INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Characterization of the promoter region of the gene for phosphoenolpyruvate carboxykinase (GTP)

Gurney, Austin Louis, Ph.D.

Case Western Reserve University (Health Sciences), 1992
CHARACTERIZATION OF THE PROMOTER REGION OF THE GENE
FOR PHOSPHOENOLPYRUVATE CARBOXYKINASE (GTP)

by
AUSTIN LOUIS GURNEY

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Thesis Advisor: Richard W. Hanson, Ph. D.

Department of Biochemistry
Case Western Reserve University
May, 1992
We hereby approve the thesis of

Austin Louis Gurney

candidate for the Ph.D.
degree.*

Signed:  

R.W. Hansen  
(Chairman)

Ruth and D. Retel

Richard E. Miller

Date  4/10/92

*We also certify that written approval has been obtained for any proprietary material contained therein.
CHARACTERIZATION OF THE PROMOTER REGION OF THE GENE FOR PHOSPHOENOLPYRUVATE CARBOXYKINASE (GTP)

Abstract
by
AUSTIN LOUIS GURNEY

The goal of the research contained within this thesis has been to identify features of the organization of the PEPCK promoter which enable the appropriate expression of the PEPCK gene. Initial research efforts focused on an examination of the functional significance of the single base difference between the sequence of CRE-1 and CRE-2. This research resulted in the observation that the cAMP responsiveness of the PEPCK promoter could be greatly increased by a single base substitution that altered the CRE-2 sequence to be identical to the CRE-1 sequence. This observation provided early evidence that the mechanism of cAMP regulation could involve the synergistic action of multiple cis elements. Subsequent research pursued identification of specific transcription factors
which are able to bind to defined regions of the PEPCK promoter. This work included the discovery that Fos and Jun are able to bind to the PEPCK promoter and acutely modulate the rate of PEPCK transcription. The action of Fos and Jun on PEPCK gene transcription involved each of the Fos/Jun binding sites, and was modulated by additional transcriptional regulatory elements within the promoter. The ability of Fos to inhibit PEPCK transcription was dependent upon P3(I), a region of the promoter which does not bind Fos/Jun heterodimers, but does bind members of the C/EBP family of transcription factors. Stimulation of PEPCK transcription by 8-Br-cAMP or by overexpression of the catalytic subunit of protein kinase A was inhibited by Fos expression. The inhibitory effects of phorbol esters and protein kinase C on PEPCK gene expression may be mediated through the action of Fos and Jun. In addition, several other transcription factors including the thyroid hormone receptor and C/EBPβ have been found to interact with the PEPCK promoter. The identification of these factors and the examination of their ability to alter the rate of transcription has expanded our knowledge of the organization the PEPCK promoter.
Dedication

This bit of shaggy doggerel is dedicated to my parents.
Acknowledgments

I wish to express my love and gratitude to my wife and friend Ümit Yalçınalp. Her contribution to this thesis is immeasurable. She has brought my life and my work a perspective that continues to enrich and enlighten. I sincerely hope that now that this protracted thesis battle is past I can firmly transcend the moral bankruptcy that ensues when one's career is allowed to become more important than one's personal life. I eagerly look forward to the continued exploration of our shared lives.

I have had the good fortune to work in a laboratory that has been graced with many talented people. I appreciate the opportunity I have had to interact with them. Dr. Edwards Park has my warmest admiration and gratitude for helping me learn to not be overwhelmed by the superficiality created when science is driven not by the desire to reach greater understandings but only by the pressure to generate manuscripts and slides to blather about at talks. I thank Ed for teaching me to laugh at the vanity and the foolishness while striving to maintain my personal integrity. I respect and admire Dr. Maria Hatzoglou for her persistent ability to think creatively and with genuine enthusiasm. I thank her for expressing confidence in me. I also wish to thank Dr. Molly Mcgrane, Dr. Summer Savon, Dr. Jinsong Liu, and Dr. Bill Roesler and Teiko Kimura for help and support during these long years.

My life during these past seven years has been made richer and more enjoyable by the presence of many good people. In particular, Bill Magner has earned my respect.
and friendship for his reasonably tireless efforts to embody
the best elements of the ridiculous religious beliefs to which
he subscribes. I also wish to thank Gerry and August for
the thousands of things that they do. They deserve the very
best and get thanked far too infrequently. I wish to thank
my old roommates, Dr. Mike Romero and Dr. Gregory
Hannon, for their friendship and artistic inspiration and for
their kindness.

I thank the Smiths, both Kent and Lisa, for everything.
We have had fun. I hope the years do not pull us apart.

I wish to thank my advisor, Dr. Richard Hanson, for
permitting me to work in his laboratory. During these past
many years, I have learned a great deal, and I am grateful
for the opportunity that has been provided to me. I am
grateful for his continued optimism and his unflagging belief
in the value of our work. I am grateful also for his diligent
efforts to promote the development of good writing skills.
Finally, I thank my advisor Dr. Richard Hanson, and the
other members of my committee; Dr. David Setzer, Dr. Pieter
deHaseth, Dr. Mulchand Patel, and Dr. Richard Miller, for
their time and energy on my behalf, and for granting me my
freedom.
Table of Contents.

Abstract ........................................................................................................... ii
Dedication ......................................................................................................... iv
Acknowledgments ........................................................................................... v
Table of contents ............................................................................................. vii
List of Figures .................................................................................................. x
Abbreviation ...................................................................................................... xii

I. Introduction .................................................................................................. 1
   A. Overview .................................................................................................... 1
   B. The Role of PEPCK ............................................................................... 3
   C. Regulation of PEPCK ............................................................................ 7
   D. Eukaryotic gene regulation ..................................................................... 11
   E. Statement of research goals .................................................................... 16

II. Materials and Methods ............................................................................... 18
   A. Materials .................................................................................................. 18
   B. Construction of vectors ......................................................................... 19
   C. Construction of vectors containing single base substitutions .............. 20
   D. Preparation of plasmid DNA ................................................................. 22
   E. DNase I footprint assay ........................................................................ 23
   F. Gel Shift assay ....................................................................................... 28
   G. Cell transfection and CAT assays ......................................................... 31

III. Single Base Substitution in CRE-2 can increase cAMP Responsiveness of the PEPCK Promoter ............................................................. 33
   A. Overview .................................................................................................. 33
   B. Results ..................................................................................................... 34

vii
(1). Point mutations alter binding to nuclear proteins from rat liver .......................... 34
(2). Point mutations alter binding to C/EBPα and CREB ........................................... 36
(3). Cyclic AMP regulation is altered ..................... 37
C. Discussion ............................................................... 40

IV. Fos and Jun are able to Bind to the PEPCK Promoter and Modulate the Rate of PEPCK Transcription .................................................. 45
   A. Overview .................................................................. 45
   B. Results ................................................................... 49
      (1). Binding of Fos and Jun to the PEPCK promoter .................................................. 49
      (2). Activation of transcription from the PEPCK promoter by Fos and Jun ............. 51
      (3). The effect of mutant Fos and Jun proteins on PEPCK transcription .................. 52
      (4). Fos blocks the induction of PEPCK transcription by cAMP ............................... 53
   C. Discussion ............................................................... 55

V. Identification of Binding Sites for Thyroid Hormone Receptor and C/EBPβ within the PEPCK Promoter ............................................. 63
   A. Overview ................................................................. 63
   B. Thyroid hormone receptor ........................................ 64
   C. C/EBPβ .................................................................. 69

VI. Discussion .................................................................. 76
   A. Overview ................................................................. 76
   B. The role of promoter architecture in PEPCK promoter function ............................ 77

viii
C. Hormonal regulation of PEPCK gene expression .... 84
D. Future directions of PEPCK promoter analysis .... 88
VII. References ........................................................................................................ 91
VIII. List of Figures .................................................................................................... 110
List of Figures

Figure 1.2. Schematic of the PEPCK promoter showing the relative positions of the protein binding sites that were identified by footprint analysis................................................................. 110

Figure 3.1. The promoter regulatory region of the PEPCK promoter showing base substitutions .............. 112

Figure 3.2. Titration of nuclear extracts from rat liver into footprint assay of promoters containing single base substitutions ................................. 114

Figure 3.3. Comparison of the binding of rat liver nuclear proteins, C/EBP and CREB to the intact PEPCK promoter and the PEPCK promoter containing the -140 G to C single base substitution ......................... 116

Figure 3.4. cAMP regulation of transcription from the PEPCK promoter modified at CRE-1 and CRE-2 ...... 118

Figure 4.1. Footprint analysis of Fos and Jun binding sites in the PEPCK promoter .................................. 120

Figure 4.2. Footprint analysis of the relative ability of binding sites within the PEPCK promoter to bind Jun homodimers and Fos/Jun heterodimers ......... 122

Figure 4.3. Alteration of PEPCK transcription by expression of Jun and Fos ......................................... 124

Figure 4.4. The effect of Fos and Jun on the level of transcription from PEPCK promoters containing block mutations ..................................................... 126

x
Figure 4.5. Effect of Fos and Jun proteins containing specific mutations on PEPCK-CAT expression .................. 128

Figure 4.6. Effect of Fos on stimulation of PEPCK expression by protein kinase A and cAMP ....................... 130

Figure 5.1. Comparison of the PEPCK TRE with the consensus TRE ....................................................... 132

Figure 5.2. Examination of thyroid hormone receptor binding by DNase I footprint analysis with oligonucleotide competition ...................................................... 134

Figure 5.3. Identification of C/EBPβ binding sites in the PEPCK promoter-regulatory region ....................... 136

Figure 5.4. Binding affinities of C/EBPα, C/EBPβ, Fos/Jun and CREB for CRE-1 ........................................... 138

Figure 6.1. A schematic of the promoter region of the PEPCK gene that depicts the relative location of protein binding elements ................................................................. 141

Figure 6.2. A model of PEPCK promoter function incorporating a proposed "linker" protein ......................... 143
Abbreviations

The following abbreviations have been used in this thesis:

ATP  adenosine triphosphate
β-Gal  β-galactosidase
8 Br-cAMP  8 bromo- cyclic adenosine monophosphate
BSA  bovine serum albumin
CIP  calf intestine phosphatase
C/EBP  CCAAT/enhancer binding protein
CRE  cAMP response element
CREB  cAMP response element binding protein
DTT  dithiothreitol
EDTA  ethylenediaminetetracetic acid
LB  Luria-Bertani medium
PEPCK  phosphoenolpyruvate carboxykinase
PNK  polynucleotide kinase
TBE  Tris-borate electrophoresis buffer
Tris  2-amino-2(hydroxymethyl)-1,3-propanediol
Chapter I. Introduction

A. Overview

Phosphoenolpyruvate carboxykinase (GTP) (EC. 4.1.1.32) (PEPCK) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate. This reaction is a rate limiting step in gluconeogenesis. Thus, PEPCK plays central role in the control of glucose metabolism and is subject to acute regulation by a wide variety of metabolic and hormonal signals. This regulation is achieved primarily through rapid changes in the rate of gene transcription. For this reason, PEPCK is an excellent model system for studying the mechanisms by which eukaryotic gene regulation is achieved.

The focus of the research of this laboratory has been to develop an understanding of the mechanisms by which PEPCK expression is coordinated to meet the metabolic needs of the animal. This effort has proceeded in a systematic fashion from analysis of changes in enzyme activity to characterization of the
changes in transcription that occur in response to metabolic fluctuations. Subsequent work has focused on identification of the role of specific hormones in modulating PEPCK transcription. The goal of the work contained within this thesis is the continuation of this process through the characterization of features of the promoter for the PEPCK gene which enable the appropriate regulation of PEPCK expression.
B. The Role of PEPCK

PEPCK was discovered in this department by Dr. Merton Utter (Utter and Kurahashi, 1953). The enzyme was first discovered in chicken liver as an activity which formed oxaloacetate and GTP from phosphoenolpyruvate, GDP, and CO2. The reaction itself is readily reversible. PEPCK is found primarily in the liver and kidney cortex, both gluconeogenic tissues, and in lesser amounts in the jejunum, adipose tissue, and the mammary gland. The enzyme is widely distributed in nature (Utter and Kolenbrander, 1972). It is present the liver and kidney of all vertebrates examined to date. The enzyme isolated from a variety of species appears to have a molecular weight of approximately 70,000 Daltons. It is believed to be a monomeric enzyme in vertebrates, and has a requirement for divalent metal cations and a free sulfhydryl group(s) (Utter and Kolenbrander, 1972).

The reaction catalyzed by PEPCK is an important control point in gluconeogenesis and in the regulation
of carbohydrate metabolism. Glucose is a major fuel, particularly for the brain, skeletal muscle, and erythrocytes. Glucose homeostasis is essential. While the breakdown of glycogen stores can provide for short term glucose needs, after several hours of fasting, gluconeogenesis is required to maintain blood glucose levels. The gluconeogenic pathway is essentially a reversal of glycolysis and utilizes many of the same enzymes. There are, however, three reactions within the glycolytic pathway that are sufficiently energetically favorable as to be essentially irreversible. These reactions are catalyzed by glucokinase, phosphofructokinase, and pyruvate kinase. In order for gluconeogenesis to proceed, these steps are circumvented by glucose 6-phosphatase, fructose 1,6-diphosphatase, and by the combined actions of pyruvate carboxylase and PEPCK. The activity of each of these enzymes is regulated in response to metabolic needs. Several of these enzymes are controlled by phosphorylation or allosteric effectors. Most, if not all, of the enzymes are also controlled at the level of gene expression (Pilkis and Claus, 1991). Details of the regulation of PEPCK expression will be described in the next section.
In addition to its role as a pace setting enzyme in the gluconeogenic pathway, PEPCK is involved in other metabolic functions. PEPCK contributes to glyceroneogenesis, the generation of α-glycerol-phosphate from lactate for triglyceride synthesis in adipose tissue and the jejunum (Reshev et al., 1970). This role is thought to account for the relatively high levels of expression of PEPCK in these tissues. PEPCK also participates in ammoniagenesis within the proximal tubule of the kidney (Pitts et al., 1972). In response to metabolic acidosis, the kidney increases production of ammonia to buffer excess protons. The ammonia is produced from glutamine by the actions of glutaminase and glutamate dehydrogenase. The glutamate substrate is derived from glutamine released from muscle. The α-ketoglutarate that is produced in this process must be further metabolized. PEPCK is ultimately involved both in the utilization of the α-ketoglutarate as a gluconeogenic precursor and in combination with pyruvate kinase in the oxidation of the α-ketoglutarate to CO₂.

There are two isoforms of PEPCK in most vertebrate species which exhibit distinct sub-cellular
localization. The two forms are localized to the cytosol and the mitochondria matrix. The relative abundance and tissue distribution of these two isoforms varies markedly between species (Soling and Kleineke, 1976). The catalytic properties of the two isozyme species are virtually indistinguishable. The proteins are, however, encoded by distinct genes (Weldon et al., 1990). The cytosolic form has been cloned from chicken and rat. A cDNA encoding the mitochondrial form has been isolated from the chicken (Weldon et al., 1990). In chicken, the two enzymes exhibit limited regions of substantial amino acid identity (80% within the core region of the proteins) and are 60% identical at the nucleotide level (Savon, Ph.D. thesis OWRU, 1991; Weldon et al., 1990). Of the two isoforms, it is the cytosolic form which is acutely regulated by hormones and diet, while the mitochondrial form is expressed constitutively. The mechanisms by which PEPCK expression is controlled have been studied most extensively with the gene encoding the cytosolic form of PEPCK from the rat. Therefore, subsequent reference to PEPCK will refer to this form of the enzyme, unless otherwise indicated.
C. Regulation of PEPCK

PEPCK gene transcription is known to be regulated by a variety of agents including glucagon (acting via cAMP) (Lamers et al., 1982), insulin (Sasaki et al., 1984), thyroid hormone (Loose et al., 1985; Giralt et al., 1991), glucocorticoids (Peterson et al., 1988), prolactin, and perhaps other agents including retinoic acid (Pan et al., 1990; Lucas et al., 1991). In the kidney PEPCK activity is regulated by metabolic acidosis and alkalosis. The major physiological regulators of hepatic PEPCK activity are insulin and glucagon, hormones of the fed and fasted state respectively. Under conditions of high carbohydrate diet, insulin levels are elevated while glucagon levels are depressed. Gluconeogenesis is inhibited and the level of PEPCK gene transcription is low. During fasting conditions, or after a high protein-low carbohydrate diet, glucagon levels increase together with the concentration of cellular cAMP and insulin levels decrease. Gluconeogenesis is required to maintain glucose levels, and transcription of the PEPCK gene is elevated. The substrates for
this process include amino acids derived from the diet or protein breakdown and glycerol derived from triglyceride breakdown. An additional substrate is lactate derived from Cori cycle activity.

PEPCK is not known to be subject to allosteric control or regulation by covalent modification. Consequently, changes in PEPCK activity are achieved through changes in the abundance of the protein. The cloning of the genes encoding PEPCK has permitted a detailed examination of the mechanisms by PEPCK activity is regulated. It has been shown that regulation is achieved primarily at the level of transcription (Lamers et al., 1982), with additional regulation achieved by changes in the stability of the PEPCK message (Hod et al., 1988). PEPCK mRNA has a very short half-life, of approximately 30 minutes (Cimbala et al., 1982). This rate of turnover permits rapid changes in message abundance through changes in the rate of transcription and accumulation of PEPCK mRNA due to stabilization of the message by cAMP and glucagon (Lamers et al., 1982; Hod et al, 1988).

Several studies have demonstrated that sequences within the PEPCK promoter region extending from -450
to +73 contain the information required for tissue specific and developmental expression, as well as correct hormonal regulation (Short et al., 1986; Benvenisty et al., 1989; McGrane et al., 1988, Magnuson et al., 1987, Trus et al., 1990, Wynshaw-Boris et al., 1984, 1986). Thus, a relatively short segment of DNA contains the information necessary to achieve a remarkably complex pattern of transcriptional regulation.

Footprint analysis identified at least eight distinct regions of the promoter which are protected by proteins present in extracts prepared from rat liver nuclei (Roesler et al, 1989). These regions are depicted in Figure 1.1. The role of these binding sites has been studied through the use of the PEPCK promoter containing deletions or block substitutions (Liu et al., 1991). The function of these modified promoters has been quantified through the use of a variety of reporter genes which have been linked to the PEPCK promoter and transfected into hepatoma cell lines.

These techniques have enabled the identification of regions which are involved in the regulation of
PEPCK gene transcription by a variety of hormones. As will be described in greater detail later in this thesis, our lab and others have identified sequences that are involved in the regulation of PEPCK transcription by thyroid hormone (-332 to -308) (Giralt et al., 1991), glucocorticoids (-350 to -420) (Peterson et al., 1988, Imai, 1990), and insulin (-416 to -402) (O'Brian et al., 1990). Multiple elements responsible for the cAMP responsiveness of the PEPCK promoter map between -350 and +1 (Liu et al., 1991). A cis-acting element, termed CRE-1 (-90 to -82), contains the consensus CRE motif, TTAGGTCA, and has been shown to convey cAMP responsiveness when linked to a neutral promoter. A second putative CRE at positions -142 to -135 differs from this sequence by a single base (G vs. C at position -140) and an additional protein binding element, P4(1), between -279 and -286 differs from the sequence of CRE-1 by two bases (-282 A vs. G and -284 G vs. A). In addition P3(1) has been demonstrated by Liu et al. (1991) to play a major role in cAMP regulation of transcription from the PEPCK promoter. Each of these protein binding elements has been shown, on the basis of competition studies, to bind similar or identical
proteins (Roesler et al., 1989).

With the identification of the cis-acting sequences involved in promoter function, it has become possible to pursue the identification of specific factors which are able to interact with these elements. The identification of these proteins is of central importance to developing an understanding of how multiple signals are able to act to establish the rate of PEPCK transcription and may lead to broader insights into general mechanisms by which eukaryotic gene expression is controlled.

D. Eukaryotic gene regulation

There are many substantial questions concerning the function of eukaryotic promoters which remain unanswered. The basic molecular mechanisms by which proteins function to enhance transcription are not well established. The mechanisms by which multiple proteins interact to coordinate transcription and the role of promoter architecture in determining the function of specific proteins remain to be determined. Other questions concerning the regulation of the
expression of transcription factors and the control of their function through post-translational modification, changes in stability, cellular localization or other means have only begun to be investigated. None the less, the past decade has seen extraordinary progress towards understanding the basic architecture of promoters through which eukaryotic gene expression is controlled. What has emerged is a view of transcription in which a finite number of families of transcription factors are responsible for the transcription of a great variety of genes. The specific pattern of gene expression controlled by a particular promoter seems to be dictated by both the specific coterie of protein recognition sequences present within the promoter regulatory region of the gene, and by the specific arrangement of these elements.

Analysis of the function of specific transcription factors has benefited from several key technical advances. The first of these was the development of sequence-specific DNA affinity columns by Tjian and co-workers (Briggs et al., 1986). This has enabled researchers to purify, in a relatively
straightforward fashion, transcription factors that are present in low abundance. A second advance has been the development of techniques to screen expression libraries for proteins which bind to specific DNA sequences (Singh et al., 1988; Vinson et al., 1988). This has enabled the cloning of genes encoding transcription factors in the absence of any direct knowledge of amino acid composition or even of molecular weight of the protein species. With these techniques, it has been possible to purify many transcription factors and clone their respective genes. Several of the earliest identified factors have now been found to be key agents in the control of PEPCK gene transcription, and will be described in detail later in this thesis.

Analysis and comparison of transcription factors has provided insight into some of the general characteristics these proteins. One important feature common to many of the factors examined to date is the presence of a well defined domain structure. The "typical" transcription factor consists of relatively distinct domains involved in DNA recognition and binding, and protein/protein interactions and
transcription regulation. In a number of cases it has been shown that these domains can be completely dissociated. This has been most strikingly demonstrated through the construction of hybrid transcription factors which possess the DNA binding specificity of one transcription factor coupled with the activation domain derived from a different transcription factor (Struhl, 1987; Hope and Struhl, 1986; Brent and Ptashne, 1985, Carey et al.,1990). This modular structure may have an evolutionary significance, perhaps allowing the rapid proliferation of novel transcription factors through the recombination of distinct DNA binding domains and transcription control domains.

The DNA binding domains of transcription factors have generally been found to utilize several basic structural motifs such as the "helix-turn-helix", "zinc finger" and the "leucine zipper". Many of the transcription factors bind to DNA as dimers. As a result, many of the DNA sequences which function as protein recognition sites exhibit palindromic character.

The term "leucine zipper" has been used to
describe a particular amino acid motif which is present in a large and rapidly growing list of transcription factors. These proteins contain within their primary amino acid sequence a domain which exhibits a strict periodic array of leucine residues spaced seven residues apart (Landschulz et al., 1988). This region has been shown to be necessary and sufficient for protein dimer formation, and occurs adjacent to a DNA binding domain rich in basic amino acids. The DNA binding domain and the leucine zipper domain have been proposed to bind DNA through a structure termed "scissor-grip" (Vinson et al., 1989). Leucine zipper proteins are potentially able to form selective heterodimers. This could potentially permit an additional level of transcriptional control through the action of heterodimer proteins exhibiting unique DNA sequence specificity and unique regulatory features. There are now numerous reports of heterodimer formation between members of the leucine zipper family, including members known or suspected to be able to interact with the PEPCK promoter. Specific examples of this will be discussed in greater detail within the results.
E. Statement of research goals

The goal of the research contained within this thesis has been to identify features of the organization of the PEPCK promoter which enable the appropriate expression of the PEPCK gene. The course of this work has included several distinct phases which have arisen as a consequence of both developments in the field of gene regulation and progress made by others within this laboratory. Initial research efforts focused on an examination of the functional significance of the single base difference between the sequences of CRE-1 and CRE-2. This research, described in chapter three, resulted in the observation that the cAMP responsiveness of the PEPCK promoter could be greatly increased by a single base substitution that altered the CRE-2 sequence to be identical to the CRE-1 sequence. This observation provided early evidence that the mechanism of cAMP regulation could involve the synergistic action of multiple cis-acting elements. Subsequent research pursued identification of specific transcription factors which are able to bind to defined regions of the PEPCK promoter. This work has included the
discovery that Fos and Jun are able to bind to the PEPCK promoter and acutely modulate the rate of PEPCK transcription. A detailed examination of the interaction of these transcription factors with the PEPCK promoter is presented in Chapter IV. In addition, in collaboration with colleagues in this laboratory, several other transcription factors including the thyroid hormone receptor and C/EBPβ have been found to interact with the PEPCK promoter (See Chapter V). The identification of these factors and the examination of their ability to alter the rate of PEPCK gene transcription has expanded our knowledge of the organization the promoter.
Chapter II. Materials and Methods

A. Materials

DNA-modifying enzymes and poly[d(IC).d(IC)] were purchased from Boehringer Mannheim. [γ-32P] ATP (6,000 Ci/mmol), [α-32P] dCTP (3,000 Ci/mmol) and [14C] 1,2 dichloroacetyl-chloramphenicol (57.9 mCi/mmol) were purchased from Du Pont-New England Nuclear. Sephadex G-50 was purchased from Pharmacia. Oligonucleotides were chemically synthesized using an Applied Biosystems 380A DNA Synthesizer. All other chemicals were of the highest purity available. Recombinant Fos and Jun proteins were expressed as hexahistidine fusion proteins in E. coli and were purified by nickel affinity chromatography (Abate et al., 1990). Recombinant Fos and Jun and mammalian expression vectors for wild type and mutant Fos and Jun proteins were the generous gift of Drs. Cory Abate and Tom Curran (Roche Institute of Molecular Biology, New Jersey). C/EBPα and C/EBPβ (11 kD fragments containing the DNA binding domains) were generously provided by Steven McKnight (Carnegie Institution in Baltimore Maryland). Recombinant CREB and C/EBPα were
produced in *Escherichia coli* as outlined by Park et. al. (1990).

**B. Construction of vectors**

Block substitutions were introduced into specific protein binding domains of the PEPCK promoter (~490 to +73) by Dr. Jinsong Liu using a variation of the method of Kunkel and co-workers (1985). The specific procedures used to introduce these block mutations into the PEPCK promoter have been described in detail previously (Liu et al., 1990). The expression vectors for the wild type and mutant Fos and Jun proteins have been described previously (Gentz et al., 1989, Sonnenburg et al., 1989). These vectors include the cDNA of c-fos or c-jun (from rat) linked to the CMV (cytomegalovirus) promoter. Jun ΔL3 and Fos ΔL3 contain leucine to valine amino acid substitution at leucine 3 within the leucine zipper domain. Fos ΔBR and Jun ΔBR contain amino acid deletions within the DNA binding domain (amino acids 139-145 and 260-266, respectively). Fos 102-308 contains a deletion of 101 amino acids from the amino
terminal and Fos 1-258 contains a carboxyl terminal truncation which removes a "repressing" region identified by Gius et al. (1990).

C. Construction of vectors containing single base substitutions

A polylinker containing the following restriction sites, Xba I, Bam HI, Kpn I, Sal I, and Bgl II was synthesized. The sequences of the complementary oligonucleotides are CTAGATCGCGAGGATCCCCCGGGGTACCAGTCGACCTCAGAGA and GATCCTCTCGAGGTGACTGTTACCCCCGCGGGGATCTGCGGAT. This polylinker was ligated into the Xba I and Bgl II sites of Pscan (Stratagene) to form P-neo. The Pvu II-Bgl II fragment of the PEPCK promoter (positions -62 to +73) was ligated into the Sal I and Bgl II sites of P-neo to form Pvu II-Bgl II neo (the 5' overhang generated by Sal I was removed by digestion with mung bean nuclease). A synthetic, double-stranded oligonucleotide was synthesized which contained the sequence found between -108 and -65 of the PEPCK promoter, including bases -108 to -105 as a four base
5' overhang which is compatible with \textit{Bam} HI. During synthesis, 2 percent random nucleotides were introduced at each position in each strand. The resulting oligonucleotide was ligated into the \textit{Kpn} I and \textit{Bam} HI sites of \textit{Pvu} II-\textit{Bgl} II neo (the 3' overhang generated by \textit{Kpn} I was removed by digestion with mung bean nuclease). This ligation resulted in the formation of a heterogeneous population of promoters which contain the sequence of the PEPCK promoter between -108 and +73 with random base substitutions in positions -108 through -65. The ligated vector was transformed into bacteria. Individual colonies were selected and the promoters were sequenced to identify single base substitutions. A promoter which was found to contain no random mutations, \textit{(-108-wt neo)} was digested with \textit{Xba} I and \textit{Bam} HI. A synthetic double-stranded oligonucleotide was synthesized which contained the sequence found between -174 and -105 of the PEPCK promoter, including bases -108 to -105 as a four base 5' overhang which is compatible with \textit{Bam} HI. A single base substitution at position -109 (T to G) resulted in the generation of a \textit{Bam} HI site. This oligonucleotide was synthesized such that there was an introduction of 2 percent random nucleotides at
each position in each strand. The oligonucleotide was ligated into the Xba I and Bam HI sites of -108-wt neo, and the resulting mutant promoters were identified by sequence analysis. Promoters which contained the desired mutations were then subcloned into the Xba I and Bgl II sites of polyCAT, a vector which contains the bacterial structural gene for chloramphenicol acetyltransferase (CAT). Site directed mutagenesis as described by Liu et. al. (1990), was used to prepare the single base substitution at position -140 and the two base substitution at positions -282 and -284 within a region of the PEPCK promoter extending from position -490 to +73.

D. Preparation of plasmid DNA

Plasmid DNA was prepared according to Maniatis et al. (1982). Small scale preparation was prepared using the alkaline lysis method from 5-10 ml overnight cultures of bacteria. Bacteria were grown in TB media (prepared by addition of 100 ml of 0.17 M KH2PO4 and 0.72 M K2HPO4 to 900 ml of autoclaved media containing
12 g Bactotryptone, 24 g Bacto-yeast extract and 4 ml glycerol) supplemented with 50 mg/L ampicillin. Large scale preparation of plasmid DNA utilized a scaled up version of the alkaline lysis method to isolate non-genomic nucleic acids followed by purification of supercoiled plasmid by CsCl₂ density gradient centrifugation. All plasmids used in transfection studies or DNase protection assays were judged to be free of both RNA and genomic DNA by ethidium bromide staining of nucleic acid after electrophoresis in 1 % agarose.

E. DNase I footprinting

DNase footprint probes of the PEPCK promoter were prepared such that one strand of the DNA was end labeled with ³²P γ-ATP and polynucleotide kinase. The following protocol was developed which enabled the generation of probes that were substantially free of contaminating genomic DNA and RNA as well as devoid of contaminants which frequently occur in probes prepared by other techniques such as residual protein, agarose, or phenol.
Ten to 30 µg of wtPTZ18RCAT was digested in 100 µl of medium salt buffer (Maniatis et al., 1982) with 10-30 units of either Xba I or Bgl II at 37 degrees centigrade for one hour. One hundred µl of 1 x CIP buffer was added directly to the DNA restriction digest mix with 2 to 3 units of calf intestine phosphatase (CIP). Incubation was continued at 42 °C for one hour. CIP was inactivated by addition of 5 µl 100 mM EGTA followed by incubation at 65 degrees centigrade for 10 minutes. The DNA was then purified by phenol-chloroform (1:1) extraction followed by chloroform-isoamyl alcohol (24:1). The DNA was then precipitated with ethanol and 0.3 M NaCl, rinsed with 70 % ethanol and dried under vacuum. The DNA was then resuspended in 20 µl of H₂O. One half µg of the DNA was then end-labelled in 100 µl of 1 x PNK buffer (Maniatis et al., 1982) with 2 µl (0.2 mCi, 6000 Ci/mMol) of ³²P γ-ATP and 1-2 units of polynucleotide kinase (PNK). The DNA was then digested with a second restriction enzyme, either Xba I or Bgl II. One hundred µl of 1 x Medium Salt Buffer was added with 10 units of restriction enzyme and the mixture was incubated at 37 degrees centigrade for 15-30 minutes. The labelled DNA was separated from unincorporated
label by passage over a one ml column of G-50 Sephadex. The G-50 Sephadex was equilibrated in 10 mM Tris pH 7.4 and 50 mM NaCl. The labelled DNA was then precipitated with two volumes of ethanol and 0.3 M NaCl, rinsed with 70% ethanol and dried under vacuum. The DNA was resuspended in 10 μl of H2O after which 1.0 μl of non-denaturing blue juice (Maniatis et al., 1982) was added. The DNA fragments were separated by electrophoresis on a 0.4 mm thickness 5% acrylamide (19:1 acrylamide to bis acrylamide) gel in 1 x TBE (Maniatis et al., 1982). Following electrophoresis, the gel was transferred to a used x-ray film (the film functions as a solid support for the gel). The gel was then covered with plastic film and was placed in a film cassette with X-omat film and a brief 1-2 minute exposure autoradiograph was obtained. By alignment of the autoradiograph with the gel it was possible to determine the location of the desired DNA fragment. This band was excised with a razor blade. The labeled DNA fragment was eluted from the gel in 100 μl of 50 mM NaCl overnight at 4 degrees centigrade. The activity of the radiolabeled probe was determined by scintillation counting.
The DNase I protection assay was conducted in the following manner. Ten to twenty thousand cpm of labelled footprint probe was incubated with nuclear extract or purified DNA binding protein in 50 μl of binding buffer (10 mM Hepes pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 50 mM NaCl, 2% PVA (optional), 0.05 mg/ml poly[di-dC]). The mixture was incubated either on ice or at room temperature for 30 min. Five μl of 100 mM MgCl₂-30 mM CaCl₂ was added immediately prior DNase I addition. If the incubation was conducted on ice, the samples were allowed to warm to room temperature before addition of MgCl₂-CaCl₂ solution. Each sample was allowed to warm for precisely the same length of time due to temperature dependent changes in DNase I activity. DNase I was added to each sample and the sample was incubated at room temperature for 45 seconds before addition of 150 μl of stop solution (150 mM NaCl, 0.7% SDS, 15 mM EDTA, 30 μg/ml tRNA). The DNase I was mixed with the sample by three strokes in and out of a pipet. The DNase I was prepared as a fresh dilution into water to a final stock concentration of 30 U/ml. The volume of DNase I added was dependent upon the specific nuclear extract and quantity of nuclear extract present within an
incubation. In general, reactions not containing added proteins or containing purified transcription factors required approximately 0.05 U of DNase I and reactions containing 10-20 µg of nuclear extract required 0.1-0.2 U of DNase I (the amount of DNase I used was a quantity sufficient to generate essentially equal numbers of short and long DNA fragments. This is achieved when the probe DNA is nicked by the DNase I less than one time per molecule). Following addition of stop solution, the sample was extracted with phenol/chloroform and the nucleic acids were precipitated by addition of two volumes of ethanol. The precipitated DNA was pelleted in a microcentrifuge. The pellets were rinsed with 70% ethanol, dried under vacuum and carefully resuspended in 8 µl of formamide blue juice (Maniatis et al., 1982). The DNA fragments were denatured by incubation of the samples at 90 degrees centigrade for 2 minutes and were then separated by electrophoresis on a 6% polyacrylamide (38:2 acrylamide to bis acrylamide) gel containing 7 M urea.
F. Gel shift analysis

The binding conditions employed in gel shift analysis were essentially as previously described (Bokar et al., 1989). In a typical experiment, twenty thousand cpm of labeled probe was incubated with a specific DNA binding protein or nuclear extract in twenty μL of binding buffer (10 mM Hepes pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 50 mM NaCl, 0.05 mg/ml poly[dI-dC], 10 mg/ml BSA (optional)). Binding reactions were conducted for 30 minutes at room temperature. All gel shift assays were subjected to electrophoresis in a 4.5% acrylamide gel (44:0.8 acrylamide to bis acrylamide) with 0.25 x TBE buffer at room temperature. The electrophoresis was conducted at 100 Volts (constant voltage) for 90 min. Gels were dried prior to exposure to film.

Probes for gel shift analysis were prepared by filling in 5' overhang sequences (Xba I compatible overhang) present on all of the synthetic oligonucleotides used in these studies with Klenow enzyme and [α-32P] dCTP and unlabeled dGTP, dATP and dTTP. The labelling reaction was allowed to proceed at room temperature for 30 minutes. The reaction
mixture was passed through a 1 ml Sephadex G-50 column (equilibrated with 50 mM NaCl) to remove unincorporated label. Probes prepared by end labelling with [γ-32P] ATP and polynucleotide kinase were not used for gel shift analysis due to the generation of labelled single-stranded species.

Oligonucleotide competition experiments were used to determine whether factors that were observed to bind to specific oligonucleotides in gel shift experiments were also able to bind to other known protein recognition sequences. In these experiments unlabeled oligonucleotides containing known protein recognition sequences were added to the gel shift binding assay along with the labeled oligonucleotide probe before addition of nuclear extracts or DNA binding proteins. Addition of unlabeled oligonucleotides to the reaction mixture after addition of DNA binding proteins was not done, as it was not effective and may be considered inappropriate. In a typical experiment between 5 ng and 50 ng of unlabeled oligonucleotide was used.

Determination of the affinity with which specific transcription factors bind to the CRE-1 region of the
PEPCK promoter was performed using the gel shift technique. The method used was essentially as previously described (Bokar et al. 1988, Cao et al. 1991). A constant amount of protein was incubated with a range of oligonucleotide concentrations. To achieve the titration of oligonucleotide, each binding reaction was prepared by addition of three separate components. The first component was composed of the DNA binding protein in 1X binding buffer as described above, in the absence of DNA probe. The second component consisted of a high concentration of probe in 1X binding buffer in the absence of any DNA binding protein. The third component consisted of 1X binding buffer and without any DNA binding protein or DNA probe. To achieve a titration of DNA probe, each binding reaction contained an identical quantity of the first component (containing the DNA binding protein) and varying amounts of the second and third component. The total volume of the final binding reactions were kept constant. To extend the range of the titration, multiple preparations of the second component were utilized that contained differing amounts of DNA probe.
To reduce the possibility of an artifactual effect due to a difference in residence time of the binding reactions in the wells of the gel prior to electrophoresis, the samples were loaded into the wells while voltage was applied across the gel. Following electrophoresis, the gels were dried and bands corresponding to bound protein-DNA complex and free probe were visualized by fluorography. The amount of radioactivity in each band was determined by excising the band and counting the radioactivity in a scintillation counter.

G. Cell transfection and CAT assays

HepG2 cells, a human hepatoma cell line, were transfected with DNA using the calcium phosphate precipitation procedure (Maniatis et al., 1982). Each transfection contained 5 μg of CAT vector, 5 μg of either wild type CMV-Fos or CMV-Jun, and 2 μg of a vector containing the β-galactosidase gene driven by the Rous sarcoma virus promoter. Approximately 4 X 10^6 cells were added to the precipitated DNA and the mixture was divided between two plates. Two days
after transfection, the cells were harvested and lysed by freeze-thawing. Analysis of CAT activity was performed as described by Ausubel et al. (1989) using an equal amount of protein from each assay (Bradford, 1976). [\textsuperscript{3}H]-Chloramphenicol and butyryl-SCoA were added to each extract. The butyrated chloramphenicol was extracted by xylenes and quantified by scintillation counting. To correct for differences in transfection efficiency the relative CAT activities of the samples were divided by their relative \(\beta\)-galactosidase activities. Each transfection was performed in duplicate.
Chapter III. Single Base Substitution in CRE-2 can increase cAMP Responsiveness of the PEPCK promoter.

A. Overview

Recent studies have identified several regions of the PEPCK promoter which bind proteins that are similar or identical to the proteins which bind to the previously characterized cAMP regulatory element, CRE-1. The binding affinities of two of these sites, CRE-2 and P4, for proteins present in rat liver are significantly lower than that of CRE-1. The DNA sequence of these elements differs from that of CRE-1 by one and two bases respectively. In order to examine the functional significance of these mismatched nucleotides the sequence of these sites was altered to be identical with the sequence of CRE-1. A single base pair change in a putative cAMP regulatory element (CRE-2) (G to C at -140) and a change of two nucleotides at P4 (G to A at -284, and A to G at -282) each result in a dramatic increase in cAMP induced gene transcription. In contrast, a mutation in the
dominant CRE (G to T at -86) greatly reduces the response of the promoter to cAMP. These alterations in the PEPCK promoter correlate directly with altered binding of CCAAT/Enhancer binding protein (C/EBP) and the cAMP response element binding protein (CREB) to both CREs. The increased cAMP stimulation of transcription from the PEPCK promoter containing the single base substitution in CRE-2 is shown to involve the synergistic action of both of CRE-1 and CRE-2.

B. Results

(1). Point mutations alter binding of nuclear factors from rat liver.

The effects on promoter function of two single base substitutions which altered the sequences within CRE-1 and CRE-2 were characterized in detail. The single base substitution within CRE-2 involves a G to C transversion at position -140, which alters the sequence between -142 and -135 so that it is identical to the sequence between -90 and -82
(Figure 3.1). The second single base substitution disrupts CRE-1 by introducing a G to T transversion at position -86. An additional promoter was prepared which contained both the G to C transversion at position -140 and the G to T transversion at -86.

The effect of these alterations in CRE-1 and CRE-2 on the binding of nuclear proteins to the PEPCK promoter region was determined by DNase I footprint analysis (Figure 3.2). The G to C substitution at position -140 resulted in a modest increase in the binding of nuclear protein(s) to CRE-2. The lower affinity with which factors present in rat liver nuclear extract bind to CRE-2 relative to CRE-1 can largely be accounted for by the single base difference at position -140, although the flanking sequences may also influence protein binding. The G to T substitution at position -86 results in a decrease in the binding of nuclear protein(s) to CRE-1. The binding pattern obtained with a promoter which contains both the -140 G to C substitution and the -86 G to T substitution is also shown. Comparison of the protein binding patterns observed with these promoters suggests that the single base substitution in one of
the two CREs does not alter protein binding to the other CRE suggesting that these two elements bind to nuclear protein(s) independently.

(2). Point mutations alter binding of C/EBPα and CREB

Recently we found that CCAAT/Enhancer Binding Protein, (C/EBPα), binds to each of these protein binding domains, although binding to CRE-2 is relatively weak (Park et al., 1990). Both CRE-1 and CRE-2 resemble the consensus binding site for CREB, a protein which has been shown to be involved in mediating the cAMP responsiveness of several genes (Short et al., 1986; Hoeffler et al., 1988; Yamamoto, et al., 1988; Roesler et al., 1988). Furthermore, we have recently found that CREB binds to CRE-1 (Park et al., 1990). While there has to date been no direct evidence for a role of C/EBPα in mediating cAMP regulation of transcription, several reports have suggested a possible connection to cAMP regulation as well as a role in the regulation of genes involved in energy metabolism (McKnight, et al. 1989, Christy, et al. 1989). Both rat liver and the JEG-3 cell line used in this study contain mRNA for both C/EBPα and CREB (unpublished observations Jack Thatcher and
Richard Hanson). Because the promoters containing base substitutions display altered affinity for the binding of proteins present in nuclear extracts, as well as altered cAMP regulation (see below), we examined the binding of C/EBPα and CREB to these promoters. The G to C substitution at -140 results in a significant increase in the ability of both C/EBPα and CREB to bind to CRE-2 (Figure 3.3), while a G to T substitution at -86 decreased the binding of both C/EBPα and CREB to CRE-1 (data not shown). Alterations in the binding of proteins present in nuclear extracts to PEPCK promoters containing base substitutions within the CREs correlated with changes in the binding of C/EBPα and CREB to these CRE elements.

(3). Cyclic AMP regulation is altered.

The relative cAMP responsiveness of the modified promoters was determined by analysis of the transient expression of PEPCK-CAT using the JEG-3 cell line, a human placental cell line previously used to study cAMP regulation of transcription (Hoeffer, et al. 1988). Transcription of a chimeric PEPCK-CAT gene containing 174 base-pairs of 5' flanking sequence was
stimulated 5 fold by 8-Br-cAMP (Figure 3.4A and 3.4B). The -140 G to C substitution within CRE-2 substantially increased responsiveness (40 fold stimulation by cAMP) of the PEPCK promoter while the -86 G to T substitution within CRE-1 resulted in the loss of cAMP responsiveness. The PEPCK promoter which contains both the -140 G to C base substitution in CRE-2 and the -86 G to T base substitution in CRE-1 has a level of cAMP responsiveness which was similar to that of the wild type promoter. This implies that the high degree of cAMP regulation that is observed with the promoter containing the -140 G to C base substitution in CRE-2 is dependent upon the function of an intact CRE-1. The level of basal expression paralleled the degree of cAMP responsiveness, with the highest level occurring with the promoter containing the -140 G to C substitution and the lowest level with -86 G to T substitution.

We also examined the effect of the -140 G to C substitution in CRE-2 when it is contained within a larger region of the promoter (-490 to +73). This segment of the PEPCK promoter has previously been shown to contain the elements necessary for the tissue
specific, hormonal, and developmental regulation of the PEPCK gene (McGrane et al., 1988). The modified promoter exhibited enhanced cAMP responsiveness as compared to the intact PEPCK promoter of the same length. Interestingly, the PEPCK promoter which extended to -490 was expressed at a lower level and was less responsive to 8 Br-cAMP than the segment of the PEPCK promoter between -174 and +73. This may be due to the presence of inhibitory elements located within the region extending from -174 to -490, as suggested by previous studies (Short et al., 1986, Klemm et al., 1990).

The observation that two CRE-1 elements could act synergistically to convey cAMP responsive transcription raises the possibility that there may be direct protein-protein contact between the factors which bind to the CREs or simultaneous involvement of the bound factors within a larger transcription complex. To examine whether the synergistic action of multiple CRE-1 elements could operate when the CREs were separated by greater distances, the sequence of the P4 element, which differs from the sequence of the CRE-1 by two nucleotides, was altered by Dr. Jinsong
Liu (-284 G to A and -282 A to G) to encode the sequence of CRE-1. The cAMP responsiveness of this promoter was examined in HepG2 cells, a human hepatoma cell line (Figure 3.4C). The introduction of a second CRE-1 sequence approximately 200 base pairs from the CRE-1 results in a promoter which exhibits substantially greater cAMP responsiveness than the native PEPCK promoter, suggesting that the synergistic action of multiple CRE elements occurs by a mechanism which has considerable flexibility with regard to the relative positioning of the CREs.

C. Discussion

CRE-1 was first identified as a cAMP responsive element within the PEPCK promoter by Short et. al. (1986), who studied a series of Bal 31 deletions within the PEPCK promoter. They determined that cAMP regulation was retained in a promoter which extended to -109, but regulation was not evident with a promoter deleted to -68. It was clear, however, that segments of the promoter containing only 109 bases of 5' flanking sequence were not fully cAMP
responsive, relative to larger segments of the PEPCK promoter. Subsequent analysis of a number of promoters which are regulated by cAMP has led to the identification of a consensus sequence CRE motif, TGACGTCA involved in mediating the cAMP response (Yamamoto et al., 1988). The CRE-1 of the PEPCK promoter differs from this consensus at a single position (TTACGTCA), at -89. Proteins present in rat liver nuclei are capable of binding to a synthetic oligonucleotide which contains the sequence of this region of the PEPCK promoter and this oligonucleotide is able to convey cAMP regulation when linked to a neutral promoter (Bokar et al., 1988). Oligonucleotides which contain specific mutations within the CRE sequence have a markedly lower affinity for this factor(s) and possess a diminished ability to convey cAMP regulation (Bokar et al., 1988).

Four of these protein binding domains (CRE-1, CRE-2, P3, P4) appear to bind similar factor(s) based on the ability of synthetic oligonucleotides containing sequence from each of these regions to compete for the binding of factors to each of the binding domains. C/EBPα is able to bind to each of
these sites. Recent work suggests that there is a family of related proteins with C/EBP-like binding specificity. CREB has also been shown to bind to the CRE sequences of the PEPCK promoter. This transcription factor has been shown to be a member of a large family of related proteins which are able to bind to CRE-like sequences. Both C/EBPα and CREB contain the leucine zipper structural motif involved in protein dimer formation (Vinson et al., 1989). In this report, changes in the binding of both C/EBPα and CREB to the PEPCK promoters containing base substitutions were shown to correlate with alterations in the cAMP responsiveness of these promoters. Experiments designed to determine whether C/EBPα and CREB can form heterodimers have not provided evidence for heterodimer formation. However, other members of the C/EBPα and CREB families may be capable of heterodimer formation (Hai et al., 1989). It seems likely that the cAMP regulation of PEPCK gene expression is controlled by a mechanism which is more complex than the alteration of the activity of a single transcription factor.

It is interesting that the alteration of a
single base in CRE-2 within the PEPCK promoter results in a significant increase in its responsiveness to cAMP. The increased cAMP responsiveness suggests that the two CRE's act synergistically. At 10.5 bases per helical turn, the two CRE's are separated by five helical turns, so that proteins which bind to CRE-1 and CRE-2 lie on the same face of the helix. This relationship may be important in achieving the synergistic cAMP stimulation of transcription through the two CREs. The two elements seem to bind to nuclear proteins independently (Figure 3.2) suggesting that cooperative binding of factors to the DNA is not involved in mediating the synergistic action of multiple CREs. It is conceivable that CRE-2 represents a cryptic element which may have degenerated in the course of the evolution of the PEPCK promoter. The demonstration that by increasing the binding affinity of this site, the function of the promoter can be modified suggests that it may be possible to alter the function of other promoters in an analogous manner. At this time, it is not possible to assemble a synthetic promoter from a collection of short cis-acting elements and accurately predict how the promoter will function. The ability to modify the
degree of hormonal regulation of a promoter by single base substitution while preserving the overall promoter architecture may prove to be a useful approach for developing promoters which meet the specific needs of effective gene therapy.
Chapter IV. Fos and Jun are able to Bind to the PEPCK Promoter and Modulate the Rate of PEPCK Transcription

A. Overview

Since the initial discovery that Fos and Jun, products of the c-fos and c-jun proto-oncogenes, bound to specific DNA sequences and modulated the rate of gene transcription, a wealth of new information has suggested a central role for these proteins in the control of eukaryotic gene expression. Fos and Jun appear to be involved in the regulation of a wide variety of cell-specific genetic programs. They are rapidly induced in hepatic cells by insulin (Messina et al., 1990; Mohn et al., 1990), and following partial hepatectomy (Mohn et al., 1990). In fibroblasts, Fos and Jun are induced by platelet derived growth factor (PDGF) (Muller et al., 1984), in cells of the hippocampus, Fos and Jun levels increase in response to electrical stimulation or specific neurotransmitters (Morgan et al., 1987; Sheng et al., 1990; Morgan et al., 1991), and in aortic smooth muscle cells Fos and Jun are induced by angiotensin II
(Naftilan et al., 1990). The levels of Fos and Jun activity are markedly increased by phorbol esters (Angel et al., 1988; Curran, 1988), agents which mimic the effect of diacylglycerol, an intermediate formed by phospholipase C, which induces the activity of protein kinase C (Schalasta et al., 1990). Inhibitors of phospholipase C repress c-fos transcription (Schalasta et al., 1990). Jun/Jun homodimers and Fos/Jun heterodimers as well as other members of the Jun and Fos families have been shown to modulate the transcription of a number of genes through a common sequence element termed an AP-1 site (activator protein 1) or TRE (TPA responsive element) (Angel et al., 1987; Lee et al., 1987; Nakabeppu et. al., 1988; Rauscher et al. 1988). This sequence motif, and the related CRE motif (cAMP response element), appear to be capable of mediating transcriptional response to multiple signal transduction pathways (Hai and Curran, 1991).

In addition to binding to AP-1 sites, Fos and Jun are each involved in interactions with other proteins. Jun forms a complex with the glucocorticoid receptor, thereby damping the ability of the receptor
to bind DNA (Yarg-Yen et al., 1990; Schule et al. 1990). Jun may be capable of binding to other steroid receptors as well. Fos may also form complexes with proteins other than Jun, and can act through sequences other than the AF-1 element (Lucibello et al., 1990; Gius et al., 1990). An interaction between Jun and the steroid hormone receptors is likely to have significant ramifications in the regulation of genes of metabolic importance. An intriguing finding is the recent observation that in a variety of cell lines Jun levels are markedly induced by deprivation of a single amino acid (Pohjanpelto et al., 1990) suggesting that it may play a role in regulating the cellular response to nutritional stress.

In this chapter, I examine the possibility that Fos and Jun may be involved in mediating the rapid changes in PEPCK gene transcription. Our initial interest in examining the role of Fos and Jun in PEPCK gene transcription is based on several observations. First, it has been shown that phorbol esters acutely down regulate expression of the PEPCK gene (Chu et. al., 1986). In addition, in a number of instances in which PEPCK gene expression is known to be repressed,
Fos expression has been shown to be elevated such as following administration of insulin (Messina, 1990) or vanadate (Bosch et al. 1990), and during liver regeneration following partial hepatectomy (Milland et al. 1990; Mohn et al., 1990; Corral et al., 1985). The promoter region of the PEPCK gene also contains elements that resemble known Fos/Jun binding sites, including a CRE between -90 and -83, as well as additional upstream elements between -285 to -252 which are similar to the consensus AP-1 motif. In this chapter I demonstrate that Fos and Jun are able to bind specifically to several sites within the PEPCK promoter and that each can modulate the rate of PEPCK gene transcription.
B. Results

(1). Binding of Fos and Jun to the PEPCK promoter.

To investigate the possibility that the PEPCK promoter contains binding sites for Fos and Jun, DNase I footprint assays were conducted using purified Fos and Jun. A segment of the PEPCK promoter extending from -490 to +73 was end labeled and incubated with Fos and Jun. Protein binding was observed at three regions of the PEPCK promoter which had been shown previously to bind proteins present in rat liver nuclei (Figure 4.1A). These regions include CRE-1, a cAMP responsive element which has also been shown to be able to bind both C/EBP and CREB, as well as P2 (-170 to -150), and the P3(II)-P4 region (-285 to -252). P4 also binds members of the C/EBP family of transcription factors. Examination of the nucleotide sequence within the P3(II) and P4 regions of the PEPCK promoter indicated the presence of two distinct sequence elements which were similar to the AP-1 consensus binding sequence. This suggested that the observed footprint pattern was the result of two adjacent binding sites. To determine whether this
region contained two AP-1 binding sites, DNaseI footprint assays were conducted utilizing probes prepared from PEPCK promoter (-490 to +73), containing specific block substitutions which disrupted the potential AP-1 sites (Figure 4.1B). The PEPCK promoter which contained a mutation in P3(II) retained Fos and Jun binding within the P4 region and vice versa (Figure 1B), thus the P3(II) - P4 region contains two adjacent AP-1 sites.

To determine the relative binding affinity of Fos and Jun for specific elements in the PEPCK promoter, DNaseI footprinting was conducted over a range of protein concentrations (Figure 4.2A). The relative order of the binding affinities for Fos and Jun to these sites is CRE-1 > P3(II) - P4 > P2; however, binding to P2 is weak, and the sequence within this region does not bear any obvious similarity to the AP-1 consensus binding sequence. Jun is known to bind to DNA as a homodimer, albeit with lower apparent affinity than the Fos/Jun heterodimer (Nakabeppu et al., 1988; Halazonetis et al. 1988), due in part to greater stability of the Fos/Jun heterodimer than the Jun homodimer (Rauscher
et al., 1988). In agreement with these observations, we noted that Jun bound to the PEPCK promoter at the same locations as Fos/Jun, but with markedly lower affinity (Figure 4.2B).

(2). Activation of transcription from the PEPCK promoter by Fos and Jun.

To examine whether the binding of Fos and Jun to the PEPCK promoter was of functional significance, PEPCK-CAT was cotransfected into HepG2 cells, together with expression vectors encoding Fos or Jun cDNA. These vectors (CMV-Fos and CMV-Jun) contain the entire open reading frame of either Fos or Jun downstream of the CMV promoter. CMV-Jun markedly increased transcription from the PEPCK promoter (Figure 4.3). In contrast, transfection of CMV-Fos inhibited transcription from the PEPCK promoter and Fos expression blocked the expected increase in transcription caused by Jun, in a concentration dependent manner (Figure 4.3). The role of each of the protein binding domains of the PEPCK promoter in the stimulation of transcription by Jun was determined by transfecting the PEPCK promoter containing defined block mutations together with CMV-Jun (Figure 4.4).
Mutation in CRE-1, P3(11) or P4 greatly reduced the ability of Jun to stimulate transcription from the PEPCK promoter. Fos inhibition of the stimulation of the PEPCK promoter by Jun was retained when the promoter contained a block mutation in CRE-1 (Figure 4.4). Interestingly, P3(1), a C/EBP binding domain immediately 3' to P3(II), was also required for full activation of PEPCK transcription by Jun and for Fos inhibition. Transcription from the promoter containing a block mutation in the P3(1) was increased in a synergistic manner by the combined action of Jun and Fos.

(3). The effect of mutant Fos and Jun proteins on PEPCK transcription.

The ability of Fos to act in a dominant negative manner to block the transactivation of PEPCK gene transcription by Jun led us to further examine the regions of the Jun and Fos proteins which were required to mediate these effects. In collaboration with Marta Giralt, PEPCK-CAT was cotransfected with expression vectors for Fos and Jun which contained defined deletions. Mutations within the DNA binding domain (Jun ΔBR) or the leucine zipper region (Jun
ΔL3) of Jun, markedly reduced the ability of CMV-Jun to transactivate PEPCK-CAT (Figure 4.5). Similar mutations within the binding region or the leucine zipper region of Fos attenuated the ability of Fos to block transcriptional activation of the PEPCK-CAT gene by Jun. However, substantial deletions from the amino terminus (Fos 102-308, lacking the first 101 amino acids), or the carboxyl terminus (Fos 1-258, lacking the last 50 amino acids) of Fos did not eliminate its ability to reduce Jun stimulated transcription from the PEPCK promoter. Thus, the DNA binding and leucine zipper domains of both Fos and Jun are required to mediate their effects on PEPCK transcription.

(4). Fos blocks the induction of PEPCK transcription by cAMP.

The binding of Fos/Jun to the CRE-1 region of the PEPCK promoter led me to examine the relationship of these two proteins to the cAMP regulation of PEPCK gene transcription. The addition of 8 Br-cAMP to the hepatoma cells which had been transfected with PEPCK-CAT did not enhance transcription from the PEPCK promoter over that noted with Jun alone. However, transfection of PEPCK-CAT together with CMV-Fos
resulted in complete loss of the stimulation of the transcription of PEPCK-CAT by 8-Br cAMP (Figure 4.6A). In the presence of Fos, 8-Br-cAMP actually decreased transcription from the PEPCK promoter. Previous work has suggested that the modest stimulation of PEPCK-CAT transcription by 8 Br-cAMP may be a reflection of limiting amounts of protein kinase A in HepG2 cells (Liu et al. 1991). Experiments in which PEPCK-CAT was transfected with SRα-PKA, a vector which contains the cDNA of the catalytic subunit of protein kinase A driven by the SRα viral promoter, demonstrated that PEPCK expression can be stimulated over 10 fold by protein kinase A. Fos expression greatly reduced but did not eliminate this stimulation (Figure 4.6B). This inhibition of transcriptional stimulation of the PEPCK promoter by PKA was substantial at the lowest amount of Fos expression vector tested.
C. Discussion

The ability of Jun and Fos/Jun to bind to the PEPCK promoter was predicted based on the presence of several sites in the PEPCK promoter which have homology to the Jun/AP-1 binding sites present in the promoters for the genes coding for collagenase (Angel et al. 1987), SV40 (Lee et al. 1987), metallothionein IIA (Lee et al. 1987), and proenkephalin (Sonnenburg et al., 1989). Transcription of these three genes is stimulated by phorbol esters. In contrast, PEPCK gene transcription is markedly inhibited by phorbol esters. A unique aspect of the regulation of PEPCK gene transcription by Jun is the fact that Fos completely blocks the stimulatory effect of Jun without diminishing its binding to the PEPCK promoter. In fact, the Fos/Jun heterodimer has a higher affinity for specific elements in the PEPCK promoter than does the Jun/Jun homodimer. Jun is one of several transcription factors which have now been shown to be capable to bind to CRE-1 in the PEPCK promoter and to also stimulate transcription when introduced into hepatoma cells. The specific regions of the PEPCK promoter which bind to Fos and Jun have previously
been shown to be important for the regulation of PEPCK gene transcription by hormones, most notably by cAMP and thyroid hormone (Giralt et al., 1991). If the negative effect of Fos on transcription of the PEPCK gene is of physiological significance, it could provide a clue to the major regulatory interaction occurring between cAMP and insulin in the transcription of the PEPCK gene.

A major question is whether Fos contributes to the effect of insulin on the PEPCK gene. Insulin has been described as the major and dominant regulatory agent in controlling transcription of the PEPCK gene (Granner et al. 1983). Other, non-physiological compounds such as vanadate, (Bosch et al. 1990), phorbol esters (Chu et. al., 1986) and lithium (Bosch and Hanson, unpublished observations) also block transcription of the PEPCK gene in hepatoma cells. Recently, we have shown that protein kinase C, when transfected into hepatoma cells, blocks the stimulatory effect of 8-Br-cAMP on transcription from the PEPCK promoter. It is interesting to note that all of these agents have also been shown to increase c-fos gene expression (Bosch et al., 1990; Curran
1988; Schalasta et al. 1990) suggesting a possible link between Fos and the action of insulin on PEPCK gene expression.

The gene for c-fos is known to be rapidly induced in hepatic cells by insulin (Messina 1990; Mohn et al. 1990), which is consistent with Fos playing a role in the regulation of PEPCK transcription. The induction of c-fos is the most rapid known effect of insulin on gene transcription (Messina 1990). However, c-fos gene expression can also be induced by cAMP (Squinto et al. 1989), the major positive regulator of PEPCK gene expression. In explaining this apparent contradictory effect of c-fos, it is necessary to separate the short and long term effects of cAMP on PEPCK gene expression. The administration of cAMP to animals results in rapid stimulation of PEPCK gene transcription. However, there is a marked attenuation of the stimulatory effect of the cyclic nucleotide on transcription with continued exposure to the cyclic nucleotide (Lamers et. al., 1982). In fact, transcription of the PEPCK gene becomes refractory to added cAMP (Sasaki et al., 1984). It is possible that this attenuation in PEPCK
gene transcription is related to the cAMP mediated induction of Fos within the cell. In this chapter we demonstrate that Fos expression in hepatoma cells will totally block the induction of transcription from the PEPCK promoter by cAMP, the C subunit of PKA, and Jun. Fos is the first and only known transcription factor which has been linked to the negative regulation of the PEPCK promoter. The full mechanism of insulin action on PEPCK transcription is complex, and at least a part of the effect of insulin is mediated by elements upstream of the Fos/Jun binding domains (O'Brian et. al., 1990). None the less, the inhibition of PEPCK transcription by Fos could provide a model for studying the dominant inhibition of this highly regulated gene.

The ability of Fos to inhibit PEPCK expression depends upon the presence of P3(1), a region which does not bind Fos/Jun heterodimers. P3(1) binds to members of the C/EBP family, and expression vectors for C/EBP transactivate transcription from the PEPCK promoter through this sequence. Recent work has shown that C/EBPβ, (also known as liver activator protein or LAP), is also able to bind the PEPCK promoter with
similar sequence specificity to C/EBPα (see chapter V). Work is currently underway to determine the identity and relative contribution of each the proteins which bind to this region of the PEPCK promoter. It is not clear how Fos interacts with the factors which bind to P3(1) to block PEPCK transcription. Both Fos and the members of the C/EBP family contain the leucine zipper structural motif, so it is possible that Fos forms heterodimers with a protein(s) which binds directly to the P3(1) region. Alternatively, Fos may bind to a protein(s) in such a manner as to prevent subsequent DNA binding, or may act in a less direct way to alter the levels or activities of transcription factors available for binding to the PEPCK promoter. This could be similar to recent models proposed for the interaction of the glucocorticoid receptor with Jun (Yang-Yen et al. 1990, Schule et al. 1990), in that Fos may function as an adaptor to modify the function of pre-existing factors.

Fos has been shown to repress expression of a variety of genes (Gius et al. 1990, Wilson et al. 1988). Analysis of the repression by Fos of Egr-1, an
immediate early gene from adenovirus, that is inducible by growth factors, identified a target sequence which was distinct from the consensus AP-1 sequence (Gius et al. 1990). Jun did not appear to be involved in the repression of Egr-1 expression by Fos. The ability of Fos to inhibit transcription was dependent upon the C-terminal 40 amino acids, and did not depend upon the DNA binding domain or the leucine zipper region. This differs from the results presented here in that the DNA binding and leucine zipper regions of Fos are clearly required for its ability to inhibit PEPCK transcription.

Both Fos and Jun are members of a family of related transcription factors, containing the leucine zipper motif, and can interact with each other to form homo- or heterodimers (Nakabeppu et al. 1988; Cohen and Curran, 1988; Cohen et al. 1989; Nishina et al. 1990; Zerial et al. 1989). In addition to Fos and Jun, it is possible that other members of this family may also regulate PEPCK gene transcription. However, Jun D and Fra-1, (Fos related antigen 1) did not alter transcription from the PEPCK promoter in experiments in which expression vectors for these proteins were
introduced into hepatoma cells together with the PEPCK promoter. Other leucine zipper transcription factors, including C/EBPα, C/EBPβ and CREB, can also bind to CRE-1 in the PEPCK promoter and the possibility exists that many of these proteins may function as heterodimers.

The levels of Fos and Jun can be regulated by phospholipase C activity through changes in the level of diacylglycerol, which allosterically activates protein kinase C (Schalasta et al. 1990). Both the cAMP and the diacylglycerol-phosphotidyl inositol second messenger systems can alter PEPCK transcription through specific cis-acting elements located within a few hundred base-pairs upstream of the transcription start site. These two pathways interact at the PEPCK promoter through competition for shared cis-acting sequences, such as CRE-1, which are capable of binding to a surprisingly large number of distinct proteins. There are reported examples of cross-talk between the signal transduction pathways. Examples include the cAMP induced translocation of protein kinase C into the nucleus (Chambier et al. 1987) and the phorbol ester induced phosphorylation of adenylate cyclase.
(Yoshimasa et al. 1987). The pivotal role of P3(1) in mediating both the stimulation of transcription by cAMP and thyroid hormone and the inhibition of transcription by Fos suggests that there are likely to be additional levels of interaction including direct protein-protein contacts between proteins bound to distinct regions of the PEPCK promoter or competition for utilization of specific transcription factors at elements in the promoter.
Chapter V. Identification of Binding Sites for Thyroid Hormone Receptor and C/EBPβ within the PEPCK Promoter.

A. Overview

Footprint analysis of the PEPCK promoter has indicated the presence of at least eight distinct regions of the PEPCK promoter which are protected by proteins present in extracts prepared from rat liver nuclear extracts. A full understanding of the mechanisms by which PEPCK expression is controlled will require the elucidation of the identity and function of the factors which interact with each of these regions. In addition to Fos, Jun, C/EBP, and CREB, several other transcription factors have been observed to interact with the PEPCK promoter during the course of my thesis research. This chapter presents the evidence generated in collaborative efforts with Dr. Marta Giralt and Dr. Edwards Park that both the thyroid hormone receptor, and C/EBPβ (also known as LAP (Liver Activation Protein)) are able to interact with the PEPCK promoter and modulate the rate of PEPCK gene transcription.
B. Thyroid hormone

Thyroid hormone is known to have major effects on a wide variety of metabolic processes in vertebrate animals, including effects on growth and development, metabolism, and temperature regulation. The hormone acts to alter body temperature through increases in oxygen and energy consumption (Glass et al., 1990). Thyroid hormone has major effects on both skeletal and cardiac muscle, altering expression of a variety of muscle specific genes including members of the myosin heavy chain family as well as the sarclemmal calcium ATPase (Izumo et al., 1988, Rohrer et al, 1988). The effects of thyroid hormone on metabolic pathways are varied. The hormone promotes increased synthesis of carbamyl phosphate synthetase, the rate limiting enzyme in the urea cycle. It also modulates HMG CoA reductase, the rate limiting enzyme in cholesterol synthesis. Thyroid hormone increases the expression of malic enzyme, alkaline phosphatase, Na⁺/K⁺ ATPase, growth hormone and numerous other genes (Glass et al, 1990). It was demonstrated in this laboratory that T3 (3,5,3'-L-triiodothyronine, thyroid hormone) increases
the rate of hepatic PEPCK gene transcription *in vivo* (Loose et al., 1985).

The recent identification of the gene encoding the thyroid hormone receptor as a cellular homolog of the v-erbA gene has permitted rapid advances in our understanding of the mechanisms of thyroid hormone action (Thompson et al., 1987). The encoded protein was found to contain limited homology to the glucocorticoid and estrogen receptors particularly within the proposed DNA binding domain, as well as substantial homology with the retinoic acid receptor. These receptors are thought to act to modify the expression of target genes through interaction of the receptor with sites within the promoter-regulatory region of these genes.

Detailed studies of the promoter elements involved in thyroid regulation of gene transcription have thus far involved only a limited number of T3 responsive genes. The PEPCK promoter offers an excellent model system for the characterization of thyroid responsive elements due to the extensive work which has gone into the characterization of the promoter and the availability of valuable promoter
deletions and mutations. Numerous studies have documented the ability of thyroid hormone to induce PEPCK transcription in vivo and in isolated hepatocytes and hepatoma cell lines (Loose et al., 1985; Iynedjian and Salvert, 1984; Hoppner et al., 1986, Muñoz et al., 1990). It has also been noted that T3 potentiates the action of cAMP. The features of the PEPCK promoter which enable this responsiveness have not previously been examined.

The sequences involved in the regulation of PEPCK by thyroid hormone have been identified through a collaborative effort in our laboratory led by Dr. Marta Giralt (Giralt et al., 1991). Analysis of the hormone responsiveness of PEPCK promoters containing 5' deletions identified a region of the PEPCK promoter necessary for thyroid hormone responsiveness located between -355 and -277. Examination of the sequence within this region indicated the presence of an element which exhibited a 10 out of 12 basepair identity with a consensus thyroid response element based on examination of several genes known to be regulated by thyroid hormone (Figure 5.1). Further, footprint analysis revealed that this region was
protected by factors present in rat liver nuclear extract. To further demonstrate the role of this putative TRE (thyroid response element) in the thyroid hormone regulation of PEPCK transcription several approaches were undertaken. The ability of this element to act as a TRE was confirmed by testing its ability to convey thyroid regulation upon a neutral promoter. We prepared a chimeric gene containing two copies of a synthetic oligonucleotide containing this sequence upstream of the promoter in SV1CAT (this vector contains the CAT structural gene driven by a derivative of the SV40 early promoter which retains only the basal promoter elements (Bokar et al., 1988)). Transcription of this chimeric gene was found to be stimulated several fold by thyroid hormone when introduced into HepG2 cells. The parent SV1CAT gene was not stimulated by thyroid hormone. This demonstrates that the putative TRE identified through the analysis of PEPCK promoters containing 5' deletions was not only necessary but also sufficient to convey thyroid hormone responsiveness. Additional experiments conducted by Marta Giralt demonstrated that the protein species which bound this element in gel shift analysis could also bind to an
oligonucleotide containing the sequence of the consensus TRE. Further, it was possible to demonstrate, using $^{125}$I labelled T3 that the protein which was observed to bind to this element in gel shift analysis bound thyroid hormone and generated a DNA-protein complex with identical electrophoretic mobility to that observed with the oligonucleotide containing the consensus thyroid response element. Finally, I was able to show through the use of the footprint technique that not only would the oligonucleotide containing the consensus TRE compete for the binding of factors to the TRE we had identified within the PEPCK promoter, but that this was the only region of the PEPCK promoter for which the binding of factors could be competed by either the consensus TRE or the putative PEPCK TRE (Figure 5.2). This suggests that there is only a single TRE within the PEPCK promoter and that it is located between -332 to -308.
C. C/EBPβ

It is now known that C/EBPα (formerly C/EBP) is one member of a family of several related proteins (Cao et al., 1991). A second member of this family has been repeatedly cloned by a number of researchers and has been variously termed NF-IL6, LAP, IL6-DBP, AGP/EBP, and most recently C/EBPβ (Akira et al., 1990; Descombes et al., 1990; Poli, et al., 1990; Chang et al., 1990; Roman, et al., 1990; Cao et al., 1991). C/EBPα and C/EBPβ have both substantial similarities and significant differences. The two proteins have highly similar leucine zipper regions and DNA binding domains. The sequence specificity of DNA binding appears to be very similar. On the other hand, the amino two thirds of the proteins do not share any discernable homology. The amino terminal domains are thought to be responsible for much of the ability of these proteins to modulate transcription. Thus, while the proteins are clearly related, the potential exists for these proteins to perform markedly different functions within the cell.

To determine whether C/EBPβ binds to sequence
elements within the PEPCK promoter region, footprint analysis was performed using purified protein produced in a bacterial expression system, provided to us by Dr. Steven McKnight. As is shown in Figure 5.3, C/EBPβ binds to the PEPCK promoter at the same positions as C/EBPα. The pattern of DNaseI protection obtained with the two proteins is virtually identical. Further, titration experiments designed to determine the relative affinity of the multiple binding sites for C/EBPβ produced results similar to those previously obtained with C/EBPα. For both proteins, CRE-1 and P3(I) displayed the greatest affinity, P4 displayed intermediate affinity and CRE-2 displayed the lowest affinity.

The ability of C/EBPβ to alter the rate of transcription from the PEPCK promoter was analyzed in cotransfection experiments utilizing a C/EBPβ expression vector. These experiments, performed by Dr. Edwards Park, demonstrated that C/EBPβ activates PEPCK transcription in hepatoma cells. The degree of stimulation was at least as great as that observed in similar experiments with C/EBPα. However, the ability of C/EBPβ to activate transcription from the PEPCK
promoter was dependent upon CRE-1 and was independent of the upstream protein binding sites. This differs from the results obtained studying C/EBPa with which the ability to activate transcription was dependent upon each of the protein binding sites, particularly P3(I) and CRE-1. Additional differences were noted in experiments examining the effect of protein kinase A on PEPCK transcription in cells cotransfected with expression vectors for C/EBPa or C/EBPβ. Protein kinase A induced transcription from the PEPCK promoter shortened to -134 to +73, when cotransfected with C/EBPa (The PEPCK promoter containing a 5' deletion from position -134 retains the CRE-1 binding site for C/EBPa and C/EBPβ but does not contain any of the upstream binding sites. It was chosen to remove possible complications due to the multiple binding sites for these factors.). However, PKA did not induce transcription of PEPCK in cells cotransfected with C/EBPβ. These results support the hypothesis that the dissimilar amino terminal domains of the two proteins may impart unique regulatory features upon the proteins. Similar experiments designed to examine the effect of protein kinase A on the activation of transcription from the PEPCK promoter by CREB
demonstrated that the ability of CREB to activate PEPCK transcription was dependent upon protein kinase A and the degree of stimulation was twice that noted with C/EBPα.

The observation that multiple proteins bind to the CRE-1 region of the PEPCK promoter prompts the question: Which protein is bound in vivo? Unfortunately, there are no techniques currently available that are able to determine the identity of a specific factor bound to a specific DNA sequence element within a mammalian cell. Therefore, we pursued two indirect approaches that could provide some information on the relative ability of C/EBPα and C/EBPβ to interact with the PEPCK promoter. The first approach utilized antibodies specific to unique regions of the C/EBPα and C/EBPβ proteins. We examined the ability of these antibodies to disrupt or retard the mobility in gel shift analysis of complexes formed between proteins present in nuclear extracts prepared from rat liver and an oligonucleotide containing the sequence of the CRE-1 region of the PEPCK promoter. I observed that antibodies to C/EBPα did not significantly alter the complexes observed in
gel shift assays formed between CRE-1 and liver nuclear proteins. This observation was repeated by Dr. Steve Nizielski in our laboratory. However, in experiments performed with antibodies to C/EBPβ he observed that 30-40 percent of the protein-CRE-1 complex could be retarded. These experiments suggest that in rat liver CRE-1 might bind C/EBPβ in preference to C/EBPα. There are several potential limitations to this interpretation. The binding conditions employed in the gel shift analysis are undoubtedly quite different from the local environment experienced by the CRE-1 region of the promoter in vivo. In addition, the gel shift technique does not allow for the possible contributions of proteins bound to other regions of the promoter in recruiting specific proteins to the CRE-1 region. The isolation of nuclear proteins may not be equally efficient for each protein species, and post-translational modifications that modulate protein function may be lost during the isolation process. In this regard, nuclear extracts have been found to contain substantial phosphatase activity which may dephosphorylate relevant protein species (Dr. Dwight Klemm, unpublished observations).
A second approach undertaken to examine the relative roles of C/EBPα and C/EBPβ involved determination of the binding affinity of these proteins for CRE-1. The technique employed utilized the gel retardation assay using a constant amount of protein and a range of DNA-probe concentrations. The bound and free bands that were obtained were excised and the radioactivity of the labeled DNA probe was determined. Scatchard analysis was used to obtain the $K_a$ of binding (see Figure 5.4A and 5.4B). The results of these experiments indicate that C/EBPβ binds to CRE-1 with greater affinity than C/EBPα. These results are similar to recent results obtained in Steven McKnight's laboratory (Cao et al., 1991). They determined the affinity of C/EBPα and C/EBPβ for an "optimal" C/EBPα binding site essentially as I have done, and found that C/EBPβ had a greater affinity for this "optimal" C/EBPα site than did C/EBPα. These results are also compatible with the results obtained with the antibody experiments. The caveats which limit the ability of this technique to predict the situation in vivo are similar to those described for the antibody experiments. None the less, both approaches suggest that C/EBPβ is able to avidly
interact with the PEPCK promoter and thus may be involved in PEPCK regulation.

Determinations of CRE-1 binding affinity were made for Fos/Jun, CREB, C/EBPα and C/EBPβ (Figure 5.4). The results of this analysis indicate that C/EBPβ, CREB, and Fos/Jun all bind to the CRE-1 region with similar affinity, with a $K_d$ on the order of $10^{-10}M$. The affinity of each of these factors is equal within the resolution of these experiments. This may have significant implications for our understanding of how the PEPCK promoter functions. If promoter elements such as CRE-1 are able to bind to multiple protein species with similar affinity, then the determination of which protein is actually bound in vivo is either a reflection of mass-action considerations or is a consequence of interactions with other components of the promoter environment. However, the conditions experienced by the proteins in vivo are obviously different from those employed in these studies, thus the proteins may actually possess different affinities for CRE-1 in vivo.
Chapter VI. Discussion

A. Overview

The overall goal of the work presented within this thesis has been to further our understanding of the PEPCK promoter. Identification of factors which can interact with the PEPCK promoter and elucidation of the function of the cis-acting elements within the promoter are essential components of this process. At the time when this thesis work was initiated, no transcription factors had been shown to bind to the PEPCK promoter. The schematic shown in Figure 6.1 summarizes much of the current understanding of the structure of the PEPCK promoter region. It is readily apparent that the promoter region is an extremely complicated arrangement of protein binding sites. This discussion presents a consideration of the features of this promoter that enable the PEPCK gene to be appropriately expressed. In particular, the observation that hormonal response patterns of the PEPCK promoter require the presence of multiple cis-acting elements is discussed. This may underlie important protein-protein interactions. The possible role of "linking" proteins in coordinating the action
of multiple transcription factors is discussed. The hormonal regulation of PEPCK gene expression is discussed with consideration of the significance of the finding that many of the protein binding elements of the PEPCK promoter are able to interact with multiple transcription factors. Finally, the discussion concludes with a consideration of future directions that could be undertaken to further our understanding of PEPCK gene regulation.

B. Promoter Architecture

The large number of protein binding sites within the PEPCK promoter could potentially enable significant protein-protein interactions to occur. This might enable an integrated response by the PEPCK promoter to the multitude of diverse signals which impact the rate of PEPCK transcription. Alternatively, it is possible that such interactions do not occur, or are not of great significance to the overall function of the PEPCK promoter. Accordingly, the PEPCK promoter could be considered as a collection of cis-acting elements, each able to interact with
specific transcription factors, and each acting relatively independently to promote or repress PEPCK transcription. Within this model, the interaction between the various elements might be restricted to competition for the "attention" of the general initiation factors of the transcriptional machinery.

Evidence suggesting that this model is an oversimplification and that there are likely to be some coordinating interactions between factors bound to distinct regions of the PEPCK promoter is provided by the observation that many of the hormonal response patterns of the PEPCK promoter require the presence of multiple cis-acting elements. The analysis of the single base substitution within CRE-2 which altered the sequence to resemble CRE-1 demonstrated clearly that multiple elements can provide a synergistically greater responsiveness to cAMP than can a single element. Interestingly, each of elements of the PEPCK promoter that are involved in cAMP regulation are positioned such that the binding sites lie on the same face of the helix. Induction of transcription by thyroid hormone involves binding of the thyroid receptor to sequences between -332 and -308, and interaction with the protein bound at P3(1) within the
PEPCK promoter (Giralt et al., 1991). P3(1), which has previously been shown to bind members of the C/EBP family of transcription factors, seems to play a central role in PEPCK promoter function, being involved in both thyroid regulation and cAMP regulation, as well as the tissue specific expression of the PEPCK gene in the liver (Patel and Hanson, unpublished observations). The ability of Jun to induce PEPCK transcription is dependent upon both the P3(II)-P4 region and CRE-1. In each of these cases, and in several other similar situations, multiple regions of the PEPCK promoter act in an apparently synergistic manner to mediate the transcriptional response. This may be due to cooperative binding of factors to these regions of the promoter. Alternatively, factors which bind to the promoter independently may subsequently interact in a simultaneous fashion with other elements of the transcriptional machinery to bring about a change in transcription. Footprint analysis was utilized to examine the effect of disruption of the P3(1) binding region on the binding of factors to each of the other binding sites within the PEPCK promoter. In collaboration with Dr. Michael Brenowitz, careful
titration of nuclear extract into footprint assays was performed. The observed binding of factors present in nuclear extracts to each of the binding sites within the native PEPCK promoter and the promoter containing the mutation in P3(1) were indistinguishable. Thus, these experiments did not produce evidence of cooperative binding between factors binding to P3(1) and factors interacting with other regions of the promoter. It is possible, however, that cooperative binding does occur in vivo but is not observed under the conditions employed in the footprint analysis. It is also possible that cooperative interactions function to promote the binding of specific factors in preference to other factors which might otherwise be capable of binding to a given site.

A simple model which can account for both the observed synergistic interaction of multiple promoter elements and the conservation of promoter architecture is depicted in Figure 6.2. In this model, a hypothetical "linking" protein acts to mediate the transmission of signals derived from multiple factors bound to the DNA to the transcription initiation complex. Such a model is attractive for several
reasons. If such a protein is required to interact with multiple bound factors in order to function, this could explain why many hormonal response patterns of the PEPCK promoter seem to require multiple promoter elements. It might also explain some of the conservation of promoter organization observed between the chicken and the rat promoter. Presumably there would be constraints on the positions within the promoter region that transcription factors could occupy that permit optimal interaction with such a linking protein. The interactions of such a linking protein with a transcription factor might be weak and transient in nature. Simultaneous interactions with multiple transcription factors might help stabilize the interaction of the linking protein with the PEPCK promoter. Thus, cooperative binding of the linking protein might offer a mechanistic explanation for the observation that multiple regions of the promoter seem to act synergistically to mediate many hormonal responses. Transient interaction with the promoter may also facilitate frequent re-coordination of the linking protein with different sets of transcription factors bound to different regions of the promoter. Alternatively there may a family of such proteins each
possessing distinct factor binding specificity and distinct capacities to alter transcription. This type of model has, as a consequence, the prediction that the rate of transcription from a given PEPCK promoter will vary with time depending upon the specific set of transcription factors that are actively involved in interactions with a linking protein(s) at a given point in time. The overall rate of PEPCK gene transcription in a cell population would then be a reflection of the average rate of transcription in the population.

Identification of the proteins that interact with the transcription factors bound to the DNA is a critical next step in the elucidation of the mechanisms by which transcription is controlled. There is some evidence that such proteins may exist. The adenovirus protein E1A is known to modulate the rate of transcription from promoters within the adenovirus genome as well as from a variety of cellular genes. The E1A protein is not able to bind to DNA directly. It has, however, been shown to possess the ability to bind to several members of the CREB/ATF and Jun families of transcription factors.
ELA is thought to alter transcription through interactions with both transcription factors such as CREB bound to upstream enhancer elements and basal transcription factors bound to the TATA box promoter element. ELA has also recently been found to form a complex with a protein kinase (Kleinberger et al., 1991). The function of this kinase activity is not known, but it may act to modify transcription factors. It is interesting to note that ELA has recently been shown to be capable of markedly repressing the rate of PEPCK gene transcription (Kalvakolanu et al., in press.). Recently, several groups have reported evidence for the existence of proteins which act to facilitate the ability of transcription factors to modify the rate of gene transcription (Flanagan et al., 1991; Dynlacht et al., 1991). These proteins, variously termed mediators or coactivators, are thought to act between an enhancer protein and general initiation factors and may prove to be similar to the "linking" protein I have proposed. It is interesting to note that recent studies of TFIID, the "TATA" binding factor, have found it to be part of a large multisubunit complex. Gel filtration and glycerol sedimentation studies suggest a molecular weight in
excess of 700 kDa (Pugh and Tjian, 1990; Tanese et al., 1991; Conaway et al., 1991). The ability of enhancer proteins to activate transcription in vitro has been shown to be dependent upon as of yet uncharacterized components of this complex (Pugh and Tjian, 1992). Another viral protein, TAX, encoded by the HTLV-1 virus, shares with E1A the capacity to bind to transcription factors such as CREB and alter the rate of gene transcription as well as the inability to bind DNA directly. Moreover, recent experiments utilizing gel shift analysis and purified proteins have demonstrated that TAX containing complexes are only observed with DNA fragments containing more than one CREB binding site (Zhao and Giam, in press). Thus, the properties of this protein are quite similar to properties proposed for the hypothetical "linker" protein in the model presented above. It will be of great interest to identify possible cellular homologs of this protein.

C. Hormonal Regulation

The past several years have seen considerable progress towards mapping the cis-acting elements involved in the hormonal regulation of a number of
eukaryotic promoters (For review Roesler et al, 1988, Ptashne, 1989; Mitchell and Tjian, 1989; Berk and Schmidt, 1990). Among these, the PEPCK promoter has been among the most intensively studied. It has been an excellent model system for a number of reasons. The reaction catalyzed by PEPCK is of obvious importance as a regulated step within a key metabolic pathway. As a consequence, the gene for PEPCK exhibits tissue specific and developmental regulation as well as significant and rapid alterations in expression in response to a wide variety of hormonal signals. We have now reached a point where it is possible to describe in rough outline many of the features of the promoter regulatory region which direct expression the PEPCK gene. For some signals, such as thyroid hormone, the intracellular pathway is relatively direct. Thyroid hormone binds to a receptor that is able to directly bind to DNA sequences within the PEPCK promoter. For other signaling systems, the pathway is more complicated. Cyclic AMP has been shown to mediate is effect on gene transcription through the action of the catalytic subunit of protein kinase A (Mellon et al., 1989). Whether protein kinase A directly catalyzes the activation of
transcription factors or instead acts to stimulate other kinase activities that then are able to alter transcription factors is not clear. CREB, a transcription factor shown to be capable of mediating a response to cAMP, has been shown to be a substrate for protein kinase A in vitro (Gonzalez, 1989; Lee et al., 1990). However, the mechanism of regulation of PEPCK gene transcription by cAMP is more complicated than phosphorylation of a single factor. Multiple regions of the promoter are involved and these regions are each able to interact with multiple factors. It is therefore likely that the mechanism of action of cAMP involves both short term actions mediated by the phosphorylation of existing factors and longer term changes mediated by changes in the relative abundance of factors.

The observation that the factors which are already known to bind to CRE-1 do so with relatively similar affinities may reflect important features of promoter function. Most of the transcription factors which have been shown to bind the PEPCK promoter are now known to be members of multigene families. Many are members of the leucine zipper super-family and may
also function as heterodimers. A conservative estimation of the number of protein species which can bind to the eight base sequence of CRE-1 might easily be over a dozen. If this degree of complexity is realized in vivo, then it is necessary to modify our conception of promoter function away from a "one hormonal response-one transcription factor" model and towards an understanding of the PEPCK promoter that recognizes the contribution of an interrelated network of factors in mediating a given hormonal "response". The actions of cAMP, for instance, may be mediated through changes in the phosphorylation state of multiple factors as well as changes in the abundance of certain factors. Changes in phosphorylation may alter the the ability of these factors to form heterodimers. It is reasonable to suspect that other hormonal signals might also lead to alterations of some of the same factors that are affected by cAMP, and thus the response to cAMP would be influenced by the impact of each of the other hormonal signals. Both Fos and Jun are subject to complex patterns of regulation in response to a variety of stimuli. The ability of Fos and Jun to bind to CRE-1 provides evidence that signal transduction pathways are able to
interact through competition for shared protein binding sites within the PEPCK promoter. Further, the ability of Fos to block the stimulation of PEPCK transcription by Jun suggests the potential power of leucine zipper proteins to form heterodimer transcription factors with markedly different properties than the component species possess as homodimers.

D. Future Directions

The long term goals of the study of PEPCK gene regulation should include the pursuit of an understanding of the molecular mechanisms by which "information" is processed and utilized to generate a "response". The current level of understanding of these mechanisms suggests that, in general, information transfer is mediated by protein-protein interactions. However, each new insight into the mechanisms by which this gene and others are regulated has also brought with it the discovery of potential complexities that were greater than anticipated. Thus, it is reasonable to suppose that aspects of PEPCK gene regulation may be mediated by mechanisms that have not yet been fully considered. There may be
significant roles played by a variety of factors that have not been well studied including: chromatin structure, local DNA structure, small diffusible molecules (such as NADH, sugars, free radical compounds, lipids, etc.), or yet uncharacterized enzymatic activities.

Further progress towards understanding the mechanisms of PEPCK gene regulation will require the development of new techniques to resolve the identity of factors interacting with the promoter in vivo. It is only when we have the ability to in some way record the actual interaction of a specific factor with the promoter in vivo that we will be able to dissect the specific interactions that are relevant to the regulation of the promoter. Knowledge of the identity of transcription factors that are bound to the promoter in vivo would greatly facilitate the development of in vitro transcription systems. Moreover, this knowledge would help permit a reasoned approach to the study of how specific protein kinase and phosphatase activities act to alter the rate of transcription.

The PEPCK promoter is likely to continue to be
an attractive model system for the study of eukaryotic gene regulation. This field has seen many dramatic advances. If the past is any guide, the future will be fascinating.
References


Brent, G. A., Larsen, P. R., Harney, J. W., Koenig, R.


Imai, E., Stromstedt, P., Quinn, P. G., Carlstedt-


Lamars, W. H., Hanson, R. W., and Meisner H. M. (1982) cAMP stimulates transcription of the gene for cytosolic phosphoenolpyruvate carboxykinase in


Loose, D. S., Cameron, D. K., Short, H. P., and Hanson, R. W. (1985) Thyroid Hormone Regulates Transcription of the Gene for Cytosolic Phosphoenolpyruvate Carboxykinase (GTP) in Rat


Morgan, J. I., Cohen, D. R., Hempstead, J. L., Curran,


Biol. Chem. 245:5979-5984.


Roman, C., Platero, J. S., Shuma, J. and Calame, K. (1990) Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. Genes & Dev. 4:1404-1415.


Schalasta, G., and Doppler C. (1990) Inhibition of


bacteriophage. Genes and Dev. 2:801-806.


Yamamoto, K. K., Gonzalez, G. A., Biggs III, W. H. and


Figure Legends

Figure 1.1. Schematic of the known protein binding sites within the promoter of the PEPCK gene. This schematic depicts the relative positions of the protein binding sites with the promoter for the PEPCK gene. The shaded boxes represent regions of the DNA within the PEPCK promoter that were protected in footprint analysis by proteins present in nuclear extracts. The alpha-numerical symbols above the boxes are the nomenclature that was chosen to represent these binding sites. This nomenclature is described in Roesler et al. (1989). The numbers below the boxes refer to the distance in basepairs from the start site of transcription. The arrow represents the start site of transcription.
Figure 3.1. The -174 PEPCK promoter showing base substitutions. The positions of known protein binding sites, previously identified by DNaseI footprint analysis are shown relative to the initiation site of transcription within the PEPCK promoter. The sequences within CRE-1 and CRE-2. The single base substitutions characterized in this report, -140 G to C and -86 G to T are depicted above CRE-2 and CRE-1 respectively.
Figure 3.2. Titration of nuclear extracts from rat liver into footprint assay of promoters containing single base substitutions. The PEPCK sequence between −174 and +73 was isolated as an Xba I - Bgl II fragment and was end labeled at the Bgl II site with T4 Polynucleotide Kinase and [γ^{32}P] ATP. The amount of nuclear extract used in each reaction is shown above each lane. The protein binding sites are indicated by the boxes at the left.
<table>
<thead>
<tr>
<th>DNA PROBE</th>
<th>WILD TYPE</th>
<th>-86 G to T</th>
<th>-140 G to C</th>
<th>-140 G to T</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUCLEAR EXTRACT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ug)</td>
<td>0</td>
<td>.4</td>
<td>1.6</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>.4</td>
<td>1.6</td>
<td>6.4</td>
</tr>
<tr>
<td>CRE-2</td>
<td>-1.49</td>
<td>-1.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NF-1/CTF</td>
<td>-1.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRE-1</td>
<td>-0.77</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3. Comparison of the binding of rat liver nuclear proteins, C/EBPα and CREB to the intact PEPCK promoter and the PEPCK promoter containing the -140 G to C single base substitution. Both the intact PEPCK promoter (-174 to +73) and the promoter containing a G to C transversion at -140 were 5' end labeled (see Figure 3.2) and incubated with either 7 µg of rat liver nuclear extract or 50 ng of 11 kD C/EBP or .5 µg of partially purified CREB as indicated at the top of the gel. The protected regions are outlined by the boxes.
Figure 3.4. cAMP Regulation of Transcription from the PEPCK promoter modified at CRE-1 and CRE-2. A. JEG-3 cells were transfected with PEPCK-CAT fusion gene and treated with 8-Br-cAMP for eight hours. The transfection and CAT assay were performed as described in Materials and Methods. The PEPCK promoters contained 174 bases of 5' flanking sequence, and the base substitutions are indicated above each pair of CAT assays. B. The transcription in the presence or absence of 8-Br-cAMP of each of the PEPCK promoters in JEG-3 cells relative to the intact promoter containing 174 base pairs of 5' flanking sequence is shown. Each value represents the average of at least three separate experiments done in duplicate. C. The basal transcription and cAMP stimulation in HepG2 cells of each of the PEPCK promoters relative to the intact promoter containing 450 base pairs of 5' flanking sequence is shown. Each value represents the average of at least three separate experiments done in duplicate.
Figure 4.1. Footprint analysis of Fos and Jun binding sites in the PEPCK promoter. A. The XbaI-BglII fragment of the PEPCK promoter (-490 to +73) was isolated from PEPCK-CAT and end-labelled on the noncoding strand at the XbaI site. The left lane shows the DNaseI digestion pattern in the absence of protein. The middle lane shows the pattern of protection obtained in the presence of 10 μg of proteins isolated from rat liver. The right lane shows the protection pattern obtained following incubation of the probe with 1 μM Fos and Jun (1:1 mixture). The binding sites are outlined by boxes at the right. B. Promoters containing block mutations within the P3(II) and the P4 region were end-labeled as in 1A. For each probe, the DNaseI pattern obtained in the absence of protein and in the presence of 1 μM Fos and Jun (1:1 mixture) is shown.
Figure 4.2. Footprint analysis of the relative ability of binding sites within the PEPCK promoter to bind Jun homodimers and Fos/Jun heterodimers. A PEPCK promoter fragment was isolated as described in Figure 1A., and incubated with the indicated amounts of Fos and Jun. The specific conditions of the binding reaction were as described in (Abate et al., 1990).
Figure 4.3. Alteration of PEPCK transcription by expression of Jun and Fos. HepG2 cells were transfected with 5 μg PEPCK-CAT, 2 μg RSV-b-gal, and the indicated amounts of CMV-Fos and CMV-Jun. After 48 hours, the cells were harvested and the CAT activity was measured as described in Materials and Methods. The results shown are corrected for β-galactosidase activity and include standard error bars.
Figure 4.4. The effect of Fos and Jun on the level of transcription from PEPCK promoters containing block mutations. HepG2 cells were transfected with PEPCK-CAT vectors containing specific block substitutions within the protein binding domains of the promoter. The sequence alterations of these promoters containing block mutations have been described previously (Liu et al., 1990). The PEPCK-CAT vectors were transfected alone, with CMV-Fos, with CMV-Jun, or with both CMV-Fos and CMV-Jun as indicated by the + and - symbols below each column. The region of the PEPCK promoter which contains a mutation is also indicated on the horizontal axis. Transfections and CAT activity were performed as outlined in Figure 4.3.
Figure 4.5. Effect of Fos and Jun proteins containing specific mutations on PEPCK-CAT expression. HepG2 cells were transfected with 5 μg PEPCK-CAT, 2 μg RSV-βgal, and 5 μg each of expression vector for the indicated mutant Fos or Jun protein. The specific mutations within these proteins have been previously described in detail (Gentz et al., 1989) and are summarized in Procedures. CAT activity was measured as described in Figure 4.3. The results shown are the average of at least three independent transfections each done in duplicate.
**Figure 4.6. Effect of Fos on stimulation of PEPCK expression by protein kinase A and cAMP.**

**A.** HepG2 cell were transfected as in Figure 3. At 40 hours after transfection, cells were exposed to .5 mM 8 Br-cAMP for 6 hours, and then harvested. The results shown are the average of three separate experiments performed in duplicate. **B.** HepG2 cells were transfected with 5 μg PEPCK-CAT, 2 μg RSV-b-gal, and the indicated amounts of CMV-Fos and SRα-PKA. Cells were not exposed to 8 Br-cAMP. SRα-PKA is an expression vector for the catalytic subunit of protein kinase A driven by the SRα promoter to direct the expression of the open reading frame for the C subunit (Muramutso et al., 1989). After 48 hours, the cells were harvested and the CAT activity was measured as described in Figure 3. The results shown are corrected for b-galactosidase activity and include standard error bars. The results are the average of at least three independent transfections each done in duplicate.
Figure 5.1. Comparison of the sequence of the putative TRE within the PEPCK promoter with the consensus TRE sequence. The antisense strand of the PEPCK promoter between -316 and -332 is compared with the direct repeat consensus sequence described by Brent et al., (1989). Identical nucleotides are indicated by asterisks.
-316 TGGGGGTCAAGGACAGG

********* *

AGGTCAGGTCA

-322

putative TRE of rat PEPCK promoter

consensus TRE
direct repeats
Figure 5.2. Examination of thyroid hormone receptor binding by DNase I footprint analysis with oligonucleotide competition. A 564-bp XbaI-BglII fragment of the PEPCK promoter (nucleotides -490 to +73) was prepared as described in Materials and Methods. The probe was incubated with 40 μg of nuclear extract. The amount of competitor oligonucleotide added is marked in the figure. The sequence of the OPT TRE oligonucleotide is the palindromic TRE described by Glass et al. (1988).
Figure 5.3. Identification of C/EBPβ binding sites in the PEPCK promoter-regulatory region. The 563 bp XbaI-BglII fragment of the PEPCK promoter (-490/+73) was end labeled with [γ-32P]-ATP and T4 polynucleotide kinase. The probe was incubated with either no protein (-), 20 µg of proteins isolated from rat liver nuclei (RlNE), 50 ng of 11 kDa recombinant C/EBPβ protein (C/EBPβ) or 10 µg of a bacterial extract containing 43 kDa recombinant C/EBPα protein (C/EBPα). The protein added is indicated above each lane. The regions protected by rat liver nuclear extract are outlined on the left (Roesler et al., 1989).
Figure 5.4. Binding affinities of C/EBPα, C/EBPβ, Fos/Jun and CREB for CRE-1. A. The relative affinities of the transcription factors were determined by Scatchard analysis. The Scatchard plots shown were obtained from gel retardation analysis conducted with increasing amounts of labeled probe added to a constant amount of protein (see Materials and Methods). B. Shown are the $K_a$ values obtained from Scatchard analysis of the binding of C/EBPα, C/EBPβ, Fos/Jun, and CREB to an oligonucleotide containing the sequence of the CRE-1 region of the PEPCK promoter. These represent data from individual experiments. Between individual experiments, several fold variation was observed in $K_a$ values. The binding affinity of C/EBPα was consistently at least an order of magnitude lower than the affinities observed for C/EBPβ, CREB, Fos/Jun.
<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>$K_a$ of binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPα</td>
<td>$2.9 \times 10^8$ M$^{-1}$</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>$7.2 \times 10^9$ M$^{-1}$</td>
</tr>
<tr>
<td>Fos/Jun</td>
<td>$3.6 \times 10^9$ M$^{-1}$</td>
</tr>
<tr>
<td>CREB</td>
<td>$3.5 \times 10^9$ M$^{-1}$</td>
</tr>
</tbody>
</table>
Figure 6.1. A schematic of the promoter region of the PEPCK gene that depicts the relative location of known protein binding elements. This schematic depicts the transcription factors that have been shown to interact with the PEPCK promoter. The numbers below the boxes refer to the distance in basepairs from the start site of transcription. Glucocorticoid receptor and Accessory factors 1 and 2 (AF-1 and AF-2) have been found to be necessary for glucocorticoid regulation of PEPCK gene transcription (Imai et al., 1990). Interaction of TFIID with the PEPCK promoter has not yet been directly demonstrated.
Figure 6.2. A model of PEPCK promoter function incorporating a proposed "linker" protein. This schematic presents a simplified view of the PEPCK promoter that emphasizes the importance of the CRE-1 and the P3-P4 regions of the promoter in directing regulated expression. A hypothetical linker protein is depicted that is hypothesized to interact simultaneously with proteins bound to both the CRE-1 and P3-P4 regions of the promoter. These interactions are represented by the symbol "x". This linker protein is hypothesized to act to modulate the rate of gene transcription through interaction with general initiation factors bound to the DNA in the TATA region of the promoter. This activity is represented by the symbol "+". The curved line (bold type) represents the DNA of the PEPCK promoter. The ovals represent proteins bound to the DNA.