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I am submitting herewith a dissertation written by Hugh A. Miller III entitled "Hepatic cAMP Dependent DNA Binding Factors." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

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Wesley D. Wicks, Major Professor

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HEPATIC cAMP DEPENDENT DNA BINDING FACTORS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Hugh A. Miller III

December 1986

DEDICATION

I wish to dedicate this dissertation to my wife, Linda, without whose continual friendship, support, and perseverance, the body of work contained within these pages would not have been accomplished.

ACKNOWLEDGMENTS

I would like to thank Dr. Wesley D. Wicks for his guidance, support, and freedom to produce this project. Special thanks go to Dr. Doris Schlichter for her support, the example she set forth for me to follow as a good, critical scientist, and for the many enlightening discussions which we had, without which, the ideas within these pages would be very dull.

I also would like to acknowledge my committee: Dr. Leaf Huang, Dr. David Brian, and Dr. John Koontz, for their guidance and constructive criticism.

A special thanks to Dr. John Koontz for being a good friend and for taking time to listen and help me during difficult times. I also wish to thank Charlie Spielholtz for his friendship and for being a good listener.

ABSTRACT

Intracellular levels of adenosine cyclic monophosphate (cAMP) have been determined to regulate the synthesis of several proteins at the transcriptional level. The presence of factors which would recognize and bind to sequences within the regulatory region of the gene for these proteins has been proposed.

The regulatory region of the gene for hepatic phosphoenolpyruvate carboxykinase (PEPCK) responsible for cAMP regulation has been determined to be within the first 621 base pairs immediately 5' of the transcribed region of the gene. Fragments produced by restriction endonuclease digestion of this region were utilized in the nitrocellulose filter binding assay in order to detect possible DNA binding factors.

Crude nuclear extracts prepared from rat liver treated with cAMP were found to specifically retain on nitrocellulose a fragment of the PEPCK gene from 71 to 111 base pairs 5' of the start site of transcription (41 base pairs in size). This region is included in a fragment from 70 to 109 base pairs from the start site which confers cAMP regulation on a heterologous gene (Short et al., 1986, J. Biol. Chem. 261: 9721-9726).

Competition binding revealed that this factor(s) in the nuclear extract demonstrated an approximate dissociation constant of 1×10^{12} when tested with the specific 41 base pair fragment.

The ability of nuclear extracts from cAMP-treated rat liver to protect guanine residues within this 41 base pair region from methylation by dimethylsulfate was demonstrated. The extract from cAMP-treated

liver nuclei protected guanines at positions 98, 94, 93, 92, and 91 on the 5' side of the start site. These guanines are close to the region that has been proposed to be the cAMP regulatory element.

These lines of evidence suggest that the identified factor(s) may be involved in the regulation of gene transcription by cAMP.

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INTRODUCTION

The mechanism of gene transcriptional regulation in both prokaryotic and eukaryotic cells is currently under investigation. The subject of this dissertation is to investigate the possibilty that adenosine cyclic monophosphate (cAMP) induces eukaryotic gene expression by a transacting factor(s) which binds to specific regions of a gene regulated by cAMP.

Cyclic AMP regulates gene expression in prokaryotic cells and its mechanism is well known. There is a protein, referred to as the catabolite activator protein (CAP), which binds cAMP and binds to selective sites in promoters of genes such as the gene for lactose (Schmitz, 1981). The CAP protein is a dimer with each monomer containing 209 amino acids (Cossart and Gicquel-Sanzey, 1982). Each monomer has a binding site for cAMP and a DNA binding site (Fried and Crothers, 1983) which include two closely spaced double helices that have been shown to interact with DNA (Pabo and Sauer, 1984).

The sequence where CAP binds in the promoter regions is a 15 base sequence that has been found in 10 promoters within E. Coli (Schmitz, 1981). Binding of CAP to this region has been shown to affect transcription either by changing the local conformation of the DNA, by actually interacting with polymerase and altering the rate of polymerase activity by protein-protein interaction, or both (Meikeljohn and Gralla, 1985). Recent results from Jay Gralla (Meiklejohn and Gralla, 1985)

suggest that CAP actually clears the promoter of other factors and allows RNA polymerase to bind and stabilizes this binding.

Changes in the levels of cAMP in eukaryotic cells generated by hormone activation of adenylate cyclase have been shown to result in changes in the messenger RNA (mRNA) levels for several genes in rat liver, including phosphoenolypyruvate carboxykinase (PEPCK) (Beal et al., 1982), tyrrosine aminotransferase (Scherer et al., 1982), ornithine aminotransferase (Merrill, 1985), and carbamoylphosphate synthetase (Groot, 1984). Genes of other tissues have also demonstrated cAMP mRNA induction such as lactate dehydrogenase in rat glioma cells (Jungmann et al., 1983), human chorionic gonadotropin in JEG-3 choriocarcinoma cells (Burnside et al., 1985), rat somatostatin (Montminy et al., 1986), and human proenkephalin (Comb et al., 1986).

These changes in mRNA levels suggest that cAMP activates transcription and there is evidence that this activation is mediated by the cAMP-dependent protein kinase (cAMP-PK) (Boney et al., 1983; Reisine et al., 1986; Montminy et al., 1986). Delivery of the catalytic subunit of cAMP-dependent protein kinase into rat hepatoma cells was shown to stimulate the activity of the enzyme tyrosine aminotransferase (TAT) in a similar fashion as an analog of cAMP, 8-Bromo-cAMP. Furthermore, the delivery of the protein inhibitor of cAMP-PK, PK-I (Whitehouse et al., 1980) was able to prevent 8-Br-cAMP from inducing TAT activity. Recently, the 5' flanking region of rat somatostatin was investigated for cAMP inducibility with transfection experiments (Montminy et al., 1986). The expression plasmid containing the flanking region attached to

chloramphenicol acetyltransferase (CAT) was delivered to pheochromocytoma cells, line A126-1B2, which have been shown to be deficient in cAMP-PK activity (Van Buskirk et al., 1985) and the CAT activity was not induced by cAMP (Montminy et al., 1986).

Regulation of gene transcription in eukaryotic cells has been demonstrated in several systems to be the result of interaction between sequences located near the promoter of the gene and specific proteins (Dynan and Tjian, 1985). In the case of SV-40 regulation, the early genes of the virus encode for a protein known as large T antigen (Tjian et al., 1978) which has been shown to bind to a region within the origin of replication (Meyers, 1981) and inhibit the further transcription of the early genes of the virus (Rio and Tjian, 1983).

In the case of the observed regulation of several genes by glucocorticoids, the soluble cytoplasmic receptor for glucocorticoid has been shown to bind to regions of the mouse mammary tumor virus LTR (MMTV) (Payvar et al., 1981) as well as other flanking regions (Renkawitz et al., 1984). This binding occurs with the receptor that has bound glucocorticoid, and has been shown to occur within regions of the DNA that contains two or more binding sites in tandem (Scheidereit et al., 1983). A consensus region for receptor binding has been determined, 5'T(C)GGTA(T)CCA(C)A(T)TGTT(C)CT 3' (Rousseau, 1984), and this consensus sequence has been found in the 5' flanking region of several eukaryotic genes such as human metallothionein II (Karin et al., 1984).

The glucocorticoid receptor as purified is a protein of 90-94 K MW (Govindan and Gronemeyer, 1984). The model for the activation of

transcription by glucocorticoids has shown that the receptor binds the hormone, translocates into the nucleus (Rousseau, 1984) and binds to the DNA. The receptor has been proposed to be regulated by a small molecule, yet unidentified, that inactivates the receptors, possibly by preventing hormone bound receptors from bindiing the DNA (Litwack et al., 1980).

The sequence that contains the receptor binding site can be moved in either direction along the DNA in relation to the promoter (Chandler, 1983) and still confer regulation. This property of the regulatory region to regulate the transcription from any position, even several 100 to 1000 base pairs away, is referred to as an enhancer (Yamamoto, 1984). Enhancer elements have also been located in SV-40 (Benoist and Chambon, 1981) and in the immunoglobulin gene region (Gruss, 1984).

The glucocorticoid receptor binding to its sequence of DNA demonstrates the use of transacting DNA binding factors in eukaryotic cells to regulate gene expression. In fact, the estrogen receptor has also demonstrated binding to regions of the flanking sequences of the rat prolactin gene (Maurer, 1985).

The mechanism of cAMP modulation of gene expression has been shown to involve the cAMP-PK. The kinase translocates into the nucleus minutes after activation by cAMP (Keuttel et al., 1985). This translocation into the nucleus would suggest that the kinase could activate a protein within the nucleus and this protein could modulate the transcription of genes by interacting with specific sequences. The SV-40 large T antigen is phosphorylated <u>in vivo</u> and removal of a phosphate

from a serine residue within the protein results in alteration of the binding affinity to the 72 base pair (bp) repeats within the SV-40 origin (Bauman, 1984).

The regulatory subunit, RII, has been proposed to be the cAMP transcriptional regulatory factor, due primarily to proposed homology between RII and the CAP protein of E. Coli (Naganime and Reich, 1985). However, this homology is primarily within the cAMP binding region (Schlichter et al., 1986). RII has also been proposed to possess topoisomerase activity (Constantinou et al., 1985), but this topoisomerase activity appears to be a contamination of the RII preparation (Granner, personal communication).

The sequences that are responsible for cAMP transcriptional induction have been demonstrated for rat hepatic PEPCK (Wynshaw-Boris et al., 1984), rat somatostatin (Montminy et al., 1986), and human proenkephalin (Comb et al., 1986). The sequences within the PEPCK flanking region have been shown to be between -108 to -62 (Short, 1986) and within the flanking region of somatostatin, between -60 to -29 (Montminy et al., 1986) and within the 5' flanking region of human proenkephalin, -107 to -71 (Comb et al., 1986).

In the case of hepatic PEPCK, these sequences can confer cAMP induction in either orientation or even on the 3' end of the structural gene (Wynshaw-Boris, 1986). This region of the PEPCK regulatory region contains a 12 base sequence; 5' CTTACGTCAGAG 3 (Short et al., 1986) which has been shown by using the Microgenie DNA sequence program (Beckman) to be contained in the flanking regions of other genes. Table I

Table I

Consensus Sequences in cAMP Regulated Genes

Gene	Sequence	Region
PEPCK	C G G C C C C T T A C G T C A G A G G C	-78/-97
Somato	C C T T G G C T G A C G T C A G A G A G	-36/-55
Proenk	G T A G G G C C T G C G T C A G C T G C	-81/-100

	Care
CONSENSUS	
100%	C C G T C A G
66% C	G G C T T A C G T C A G A G G C

demonstrates the three sequences that have been showhn to confer cAMP response to reporter genes upon transfection. These genes contain a 12 base region that is highly conserved, however this 12 base region was not sufficient to confer cAMP regulation on a heterologous gene (Montminy, 1986; Birnberg, unpublished observations), suggesting that more of the sequence is needed than is contained in these 12 bases.

With this background, it seemed reasonable to investigate the possibility that a factor(s) extracted from nuclei will interact specifically with a region of DNA from the PEPCK flanking sequences. At the time this study was undertaken, the PEPCK responsive region was known to be within 548 bases of the 5' flanking region (Wynshaw-Boris, 1984). Therefore this region of DNA was obtained and nuclear extracts prepared from both control and 8-Br-cAMP treated rat livers as a source of the putative factor(s). These extracts were used in the nitrocellulose filter binding assay (Riggs et al., 1970) to identify any sequences which might be specifically retained by the cAMP-treated protein extracts. Once the sequences that were retained on nitrocellulose filters were identified, chemical footprinting methods, i.e., dimethyl sulfate protection (Gilbert et al., 1976), were used to confirm the filter assay and also to describe more precisely the region of interaction between the factor(s) and the DNA.

Comparison of the consensus sequences of the three regions of DNA that have been shown to confer cAMP regulation in transfection experiments (Short et al., 1986; Montminy et al., 1986; Comb et al., 1986). A consensus sequence is shown at both 100 and 66%.

CHAPTER 2

MATERIALS AND METHODS

Materials

All buffers were made with chemicals from either Fissher or Sigma. Radioactive nucleotides, dCTP- -^{32}P, (3200 Ci/mmole), were obtained from ICN Corp. The gamma ^{32}P ATP was synthesized with a kit from Promega-Biotec Corp. using ^{32}Pi from ICN Corp. The acrylamide and urea used in the sequencing gels were purchased from IBI Corp. Restriction enzymes, polynucleotide kinase and Klenow fragment were obtained from either BRL Corp. or IBI Corp. Restriction enzymes were stored at -20°C.

DNA Preparation

The DNA that was used as a source of restriction fragments for the filter binding, agarose gel, and protection experiments was obtained from Dr. R. Hanson at Case Western Reserve. Dr. Hanson kindly supplied a plasmid, pBH 1.2, which contains 1.2kb of the genomic clone of rat hepatic phosphoenolpyruvate carboxykinase (PEPCK) in a Bam HI/HindIII fragment cloned into pBR 322 (Wynshaw-Boris et al., 1984). This insert contains the region from -548 to +638 of the PEPCK gene.

The plasmid pBH 1.2 was obtained in E. Coli strain HB101. The plasmid was isolated from chloroamphenicol amplified cultures (Takemoto, unpublished observations). The bacteria contining the plasmid were plated on LB agar plates containing 40ug/ml ampicillin (Maniatis et al., 1982). Single colonies were isolated and each was used to inoculate a

100ml LB liquid culture. The next day the liquid culture was used to inoculate a 1 liter culture at a ratio of 1:20.

The inoculate was allowed to grow by agitation at 37°C until the culture had an absorbance of 0.400 at 550nm at which time solid chloroamphenicol was added to 200mg/liter culture and the culture allowed to incubate overnight.

The bacteria were then harvested by centrifugation at 6000 rpm for 20 min at 4°C. The pellet was resuspended in 8ml/liter culture in Tris-sucrose solution {0.05M Tris-Cl, pH 8.0, 25% sucrose (w/v)} and transferred to a 50ml Sorval tube. To the suspended bacteria, 0.8ml/liter culture of 4mg/ml lysozyme (Sigma) and 0.8ml/liter culture of 0.5M EDTA (pH 8.0) was added. After vortexing gently, the mixture was incubated on ice for 20 min followed by the addition of 5.6ml/liter culture of Triton buffer {0.05M Tris-Cl (pH 8.0), 0.062M EDTA (pH 8.0), 2% Triton X-100}. The mixture was gently inverted and kept on ice 30 min followed by incubation at room temp. for 15 min. The solution was then centrifuged at 16,000 rpm for 1 hr at 5°C.

The supernate was passed through prewet gauze {wet in TE (10mM Tris-Cl (pH 8.0), 1mM EDTA (pH 8.0)} into a graduated cylinder. The lysate volume was measured and cesium chloride, (CsCL, BRL), was added at 1g/ml lysate, the solution was mixed gently by inversion and the CsCl was allowed to dissolve completely by incubation for 20-30 min at 4°C. The cleared lysate solution was added to 15ml Beckman heat-sealable centrifuge tubes at 10ml/tube with 600ul of ethidium bromide, (EtBr, Fisher), 10mg/ml) and the tube was filled to capacity with mineral oil

and sealed. After mixing by inversion, the tubes were centrifuged in a fixed angle rotor (Beckman Ti-65) at 38,000 rpm for at least 40 hrs. at 19°C (Maniatis et al., 1982).

The plasmid appeared as a band of EtBr stained material usually within the first third of the CsCl gradient with the genomic band visible above the plasmid band and RNA appearing as a red stained precipitate. To harvest the plasmid, the appropriate band was visualized with a long wave UV lamp and the boundaries of the plasmid marked on the tube. The sample was harvested through a 20 gauge needle that was inserted below the band with the bevel up and approximately 2/3 through the tube. The tube was draineed into a 30 ml glass Sorval tube to a level equal to the thickness of the bank +2mm.

Excess EtBr was removed by mixing the collected band with 1 volume of 1-butanol followed by vortexing and aspiration of the butanol layer (top) twice. The cleared solution was dialyzed against 10mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) for at least 4 hrs at room temperature with several changes to remove the remaining CsCl from the plasmid preparation. The protein was extracted from the plasmid preparation with 1 volume of phenol (BRL) {water soluble phenol is extracted with 1M Tris-CL (pH 8.0) and 0.1M Tris-Cl until the pH of the phenol is approximately 7.9 and the phenol is stored under TE at 4°C and is stable for 1 month} followed by extraction with 1:1 phenol:chloroform (1:25 with Isopropanol), and 1 volume of chloroform. The extractions were carried out by mixing and centrifugation at 6000 rpm for 8 min and removal of the aqueous layer (top). The remaining organic solvents were removed with

water-saturated ether followed by careful evaporation of the ether by blowing nitrogen through the solution.

The plasmid preparation was precipitated using 1/2 volume 7.5M ammonium acetate and 2-3 volumes of cold 95% ultrapure ethanol (Bethesda Research Laboratories). The precipitation was carried out at -70°C for at least 1 hr followed by centrifugation at 12,000 rpm for 30 min. The purity of the plasmid preparation was checked by first measuring the OD at 260nm (concentration is approximated by the following relationships: for double stranded DNA 1 OD at 260nm = 50ug/ml, for single stranded DNA, 1 OD at 260nm = 20ug/ml) (Maniatis et al., 1982). The protein/DNA ratio was estimated by the ratio of OD 260/OD 280nm with a 'pure' preparation of DNA giving a 260/280 ratio of 1.9 to 2.0 (Maniatis et al., 1982). The purity of the preparation was also examined by use of horizontal agarose gel electrophoresis. The samples were applied to a horizontal agarose gel cast in 1X Tris-borate-EDTA buffer (TBE), {0.089M Tris-borate, 0.002M EDTA (pH 7.6-8.0)}. The gel was subjected to a constant voltage (usually 120-150 volts) until the tracking dye {0.25% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose in water} was 0.5mm from the bottom. The gel was inclubated in a solution of 0.1ug/ml EtBr in TBE for 15-30 min followed by analysis usinng a UV translumi-nator. A photographic record was made with a Polaroid system (type 665 film, 4.5 fstop, 1 sec exposure) (Maniatis et al., 1982).

In order to verify the identify of the cloned fragments of PEPCK, a 24 base pair oligonucleotide was synthesized by Biosearch, Inc. (San Rafael, California). The sequence of this oligonucleotide was derived

from the known sequence of the PEPCK flanking region (Wynshaw-Boris et al., 1984) and corresponded to the region from -35 to -58 in the PEPCK flanking region (figure 1). To verify that the correct clone was in pBH 1.2, the plasmid was restricted with Bam HI and Bgl II (standard restriction assay involved addition of 1-5ug of DNA, 1/10 volume of 10X buffer either recommended or supplied with the enzyme, and 1-5 units of restriction enzyme). Incubations were carried out at 37°C and the restriction digest analyzed on a 1% agarose gel (figure 2A) alongside uncut plasmic and a size standard which consisted of oligonucleotides in 123 base pair increments (Bethesda Research Lab). The gel was transferred to nitrocellulose by the method of Southern (Maniatis et al., 1982) and the nitrocellulose probed with the end-labeled 24 bp oligonucleotide {labeling carried out with T4 polynucleotide kinase and gamma ^{32}P labeled ATP in a buffer of 0.1M Tris-Cl (pH 7.6), 1mM $MgCl_2$, 20mM 2-mercaptoethanol at 37°C for 1 hr (Woods, 1984)}. Hybridizations were carried out at 42°C in 6X SSC, 1X Denhart's, 0.5% SDS, 100ug/ml sheared, denatured salmon sperm DNA (Sigma), and 0.05% sodium pyophosphate overnight. The hybridization was washed in 6X SSC and 0.05% sodium pyophosphate at 37°C, followed by a 10 min wash at 47°C (Woods, 1984).

Figure 2B demonstrates hybridization analysis of Bgl II and Bam HI digestion of pBH 1.2. The 24mer probe hybridized to the uncut pBH 1.2 (A), the linearized plasmid (B), and to the 621 bp insert (D). The probe did not hybridize to vector (D) or the 123 bp ladder (C).

5'AGGGGCAGGGCTGTCCTCCCTTCT 3'

Synthetic 24mer

Figure 1. Sequence of synthetic 24mer.

Oligonucleotide prepared by Biosearch (San Rafael, CA) to correspond to the region from -35 to -58 within the PEPCK gene 5' flanking region.

Figure 2. Analysis of plasmid pBH 1.2.

A: 1% agarose gel analysis of plasmid pBH 1.2 demonstrating the uncut plasmid (A), linearized plasmid digested with Bgl II (B), 123 base pair ladder (C), and a Bgl II-Bam HI digest of the plasmid yielding a 621 bp insert (D).

B: Autoradiogram of the above agarose gel after Southern transfer to nitrocellulose and hybridization to end-labeled 24mer.

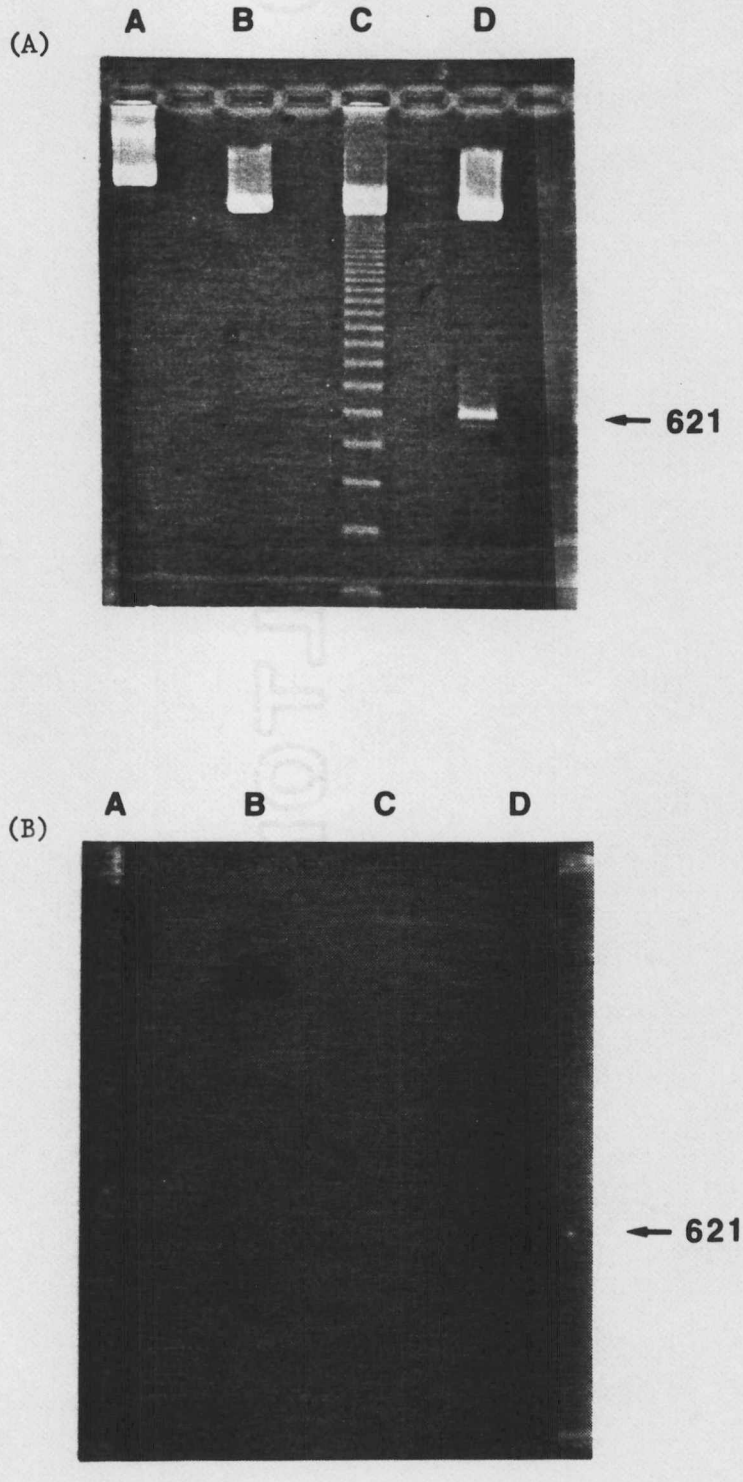


Figure 2

Preparation of Subfragments of pBH 1.2

The plasmid pBH1.2 and subfragments of the plasmid were utilized in the nitrocellulose filter binding assay (see below). To prepare the subfragments of pBH 1.2, the plasmid was digested with restriction enzymes and the fragments isolated according to the scheme outlined in figure 3. After the necessary restriction digest, the sample was loaded on a horizontal agarose gel and subjected to electrophoresis as described above. Upon visualization of the bands with EtBr, the bands were removed with a razor blade and soaked in electroelution running buffer {20mM Tris-Cl (pH 8.0), 0.2mM EDTA, 5mM NaCl}. The fragments were electroeluted from the slice of agarose in the above buffer in an electroelution device (IBI). This device used a "v" shaped tube into which was placed 75ul of a high salt buffer, either 3M or 7.5M ammonium acetate in water with 0.01% bromophenol blue as a detection dye. A 7.5M salt cushion gave a better recovery of fragments less than 500bp in size. After electroelution, usually 30-60 min at 60 volts, depending upon the size of the fragment, the solution in the "v" tube, approximately 410 ul, was removed and mixed with 2.5 volumes of cold ethanol to precipitate the DNA. Prior to precipitation, the DNA concentration was approximated by measuring the OD at 260nm of the solution from the "v" tube against a blank solution from a "v" tube lacking DNA.

The electroelution procedure usually gave a recovery of 70-80% as approximated by measuring the OD at 260nm prior to ethanol precipitation. After precipitation, the concentration of DNA present was always estimated to be 80% of that prior to precipitation. The purified

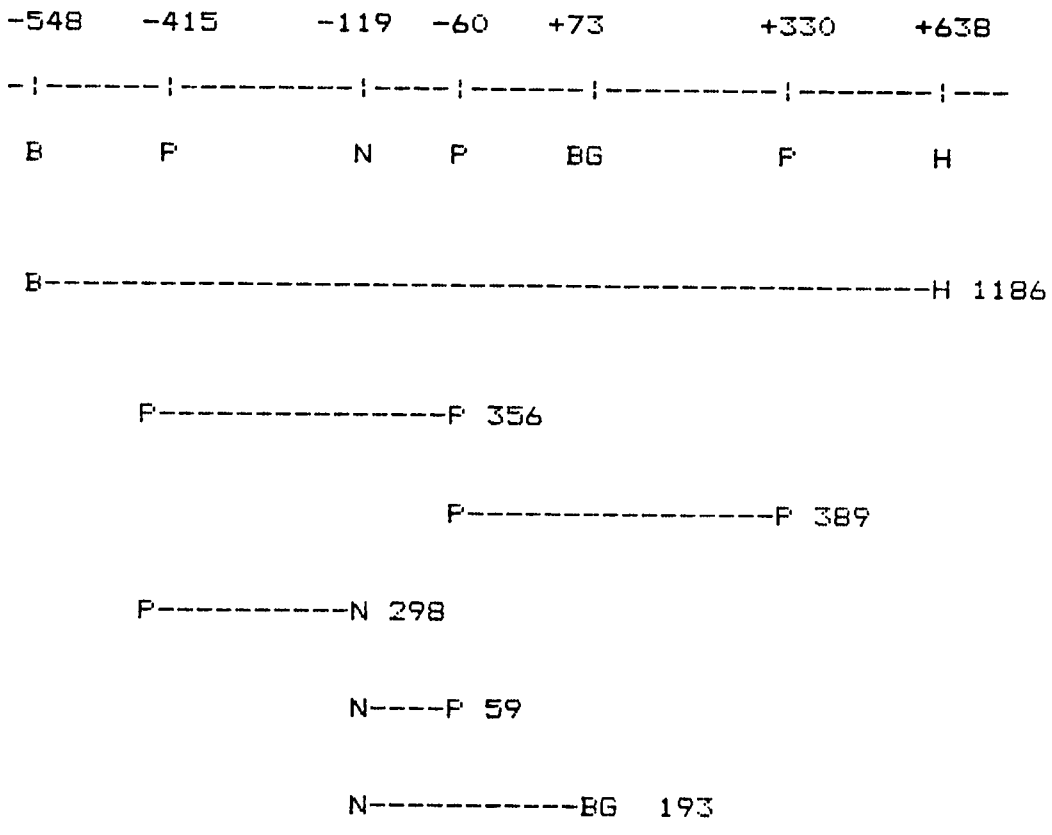


Figure 3. Preparation of subfragments of pBH 1.2.

The plasmid pBH1.2 was digested with the restriction enzymes: Pvu II (P), Bam HI (B), Nco I (N), and Hind III (BG). The Hind III site was derived from a plasmid constructed that contained the Bam HI/Bgl II insert (-548 to +73) in Hind III sites. Each fragment was isolated from agarose gel after digestion.

fragments were end-labeled by the exchange reaction with T4 polynucleotide kinase (Woods, 1984) as described above. The specific activity of the end-labeled DNA usually ranged from 5×10^5 to 5×10^6 cpm/ug DNA. The end-labeled fragment was suspended in the binding buffer used for the assay at a concentration of 2ng/ul, stored at -20°C, and used within 4 days of labeling.

Preparation of 50mer

In order to further investigate the binding of the cAMP-treated extract to the region from -119 to -58, a synthetic 50mer was designed which was complementary to the region from -111 to -71 in the PEPCK flanking region. This 50mer also was designed with Sal I and Ava II ends to allow ligation into multimers for cloning purposes, figure 4.

The 50mer was prepared in a Biosearch SAMI automated synthesis device which used the phosphoramidite method (Beaucage and Caruthers, 1981). Each strand was synthesized separately and its size was analyzed by agarose gel electrophoresis using Nusieve agarose, which allowed for separation between 10 and 200 base pairs. Figure 5 shows that the 50mers were larger than a previously analyzed 24mer. For further purification, each strand was loaded onto a 20% polyacrylamide, 7M urea gel and the gel run until the bromophenol blue dye was at the bottom. The largest size oligomer was detected by fluorescent shadow casting (Vlasuk, 1984) and the DNA extracted from the appropriate gel slice after grinding the polyacrylamide into small chunks and incubating overnight at 37°C in distilled water. The polyacrylamide was removed by centrifugation and the oligonucleotides were lyophilized.

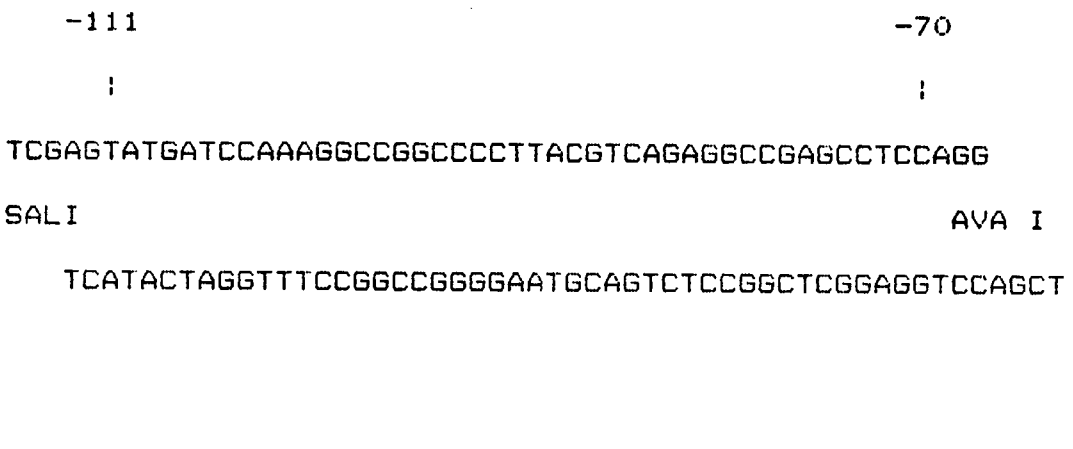


Figure 4. Sequence of synthetic 50mer.

The sequence of the two strands of the 50mer, corresponding to the region from -111 to -70 in the PEPCK flanking region. The 50mer has Sal I and Ava I ends.

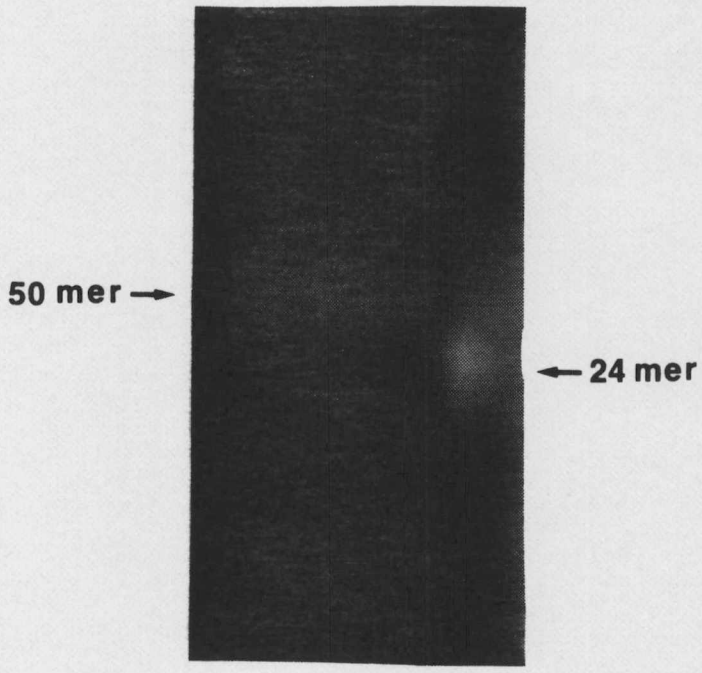


Figure 5. Sizing of 50 base pair oligonucleotides.

Each strand of the synthetic 50mer was analyzed on a 4% Nusieve agarose gel and stained with ethidium bromide. The 24 base pair oligonucleotide examined previously was also run on the gel to determine if the 50mers were larger than the 24mer.

The oligonucleotides were mixed together at 90°C for 2-3 hours and then allowed to cool gradually for annealing. After annealing, the individual strands and the annealed mixture were analyzed by end-labeling with polynucleotide kinase and ^{32}P-γ-ATP (Woods, 1985). The extent of annealing was analyzed on a 20% polyacrylamide gel (figure 6) and demonstrated that the annealed 50mer migrated slower than the two individual 50mers with just slight contamination of unannealed strands. This analysis does not describe the extent of annealing since the amount of labeled, annealed 50mer applied was significantly less than the individual strands.

Validation that the 50mer had the correct sequence was obtained by Maxam-Gilbert chemical sequencing reactions (Maxam and Gilbert, 1981). Figure 7 demonstrates the sequence of 40 of the 50 bases contained in each strand, which fit with the predicted sequence.

Preparation of Nuclear Extracts

The nuclear extracts which were examined for the presence of specific DNA binding factors were prepared according to the procedure of Bogmeyer et al. (1984). This procedure makes use of 0.35 M NaCl in order to extract proteins from isolated, intact nuclei. The source of the nuclei for extraction was either whole Sprague Dawley rat livers or tissue culture cell lines maintained *in vitro*.

For preparation from whole rat liver, rats were injected 1 hr prior to excision of the liver with either phosphate buffered saline (PBS), control, or PBS containing 1 mg/100 grams of body weight 8-Br-cAMP and

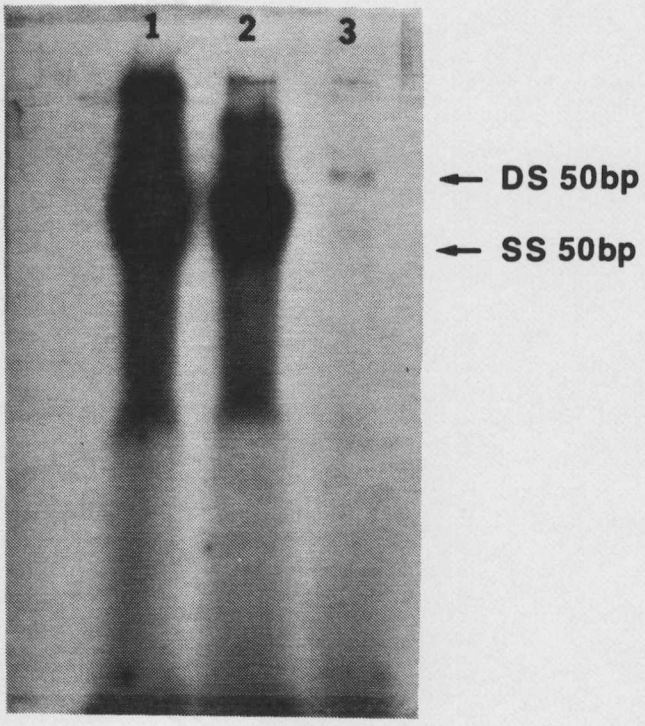


Figure 6. Characterization of annealed 50mer.

Individual synthetic oligomers and annealed 50mer were end-labeled and analyzed on a 20% polyacrylamide gel. Lane 1 and 2 contain the separate strands and land 3 contains the annealed 50mers.

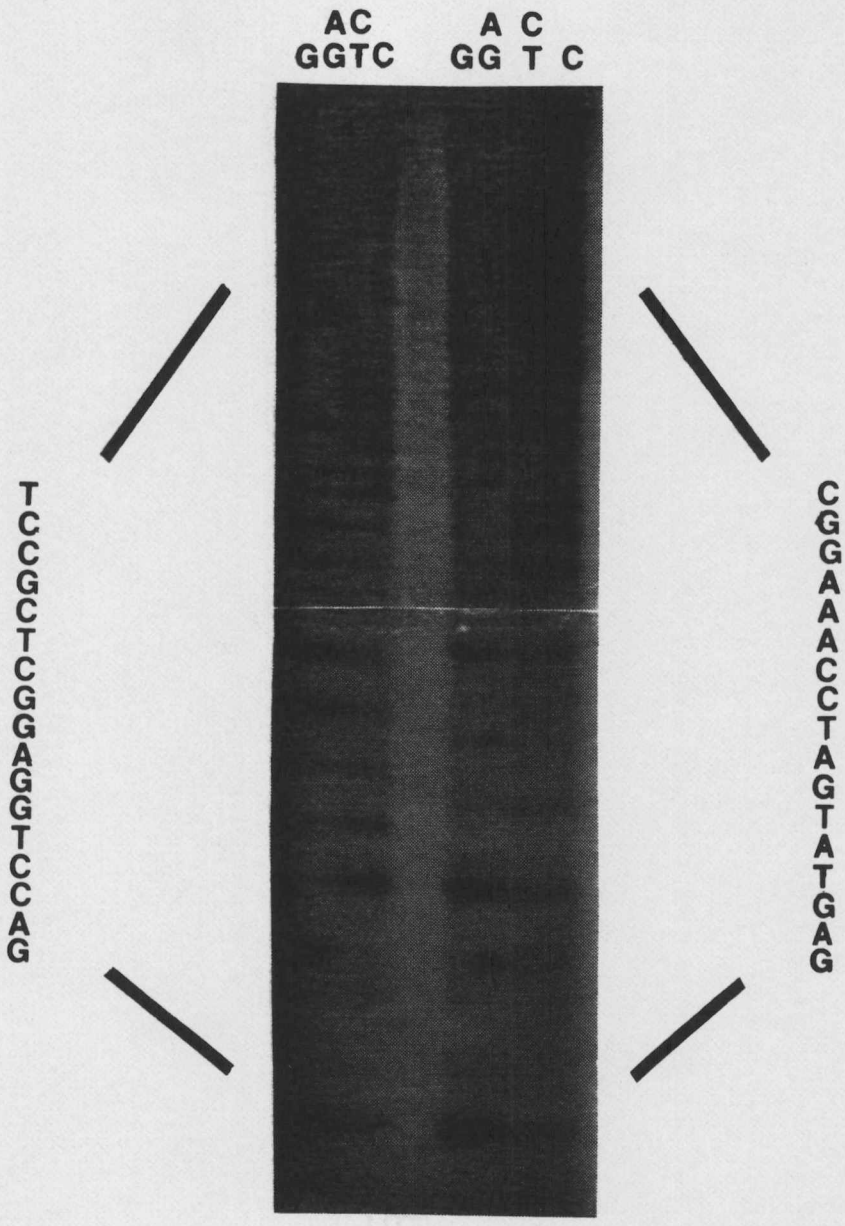


Figure 7. Sequence of each strand of synthetic 50mer.

Each strand was end-labeled with polynucleotide kinase and subjected to the Maxam-Gilbert chemical sequencing reactions (G, A+G, T+C, C) and analyzed by a 20% denaturing polyacrylamide gel.

1mg 3-isobutyl-1-methyl xanthine (MIX), cAMP. When tissue culture cells were used as the source of nuclei, the cells were incubated with cAMP or without (control) with 2mM 8-Br-cAMP in serum free medium (4 ml/100 cm^2 plate) for 1 hr prior to harvesting the cells.

Control and cAMP-treated rat liver were homogenized at 4ml/gram in a Dounce homogenizer in homogenizing buffer, HB {10mM Hepes (pH 7.5), 0.5mM spermidine, 0.15mM spermine, 5mM EDTA, 0.25mM EGTA, 50mM NaF, 7mM 2-mercaptoethanol, 1mM PMSF} and 0.5M sucrose. After centrifugation at 12,000 xg (SS34-Sorval), the cells were lysed in HB containing 0.1% Nonidet P40 (NP-40) and the nuclei pelleted at 12,000 xg for 30 min. This process was repeated and the nuclei were washed twice in HB buffer containing 0.35 M sucrose. The washed nuclei were then preextracted with HB buffer containing 0.05M NaCl and 10% glycerol for 15 min at 4°C by slowly mixing the nuclei on a rotating motor with a clamp to hold the tubes containing the nuclei. After pelleting as above, the nuclei were extracted in HB buffer with 0.3M NaCL and 10% glycerol for 1 hr as described above. The nuclei were pelleted at 12,000 xg, the supernate brought to 45% ammonium sulfate, and the ammonium sulfate dissoolved at 4°C for 30 min. The precipitated proteins were collected at 17,000 xg for 30 min. The protein pellet was redissolved in HB containing 0.35M sucrose and the extracts were aliquoted at -70°C and thawed once prior to use.

When tissue culture cells were used as the source of nuclei, the extraction procedure was the same as above except that the cells were removed from the plate with a rubber policeman and lysed in 0.1% NP-40

in HB without the use of a Dounce homogenizer. The centrifugations were carried out in a microfuge at 17,000 xg and the extract pellet from cultured cells was usually resuspended at 100ul/100 cm^2 plate of cells.

The protein yields from the nuclear extracts, as determined by the Lowry procedure (Lowry et al., 1951), averaged 15 ± 5 pg/nuclei from rat liver and 20 ± 10 pg/nuclei from cultured cells. Polyacrylamide-SDS gel analysis (Laemmli, 1970) of the extracts (figure 8) showed a large number of polypeptides ranging from 15,000 to 100,000 daltons in size. There appeared not to be a qualitative difference between the extracts; however, there may be quantitative differences at a few bands.

DNA Nitrocellulose Filter Binding Assay

The assay chosen to detect possible interaction between a factor in the nuclear extracts and the DNA fragments from the flanking region of the PEPCK gene was the nitrocellulose filter binding assay (Riggs, 1970). This assay utilizes the differential ability of nitrocellulose to retain protein very efficiently but double stranded (ds) DNA is poorly retained (Woodbury, 1983). Thus, if labeled dsDNA fragments are retained on nitrocellulose in the presence of proteins, it must be due to an interaction between the DNA and the proteins.

The assay conditions chosen were modified from Nowock et al. (Nowock and Sippel, 1982) by addition of 5mM EDTA and 50mM NaF as phosphatase inhibitors in the event that the factor(s) is phosphorylated. The assay consisted of mixing end-labeled DNA (1-10ng) with nuclear protein extracts (0-10 ug protein) in a total of 100ul binding buffer

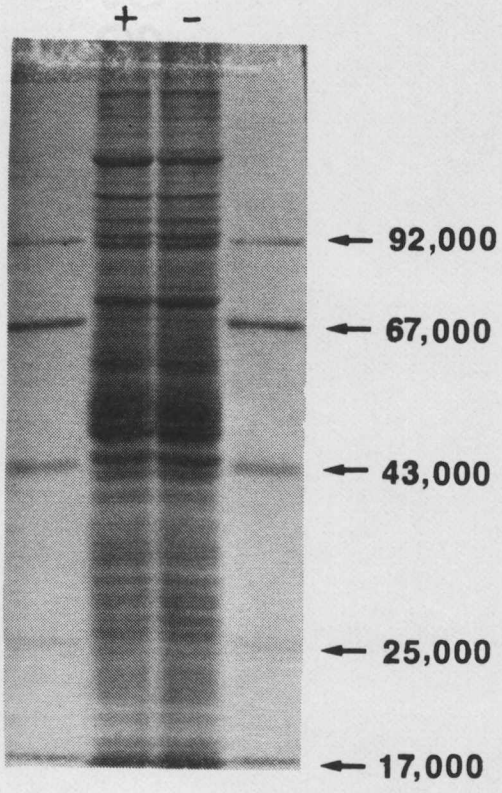


Figure 8. SDS PAGE analysis of liver 0.35M salt nuclear extracts.

Extracts (100ug) prepared from either cAMP-treated (+) or control (-) rat liver nuclei were analyzed on an 8.5% SDS-Polyacrylamide gel.

{10mM Hepes (pH 7.5), 10mM $MgCl_2$, 5mM EDTA, 2mM DTT, 5ug/ml BSA, 50mM NaF, 50mM NaCl, and 10X by weight of linearized, nonspecific DNA (usually pUC-18 plasmid digested with Hind III)}. The mixtures were incubated for 30 min in siliconized 96 well microtiter plates. Siliconizing was performed under vacuum with dichlorodimethyl silane overnight. The plates were washed with deionized water and allowed to dry before use.

The samples were transferred to a 96 well nitrocellulose plate system (Millititer, Millipore) presoaked in binding buffer without BSA and DTT (wash buffer) for at least 1 hr prior to filtration. The nitrocellulose filters were washed with 1.5 to 2 ml of wash buffer and the filters dried under a heat lamp for 1 hr. After drying, each filter was punched from the 96 well plate and placed into 5 ml polypropylene vials and the amount of labeled DNA retained on each filter determined by counting Cerenkov radiation using a Beckman scintillation counter with a full window (0 to 1000).

The conditions such as salt and nonspecific DNA concentration necessary for detection of a differential binding signal between control and cAMP-treated nuclear extracts were determined experimentally (see Results). Each data point from the nitrocellulose filter assay was the average of triplicate determinations (i.e., three filters at the same protein concentration per assay).

Agarose Gel Binding Assay

The agarose gel binding assay utilized was developed by J. Berman (1986). The assay makes use of the observation that a DNA fragment

complexed with protein exhibits retarded migration during polyacrylamide gel electrophoresis (Garner and Revzin, 1981). This method has been used to study protein-DNA complexes in several systems (Hendrickson, 1985). The agarose gel assay exhibits similar retarding of DNA by a protein; however, the use of agarose allows the assay to be performed on a wider range of DNA fragment sizes than is possible with polyacrylamide gel systems.

DNA that was to be used for the agarose gel assay was labeled either by polynucleotide kinase (see above) or by the fill-in reaction of DNA polymerase I Klenow fragment (Maniatis et al., 1982). The DNA (1ug) was reacted with alpha ^{32}P-dCTP along with the proper unlabeled dNTP's as determined by the sequence of the 5' overhang remaining after a 4 or 5 base restriction enzyme digestion, in polymerase reaction buffer {7mM Tris-Cl (pH 7.9), 7mM $MgCl_2$, and 50 mM NaCl, 1mM DTT), for 1 hr at RT. The reaction was stopped by addition of 5mM EDTA and the unincorporated nucleotides removed either by chromatography through a Biogel P-6 (Biorad) column contained in a 3ml syringe using TE buffer containing 0.1M NaCl, or by ethanol precipitation with 7.5M ammonium acetate. The Klenow labeling resulted in a specific activity of approximately 1 x 10^6cpm/ug.

The agarose gel assay consisted of mixing 0-10ng of labeled digested plasmid or fragments, 10X by weight of competitor DNA (usually the parent plasmid devoid of PEPCK inserts), and 0 to 2ug protein extracts in a total volume of 25ul {10mM Hepes (pH 7.5), 50mM NaF, 50mM NaCl, 10mM $MgCl_2$, 5mM EDTA, 2mM DTT, and 12% (v/v) glycerol}. The

reaction was incubated 15-30 min at RT and the reaction volume was applied to wells of an agarose gel prepared in Tris-acetate-EDTA (TAE) buffer, {10mM Tris-acetate (pH 7.9), 2mM EDTA}. The gel was loaded dry and carefully placed into the electrophoresis buffer chamber (TAE) and flooded with buffer. The assay was subjected to electrophoresis for 2 hrs at 90 volts at 4°C with recirculation of the buffer between chambers using a peristalic pump.

The use of tracking dye has been known to interfere with protein-DNA complexes (Berman, 1986); therefore, a single lane at one end of each gel was used with tracking dye to allow determination of when to terminate electrophoresis.

The assay consisted of observing shifts in the migration of specific bands of labeled DNA (see Results). The specificity of the response was determined by the absence of shifts of bands corresponding to nonspecific DNA.

In Vitro Dimethyl Sulfate Protection

Dimethyl sulfate (DMS) is a chemical which methylates nitrogen #7 of the guanine ring and is used in the Maxam-Gilbert chemical DNA sequencing method (Maxam and Gilbert, 1981). Walter Gilbert (Siebenlist and Gilbert, 1980) showed that when protein and DNA interact, any guanine residues which lie within the site of binding of the protein to the DNA will be protected from methylation by DMS. Treatment of the DNA strand methylated by DMS with 1M piperidine results in cleavage of the phosphodiester bond at the base which has been methylated and will

produce a ladder of DNA strands, observed on a denaturing polyacrylamide gel, whose size represents the location of a guanine residue in the DNA.

If a protein interacting with the DNA at a specific site leads to protection from DMS then the result will be the absence or reduction in intensity of that size DNA fragment which corresponds to the protected base.

DNA fragments utilized for DMS protection require that only one strand be labeled with ^{32}P and this was accomplished by specific end-labeling with the large fragment of DNA-Polymerase I (Klenow) as above (Manitias et al., 1982). The labeled DNA was reacted with protein extracts under the same conditions used for the nitrocellulose filter assay (see above). After the 30 min reaction at RT, the reactions were cooled to 0°C on ice for 10 minutes followed by the addition of 1-3ul of DMS and incubation for 4-10 min at 20°C. After the reaction, 25ul of stop solution (1.5M sodium acetate, pH 7.0, 1.0M 2-mercaptoethanol, 100ug/ml tRNA) was added followed by 350ul of cold absolute ethanol (IBI), and incubation in a dry ice-ethanol bath for 10 min. The DNA was precipitated by centrifugation at 17,000 xg for 20 min at 4°C.

The pellet was resuspended in 200ul of 0.3M sodium acetate (pH 7.0) and the proteins were extracted once with phenol and then ether (Maniatias, 1982). The DNA was precipitated by addition of 750ul of cold 95% ethanol (IBI) and precipitation as described above. The precipitated DNA was washed with 1ml of ethanol and dried under vacuum.

The pellet was resuspended in 100ul of freshly diluted 1M piperidine, tightly sealed and incubated at 90°C for 30 min to allow for

strand cleavage. The samples were frozen in dry ice-ethanol and lyophilized. The lyophilized pelleet was resuspended in 10ul of distilled water and lyophilized. After addition of 200ul water, the samples were lyophilized overnight.

The next day the sample was resuspended in a small volume (1-2ul) of loading dye {80% (w/v) formamide, 10mM NaOH, 1mM EDTA, 0.1% (w/v) xylene cyanol and bromphenol blue} and was heated to 90°C for 2 min and 1ul was loaded on a 7M urea-polyacrylamide sequencing gel (Maxam and Gilbert, 1982). A 40cm, 20% gel was used for short (>100bp) fragments and a 40cm or 80cm, 8% gel was used for fragments between 100 and 250 bases in length.

The gels were run at 50°C ± 5°C in TBE buffer, the glass plates separated, and the gel left attached to one of the plates. When an 8% gel was used, the small plate was treated with bind silane and the larger one with repel silane. The separated gel was covered with plastic wrap and subjected to autoradiography using XAR-5 film and in some cases a Dupont intensifier screen at -70°C. The length of exposure was determined empirically.

CHAPTER 3

RESULTS AND DISCUSSION

Nitrocellulose Filter Binding Assay

Parameters. The nitrocellulose filter assay depends upon the interaction between proteins and double stranded DNA for retention of the DNA on tthe filters. In order to demonstrate this principle, cAMP-treated nuclear extracts were reacted with plasmid pBR322 which had been linearized with Hind III and end-labeled with gamma-^{32}P-ATP and polynucleotide kinase. The labeled DNA was reacted with increasing amounts of extract protein and passed through nitrocellulose filters. The pBR322 DNA (10ng) (figure 9) exhibits protein dependent binding within the range of protein concentrations tested (2.3 to 23ug). This demonstrates that the assay allows detection of DNA-protein binding and also shows that the 0.35M nuclear extracts contain many proteins which bind DNA nonspecifically. The detection of differential binding between cAMP and control treated nuclear extracts required reducing this amount of nonspecific DNA binding. This was achieved by using lower concentrations of protein (less than 5ug) and including a 10 fold (by weight) excess of unlabeled, nonspecific DNA. This fragment was utilized to detect differential binding between control and cAMP treated extracts. The initial conditions to detect binding were those used by Sippel et al. (1984), which include 100mM Na+ (combination of 50mM NaF and 50mM NaCl) and 10 fold, by weight, of unlabeled competitor, linearized pUC-18 DNA.

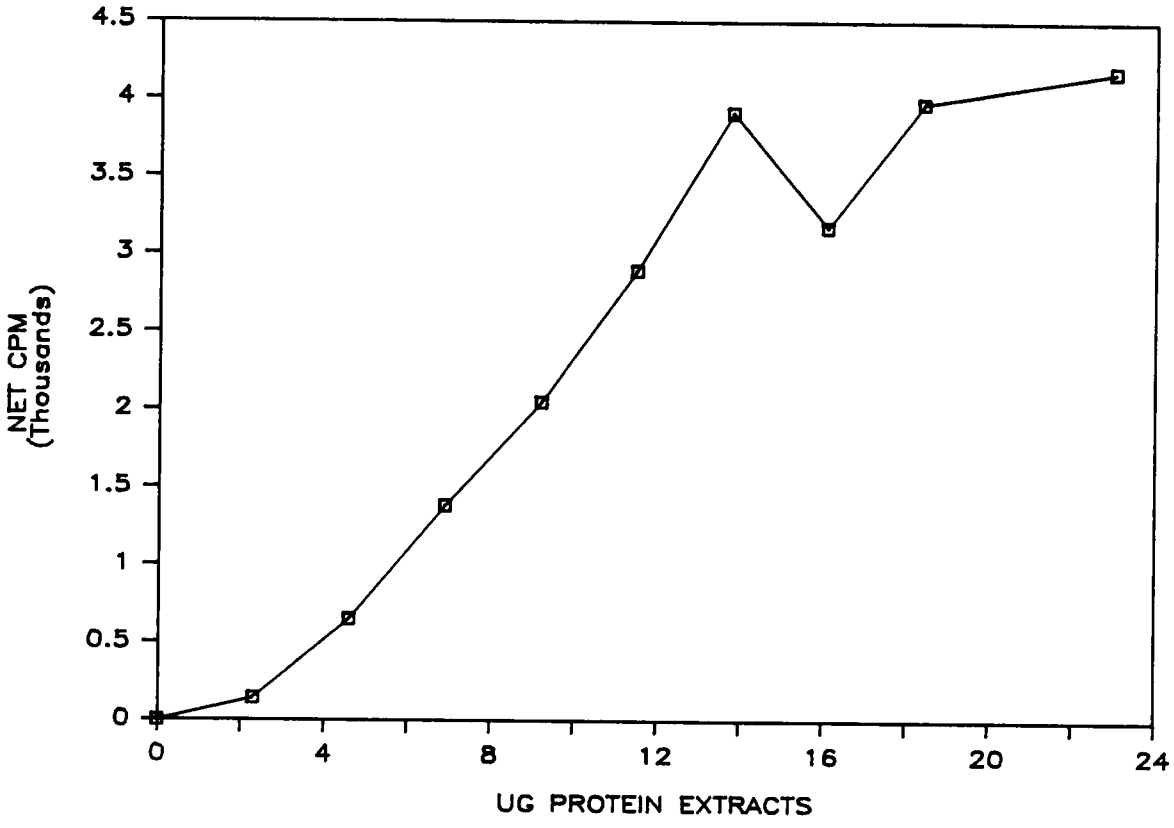


Figure 9. Analysis of filter binding assay.

Plasmid pBR322 was linearized and end-labeled, 10ng; of labeled plasmid was reacted with various protein amounts of cAMP-treated rat liver crude 0.35M salt nuclear extracts and passed over 0.45um nitrocellulose filters and dried and counted. Data is expressed as cpm retained on the filters.

Detection of cAMP-dependent binding component. The plasmid pBH 1.2 was digested with Bam HI and Hind III and the 1.2kb BH fragment (-548 to +638) was isolated by agarose gel and electroelution (figure 10). This fragment was utilized to detect differential binding between control and cAMP treated extracts. The initial conditions to detect binding were those used by Sippel et al. (1984), which include 100mM Na+ (combination of 50mM NaF and 50mM NaCl) and 10 fold, by weight, of unlabeled competitor, linearized pUC-18 DNA.

Figure 11 shows that binding of the 1.2 kb fragment with the cAMP-treated nuclear extracts is higher than that observed with the control extracts up to 0.7ug of protein. Beyond that point the binding exhibited by both control and cAMP-treated extracts becomes similar. This type of protein-dependency curve is to be expected if one assumes that the factor(s) which causes retention of the BH 1.2 fragment has a high affinity for the PEPCK flanking region after cAMP treatment but is also present in control extracts, either in a low affinity form or at a lower concentration. The existence of such specific binding in the control extracts could be due to the fact that rats which are used for the source of the extracts exhibit slightly elevated levels of cAMP due to the stress impressed on the animals in handling.

The ability of this assay to detect a differential DNA binding activity between control and cAMP-treated nuclear extracts depends upon two parameters: the first is the concentration of NaCl present in the reaction mixture. Proteins which bind to DNA exhibit two types of interactions with the DNA molecule: 1) ionic interactions are the

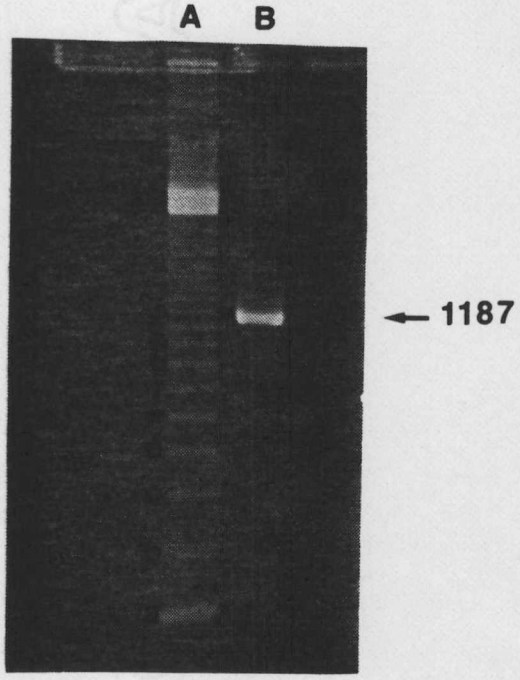


Figure 10. Preparation of 1.2kb BH fragment.

Isolation of the 1.2 kb Bam HI/Hind III fragment from pBH1.2. The plasmid was digested with Bam HI and Hind III and the 1.2 kb fragment was isolated on a 1.5% agarose gel. The 1.2 kb fragment was isolated from the agarose by electroelution and anallyzed on a 1.5% agarose gel. Lane A: 123 bp ladder, lane B: 1.2 kb fragment from pBH 1.2.

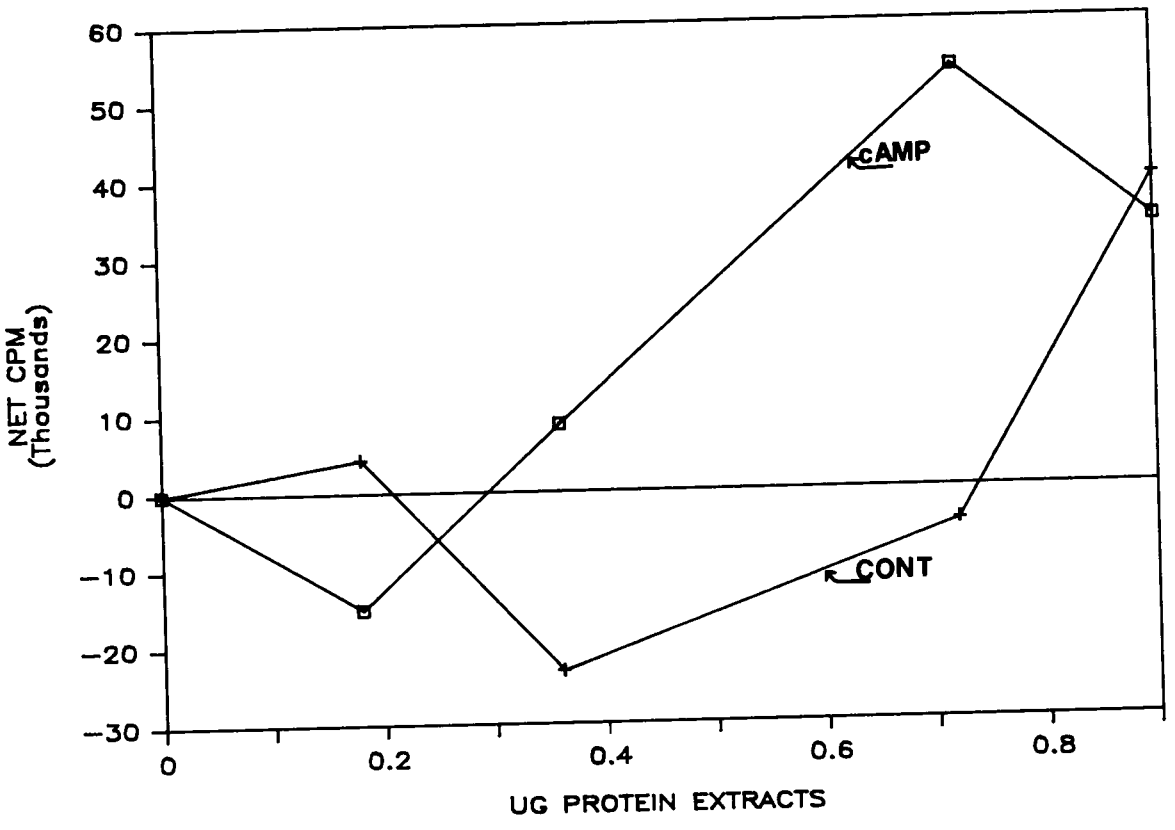


Figure 11. Net binding of cAMP-treated extracts to BH 1.2.

Labeled BH 1.2 was reacted with various protein amounts of cAMP-treated extracts in the presence of 100ng competitor DNA and 100mM Na+. Data is expressed as net cpm, the background (15% of input) has been subtracted.

primary result of interactions between the negatively charged phosphates on the DNA backbone and positively charged residues on the proteins, and 2) specific hydrogen bonding between the bases in the major and minor groove of the helical molecule and amino acids in the proteins (Von Hippel, 1985). By varying salt concentrations, interactions which arise due to ionic interactions will be decreased, thus increasing the opportunity to detect interactions with specific base pairs through hydrogen bonding.

A second variable that was found to be required for detection of a differential binding signal was the presence of nonspecific DNA in the reaction mixture. This was found to decrease nonspecific binding presumably due to binding of the bulk of DNA binding proteins which interact with DNA primarily by ionic means.

Salt dependency. To test whether the higher binding observed with the cAMP-treated extracts is sensitive to salt, 0.72ug of each extract was reacted with 10ng of labeled BH 1.2 and 100ng pBR322 at various salt concentrations between 200 and 200 mM.

Figure 12 shows that the net binding of the cAMP extracts (binding in the control extracts is subtracted) decreases as the salt concentration during the assay is increased. This decrease in binding is expected if the factor(s) behaves in a similar way to other DNA-binding proteins, where the addition of 100mM Na+ suppresses the nonspecific interactions; however, 200mM will also interfere with the ionic part of the specific interaction (von Hippel, et al., 1984).

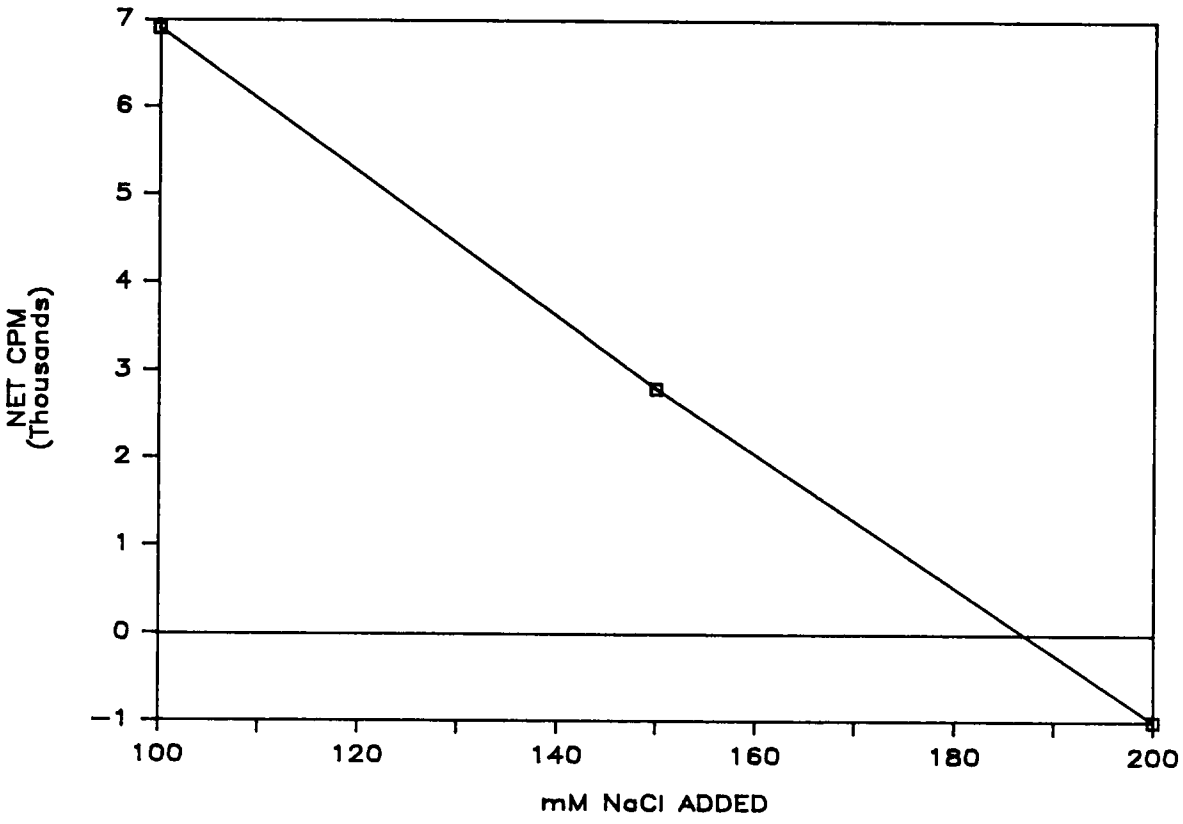


Figure 12. Salt dependency with cAMP-treated extracts.

Labeled BH 1.2 was reacted with 0.72ug of cAMP-treated extracts in the presence of 100ng competitor and various concentrations of NaCl. Data is expressed as net cpm of cAMP-treated extracts, binding to the control extracts has been subtracted.

Another test of the specificity of this binding event would be to investigate the dependency on the amount of nonspecific DNA required to detect the differential binding. Since the crude nuclear extracts would contain several DNA binding components, it is necessary to include competitor DNA in order to bind those proteins and allow detection of interaction of the specific factor(s) with the labeled DNA.

Unlabeled, nonspecific competitor dependency. Labeled BH 1.2 was reacted at 10ng with 0.72ug protein of both control and cAMP-treated extracts in the presence of 100mM Na+ and various amounts of unlabeled pBR322 DNA.

The net binding observed with the cAMP extracts when tested with various amounts of nonspecific pBR322 is demonstrated by figure 13. These results show that there are two regions of large differential between the control and cAMP-treated extracts: at 40ng competitor ($p > 0.005$) and at 100ng ($p > 0.005$). It was decided to use the most restrictive conditions, 100ng, to test for the presence of any specific factor. These conditions are narrow due to the complexity of the rat liver nuclear extracts.

Competition binding to BH 1.2. To test the specificity of the interaction detected between the 1.2 kb PEPCK fragment and the cAMP extracts, competition binding was performed with labeled 1.2 kb fragment in the presence of 0.72ug of cAMP-treated liver nuclear extracts together with increasing amounts of unlabeled competitor DNA: either additional linearized pUC-18 or unlabeled BH 1.2. Figure 14 demonstrates

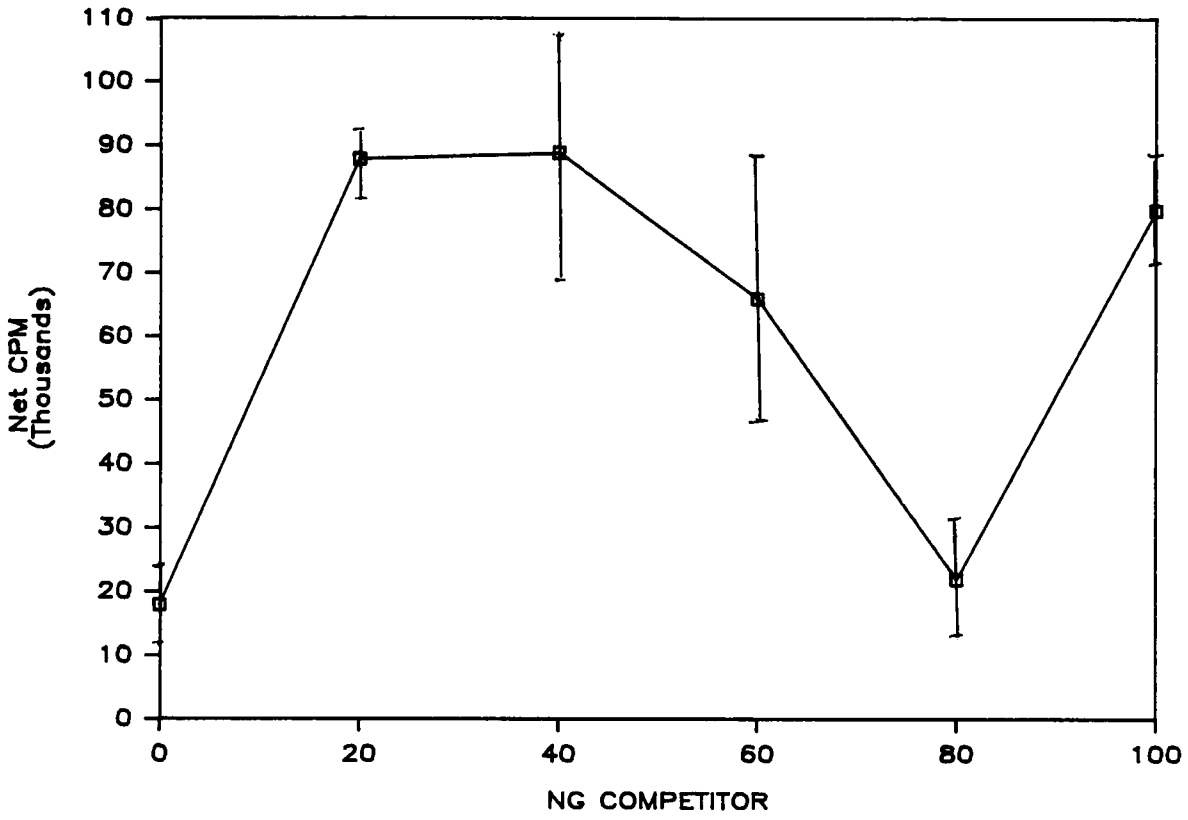


Figure 13. Competitor DNA dependency binding to BH 1.2.

Isolated BH 1.2 was end-labeled and reacted with 0.72ug of both control and cAMP-treated rat liver nuclear extracts in the presence of various amounts of unlabeled, linearized pUC-18. The results are shown as the net CPM between the control and cAMP-treated extract binding. The background of the assay was actually higher than the binding observed with control extracts.

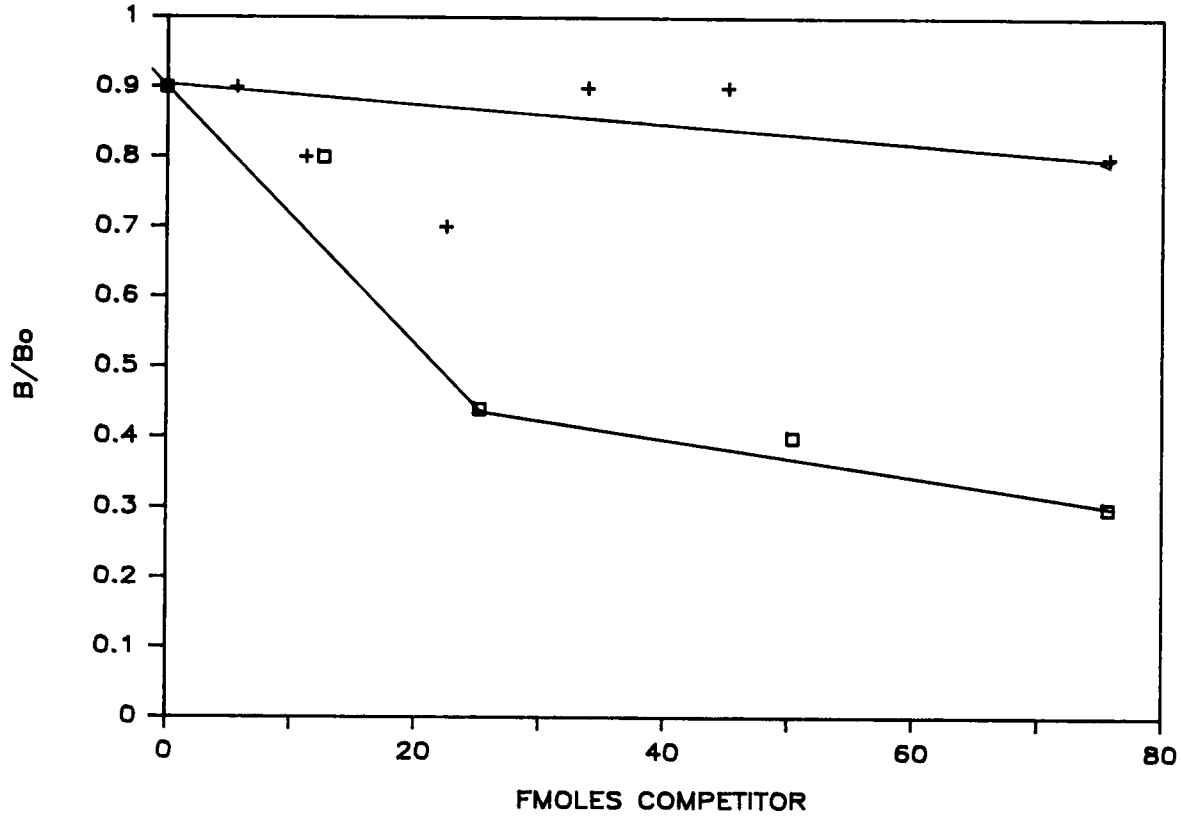


Figure 14. Competition binding to BH 1.2.

Labeled BH 1.2 was reacted with 0.72ug of cAMP-treated liver nuclear extracts with 200ng linearized pUC-18 and 100mM Na+, and additional amounts of either unlabeled BH 1.2 ([]) or linearized pUC-18 (+). The binding is expressed as a ratio of binding without added competitor (Bo, 10%) to binding in the presence of competitor (B). The background averaged 10%.

the results of this competition where the binding observed in the presence of additional competitor is expressed as a ratio of binding with competitor (B) to binding without added competitor (Bo), where B/Bo is 1 without additional competitor. The unlabeled, specific BH 1.2 fragment was able to compete with labeled BH 1.2; however, additional pUC-18 (initial amount in the assay is 100ng, 56 fmoles) up to 76 fmoles (a total of 132 fmoles) was unable to compete for binding to the 1.2 kb fragment exhibited by the cAMP-treated extracts. This supports the conclusion that the observed binding of the 1.2 kb fragment is specific.

Table II demonstrates the consistency of the binding observed to the 1.2 kb BH fragment. Each experiment was performed with different 0.35M NaCl extracts of stored nuclei (frozen in liquid Nitrogen) from the same preparation of rat liver nuclei. These data demonstrate that the observed binding is independent of the extraction procedure and that the binding component is stable in frozen nuclei, at least for a short time.

Binding was carried out with 10ng labeled BH 1.2 and 100mM Na+ in the presence of 100ng competitor DNA. Each point represents the average of triplicate determinations.

Defining the Site of Binding Within the 1.2kb PEPCK Fragment

Pvu II digest of 1.2kb fragment. In order to further define the site of binding within this 1.2 kb fragment, restriction digests with Pvu II were used to produce two fragments, -415 to -60 and -59 to +330. These two fragments were isolated by electroelution and the sizes

Table II

Binding of 1.2kb BH Fragment to Liver Nucler Extracts

Experiment	cpm x 1000 +cAMP	Control	Difference
1	15.3 ± 1.2	10.6 ± 0.8	4.7
2	15.3 ± 1.1	11.0 ± 0.9	3.6
3	19.7 ± 1.3	11.6 ± 1.0	8.1

confirmed by agarose gel (figure 15). Each isolated fragment was tested with the rat liver nuclear extracts in order to define the region where the cAMP-dependent binding occurs.

Figure 16 depicts the results of testing several protein concentrations of the nuclear extracts with the isolated Pvu II fragments. Panel A shows that the region from -415 to -60 exhibits differential binding with the cAMP-treated extracts whereas panel B shows that the region from -59 to +330 exhibits similar binding in both control and cAMP-treated extracts. This result can be explained by the fact that the region from -59 to +330 contains the promoter sequences which recognize the promoter binding factors as well as RNA Polymerase II (von Hippel et al., 1984). The presence of these proteins does not depend upon the hormonal state of the cells, therefore they would be expected to be contained in both control and cAMP-treated extracts.

Based upon these experiments it seems reasonable to suggest that the region of interaction between the cAMP-treated extracts and the

Figure 15. Preparation of Pvu II fragments.

Isolatioon of the Pvu II fragments from pBH1.2. The plasmid was digested with Pvu II and the fragments corresponding to 356 and 390 bp were separated by 1.5% agarose gel. The individual fragments were isolated from the gel by electroelution and analyzed on a 1.5% gel. Lane A: 123 bp ladder (a-123bp, b-246bp, c-369bp, and d-492bp); B: 356 bp; C: 390 bp fragment.

Figure 16. Protein dependent binding to Pvu II fragments.

A: Binding of protein extracts from control and cAMP-treated rat liver nuclei to labeled 356bp fragment (-415 to -60) in the presence of 100ng linearized pUC-18 and 100mM Na+. Background was 12%.

B: Binding of protein extracts from control and cAMP-treated rat liver nuclei to labeled 390bp fragment (-59 to +330) in the presence of 100ng linearized pUC-28 and 100mM Na+. Background was 15%.

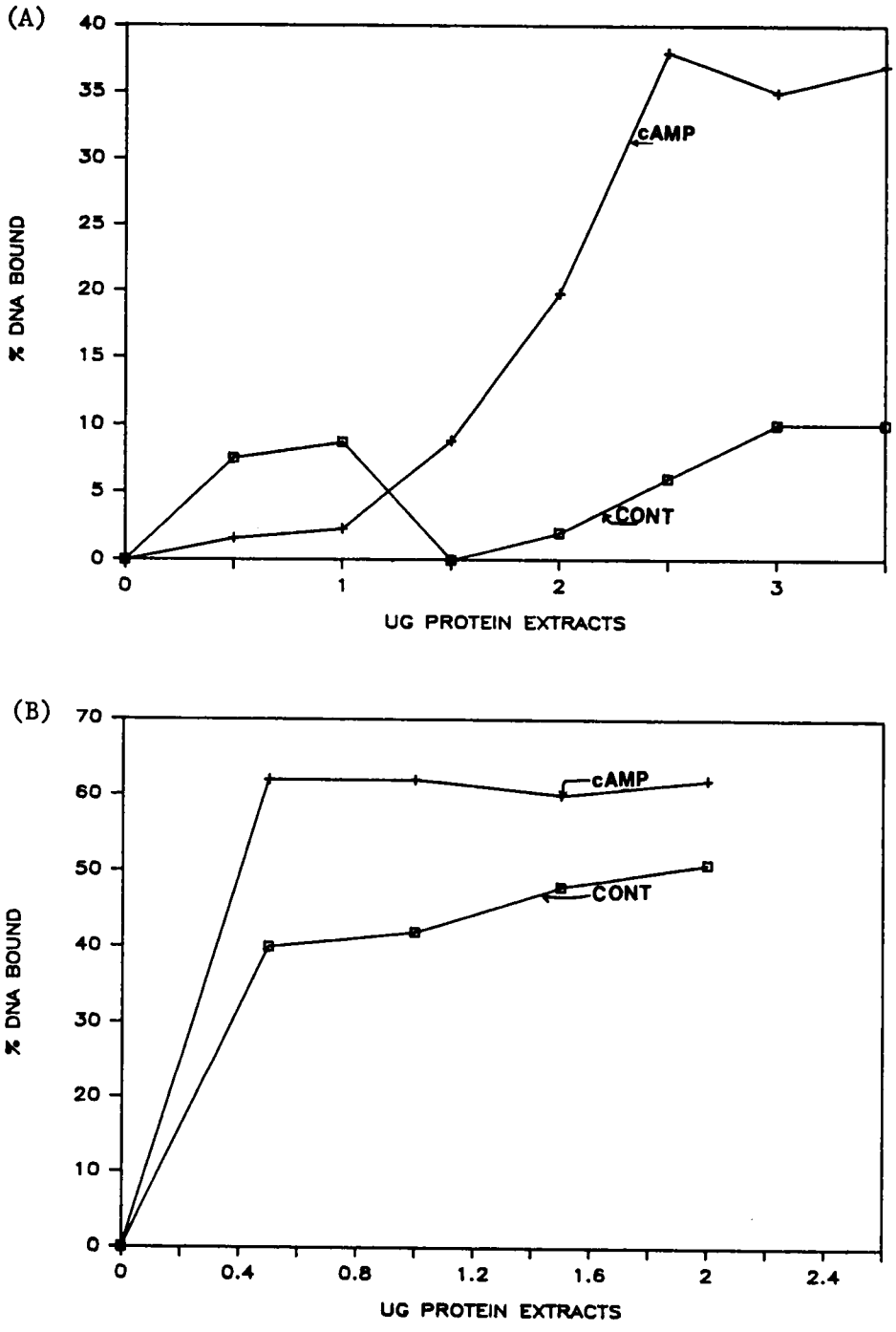

(A)
40
35
30
25
20
15
10
5
0
% DNA BOUND
cAMP
CONT
0
1
2
3
UG PROTEIN EXTRACTS
(B)
70
60
50
40
30
20
10
0
% DNA BOUND
cAMP
CONT
0
0.4
0.8
1.2
1.6
2
2.4
UG PROTEIN EXTRACTS

Figure 16

PEPCK flanking region is within the sequences from -415 to -60 (356mer). Figure 17 shows a complete protein dependency curve for binding to the 356bp fragment. This protein dependency curve demonstrates that the binding is saturable between 2 and 3ug of protein. From these data it also becomes apparent that the differential binding occurs at low (1-4ug) protein and at higher protein amounts, the binding increases in both cAMP-treated and control extracts. This would suggest that the control extracts either contain the factor(s) in a low affinity form or the amount of the factor(s) is low in the control extracts (see section on binding to 1.2kb).

This figure also demonstrates that at low protein inputs, less than lug, the signal to noise ratio is high in the assay, producing the higher noise apparent at low protein concentrations.

The question of biological replication was addressed using the 356mer and testing extracts prepared from several sets of rat livers and rat hepatoma cells.

Table III shows the results of testing nuclear extracts from three different sets of cAMP-treated and control rat livers as well as from three different sets of control and cAMP-treated H-35 rat hepatoma cells (100mm confluent culture) with the 356mer. As these data demonstrate, the differential binding observed in the cAMP-treated rat livers is reproducible across several biological replicates. This table also implies that the factor(s) is present in a rat hepatoma derived cell line (H-35) and that the control binding in these cells is lower than in the rat livers. This lower control binding could originate from the

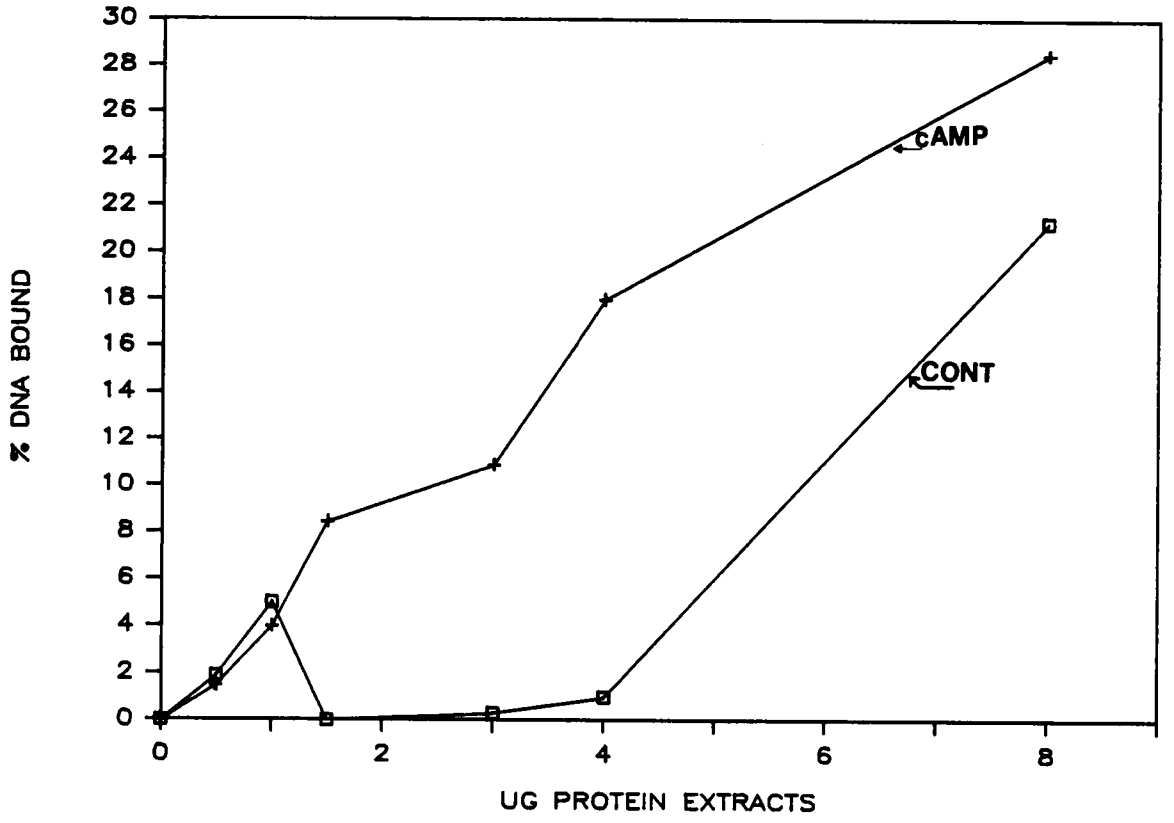


Figure 17. Protein dependent binding to 356mer.

Binding of protein extracts from control and cAMP-treated rat liver nuclei to labeled 356bp fragment (-415 to -60) in the presence of 100ng linearized pUC-18 and 100mM Na+. Background was 12%.

Table III

Differential Binding of 356mer to Extracts
from Rat Liver and Hepatoma Cells

Experiment No.	cAMP	% DNA Bound to Nuclear Extracts	
		Rat Liver	H-35 Cells
1	-	5	0
	+	35	14
		30	14
2	-	10	0
	+	35	14
		25	14
3	-	1	7
	+	19	18
		18	11
Average Net %:		24	13

fact that the cells are deprived of serum (i.e., growth factors) during the 1 hr incubation and therefore the cAMP level would be expected to be low. This absence of control binding with serum-starved cells suggests that the binding observed in the rat liver control nuclear extracts is due to a low level of the activated factor and not necessarily just binding of a low affinity form. End labeled 356mer (-415 to -60), 10ng, was reacted with 100ng linearized pUC-18 in 100mM Na+ and either 1.5ug (H-35 cells) or 3ug (liver) of both control (-) and cAMP-treated (+) nuclear extracts. The background averaged 10% of the input radioactivity. Each value represents an average of triplicate determinations with a SD of 10%.

Binding to NcoI/Pvu II fragment. The next question was to define more precisely the region of binding within the 356bp fragment. This fragment contains a Nco I site at -119, therefore it was decided to first label the 356mer with polynucleotide kinase and then to digest with Nco I and isolate the two fragments on an agarose gel (figure 18) followed by electroelution. These two fragments were used to test the binding to the rat liver nuclear extracts. This method of preparation allowed detection of binding; however, the amount of each fragment could not be estimated correctly due to the preparation procedure. It is surmised that the amount of labeled DNA input into each reaction was between 2-5 ng.

The results of testing these two fragments are shown in table IV. The fragment including the region from -119 to -61, exhibited

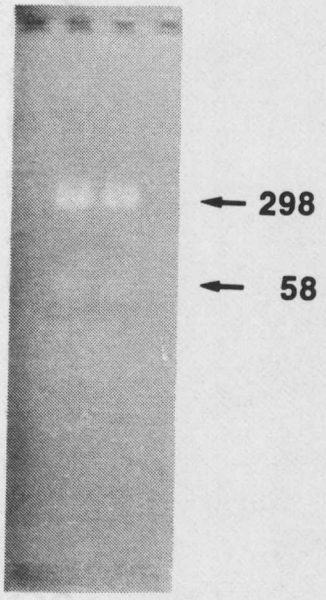


Figure 18. Digestion of Pvu II 356mer with Nco I.

The isolated Pvu II 356mer bp fragment was end-labeled with polynucleotide kinase, digested with NcoI, and the digestion separated by 2% agarose gel. Fragment 1 is the 298 bp (-415 to -118) and fragment 2 is the 59 bp (-119 to -61). Each fragment was isolated by electroelution and used in the filter binding assay.

Table IV

Net CPM Bound by Nco I Digestion Fragments of 356mer

	% Bound			
	209mer		598mer	
ug Protein	Cont	cAMP	Cont	cAMP
1	0	0	10	60
2	0	0	15	80

significant cAMP dependent binding whereas the fragment from -415 to -120 revealed no binding to either control or cAMP-treated nuclear extracts under these conditions.

These results suggest that the site of binding of the cAMP-treated extracts is within the 58bp fragment from -119 to -61. This fragment lies just 5' of the known promoter region which contains the CAAT and TATAA boxes (Wynshaw-Boris et al., 1984). In fact, this region of binding was found prior to Hanson reporting that the cAMP regulatory element (cAMP) is between -109 and -61 (Wynshaw-Boris et al., 1986).

It was found to be difficult to obtain usable amounts of the 58bp fragment from pBB0.6 (plasmid used to obtain 356mer, this plasmid contains the PEPCK flanking region from -548 to +73), since it represents only 1.3% in size of pBB0.6. Therefore it was decided to have a synthetic fragment generated to assay the binding detected in this region.

Binding was performed with labeled eluted fragments in the presence of 100mM Na+ and 100ng competitor DNA. Each percent bound represents triplicate determinations. SD varied by 10%.

Synthetic 50mer

Binding to the synthetic 50mer. A synthetic 50 base pair oligonucleotide, complementary to the region from -111 to -71 was synthesized, purified, and labeled as outlined in Methods. The extent of binding of both control and cAMP-treated rat liver crude nuclear extracts to the synthetic 50mer was tested using the nitrocellulose binding assay.

The 50mer was end-labeled with polynucleotide kinase and then reacted with 0.025 to 1ug protein extract in the presence of 100ng linearized pUC-18 DNA and 100mM Na+. Figure 19 demonstrates the percent of input 50mer bound at each protein concentration tested with both control and cAMP-treated extracts. These data suggest that binding to the 50mer saturates between 0.5 and 0.6ug of protein extracts. This saturation is in contrast to that observed with the 356mer (figure 17) where protein saturation occurred between 1.5 and 2ug. This difference in saturation between the two fragments can be explained by the apparent dependence that the size of the fragment has on complex retention during filtration onto nitrocellulose (see Conclusion).

Analysis of the specificity of binding to the 50mer of 1.0ug of the cAMP-treated extracts was performed by way of a competition assay. Binding to the 50mer was tested in the presence of 0.5 to 25 fmoles of

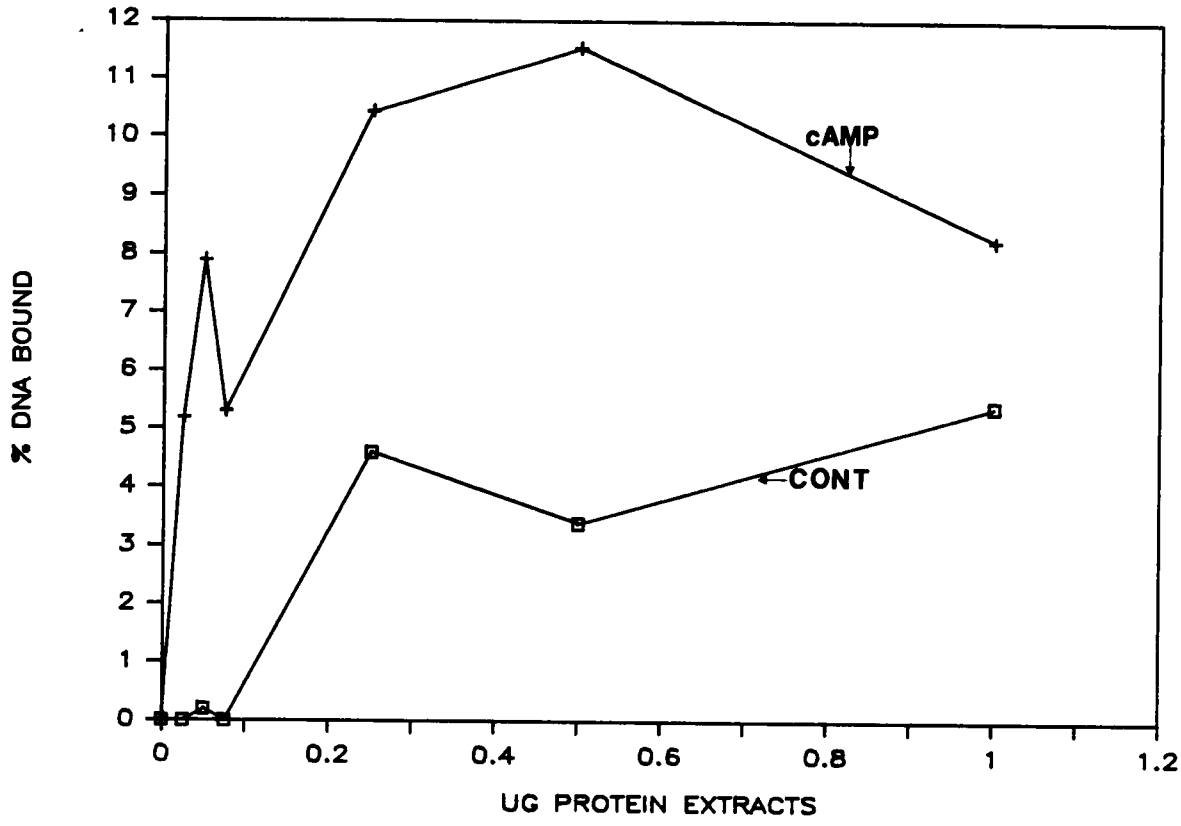


Figure 19. Protein dependent binding to 50mer.

Labeled 50mer was reacted with protein extracts from control and cAMP-treated nuclear extracts in the presence of 100ng pUC-18 DNA and 100mM Na+. Background was 15% of input. The triplicate determinations varied by 10%.

various fragments of DNA. The fragments used were the following: a 322bp fragment isolated from pUC-18 by Pvu II digestion, a 298 bp fragment isolated from pBB0.6 which includes the region from -59 to +73 of the PEPCK genomic clone along with 166 base pairs of the prokaryotic gene for chloroamphenicol acetyltransferase (plasmid pBB0.6), the 356bp Pvu II fragment containing the region from -415 to -60, and a 193mer containing the region from -119 to +73.

Figure 20 demonstrates the results of binding to the labeled 50mer in the presence of several fragments of DNA. Competition with unlabeled 50mer demonstrates that the concentration required to reduce the binding by 50% is approximately 1.0 fmoles (which corresponds to a rough affinity constant of $1 \times 10^{12}\ M^{-1}$) and the 356mer and 193mer also appear to have the same affinity. Both of these fragments contain the region from -111 to -71 of the PEPCK gene.

The competition by the 298mer (containing sequences -59 to +73 of PEPCK) suggests that the cAMP-dependent factor may have some interaction with the known promoter region of the PEPCK gene. This competition occurs at 5 fmoles ($2 \times 10^{11}\ M^{-1}$), 5-fold more 298mer than 50mer required to reduce binding 50%. This result would suggest that if there are protein-protein interactions, the affinity difference between the binding to the cAMP region and promoter factors would be 5 fold. This interaction may be due to an interaction with other factors which have been shown to interact with the promoter region.

No significant competition with the 322mer suggests that the interaction detected in the cAMP-treated extracts is specific for the region from -111 to -71 of the PEPCK flanking region.

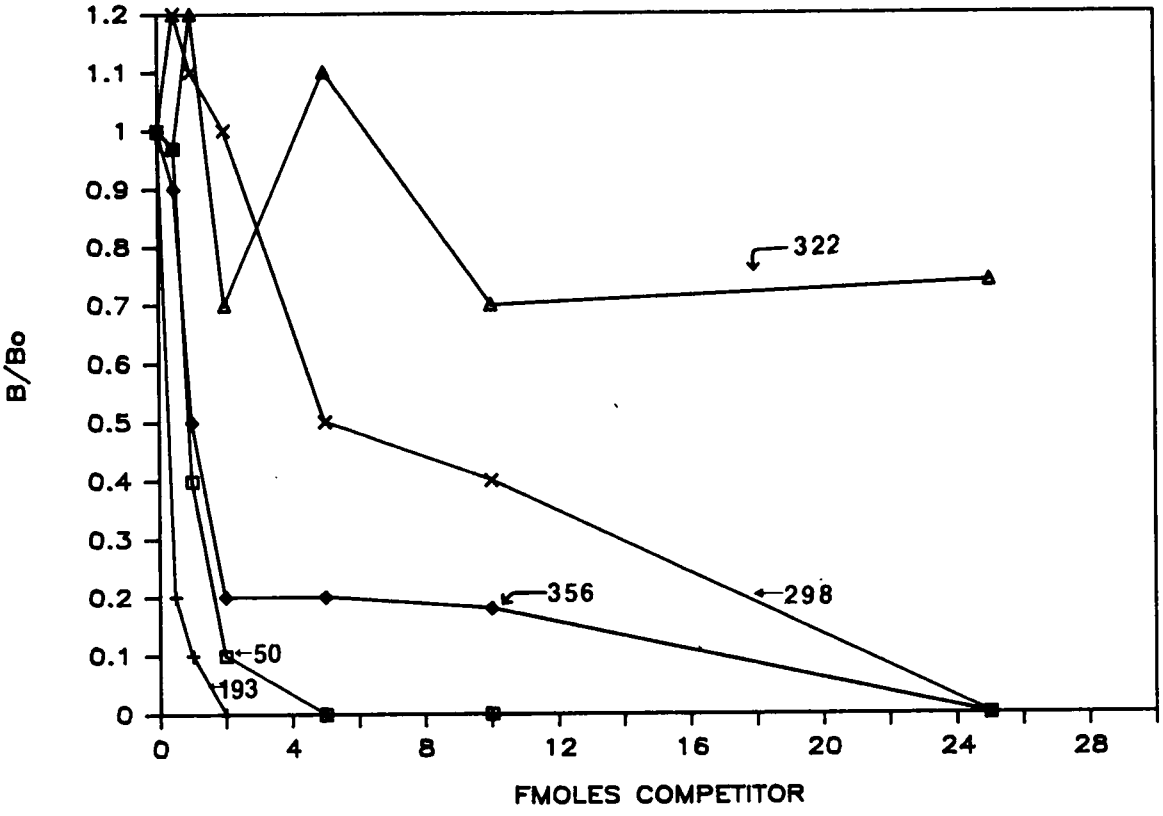


Figure 20. Competition binding to 50mer.

Labeled 50mer was reacted with 1.0ug of protein extracts from cAMP-treated rat liver nuclei in the presence of 100ng pUC-18 DNA and 100mM Na+. Binding to 50mer was performed in the presence of various fmoles of cold competitors (B): 322bp fragment from pUC-18; 298bp fragment from BB0.6; 356bp pvu II fragment from BH1.2; and unlabeled 50mer. Background binding was 12% and 26 fmoles of the 50mer was bound in the absence of added competitor (Bo).

The agarose gel shift assay was used with the 50mer in an attempt to confirm the presence of the cAMP-dependent DNA binding component. This assay proved difficult to analyze due to variations in the size of the fragment retarded by the cAMP-treated rat liver nuclear extracts (data not shown).

Another method of confirmation of the results from the nitrocellulose assay was utilized, that of dimethyl sulfate protection.

Chemical footprinting by dimethyl sulfate protection was attempted on the synthetic 50mer; however, the results were made difficult by the small size of the DNA (data not shown). These results showed that if the 50mer was labeled at the Ava II end (-111) and then DMS protection performed with both control and cAMP-treated extracts, guanines from -92 to the other end of the strand (-71) showed protection with 3ug of cAMP-treated extracts but required 24ug of control extracts to produce a similar pattern of protection. The fact that over half of the 50mer strand tested revealed protection, therefore raising a question of specificity of the protection, suggested that the 50mer is too small a fragment of DNA for obtaining an accurate footprint.

Therefore, it was decided to analyze a larger fragment of DNA containing the binding region between -111 and -71. The isolated 193 bp fragment (figure 21) used in the competition experiment with the synthetic 50mer (figure 20) that contains 193 bases off the PEPCK 5' flanking region (-119 to +73) was demonstrated to compete binding to the 50mer, therefore it was decided to use this fragment for the DMS protection experiments. Prior to these experiments, protein dependency and

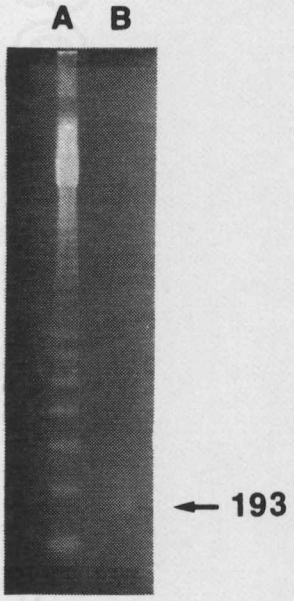


Figure 21. Isolation of 193bp fragment.

The plasmid pBB0.6 containing the 621bp PEPCK flanking region (-548 to +73) was digested with Hind III and Nco I and the fragment isolated by electroelution from 2% agarose. Analysis of the isolated fragment by 2% agarose, where Lane A is the 123 bp ladder and Lane B the isolated 193 bp.

competition was performed to further demonstrate that this fragment contains the specific cAMP-dependent binding region. Both of these analysis showed that the 193mer does demonstrate differential binding to the cAMP-treated rat liver nuclear extracts and that this binding could be competed with unlabeled 50mer but not with unlabeled 322mer from pUC-18 (data not shown). This competition also demonstrated that the 298mer (-59 to +73 of PEPCK and 166bp of CAT) also competed binding to the 193mer, again suggesting that either the cAMP-dependent factor(s) has another binding site in the promoter region or the factor(s) has interaction with other promoter binding components (see competition with 50mer above).

Dimethyl Sulfate Protection

The isolated 193mer was end-labeled on one strand (Nco I) with the Klenow fragment and both alpha-^{32}P-dCTP and alpha-^{32}P-TTP and incubated with 1, 2, and 3ug of protein from both control and cAMP-treated rat liver crude nuclear extracts. The reactions were processed as described in Methods and analyzed on an 8% denaturing polyacrylamide gel alongside the Maxam-Gilbert sequence reactions (Maxam and Gilbert, 1982).

Figure 22 shows the results of the footprinting of the 193 bp fragment using 2ug of control and cAMP-treated rat liver extracts. The lanes marked G, G+A, C+T, and C are the Maxam-Gilbert sequencing reactions, the land marced (c) is the DMS reaction in the absence of protein, and the lanes marked (-), control extracts and (+), cAMP-treated extracts.

Figure 22. Dimethyl sulfate protection of 193bp PEPCK fragment.

The 193 bp fragment was labeled at the NcoI end with Klenow fragment and 60ng of the labeled fragment (100,000cpm) was reacted with 2ug of nuclear extracts from control (-) and cAMP-treated (+) rat liver. After incubation at Rt for 30 min and and cooling to 0° for 10 min, 1-3ug dimethyl sulfate was added. After exposure to dimethyl sulfate, precipitation and backbone cleavage, the reactions were applied to an 80cm, 8% polyacrylamide, 7M urea gel and electrophoresed in TBE buffer. The gel was analyzed by autoradiography using a Kodak intensifying screen at -70°C for 4 days.

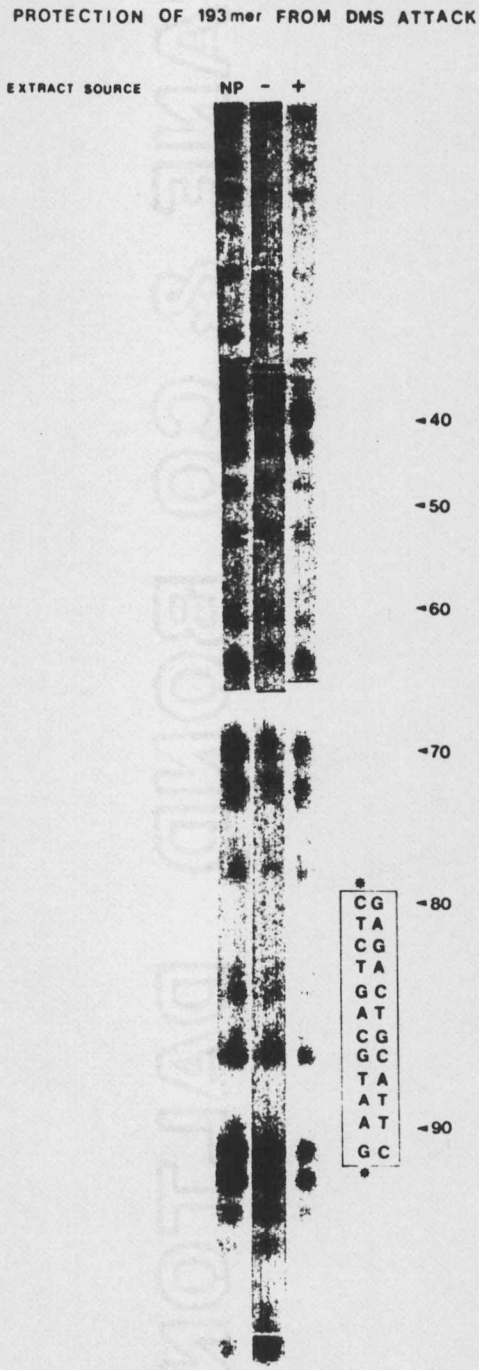

PROTECTION OF 193mer FROM DMS ATTACK
EXTRACT SOURCE
NP
-
+
-40
-50
-60
-70
-80
-90
CTCTGACGTAAG
GAGACTGCATTC

Figure 22

This analysis reveals that the guanines at positions -97, -94, -93, -92, and -91 appear to be protected in the cAMP-treated extracts. Guanine at -94 appears to be over 80% protected whereas at -97, -93, -92, and -91 the protection is, at best, partial.

Densitometry scanning (Hoeffer densitometer) was performed with these lanes and the results are shown in figure 23. The areas under each densitometric scans were normalized to correct for unequal counts loaded. Normalization was performed by using the intensity of the guanine at -112, which demonstrated similar intensity in each lane in this and a second experiment. The corrected areas were than expressed as a ratio of the area of each guanine in the presence of protein to the area of the corresponding guanine without protein. These ratios, as shown in figure 23, clearly show that the guanines at positions -94, -93, -92, and -91 demonstrate protection (ratio less than 1), whereas the densitometer determined that the guanines at position -87 and -84 show hypermethylation with the cAMP-treated extracts (cAMP/NP ratio larger than cont/NP ratio).

The protection of the 193mer was performed twice and the composite result of these experiments is shown in figure 24.

The normalized areas of each guanine from the two experiments and two different protein concentrations were compared and the ratios determined as in figure 23. These ratios were averaged for the two experiments (except for the guanines at positions -105, -104, and -98; which are from only one experiment, due to difficulty in obtaining areas for these guanines for each experiment) and these areas are expressed as the

Figure 23. Densitometry scans of guanines from 193mer protection with 2ug protein extract.

The normalized areas of the guanines from -117 to -79 of the PEPCK gene analyzed by DMS protection. The areas are expressed as the ratio between control:no protein lanes [///] and cAMP-treated:no protein lanes [XXX]. By definition, a ratio less than 1 reflects protection.

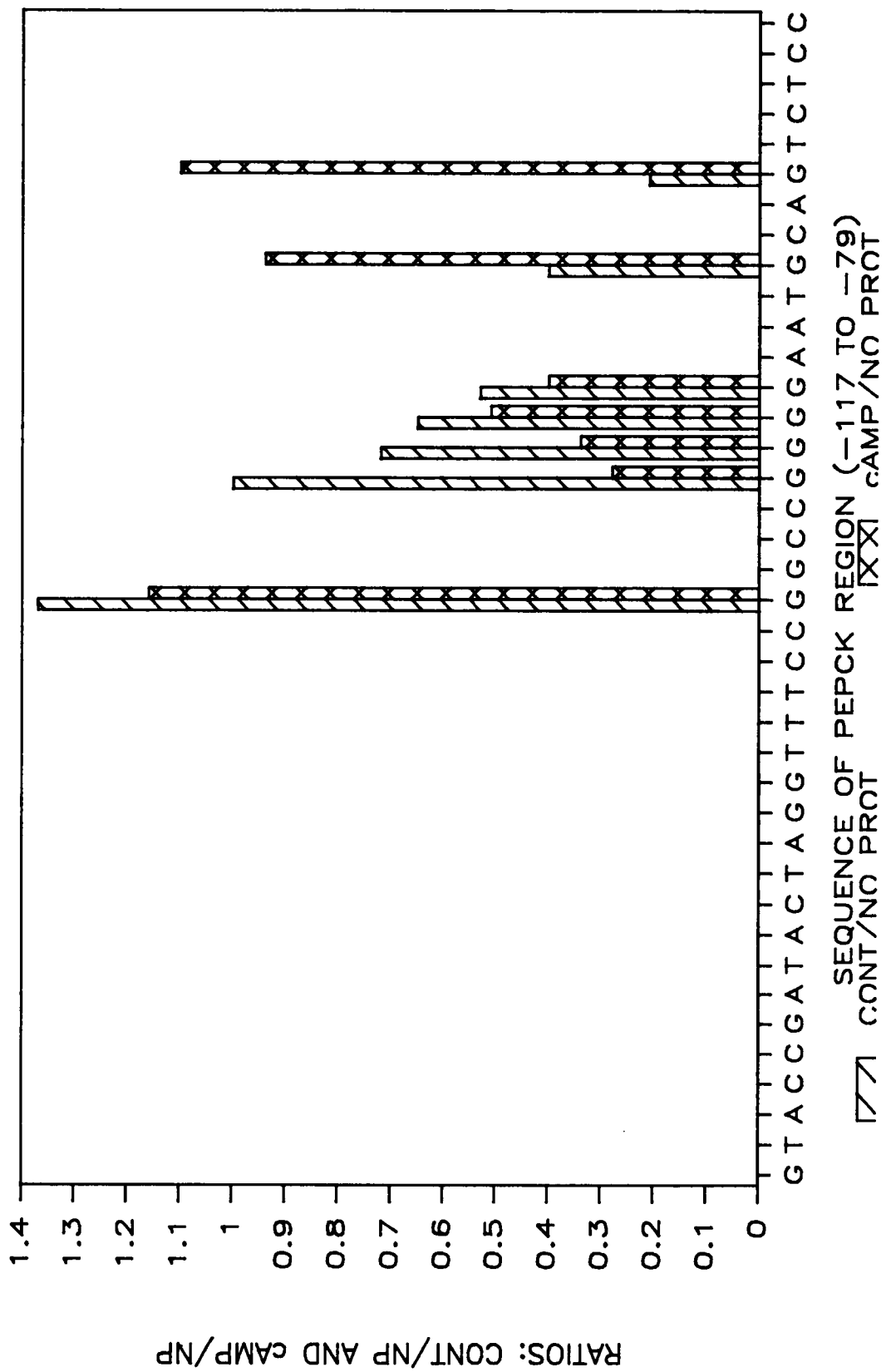

RATIOS: CONT/NP AND cAMP/NP
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
GTACCGATACTAGGTTTCCGGCCGGGGAATGCAGTCTCC
SEQUENCE OF PEPCK REGION (−117 TO −79)
CONT/NO PROT
cAMP/NO PROT

Figure 23

Figure 24. Protein dependent DMS protection of PEPCK gene region (-117 to -79).

The ratios of the corrected areas for the guanines from two different protection experiments with the 193mer, expressed as the difference between the cont/no protein and cAMP/no protein ratio for 1ug [///] and 3ug [XXX] of nuclear extracts.

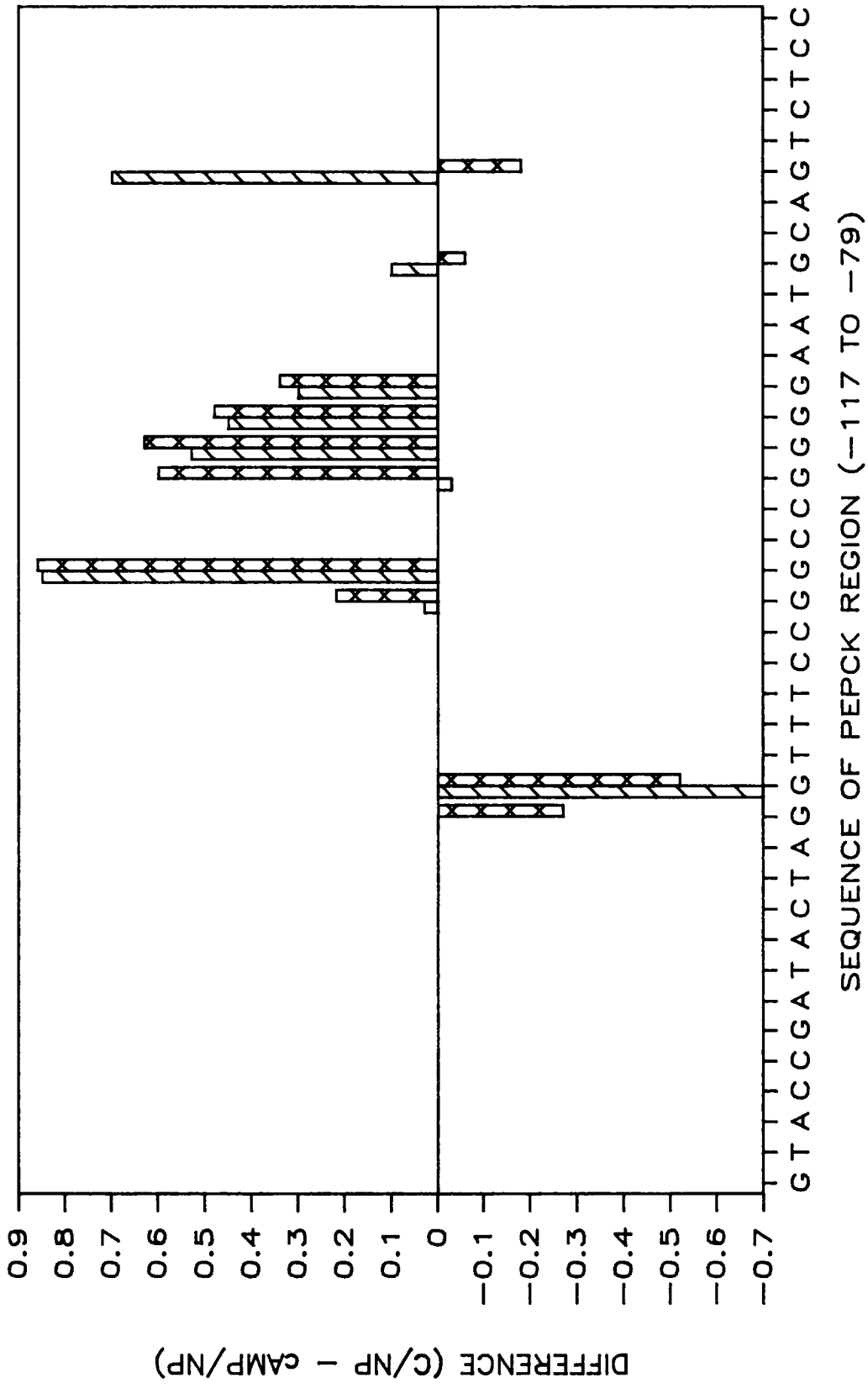
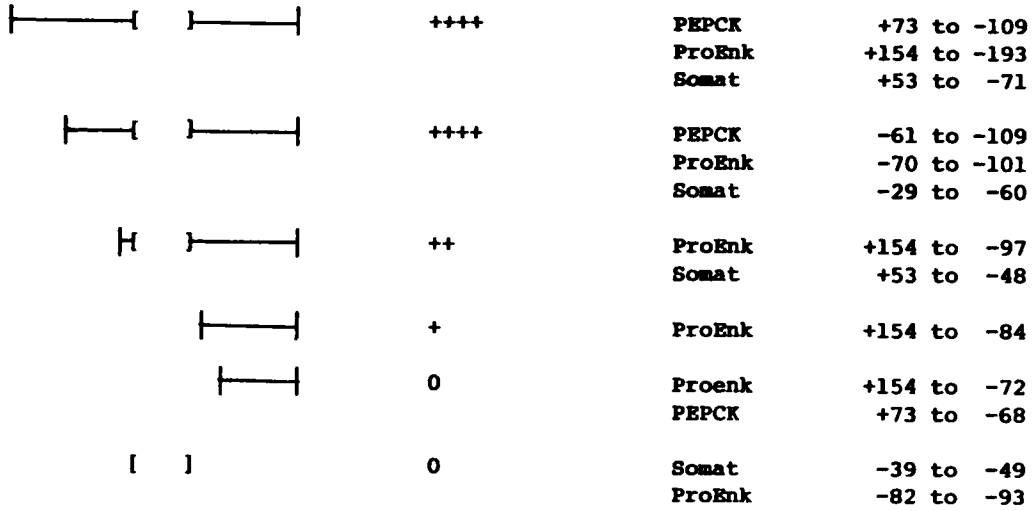

DIFFERENCE (C/NP − cAMP/NP)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
−0.1
−0.2
−0.3
−0.4
−0.5
−0.6
−0.7
GTACCGATACTAGGTTTTCCGGCCGGGGAATGCAGTCTCC
SEQUENCE OF PEPCK REGION (−117 TO −79)

Figure 24

difference between the control/np ratio and the cAMP/np ratio. Differences larger than one represent protection and differences less than one represent hypermethylation. This composite demonstrates that the guanines at positions -97, -93, -92, and -91 show protection regardless of the protein used (1 or 3ug). Positions -98 and -94 suggest that more protection occurs at higher protein (3ug), but the meaning of this is not clear at present.

The protected region appears to be on the 5' side of the 12 base sequence that is considered the consensus sequence (CTTACGTCAGAG) (Wynshaw-Boris et al., 1986) with only one G within the sequence protected (-91) and two other G residues demonstrating hypermethylation (-87 and -84). These results strongly suggest that the identified nuclear factor(s) binds to a region of the cAMP responsive sequences outside of the so-called consensus sequence, which has been demonstrated not to confer cAMP-regulation on its own (Comb et al., 1986).

Analysis of deletion/transfection experiments carried out with the flanking regions of PEPCK (Short et al., 1986), rat somatostatin (Montminy et al., 1986) and human proenkephalin (Comb et al., 1986) (figure 25) reveals that the region just 5' of the consensus sequence is important for a full cAMP response as measured by transfection using heterologous reporter genes. Deletion of a few bases 5' of the core sequence produces half of the potential response and deletion just 3' of the core sequence reduces the response even further in somatostatin and proenkephalin. To date, the region surrounding the core sequence in PEPCK has not been mapped to this detail; however, if a correlation is



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SOMAT: Montminy, M., Sevarino, K., Wagner, J., Mandel, G., and Goodman, R. (1986) Proc. Natl. Acad. Sci. 83, 6682-6686.

PROENK: Comb, M., Birnberg, N., Seasholtz, A., Herbert, E., and Goodman, H. (1986) Nature 323, 353-356.

Figure 25. Comparison of 5' deletion/transfection experiments.

Comparison of deletion/transfection experiments among the three genes where the cAMP-responsive element has been found. PEPCK--Short et al., 1986, Somat--Montminy et al., 1986, and ProEnk--Comb et al., 1986.

made based upon the conserved sequences between these three genes (table 1, p. 6), then a prediction can be made that the region just 5' of the consensus sequence in PEPCK should be important for full cAMP-response (figure 26), and this is the region which contains the protected guanines.

Analysis by the DMS protection does not reveal the total area of the sequence that would interact with the putative factor(s); however, it does strongly support the presence of an trans-acting factor(s) which interacts with the same region of DNA that confers cAMP-responsiveness to both homologous and heterologouss promoters (Short et al., 1986).

Binding with Extracts from Heterologous Cells

Binding to 356bp Pvu II fragment. Nuclear extracts from mouse fibroblast cells (L-cells) were prepared according to the procedure outlined in Methods. The investigation of the mouse L cells was performed as a result of experiments with expression plasmids containing the 5' flanking region of PEPCK (-548 to +73) (Wang and Huang, 1986; Liu, unpublished observations). Transfection of L cells with these expression plasmids demonstrated regulation by cAMP of the expression of the reporter genes. Since other tissues have demonstrated that cAMP will regulate gene expression (Montminy et al., 1986; Comb et al., 1986), the universality of the presence of the cAMP-dependent DNA binding factor will need to be tested.

Table V demonstrates the extent of binding observed by using the 356bp Pvu II fragment and 0.35M nuclear extracts from rat liver, rat

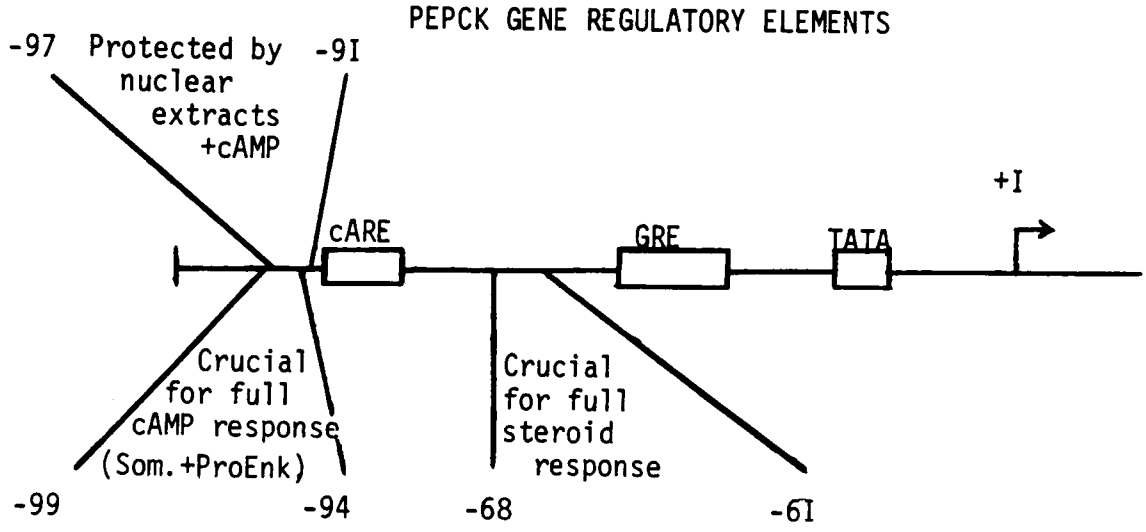

Figure 26. Analysis of regulatory elements in PEPCK 5' flanking region.

Table V

Binding of 356 bp Pvu II PEPCK Fragment to Nuclear Extracts from Different Cells

Experiment	cAMP	% DNA Bound		
		Liver	H-35	L-Cells
1	-	2 ± 2	0	7 ± 2
	+	11 ± 3	14 ± 2	28 ± 6
2	-	0	0	1 ± 2
	+	17 ± 7	14 ± 6	11 ± 2
3	-	10 ± 3	7 ± 2	9 ± 3
	+	35 ± 5	18 ± 7	30 ± 8
Average Difference		+13	+13	+21

hepatoma, and mouse L cells. This table shows that the net binding to the different nuclear extracts was consistent, and it appears that mouse L cells also contain the cAMP-dependent DNA binding activity. Binding of end-labeled 356mer to 0.35M NaCl extracts of nuclei from rat liver, rat hepatoma (see Table III, p. 49), and mouse fibroblast cells. Experiments 1 and 3 represent binding to 1.5ug protein and experiment 2 is to 2ug protein. Binding was performed in the presence of 100mM Na+ and 100ng linearized pUC-18 DNA. Data is expressed as percent DNA bound (bound cpm-background cpm/input cpm-background cpm). The background averaged 10% of input cpm. Each value represents the mean of three determinations per experiment.

To further analyze the binding observed in the mouse L cells, the agarose gel shift assay was used. This assay was performed on Hind III digested pBB0.6 (containing the PEPCK region -548 to +73) which produced the 621bp PEPCK insert and the rest of the plasmid (chloroamphenicol acetyltransferase gene from bacteria, SV-40 enhancer and polyadenylation site, and pBR322 sequences). The specificity of the L cell binding was tested by end-labeling the digested plasmid with Klenow fragment and dCTP-γ-^{32}P and reacting with both control and cAMP-treated L cell nuclear extracts in the presence of increasing amounts of unlabeled, linearized pUC-18 DNA.

Figure 27 demonstrates that the inclusion of up to 50ng of competitor was required to disrupt the complex between the control extracts and the 621bp fragment but it took up to 180ng of competitor to disrupt the complex between the cAMP-treated extracts and the 621 bp PEPCK fragment.

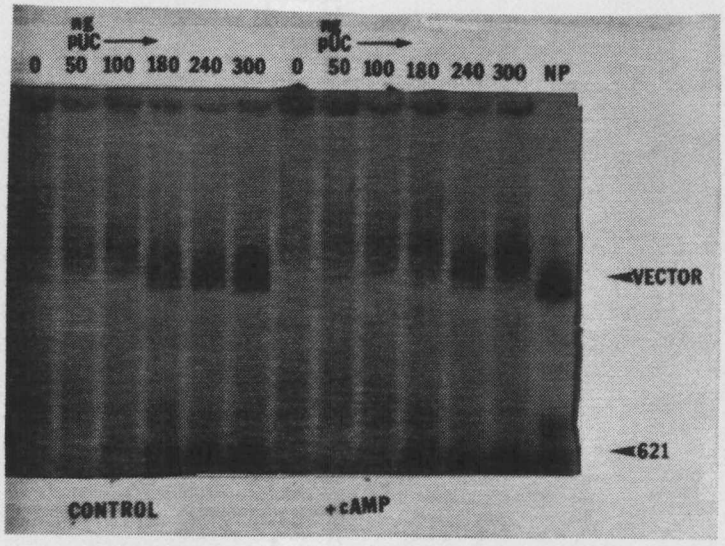

Figure 27. Agarose gel competition with L-cell extracts.

Plasmid pBB0.6 was digested with Hind III and end-labeled with Klenow fragment and dCTP-γ-^{32}P. 10ng of the mixture was reacted with 2ug of both control and cAMP-treated nuclear extracts from mouse L cells in the presence of various amounts of linearized pUC-18 DNA and then subjected to agarose gel analysis (1%). The gel was dried and autoradiographed.

The migration of the vector appears to be varied due to incomplete digestion of the plasmid.

Further analysis with this method revealed that the cAMP-dependent binding in the L cell extracts may require further investigation by competition and footprinting analysis to determine the specificity and whether the factor(s) identified in these cells also protects the same region of the PEPCK 5' flanking sequences.

CHAPTER 4

CONCLUSION

The data presented within this thesis support the conclusion that there is a factor(s) within cAMP-treated rat liver nuclei that specifically binds to a region of the 5' flanking sequences of the rat hepatic phosphoenolpyruvate carboxykinase gene.

Figure 28 is a summary of the results of the filter binding assay with a series of overlapping restriction fragments from the PEPCK gene 5' flanking region. This summary allows the optimal site of binding of the cAMP-dependent DNA binding factor to be defined to a specific portion of the 5' flanking region, somewhere within the region from -71 to -111.

The nitrocellulose filter assay, developed by Riggs (Riggs et al., 1970) has been utilized by other groups to identify DNA-binding proteins from various tissues. Riggs utilized the assay in order to investigate the interaction between the lac repressor and its operator and obtained a dissociation constant of 2×10^{13} M^{-1} for this interaction (Riggs et al., 1970). Nowock and Sippel used the assay to detect four sites within the chicken lysozyme flanking region that bind a factor(s) from chicken oviduct nuclear extracts with an apparent Kd of 6×10^{12} M^{-1} (Nowock and Sippel, 1982). Felsenfeld's group also utilized the assay to determine the binding of a factor(s) from erythrocyte nuclei that binds to a region within the chicken beta-globin gene. This factor(s)

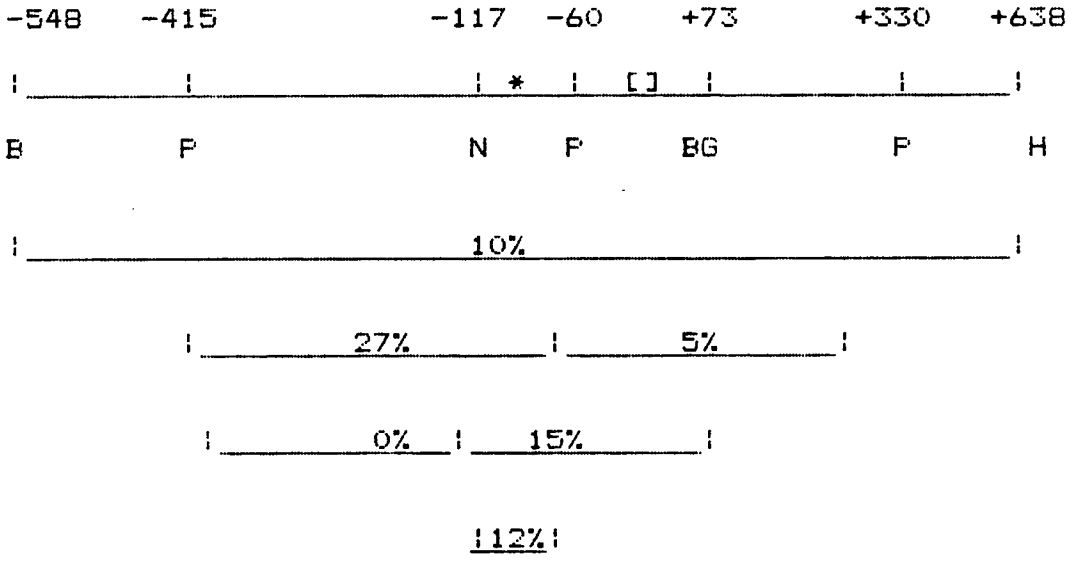

Figure 28. Net percent binding to fragments of PEPCK gene 5' flanking region.

Net percent binding of rat liver 0.35M Na+ nuclear extracts to various fragments derived from the PEPCK gene 5' flanking region. Each fragment was purified by electroelution from agarose. The percent binding shown represents that at protein saturation for each fragment. The cARE region is designated by the "*" and the promoter region by the "[]". Restriction enzymes: B-Bam HI, P-Pvu II, N-Nco I, BG-Bgl II, and H-Hind III.

also exhibits an approximate disssociation constant of 1.5×10^{12} M^{-1} (Emerson et al., 1985).

The above set of dissociation constants was obtained by competition binding assays, assuming 1 protein binding site per DNA molecule. The cAMP-dependent binding factor(s) reveals a competition dissociation constant of approximately 1×10^{12} M^{-1}, which is in the range of the constants exhibited by the other specific DNA binding factors.

The nitrocellulose filter assay does have peculiarities with which one must deal. In fact, von Hippel has pointed out that this assay, even if one is using purified proteins, does not exhibit full efficiency of binding (Woodbury and von Hippel, 1983). The molecular basis for this inefficiency of retention of protein-DNA complexes on the filter may be due to interactions among the proteins and the filter which could cause dissociation of the protein from the DNA upon filtration. During the course of the investigation using various sized fragments of the PEPCK flanking region, another peculiarity was noticed.

When the number of fmoles bound at saturation was calculated for each fragment tested, it was apparent that as the size of the DNA fragment decreases, the number of fmoles bound by the cAMP-treated extracts increases (Table VI). This increase in fmoles bound correlated with the ratio of the sizes of the DNA molecules. The molecular basis for this phenomenon is not apparent but one might speculate that the larger the fragment of DNA, the greater shear force the DNA would encounter upon filtration. A combination of this greater shear force and filter interactions with the proteins, see above (Woodbury and von Hippell, 1983)

Table VI

Relationship of Fragment Size and Net fMoles Bound

Size	Net fMoles Bound	ug Protein	Ratio of Size to 1187bp Fragment	n
1187	1	0.75	1.10	3
356	4	1.5	3.4	5
193	7	1.0	6.0	2
58	26	2.0	20.0	1
50	27	0.8	24.0	2

might allow for the apparent size-dependent dissociation of the DNA-protein complex, assuming the factor identified is a protein. The net fmoles bound at saturation by each fragment tested with the nitrocellulose filter assay. The net fmoles bound were calculated by correcting for the background cpm demonstrated for each experiment. n represents the number of observations included.

The nature of the factor that has been detected is not clear but it is present in protein rich extracts and it does behave like a DNA binding protein. Based on analysis of steroid hormone actionn and the most likely relationship to the cAMP-dependent protein kinase (Yamamoto, 1984; Boney et al., 1983) it seems probable that the factor is a protein. Indeed, the two working models of cAMP action, the possibility that the factor is actually the regulatory subunit RII (Nagamine and Reich, 1985) or that it is a phosphoprotein substrate of the cAMP-dependent protein kinase (Schlichter et al., 1986), suggest that it should be a protein. As to whether the factor is RII or another phosphoprotein, the only evidence collected comes from experiments which were performed with RII prepared from skeletal muscle.

RII was purified from beef heart and both phospho and dephospho forms demonstrated detectable binding to the 1.2 kb BH fragment. However, both unlabeled BH 1.2 and pUC-18 DNA demonstrated equal ability to compete for binding to the labeled BH 1.2 (figure 29). This result is in contrast to the differential competition demonstrated by cAMP-treated rat liver nuclear extracts (see figure 14, p. 41). These experiments do not totally rule out rat liver RII as a candidate since there are

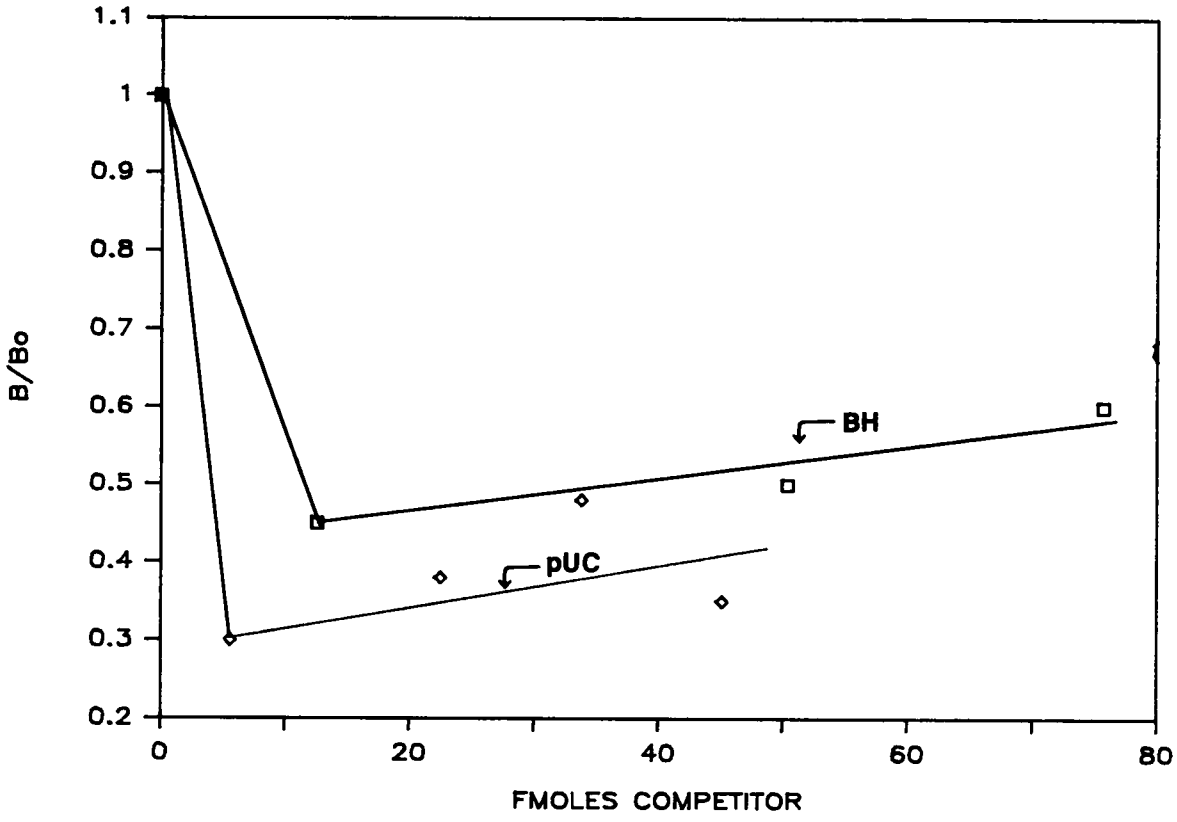

Figure 29. Competition binding with beef heart phospho RII.

Skeletal muscle RII was purified by the method of Ramseyer et al. (1976) and was phosphorylated in vitro by the catalytic subunit of the cAMP-dependent protein kinase. The prepared RII (0.75ug) was reacted with 10ng of labeled BH 1.2 in 100mM Na+ but without added competitor (12% binding above background at Bo). Incubations were carried out in the presence of either unlabeled BH 1.2 or pUC-18 DNA (B). Each point represents triplicate determinations with a background of 18%.

differences between RII from skeletal muscle and rat liver (Rymond and Hofmann, 1982). However, the recent report from Granner's lab that the topoisomerase activity supposedly present in rat liver RII (Constantinou et al., 1985) can be fully separated from RII activity (Granner, unpublished observations) supports the notion that RII does not seem likely to be the cAMP-dependent transcriptional factor.

The site of binding demonstrated for the cAMP-dependent factor in the present work lies within the region (-109 to -68) shown by Hanson's group to confer cAMP-responsiveness on heterologous genes (Short et al., 1986). Dimethyl sulfate protection (DMS) revealed that guanines in the region from -97 to -91 were partially protected from methylation by incubations with the cAMP-treated extracts. The fact that only partial protection was observed presumably arises from the fact that the labeled fragment DNA is in great excess over the amount of factor present. In fact, the protein/DNA ratio in the protection experiments, 2ug/60-90ng, when compared with the filter assay, 1ug/10ng at saturation, shows that during the protection experiment, the protein concentration was significantly below saturation. The footprinting reaction also revealed sites of apparent hypermethylation in the region between -90 and -80. Such a combination of protected and hypermethylated sites within binding regions of specific DNA binding factors has been demonstrated previously. For example, Ptashne's group has shown that incubation of the yeast regulatory factor, GAS4, with its specific binding region produces both partially protected and hypermethylated guanine residues (Giniger et al., 1985). Incubation with the SV-40 transcription factor also

causes both protected and hypermethylated guanines in the specific binding region (Gidoni et al., 1986).

The DMS protection data also suggest that the cAMP-dependent factor may exhibit its greatest affinity for a region of the DNA just upstream (5)' of the so-called cAMP regulatory element (cARE) as identified by deletion experiments (see figure 25, p. 68).

Among the three genes (Short et al., 1986; Montminy et al., 1986; Comb et al., 1986) where the cAMP-responsive region have been mapped, there appears to be strong homology around the core sequence. The results of the deletion experiments allow one to call the following region the total consensus sequence.

```
PEPCK 5' GGCCGGCCCCTTACGTCAGAGGG 3'

SOMAT    CCTCCTTGGCTGACGTCAGAGAG

PROENK   GGCGTAGGGCCTGCGTCAGCTGCA
         ** *   ***   **** *
```

The same or similar binding factors could be envisioned to bind to this region.

With regard to the possible universality of the identified facctor, experiments with cAMP-treated mouse L cells revealed the presence of the binding factor using the 356mer as the probe (see Table V, p. 71). Demonstration of just how universal the factor may be, however, awaits further experiments with regulated regions of other genes and extracts

from other cell types. The procedures outlined in this thesis should allow for this analysis to be relatively straightforward.

The most enticing project is the purification of the factor. Once even partially purified, the protection experiments should become more clear and allow for detailed protein dependency determinations. Purification will also allow determination of whether the factor is a substrate of the cAMP-dependent protein kinase.

In any event, the results of this work have provided clear evidence for the existence of a factor, presumably a protein, which could very well prove to mediate the effects of cAMP on eukaryotic gene expression.

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