Purification of the Proteins that Bind to the *gp-2* Promoter *in Dictyostelium discoideum.*

by

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Biology (Molecular and Cellular Biology Section)

(ABSTRACT)

During *Dictyostelium* development, glycogen degradation is a crucial event that provides glucose monomers that are utilized to synthesize the essential structural components for cellular differentiation. The degradation is catalyzed by the product of the glycogen phosphorylase-2 gene. Cloning and sequencing of the *gp-2* gene revealed several repeated sequences in the promoter region that are putative regulatory sites. I present here the purification of a DNA binding protein that binds to the 3' "C" box sequence in the gp-2 promoter using a DEAE Sephacel resin and specific "C" DNA affinity column chromatography. With undifferentiated amoebae cell extract, a DNA binding protein migrated at 0.40 Rf and with 17 hr differentiated cell extract, the protein migrated at 0.32 Rf. Both the 0.32 and 0.40 Rf proteins were purified to homogeneity and showed to consist of three subunits of 18 kD, 35 kD and 62 kD (for 0.40 Rf) or 81 kD (for 0.32 Rf). Amino acid sequence analysis showed identity between a region of the 62 and 81 kD

subunits. I conclude that the difference in the shifted 0.40 Rf and 0.32 Rf bands in EMSA is due to the 62 and 81 kD subunits. A southwestern blot analysis of the 17 hr cell extract demonstrated that the DNA binding activity resides in the 81 kD polypeptide. The effect of pH and phosphatase inhibitors on a "conversion" between the 0.32 and 0.40 Rf bands was examined. The results suggest that phosphorylation may be involved in the "conversion" reaction. This study suggests that the purified protein may be a trans-acting factor that is involved in *gp-2* regulation.

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1. Introduction

The developmental processes of multicellular organisms involve a number of specific and complex series of biochemical events that need precise control mechanisms. One of the important mechanisms the cell utilizes to control these events is gene expression. One method by which gene expression is regulated is the process of differential gene transcription. The cell achieves gene regulation by activating or repressing the transcription of particular genes by interaction with certain DNA binding proteins called transcription factors. Once transcription is initiated, other steps of gene expression will follow. Thus, the study of transcription factors is an important approach to the understanding how genes are regulated. Understanding how transcription factors interact with genes in a temporal manner in order to regulate cellular differentiation is an elaborate and complicated goal. As a result of fertilization, division, and differentiation, a unicellular fertilized egg becomes a complex, multicellular organism. Cells must require complex and precise biochemical controls to achieve this process. Therefore, studying the molecular basis of cellular differentiation and developmental processes in eukaryotes may be simplified by the use of a model system.

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model to study developmental process in eukaryotes, especially gene expression and regulation. *Dictyostelium* is a particularly useful experimental system because of (1) the well characterized developmental cycle in which one cell type differentiates to two different cell types, (2) the accessibility to mass culture of cells for protein purification, and (3) the availability of a DNA mediated transformation system. Since its isolation by Raper for the

first time in 1935, the organism has become frequently used as a model system to provide information about multicellular development.

1.1: The Life Cycle of *Dictyostelium*

Dictyostelium naturally exists as single haploid amoebae that are genetically, morphologically, and biochemically identical (Fig 1). Under harsh conditions, as for example the scarcity of a food supply, these free living amoebae are signaled to differentiate. They immediately start to synthesize and secrete cAMP which in turn signals the migration of cells toward an aggregation center (Konijn et al., 1967). After about 16 hr, the cells finally form a multicellular structure called a slug. The slug behaves as a multicellular organism as it enters the culmination stage and moves toward a heat or light source that may provide suitable conditions for spore formation. There are two different cell types determined in the slug. The anterior cells, called the prestalk cells, will later form a stalk for a fruiting body, the terminal differentiated structure (Bonner, 1959). Posterior cells of the slug, called the prespore cells, will form a spore mass in the final stage of differentiation. Each slug is then transformed into a fruiting body composed of a tall stalk supporting a cap filled with the spore mass. When environmental conditions become more suitable for growth, the spores germinate and the resulting free-living amoebae start a new life cycle while the stalk cells die. The whole life cycle is complete within 24 hr (Loomis, 1982).

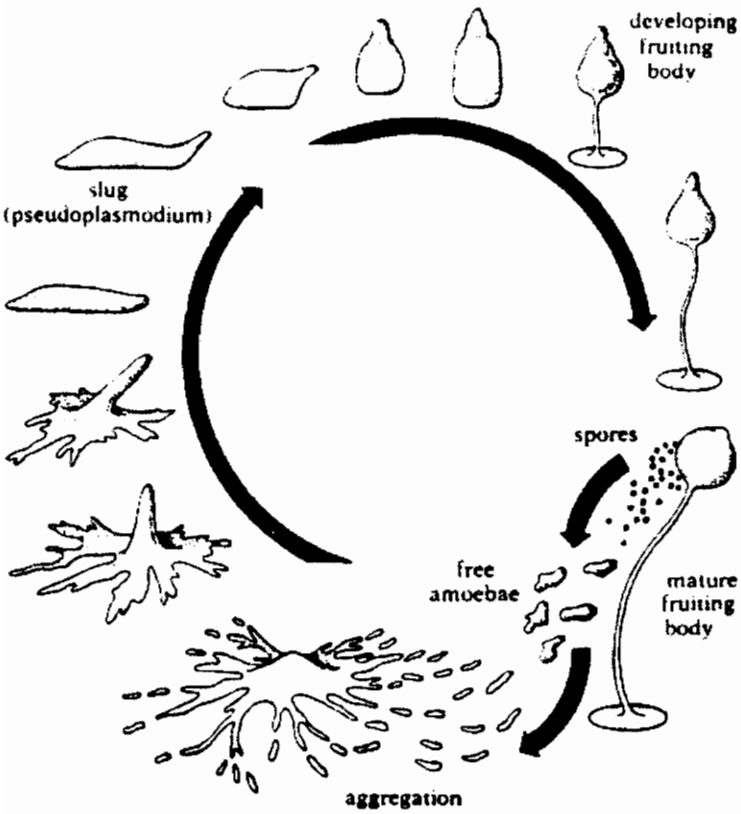

Figure 1. The life cycle of *Dictyostelium discoideum*.

1.2: Glycogen Phosphorylase in *Dictyostelium discoideum*

Glycogen degradation is an important event that occurs during *Dictyostelium* cell differentiation. The process is catalyzed by the enzyme glycogen phosphorylase (1,4-alpha-D-glucan:orthophosphate alpha-glucosyltransferase; EC 2.4.1.1). During the culmination stage, the enzyme catalyzes the conversion of glycogen to glucose-1-phosphate, the precursor for the synthesis of several end products of development, primarily cellulose for cell wall construction (Gustafson and Wright, 1972; Marshall et al., 1970; Wright et al., 1968).

In *Dictyostelium discoideum*, glycogen phosphorylase exists in two forms, GP-1 and GP-2 (Rutherford et al., 1988). The enzyme activity of GP-1 is dependent on 5' AMP. This is present in vegetative amoebae and maximal in the early stages of development. As development progresses, GP-1 activity decreases throughout the subsequent stages (Brickey et al., 1990; Naranan et al., 1988; Rutherford et al., 1986). GP-2, a 5' AMP independent form, is not present in early development, but ultimately high activity is expressed at culmination (Firtel and Bonner, 1972). Exogenous cAMP appears to induce the expression of gp-2 mRNA, protein, and enzyme activity (Sucic et al., 1993). Maximal GP-2 activity coincides with a decrease in glycogen level and the conversion of the degradation products into the structural components of the spore and stalk cells (Cloutier and Rutherford, 1987; Thomas et al., 1968; Wright and Kelly, 1981; Wright et al., 1968). In most eukaryotic systems in which glycogen phosphorylase has been identified, the inactive form is converted biochemically into the active form through a cAMP-mediated phosphorylation. However, the two forms of the enzyme in *Dictyostelium discoideum*, are the products of two separate genes. Both genes have been cloned and sequenced (Rogers et al., 1992; Rutherford et al., 1992).

Several studies suggest that *gp-2* expression is developmentally regulated: (1) The increase of phosphorylase activity at the late stages of development did not occur if RNA and protein synthesis were inhibited (Firtel and Bonner, 1972). (2) The correlation between the ^{35}S-methionine incorporation into the phosphorylase protein during development and the increase in enzyme activity suggested that the enzyme was newly synthesized at initiation of development (Thomas and Wright, 1976). And (3) Evidence that the enzyme was synthesized during development and was regulated at the mRNA level (Higgins and Dahmus, 1982). Because of my interest in the molecular mechanism regulating cell differentiation I will emphasize the *gp-2* gene in this thesis.

1.3: Analysis of the *gp-2* Promoter and Initial Attempts to Find Proteins that are Involved in its Regulation.

To understand the regulatory mechanism that control expression of the *gp-2* gene, it is important to study the cis-acting regulatory elements of the gene itself as well as the trans-acting factors that regulate the gene. For the first aspect, a structural gene and promoter element of *gp-2* were isolated and analyzed (Rutherford et al., 1992). Figure 2 shows the promoter sequence, including several repeated sequences, three "TATA" boxes, two "TAG" boxes, and two "C" boxes. By the use of a promoter deletion analysis and a luciferase reporter gene, Sucic et al. (1993) showed that these repeated sequences are important cis-acting elements in transcriptional regulation of *gp-2*.

```
        >d7
-1216   GAATTCATTATAAACTATGGTGAAAGAGCCATTTTCAATTTCTCAAGAAATAAGAGTGAA   -1157
-1156   GCTGAAAAGAAAGAAATCTCACCAAATTGGTTAACAAGCTCTTTACATGATCATGTAAAA   -1097
-1096   ATAGATTATTGGATCTTGGTTGACTATTACAATAATACCATTGAAAAAGGCTCTTGGAAA   -1037
-1036   GAAGAAGATGAATCACTTATCTATATAACTAGTGTTTACTTTTATCTTGTCGAATCTCTC   -977
 -976   ATAAACACTATTCAAACCGCTACTGAACGTAGATTATTCTCTAGTAAACAAATACTTCAA   -917
 -916   TCACTTGAAAATCGTGAATCAACTTTCAAATCAATCTTTAATAGACAACAACGTTCTTCC   -857
 -856   CTCAATCAAGAAACTATTAAAAAATTGGAACAATTATTAATTAAATTTGAAAAAATCATT   -797
 -796   TAATTTCAAAATCTTTTAAAGATTTTAGAGGTTATTAAAAAAAAAAAAAAAAAAAAAAAA   -737
 -736   TCCAAAAAAAAAATCCATAAAGTCCAAAATCAAATAATTTAAATTTAAATTATTTTTAAA   -677
                                  >d1-9
 -676   TTAAAAACTAATCAAAAAATAAAAAAAATAATAAAAATAATAAATAAACAAATAAAAATA   -617

 -616   TTAAAAATTAATTATAATAAAGAACAAATAAATTATTGATTGTGTTTTTAAAAATAATAA   -557
                >d11
 -556   TAATTCAAAAAAAAAAAAAAAAAAAAAATTAAAATTAAAAAAATAATTATAATAATTATA   -497
              >d10
 -496   ATAATAAAAATAATAGTAATAATAATGAAAATAATTAAAAATGGATAAACTAAAAATGGA   -437
                                                          >d23
 -436   ATTTTTTTTTTTTTTTTTTTTTTTATTAATTTTTTTTAATAATGTAGTTTACAATGTATT   -377
                >d29  >d27
 -376   ATTCTACCCACTATTGTTGGAAAAAAAAAACAGTGCAAACTCACCCACTCACAATTTTTT   -317
                                  #
 -316   AAACACAAATAAAAAAATTTTAGTGGTATCTGTGAAAAAATAGCTCCATACAAAAAACAA   -257
                >d2.7
 -256   ATTTTATCAAACACCACCAAATATATTATTTTTATTTAAATTAATTTACTTTTTTTTTTT   -197
                                  >d2
 -196   TATTAATATTATATTTTTTTTTTTTTTTTTTTTTTTTTATATTTTGGTTTTTTTCATTATT   -137
 -136   TATTATATTATTATTTTTTTCATCAATAAATTAATATTTCTTTGTATTTCTTTTTATATT   -77
  -76   ATTCATTAAAAAAAAAAAAAATAAAAAAAAAAACAAAATAAAAAAAAAAATAAAATAATAA   -17
                        M  E  E  K  T  S
  -16   AACAAATTAAAAAAAAATGGAAGAAAAAAGAAGT   17
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Figure 2. The nucleotide sequence of the *gp-2* promotor.
Several repeated sequences, the "TATA" boxes (-608 bp to -600 bp, -513 bp to -506 bp, and -504 bp to -497 bp), "TAG" boxes (-461 bp to -452 bp, and -446 bp to -437 bp), and "C" boxes (-371 bp to -365 bp, and -334 bp to -328 bp) are shown overlined. The (>) symbol locates the first nucleotide of each sequence deletion analysis. The transcription starting site is indicated by (#). The numbers to the side of the sequence refer to the translation starting site.

These studies suggested that the TAG boxes may play important role in the regulation of the *gp-2* gene by cAMP, an extracellular signaling molecule (Sucic et al., 1993). Another result from our laboratory showed that, if the 5' "C" box was deleted, the reporter gene activity decreased 100 fold. The deletion of both of the "C" boxes resulted in complete loss of reporter gene activity. Therefore, in order to study the trans-acting elements of the *gp-2* gene, the 3' "C" box sequence was used as a probe in an attempt to find DNA binding proteins that were involved in *gp-2* gene regulation.

In this thesis, I present the purification of a DNA binding protein that may be involved in *gp-2* gene regulation during the developmental process in *Dictyostelium discoideum*. The remarkably successful techniques for DNA binding protein purification, sequence-specific affinity chromatography and the band shift technique to identify the proteins that bound to the *gp-2* "C' box sequence were utilized in the purification procedures. Also the effects of pH and phosphatase inhibitors on an apparent "conversion" between two forms of the DNA binding proteins were examined.

2. Materials and Methods

2.1: Cell Culture and Extract Preparation

Dictyostelium discoideum strain AX3K was grown in HL5 medium as described previously (Rogers et al., 1994). The cells were maintained at a density not exceeding 5 x 10^6 to 1 x 10^7 cell/ml. For large scale protein purification, 30, 2-liter flasks containing 500 ml HL5 at 1 x 10^7 cell/ml were harvested by centrifugation in an IEC flow-through clinical centrifuge. The cell pellet (approximately 250-300 grams wet weight) was washed with 1 liter MES (N-morpholinoethanesulfonic acid) buffer (20 mM MES (pH 6.8), 0.2 mM $CaCl_2$, 2 mM $MgSO_4$) and collected by centrifugation for 5 min at 1,300 x g. The cells were resuspended in 5 volumes of MES buffer and 500 ml of the resuspended cells were transferred to 2 liter flasks. The cells were then placed on a rotary shaker (New Brunswick Model G26) and shaken at 130 rpm at 23°C for either 6 hr for 0.4 Rf band purification or 17 hr for 0.32 Rf band purification. The cells were then harvested as described above, resuspended in 1 liter of milli-Q water, placed in 1 liter centrifuge bottles and centrifuged at 1,300 x g for 5 min. The pellet was resuspended in 5 volumes of buffer A (25 mM Tris (pH 7.9), 0.5 mM EDTA, 150 mM sucrose, 2% Nonidet P40) and placed on ice for 15 min to allow the cells to rupture, then centrifuged at 10,000 x g for 10 min. The supernatant was removed and stored at -80°C. The pellet was resuspended in two volumes of the original cell pellet of buffer B (20 mM Tris (pH 7.9), 5 mM DTT, 5 mM NaEDTA, 1.0 M NaCl, 5 ug/ml PMSF, 5 ug/ml leupeptin), incubated on ice for 30 min,

then centrifuged at 150,000 x g for 60 min. The supernatant was removed and stored at -80°C.

2.2: DNA Electrophoretic Mobility Shift Assay (EMSA)

The double-stranded C box oligonucleotide was labeled with ^{32}P-deoxynucleotide triphosphates at 5' overhangs using the Klenow fragment of DNA polymerase I and passed over Bio-spin 6 columns (Bio-Rad) to remove unincorporated nucleotides. The labeled probe (30,000 cpm, 0.1 to 1.5 ng) was added to a mixture of 3 ul of extract and 7.5 ul of reaction buffer (22 mM Tris-HCl, K_2HPO_4/KH_2PO_4, Imidasole, or MES containing 8.5% glycerol, 43 mM NaCl, 4.4 mM $MgCl_2$, 4.4 mM EDTA, 2.2 mM dithiothreitol, 4.4% Nonidet P40 at various pHs). The nonspecific competitors, poly dI-poly dT or poly dA-poly dT (0.1 to 1.0 ug), were included in some assays. The reaction mixture was incubated at room temperature for 20 min before adding 2 ul of loading dye (250 mM Tris-HCl (pH 7.9) 50% glycerol, 4 mg/ml bromphenol blue, 4 mg/ml xylene cyanol). Gel electrophoresis was then performed in 5% polyacrylamide in TBE buffer (90 mM Tris-borate (pH 8.5), and 2 mM EDTA) at 200 volts for 70 min at room temperature. The gel was dried and autoradiographed for 12 to 24 hr.

2.3: Effect of pH on Binding of Proteins to DNA

Bio spin-6 columns were equilibrated by adding 5 ml of either 20 mM Tris buffer (pH 7.9) or 20 mM MES buffer (pH 6.3), drained by gravity, then centrifuged at 7,500 x g for 4 min. A 60-100 ul sample of 17 hr shaking cell extract prepared in buffer A, as described above, was applied to the column. The spin column was centrifuged again, as above, and the eluate was assayed by EMSA either in Tris or MES reaction buffer or passed through another Tris spin column to bring the pH back to 7.9 before being subjected to the EMSA. For analyzing the effect of phosphatase activity, extracts were incubated with phosphatase inhibitors (1 mM final concentration) in either Tris or MES reaction buffer.

2.4: Protein Purification

2.4.1: DEAE Sephacel Batch Assay

The thawed cell extract (as described above) was brought to 10 ug/ml with phenylmethylsulfonyl fluoride (PMSF) and then protamine sulfate was slowly added to a final concentration of 6 gm/liter packed cells. After stirring at 4°C for 15 min, the extract was centrifuged at 20,000 x g for 30 min. The supernate was removed, passed through glass wool, applied to DEAE Sephacel resin (Pharmacia, 1 g resin/6 ml packed cells) in a 1 liter bottle and shaken on the rotary shaker at 150 rpm for 60 min at 23°C. The resin was poured into a column (5 cm x 6 cm) and washed with 20 mM Tris buffer (pH 7.9) until the optical density reached a basal level. Two flow-through volumes (about 1.5 liters total

volume) were collected during this washing (FT1 and FT2). Bound proteins were eluted from the resin with 20 mM Tris (pH 7.9) containing 0.3 M NaCl (flow rate 20 ml/min). This material (the 300-1 fraction) was collected until the maximum absorbance started to decline (as measured by a column monitor), then another fraction was collected (the 300-2 fraction) until the absorbance reached the base line. Finally 20 mM Tris (pH 7.9) containing 1 M NaCl was applied to elute the remainder of the proteins. The active fraction (300-1) was brought to 0.4 M NaCl before being stored at -80°C.

2.4.2: DNA Affinity Chromatography

2.4.2.1: Preparation of DNA

An oligonucleotide with the sequence 5' GATCCAGTGCAAACTCACCCACT-CACAAT 3' (the 3' "C" box) and its complementary strand were annealed and 5' phosphorylated as follows. The oligonucleotides (100 nmol each) were combined in 435 ul of 58 mM Tris-HCl (pH 7.6) and 12 mM $MgCl_2$. The mixture was heated to 88°C for 5 min, 65°C for 10 min, 37°C for 10 min, and room temperature for 5 min (Kadonaga, 1986). Then ATP (final concentration 3 mM) and 50 ul (100U) T4 kinase were added to give an approximate total volume of 500 ul. The mixture was incubated at 37°C for 2 hr, 250 ul 5M NH_4OAc added, and the DNA was precipitated with ethanol. The pellet was resuspended in 225 ul H_20, plus 25 ul 500 mM Tris-HCl (pH 7.6) containing 100 mM $MgCl_2$, and incubated 88 C 5 min, 65 C 10 min, 37 C 10 min, and room temperature 5 min. The mixture was brought to 1 mM ATP, 10 mM dithiothreitol, then 75 Weiss units of T4 ligase were added to give 267 ul final volume. The reaction tubes were incubated overnight at 17 C, then extracted with phenol and chloroform, and precipitated with

ethanol. The pellet was dried in a speedvac, then dissolved in 125 ul H_2O. A 1 ul sample was analyzed by agarose gel electrophoresis to determine the extent of ligation while the rest was stored at -20°C. The 1.8% agarose gel was performed in TBE buffer at 100 volts for 1 hr. The results showed oligomers ranging from 3 mers to 30 mers with the majority of 5-6 mers.

2.4.2.2: Coupling the DNA to CNBr Sepharose-4B

A 5 g sample of CNBr-sepharose-4B (Pharmacia) was added to 500 ml of 1 mM HCl for 10 min. The resin was then washed by vacuum filtration with 1 liter of 1 mM HCl, 500 ml H_2O, then resuspended in 20 ml H_2O. The oligonucleotide solution and 2.5 ml 100 mM K_2HPO_4/KH_2PO_4 (pH 8.0) were added. After rotation at room temperature for 20 hr, the mixture was transferred to a vacuum filter, washed twice with 500 ml H_2O, and 500 ml 1 M ethanolamine (pH 8.0). The resin was transferred to a 50 ml tube, 1 M ethanolamine was added to give a smooth slurry and the resin was rotated for 4-6 hr at room temperature. The solution was transferred to a vacuum filter, washed with 500 ml 10 mM K_2HPO_4/KH_2PO_4 (pH 8.0), 500 ml 1 M K_2HPO_4/KH_2PO_4 (pH 8.0), 500 ml 1 M KCl, 500 ml H_2O, and 500 ml storage buffer (10 mM Tris (pH 7.8), 1 mM EDTA, 0.3 M NaCl, 0.04% sodium azide). The resin was resuspended in storage buffer, packed in a column (1.5 cm x 10 cm) and stored at 4°C (Latchman, 1993).

2.4.2.3: C Box Affinity Chromatography

Before use the column was equilibrated by pumping 5 column volumes of 20 mM Tris (pH 7.8) containing 300 mM NaCl buffer over the column at room temperature. The

300 mM eluate from the DEAE Sephacel column (300-1) was thawed and passed over the column at 2 ml/min. After 3, 20-ml fractions were collected the bound proteins were eluted for 45 min with a linear 0.3-1.4 M NaCl gradient in 20 mM Tris (pH 7.8) at the rate of 1 ml/min as formed by a Waters 650 protein purification system. The eluate was collected as 10 ml fractions. DNA binding activity was assayed immediately after the gradient ended; a 1 ml sample was removed from each fraction for SDS-PAGE and the rest of the fraction was stored at -80°C.

2.5: Deoxycholic Acid-TCA Precipitation

2.5.1: One ml Samples.

Deoxycholic acid (DOC) was added to a 1 ml sample of each affinity fraction to 0.015% final concentration (Peterson, 1977). The tubes were placed on ice for 30 min then 100% TCA was added to 10% final concentration. The samples were incubated at -20°C for 15 min and centrifuged at 14,000 rpm for 15 min. The supernatant was removed, the pellet was washed once with 1 ml 10% TCA and once with 1 ml ice cold acetone. After centrifugation as above, the pellet was air-dried and stored at -20°C.

2.5.2: Pooled Samples.

Active affinity fractions were pooled (about 40 ml total volume), DOC and TCA was added as described above and the tubes were centrifuged at 3,100 x g for 45 min

(Mahuran, 1983). The pellet was washed with 2 ml 10% TCA, then with 3 ml ice cold acetone and after centrifugation at 3,100 x g 30 min, the supernatant was removed and the pellet was air-dried and stored at -20°C.

2.6: SDS-PAGE and Electroblotting

Samples were denatured in the presence of 5% ß-mercaptoethanol and 2% SDS prior to separation by SDS-PAGE. Gel electrophoresis using a 12% resolving gel was performed at 111 volts for the stacking gel and 225 volts for the resolving gel for a total of 2 hr. The electrophoresis buffer was 50 mM Tris (pH 7.9), 2.6 mM EDTA, 0.5 M glycine, and 0.1% SDS. The gel was then fixed with 300 ml 12.5% TCA, stained by adding 30 ml 0.5% Brilliant blue G or alternatively silver stained (Merril, 1981). For electroblotting, the gel was first rinsed for 5 min in CAPS (Cyclohexylamino-propanesulfonic acid) transfer buffer (10 mM CAPS (pH 10) in 10% methanol). Proteins were transferred onto a PVDF membrane in CAPS transfer buffer at 0.5 Amp for 60 min at 4°C. The membrane was then washed 3 times with Milli-Q water at room temperature, stained with 0.1% Brilliant blue R-250 in 50% methanol for 2 min, then destained in 50% methanol, and air-dried. Protein bands were excised from the gel and stored at -20°C for peptide micro-sequencing (Matsudaira, 1993).

2.7: Electroelution and Protease Digestion

Preparative SDS-PAGE was performed as described above, the gel was stained with 0.1% Brilliant Blue R in 50% methanol for 1 hr, destained in 50% methanol, then the protein bands were excised and stored at -20°C. The gel slices were thawed and protein eluted using an electroelution apparatus (Amicon micro-electroeluter) according to the manufacturer's directions. Gel electroelution into a Centricon 3 microconcentration cell (Amicon) was performed with solution F buffer (25 mM Tris, pH 7.9, 1.3 mM EDTA, 1.8% glycine, and 0.05% SDS) at 200 volts for 2 hr. For cleavage of the eluted proteins, either trypsin or protease *Staphylococcus aureus* , strain V8, was added at a ratio of 1:40 (protease : target protein on a weight basis) to the solution in the spin column which was then concentrated by centrifugation at 7,500 x g for 5 hr at 4°C. The concentrated sample incubated at 37°C with protease in the presence of 1.8% SDS and 1% 2-mercaptoethanol for an additional 90 min. The digested products were then separated in a 15% polyacrylamide gel by SDS-PAGE prior to electroblotting.

2.8: Southwestern Blot Analysis

The proteins were electroblotted onto a nitrocellulose membrane as described above. The membrane was then placed in 50 ml Blotto (5% nonfat powder milk, 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA,1 mM DTT) and placed on a rocking platform at room temperature for 60 min. The membrane was washed three times for 5 min

each with 50 ml of binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT). The DNA binding reaction was carried out for 3 hr in 25 ml of binding buffer containing 50,000 cpm/ml of ^{32}P-labeled C box oligonucleotide and 1 ug/ml of nonspecific poly dI-poly dC. The membrane was washed with 50 ml binding buffer 4 times for 5 min each, dried and autoradiographed at -80°C for 12 to 24 hr (Wayne, 1991).

2.9: Amino Acid Sequencing

The purified peptides resulting from the proteolytic cleavage of the native protein were sequenced using an Applied Biosystems Model 477A Sequencer with on-line identification of phenylthiohydantions by Laura Douglas, at the protein sequencing facility in Virginia Polytechnic Institute and State University.

3. Results

3.1: DNA-Binding Protein Expression during Development.

To determine the expression pattern of DNA binding proteins during development, EMSA was performed on nuclear and cytosolic extracts prepared from cells at various stages of development. If extracts from vegetative amoebae were incubated with the radioactive "C box" probe and assayed by EMSA, a retarded band at 0.40 Rf was observed (Fig 3, lanes 1-3). The undifferentiated amoebae were then shaken in non-nutrient media to trigger multicellular differentiation, and cells were removed for extract preparation at different time points. Using extracts obtained from the 1 and 4 hr time points, the 0.4 Rf retarded band was still present (Fig 3, lanes 4 and 5). However, with the 17 hr cell extract, which coincided with the late aggregation stage of development, a retarded band appeared at 0.32 Rf and the 0.40 Rf band was no longer present (Fig 3, lane 6). The specificity of DNA binding was determined by competition with the unlabeled C box probe (not shown). With a 133-fold excess of unlabeled probe, the binding of labeled probe was decreased over 90% for both the 0.4 Rf band of the amoebae cell extract and the 0.32 Rf band from the 17 hr cell extract. This shows the temporal expression of the proteins that caused shifted bands in EMSA. The 0.40 Rf band was observed in amoebae stage and the 0.32 Rf band was expressed in differentiated cells.

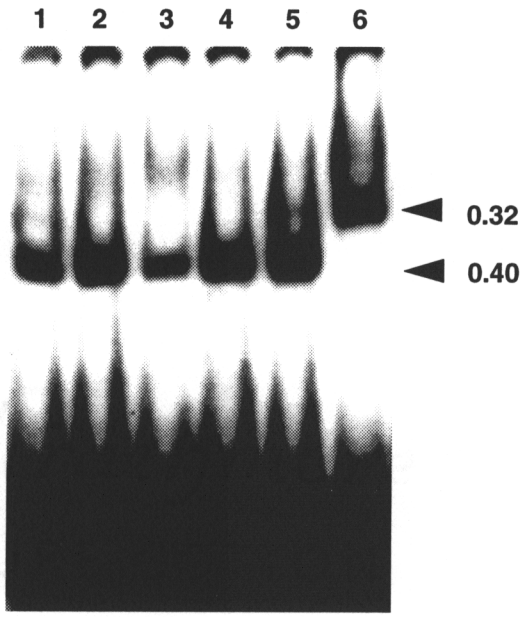

Figure 3. EMSA of nuclear proteins from both undifferentiated cells and differentiated cells that bind to "C" probe during developmental time points. Lanes 1, 2, 3 are extracts from vegetative amoebae during growth at cell densities of 4, 6, and 9 x 10^6 cell/ml, respectively. Lanes 4, 5, 6 are extracts obtained from cells that have been in non-nutrient buffer for 1, 4, and 17 hours. Numbers to the right of the lanes are the Rf (distance migrated by the retarded band divided by the distance migrated by the unbound DNA probe).

3.2: "Conversion" Between the 0.32 Rf and 0.40 Rf Band.

3.2.1: pH Effect.

In my initial attempts to purify DNA binding proteins, I tested the effectiveness of several chromatography resins, including hydroxyapatite and CM Sephadex. Because the capacity of these resins is greater at lower pH, I performed some of these analyses at pH 6.0 to 6.5. In some experiments the extract from differentiated cells was prepared in Tris buffer at pH 7.9 as described in Materials and Methods, then applied to a column that had been equilibrated at pH 6.0 to 6.5. In this case the sample applied to the column showed the normal 0.32 Rf band in the EMSA while the fractions eluted from the column showed a low level of the 0.32 Rf and the presence of a 0.40 Rf band. If, however, the hydroxyapatite chromatography was performed in K_2HPO_4/KH_2PO_4 buffer (pH 7.9), the 0.32 Rf band was observed in the eluted fractions. Thus, chromatography with hydroxyapatite or CM Sephadex at low pH resulted in loss of a shifted band that was a characteristic of differentiated cells (at 0.32 Rf), and the appearance of a band that had been observed only in undifferentiated cells (at 0.40 Rf). This result provided the possibility of a pH dependent "conversion" between the 0.32 and 0.40 Rf bands.

To test this hypothesis, the DNA binding activity in 17 hr cytosolic cell extracts was analyzed by the EMSA in several different buffers with a range of pH's. At pH 7.0 or 7.5, retarded bands appeared at 0.22 and 0.32 Rf (Fig 4, lanes 5, 6, 9, 10, 13 and 14). At pH 6.5, a retarded band located at 0.4 Rf was observed and also the intensity of the 0.22 and 0.32 Rf bands was diminished (lanes 4, 8 and 12). If the pH was decreased to 6.0, the EMSA showed nearly complete loss of the upper bands and a corresponding increase in

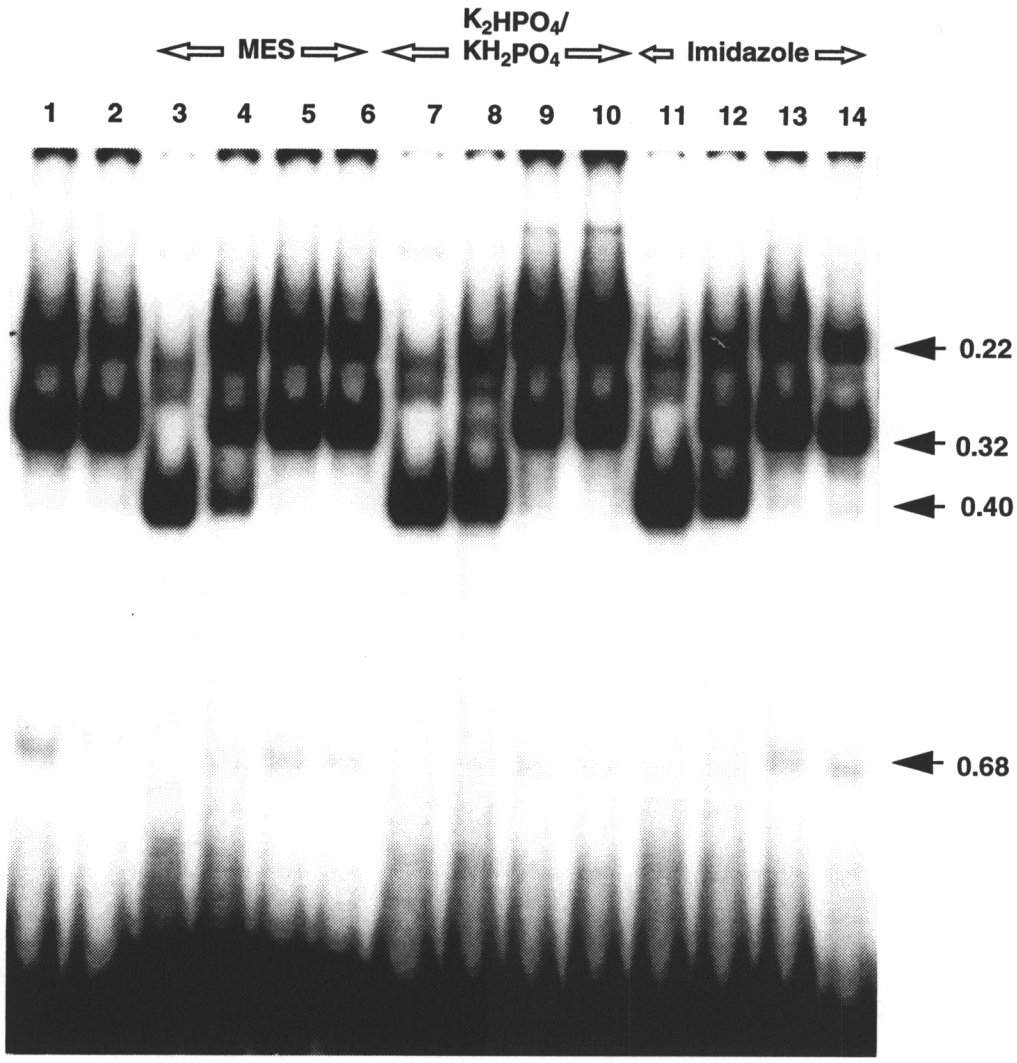

Figure 4. EMSA of 17 hr cytosolic cell extracts assayed in different buffers with a range of pH. The reaction buffers in which the assays were performed are shown above the lane numbers. Lanes 1, 2 show assays in Tris and Hepes reaction buffer (pH 7.9), respectively. Lanes 3, 7, 11 were performed at pH 6.0, lanes 4, 8, 12 at pH 6.5, lanes 5, 9, 13 at pH 7.0, and lanes 6, 10, 14 at pH 7.5. Numbers to the right are Rf.

the intensity of the 0.40 Rf band (lanes 3, 7, and 11). The same result was observed for MES, K_2HPO_4/KH_2PO_4, and imidazole buffers. The 0.22 Rf band, as a result, could be part of the conversion of the 0.32 Rf to 0.40 Rf band. However, there was no extensive study on the 0.22 Rf band to show its function or the components that cause the shifted band.

Because these DNA-binding proteins are present in both cytoplasmic and nuclear fractions, I tested if the effect of pH on the migration of the retarded bands occurred with both cytoplasmic and nuclear extracts (Fig 5). No effect was found on nuclear (lanes 2, 6, and 10) or cytoplasmic (lanes 1, 5, and 9) extracts from the amoebae stage. In each case, the characteristic 0.40 Rf band was present. With 17 hour cytoplasmic extracts, EMSA at pH 6.3 again resulted in the loss of the 0.32 Rf band, and appearance of the 0.40 Rf band (compare lanes 3, 7 and 11). However, with 17 hr nuclear extracts, there was no conversion of the 0.32 to 0.40 Rf band (compare lanes 4, 8 and 12). Thus at low pH, EMSA of 17 hr nuclear extract showed no *in vitro* conversion of the 0.32 Rf band, while in cytoplasmic extracts the conversion was readily apparent. Attempts to identify the "factor" that is responsible for the pH effect by chromatography of cytoplasmic extracts have thus far been unsuccessful.

I next tested if the conversion of the 0.32 Rf band to the 0.40 Rf band in the 17 hr cytosolic extract was reversible. Two samples were used, (1) partially purified fractions of a hydroxyapatite chromatography in which the active 0.32 Rf band was eluted at approximately 300 mM K_2HPO_4/KH_2PO_4 (pH 7.9) (Fig 6, lanes 1 to 8) and (2) an unfractionated 17 hr cell extract in 20 mM Tris (pH 7.9) (lanes 9 to 18). EMSA of the two samples in MES (pH 6.3) or Tris (pH 7.9) reaction mixture showed the usual conversion from 0.32 to 0.40 Rf bands with the extract in 20 mM Tris (pH 7.9) (lanes 9 and 10) but

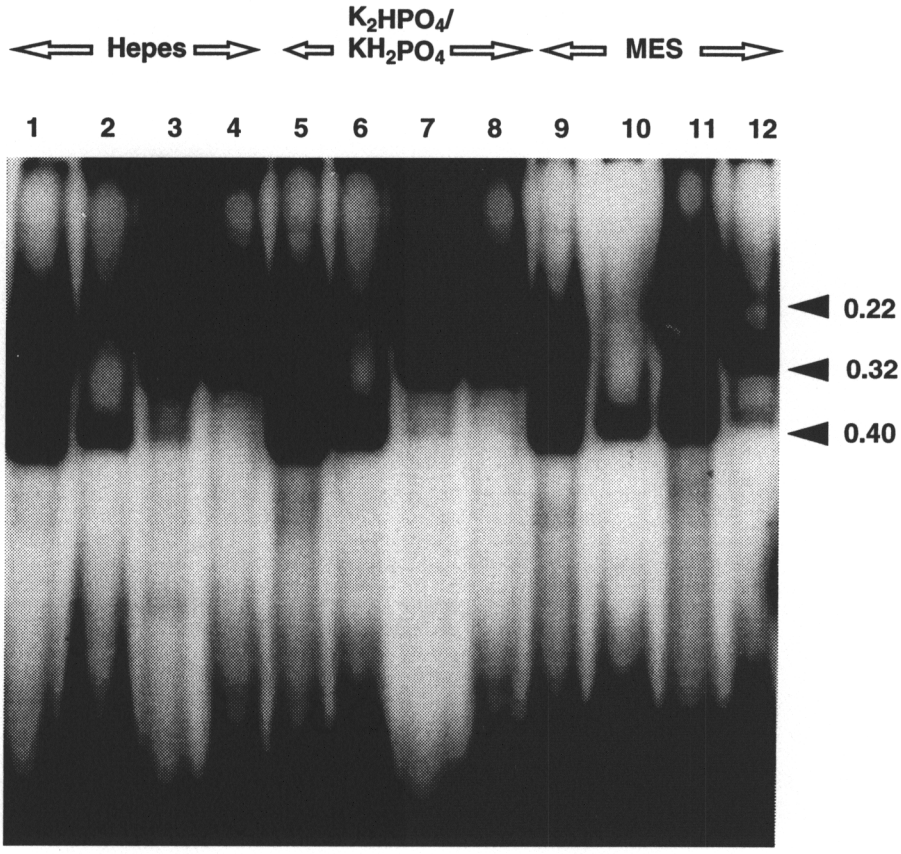

Figure 5. EMSA of cytosolic and nuclear extracts performed in different buffer conditions. The reaction buffers, Hepes (pH 7.9), K_2HPO_4/KH_2PO_4 (pH 7.4), and MES (pH 6.3) are indicated above the lane numbers. Lanes 1, 5, 9 and 3, 7, 11 are cytosolic extracts from amoebae and 17 hr cells, respectively. Lanes 2, 6, 10 and 4, 8, 12 are nuclear extracts from amoebae and 17 hr shaking cells.

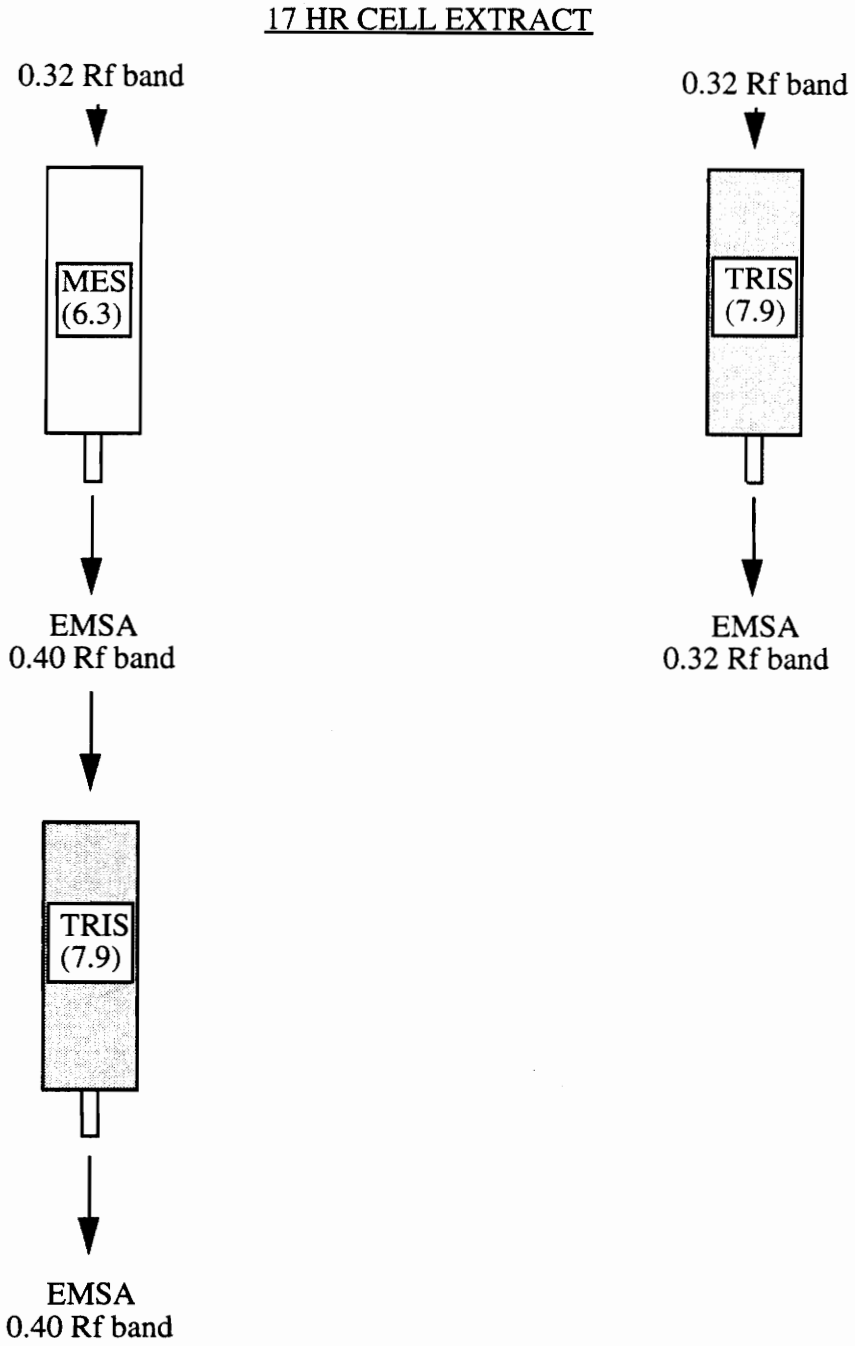

Figure 6. The test of reversibility of the "conversion" of the two forms of proteins.

Figure 6a. Diagram for test of the reversibility of the "conversion" from the 0.32 Rf band to the 0.40 Rf band.

Figure 6b. Test of the reversibility of the "conversion" from 0.32 to 0.40 Rf. The hydroxyapatite fraction that contained 0.32 Rf activity was used in the assay from lanes 1 to 8. A 17 hr cell extract (as described in Materials and Methods) was used in the assays from lanes 9 to 18. The - or + symbol refers to the presence of reaction buffer in which the assay was performed (Tris (pH 7.9), MES (pH 6.3)). One aliquot of the extract was passed through a Bio-spin 6 column containing 20 mM MES buffer (pH 6.3), to lower the pH and allow conversion to occur. This extract was then assayed by EMSA in Tris or MES reaction buffer as shown in lanes 3, 4, 11, 12. Another aliquot of the same extract was passed over an identical spin column equilibrated in 20 mM Tris buffer (pH 7.9) and then assayed by EMSA as shown in lanes 5, 6, 13, 14. A sample of the eluate from the first MES spin column was then passed over a second spin column that had been equilibrated in 20 mM Tris buffer (pH 7.9), then assayed as shown in lanes 7, 8, 15, and 16. The sample without the spin column treatment were assayed in lanes 1, 2, 9, 10, 17, and 18. All samples were incubated in the reaction mixture for 30 min before loading on the gel, except in lanes 17 and 18, the reaction mixture was added then immediately loaded on the gel. Lanes 19 and 20 are the same as lanes 7 and 15 respectively.

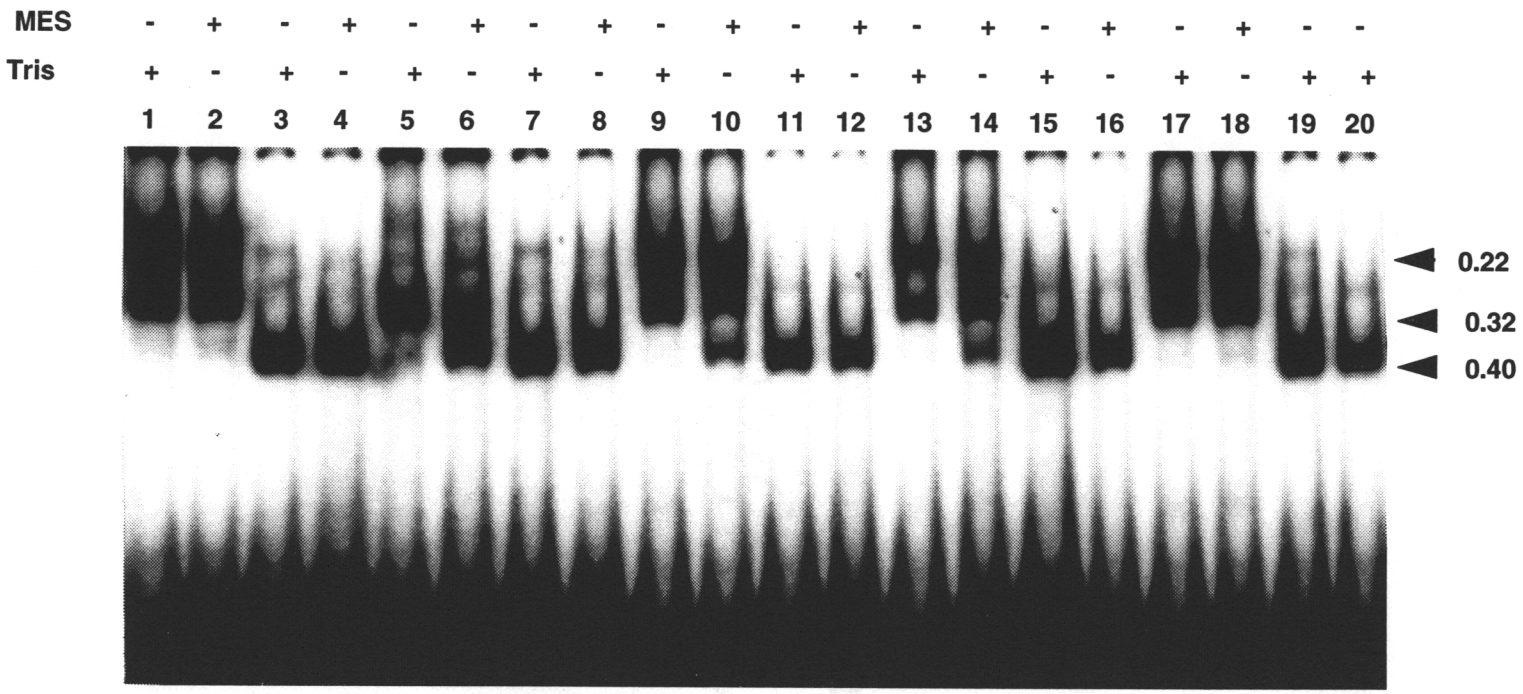

Figure 6. Test of the reversibility of the "conversion" from 0.32 to 0.40 Rf band.

no conversion with the extract in 300 mM phosphate buffer (pH 7.9) (lanes 1 and 2). Likewise, no loss of the 0.32 band was observed if the sample in Tris buffer was placed in MES reaction mixture and then immediately loaded on the gel (the zero time control) (lanes 17 and 18). The lack of conversion of the hydroxyapatite fraction in the MES reaction mixture was found to be due to the 300 mM phosphate buffer (pH 7.9) that was carried into the reaction mixture with the sample. The final buffer concentration in this case was 78 mM phosphate and 14 mM MES, with a resulting pH of 7.9. This explained why the 0.32 Rf band was retained during the hydroxyapatite chromatography when high pH phosphate buffer was used.

The pH of both the hydroxyapatite fraction and the sample in 20 mM Tris (pH 7.9) was then lowered by passing them through a Bio-spin 6 column containing 20 mM MES buffer (pH 6.3) to allow the conversion to occur. Another aliquot of the same extract was passed over an identical spin column equilibrated in 20 mM Tris-HCl (pH 7.9). Extract that had been passed through the MES spin column at pH 6.3 showed only the 0.4 Rf band whether assayed in Tris or MES reaction buffer (lanes 3, 4, 11, and 12). Thus, once the sample was converted to the 0.40 Rf band, it would not shift back to the 0.32 Rf position even when assayed in the high pH Tris reaction buffer. Extracts that were passed over the spin column equilibrated in Tris (pH 7.9), showed the same pattern as extract that was not subjected to spin column treatment; that is, the appearance of the 0.40 Rf band only in the MES reaction buffer (lanes 5, 6, 13, and 14). This result shows that there was no effect on the extract of simply passing it over a spin column. To test further if the 0.40 Rf band could convert back to the 0.32 Rf band, I passed the eluate from the MES spin column over a spin column equilibrated in 20 mM Tris (pH 7.9). When this sample was assayed by the EMSA, no conversion back to the 0.32 Rf form was observed (lanes 7, 8, 15, and 16). I conclude that once the protein is converted from the 0.32 to the 0.40 Rf form, it cannot be reconverted back to the 0.32 Rf by restoring the high pH environment.

3.2.2: Effect of Phosphatase Inhibitors on the "Conversion"

Because phosphorylation/dephosphorylation is one of the most common mechanisms that cells use for the regulating the activity of DNA binding proteins, I tested the effect of three phosphatase inhibitors on the apparent conversion of the 0.32 to 0.40 Rf bands. In Fig 7, a 17 hr extract was assayed with EMSA in either Tris (pH 7.9) or MES (pH 6.3) reaction buffer. Again, assay at low pH resulted in loss of the 0.32 Rf band and appearance of the 0.40 Rf band (compare lanes 1 and 2). Samples of the same extract were assayed in MES reaction buffer containing the tyrosine phosphatase inhibitor, sodium vanadate (lane 3), the serine and threonine phosphatase inhibitors, okadaic acid (lane 4) and calyculin A (lane 5), and the general phosphatase inhibitor, sodium fluoride (lane 6) (Gordon, 1991). In the presence of sodium vanadate there was little conversion of the 0.32 to 0.40 Rf bands as compared to the control, while the other phosphatase inhibitors had no effect.

Fig 8 shows the effect of sodium orthovanadate on the time course of conversion of the 0.32 to 0.40 Rf bands in low pH buffer. Samples from 17 hr cell extracts were incubated for various time periods, in EMSA reaction buffer containing either Tris (pH 7.9) or MES (pH 6.3) and in the presence or absence of 1 mM sodium vanadate. The reactions were then terminated with loading dye (containing 250 mM Tris-HCl (pH 7.9)) and immediately loaded on the gel. Figure 8 shows that little or no conversion occurred over the 30 min time period at pH 7.9 (lanes 3, 14, 17 and 20) while at pH 6.3 nearly complete conversion occurred by approximately 10 min (compare lanes 2, 5, 7, 9 and 11). In the presence of vanadate, however, only 41 % conversion had occurred at 10 min (lane 10). After 20 and 30 min, complete conversion had occurred at pH 6.3 (lanes 16 and 19), while in the presence of sodium vanadate only 69 % and 90 % of the 0.4 Rf band was present (lanes 15 and 18).

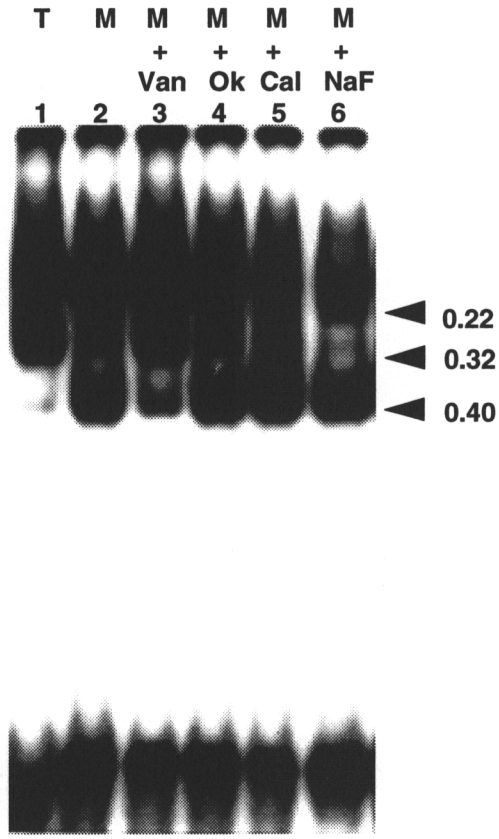

Figure 7. The effect of phosphatase inhibitors on the apparent conversion of the 0.32 to 0.40 Rf band. EMSA of a 17 hr cell extract was performed in either Tris (T, pH 7.9) or MES (M, pH 6.3) reaction buffer. Samples of the same extract were assayed in the Mes reaction buffer containing sodium orthovanadate (Van) in lane 3, okadaic acid (Ok) in lane 4, calyculin A (Cal) in lane 5, and sodiumfluoride (NaF) in lane 6. The samples were incubated in the reaction buffers for 30 min before the loading dye solution was added.

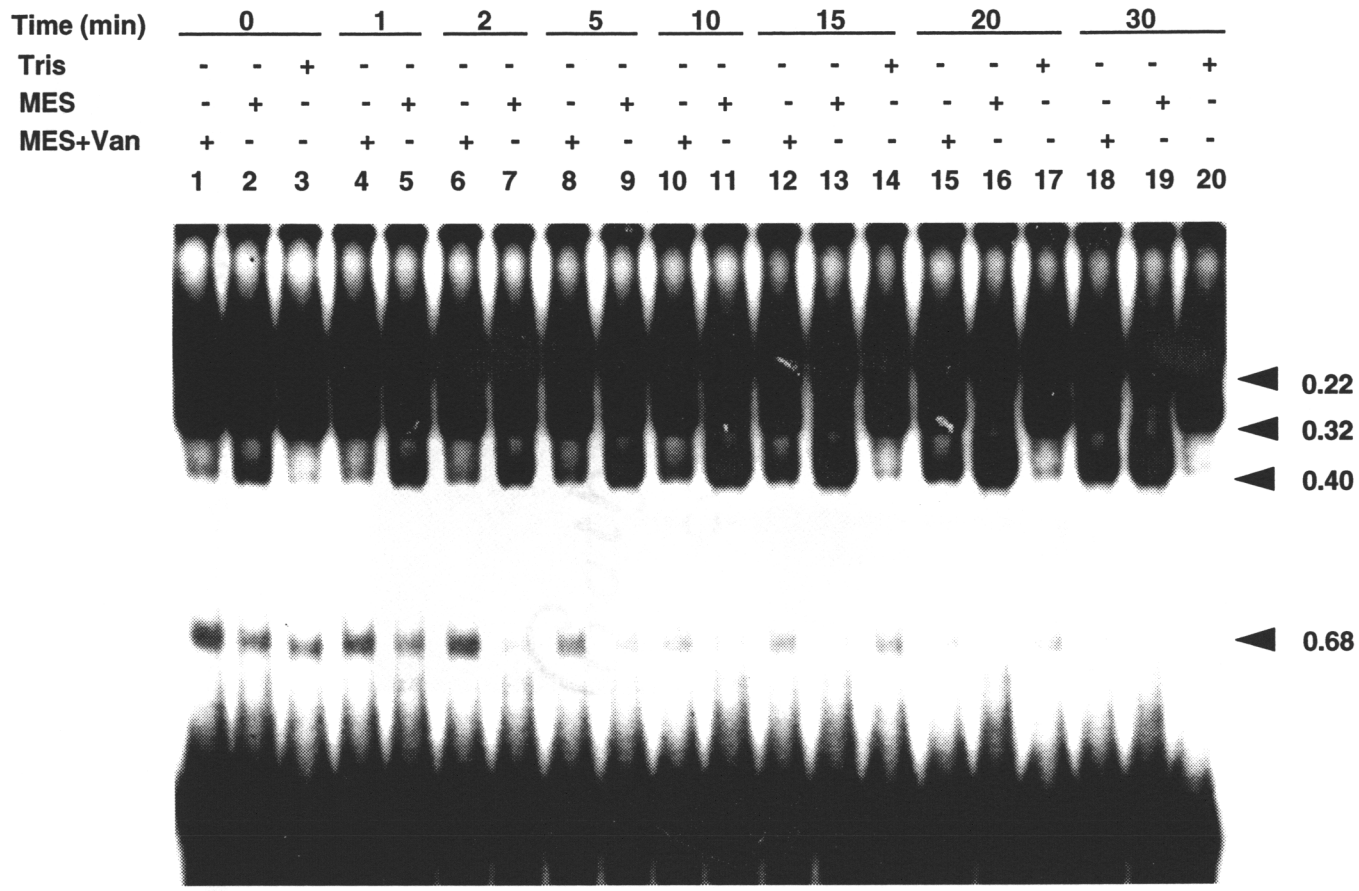

Figure 8. The effect of sodium orthovanadate on the time course of the conversion of the 0.32 to 0.40 Rf band. The 17 hr cell extract was assayed in Tris (pH 7.9), MES (pH 6.3) or MES reaction buffer containing 1 mM sodium orthovanadate. The - or + symbol indicates the reaction mixture in which the EMSA was performed. The underlined numbers indicate the incubation time (min) of the samples in the reaction mixture.

These results suggest that the tyrosine phosphatase activity caused a dephosphorylation on the 0.32 Rf band and that dephosphorylated 0.40 Rf band was observed as a result of the "conversion".

3.3: Purification of 0.32 and 0.40 Rf Band Proteins

In an attempt to purify DNA binding proteins that are involved in *gp-2* gene regulation, cell extracts were prepared as described in Materials and Methods then separated on anion-exchange and DNA affinity columns. The extract was first subjected to chromatography on DEAE Sephacel and the DNA binding activity in each fraction was determined by EMSA. If undifferentiated cell extract was used, a 0.40 Rf band was retained by the resin and eluted in the 300 mM NaCl fraction-1 (Fig 9a, lane 4). If the 17 hr cytosolic extract was used, the 0.32 Rf band was eluted in the 300-1 salt fraction (Fig 9b, lane 4). The 300-1 fraction, with an approximate volume of 100 ml, was divided into two fractions and each was passed over separate C box affinity columns (see Materials and Methods). After removing the non-bound proteins by washing with several column volumes, a linear gradient was applied from 0.4 M to 1.4 M NaCl. After the salt gradient, all affinity fractions were assayed by the EMSA. Elution of the DNA-binding activity at approximately 0.7 M NaCl was found with either 6 hr or 17 hr extracts (Fig 10a, lanes 9, 10, 11, and Fig 10b, lanes 4, 5, 6 respectively).

To determine the extent of purification by affinity chromatography, 1 ml samples were removed from each affinity fraction, TCA-precipitated (see Materials and Methods) and then analyzed by SDS-PAGE. From 6 hr extracts, three peptides of 62, 35, and 18 kD

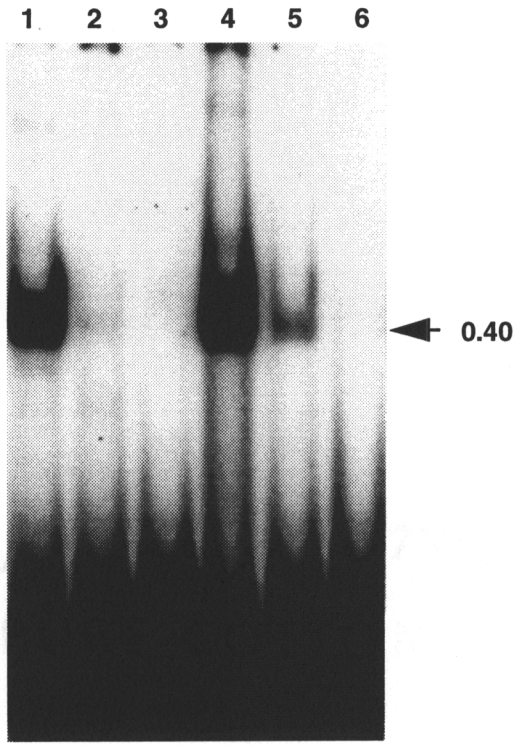

Figure 9. EMSA of the DEAE Sephacel Batch Chromatography.

Figure 9a. The assay was performed as described in Materials andMethods using the 6 hr cell extract. The 0.4 Rf band activity in lane 1 was from the sample that was applied to the resin. Two flow-through fractions were assayed as FT-1 in lane 2, and FT-2 in lane 3. Lanes 4, 5, and 6 are the 300 mM NaCl eluate fraction-1, fraction-2, and the 1 M NaCl eluate fraction, respectively.

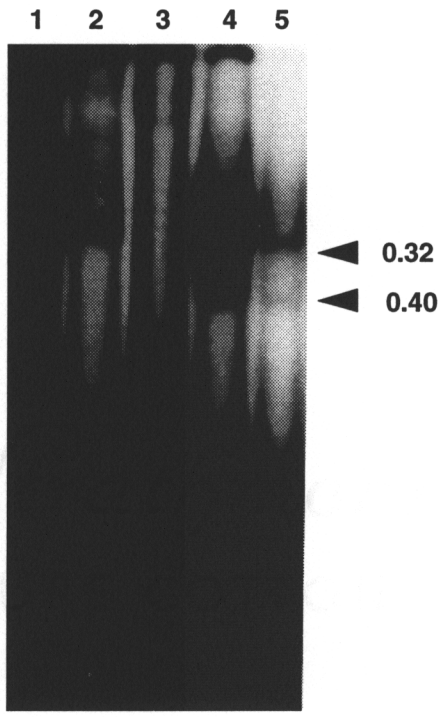

Figure 9b. The same experiment was performed using the 17 hr cell extract. Lane 1 showed the 0.32 Rf band activity in the sample that was applied to the resin. The assay of the flow-through fractions is shown in lanes 2 and 3. The bound protein was eluted off the resin at 300 mM NaCl fraction-1 in lane 4 and fraction-2 in lane 5. The 1 M NaCl fraction was not included in this assay.

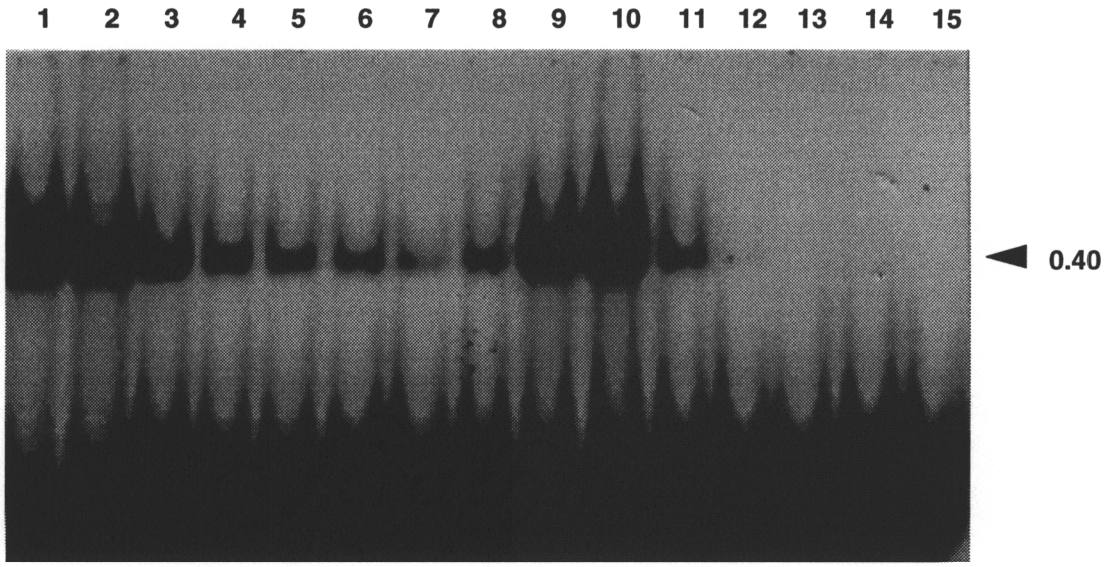

Figure 10. EMSA of fractions from DNA affinity column chromatography, with extracts from 6 hr and 17 hr cells.

Figure 10a. Lane 1: the 0.3 M fraction from DEAE Sephacel chromatography of 6 hr cell extract that was applied to the affinity column; Lane 2: the activity in the flow-through fraction, showing that the column was overloaded; Lanes 3, 4, and 5: three 20 ml flow-through fractions that were collected after the extract was loaded; Lanes 6 to 15: the fractions collected during salt gradient. The DNA binding protein was eluted from the column at about 0.7 M NaCl, as shown by the 0.40 Rf band in lanes 9, and 10.

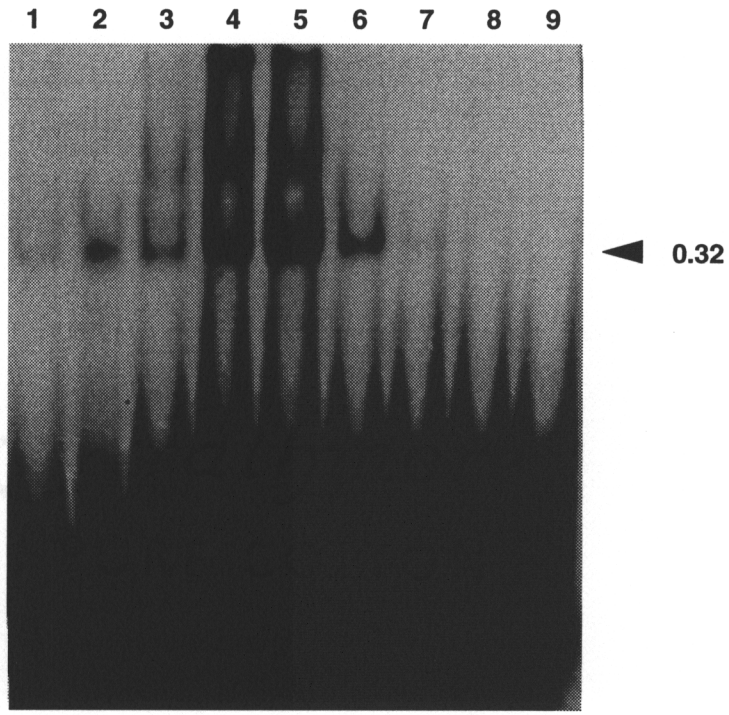

Figure 10b. The same chromatography was performed with the 17 hr cell extract. The figure showed the gradient fractions 1 to 9 assayed by the EMSA. The 0.32 Rf band activity also eluted at about 0.7 M NaCl concentration as shown in lanes 4 and 5.

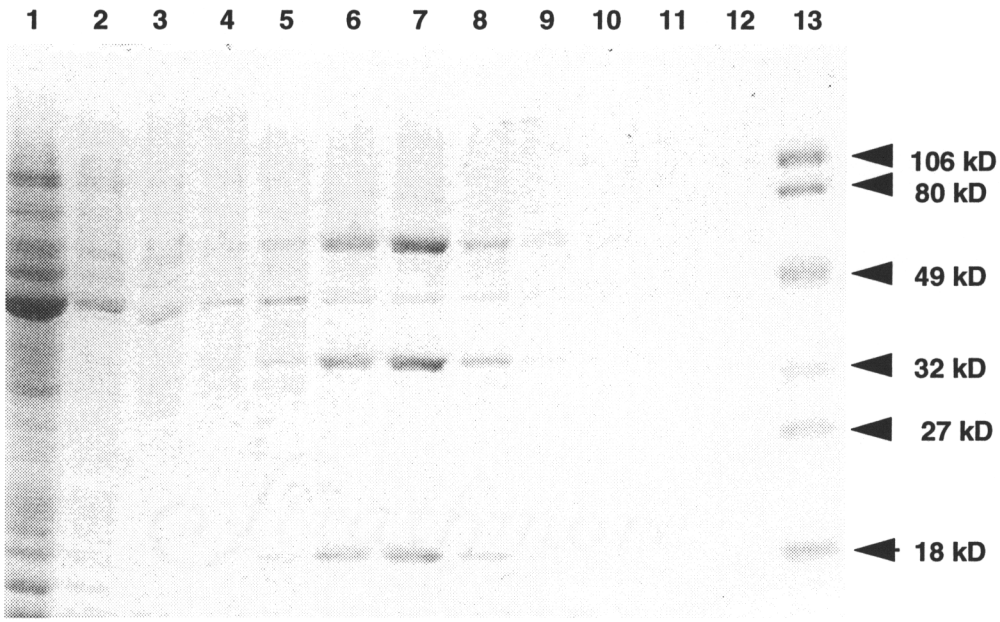

Figure 11. SDS-PAGE of the "C" box DNA affinity fractions.

Figure 11a. A 1 ml sample was taken from each of the 10 ml 6 hr affinity fractions, DOC-TCA precipitated, then analyzed by SDS-PAGE. The gel was stained with Brilliant Blue G. Lanes 1 and 2 are concentrated samples from affinity flow-through 1 and flow-through 2; lanes 3 to 12 are the eluted fractions corresponding to lanes 6 through 15 of Fig 10a. Three peptides of 62, 35 and 18 kD were present in lanes 6, 7 and 8. Their relative sizes were determined by comparison to the protein standard marker in lane 13.

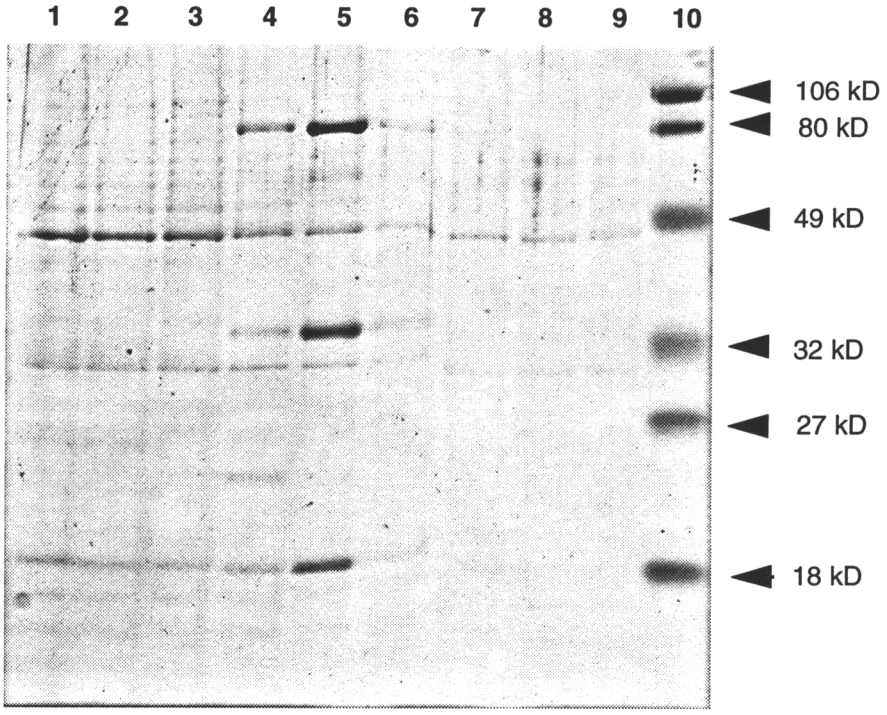

Figure 11b. SDS-PAGE of the 17 hr DNA affinity chromatography. The same experiment as in Fig 11a was performed. The lane numbers correspond to those shown in Fig 10b. An 81 kD band was present in the same fractions as the 35, and 18 kD peptides, lanes 4, 5 and 6, which coincided with the 0.32 Rf band activity in affinity chromatography. A standard marker was run on lane 10.

were apparent in the fractions that coincided with the 0.40 Rf activity as determined by EMSA (Fig 11a, lanes 6, 7 and 8). If the 17 hr extract was used, the SDS-PAGE showed a new band at 81 kD and the 62 kD was no longer present, while the two smaller subunits, 35 and 18 kD remained (Fig 11b, lanes 4, 5, and 6). Thus, 62 and 81 kD peptides bands appeared to be related to the migration of the retarded bands in the EMSA of 0.40 and 0.32 Rf, respectively. In addition, both the shifted bands of the EMSA and the purified DNA binding proteins showed temporal expression during development.

3.4: Amino Acid Sequencing

In order to obtain a sufficient amount of the purified protein for microsequencing, I prepared extract from 750-1,000 gm (wet weight) of cells. This material was carried through the DEAE Sephacel and C box affinity steps as described above. Fractions containing DNA binding activity from 10 separate affinity columns were pooled (approximately 400 ml), TCA-precipitated, separated on 12% SDS-PAGE, and transferred onto PVDF membrane for peptide sequencing. With the first attempt to sequence the intact peptides, all were found to be blocked at the amino terminus. Therefore, each peptide band was subjected to proteolytic cleavage prior to electroblotting (see Materials and Methods). The digested products were separated using 15% polyacrylamide gel by SDS-PAGE and then transferred onto PVDF membrane. The peptides were then analyzed for amino acid sequences. The summary of sequences obtained from either trypsin or V8 digestion is shown in Table 1. Identical sequences were observed between two fragments resulting from V8 digestion of the 81 kD and 62 kD peptides.

Table 1. Amino acid sequences of protease-digested products of each purified polypeptide. Identical sequences from digested fractions of the 81 kD and 64 kD poly peptides are shown (underlined).

Polypeptide	Protease	Amino acid sequence
81 kD	V8 V8	VMGKTGDRLFGKSAAELYQM GAPLVMNDQFTAMKGVRNKQ
62 kD	V8 Trypsin	GAPLVMNDQFTAMKGVRNKQ VMISPDQTQFTPHYNFKKIA
35 kD	V8 V8/trypsin Trypsin	GSTDEPTYSLDLDIRDGTGQ NQYYSFVLKPALQRQAPQQQEQLTTIQGYLVSYH AQPVVEMQIFTFQNLPMQPG

3.5: Southwestern Blot Analysis

To determine which of the subunits contained DNA binding properties, southwestern blot analysis was performed using the affinity fractions from 17 hr extracts. The pooled affinity fractions containing DNA binding activity were precipitated, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was then probed with the labeled C box DNA both in the presence and the absence of non-specific poly dI-poly dC. Autoradiography of the membrane (Fig 12) showed that only the 81 kD subunit bound to the DNA probe. The specificity of the binding reaction was determined by including non-specific poly dI-poly dC. A 2.5×10^9 fold excess of the competitor did not compete with the binding (result not shown).

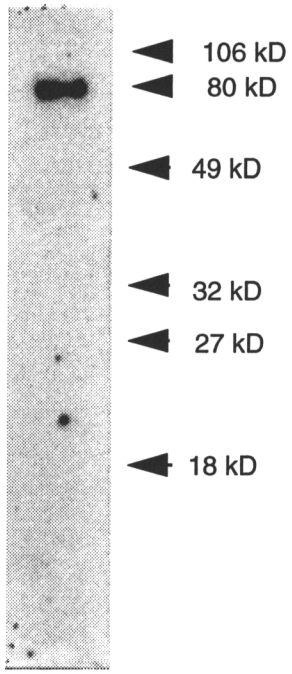

Figure 12. Autoradiograph of the southwestern blot analysis of the purified protein from the 17 hr cell extract. The purified protein was blotted onto a nitrocellulose membrane, then probed with ^{32}P-labeled "C" oligonucleotide. The autoradiograph showed that the 81 kD subunit contained the DNA binding activity.

4. Discussion

My investigation of the protein(s) that bind to the 3' C-box of the *gp-2* promoter showed two typical shifted bands on EMSA, a 0.40 Rf band in undifferentiated cell extract, and 0.32 Rf band in 17 hr differentiated cell extract. To characterize the protein(s) that result in the gel shift, the extracts were purified by DEAE Sephacel chromatography and "C" box specific DNA-affinity chromatography. Using 6 hr cell extract for the 0.40 Rf band and 17 hr cell extract for the 0.32 Rf band, the proteins were purified to homogeneity and found to consist of 18 kD, 35 kD, and either 62 kD (for 0.40 Rf) or 81 kD (for 0.32 Rf) subunits. Amino acid sequence analysis of a segment of the 62 kD and 81 kD polypeptide shows identity (Table 1). Therefore, I conclude that the difference in the shifted 0.40 Rf and 0.32 Rf bands in EMSA is due to the 62 kD and 81 kD subunits.

My first attempt to purify the 0.32 Rf band using CM sephadex chromatography at pH 5.8 surprisingly revealed the loss of the 0.32 Rf and the appearance of the 0.40 Rf band. The possible conversion between these two forms of the protein was studied with the EMSA at various pHs and in the presence and absence of phosphatase inhibitors. I found that the protein from differentiated cells producing the 0.32 Rf band could be "converted" to a form yielding the 0.40 Rf band by a brief exposure to pH 6.0 (Fig 4, lanes 3, 7, and 11). In addition, the conversion could be inhibited by an inhibitor of tyrosine phosphatase activity (Fig 7 and 8).

To explain these results, I will briefly examine four possible models for the *in vitro* expression of the 0.32 and 0.40 Rf bands: (1) The gel shift bands at 0.40 Rf (in undifferentiated cell extract) and 0.32 Rf (in 17 hr cell extract) are due to the same protein. In undifferentiated cells, the protein exists as an unphosphorylated form with one subunit

that migrates at 62 kD in SDS-PAGE, and causes a 0.40 Rf band in EMSA. When cells are triggered to differentiate, the protein becomes highly phosphorylated resulting in a change in the shift of the 0.40 to 0.32 Rf band in EMSA and migration in SDS-PAGE corresponding to 81 kD. Phosphorylation is a known regulatory mechanism for DNA binding activity and has been shown to alter the pattern of shifted bands in the EMSA (Carty, 1994). Likewise, phosphorylation can increase a protein's apparent molecular weight on the SDS polyacrylamide gel (Dahmus, 1981). However, it seems unlikely that phosphorylation could result in a difference of 19 kD in SDS-PAGE. (2) The two proteins are products of the same gene and the expression of the large subunit is transcriptionally regulated by RNA processing. Alternative splicing of a single gene could generate the 62 kD and 81 kD subunits and consequently the differences in the gel shift pattern. (3) The 62 kD and 81 kD subunits are the product of two separate, but closely related genes. Although I found a stretch of 20 amino acids that were identical in the two subunits, this simply represents a protein domain that is highly conserved. The 0.32 Rf band protein is a phosphorylated form and contains an extra 19 kD amino acid sequence over the 0.40 Rf band protein. The 81 kD subunit is expressed as the cell undergoes differentiation. If the pH is artificially lowered, the protein becomes dephosphorylated and a proteolytic cleavage site is revealed. Because the cleavage is irreversible, it would explain my observation (Fig 6, lanes 7, 8, 15, and 16) that when the 0.32 is converted to 0.40 Rf at low pH, it would not convert back to the 0.32 Rf as the pH was restored to pH 7.9. (4) The most likely possible model is that the two proteins are the product of the same gene and expressed throughout the whole life cycle. At the amoebae stage, the protein is contained in a low pH environment due either to compartmentation, or an acidic cytoplasm. As a result of the low pH or the lack of an appropriate kinase activity, the protein exists as a non-phosphorylated form. The large subunit is synthesized as a precursor, but is immediately cleaved to a polypeptide of 62 kD. Upon cell differentiation, the pH of the environment increases,

again due to compartmentation, or a general increase in cytoplasmic pH. At high pH the protein is phosphorylated and is retained as the 81 kD peptide. The lack of cleavage in differentiated cells could be due to the phosphorylation state of the protein or to the inactivity of the protease at the high pH. Artificially lowering the pH *in vitro* mimics the *in vivo* environment of the undifferentiated cell, resulting in dephosphorylation and cleavage of the 81 kD subunit, and, therefore, restoration of the 0.40 Rf gel shift band.

Although the latter model is complicated, there is precedent for this idea in the literature. For example, it has been shown that as development proceeds, an increase of cytoplasmic alkalinization occurs in *Dictyostelium* upon cAMP stimulation (Aerts et al., 1987; Van Duijn and Inouye, 1991). Furthermore, an artificial raising of the intracellular pH alone is sufficient to increase the rate of protein and DNA synthesis in *Dictyostelium* (Aerts et al., 1985). Others have shown that pH may influence the efficiency of mRNA translation rates through the transition in cytoskeletal association of essential protein synthetic cofactors (Edmonds et al., 1995).

Purification of *gp-2* promoter DNA binding proteins shows the active molecule consists of three polypeptide subunits of 81 kD (differentiated cell) or 62 kD (undifferentiated cell), 35 kD, and 18 kD. From southwestern blot analysis using differentiated cell extract, a DNA binding activity resides only in the 81 kD subunit. There is similarity between the results presented here and those for replication protein-A (RP-A) from *Drosophila melanogaster* (Marton, 1994). RP-A is a heterotrimeric, single stranded DNA binding protein, that has been identified in several eukaryotic systems including human, *Saccharomyces cerevisiae*, calf thymus, the trypanosomatid, *Crithidia fasciculata*, and *Xenopus laevis* . In addition to being required for DNA replication (both initiation and elongation), RP-A is also involved with *in vivo* DNA repair and recombination (Longhese, 1994) and gene regulation (Luche, 1992). The structure of the heterotrimeric protein is highly conserved among species, consisting of three polypeptide subunits with

approximately 70 kD, 34 kD, and 12 kD molecular mass. The single-stranded DNA binding activity resides in the 70 kD subunit (Kenny, 1990; Wold, 1989). Because of the similarity of the size of the subunits, and the fact that the DNA binding activity resides in the large subunit, I compared the sequence of peptides from the 62 and 35 kD subunits of the *Dictyostelium* protein to the sequence of the RP-A from several organisms. The sequence of a large subunit of RP-A is very similar in most species in which it has been identified, while that of the 34 kD subunit shows more variability between organisms. Although I found some similarity between the peptides, the sequence comparison does not support the conclusion that the *Dictyostelium* protein is RP-A. In fact no protein with high sequence identity was identified through a Gene Bank search, and RP-A was not included in the 100 proteins showing in the highest sequence similarity. I tentatively conclude that the similarity of the subunit structure between the *gp-2* binding protein and RP-A is coincidental.

There is significant homology in the C box sequence that was used in the assay and affinity purification of the 0.32/0.40 Rf band protein and a short C-rich element 'ACACCCA' of the cis-acting regions of other late gene promoters in *Dictyostelium* . This nucleotide sequence was found to be conserved in the cis-acting region of the prespore-specific genes, SP96, SP60, Dp87, and also in the 5' region of the spore-coat protein gene SP70. There are no corresponding elements present in the *gp-1* promoter. This suggests that the presence of the cis-acting element plays an important role in gene regulation (Tasaka et al., 1992). Another homologous sequence was found to act as a specific binding site for the G box binding factor (GBF), the first transcription factor to be purified in *Dictyostelium* (Schnitzler et al., 1994). GBF was shown to be one of the components required for post-aggregative development and late gene activation by cAMP. It is a 92 kD polypeptide that binds to several of the CA/GT-rich late regulatory elements. Each of the binding sites consists of two half-sites of the rough consensus

(T/G)G(G/T)G(T/G)G(T/G) or the complementary strand. Despite the similarity of the specific DNA binding sites, the purified 81 kD subunit from differentiated cells appears to be a different peptide from GBF. Comparison of amino acid sequence from three regions of the 81 kD subunits showed little or no sequence identity.

To understand more about this DNA-binding protein, further studies could be done as follows: (1). Test the effect of known protease inhibitors on the conversion of the two forms of the purified protein and crude extract. (2). Test the effect of pH on the conversion of the 0.32 Rf band to the 0.40 Rf band with a sample of the purified proteins. (3). Cloning the genes that code for the purified DNA-binding proteins by using degenerate oligonucleotides designed from the obtained amino acid sequences as a primers for PCR. The PCR products will then be used to screen a cDNA libraly from different stages of development. (4). Knockout the specific gene to observe the effect on the gp-2 expression and on the development of cells.

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