

## 1. Introduction

### 1.1. The Life cycle of *Dictyostelium discoideum*.

Understanding the mechanisms by which cells enter and successfully achieve the differentiated state is an important aspect in the study of eukaryotic biology. The cellular complexities of higher organisms make the study of cell-specific differentiation a formidable task. *Dictyostelium discoideum* is a cellular slime mold that provides a simple eukaryotic system for such a study. *Dictyostelium* produces only two types of differentiated tissue, is haploid (facilitating mutational analysis) and can be easily induced to undergo a developmental phase that is completed after only 24 hours (Fig 1.).

In the presence of adequate levels of nutrients, cells of *Dictyostelium* exist as undifferentiated, single cells capable of dividing indefinitely by mitosis every 4 to 10 hours. The developmental phase of the life cycle can be induced by starvation. A single cell that first responds to the depleted levels of nutrients attracts other starving cells chemotactically with pulses of extracellular cAMP. The cAMP signal is amplified by cells migrating to the “aggregation center”. The formation of the cell aggregate marks the beginning of the developmental phase in which cells differentiate into prestalk or prespore cells [8]. The cell fate is largely dependent on the stage of the cell cycle at the time differentiation is induced [9]. During axenic growth in liquid medium, cell proliferation by mitosis is devoid of the G1 phase. When induced to differentiate, a cell in the middle of the G2 phase is arrested until the tipped aggregate stage and then released to undergo one round

of cell division. Such cells are destined to differentiate as prespore cells. Cells induced in the M, S or early G2 on the other hand, complete one round of mitosis prior to aggregation and form the prestalk cells. Culmination of the developmental phase occurs with the formation of the fruiting body that contains stalk cells and spore cells. The spores are dispersed into the environment and germinate into undifferentiated cells under favorable conditions [10-14].

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## 1.2. The Roles of cAMP and DIF-1 in *Dictyostelium* Development.

Extracellular morphogens cAMP and DIF-1 play a significant role in *Dictyostelium* development. The role of cAMP as a primary messenger during early development has been described in section 1.1. (The Life Cycle of *Dictyostelium discoideum* ). Vegetative cells of *Dictyostelium* are capable of sensing cell density as well as nutrient resources in their environment. A protein known as prestarvation factor (PSF) is secreted by amoebae. Increased density of *Dictyostelium* cells results in elevated levels of PSF. At these high levels, PSF can induce the expression of several genes involved in signal transduction [15-19]. These include genes for the cAMP receptor CAR1 [20, 21], phosphodiesterase (PDE), phosphodiesterase inhibitor (PDI) [22, 23] and adenylyl cyclase [24]. When cAMP binds its cell surface receptors, it activates adenylyl cyclase that in turn produces cAMP within the cells. Intracellular cAMP is secreted by the cells where it diffuses to neighboring cells and stimulates them [25]. The binding of cAMP to its receptor eventually results in phosphorylation of the receptor and consequently its dissociation from G proteins. Unbound extracellular cAMP is degraded by cAMP-phosphodiesterases. This enables cells to respond to the next cAMP stimulus [26, 27]. Thus a pulsatile pattern of cAMP levels is established and maintained until aggregation is achieved.

In addition to their role in chemoattraction, extracellular cAMP pulses are also involved in modulating the expression of genes during early development. Some genes such as those involved in aggregation are induced whereas others such as discoidins I and II are repressed [22, 26]. As cells progress through the developmental phase, expression of new genes becomes essential and that of others such as those required for aggregation needs to be repressed. The concentrations of cAMP required for the induction of genes during development

are about 10 – to 100-fold higher than during aggregation [28-31]. In contrast to aggregation however, cAMP signaling during development is continuous [32, 33].

The effect of cAMP on gene expression is cell-type dependent. Prespore-specific genes require a continuous stimulus from extracellular cAMP [34-38]. On the other hand, pre-stalk genes are induced by cAMP initially but inhibited by it at later stages. The role of DIF-1 in *Dictyostelium* development is seen in the differentiation of prestalk cells. The extracellular matrix (ecm) genes *ecm A* and *ecm B* are prestalk specific. The *ecm A* gene is expressed in prestalk cells during the formation of the early aggregate and requires both cAMP and DIF-1 for induction. The expression of the *ecm B* gene on the other hand occurs during culmination and is induced by DIF-1 but repressed by cAMP [39].

*Dictyostelium* cells express different cAMP receptors at different times during development. Some receptors are known to be expressed in a cell type specific manner. It has therefore been suggested that these variations in the expression of cAMP receptors facilitate the ability of cAMP to exert variable influences on gene expression in different cell types [27].

The molecule DIF-1 is considered to be the most active morphogen amongst a group of closely related chlorinated alkali phenones. Accumulation of DIF in *Dictyostelium* cell aggregates begins at about 10 hours of development [40]. Following an initial induction by cAMP, DIF-1 induces vegetative *Dictyostelium* cells to differentiate into prestalk cells [40, 41]. DIF-1-induced gene products accumulated in prestalk cells *in vitro* [42]. If cells were disaggregated after 10 hours of development, DIF-1 repressed the expression of prespore-specific genes [43]. This suggested that the role of DIF-1 in

differentiation was the promotion of stalk cell differentiation by the induction of prestalk-specific genes and repression of prespore-specific genes [44].

### **1.3. The Glycogen phosphorylase-2 Gene in *Dictyostelium discoideum***

One of the early events occurring during cellular differentiation in *Dictyostelium discoideum* is the degradation of glycogen by glycogen phosphorylase-2. The resultant glucose precursors are required for the synthesis of structural components in the differentiated cells [4-6]. This makes the regulation of glycogen metabolism an integral event in the differentiation process. *Dictyostelium* has two isozymes of the enzyme glycogen phosphorylase, GP1 and GP2. It has been previously shown by our laboratory that GP1 and GP2 are encoded by separate genes and that the expression of each gene is developmentally regulated. The derived amino acid sequences of the two genes show a 52% identity, however, their genomic and 5' upstream non-coding sequences show no significant homology. The expression of *gp-1* occurs only in undifferentiated cells whereas *gp-2* is expressed only during cell differentiation. It has been shown that the regulation of gene expression occurs predominantly at the level of transcription and hence, I have concentrated my efforts on studying the transcriptional regulation of *gp-2* [45-49]

GP2 activity is first detected in prestalk cells. The glucose thus produced is used for the synthesis of cellulose for the construction of the stalk. GP2 activity in the prespore cells is detected a few hours later when the prespore mass is lifted off the substratum by the lengthening of the stalk. Maximal expression of prespore GP2 occurs when the prespore mass reaches the apex of the stalk. This coincides with the completion of cellulose synthesis for the spore wall [50, 51].

To determine the significance of *gp- 1* and *gp- 2*, clones that carried a null mutation of either one of these genes were obtained by homologous recombination. It was found that *gp- 1*<sup>-</sup> mutants accumulated 17- to 28- fold higher levels of glycogen than wild-type clones during the late stationary phase. This indicated that undifferentiated cells lacked an alternative mechanism for glycogen degradation. In *gp-2* minus strains on the other hand, the posttranslational regulation of GP1 appeared to be modified to compensate for the lack of GP2. The existence of an alternative pathway for glycogen breakdown during *Dictyostelium* development demonstrated the importance of GP2 function for cellular differentiation in this organism. In contrast to wild-type clones, the GP1 protein was detected in equal amounts in both cell types in *gp- 2* null mutants. Thus, both the temporal as well as cell-type-specific expression of *gp- 1* appeared to be affected by the loss of *gp- 2*. This indicated an overlap between the regulatory pathways of the two genes at the translational and/or transcriptional levels [52].

The *gp- 2* gene is expressed in both the cell types of *Dictyostelium* in response to an intricate mechanism of regulation imposed by morphogens such as cAMP, NH<sub>4</sub><sup>+</sup>, adenosine and Differentiation Inducing Factor (DIF). The gene can be induced by both DIF and cAMP, however, induction by DIF can occur only in the presence of previous cell-cell contact. In addition, induction of the gene by cAMP can be inhibited by DIF. Gene induction by either cAMP or DIF can be inhibited by NH<sub>3</sub>. Finally, adenosine inhibits the DIF induction but not cAMP induction of the gene [1, 53-56].

Like most *Dictyostelium* gene promoters, the promoter sequence of *gp-2* has an AT bias (88%) [7]. Repeat sequences consisting of three TA-, two TAG- and two C-rich repeats referred to as the TA-, TAG- and C-boxes respectively are

also present within this sequence (Fig. 1). The *gp2* gene was cloned [57] and the 5' untranscribed region was subjected to deletional analysis to identify regulatory regions [2]. Interestingly, the TAG- and C- boxes were found to lie within regions containing *cis*-acting regulatory

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elements. To better define these regulatory elements, the *gp2* promoter was further analyzed by site-directed mutagenesis. The C-box element was found to be involved in cAMP–induction of *gp2*.

#### **1.4. The 3’C-Box Element of *gp2*.**

The 3’C-box element was investigated previously by site-directed mutagenesis<sup>1</sup>. A mutation of the 3’ C-rich repeat and adjacent sequences (construct C2M) resulted in a 50-fold drop in levels of reporter gene expression as compared to the wild-type. An internal deletion in C2M in which the 5’ C-box and intervening sequences between the two C-boxes were deleted (construct NP) had no further effect on the levels of reporter gene expression. This led to the conclusion that the 3’ C-box and adjacent sequences are involved in the transcriptional regulation of *gp2*. It is important to note that this study did not investigate the effect of mutating the upstream C-box in the presence of an otherwise intact promoter. Therefore, no conclusions could be drawn about the significance of the upstream C-box based on this study.

The expression profiles of C2M and NP clones were compared to those of WT clones during the course of development<sup>1</sup>. It was found that mutations within these constructs had no effect on the temporal regulation of *gp2*.

Because *gp2* is regulated by cAMP, the induction of the mutant promoter constructs by this morphogen was analyzed<sup>1</sup>. The wild-type construct showed a 10- to 20-fold induction in response to extracellular cAMP. However, C2M showed no response to extracellular cAMP. This clearly indicated that a cAMP inducible element was present in the region containing the 3’ C-box motif.

### 1.5. The Role of TF1, the *Dictyostelium* homolog of Replication Protein A.

The definition of regulatory elements helped in the identification and purification of proteins that bound to them. Cytoplasmic and nuclear cell extracts were obtained from *Dictyostelium* cells at 2 hour intervals and analyzed by electrophoretic mobility shift assays (EMSA) using various oligonucleotides representing the region from -543 to -317 bp in the *gp- 2* promoter. A DNA-binding activity was detected in nuclear extracts of undifferentiated cells using an oligonucleotide from -347 to -317. The 3' C-box element is present within this sequence. This binding activity with the 3' C-box oligonucleotide gave a 0.4 R<sub>f</sub> band on EMSA. A second DNA-binding activity was observed for the same oligonucleotide in nuclear extracts from differentiated cells. The activity in differentiated cells corresponded to a band of 0.32 R<sub>f</sub> in EMSA.

The DNA-binding proteins were purified first by ion-exchange chromatography using a DEAE Sephacel column. Active fractions were detected by EMSA using a radiolabeled -347 to -317 fragment. The active fractions were pooled and separated by affinity chromatography using a -347 to -317 ligand. Bound proteins were eluted using a NaCl gradient and active fractions detected by EMSA as before. The subunit molecular weights of the DNA-binding proteins were determined by SDS-PAGE. In undifferentiated cells, the 0.4 R<sub>f</sub> band corresponded to three peptides of molecular weights 18kDa, 35kDa and 62kDa. The 0.32 R<sub>f</sub> band from differentiated cells corresponded to peptides of molecular weights 18kDa, 35kDa and 81kDa. Peptide sequencing indicated that the proteins purified from the two types of cell extracts were different forms of the same protein. Thus the large subunits from the undifferentiated and differentiated extracts were responsible for the R<sub>f</sub> shift. Gel filtration indicated that the protein, now called TF1, bound the DNA as a monomer with a size corresponding to the

sum of the three subunits. To determine which of the TF1 subunits was involved in binding DNA, active affinity fractions from differentiated cells were subjected to Southwestern blot analysis. The autoradiograph showed that the 81kDa subunit bound the radiolabeled -347 to -317 oligonucleotide.

The *tfl* gene was cloned and sequenced. Sequence comparison between TF1 and available sequences in databases revealed that TF1 was the *Dictyostelium* homolog of replication protein A (RPA). RPA is a heterotrimeric protein that has been purified from other eukaryotic systems and known to function in both DNA replication as well as gene regulation [58-60]. RPA protein structure appears to be conserved among the various species, consisting of three peptides of molecular weights 70 kDa, 34kDa and 12kDa. As in the case of TF1, the large subunit (70kDa) is responsible for DNA-binding. Northern analysis of *tfl* expression showed that the mRNA of the large subunit was present at a constant level during development. The size of this mRNA is also constant at all stages of development in spite of the fact that the size of the peptide increases from 62kDa to 81kDa during differentiation. This indicated that the large subunit of *Dictyostelium* RPA is modified at the posttranslational level [61].

## **1.6. A Statement of Results.**

This dissertation describes the definition of *cis*-acting transcriptional regulatory elements of the *gp2* promoter by site-directed mutagenesis and the subsequent identification, purification and characterization of a new *Dictyostelium* DNA-binding protein called TF2.

## 2. Materials and Methods

### 2.1. Site-directed Mutagenesis

#### 2.1.1. Generation of Mutant Constructs

The construction of C1M and C12M is described here; the construction of all other constructs has been previously reported<sup>2</sup>. A 1146 bp fragment extending from +495 to -651 upstream of the *gp2* coding region was cloned previously in pBluescript II SK+ (Stratagene) to form construct p1.9Blue. The p1.9Blue construct was used as a template for PCR amplification of an 800bp region that I refer to as the ‘full length promoter’. The full-length promoter is defined as the shortest fragment of the *gp2* promoter that is sufficient for inducing wild-type levels of luciferase reporter gene expression as determined by 5’ deletion analysis. The amplification was carried out in two separate but overlapping PCR reactions (Fig. 3). The primers used for PCR included T7, Primer2, Primer3 and GP2XB carrying restriction enzyme sites not present in the *gp2* promoter sequence. Primers 2 and 3 had a 16 base pair overlap and were designed to bind at the region containing the 5’ C box. The mutant sequence carried by primers 2 and 3 was the *EcoRI* restriction site. Two PCR products were obtained using the p1.9Blue template, one from T7 and Primer2 and the other from Primer3 and GP2XB. Each PCR product was subcloned into separate vectors. The T7/Primer2 product was removed by digestion with with *KpnI* and *EcoRI* and cloned in-frame with the Primer3/GP2XB subclone cut with *EcoRI* and *BamHI*. This gave rise to construct p2C1M that contained a full length promoter fragment

carrying a mutant 5' C box sequence. The p2C1M construct was digested with *KpnI* and *XhoI* to obtain an 800 base pair mutant promoter. This fragment was then cloned into compatible sites in a pVTDH vector containing a luciferase reporter gene (Fig. 5). This strategy gave rise to C1M (Fig. 4). In order to construct C12M, primers 3 and GP2XB were used to amplify a fragment using a template called F16. The construction of F16 has been described previously<sup>2</sup>. F16 contains the full length *gp2* promoter fragment in pVTDH with a mutation of the 3' C box sequence<sup>1</sup>. The PCR product from F16 was cloned in frame with the insert in the T7 and primer 2 PCR product subclone. This resulted in the generation of a clone containing a full length *gp2* promoter with both the 5' and 3' C boxes mutated. This full length fragment was cloned into pVTDH to yield C12M (Fig. 4).

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### 2.1.2. Transformation of *Dictyostelium* cells

The luciferase reporter gene vector pVTDH (Fig. 5) is extrachromosomally maintained and encodes G418 (neomycin) resistance. The *gp2* promoter / pVTDH constructs were used to transform *Dictyostelium* strain AX3K amoebae by the calcium phosphate precipitation / glycerol shock method described by Nellen *et al* [62]. Transformed *Dictyostelium* cells were plated on lawns of *E. coli* B/r G418<sup>r</sup> cells growing on DM(50ug/ml G418) plates. The transformed amoebae produced plaques in the *E. coli* B/r G418<sup>r</sup> lawns after about 1 week. Isolated plaques were picked and used to inoculate well plates containing HL5 (10 ug / ml G418) medium. The cells were then transferred to 20 ml of HL5 (10 ug / ml G418) medium and allowed to grow axenically until they reached a density of about  $1 \times 10^6$  cells / ml and then harvested for the developmental assays. Cell harvesting involved centrifugation at 1000 x g for 5 min followed by washes with ice-cold, milli-Q water. The harvested cells were resuspended in differentiation buffer (20 mM KCl, 2.5 mM MgCl<sub>2</sub>, 49 mM KH<sub>2</sub>PO<sub>4</sub>, 7.9 mM Na<sub>2</sub>HPO<sub>4</sub>) to a concentration of  $3 \times 10^7$  to  $6 \times 10^7$  cells / ml. To induce development, 0.5 ml of cells were plated on Gelman GN-6 cellulose acetate filters supported by Gelman absorbent pads saturated with starvation buffer. The plated cells were incubated at 22<sup>o</sup> C to allow development to the desired stage. The developing cells were harvested at 0 and 20 hours (2nd finger stage) with 1 mM sodium orthovanadate. Cells were subjected to centrifugation at 1000 x g for 5 min to obtain pellets that were then resuspended in 100 µl of 30 mM glycyl-glycine pH 8.3 (supplemented with 5 ug / ml Leupeptin and 5 ug / ml Pepstatin A) and stored at -80<sup>o</sup> C.

### **2.1.3. Luciferase Assays**

Frozen cell pellets were lysed by thawing then centrifuged at  $14,000 \times g$  for 10 min. The supernatant was then removed and used for measuring luciferase activity. To 100  $\mu\text{l}$  of luciferase assay mix (0.54 M glycyl-glycine, 4 mM  $\text{MgCl}_2$ , 2.1 mM ATP), 10  $\mu\text{l}$  of cell extract and 100  $\mu\text{l}$  of 0.6 mM Luciferin was added and the Luciferase units measured using a Berthold Luminometer (Lumat LB 9501). Luciferase activity is expressed as Luciferase units per microgram of total protein. Total protein concentrations of the cell extracts were determined by the Bradford method using BSA Fraction V (Sigma) as a standard.

## **2.2. Protein Purification and Characterization**

### **2.2.1. Cell Culture and Preparation of Cell-free Extracts**

*Dictyostelium discoideum* strain AX3K, was grown to stationary phase in HL5 medium as described previously. The cells were freed of the nutrient medium by centrifugation at  $1,000 \times g$  for 5 min followed by resuspension in Milli-Q water. The washing step was repeated and the cell pellet was resuspended in five volumes of Buffer A (0.5 mM EDTA, 150 mM sucrose, 2% Nonidet P40, 25 mM Tris-HCl, pH 7.8) and 0.2% PMSF followed by incubation on ice for 15 min to allow cell lysis. The lysate was subjected to centrifugation at  $10,000 \times g$  for 10 min. The supernatant thus obtained was aliquoted and stored at  $-80^\circ\text{C}$ .

### **2.2.2. DEAE Ion-exchange Chromatography**

About 100 ml of DEAE Sephacel resin was poured into a 98 cm<sup>3</sup> column and equilibrated in 20 mM Tris-HCl (pH 7.8). Thawed cytoplasmic extract was centrifuged at 20,000 *x g* for 20 min and the supernatant mixed with an equal volume of 20 mM Tris-HCl (pH 7.8) prior to loading onto the equilibrated DEAE column. Bound proteins were eluted from the resin using a linear gradient ranging from 20 mM Tris-HCl (pH 7.8) to 0.8 M NaCl in 20 mM Tris-HCl (pH 7.8) over 2 hours (flow rate 5 ml / min) and collected using a fraction collector. Two flow through fractions and about 1ml of the material loaded on the column were saved for use as controls in EMSA for the detection of active fractions. All fractions were stored at 4<sup>0</sup>C.

### **2.2.3. Concentration of Active DEAE Fractions**

DEAE chromatography fractions determined to be active by EMSA were pooled and brought to 80% saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The suspension was centrifuged at 20,000 *x g* for 20 min and the pellet resuspended in 20 mM Tris-HCl (pH 7.8). The resuspension was then dialyzed overnight at 4<sup>0</sup> C against 1 l of Buffer A using membrane tubing from SPECTRA/POR<sup>®</sup> (MWCO 12-14 000).

### **2.2.4. Preparation and Use of a DNA Affinity Column**

The DNA affinity column was prepared as described by Kadonaga and Tjian [63]. An oligonucleotide with the sequence 5'GATCTGTAGTTTACAATGTATTATTCTACCCACTATTGTTGGA3' and its complementary strand were annealed, 5' phosphorylated, and ligated. The ligated oligomers were then coupled to 5 g of CNBr-sepharose-4B (Pharmacia), and stored at 4<sup>0</sup> C until use. The concentrated DEAE fraction pool was centrifuged at

144,800  $\times g$  for 30 min and the supernatant loaded onto the affinity column at 0.5 ml / min. Once the sample was completely loaded, 20 mM Tris-HCl (pH 7.8) was passed through the column at 1 ml / min until the absorption profile of the eluting material returned to baseline. A linear gradient from 20 mM Tris-HCl (pH 7.8) to 0.3 M NaCl in 20 mM Tris-HCl (pH 7.8) was used over 1 hour to elute bound proteins from the column. DNA binding activity was determined by EMSA.

### **2.2.5. Gel Filtration Chromatography**

Fractions from the affinity column that contained DNA binding activity were transferred into Centricon-10 spin concentrators (Amicon) and centrifuged to reduce the volume to about 100 microliters. The samples were then removed and pooled to a single Centricon-10 column. The sample was reduced to about 100  $\mu$ l, then 25 mM Tris HCL (pH 8.5) containing 0.3 M NaCl was added to bring the volume to 550  $\mu$ l. The sample was then centrifuged in a microfuge at 14,000  $\times g$  for 10 min and 500  $\mu$ l was removed and loaded on the 300SW column. A 300SW gel filtration column (8.0 mm  $\times$  300 mm, Waters) was equilibrated with 25 mM Tris HCl, (pH 8.5) containing 0.3 M NaCl until the conductivity of the eluant was the same as the solution being applied to the column. After 500  $\mu$ l of the sample was applied, the column was pumped with the same buffer at a flow rate of 0.3 ml / min.

### **2.2.6. Electrophoretic Mobility Shift Assays (EMSA)**

The “probes” were prepared by annealing two complimentary oligonucleotides to produce 5’ overhangs then labeled with  $\alpha$ -<sup>32</sup>P-deoxynucleotide triphosphates using the klenow fragment of DNA polymerase I. Unincorporated nucleotides were removed by passing the labeling reaction

through Bio-spin 6 columns (Bio-Rad). The reaction mixture contained 22 mM Tris-HCl (pH 7.9), 8.5 % glycerol, 43 mM NaCl, 4.4 mM MgCl<sub>2</sub>, 4.4 mM EDTA, 2.2 mM dithiothreitol, 4.4 % NP40 and 30,000 cpm (approximately 0.1 to 1.5 ng) of <sup>32</sup>P-labeled probe in a total volume of 11.2 µl. Approximately 5 – 25 µl of the protein sample were incubated with the probe for 30 min at room temperature. Following incubation, 2 µl of loading solution (250 mM Tris-HCl, pH 7.8, 50 % glycerol, 4 mg / ml bromophenol blue, 4 mg / ml xylene cyanol) was added before loading on a 5% polyacrylamide gel (acrylamide:bis, 37.5 : 1) in 1X TBE (90 mM Tris, 65 mM boric acid and 2.5 mM EDTA). Electrophoresis was carried out at constant voltage (200 V) for 70 min at room temperature in 1X TBE running buffer. In competition assays, the unlabeled competitor probe was incubated with the extract for 10 min before addition of the radioactive probe.

### **2.2.7. Preparation of Peptides for Sequencing By Mass Spectroscopy**

Active affinity chromatography fractions were pooled and concentrated using Centricon-10 spin columns (Amicon, MWCO 10kDa). The concentrated pool was analyzed by SDS-PAGE using a 7.5% gel. The gel was fixed overnight in 50% ethanol in 10% acetic acid and then washed in a solution of 50% methanol in 5% acetic acid for 30 min. This was followed by a second wash in the same solution for 45 min. Protein bands were visualized by Coomassie staining (0.1% Coomassie, 45% methanol, 10% acetic acid) for 5 hours followed by destaining in 50% methanol in 5% acetic acid, and then stored in 5% acetic acid. The gel was photographed and sent to the W.M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia for Mass Spectrometric analysis.

### **2.2.8. Selection Of cDNA Clones**

Partial peptide sequences of TF2 obtained by Mass Spectroscopy were compared to available sequences from a *Dictyostelium* cDNA library (*Dictyostelium* developmental cDNA project at the Institute of Biological Science, University of Tsukuba, Japan) [64]. Two cDNA clones SSL494 and SSD214 containing 1 kb and 0.8 kb inserts respectively, were found to contain several TF2 peptide sequences (see Fig. 14). The sequence of clone SSD214 is present within the sequence of SSL494. Lyophilized stocks of SSD214 and SSL494 were a generous gift from the Japanese *Dictyostelium* cDNA project team.

### **2.2.9. Probe Synthesis for Southern Blot Analysis**

An 800 bp cDNA insert from SSD214 was used as a template for probe synthesis. The probe was labeled using the random prime labeling method. The cDNA insert was obtained by double digestion of 5 µg of SSD214 with 10 units of *Sal* I (Boehringer Mannheim) and 10 units of *Not* I (New England Biolabs) in a total reaction volume of 20 µl. The cDNA insert was gel purified using Quantum Prep Freeze N' Squeeze spin columns (Bio-Rad) and labeled with fluorescein-11-dUTP using the ECF random prime labeling kit from Amersham Pharmacia Biotech.

### **2.2.10. Southern Blot Analysis**

Genomic DNA was prepared from amoebae using the QIAamp Blood kit (Qiagen). In three separate reactions, 10 µg of genomic DNA was digested with 50 units of *Cla* I (Boehringer Mannheim), 50 units of *Eco* RI (Boehringer Mannheim) or 40 to 60 units of *Hind* III (Boehringer Mannheim) in total reaction volumes of 50 µl. The digestion products were separated on a 1% TAE gel of 0.4

cm thickness. One  $\mu\text{g}$  of undigested genomic DNA and 1  $\mu\text{g}$  of lambda Sty I marker were also loaded on the gel. The DNA bands were visualized by staining with ethidium bromide.

In order to calculate the molecular weights of digestion products, a standard curve for molecular weights was plotted based on the  $R_f$  values of the bands from the marker lane. The lambda Sty I marker and undigested DNA lanes were then removed from the gel.

The gel was prepared for DNA transfer to nylon membrane by gentle agitation in depurination solution (250 mM HCl) for 10 min at room temperature followed by denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 25 min at room temperature and finally neutralized in a solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5) at room temperature for 30 min. Digested DNA bands were transferred to a Nylon membrane (Hybond-N<sup>+</sup>, Amersham) using the PosiBlot 30-30 Pressure Blotter and Pressure Control Station (Stratagene). The transfer buffer consisted of 10X SSC (0.15 M C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub> 1.5 M NaCl) + 10X SSPE (3 M NaCl, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O and 20 mM Na<sub>2</sub>EDTA). Following transfer, the DNA was immobilized on the membrane using a Stratalinker<sup>®</sup> UV crosslinker (Stratagene<sup>®</sup>) on a setting of 120,000 microjoules / cm<sup>2</sup> for 25 to 50 seconds.

The blot was incubated for 1 hour in a prehybridization solution (0.3 ml / cm<sup>2</sup>, 5X SSC, 0.1%<sup>(w/v)</sup> SDS, 1:20 dilution of liquid block (from ECF random prime labeling and signal amplification system, Amersham Pharmacia, Biotech) and 5%<sup>(w/v)</sup> dextran sulphate) preheated to 60<sup>0</sup> C. A 100 ng sample of denatured SSD 214 probe was added to the prehybridization solution and incubated overnight at 60<sup>0</sup> C. Unbound probe was removed by rinsing with 1X SSC+

0.1%<sup>(w/v)</sup> SDS at 60<sup>0</sup>C for 15 min followed by 0.5X SSC + 0.1%<sup>(w/v)</sup> SDS at 60<sup>0</sup> C for 15 min.

Bound probe was detected using the ECF signal amplification system (RPN 5750, Amersham Pharmacia, Biotech). The blot was rinsed briefly in an excess (2 ml/cm<sup>2</sup>) of buffer A (100 mM Tris-HCl, 300 mM NaCl pH 7.5) at room temperature. This was followed by incubation in 1 ml / cm<sup>2</sup> 1:10 diluted liquid blocking agent (from ECF random prime labeling and signal amplification system, Amersham Pharmacia, Biotech) in buffer A. Finally, the blot was incubated in a solution of anti-fluorescein AP conjugate diluted 5000-fold in freshly prepared 0.5% <sup>(w/v)</sup> bovine serum albumin in buffer A at room temperature for 1 hour. Unbound conjugate was removed with 2-5 ml / cm<sup>2</sup> 0.3% <sup>(v/v)</sup> Tween 20 in buffer A in 3 washes of 10 min each. The blot was incubated with detection reagent (ECF signal amplification system, Amersham Pharmacia, Biotech) for 1 hour and then scanned using a storm phosphoimager.

### **2.2.11. Extraction of RNA**

AX3K cells in stationary phase were washed and plated on cellulose acetate filters supported by pads saturated with 1X MES•LPS (7 mM MES buffer, 20 mM KCl, 5 mM MgSO<sub>4</sub>, pH 6.5). Each filter was inoculated with approximately 8 X 10<sup>7</sup> cells. For 0 hour RNA samples, the cells were immediately harvested after plating on filters. For all other RNA samples, the plated cells were incubated at 22<sup>0</sup> C. When the cells reached the desired developmental stage, they were washed off the filters with mQH<sub>2</sub>O and centrifuged at 1,300 x g for 2 min. The cell pellet was lysed in 1 ml of RNApure™ (GenHunter corporation) and incubated on ice for 10min. The cell lysate was subjected to chloroform extraction and finally resuspended in diethyl

pyrocarbonate (DEPC)-treated mQH<sub>2</sub>O. The concentration of the RNA samples was determined by spectroscopy. Samples were stored at -80<sup>0</sup> C.

#### **2.2.12. Probe Synthesis for Northern Blot Analysis**

A probe for screening Northern blots was generated by random oligonucleotide-primed synthesis involving the incorporation of  $\alpha^{32}\text{P}$ -dATP for labeling. The SSD214 cDNA was used as a template. Unincorporated nucleotides were removed using a Biospin 6 (BioRad) column. Activity of the labeled probe was measured using a scintillation counter.

#### **2.2.13. Northern Blot Analysis**

A 1% formaldehyde agarose gel was prepared in 10X MOPS running buffer (0.4 M 3-(N-Morpholino)propanesulfonic acid, pH 7, 0.1 M sodium acetate, 0.01 M EDTA). Twelve ug of RNA was loaded per lane. The volume of the RNA sample was adjusted to 3.7  $\mu\text{l}$  and then mixed with 13  $\mu\text{l}$  of cocktail (1.7  $\mu\text{l}$  10X MOPS running buffer, 3  $\mu\text{l}$  12.3 M formaldehyde, 8.3  $\mu\text{l}$  formamide). Following a 15 min incubation at 55<sup>0</sup>C, the sample was loaded on the gel with 3.3  $\mu\text{l}$  of formaldehyde loading buffer (1 mM EDTA, pH 8, 0.25%<sup>(w/v)</sup> bromophenol blue, 0.25%<sup>(w/v)</sup> xylene cyanol, 50%<sup>(v/v)</sup> glycerol). Duplicate samples of RNA were loaded in the other half of the gel for staining with ethidium bromide. Electrophoresis was carried out at 5V/cm for approximately 3 hours. RNA was transferred to a Nylon membrane (Hybond-N<sup>+</sup>, Amersham) using the PosiBlot 30-30 Pressure Blotter and Pressure Control Station (Stratagene<sup>®</sup>). The transfer buffer used was 10X SSC. Following transfer, the DNA was immobilized on the membrane using a Stratalinker<sup>®</sup> UV crosslinker (Stratagene<sup>®</sup>) on a setting of 120,000 microjoules / cm<sup>2</sup> for 25 to 50 seconds.

UV cross-linked membrane was wetted in 6X SSC and incubated in hybridization solution (1 ml /10 cm<sup>2</sup>, 5X SSC, 5X Denhardt's solution, 1%<sup>(w/v)</sup> sodium dodecyl sulfate, 50%<sup>(v/v)</sup> formamide, 1.2 mg Herring sperm DNA) at 42<sup>0</sup> C for 15 min. Incubation was continued overnight after addition of 100 ng (2.3 X 10<sup>6</sup> dpm) of SSD214 probe. The membrane was washed under high stringency conditions and exposed to x-ray film.

#### **2.2.14. Generation Of *tf2* Knock-out Construct.**

BSR, the blasticidin resistance gene, was removed from the pBSR19 vector (gift from Dr. William Loomis) using the restriction enzymes *Pst* I (NEB) and *Sma* I (NEB). The SSL 494 cDNA vector was digested with *Nsi* I (NEB) and *Eco* RV (NEB) enzymes that cut within the cDNA sequence. Ligation of the BSR gene into the digested SSL 494 vector was facilitated by the compatibility between *Pst* I and *Nsi* I overhangs and the blunt ends generated by *Sma* I and *Eco* RV enzymes (see Fig. 17). The ligation products were introduced into transformation competent bacterial cells by electroporation. Transformants carrying the correct ligation products were selected by PCR using primers NW3 and NW4 that bind internal cDNA sequence (see Fig. 17). If the BSR gene was not inserted in the desired location, a 430 bp product was obtained (see Fig. 18). Clones in which the BSR gene had ligated at the desired position gave a 1.6 kb PCR product.

#### **2.2.15. Transformation and Selection of *tf2* Knock-out Mutants**

AX3K cells in stationary phase were removed from nutrient medium by centrifugation as described above and resuspended in ice cold electroporation

buffer (10 mM NaPO<sub>4</sub>, pH 6.1, 50mM sucrose). About 8 X 10<sup>6</sup> cells were mixed with 10-25 µg DNA and electroporated in 0.4cm cuvettes at 3 mF and 2.5 Kv / cm. The cells were distributed equally to four petri dishes containing 10 ml of HL5 and incubated at 22<sup>0</sup> C. The following day, 50 µg of Blasticidin S was added to each plate for selection of transformants. The plates were monitored daily for 3 to 5 days until colonies formed. The colonies were transferred to lawns of B/r bacteria on DM agar plates. Plaques formed by transformed clones were transferred to well cultures and eventually to flasks containing HL5 medium. Transformed clones were screened by PCR using cDNA internal primers (see Fig. 17).

### 3. Results

#### 3.1. Identification of *Cis*- Acting Elements in the *gp2* Promoter.

Several mutant constructs of the *gp2* promoter were generated as illustrated in Figs. 4 and 6 and described in section 2.1. Constructs WT (wild type) and C2M (containing a mutant 3' C box) were previously analyzed<sup>2</sup>. Stable transformants of constructs C1M, C12M, A55M, TTM, T72M, TXM and XXM as well as WT and C2M (as controls) were generated by transformation. The calcium phosphate precipitation / glycerol shock method described by Nellen *et al* [62] was used. This technique yielded the best transformation efficiency, about 1 in 10<sup>7</sup> cells. Transformation efficiency by electroporation was found to be lower (data not shown). The transformation process took about 1 week after which the cells were plated on DM agar (+G418, 10 µg / ml) plates in the presence of B/r bacteria. After about 3 to 4 days, “plaques” were seen in the bacterial lawns. These are simply clearings created in the bacterial lawns by the transformants feeding on them. The plaques were transferred to 1 ml of HL5 medium (supplemented with 10 µg / ml G418) in well plates and incubated at 22<sup>0</sup> C. The cultures were allowed to grow to confluency and then used to inoculate 20 ml of HL5 (+10 µg / ml G418) in flasks and incubated at 22<sup>0</sup> C. These cultures were allowed to grow to a density of about 1 X 10<sup>6</sup> cells / ml and then transferred to 100 ml of HL5 (+10 µg / ml G418). When the 100 ml cultures reached a concentration of 1 X 10<sup>6</sup> cells / ml (in about 7 to 10 days), they were used to set up the developmental assays described in section 2.1.2.

**Go to Fig. 6**

As described in section 2.1.2, each clone was plated on multiple filters and allowed to undergo development. Cells were harvested at 0 hours and 20 hours and stored as pellets at  $-80^{\circ}$  C. Following cell lysis by freeze-thaw, the membrane and cytoplasmic fractions were separated by centrifugation and assayed for luciferase activity. A standard curve for luciferase activity was plotted to determine the linear range for luciferase readings. Cytoplasmic and membrane fractions were diluted appropriately to obtain readings within the linear range ( $1 \times 10^5$  to  $1 \times 10^7$  LU). I discovered that both fractions contained equal levels of luciferase activity. I took 3 measurements of each fraction. Specific activity measurements were calculated based on the activities of the cytoplasmic fractions (Tables 1 and 2).

At least 3 independent clones of each construct were assayed for specific activity of the reporter gene (Tables 1 and 2). To avoid errors between assays carried out on separate days, aliquots of clone WT8 (analyzed previously<sup>2</sup>), were prepared in excess and stored at  $-80^{\circ}$  C. One aliquot of WT8 lysate was assayed with new clones on each day to ensure that a constant activity was obtained for WT8 under the assay conditions.

As seen in Fig. 7, mutation of either of the C boxes resulted in a nearly 95% drop in reporter gene levels. Mutation of either one or both TAG-boxes resulted in a nearly complete loss of reporter gene expression. These data showed that the C- and TAG- boxes were present within the borders of *cis*- acting regulatory elements. Constructs that carried minimal mutations such as A55M and T72M showed less dramatic yet significant drops in luciferase levels. This suggested that boundaries of the *cis*-acting elements were not limited to the repeat sequences but extended beyond them.

### **Go to Tables 1, 2 and 3 and Fig. 7**

A considerable variation is seen in the specific activities within clones of the same construct (see Table 3). Such variations are typical of luciferase assays in *Dictyostelium* and could be the result of variations in copy numbers between clones. However, these differences within clones of the same construct are insignificant compared to the differences between mean specific activities of different constructs. For example, the error bars for WT clones are about 30% but the mean specific activity for TTM clones is close to 0%. Therefore, even at 70% of their mean value, WT clones are nearly 70% higher than TTM clones.

### **3.2. Identification of a Protein that Binds the 5' C Element of the *gp2* Promoter.**

During the course of purification of TF1 (a protein that bound to the 3' C box of the *gp2* promoter) [61] (also see section 1.5), an additional band that bound to the 5' C box probe was observed eluting from the DEAE column. The latter protein eluted at lower ionic strength from the column and produced a greater shift in EMSA than did TF1.

### **Go to Fig. 8**

In order to determine the specificity of this protein (termed TF2), I radiolabeled several sequences from the *gp2* promoter that had been identified as potential regulatory sites by deletion analysis [2] or were similar to sequences thought to be regulatory elements in other *Dictyostelium* genes [65-68]. DEAE fractions that contained the 5' C box binding activity were assayed by EMSA with all of the radiolabeled probes. The positions and sequences of the probes (B1, N, B3, TAG, 5'C, 3'C and SS) are shown in Fig. 8. The probes were designed to span the length of the *gp-2* promoter and also to distinguish between binding activities at the regions containing the two C boxes. The 5'C and 3'C probes therefore, include either the upstream or downstream C-box, respectively.

Fig. 9 shows the results from EMSA using three DEAE fractions. A band corresponding to a  $R_f$  value of 0.39 was detected using the 5'C probe. This band was not detected by the other probes. The specificity of the 0.39  $R_f$  band for the 5'C probe was tested by competition analysis with the other oligonucleotides. Reactions containing an active DEAE fraction and the radiolabeled 5' C probe were prepared. A second probe that was unlabeled was added to each tube to serve as a competitor. The unlabeled probes were in 50-fold excess of the labeled probe. Fig. 10 shows that only the unlabeled 5'C probe competed for binding. This demonstrated the specificity of the 0.39  $R_f$  band for the 5'C probe.

**Go to Fig. 9 and 10**

### **3.3. Purification of the 5' C Box-Binding Protein(s).**

Cell extracts, prepared as described in Materials and Methods, were first subjected to ion exchange chromatography using a DEAE Sephacel column (Fig. 11). A linear gradient to Buffer A containing 0.8M NaCl was used to elute bound proteins. DNA-binding activity in the fractions was detected by EMSA using radiolabeled 5' C probe (see Fig. 8). The 0.39 R<sub>f</sub> band eluted at approximately 0.5 M NaCl. Because the EMSA provides only a qualitative measure of DNA binding activity, determination of the percentage recovery during the purification procedure was not attempted. It is unlikely that the binding activity seen in the flow-through fractions is a different form of the protein, because when the same column was used with less extract all of the binding activity was recovered in the eluted fractions.

Active DEAE fractions were pooled, concentrated, and purified further by DNA-affinity chromatography using a 5' C ligand to separate the protein(s) of interest (Fig. 12). The 0.39 R<sub>f</sub> band eluted from the column at approximately 0.5 M NaCl. SDS-PAGE analysis of the active fractions from the affinity column produced two bands corresponding to 30 and 28 kDa.

In order to determine which of the two bands contained the 5' C box binding activity, I pooled and concentrated the active fractions from the affinity column and applied the concentrate to a 300 SW gel filtration column. As shown in Fig. 13, fractions that demonstrated 5' C box binding activity (the EMSA to the left of the figure) coincided with fractions containing the 28 kDa polypeptide (the SDS gel to the right of the figure). This result was obtained in seven separate purification runs, and therefore, I concluded that the 5' C box binding activity

### **Go to Figs. 11, 12 and 13**

resided in the 28 kDa polypeptide. TF2 eluted from the gel filtration column at a position corresponding to an apparent molecular weight of 53 kDa (Fig.13, insert). SDS-PAGE analysis of these same fractions showed an apparent subunit molecular weight of 28 kDa. Therefore, I concluded that TF2 exists as a homodimer under non-denaturing conditions.

#### **3.4. Amino Acid Sequencing and Identification of a TF2 cDNA Clone.**

Active fractions from ten affinity columns were pooled and subjected to N-terminal sequence analysis by Edman degradation. No sequence was obtained indicating that the amino terminus was blocked. To obtain internal amino acid sequence, active fractions from two affinity columns were pooled, then separated by SDS-PAGE and transferred to a PVDF membrane. The TF2 band was excised from the membrane and subjected to protease digestion with trypsin. Peptides thus obtained were separated by reverse phase HPLC and sequenced by Edman degradation. Commercially obtained carbonic anhydrase and *Dictyostelium* 5' Nucleotidase were also sequenced simultaneously as controls. These same samples were also subjected to Mass Spectrometric analysis to obtain partial peptide sequences. Peptide sequences obtained by Edman degradation were in agreement with those from Mass spectroscopy.

The peptide sequences obtained for TF2 were used to design degenerate oligonucleotides for use in PCR amplification of genomic DNA. In addition, the sequences were used to screen the *Dictyostelium* Developmental cDNA Library [64]. Two clones were obtained, SSD214 and SSL494 containing 800 bp and

1000 bp inserts, respectively. The sequence of the SSD214 cDNA was present within that of SSL494.

The nucleotide sequence and derived amino acid sequence of TF2 is shown in Fig. 14. An open reading frame exists starting at the ATG (+77) through position +795. The ATG at position +77 is preceded by a stretch of 21 A's, a result that is characteristic of most of the start sites of translation in *Dictyostelium* genes. Eight of the peptide sequences from Mass Spectrometric analysis were found in the derived protein sequence, thus confirming the identity of this cDNA clone as the sequence encoding the TF2 protein. These sequences are underlined in Fig. 14. Although some errors were encountered in the sequencing of the TF2 peptides, those sequences shown in Fig. 14 showed 100% identity to the derived amino sequence from the cDNA clones.

The derived protein has an apparent molecular weight of 26,378 Daltons, with 29 basic amino acids, 26 acidic amino acids, 88 hydrophobic amino acids, and 64 polar amino acids, and an isoelectric point of 8.5. As shown in Figs. 12 and 13 the subunit apparent molecular weight of TF2 as determined by SDS-PAGE is approximately 28 kDa. Therefore, it is likely that the SSL494 cDNA represents the total coding region of *tf2*. The SSL494 cDNA sequence and its corresponding protein sequence were compared to available sequences in the NCBI Blast databases. These searches did not reveal any homology to known *Dictyostelium* proteins. The highest degree of homology, up to 26%, was to the 3-oxoacyl-(acyl-carrier protein) reductase from *Aquifex aeolicus* [69] Thus TF2 appears to be a novel *Dictyostelium* DNA-binding protein.

**Go to Fig. 14**

### 3.5. Southern Analysis of the TF2-Encoding Gene.

To determine if alternate forms of the TF2-encoding gene existed, I performed Southern analysis on genomic DNA obtained from *Dictyostelium* cells. The DNA was digested with *Cla*I, *Eco*RI or *Hind*III restriction endonucleases. These enzymes were chosen based on the frequency of occurrence of their recognition sites in the TF2 cDNA and the *Dictyostelium* genome. All three enzyme sites are present infrequently in the *Dictyostelium* genome with the *Cla*I site occurring once and the other two enzyme sites being absent in the TF2 cDNA sequence. Under moderate stringency conditions, using a SSD214 cDNA fragment as a probe, the blot revealed two bands in the *Cla*I lane and one band each in the *Eco*RI and *Hind*III lanes (Fig. 15). These data suggested that there was only form of the *tf2* gene.

### 3.6. Expression of TF2 During Cell Differentiation.

To plot the pattern in which the *tf2* gene was expressed during the developmental life cycle of *Dictyostelium*, I extracted RNA from undifferentiated and differentiated stages and subjected it to Northern analysis using a radiolabeled probe derived from the SSD214 cDNA. The *tf2* message was nearly undetectable in undifferentiated cells (Fig. 16). During development a gradual increase in levels of the message occurred with a peak in expression at about 8 hours of development followed by a gradual decrease in the late stages. The SSD214 probe was then removed and the blot was reprobbed with either *Dictyostelium Actin 8* or *Dictyostelium 5'Nucleotidase* probe as a control. The *Actin 8* expression was found to be constitutive during development, in agreement with the literature [70], while the *5'Nucleotidase* gene was

developmentally regulated with maximal expression at about 5 hours of development (not shown)<sup>4</sup>.

### **3.7. Attempt at Generation of TF2 Null Mutants.**

A “knock-out vector” was generated in which the TF2 cDNA was interrupted internally by the insertion of a Blasticidin S-resistance gene (Fig. 17). A linear DNA fragment consisting of the Blasticidin S- resistance gene at the center and flanked on either side by 431 bp 5' and 215 bp 3' *tf2* cDNA sequences was removed from the vector, gel purified, then introduced into *Dictyostelium* AX3K cells by electroporation. Following selection with Blasticidin S, resistant clones were screened by PCR to confirm the insertion of the Blasticidin S- resistance gene in the desired location. A PCR product of 430 base pairs was expected from a wild-type *tf2* gene whereas a 1.6 kb product was expected from a disrupted form of the gene. Although blasticidin resistant clones were recovered, none contained a disrupted *tf2* genomic sequence. One explanation of this result is that such a gene disruption is lethal. Another explanation is that the BSR gene together with its promoter and terminator directed the integration of the fragment into another location on the genome. The linear knock-out fragment containing the Blasticidin S-resistance gene flanked by the cDNA of choice (in this case *tf2* cDNA), was designed to provide specificity for the gene of interest. The promoter and terminator provided were from two different *Dictyostelium* actin genes. However, it is possible that the AT-richness inherent in all *Dictyostelium* promoters as well as the AT-bias of gene terminators in general led to the integration of the knock-out construct into one of the several actin genes. A null mutation in any one actin gene is unlikely to be lethal and may explain the viability of the non-specific transformants I obtained.

**Go to Figs. 15, 16, 17 and 18**

#### **4. Discussion**

Because of the temporal expression of *gp2*, and its expression in both spore and stalk cells I thought this gene would offer a good model for the study of transcriptional regulation during development. Previous deletion analysis had mapped regions in the *gp2* promoter that contained transcriptional regulatory elements. The mapped regions were found to contain A/T, A/G or C-rich repeat sequences that have been previously referred to as TA-, TAG- and C-boxes respectively [2]. The next step was to determine the boundaries of the regulatory elements in *gp2*. To achieve this objective, I decided to carry out site-directed mutagenesis (SDM) of the mapped regions. An advantage of this approach over deletion analysis was its ability to study downstream elements such as the C-boxes in the presence of upstream regulatory elements. SDM constructs had minimal differences in their size. A random deletion of 2 base pairs was observed in C2M and C12M (Fig. 4). The discovery that the TAG- and C-boxes in the *gp2* promoter are present within the boundaries of transcriptional regulatory elements is not surprising because similar repeat sequences have been identified as *cis*-acting elements in other developmentally regulated *Dictyostelium* genes [66, 68, 71-77]. SDM data from TAG-box(es) mutants provided an interesting insight into the definition of these *cis*-acting elements. Mutation of one or both TAG-boxes (constructs TXM and XXM respectively) resulted in a nearly 100% drop in reporter gene levels. Even when point mutations were introduced within each

TAG box (construct T72M), significantly reduced levels of reporter gene expression (76% of wild-type levels) were observed. However, levels from TTM in which the TAG boxes were left intact but sequences flanking them were mutated, showed nearly no expression of the reporter gene. This indicated that the TAG-repeat sequences were an integral part of the TAG-regulatory element(s) but the boundaries of the element(s) were not restricted to the repeat sequences. In fact, these data suggest that regions adjacent to the repeat elements may play a significant role in complex formation by providing specificity for protein binding and thus directing transcription factors to the correct regulatory elements.

I was surprised to discover that the protein binding the 3' C box of *gp2* was, in fact, known also to be involved in the initial steps of DNA replication. Thus, TF1 (the *Dictyostelium* homologue of Replication Protein A) appears to act as a replication factor in undifferentiated cells, and as a transcription factor during cell differentiation. When TF1 is recruited into the transcriptional apparatus as cell differentiation commences, it binds to a C-rich sequence, the 3'C box [61]. Such C or G rich sequences are rare in the AT-rich stretches that are found not only in the *gp2* promoter but in most *Dictyostelium* promoters (88% AT-rich for *gp2*) [7]. As described in this dissertation, I have shown that the 5'C-box serves as the binding site for TF2. The fact that developmental expression of the reporter gene was abrogated in the absence of either C-box suggests that protein binding must occur at both C-boxes to allow full induction of *gp2*. This “double-occupancy” requirement of the C-boxes was also predicted based on footprint analyses [78] and deletion analysis [2] of the *gp2* promoter. In addition, footprint analysis indicated that *gp2* is regulated by DNA-protein interactions occupying at least three large domains in its promoter. One of these domains is occupied by both TF1 and TF2. Because *Dictyostelium* promoters are in large part comprised of long stretches of A's or T's, it seems likely that the regulatory sites are G or C

rich. Binding of proteins to these sites may augment structural changes in the shape of the DNA, thus enhancing additional binding of auxiliary transcription factors. I propose that founder *trans*-acting factor(s) bind to specific C or G regions of the *gp2* promoter and enhance the specificity for additional formation of protein complexes. Because TF1 and TF2 bind to two of these C rich sequences they may act to seed the assembly of the protein complexes found in their vicinity.

In addition to enhancing the binding of other factors, proteins binding the 5' and 3' C boxes of the *gp2* gene may interact directly [2]<sup>2</sup>. For example, it has been shown previously that 5'C deletion constructs showed no cAMP responsiveness in the levels of reporter gene expression, while temporal expression was not affected. When both the 5' and 3' C boxes were deleted the reporter gene was not expressed. However, SDM of the 3' C box in the presence of a normal 5' C box resulted in correct temporal expression of the reporter gene, but loss of cAMP responsiveness [2, 79]<sup>2</sup>. In addition, EMSA of DEAE-separated fractions demonstrated a supershift in fractions containing both TF1 and TF2, thus generating a retarded band that shifted higher than either of the factors individually<sup>3</sup>. Thus both *in vivo* and *in vitro* results suggest an interaction between the 5' and 3' C boxes.

Although the C boxes were the most obvious candidates for regulatory sites in the *gp2* promoter, I hypothesize that the strings of A's and T's that surround the C boxes may also be involved. Between the two C box elements, there exists a tract of 10 adenines. From footprinting analyses, it has been shown that this sequence is protected from nuclease digestion. Other members in the

### Go to Fig. 19

research group have recently identified a protein that binds to this A-tract sequence using EMSA of DEAE elution fractions<sup>3</sup>. Because oligo(dA) tracts have been implicated in causing bending in DNA [80] I hypothesize that a bend is augmented by the binding of this oligo(dA)tract binding factor. This results in looping the DNA and bringing TF1 and TF2 into contact. By looping the DNA, these two sites are brought into close proximity, thus facilitating interaction between these elements in generating cAMP-responsiveness and activation of the gene during cell differentiation (Fig. 19).

Bonfils *et al* [81] have recently reported on the promoter analysis and identification of *trans*-acting factors involved in the regulation of the *ribonucleotide reductase* gene *rnr B* in *Dictyostelium discoideum*. By deletion analysis they showed that the expression of *rnr B* in undifferentiated cells requires an A/T-rich element. In differentiating cells however, *rnr B* induction was dependent on two G/C-rich elements. As in the case of the C-boxes of *gp2*, one of the G/C elements of *rnr B* binds a nuclear factor and the other binds a cytosolic factor. Thus the mechanism by which *rnr B* is regulated appears to draw parallels with the *gp2* system. Both genes contain A/T-rich and G/C-rich regulatory elements. The G/C-rich elements are involved in gene regulation during differentiation in both genes and also one element binds a nuclear factor and the other a cytosolic factor.

A 92 kDa G-box binding factor, GBF, is a *Dictyostelium* transcription factor that binds several G/C-rich elements to induce gene expression [66, 67, 71, 82, 83]. The sequences of the C-boxes of *gp2* were similar to the consensus GBF

binding sites. To determine if GBF bound the *gp2* C-box sequence, McCaffery *et al*<sup>2</sup>, screened various cellular and chromatographic fractions with wild-type or mutant 3' C-box oligonucleotides in mobility shift assays. Two proteins, corresponding to 27 kDa and 110 kDa were detected that bound the wild-type but not mutant 3' C-box probe. Western analysis using GBF antiserum showed that the 92 kDa GBF protein was not present in these fractions. Instead GBF was found in both cellular and column fractions that did not demonstrate 3' C-box binding activity. This indicates that GBF does not bind the 3' C-box directly. If in fact it does bind, additional factors are involved.

Because *gp-2* is induced at a time when the cells enter into a program of differentiation, the transcriptional machinery involved in its expression plays a crucial role in the transition to the differentiated state. The identification of *cis*-acting transcriptional regulatory elements and the discovery of TF2 and its subsequent cloning, have provided clues to how such genes are regulated. It is probable that there are additional regulatory elements and proteins that are essential for *gp2* induction and concomitantly for entry and successful attainment of the differentiated state.

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## Footnotes

- <sup>1</sup> W. Wu, Investigation of the Function of a C-rich Region in the Transcriptional Regulation of the *gp-2* Gene, M.S. Thesis, Dept. of Biology, Virginia Polytechnic Institute and State University, December, 1995.I.
- <sup>2</sup> McCaffery, N. Warner, R. Favis, W. Wu, E. Brittle and C. L. Rutherford, manuscript in preparation.
- <sup>3</sup> C. L. Rutherford, unpublished results.
- <sup>4</sup> C. Chanchao, C. Eristi and C. L. Rutherford, manuscript in preparation.

## VITA

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- A Nuclear Factor and C-Rich cAMP-Responsive Elements Regulate Expression of the *Dictyostelium gp2* Gene During Development - I. McCaffery, N. Warner, R. Favis, W. Wu, E. Brittle and C. L. Rutherford (in preparation)

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- Graduate Student Assembly travel award, Virginia Tech, August, 1998
- Department of Biology travel support, Virginia Tech, August, 1998

#### Abstracts:

- TF II : A *Dictyostelium* protein that binds to a transcriptional regulatory element-N. Warty, I. McCaffery and C. L. Rutherford (Poster presentation, Virginia Tech Research Symposium, March 1999)

- Identification of *cis*- acting elements in the *gp- 2* gene of *Dictyostelium discoideum* -N. Warty, I. McCaffery, W. Wu and C. L. Rutherford (Oral presentation, American Society for Microbiology, VA Branch, Richmond, VA, January, 1998)
- TFII: A *Dictyostelium* protein that binds to a transcriptional regulatory element- N. Warty, I. McCaffery and C. L. Rutherford (Poster presentation, International *Dictyostelium* meeting, Irsee, Germany, August 1998)
- Regulation of the Glycogen Phosphorylase Gene During Cell Differentiation in *Dictyostelium* by the Human Homologue of Replication Protein A- C. L. Rutherford, R. Favis, I. McCaffery and N. Warty (Poster presentation, Johnson and Johnson Focused Giving Symposium, New Brunswick, NJ, December 1998)
- Regulation of the Glycogen Phosphorylase Gene During Cell Differentiation in *Dictyostelium* by the Human Homologue of Replication Protein A- C. L. Rutherford, R. Favis, I. McCaffery and N. Warty (C. L. Rutherford, invited speaker, Janssen Research Foundation, Spring House, PA, December 1998)
- Transcriptional regulation of the glycogen phosphorylase- 2 gene during development in *Dictyostelium discoideum* – N. Warty, I. McCaffery and C.L. Rutherford (Poster presentation, Virginia Tech Research Symposium, April 1997)
- Transcriptional regulation of the glycogen phosphorylase- 2 gene during development in *Dictyostelium discoideum* – N. Warty, I. McCaffery and C.L.

Rutherford (Poster presentation, Virginia Academy of Science conference, Blacksburg, VA, May 1997)

- Transcriptional regulation of the glycogen phosphorylase- 2 gene during development in *Dictyostelium discoideum* – E. Brittle, I. McCaffery, N. Warty, R. Favis and C.L. Rutherford (Poster presentation, Pfizer, Groton, CT, September 1997)
- Investigation of the Transcriptional Regulation of the *glycogen phosphorylase- 2* Gene in *Dictyostelium discoideum* – I. McCaffery, W. Wu, N. Warty and C. L. Rutherford (Poster presentation, International Dictyostelium conference, Sendai, Japan, October 1996)

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