAATAATATTAATAATATTAATAATTTTATTTATTTATTTATTTATTTATTTATTTATTTATT

TATTTATTTATTTATTTATTTATTTATTTAGTGGCA...145bp...GAAGCATCTAAACGTGGTATCTATCATCG +990

Primer I3

+950 Primer I4
ATATGGATTTTGAAGCATCTAAACGTCCTATCTATCATCGTC.. .270bp....AAAATATAAAGTAGTAATTAATTTATTAA

AAAATCAATAGATTAATACTAATAAAATAAATACTAAATATATATTTTTATTTTTTATTTTTGAAAAAAAAAAAAAAA

AAAGGGAGTATTAA....160bp....ATCAAAGATGACAGTTGTTGCATTTATAATTATGCCAGC

Primer IV1

Fig. 2-7: rtPCR of Total RNA

(**A**) Agarose gel of amplified cDNA. Lane 1: Primers X11 and I1 flanking intron number 1. Lane 2: Negative control: primers correspond to a region of glycogen synthase with no predicted intron . Lane 3: Primers I3 and I2 flanking intron number 2. lane 4) Primers IV1 and I4 flanking intron number 3. Lanes marked with a **D** contain *Dictyostelium* genomic DNA alone while the **C** lanes represent total RNA extracts that have been treated with reverse transcriptase. Total RNA samples were not treated with DNase so that in some lanes the DNA band visible. (**B**) Diagram of the sequences amplified. The missing sequence in the middle is indicated by dots and a number. The length of the arrows correspond to the length of the primers. The underlined sequences represent the introns.

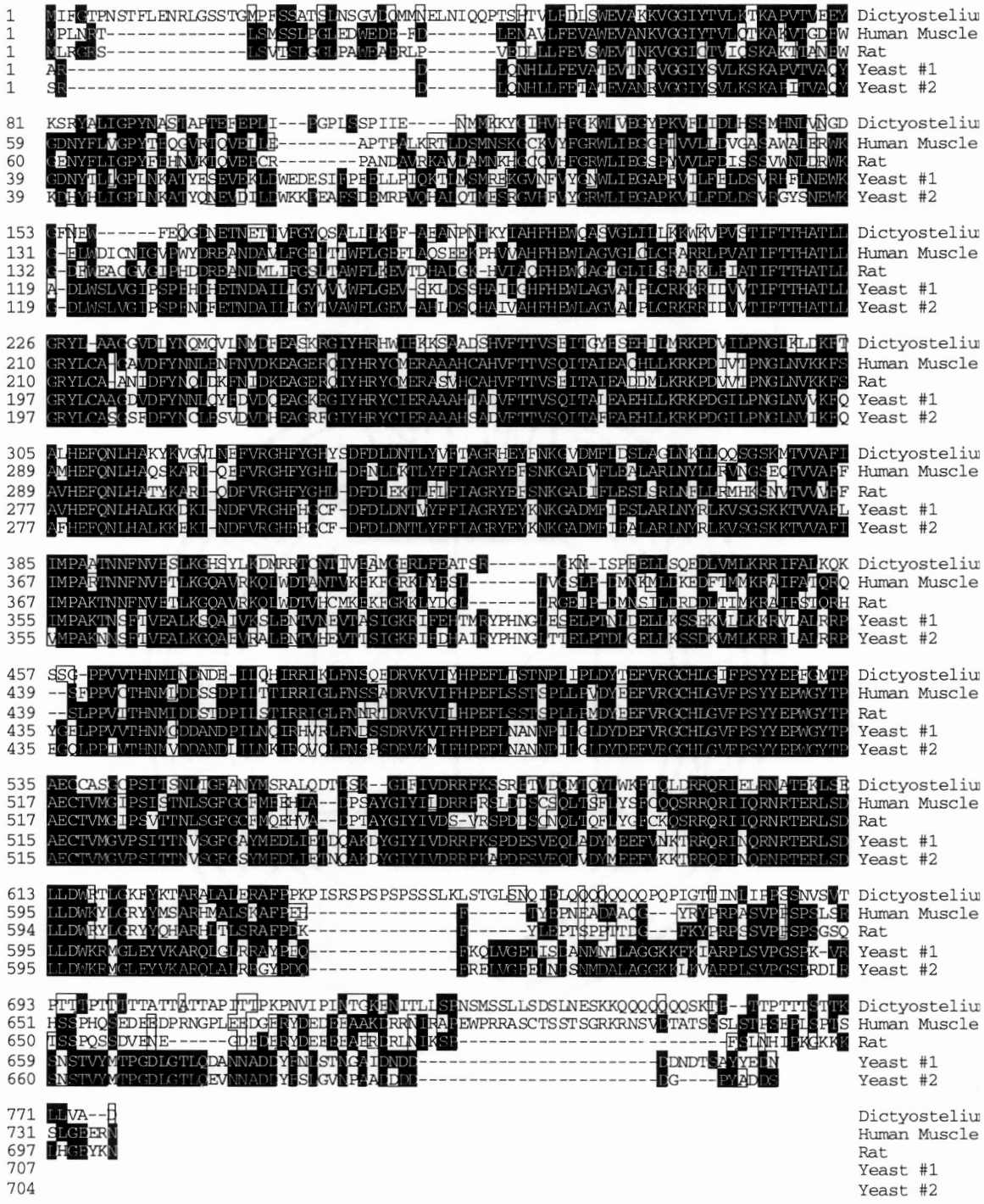


Fig. 2-8: Amino acid comparison of *Dictyostelium*, human muscle, rat liver, and the two forms of *S. cerevisiae* glycogen synthases.

Identities with the consensus sequence are shown as white characters with a black background; amino acids with high similarity are boxed and gaps are indicated by dashes

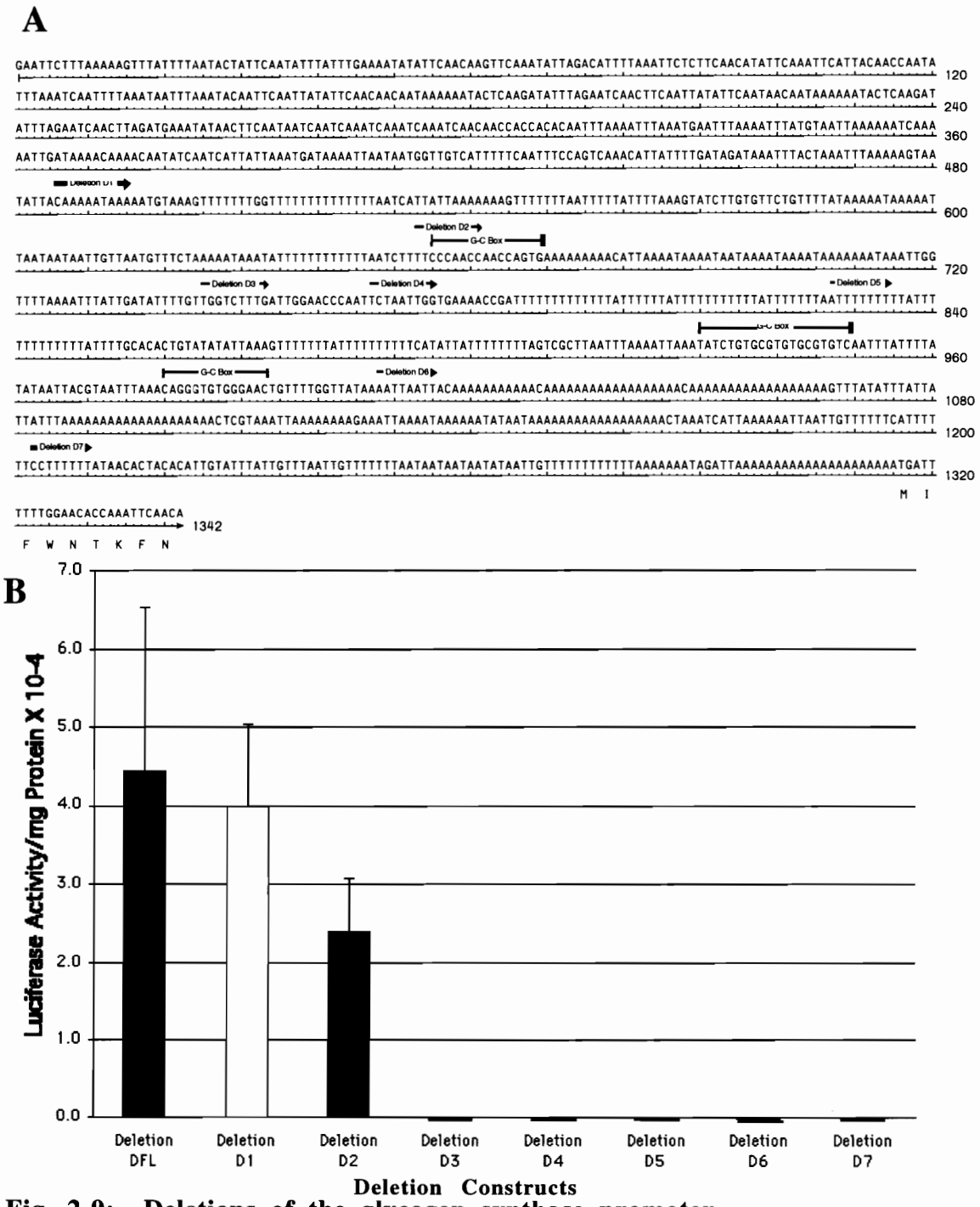


Fig. 2-9: Deletions of the glycogen synthase promoter.

(**A**) Schematic diagram of the glycogen synthase promoter and the deletions used to drive the reporter vector. The promoter fragments were ligated into the luciferase reporter gene vector as described in methods. (**B**) Results of luciferase assays on transformants carrying the glycogen synthase promoter/luciferase constructs. The specific activity of luciferase for each construct is shown. DFL represents the full length promoter fragment. The specific activities shown represent the average of between 6 and 14 replications.

A

WT 5'	TTTTCCCAACCAACCAGTGAAA
M 4 5'	TTTTCCCAACCAAC***G***AG***G***GAAA
M 7 5'	TTTTCCC***G***ACCAACCAG***G***GAAA
M 9 5'	TTTTCCCAACCAACCAG***G***GAAA
M10 5'	TTT***C***CCCA***TG***CAACCAG***G***GAAA
M18 5'	TTTTCCCAACCA***T***CCAG***G***GAAA
M25 5'	TTTTCCC***T***ACCAACCAG***G***GAAA
M35 5'	*Δ* TTTCCCAACCAACCAGTGAAA
M38 5'	TTTTCCCAACCA***C***CCAGTGAAA
M43 5'	TTTT***AT***CAAC***G***AACCAG***G***GAAA
M45 5'	TTTTCC***TG***ACCAACCAG***G***GAAA

B

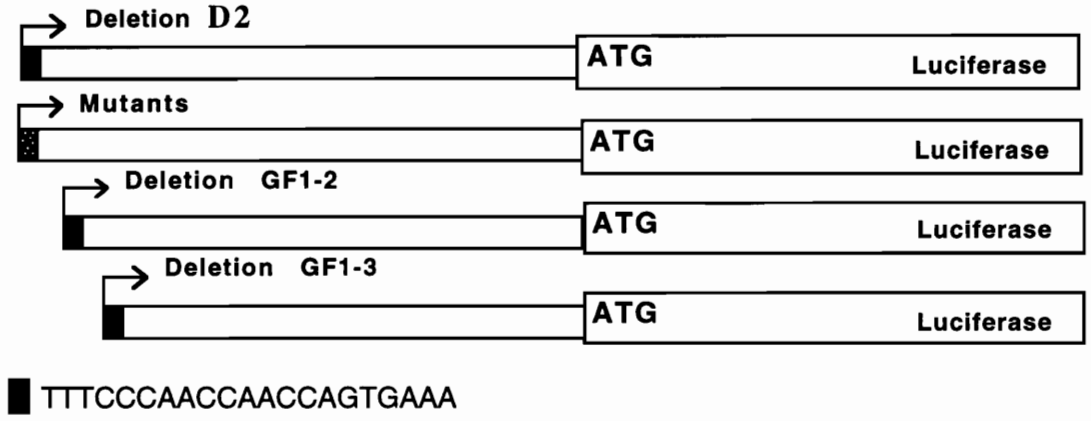


Fig. 2-10: Other Luciferase Constructs

(**A**) Mutant constructs that have been created to determine the bases that are essential for transcriptional regulation of glycogen synthase. Bold nucleotides in italics differ from wild type. (**B**) Additional luciferase constructs GF1-2 and GF1-3 in which the C-box shown above was added to the 5' end of constructs D3 and D4 constructs as shown in Fig. 2-9. Mutant constructs are altered forms of D2, described in Fig. 2-9.

Chapter 3: Enrichment PCR Cloning of Glycogen Synthase

Abstract

The cloning of the 3' coding sequence is described in this chapter. I developed a new PCR technique, enrichment-PCR, that relies on intrinsic properties of *Dictyostelium* genomic DNA. Due to the high A+T content of the *Dictyostelium* genome a polyT primer and a gene-specific primer can be used to amplify an unknown DNA fragment, flanking a known sequence.

Introduction

Several techniques exist for amplifying unknown DNA sequences that are adjacent to known sequences. Most of these amplification techniques require the DNA to be modified prior to amplification. For example, "inverse PCR" and "panhandle PCR" both require restriction enzyme digestion and ligation prior to amplification (Ouchmann et al. 1988; Jones and Winistorfer 1993). I have developed a modification of the 3'RACE System (GIBCO BRL, Grand Island, NY) that utilizes intrinsic properties of *Dictyostelium* DNA and does not require alteration of the DNA. *Dictyostelium* genomic DNA is highly A+T rich. The protein coding regions of genes are approximately 30-40% G+C (Kimmel and Firtel 1982), while the noncoding flanking regions are only 5-20% G+C (Jacobsen et al. 1974). In addition, most *Dictyostelium* genes have polyA stretches at either end of their coding sequences. Due to the high percentage of A+T, it has been estimated that approximately 15,000 oligo $(dA)_{25}$ stretches exist in the genome (Firtel et al. 1976) I have developed a new technique called "enrichment PCR" (Fig. 3-1), that takes advantage of the large number of polyA stretches in the genome. Amplification of unknown flanking regions can be performed using a gene specific primer (GSP) and a polyT primer.

Methods

Enrichment PCR amplification

PCR was performed with a DNA Thermal Cycler (Perkin Elmer, Norwalk, CT). Initial amplification was performed in a 100 µl reaction mixture containing 1 µg of *Dictyostelium* AX3K genomic DNA, 200 µM each of dATP, dCTP, dGTP and dTTP, 50 pmoles of GSP (5'TATGCCAGCTCGAACTAATAATTTC3'); 50 mM KCl, 2.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 8.3. The PCR cocktail was overlaid with 100 µl mineral oil, then denatured at 94°C for 3 min. *Taq* DNA Polymerase was then added (2.5 Units; Boehringer Mannheim, Indianapolis, IN), and 50 amplification cycles, with GSP alone, were carried out as follows: 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min. Between the first and second rounds of amplification the tubes were incubated at 80°C to avoid nonspecific amplification. A second round of amplification was then performed after adding an additional 2.5 units *Taq*, and 50 pmoles polyT primer (5'GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT3'). Thirty additional amplification cycles with GSP and polyT primer were carried out as follows: 94°C for 45 sec, 50°C for 45 sec, and 72°C for 2 min with a 10 min extension step at 72°C. The amplification products were purified using a QIAquick-spin PCR purification kit (Qiagen, Chatsworth, CA) and eluted in a 50 µl final volume. The purified product was used for sequencing without further modification. A sample of the purified amplification product (200 ng) and 10 pmoles of sequencing primer were heated to 99°C for 5 min. The mixture was quick chilled in ice water for 10 min and the products were sequenced according to the USB protocol (Sequenase 2.0; USB, Cleveland, Ohio).

Results

Enrichment PCR was used to amplify a previously unknown 1200 bp fragment of the *Dictyostelium* glycogen synthase gene. The abundance of polyA sites in the genome necessitate initial enrichment for the DNA fragment of choice. This enrichment is achieved by first carrying out linear amplification at high temperatures with the gene specific primer (GSP) alone. As seen in Fig. 3-2, lane 4, amplification with both polyT and GSP primers without first enriching for target sequence yields products that are nearly undetectable. One of these fragments is approximately the size expected for the glycogen synthase gene fragment (1200 bp); however, none of the unenriched bands reacted with a known glycogen synthase probe during Southern analysis (data not shown). After enrichment, a second round of PCR amplification was performed, using both the GSP and polyT primers. This procedure yielded a 1200 bp PCR product that was shown to react with a known glycogen synthase probe (Fig. 3-2, lane 5).

Discussion

As seen in Fig. 3-3, the derived amino acid sequence of the amplified PCR product demonstrates significant similarity to the amino acid sequence of human glycogen synthase. These results verify that the desired product was amplified. Although the PCR products that I obtained were pure enough to be used directly for sequencing, this technique could be combined with nested PCR to increase specificity.

To ensure amplification of the fragment of interest, a gene specific primer must be used that has a sufficiently high Tm to confer specificity. To ensure specificity after the second amplification, the gene specific primer should give little or no background after the linear PCR step of the procedure (personal observation).

This technique should simplify PCR cloning of unknown flanking regions in organisms with A+T rich regions. This technique could possibly be adapted to other organisms with highly repetitive sequences.

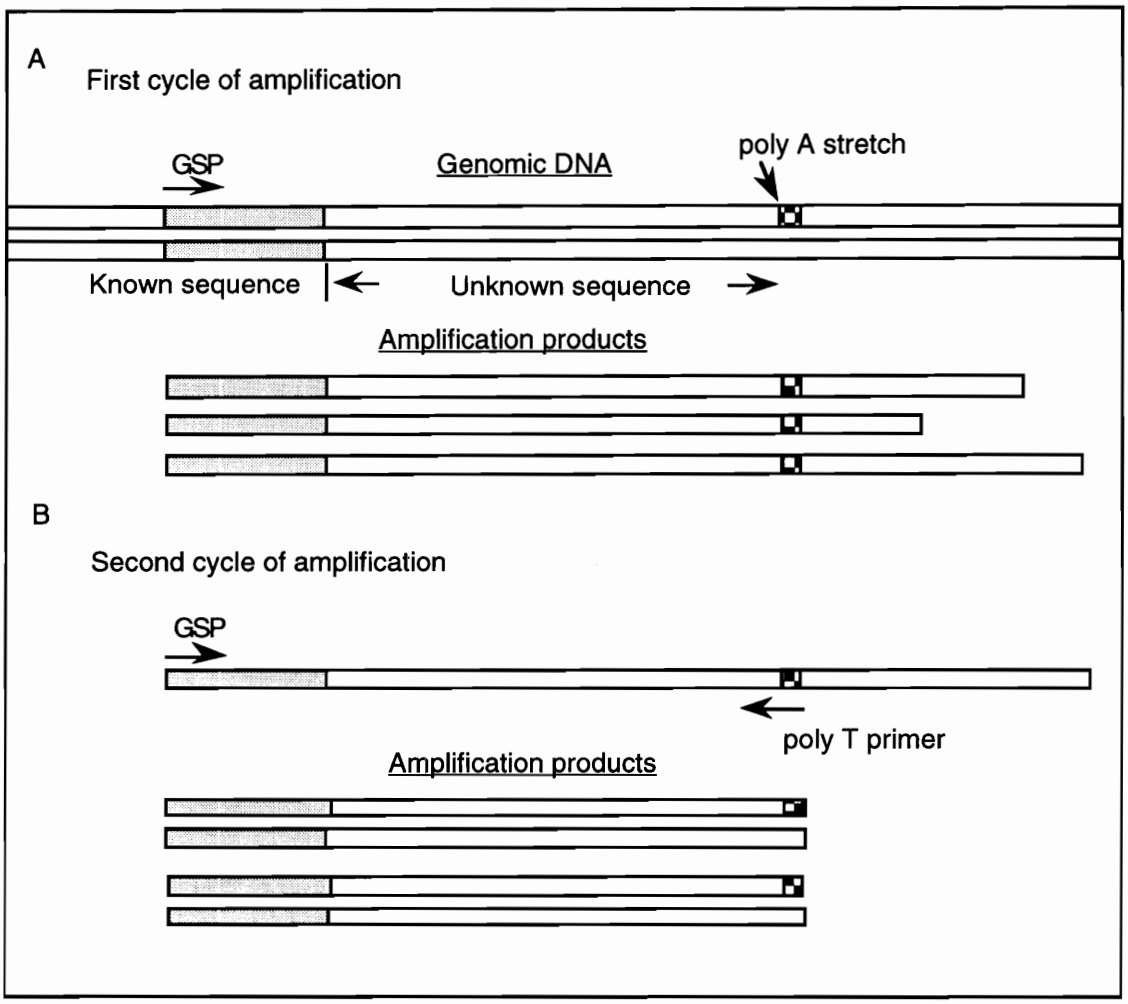


Fig. 3-1: Enrichment-mediated PCR amplification of an unknown DNA fragment that flanks a known sequence.

(**A**). 1 µg genomic DNA was amplified for 50 cycles with GSP alone. This single primer amplification resulted in the linear single stranded amplification of the target DNA. (**B**). PolyT primer was added and the reaction mixture was amplified 30 additional cycles. The addition of the PolyT primer exponentially increased the yield of the target DNA.

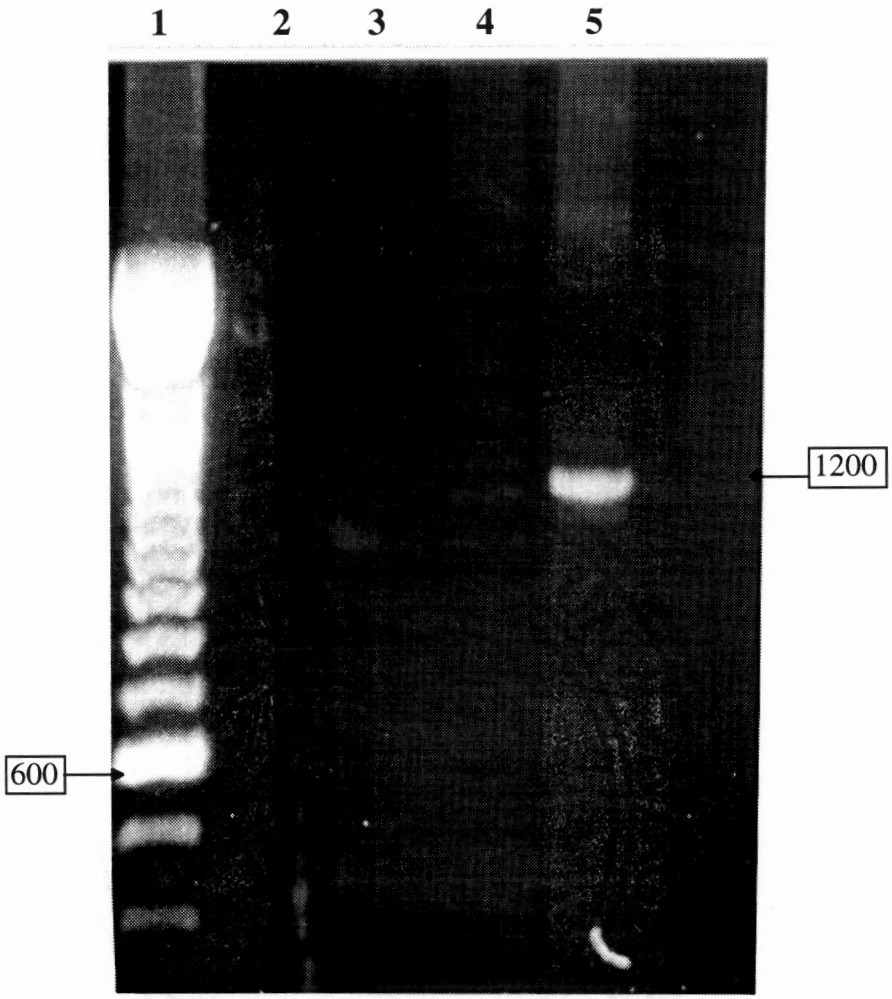


Fig. 3-2: Ethidium bromide staining of PCR amplification products.

All amplifications were carried out on *Dictyostelium* genomic DNA. 10 µl of a 100 µl reaction were added to each lane. Lane 1: 2.5 µg of a 100 bp DNA ladder (GIBCO BRL, Grand Island, NY). Lane 2: 50 cycles of PCR amplification using GSP alone. Lane 3: 30 cycles of PCR amplification with polyT primer alone. Lane 4: PCR products following amplification with both GSP and polyT primers present without a first cycle of amplification. Lane 5: PCR products obtained after a first cycle amplification with GSP alone followed by a second cycle of amplification with both GSP and polyT as primers.

Fig. 3-3: Derived amino acid sequence of enrichment-mediated PCR product compared to human muscle glycogen synthase.

The derived amino acid sequence of the enrichment-mediated PCR product (top) was compared to the sequence of human muscle glycogen synthase from amino acids 461-506 (bottom). The double lines indicate positions of identical amino acids, the double dots indicate positions of highly similar amino acids, single dots show positions of low similarity, and the amino acids without dots are not related. This sequence comparison shows 87% similarity and 78% identity between the two sequences.

Chapter 4: Construction of an Extrachromosomal Luciferase Vector[1]

Abstract

We have constructed an extrachromosomal luciferase reporter vector for *Dictyostelium* using a 626 bp fragment from the nuclear-associated plasmid Ddp2. The *ori* from Ddp2, localized within this fragment, was used to provide an autonomous replication sequence for the reporter vector. This reporter vector was stably maintained in *Dictyostelium* AX3K cells. The vector molecule was also found to exist in a relatively low copy number in the transformed cells, compared to other *Dictyostelium* vectors.

[1] Represents work done in conjunction with Dr. Y. Yin Currently at Harvard, Dana Farber Cancer Institute, Boston Mass. 02215

Introduction

Analysis of *cis*- and *trans*- acting regulatory elements of developmentally important genes has been a focal point for uncovering the molecular machinery that controls the genetically programmed cell differentiation process. For *cis* element analysis, fusion of a DNA sequence with a reporter gene, such as the luciferase or lacZ gene, is frequently used to test for the regulatory function of the segment. In order to introduce the fusion gene into a recipient cell and maintain it in the cell for functional analysis, the fusion must be carried in a plasmid vector. However, all of the currently available reporter vectors for *Dictyostelium* undergo integration into the chromosomes of the recipient cell after transformation. This is apparently due to the lack of a *Dictyostelium ori* in these vectors. The integration appears to occur randomly at multiple sites on the chromosomes. This results in several potential problems. For example, the presence of an endogenous regulatory region that is adjacent to the integration site might influence the expression of the reporter gene. In addition, random and multiple integration events might generate various recombined structures at different integration sites. Moreover, inability to recover the vectors from *Dictyostelium* transformants makes it difficult to analyze aberrant luciferase constructs, which might be due to the genetic recombination between a vector sequence and a sequence in the genome. An extrachromosomally-maintained vector containing a *Dictyostelium ori* will overcome these problems.

A number of nuclear-associated plasmids have been found in *Dictyostelium* (Metz et al. 1983; Noegel et al. 1985; Hughes et al. 1988; Orii et al. 1989). These plasmids could provide useful sequences for construction of various vectors that can be used to target a DNA fragment of interest into the nucleus. Most importantly, no sequence identity has been detected between the plasmids and *Dictyostelium* genome (Noegel et al. 1985; Hughes

et al. 1988), including chromosomal DNA, extrachromosomal rDNA and mitochondrial DNA. The *ori* from one of these plasmids, Ddp2 (*Dictyostelium discoideum* plasmid 2), has been localized within a 626-bp fragment (Chang et al. 1990; Leiting et al. 1990; Slade et al. 1990). This fragment can be used to provide an autonomous replication sequence for the construction of various extrachromosomally-replicating vectors. We report here the construction of an extrachromosomally-maintained luciferase reporter vector for *Dictyostelium*, that is based on the 626 bp Ddp2 *ori*.

Results and Discussion

Plasmid construction

The pVTL2 extrachromosomal luciferase reporter vector was constructed by fusion of a MCS-flanked luciferase gene with the backbone of plasmid p71d2-SB which is a derivative of plasmid p71d2 (Hughes et al. 1992). The luciferase gene, which was fused to *Dictyostelium sp70* gene terminator (Haberstroh and Firtel 1990), was prepared from plasmid pVT2K (a plasmid containing pBluescript II SK^+ vector backbone and an inserted luciferase gene) by restriction digestion with *Bss*HII, followed by gel purification. The ends of the *Bss*HII-digested fragment were filled with Klenow fragment of DNA polymerase I (PolIk). Plasmid p71d2 (Hughes et al. 1992) was digested with *Sal*I and *Bam*HI, filled in with PolIk, and religated to eliminate the *Sal*I and *Bam*HI sites, giving plasmid p71d2-SB. This plasmid then was digested with *Hin*dIII, filled in with PolIk, and ligated with the *Bss*HII-digested and PolIk-filled luciferase gene-containing fragment to give pVTL2.

Fig. 4-1A shows the structure of the pVTL2 vector that contains: (**1**) a luciferase reporter gene whose transcription is terminated by a *Dictyostelium* terminator from the *sp70* gene (Haberstroh and Firtel 1990), (**2**)*Dictyostelium ori* and the *REP* gene from *Dictyostelium* plasmid Ddp2, (**3**) a G418 resistance-encoding gene whose expression is under the control of *Dictyostelium act 6* promoter and *act 8* terminator, and (**4**) an Amp^R gene and an *ori* for use in *Escherichia coli* cells, which are derived from plasmid pGEM3Z (Promega). Both the *REP* gene and the *ori* from Ddp2 are necessary for extrachromosomal

maintenance of the Ddp2 derivatives (Leiting and Noegel 1988; Chang et al. 1990). The pVTL2 vector has two multiple cloning sites (MCS); one at the 5' end and the other at the 3' end of the luciferase reporter gene. This feature enables us to insert either a 5' or 3' *cis*-acting regulatory element into the vector at a site that is either 5' or 3' of the reporter gene. Thus, the vector is useful for studies with both 5' regulatory elements and 3' regulatory elements in the presence of a 5' promoter region. Fig. 4-1B shows the nucleotide sequence of the regions that flank the luciferase gene. The presence of T7 and T3 promoter sequences provide primer binding sites for DNA sequencing, and thus enables confirmation of a correct fusion of an inserted DNA element. The pVTL2 construct is a translational vector due to the presence of the initiation codon for translation (AUG) from the luciferase gene. This enables us to study regulatory elements that do not contain an ATG.

The extrachromosomal maintenance of the vector was demonstrated by Southern hybridization of plasmid DNA prepared from the AX3K transformants. A *Cla*I-*Hin*dIII fragment containing the Ddp2 ori was used as probe. Fig. 4-2A shows the results from a representative experiment in which pVTL2 plasmid DNA from six independent *Dictyostelium* AX3K transformants, that were allowed to grow for 50 generations, (lanes 1-6) gave the same banding pattern as the pVTL2 plasmid DNA prepared from *E. coli* host cells (lane 8). No hybridization was detected in the DNA prepared from untransformed AX3K cells (lane 7) nor from any chromosomal DNA. The results indicate that the pVTL2 vector was maintained extrachromosomally in AX3K cells. The slowest migrating band in each of the six clones is likely the open-circle form of the plasmid. The faint bands between the open-circle band and the supercoiled band can be regarded as the linear form and partially-coiled forms of the plasmid resulting from incomplete neutralization during the preparation of the vector plasmid DNA. To further demonstrate the pVTL2 vector was retained in an unaltered form in *Dictyostelium* AX3K transformants, plasmid DNA prepared from the AX3K transformants was cleaved with *Xho*I followed by Southern

analysis using the same Ddp2 *ori* sequence probe as described above. *Xho*I digestion of pVTL2 should produce two fragments of 5 Kb and 8 Kb. Because the 5 Kb segment contains the *ori* sequence, a corresponding band of 5 Kb should hybridize with the probe. Fig. 4-2B shows that the results were as expected, a 5 Kb band from *Xho*I digested DNA prepared from AX3K cells and *E. coli* host cells. The AX3K cells were passed through 50 generations before they were used for the preparation of plasmid DNA. Therefore, the pVTL2 vector was quite stable in the AX3K transformants as an extrachromosomally-replicating element. The plasmid DNA from both the AX3K transformants and the *E. coli* host cells was also examined with another restriction enzyme, *Xba*I. Identical banding patterns were observed (data not shown). Homologous recombination has been observed in *Dictyostelium* between a transforming DNA in an integrating vector and its complementary sequence in the chromosome (De Lozanne and Spudich 1987; Witke et al. 1987; Rogers et al. 1993). The presence of *Dictyostelium act 6* promoter and *act 8* and *sp70* terminators (see Fig. 4-1 legend) in the pVTL2 vector provides potential sites for homologous recombination between the vector and *Dictyostelium* genome. However, our Southern analysis of the pVTL2 vector plasmid from AX3K transformants revealed that the vector was stably maintained in an unaltered form for 50 generations.

Copy number of pVTL2 vector in AX3K cells

The copy number of Ddp2 in its native strain is approximately 300 copies per cell (Hughes et al. 1988). The Ddp2 derivatives have been shown to have copy numbers of between 100-300 per cell (Hughes et al. 1988; Leiting and Noegel 1988; Chang et al. 1990). The copy numbers of these derivatives are somewhat lower than the native plasmid, but still relatively high. For a reporter vector, low copy number is preferred, simply because most of the elements to be tested are associated with genes that have low

copy numbers themselves. We thought it was possible that the relatively large size of the pVTL2 construct (13 Kb) might result in a low copy number vector plasmid. To determine the copy number of the pVTL2 vector, both nuclear DNA (containing the pVTL2 vector) and the pVTL2 plasmid DNA were separately prepared from the AX3K transformants and the *E. coli* host cells. The two DNAs were probed with the Ddp2 *ori* and the resulting hybridization densities were compared (Fig. 4-3). The results revealed that the copy numbers of four independent clones were in the range of 10-50 copies per cell. The average copy number of the four clones is less than 1/3 of the lowest copy number of the previously constructed vectors based on Ddp2 (Chang et al. 1990). It is possible that the copy numbers of the pVTL2 vector could be further reduced by decreasing the G418 selective pressure for the transformants. For this study, 5 μg/ml G418 was used. However, we have successfully maintained transforming DNA in AX3K cells by reducing the G418 concentration (in HL5 medium) to 1 μg/ml. Many Ddp2 derivatives can be maintained stably in transformants without G418 selective pressure (Chang et al. 1990; Hughes et al. 1992). However, the pVTL2 vector is larger than the Ddp2-based plasmids, and therefore, it may be more difficult to maintain it for many cell generations in the absence of G418 selective pressure.

Conclusions

1) We have constructed an extrachromosomal luciferase reporter vector for *Dictyostelium*. This is the first extrachromosomal reporter vector that has been described for the *Dictyostelium* system. Availability of such a vector should be beneficial for studying *cis*-acting regulatory elements in *Dictyostelium* genes.

2) We have demonstrated stable extrachromosomal maintenance of the pVTL2 reporter vector in *Dictyostelium* AX3K cells. This demonstrated the usefulness of the vector, since the stable maintenance of the vector is crucial in order for regulatory elements to function correctly without alterations.

3) Copy number of the pVTL2 vector was determined to be in the range of 10-50 copies per cell. This is an improvement in lowering the high copy number of *Dictyostelium* vectors.

4) We have demonstrated the utility of this extrachromosomal vector using glycogen synthase promoter fragments of various sizes (**Chapter 2**).

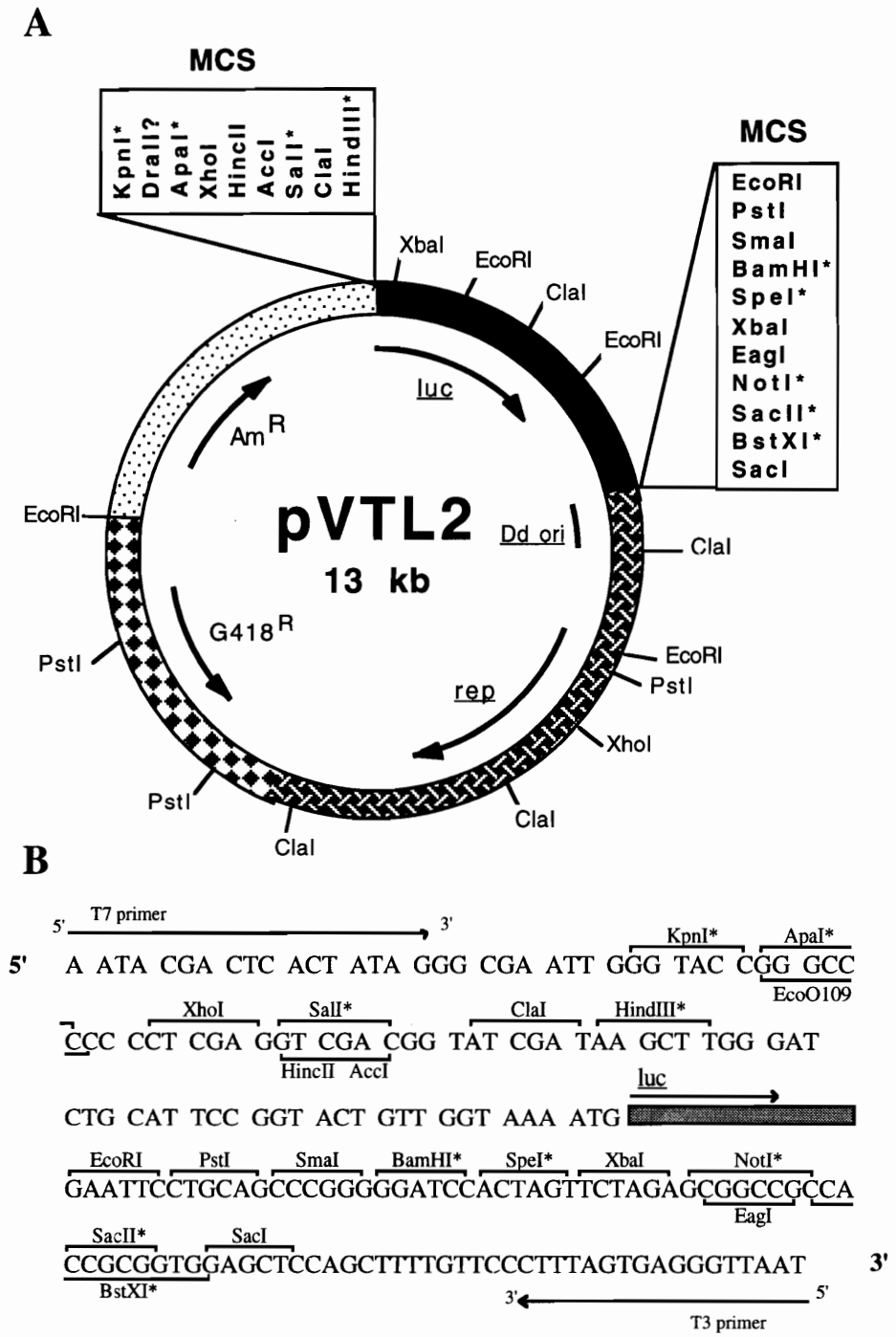


Fig. 4-1: Structure of the pVTL2 vector.

(**A**) Construction of the pVTL2 plasmid. Restriction sites marked with an asterisk(*) are unique in the MCS of pVTL2. The number of *Dra*II sites in pVTL2 is undetermined. (**B**) Sequence surrounding the luciferase gene. The 5' region contains the T7 primer, while the 3' region has the T3 binding sequence. The start codon (AUG) of luciferase is present in the construct.

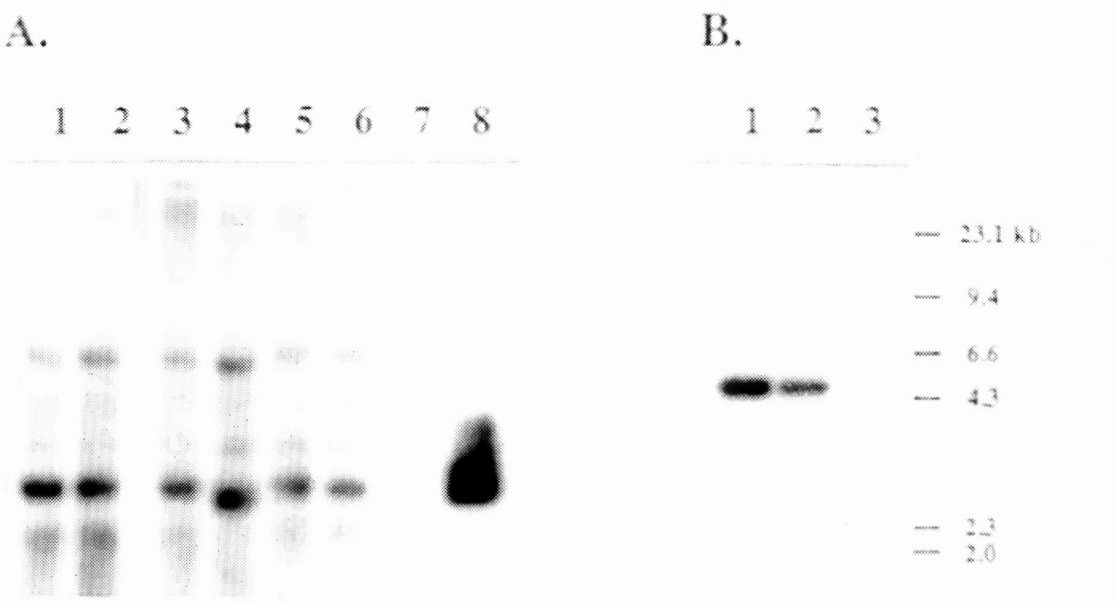


Fig. 4-2: Southern analysis of the pVTL2 vector.

(**A**) Undigested pVTL2 plasmid DNA. A mini-scale *Dictyostelium* plasmid preparation procedure was used to isolate plasmid DNA from cells grown on DM/G418 plates. The DNA was electrophoresed on a 0.8% agarose gel, transferred onto a nitrocellulose membrane (nitroplus 2000, MSI), hybridized with a ^{32}P-labeled *Cla*I-*Hind*III fragment containing the Ddp2 *ori* sequence, and exposed to an X-ray film. **Lanes 1-6**: DNA prepared from six independent pVTL2 transformants. Lane 7: DNA from untransformed *Dictyostelium* AX3K cells. Lane 8: the pVTL2 plasmid isolated from the *E. coli* host cells. (**B**) Lanes: 1: *Xho*I-digested pVTL2 DNA from the *E. coli* host cells; 2: *Xho*I-digested plasmid DNA from an AX3K/pVTL2 transformant l; 3: *Hin*dIII-digested l DNA marker. Digested pVTL2 plasmid DNA. Cells were cultured in HL5 (Hughes et al. 1992) containing 5 µg G418/ml. Preparation of nuclei was performed according to the established procedure (Firtel and Bonner 1972). Plasmid preparation columns (Qiagen) were used to isolate plasmid DNA from the nuclei according to the manufacturer's instructions.

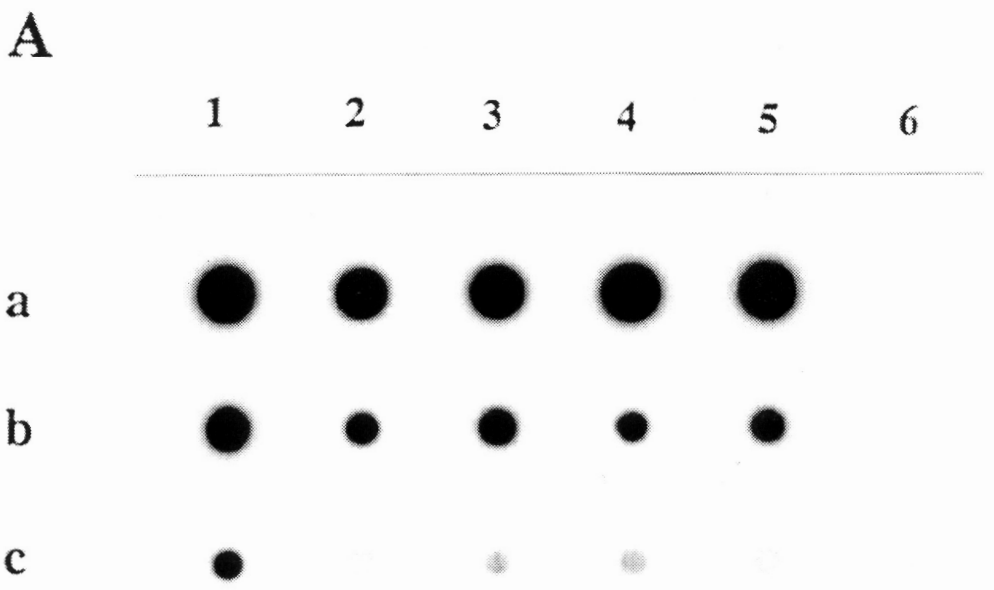


Fig. 4-3: Copy number of the pVTL2 vector.
Southern hybridization of a dot blot. Cells were grown in HL5 medium containing 5 g G418/ml. The Welker procedure was used to isolate nuclear DNA (Welker et al. 1985). The dot blot was prepared using a Hybri-Dot Filtration Manifold from BRL. Before blotting, DNA was denatured with 0.1 vol. of 3M NaOH at 65°C for 30 min, followed by a neutralization step with 0.1 vol. of Na-acetate, pH 4.8, at room temperature. A final volume of 0.5 ml DNA solution in 6X SSC was loaded onto a nitrocellulose membrane (nitroplus 2000, MSI). The membrane then was hybridized with ^{32}P-labeled Ddp2 *ori* sequence. Column 1 contains 26 (a), 2.6 (b) and 0.26 (c) ng of plasmid pVTL2, and column 2, 3, 4 and 5 were loaded with 1.0 (a), 0.1 (b) and 0.01 (c) g nuclear DNA from four independent clones, LV103, LV104, LV121 and LV122, respectively. Row a, b and c of column 6 contain 1.0, 0.1 and 0.01 g nuclear DNA, respectively, from untransformed AX3K cells. The copy number of pVTL2 in AX3K transformants was determined based on the following: the size of pVTL2 (13 Kb) is 2.6×10^{-4} as large as the size of *Dictyostelium* genome (5×10^4 Kb), 1 g nuclear DNA from the AX3K transformants should give the same hybridization density as 0.26 ng pVTL2 plasmid DNA from the *E. coli* host cells, if there is only one copy of pVTL2 per cell. The hybridization density of the dot blot was determined by a video densitometer (version 620, BioRad).

Chapter 5: A Blue/White Selectable Cloning Vector (pNBL) With the Facility for the Generation of Defined, Sequential Deletions Within a Nucleic Acid Sequence *

Abstract

We have developed a deletion cassette that can expand the functionality of any commonly used vector without significantly altering the vector. The deletion cassette confers the ability to make small specific sequential deletions of the DNA flanking the cassette. In order to demonstrate the utility of this vector we used the pBluescript II SK$^+$ cloning vector. The deletion cassette does not alter the utility of the pBluescript II SK$^+$ vector leaving the multiple cloning site intact and maintaining blue/white selection of this vector. We have shown that this altered vector (pNBL) now has the ability to create 2, 4, 5 or 9 bp deletions. We propose additional deletion cassettes that would greatly increase this vector's deletion capacity.

* Represents Work done in conjunction with Dr. Ian McCaffery; Currently at VPI&SU, Department of Biology, 2029 Derring Hall, Blacksburg, 24060

Introduction

The analysis of putative regulatory elements within a region of DNA typically involves deletion analysis using the exonuclease III/mung bean nuclease deletion system (**Chapter 2**). Initially large deletions are generated and regulatory elements crudely mapped *in vivo* using a reporter gene system (Sambrook et al. 1989). In addition, internal deletion analysis is often employed to examine the function of individual regulatory elements. However, in order to analyze *cis*-acting elements it is necessary to define regulatory elements precisely. While the exonuclease III/mung bean nuclease deletion system is a routine procedure, the irregular rate and sequence dependency of exonucleolytic digestion (personal observation; see Fig. 2-4) makes the generation of the large numbers of random deletions required time consuming and expensive. Another common way to generate small defined deletions is through the use of Polymerase Chain Reaction (PCR). PCR-generated deletions have several drawbacks. Chiefly, multiple primers must be generated followed by amplification and subcloning, which is not only expensive and time-consuming but is restricted by the DNA sequence dependency of the primer design. In addition, the error rate of most thermostable polymerases can be a problem for large inserts. Here we describe a system which allows the rapid generation of small defined sequential deletions thereby facilitating the fine analysis of regulatory elements. This system is not limited by the composition of the sequence to be deleted. Additionally, this system may be used for insertional mutagenesis by generating small defined sequence duplications.

A number of restriction enzymes possess the property of cleaving DNA at a specific site that is distant from the recognition sequence. We have exploited this property by the insertion of a small cassette containing a tandem arrangement of the recognition sequences of two of these 'distant-cutters', *Bsg*I and *Bsm*BI. This array of sites is organized such that the recognition sequences remain intact following digestion with both enzymes. Since each enzyme cleaves at a different distance from the recognition site the sequence between the sites of cleavage is deleted upon digestion with both of the enzymes. The enzyme recognition sites remain intact following deletion, therefore the cassette can be used for sequential deletions. The system offers further levels of versatility since different sized deletions can be produced depending on the enzyme(s) sites inserted and the method of repair chosen prior to re-ligation of the vector *i.e.* mung bean nuclease or T4 DNA polymerase (Fig. 5-2). Deletion cassettes of this nature can expand the functionality of any commonly used vector without significantly altering it. In order to demonstrate the utility of this cassette it was inserted into the pBluescript II SK^+ vector, notably leaving the comprehensive multiple cloning site intact and maintaining the blue/white selection feature of this vector. We have shown that this altered vector (pNBL) now conveys the capacity to create small defined deletions.

Results

The restriction enzymes *Bsg*I and *Bsm*BI cleave DNA to produce staggered ends at sites that are distant from their recognition sequences. *Bsg*I produces a 2 bp 3' overhang by cutting 14 bp and 16 bp from the recognition sequence while *Bsm*BI produces a 4 bp 5' overhang by cutting 1 bp and 5 bp from the recognition sequence (see Fig. 5-1). These enzymes were inserted as a deletion cassette into the *Kpn*I and *Sal*I sites of the cloning vector pBluescript II (SK)+ (Stratagene Inc.) (Methods/Fig. 5-1). The insertion of the cassette results in the disruption of the *Kpn*I site. This site was restored downstream of the deletion cassette regenerating the multiple cloning site. The overall size of the deletion cassette was designed to maintain the reading frame of the *lacZ* gene retaining blue/white selection of recombinants of this vector (pNBL). As shown in Fig. 5-2, the recognition sites of these enzymes are organized such that digestion with both of these enzymes results in a deletion of either 5 bp or 9 bp depending on whether the vector is repaired with T4 DNA polymerase or mung bean nuclease. Even smaller deletions, 2 bp and 4 bp, are produced when single digestions are performed with *Bsg*I and *Bsm*BI respectively, followed by treatment with mung bean nuclease.

In order to demonstrate the utility of this vector, pNBL was digested with both enzymes individually and in combination and blunt ended with T4 DNA polymerase (Methods). Since the restriction enzymes cut into the multiple cloning site of pNBL we used the disruption of restriction enzyme recognition sites to show that the proper deletions had been created (Fig. 5-3). Digestion with *Bsg*I alone followed by T4 digestion and religation destroys the *Sal*I site by creating of a 2 bp deletion (Fig 5-2 and Fig. 5-3 lane 14). Digestion with *Bsg*I, and *Bsm*BI followed by treatment with T4 creates a 5 bp deletion destroying both the *Kpn*I and the *Sal*I sites (Fig. 5-2 and Fig. 5-3 lanes 4 and 7). These treated vectors were also redigested with *Bsg*I and *Bsm*BI to show that they retained

the capacity to make further deletions. Fig. 5-3; lanes 8, 13 and 2, 9 show that the *Bsg*I and *Bsm*BI sites are still present, therefore the construct retains the capacity to be deleted further.

Methods

Creation of pNBL

50 pmoles of the two primers NBL1 (5' TCGACGGTACCGAGACGCTGCACTCGAGGGCCCGTAC 3') and NBL2 (5' GGGCCCTCGAGTGCAGCGTCTCGGTACCG 3') were combined in a total volume of 50 µl of annealing buffer (1 M NaCl, 0.1 M Tris-HCl pH 7.8, 1 mM EDTA) and overlaid with mineral oil. The annealing mixture was placed in a water bath at 99°C which was switched off and allowed to cool to room temperature overnight. The plasmid pBluescript II SK$^+$ was digested with *Kpn*I and *Sal*I and gel-purified. The vector (100 ng) and the annealed primers were ligated in molar ratios (vector:oligo) 1:1, 1:10, 1:100, 1:1000 at 16°C overnight and used to transform *E. coli* by electroporation (**Chapter 2**). Clones were digested with *Bsg*I to identify clones that contained the deletion cassette since *Bsg*I is now unique in the recombinant vector (data not shown). In order to create deletions, pNBL was digested and blunt ended with T4 or mung bean nuclease according to manufacturers instructions.

Conclusions

We have created a vector that provides the ability to make small sequential deletions in any DNA sequence adjacent to it. This vector should facilitate analysis of putative regulatory elements since it is easy to control the size and location of the deletion created. In addition, different sized of the deletions can be created using different restriction enzyme sites. For example, *Bsg*I and *Bbv*I can create a maximum deletion of 16 bp. As other enzymes become available the deletion capacity of this type of deletion vector could be greatly increased. The added restriction enzyme sites must be unique in the vector and target DNA after insertion. One additional advantage of pNBL is that it is not a specialized deletion vector; it can be used as a cloning vector without utilizing the deletion capacity of the vector.

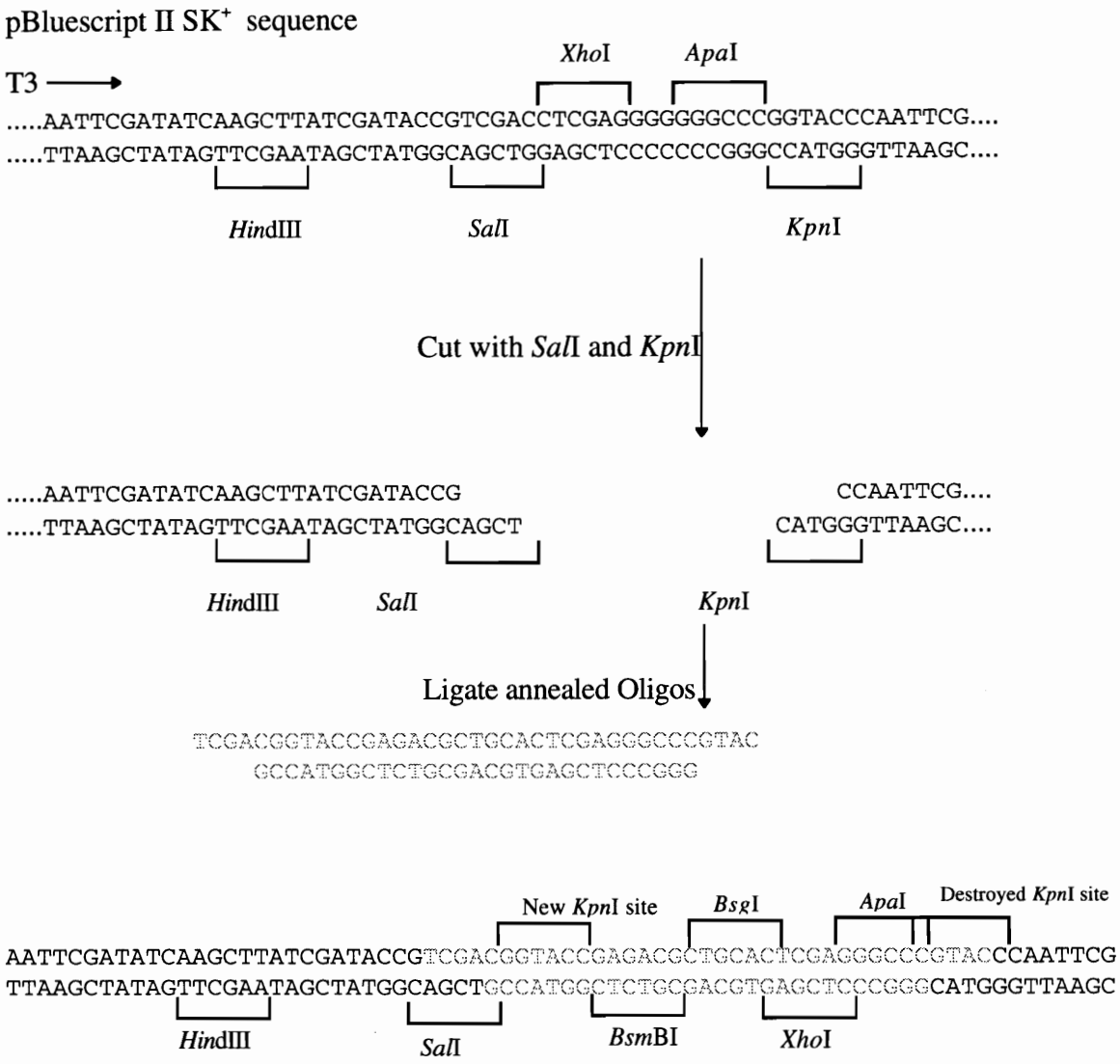
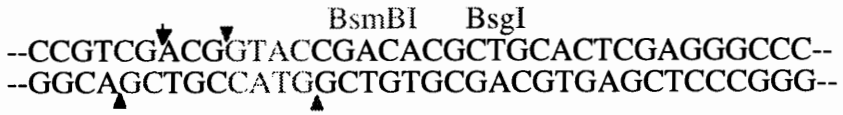


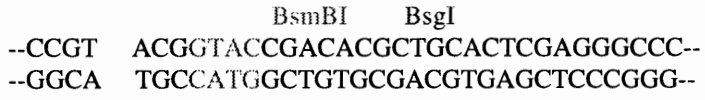
Fig. 5-1: Construction of pNBL/Multiple Cloning Site of pNBL.
The vector was constructed in such a way as to maintain all of the cloning functions of pBluescript II SK+. A total of 12 bp were added to maintain blue/white selection. We chose to recreate the *Xho*I and *Apa*I sites downstream of *Bsg*I and *Bsm*BI to allow cloning on both sides of the deletion cassette. This arrangement of sites should allow the creation of internal promoter deletions.

pNBL

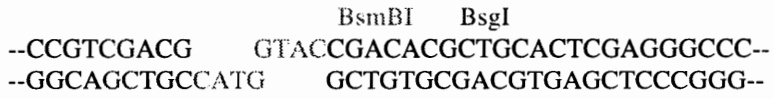


1) Cut with BsgI alone (2 bp deletion)

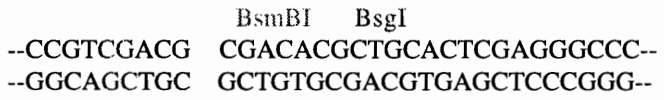
Mung Bean Nuclease or T4 polymerase



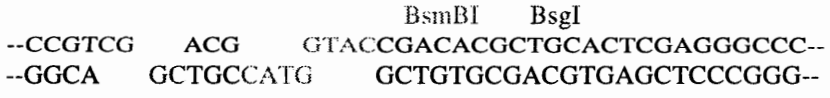
2) Cut with BsmBI alone (4 bp deletion)



Mung Bean Nuclease



3) Double digestion (5 or 9 bp deletion)



Mung Bean Nuclease
(9 bp deletion)

T4 Polymerase
(5 bp deletion)

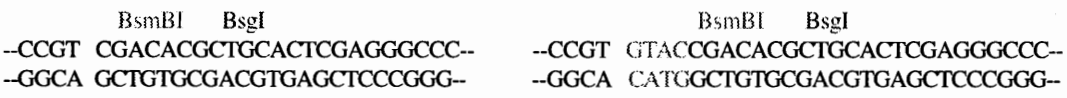


Fig. 5-2: Schematic of Deletions Created When Restriction Enzymes are Used Singly or in Combination.

The restriction enzymes are indicated above their recognition sites. BsmBI and BsgI are differentiated by the shade they are written in, the site of cleavage of each restriction enzyme is indicated by being the same shade as the restriction enzyme. The different deletions that are created by digestion with one or both enzymes are indicated.

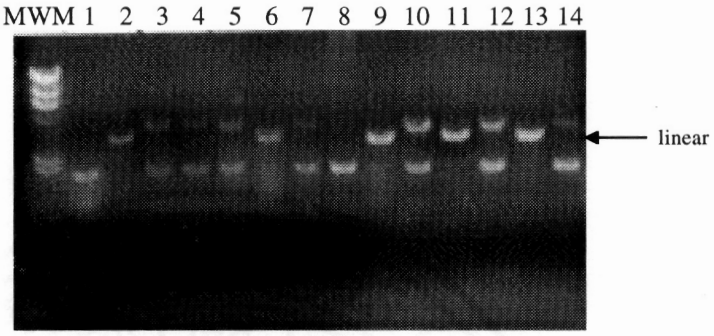


Fig. 5-3: Verification of Deletions Using Restriction Enzymes.

Molecular weight markers (MWM) are *Hin*DIII cut λ DNA. **Lanes 1-7** represent pNBL deletions generated by *Bsm*BI, *Bsg*I double digested, followed by T4 polymerase treatment. Lanes 1, 3, 5 represent deleted pNBL incubated in the appropriate restriction buffer without restriction enzyme. Lane 2 *Bsm*BI digested deleted pNBL DNA. Lane 4 *Kpn*I digested deleted pNBL DNA. Lane 6 *Bsg*I digested deleted pNBL DNA. Lane 7 *Sal*I digested deleted pNBL DNA. **Lanes 8-14** represent *Bsg*I digested, T4 polymerase treated pNBL. Lanes 8, 10, 12 represent deleted pNBL incubated in the appropriate restriction buffer without restriction enzyme. Lane 9 *Bsm*BI digested deleted pNBL DNA. Lane 11 *Kpn*I digested deleted pNBL DNA. Lane 13 *Bsg*I digested deleted pNBL DNA. Lane 14 *Sal*I digested deleted pNBL DNA.

Chapter 6: Knock-Out Strategies For Glycogen Synthase

Abstract

A number of knock-out strategies were used in an effort to generate a glycogen synthase (-) cell line. The vectors employed and the screening techniques utilized are discussed as well as strategies that could be used if further study is pursued.

Introduction

The creation of a gene disruption of glycogen synthase would enable us to answer a number of fundamental questions regarding glycogen metabolism, cellular development and molecular controls. Gene disruptions of the genes encoding glycogen phosphorylase-1 (GP1) and glycogen phosphorylase-2 (GP2) showed that these genes are coregulated (Rogers et al. 1993). The loss of GP2 led to the up regulation of GP1. It would be interesting to determine whether the expression patterns of GP1 and GP2 are altered in a glycogen synthase (-) background. In addition, previous studies have suggested that glycogen is required for the production of other carbohydrates during development (see **Chapter 2**). A study of the carbohydrate content of the mutant cells would define whether an alternative pathway is available for carbohydrate production. In addition, if the knockout causes a phenotypic change, mutant forms of the glycogen synthase enzyme could be screened to see if they can rescue the phenotype to identify structure-function relationships in the protein. For example, a mutant enzyme either lacking or containing a lower number of the CAA repeats present in the 3' end of the gene could be used to determine if the large stretches of CAA repeats have any functional activity in the cell.

The use of gene disruption to study gene function is routine in *Dictyostelium*. The most common form of gene disruption currently being utilized in *Dictyostelium* is restriction enzyme-mediated integration (REMI) (Kuspa and Loomis 1992). This REMI technique works by introducing the linear plasmid along with the restriction enzyme used to linearize it into *Dictyostelium* amoebae. Surprisingly, the plasmid is often integrated into the restriction site used. The resulting library is then screened for mutations that give a desired phenotype. While this technique is particularly powerful at obtaining previously

undiscovered genes whose disruption results in a specific phenotype, it is not suited for targeting a specific gene.

Other knock-out techniques that have been used to disrupt *Dictyostelium* cells were considered as well. The use of antisense RNA to disrupt gene expression is a popular technique in many systems including *Dictyostelium* (Rosenberg et al. 1985; Liu et al. 1992; Erickson 1993; Fang et al. 1993; Heinrich et al. 1993). Antisense RNA has also been identified as the natural method that *Dictyostelium* cells use to regulate the expression of the EB4 gene (Hildebrandt and Nellen 1992). Despite its proven usefulness in attenuating *Dictyostelium* genes, the technique has limitations. For years *Dictyostelium* researchers have had difficulty disrupting genes through antisense, that are expressed late in development (McCaffery personal communication). It has recently been shown that some antisense transcripts expressed late in development do not seem to bind their target sequences and are therefore ineffective (Oberosler et al. 1995). For this reason as well as technical reasons the use of antisense RNA was not pursued. The technique that I decided to use was the creation of a gene disruption through homologous recombination. Homologous recombination occurs at relatively high frequencies in *Dictyostelium* with published frequencies varying from (<.5 to >30 %) (De Lozanne and Spudich 1987; Rogers et al. 1993).

Methods

Transformation ($CaPO_4$) of *Dictyostelium* Cells

Cells were grown to stationary phase (over $2x10^7$ cells/ml) in HL5 (Rutherford and Cloutier 1986). $1x10^7$ cells were added to 12.5 ml of MES-HL5 pH 7.1 (1.4% glucose, 0.7% yeast extract, 1.4% peptone, 10 mM N-morpholinoethanesulfonic acid) in a petri dish and allowed to adhere overnight. Fresh MES-HL5 was added with the DNA solution (400 µl of sterile MilliQ H_2O, 12 µg DNA (1 µg/µl stock), 500 µl 2 x HBS (pH 7.1) and 100 µl 1.25 M $CaCl_2$). The DNA solution was allowed to precipitate at room temperature for 45 min, then added dropwise to the plates and swirled. The plates were incubated at 22°C for 12 hours. The cells were glycerol shocked with 3 ml of 15% glycerol solution in 1 x HBS for 3 min. The glycerol was removed and 12.5 ml HL5 was added. The next day the cells were selected using streptomycin (50 µg/ml) and G418 (10 µg/ml). After 2 days of selection they were plated on DM plates and selected as described (see **Chapter 2**)

Results

Originally a knockout construct was made by insertion of a single fragment of the glycogen synthase coding region into the pDdNeoII vector (Witke et al. 1987) (data not shown). This strategy was based on insertional mutagenesis in which the glycogen synthase gene would be disrupted by insertion of a large fragment of DNA into the center of the coding region. However, this strategy could have led to reversions through deletion of the plasmid since the entire sequence of the glycogen synthase gene was still present. Therefore, this strategy was abandoned in favor of a double homologous recombination approach that should replace the center portion of the gene with the G418 resistance cassette. This double homologous recombination has the advantage in that the center part of the gene is lost so that reversions are impossible (for a review see Sedivy and Joyner 1992). A knock-out construct was made based on a proven knock out plasmid (p60) (Rogers et al. 1993) (obtained from Yizhong Yin, currently at Dana Farber Cancer Center, Harvard). A 1212 bp fragment of *Dictyostelium* genomic DNA was amplified by PCR from a genomic clone and inserted into the *Hin*dII site of p60. This DNA segment contains a fragment stretching from -674 to +538 bp. An additional 688 bp (+1852 to + 2540) of glycogen synthase coding region was amplified by PCR from genomic DNA and cloned into the *Eco*RI site creating pGS-KO (Fig. 6-1). The orientation of both inserts was verified by sequencing (see Methods/**Chapter 2**). If the construct performed as planned a 1314 bp fragment of the center of glycogen synthase coding region should have been replaced by the G418 cassette, containing the gene (Aph3'II) encoding for aminoglycoside 3'-phosphotransferase. In order to reduce the number of false positives obtained by random integration of the transformation construct, a promoterless knockout vector, pGS-PL, was also constructed (Fig. 6-2). This vector puts the G418 resistance gene under the control of the glycogen synthase promoter. The glycogen synthase promoter is truncated

so that only a plasmid that integrated into the glycogen synthase gene, or randomly integrated next to an active promoter, will survive selection in G418. This method has been shown to increase the chances of an individual clone being a targeted integration by 50 fold (Bradley et al. 1992). However, this approach was unsuccessful. The positive control that contained a full-length glycogen synthase promoter driving the expression of the Aph3'II gene was unable to produce G418-resistant transformants (data not shown). The conclusion was that the glycogen synthase promoter is not strong enough to drive sufficient Aph3'II expression, therefore the promoterless construct could not rescue a targeted integrant. It was decided to pursue the glycogen synthase knockout using the pGS-KO construct (Methods).

Screening

Because the technique selected generates a large number of random integrations, a screening technique that was quick and easy to perform was needed. A number of screening techniques were tested. The use of iodine vapor to screen for mutations in the glycogen metabolism pathway has been successful in a number of other organisms, ranging from *E. coli* to *S. cerevisiae* (Leung and Preiss 1987; Romeo et al. 1991; Rowen et al. 1992). The transformants are plated and the plates inverted over iodine crystals, allowed to stain for ~10 min, and the color intensity of each transformant was compared. Since glycogen stains brown when exposed to iodine vapor, glycogen (-) mutants should exhibit little or no staining. This iodine vapor technique is quick, easy and cheap. When *Dictyostelium* transformants were exposed to iodine vapor the spore head stained a very dark brown. It has been shown previously that little to no glycogen is present in the mature spore (Rutherford 1976). The iodine staining observed must have been due to a factor other than glycogen. Because iodine is not specific for glycogen in *Dictyostelium*, it was discarded as a screening technique. The second technique tested was screening by the PCR. A summary of the technique that I developed to perform PCR on whole

Dictyostelium cells is included in Fig. 6-3. This technique utilized one primer from the fragment that should have been deleted if homologous recombination occurred, and another primer orientated towards the first primer. This technique has the disadvantage of producing a band if the gene is intact but no band if the gene is disrupted. In order to use this screening procedure each clone had to be streaked out one additional time to avoid any contaminating untransformed cells that might be on the plate and give a false negative band. To circumvent the problems with this PCR screening technique I developed a third screening technique that can be used on pools of cells. This third technique involves the use of one primer that is specific to the G418 cassette and a second primer that is 5' of the 1.2 kb fragment included on the vector. A positive colony would produce a band 1.5 kb in size. The only way amplification could occur would be if the vector integrated close to the gene-specific primer.

I have screened approximately 350 G418 resistant colonies for a glycogen synthase mutant and found no transformants. There are two different possibilities for this result: I may not have screened enough colonies or the glycogen synthase disruption could be lethal. Since *Dictyostelium* cells are haploid, disruption of an essential gene would be lethal.

One of the problems that I encountered was obtaining large quantities of transformants. Transformation efficiencies varied between 3-9 transformants/30 μg DNA using electroporation. Together with Laura Gubanc, an undergraduate researcher, I tested many different parameters of electroporation of *Dictyostelium* cells. These electroporation tests are summarized in her undergraduate honors thesis. Calcium chloride-mediated transformation increased the number of transformants/μg by around 225 fold over the electroporation. Consequently, we have switched to calcium chloride method of transformation.

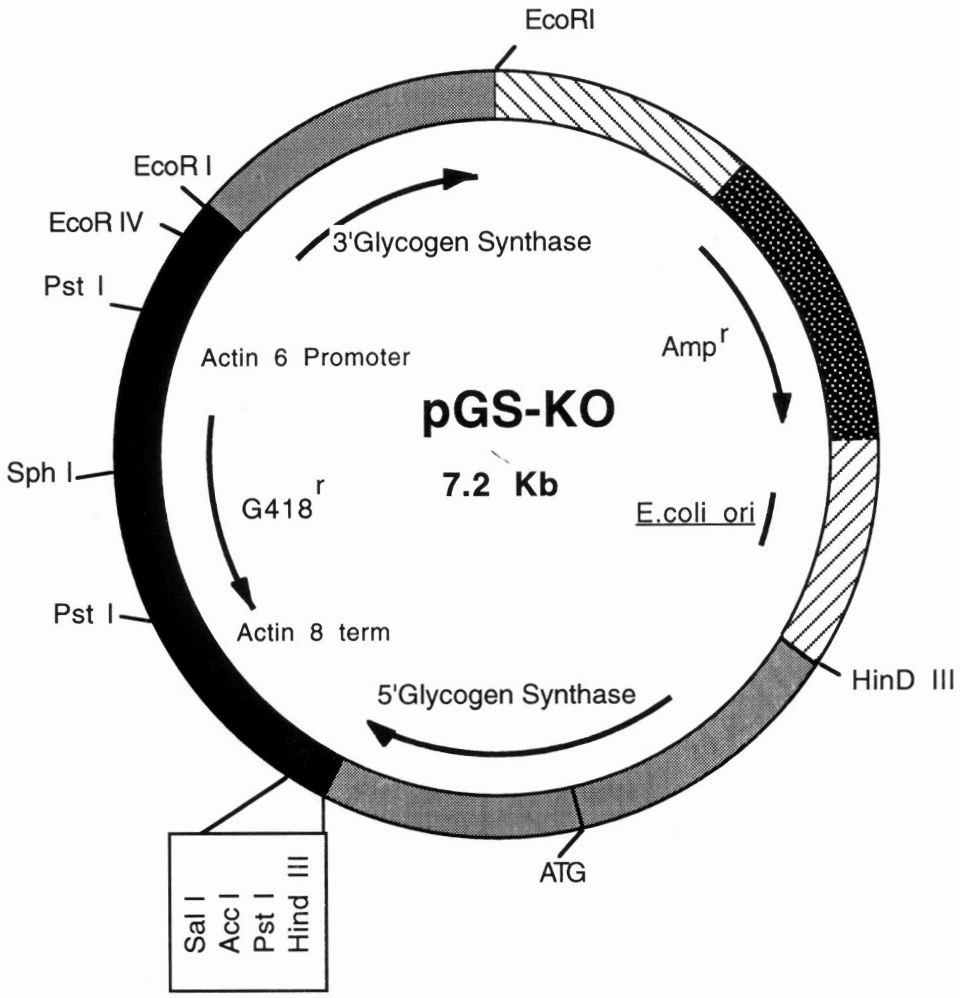


Fig. 6-1: Glycogen Synthase Knockout Vector.
The knock-out vector GS-KO is shown above. A 1.2 kb fragment of glycogen synthase 5' coding and upstream noncoding region was cloned into the *Hin*dIII site of p60. A 0.7 kb fragment of glycogen synthase 3' coding sequence was cloned into the *Eco*RI site of p60. The two fragments of glycogen synthase surround the G418 cassette, should replace the center portion of the glycogen synthase gene. The G418 cassette has its own promoter and terminator and can act independently of the rest of the vector.

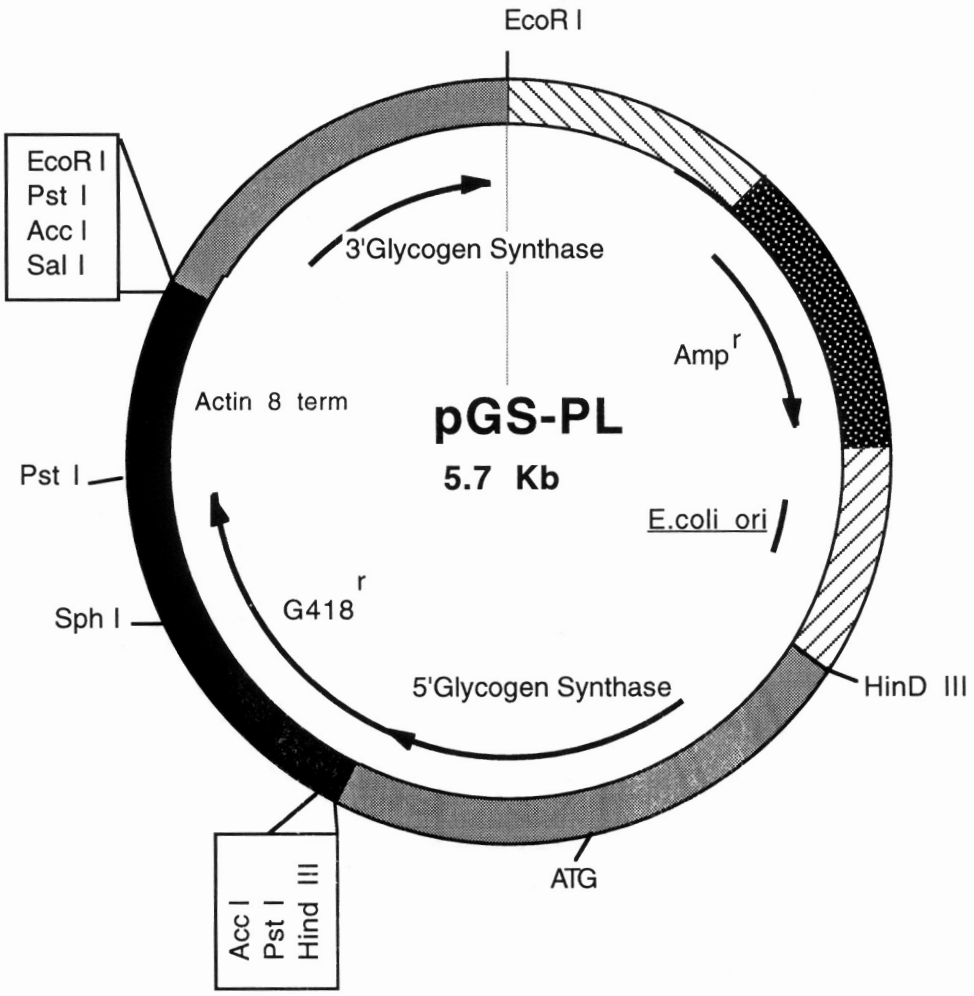
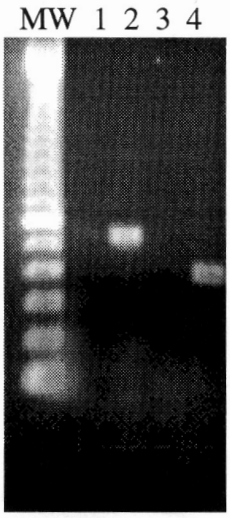


Fig 6-2: Promoterless Knockout vector.
A fusion of glycogen synthase 5' portion of the gene with the G418 cassette deleting the *act* 6 promoter that previously drove G418 activity. This fusion puts the activity of the G418 gene under the control of the glycogen synthase promoter. Additional constructs containing smaller, inactive, glycogen synthase promoter fragments were made to force the gene to recombine homologously.



Amoebae were selected from purified plaques using a toothpick
A toothpick was swirled into MilliQ H_2O
H_2O was overlaid with Mineral Oil

The cells were Frozen at -70°C; 10-15 min.

Frozen tubes were placed in the Thermocycler, set at 80°C
Primer/Buffer/*Taq* mix was added under the mineral oil
PCR was performed as usual

Fig. 6-3: PCR on Whole *Dictyostelium* Cells

In order to screen large numbers of *Dictyostelium* transformants by PCR, a technique was developed to be able to screen whole cells. Lane 1 and 3 contain untreated Dictyostelium amoebae. Lanes 2 and 4 contain Dictyostelium amoebae that were frozen and thawed before PCR. Primer sets were tried on *E. coli* to make sure no contaminating DNA was being amplified. The PCR technique is summarized below the gel.

Chapter 7: Green Fluorescent Protein[¥]

Abstract

I have constructed a green fluorescent protein (GFP) vector pNV based on the pVTL2 vector. This reporter gene is useful because the cells can be monitored *in vivo*. I made a construct that contains the glycogen synthase promoter fragment D1 (see **Chapter 4**) fused to the GFP coding region, creating the vector p2NV.

[¥] Represents work done in conjunction with Reyna Favis currently at VPI&SU, Blacksburg VA

Introduction

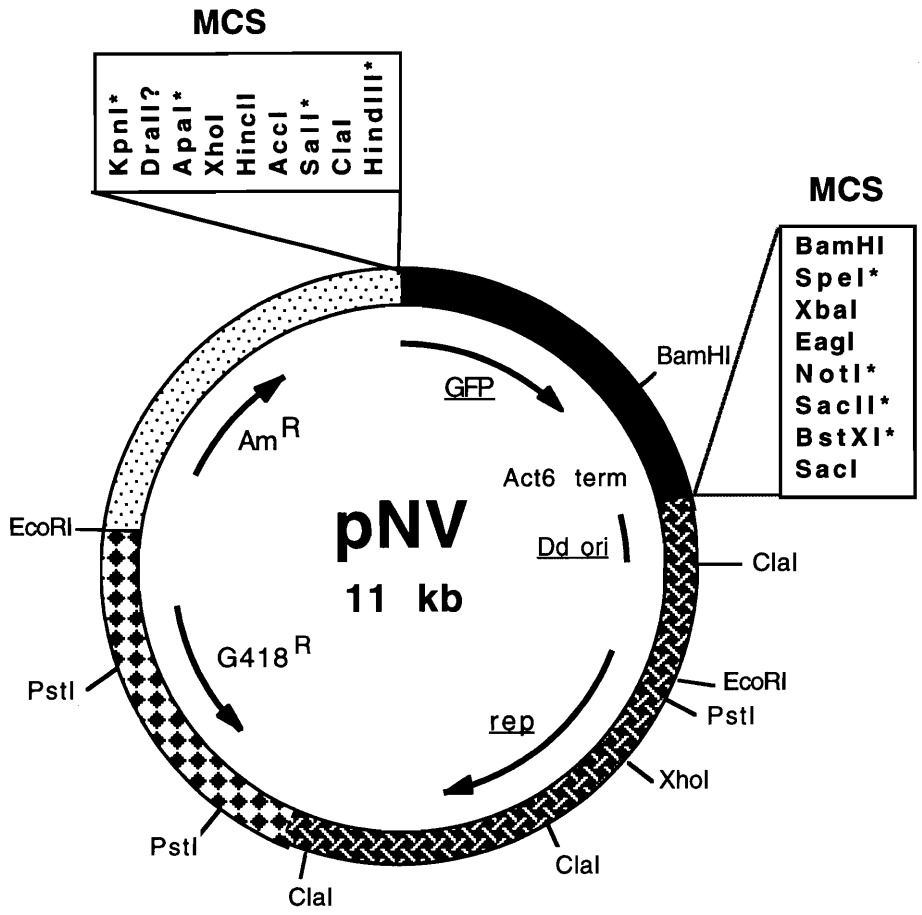
It has been shown that the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* produces fluorescence when expressed in different organisms (Chalfie et al. 1994). This protein can be exploited for use as a reporter. As a reporter GFP has some advantages not seen with other reporter systems. The main advantage of GFP is its ability to fluoresce when exposed to UV light (Chalfie et al. 1994). Since no cofactors are necessary for producing fluorescence the cost is reduced and error associated with the reporter system is also reduced. In addition, the error associated with variable penetration of histochemical stains is eliminated. Different cell type staining patterns have been obtained for the glycogen phosphorylase-2/β-galactosidase reporter constructs, depending on the detergent (Triton-X 100 or NP-40) used to permeabilize the cells (Yin 1993). Because only the cells that produce GFP fluoresce, it is possible to determine the cell type specificity of the promoter of interest. The organism expressing GFP can be monitored frequently without apparent harm.

Methods/Results

The vector pVTL2 was selected as a vector backbone because it is maintained as an extrachromosomal vector. The luciferase gene was removed from pVTL2 by digestion with *Hin*D III and *Bam*H I and the truncated vector was gel-purified. The coding region for GFP was obtained from TU#65, which is a pBluescript II KS (+) derived vector (acquired from M. Chalfie at Columbia University, NY). The coding region of GFP was PCR amplified from TU#65 using GFP5 (5'GGGGAAGCTTGGAAAATGAGTAAAGGAGAAGA 3') and GFP3 (5'GGGGGATCCTTATTATTTGTATAGTTCATCC 3'); both primers encode restriction sites on their ends corresponding to *Hin*dIII and *Bam*HI respectively. After the PCR product was digested and gel purified it was inserted into the pVTL2 plasmid creating pGFP. The *Hin*dIII and *Bam*HI sites remained unique in this vector. Next the *act*8 terminator was amplified by PCR from pVTL2 using the T7 primer and a terminator specific primer, lucT (5' GGGGGGATCCTCATAAAGGCCAAGAAGGGCGG 3'). This terminator fragment was gel-purified, digested with *Bam*HI and inserted into the *Bam*HI site of pGFP, producing pNV. A map of pNV is shown in Figure 7-1. The orientation of the terminator was verified by PCR (data not shown). In order to show that the vector was functional, promoter fragment D1 (see **Chapter 4**) was used to drive the reporter gene. This promoter fragment was cut out of pVTL2 using *Hin*dIII and *Kpn* I and inserted into pNV creating p2NV. The vectors pNV and p2NV were transformed into *Dictyostelium* cells through electroporation (see **Chapter 2**/Methods). Unfortunately, *Dictyostelium* cells are autofluorescent at wavelengths that correspond closely to those of GFP (Uchiyamaet al. 1993). This project was dropped because of the inability to visualize any expression of GFP over background. Recently other laboratories have developed the GFP vector for *Dictyostelium*, and have been able to overcome the background problem by

using a confocal microscope and narrowing the wavelength of the incoming light (Abe et al. 1995; Fey et al. 1995). Although a confocal microscopes is not currently available for us to be able to utilize this vector as a reporter system it may be possible to perform assays in collaboration with other laboratories. New forms of green fluorescent protein are being created (Clontech, Palo Alto, CA) that fluoresce at different wavelengths and may make it possible to use this vector without a confocal microscope in the future.

A.



B

Same sequence as pVTL2 GFP coding

MCS.....GGTCGACGGTATCGATAAGCTTGGAAA**ATG**AGTAAAGGAGAAGA.....

*Sal*I* *Cla*I *Hin*dIII*

Fig. 7-1: Map of GFP Reporter Vector pNV.

(**A**) Construction of the pNV plasmid. Restriction sites marked with an asterisk(*) are unique in the MCS of pNV. The number of *Dra*II sites in pNV is undetermined. (**B**) Sequence surrounding the GFP gene. The 5' region contains the T7 primer binding sequence, while the 3' region has the T3 binding sequence. The start codon (AUG) of GFP is present in the construct. The sequence surrounding the GFP gene is identical to the sequence surrounding the luciferase gene, from pVTL2 except the *Bam*HI site is not unique in pNV.

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CURRICULUM VITAE

Brian D. Williamson

Department of Biology
Virginia Polytechnic Institute and State University
2119 Derring Hall
Blacksburg, VA 24060

Phone (703) 231-8940
e-mail: Rutherfo@vt.edu

Date of Birth: May 28, 1969
Marital Status: Married

EDUCATIONAL BACKGROUND

B.S. Animal Science, 1991
Virginia Polytechnic Institute and State University,
Blacksburg, VA
Class Rank: 1 of 43

Candidate--Ph.D. Program, Molecular Biology, (from Aug., 1991)
Virginia Polytechnic Institute and State University.
Advisor: Dr. Charles L. Rutherford

ACADEMIC HONORS AND PROFESSIONAL SOCIETIES

Deans List for 4 semesters at VPI&SU
Alpha Zeta Agricultural Honors Fraternity
Recipient, Award for Scholarship, American Society of Animal Scientists
Phi Sigma Biological Honors Society
Member, American Society for Cell Biology 1994
Member, American Society for the Advancement of Science 1992

RESEARCH/LAB EXPERIENCE

August 1991 to present: Dissertation Research
Research interests: Molecular biology of cell differentiation in *Dictyostelium discoideum*; cloning and characterization of glycogen synthase in *Dictyostelium discoideum.*

Proficient in the following techniques:
Library construction and screening, cloning and subcloning, sequencing, PCR amplification, site directed mutagenesis, Southerns, Northerns, vector construction, reporter gene assays, deletion analysis of promoter, cell culture and maintenance, DNA mediated transformation (*E. coli* and *D. discoideum*).

TEACHING EXPERIENCE

Graduate Teaching Assistant at VPI&SU in:
General Biology Laboratory, 1991-92
Principles of Biology Laboratory, 1992
General Microbiology Laboratory, 1992-Present

ABSTRACTS/PRESENTATIONS

Microbiology Seminar, VPI&SU, October 1992.

American Society for Cell Biology, Annual Meeting, New Orleans, Louisiana. December 1993. "Cloning and Characterization of Glycogen Synthase in *Dictyostelium discoideum*"

Graduate Research Symposium, Blacksburg, Virginia. April 1994.
"Cloning and Characterization of Glycogen Synthase in *Dictyostelium discoideum*"

International *Dictyostelium* Conference, Dourdan, France. June 1995.
"Glycogen Synthase in *Dictyostelium discoideum*"

GRANTS RECEIVED

Graduate Student Assembly Travel Grant, 1995	$550
VPI&SU Graduate Research Development Program, 1995	$700

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MANUSCRIPTS IN PREPARATION

Williamson, B.D., Rogers, P.V., Rutherford, C.L. (1995) "Cloning and Characterization of Glycogen Synthase in Dictyostelium discoideum".

Williamson, B.D., McCaffery, I., Rutherford, C.L. (1995) "A Blue/White Selectable Cloning Vector (pNBL) With the Facility for the Generation of Defined, Sequential Deletions Within a Nucleic Acid Sequence".