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.
Analysis of regulatory elements in the phosphoenolpyruvate carboxykinase (GTP) gene in transgenic mice

Patel, Yashomati Mulchand, Ph.D.

Case Western Reserve University, 1994
ANALYSIS OF REGULATORY ELEMENTS IN THE
PHOSPHOENOLPYRUVATE CARBOXYKINASE (GTP) GENE IN
TRANSGENIC MICE

by

YASHOMATI M. PATEL

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

Advisor: Richard W. Hanson, Ph. D.

Department of Nutrition
CASE WESTERN RESERVE UNIVERSITY
JANUARY, 1994
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

Yashomati M. Patel

candidate for the Ph.D.
degree.*

(signed) R.W. Hanson
(chair)

Edith Jirgis

David Sainsbury

date 11-29-93

*We also certify that written approval has been obtained for any proprietary material contained therein.
I grant to Case Western Reserve University the right to use this work, irrespective of any copyright, for the University’s own purposes without cost to the University or to its students, agents and employees. I further agree that the University may reproduce and provide single copies of the work, in any format other than in or from microforms, to the public for the cost of reproduction.

[Signature]

 Vishnu M. Patel
Analysis of Regulatory Elements in the Gene for the Cytosolic form of Phosphoenolpyruvate Carboxykinase from the Rat using Transgenic Mice

Abstract by
Yashomati M. Patel

The regulation of the gene for the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK) from the rat, is primarily at the level of transcription. A -460/+73 base pair segment of the PEPCK gene can direct a similar pattern of expression as the endogenous PEPCK gene. Specific protein binding domains within this region have been identified by footprint analysis. The regulatory elements in the PEPCK promoter which are responsible for its tissue-specific and hormonal regulation were investigated using transgenic mice. Chimeric PEPCK-bovine growth hormone (bGH) genes containing a -460/+73 base pair segment of the PEPCK promoter with mutations in putative regulatory regions were microinjected into fertilized eggs to produce transgenic mice. The tissue specific, developmental, hormonal and dietary regulation of the various chimeric PEPCK-bGH genes were analyzed in transgenic mice. Hepatic expression of a PEPCK-bGH gene was reduced 60% by a mutation in the P3(I) region (-248 to -239). This region binds members of the CCATT/Enhancer Binding Proteins
(C/EBP) family of transcription factors and is known to be involved in the cAMP induction of PEPCK gene transcription. Renal expression of the transgene was not detectable from a PEPCK-bGH gene with a mutation in the P2 region (-200 to -164). This region has been shown to bind Hepatic Nuclear Factor I (HNF-I), a protein that is present in both the liver and kidney. A mutation in the CRE region (-95 to -74) resulted in increased expression from a PEPCK-bGH gene in both the liver and kidney. Renal expression was increased in mice containing a PEPCK-bGH gene with a mutation in the P3(l) region.

Mutations in the CRE, P1 or P3(l) regions of the PEPCK promoter resulted in the elimination of cAMP induction of PEPCK-bGH expression in the liver. In contrast, none of the mutations in the PEPCK promoter interfered with the induction of transcription from the PEPCK promoter due to feeding mice either a diet high in protein (64% by weight) but devoid of carbohydrate or a high fat (37% by weight) carbohydrate-free diet or the deinduction of transcription due to feeding mice diets high in carbohydrate (80% by weight). My findings suggest that the dietary regulation of PEPCK gene expression is based on the percentage of carbohydrate in the diet. The dramatic increase in hepatic PEPCK gene expression seen at birth was present for all PEPCK-bGH genes regardless of the block mutations present. The findings from these studies indicate that there is a different mechanism of transcriptional regulation of the PEPCK gene due to acute and chronic alterations in hormone status of an animal.
Based on these findings, models for the transcriptional control of liver and kidney specific PEPCK gene expression are proposed. The models suggest a role for transcriptional suppression as a form of regulation of the PEPCK gene. The models also incorporate the interaction of transcriptional elements, either directly or indirectly via a linking protein to modulate transcription of the PEPCK gene.
DEDICATION

This thesis is dedicated to my parents, whose encouragement and love have been a constant source of inspiration.
ACKNOWLEDGEMENTS

I would like to thank Dr Richard W. Hanson, my advisor, for his constant enthusiastic support. I would also like to thank the members of my thesis committee, Dr. Douglas Kerr, Dr. Edith Lerner and Dr. David Samols for providing constructive comments throughout my thesis project. I would like to express my deep appreciation to Dr. Jeung Yun for her contribution to this work.

I would like to thank the many people (past and present) in Richard's laboratory: Debra Crawford, Colleen Croniger, Luba Dumenco, Tom Ferkol, Greg Grossman, Parvin Hakimi, Maria Hatzoglou, Teiko Kimura, Pam Lechner, Jinsong Liu, Yan Liu, Molly McGrane, Maria Molas, Frank Mularo, Steve Nizielski, Jose Carlos Perales and Berta Warman.

Finally I would like to thank my friends in the department: Craig Cameron, Dan Hejlik, Terry Kinzy, Ted Liu, Sharon Niak and Jo Secnik.
# TABLE OF CONTENTS

Title Page ................................................................. i
Abstract ................................................................. ii
Dedication ................................................................. v
Acknowledgement ......................................................... vi
Table of Contents ......................................................... vii
List of Figures ............................................................ xi
List of Tables ............................................................. xiv
Abbreviations ............................................................. xv

I. Introduction .......................................................... 1
   A. Background ......................................................... 1
      1. Gluconeogenesis and PEPCK .................................. 1
      2. Tissue distribution of PEPCK and its
         metabolic role .................................................. 5
         a. Liver ......................................................... 6
         b. Kidney ...................................................... 7
         c. Adipose Tissue .............................................. 7
      3. Factors involved in the regulation of PEPCK
         gene transcription ............................................. 8
         a. Tissue specific expression ............................... 9
      4. Mechanism by which hormones regulate
         PEPCK gene expression ..................................... 18
         a. Glucagon ................................................... 18
         b. Insulin .................................................... 20
         c. Glucocorticoids .......................................... 22
     
    5. Nutritional regulation of PEPCK ............................. 23
    6. Developmental regulation of PEPCK ......................... 25
7. Analysis of PEPCK gene regulation using transgenic mice ........................................ 28

B. Rationale of Thesis Research ........................................ 32

II. Materials and Methods ................................................ 34

A. Materials ................................................................. 34

B. Methods ................................................................. 35

III. Results ................................................................. 55

A. Characterization of mice with PEPCK-bGH genes containing block mutations in the PEPCK promoter ........................................ 55

1. Characterization of PEPCK-bGH genes ........................................ 55

2. Identification of founder mice ........................................ 55

3. Determination of orientation and copy number of transgenes .......... 58

4. Expression of the PEPCK-bGH gene in transgenic mice ............... 65

5. Determination of bGH in the serum of transgenic mice ................. 70

B. The effect of specific mutations in the PEPCK promoter on the tissue-specific expression of the PEPCK-bGH genes ........................................ 75

1. CRE mutation ......................................................... 75
2. P1 mutation ................................................. 76
3. P2 mutation .................................................. 81
4. P3(l) mutation .............................................. 81
5. CRE/P3(l) mutation ........................................ 82

C. Regulatory elements in the PEPCK promoter
   responsible for the transcriptional response
   of the PEPCK-bGH gene to diet ...................... 83

D. Regulation of PEPCK-bGH expression
   by cAMP ..................................................... 96

E. Analysis of the insulin response element ............ 97

F. Regulatory elements in the PEPCK promoter
   responsible for the developmental expression
   of the PEPCK-bGH gene in liver ..................... 100

IV. Discussion .................................................. 107

A. System ....................................................... 108

B. Tissue specific regulatory elements in the PEPCK
   promoter ..................................................... 110

   1. Liver ..................................................... 111
   2. Kidney .................................................... 113

C. Dietary regulation ........................................ 114
D. Diabetes.................................................. 118

E. Developmental expression.......................... 119

F. Proposed models of the tissue specific regulation
   of PEPCK gene transcription in the liver and
   kidney.................................................. 122

1. Liver.................................................... 125
2. Kidney................................................... 130

G. Future studies........................................... 134

V. References.............................................. 137
LIST OF FIGURES

1. Gluconeogenic pathway in the liver ......................... 2

2. DNase I footprint analysis of the PEPCK promoter ........................... 10

3. Protein binding sites on the PEPCK promoter .................. 14

4. Alterations in the expression of gluconeogenic enzymes, the insulin/glucagon ratio and cAMP in the liver ......................................................... 26

5. Construction of the PEPCK-bGH genes .......................... 38

6. Schematic representation of the PEPCK-bGH genes ................. 41

7. Generation of transgenic mice that contain the PEPCK-bGH gene ......................................................... 44

8. Analysis of linear hybridization of hepatic RNA .................. 51

9. Analysis of DNA from mice produced by microinjection of the PEPCK-bGH gene ......................................................... 59

10. Possible orientation of the PEPCK-bGH gene in the genomes of transgenic mice ................................. 61
11. Southern blot analysis of DNA from transgenic mice containing a PEPCK-bGH gene .......................... 63

12. Northern blot analysis of total RNA from different tissues of mice containing PEPCK-bGH genes ........................................... 68

13. Slot blot analysis of RNA from livers of mice containing a PEPCK-bGH gene ................................. 71

14. Growth rate of mice containing PEPCK-bGH genes ............................................................................ 73

15. The effect of mutations in regulatory elements in the PEPCK promoter on the level of expression of the PEPCK-bGH gene in the livers and kidneys of transgenic mice .......................................................... 79

16. Tissue specific expression of bGH mRNA in mice containing a PEPCK-bGH gene with specific block mutations in the PEPCK promoter ................................................................. 84

17. Dietary regulation of hepatic and renal bGH mRNA in mice containing a PEPCK-bGH gene with a block mutations in the PEPCK promoter ................................................................. 87

18. The effect of a high fat, carbohydrate-free diet on the level of expression of the PEPCK-bGH gene in the livers of transgenic mice ................................................................. 94
19. The effect of cAMP on bGH in the livers of mice containing a PEPCK-bGH gene with block mutations in regulatory elements of the PEPCK promoter.......................... 98

20. Dietary regulation of hepatic bGH mRNA in mice containing a PEPCK(355)-bGH .................... 101

21. The expression of bGH mRNA in the livers of transgenic mice containing block mutations in the promoter of the PEPCK-bGH gene during fetal development...................... 104

22. Proposed model of the PEPCK promoter......................... 123

23. Proposed model of transcriptional regulation from the PEPCK promoter in the liver.......................... 126

24. Proposed model of transcriptional regulation from the PEPCK promoter in the kidney............. 131
LIST OF TABLES

1. Summary of regulatory elements and the transcription factors that interact with the PEPCK promoter ........................................... 12

2. Oligonucleotide primers used to produce block mutations in regulatory elements of the PEPCK promoter .......................................................... 36

3. Frequency and expression of PEPCK-bGH genes in transgenic mice ................................................................. 56

4. Properties of specific lines of transgenic mice containing PEPCK-bGH genes ................................................................. 66

5. Relative expression of the transgene containing block mutations in the PEPCK promoter in the liver and kidney of transgenic mice ............................................. 77

6. Composition of experimental diets ............................................. 90

7. Body weight, weight gain and energy intake of transgenic mice that were fed lab chow or
ABBREVIATIONS

PEPCK  Phosphoenolpyruvate carboxykinase (GTP)
        (EC 4.1.1.32)
CRE    cAMP Regulatory Element
cAMP   Adenosine-3':5' monophosphate
CREB   cAMP Regulatory Element Binding Protein
C/EBP  CCAAT/Enhancer Binding Protein
NF-I   Nuclear Factor-I
HNF-I  Hepatic Nuclear Factor-I
IRS    Insulin Response Sequence
GRE    Glucocorticoid Response Element
Bt$_2$cAMP N$^6$,2'-o-dibutyryl-adenosine,3':5'-monophosphate, cyclic
TRE    Thyroid Regulatory Element
bGH    bovine Growth Hormone
SDS    sodium dodecylsulfate
EDTA   ethylenediamine tetracetic acid
Tris   tris hydroxymethyl aminomethane
I INTRODUCTION

A. Background

1. Gluconeogenesis and PEPCK

Glucose homeostasis in all vertebrates requires de novo glucose synthesis, from precursors such as lactate, pyruvate, alanine and glycerol. Gluconeogenesis is of special significance during fasting and in periods of metabolic acidosis or whenever the percentage of carbohydrate is reduced in the diet. There are four enzymes that are unique to gluconeogenesis (Fig 1). The enzymes that catalyze these reactions are glucose-6-phosphatase, fructose-1,6-diphosphatase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase. The rate limiting step in gluconeogenesis is the GTP dependent decarboxylation of oxalacetate to pyruvate which is catalyzed by phosphoenolpyruvate carboxykinase (PEPCK) (GTP) (E.C. 4.1.1.32). PEPCK was first described by Utter and Kurahashi in 1954 from chicken liver mitochondria. In 1963 Nordlie and Lardy reported the presence of both a mitochondrial and cytosolic form of PEPCK in the livers of several mammalian species. The ratio of the two forms of PEPCK differ among species. The rat and mouse contain 90% cytosolic and 10% mitochondrial PEPCK in the liver (Hanson and Garber, 1972). In contrast, the chicken contains 100% mitochondrial PEPCK in the liver (Hod et al., 1982) and in humans, there is 50% cytosolic PEPCK and
Figure 1. Pathway of Gluconeogenesis in the Liver
50% mitochondrial PEPCK. The significance of the ratio of the two forms of PEPCK is determined by the availability of precursors to the gluconeogenic pathway. In animals, the relative ratio of the two forms of PEPCK is the same in all tissues.

The difference in the metabolic roles of the two forms of PEPCK is based on the precursor used to synthesize glucose. When pyruvate is the precursor of the gluconeogenic pathway, oxalacetate (OAA) is formed in the mitochondria by the enzyme pyruvate carboxylase. The OAA is oxidized to form malate which is then transported to the cytosol via a shuttle. The malate is then converted to OAA by the enzyme NAD-malate dehydrogenase. The NADH generated in the cytosol by this reaction provides the reducing equivalents required to form glyceraldehyde-3-phosphate. The OAA is then converted to phosphoenolpyruvate (PEP) by the cytosolic form of PEPCK.

In contrast, if lactate is the precursor in the gluconeogenic pathway, the necessary reducing equivalents are produced in the cytosol when lactate is oxidized to form pyruvate. Then OAA in the mitochondria is converted to PEP by the mitochondrial form of PEPCK. Therefore when lactate is the precursor of glucose synthesis, it is not necessary to transport reducing equivalents (NADH) from the mitochondria to the cytosol.

The two forms of PEPCK differ in their regulation. Cytosolic PEPCK is regulated by a variety of hormones, dietary status of the animal and during development while the mitochondrial form of PEPCK
is constitutively expressed. The mitochondrial form of PEPCK is not regulated by hormones or dietary status (Nordlie et al., 1965). The remaining section of this chapter will discuss the regulation of the cytosolic form of PEPCK from the rat.

PEPCK is a monomeric protein of 70,000 daltons (Beale et al., 1965). The cytosolic form of PEPCK has no known allosteric regulators. The factors which alter the activity of PEPCK, alter the rate of its synthesis. Changes in the level of PEPCK parallel the changes in its mRNA concentration. Although transcription is the primary mode of regulation of PEPCK expression, the PEPCK mRNA is stabilized by cAMP in the liver (Hod and Hanson, 1988) and acid/base status in the kidney (Kaiser et al., 1992).

2. Tissue distribution of PEPCK and its metabolic role

PEPCK is present primarily in the liver (Utter and Kurahashi, 1954), kidney cortex (Henning et al., 1966), and adipose tissue (Ballard et al., 1967) and expressed at marginal levels in lung (Hanson and Garber, 1972), the jejunum of the small intestine (Anderson, 1970), lactating mammary gland (Garcia-Ruiz et al., 1983) and the brain (Hanson and Garber, 1972). The role of PEPCK in each tissue expressing significant levels of the enzyme differs. In this section I will review the metabolic role of PEPCK in tissues in which it has been characterized.
a. LIVER- The factors which stimulate gluconeogenesis, also stimulate the transcription of the PEPCK gene in the liver. In the liver, PEPCK gene transcription is stimulated by glucagon (acting via cAMP) (Lamars et al., 1982), glucocorticoids (Granner et al., 1983), thyroid hormone (Loose et al., 1985), retinoic acid (Lucas et al., 1992), starvation and feeding animals diets high in protein and devoid of carbohydrate (Peret et al., 1981; Lamars et al., 1982). PEPCK gene transcription is inhibited by insulin (Granner et al., 1983) and feeding animals diets high in carbohydrate (Lamars et al., 1982). Hepatic PEPCK gene expression is also regulated during development (Ballard and Hanson., 1967).

PEPCK gene expression is not uniform throughout the entire liver. Previous studies have indicated that the enzymes of carbohydrate metabolism are localized to specific zones within the mammalian liver. PEPCK is expressed in a gradient from the periportal to the pericentral region of the liver (Guder et al., 1976; Wals et al., 1988). The activity of PEPCK is highest in the periportal region of the liver (Wals et al., 1988). This zonation serves to divide the opposing pathways of glycolysis and gluconeogenesis. The enzymes of glycolysis (glucokinase and pyruvate kinase L) (Guder and Schmidt, 1976; Katz et al., 1977) are concentrated in the pericentral region of the liver whereas enzymes of the gluconeogenic pathway together with the urea cycle enzymes (Moorman et al., 1988), are present at higher concentrations in the periportal region of the liver (Moorman et al., 1988). The periportal region of the liver
receives a greater supply of nutrients and oxygen than the pericentral region (Jungermann et al., 1978).

b. KIDNEY- The level of PEPCK expression is similar in the liver and the kidney of mammals. In the kidney, PEPCK functions in gluconeogenesis and ammoniagenesis. The kidney only produces significant quantities of glucose during prolonged starvation and metabolic acidosis. During metabolic acidosis, the acid-base balance of the urine is maintained by generating two molecules of ammonia from the conversion of glutamine to glutamate, and then glutamate to α-ketoglutarate in the kidney cortex. The α-ketoglutarate enters the citric acid cycle to produce OAA which is then converted into PEP by PEPCK.

PEPCK gene expression has been localized to the proximal convoluted tubules of the kidney cortex (Guder and Schmidt, 1974). The factors which regulate PEPCK in the kidney, acid-base status, glucocorticoids and cAMP, regulate the transcription rate as well as the stabilization of the PEPCK mRNA in the kidney.

c. ADIPOSE TISSUE- Adipose tissue contains significant levels of PEPCK but does not produce glucose. In 1970 Ballard et al proposed that PEPCK functions in glyceroneogenesis, a metabolic pathway which regulates the level of free fatty acids (FFA) re-esterification. In the glyceroneogenic pathway, pyruvate or lactate can be utilized as precursors in adipose tissue to synthesize 3-
glycerophosphate. The availability of 3-glycerophosphate contributes to
the increased re-esterification of FFA in the presence of pyruvate which
has been documented (Ballard et al., 1970). This pathway involves two
gluconeogenic enzymes, PEPCK and pyruvate carboxylase. PEPCK is
induced by fasting and adrenalectomy and deinduced by
glucocorticoids. The hormonal response of adipose tissue PEPCK to
glucocorticoids contrasts to the hormonal response in the liver and
kidney.

3. Factors involved in the regulation of PEPCK gene
   transcription

The analysis of transcriptional regulation of the PEPCK gene has
been facilitated by the cloning and sequencing of the cytosolic form of
PEPCK from the rat (Beale et al., 1985). The rat PEPCK gene is 6 kb in
length and contains 10 exons and 9 introns (Beale et al., 1985). The
gene contains one transcriptional start site utilized in all tissues
expressing the PEPCK gene. The PEPCK gene encodes a 2.8 kb
mRNA in all tissues studied to date (Yoo-Warren et al., 1983; Beale et
al., 1985).

The promoter regulatory region of the rat PEPCK gene has been
isolated and extensively characterized. DNase I footprint analysis was
performed on a -550/+73 bp fragment of the PEPCK promoter (Roesler
et al., 1989). This study identified multiple protein binding regions within
this segment of DNA, referred to as P1 to P6 and CRE (Fig. 2) (Roesler et al., 1989). Based on sequence homologies, protein binding studies, footprint analysis using nuclei from different tissues and transient transfection assays, putative proteins binding to these regions were identified and potential functions assigned to these regions. Table 1 lists protein binding regions characterized within a -550/+73 bp fragment of the PEPCK promoter. The location, putative transcription factors and the function based on several lines of evidence are also presented in Table 1. A schematic representation of a -460/+73 bp segment of the PEPCK promoter is given in Figure 3. The regulatory elements and putative protein binding regions are also presented.

a. Tissue specific expression

LIVER- DNase I footprinting studies using nuclei from different rat tissues demonstrated different protein binding patterns at the P2 and P3(I) regions of the PEPCK promoter (Roesler et al., 1989). The P3(I) region (-248 to -230) binds a protein present in rat liver nuclei. The sequence of the P3(I) region has a significant degree of homology to the DNA binding sequence of the CCAAT/Enhancer Binding Protein (C/EBP) family of proteins (Landschulz et al., 1988). C/EBP belongs to a class of transcription factors that contain a leucine zipper motif (Landschulz et al., 1989). The leucine zipper proteins can form homodimers and heterodimers between members of the family.
Figure 2. DNase I footprint analysis of the PEPCK promoter

A 620 base pair BamHI-Bgl II fragment of the PEPCK promoter was end labeled with $[\gamma-^{32}P]$ATP. The probe was incubated with no protein, and either 3 or 6 μg of protein isolated from rat liver, kidney, spleen or brain nuclei. The regions protected by proteins are indicated. CRE, cAMP responsive element. This figure was taken from Roesler et al., 1989.
Table 1. Properties of transcription factors that bind to elements in the PEPCK promoter
<table>
<thead>
<tr>
<th>Binding sites</th>
<th>Locations</th>
<th>Binding proteins</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRE</td>
<td>-78/-87</td>
<td>CRE, C/EBPα, C/EBPβ, DBP, Fos/jun</td>
<td>Basal, tissue-specific and cAMP</td>
</tr>
<tr>
<td>P1</td>
<td>-87/-123</td>
<td>NF-I</td>
<td>Basal</td>
</tr>
<tr>
<td>P2</td>
<td>-164/-200</td>
<td>HNF-I</td>
<td>Tissue-specific</td>
</tr>
<tr>
<td>P3(l)</td>
<td>-230/-248</td>
<td>C/EBPα, C/EBPβ, DBP</td>
<td>Basal, tissue-specific and cAMP</td>
</tr>
<tr>
<td>P3(II)</td>
<td>-249/-260</td>
<td>Fos/jun</td>
<td>Basal and cAMP</td>
</tr>
<tr>
<td>P4</td>
<td>-268/-285</td>
<td>C/EBP, Fos/jun</td>
<td>Basal and cAMP</td>
</tr>
<tr>
<td>TRE</td>
<td>-308/-332</td>
<td>Thyroid receptor</td>
<td>Thyroid regulation</td>
</tr>
<tr>
<td>GRU</td>
<td>-350/-456</td>
<td>GR, AF1, AF2</td>
<td>Glucocorticoid and insulin</td>
</tr>
</tbody>
</table>
Figure 3. Protein binding sites on the PEPCK promoter

The location of regulatory elements (designated as boxes) on the PEPCK promoter (-460 to +73 bp) and the transcription factors known to bind to these sites is shown. The abbreviations for the elements are; CRE, cAMP response element; TRE, thyroid hormone response element; GRU, glucocorticoid response unit and IRS, insulin regulatory sequence. The transacting factors are; CREB, cAMP response element binding protein; C/EBP, CCATT/ enhancer binding protein; NF-1, nuclear factor-1; HNF-1, hepatic nuclear factor-1; TR, thyroid hormone receptor.
Heterodimerization of proteins allows a higher degree of complexity of regulation of gene transcription.

Three members of the C/EBP family of proteins, C/EBPα (Landschulz et al., 1988), C/EBPβ (Descombes et al., 1990) and D element binding protein (DBP) (Mueller et al., 1990) have been shown to bind to the P3(I) region as well as the CRE and P4 regions of the PEPCK promoter. All three C/EBP proteins stimulate transcription from the PEPCK promoter when vectors containing genes coding for these proteins are cotransfected into hepatoma cells (Park et al., 1990; Roesler et al., 1992; Park et al., 1993).

The C/EBP family of proteins are expressed at high concentrations in the liver and adipose tissue (Birkenmeier et al., 1989); tissues in which PEPCK is expressed. C/EBPβ mRNA is expressed in a gradient from the periportal to the pericentral region of the liver in a pattern similar to the expression of the PEPCK gene (Park et al., 1993). Another member of the C/EBP family of proteins, C/EBPα has a developmental pattern of expression similar to the PEPCK gene (Birkenmeier et al., 1989). C/EBPα mRNA is present at low levels in the liver just before birth and increases dramatically at birth (Birkenmeier et al., 1989). It is possible that the expression of C/EBPα signals the onset of hepatic PEPCK gene transcription. The data from footprinting studies and transfection assays suggest a primary role for the C/EBP family of proteins in the liver specific and developmental expression of the PEPCK gene.
KIDNEY - In the kidney, acidosis (Alleyne et al., 1969), starvation (Iynedjian et al., 1974) and glucocorticoids (Langshaw et al., 1972) induce while alkalosis and feeding starved animals (Iynedjian et al., 1974) inhibit PEPCK levels in the kidney. The effects of starvation and refeeding on PEPCK levels in the kidney are evident only after longer time periods (Iynedjian et al., 1974), unlike their acute effects on PEPCK levels in the liver. This effect is due primarily to alterations in the acid-base balance. As stated previously, renal PEPCK is involved in the pathway which uses glutamate as a precursor to generate urinary ammonia in acidotic animals.

Proteins present in nuclei from rat kidney were shown to bind to the P2 region (-200 to -184) of the PEPCK promoter using footprinting assays (Roesler et al., 1989). The transcription factor, Hepatic Nuclear Factor I (HNF-I) has been shown to bind to the P2 region of the PEPCK promoter (Gurney and Hanson, unpublished observations). HNF-I was originally isolated from liver extracts and thus referred to as a hepatic factor (Kuo et al., 1990). Many liver specific genes contained HNF-I binding sites that are required for their expression in the liver (Costa et al., 1988). Subsequent studies demonstrated that HNF-I is expressed not only in the liver but also in the kidney and small intestine (Mendel and Crabtree, 1991). The kidney actually expressed higher levels of HNF-I mRNA than the liver (Mendel and Crabtree, 1991). HNF-I binds to DNA as a dimer and requires another protein, DCoH, (dimerization cofactor of HNF-I) for dimerization (Mendel et al., 1991). DCoH mRNA is
expressed in kidney, liver and small intestine (Mendel et al., 1991), the same tissues that express HNF-I and PEPCK mRNAs.

During development, renal PEPCK mRNA is present as early as day 16 of gestation. HNF-I mRNA is also present in the kidney before birth (Lazzaro et al., 1992). The data suggest that HNF-I is involved in the expression of the PEPCK gene in the kidney and that it is also involved in the developmental expression of renal PEPCK.

4. Mechanism by which Hormones Regulate PEPCK Gene Expression

PEPCK is regulated by a variety of hormones in a tissue dependent manner. The changes in PEPCK activity are mediated primarily by changes in the rate of PEPCK gene transcription, although there is evidence that the stability of PEPCK mRNA can be influenced by hormones. In this section, I will review the tissue dependent regulation of the PEPCK gene and the putative regulatory elements and transcription factors that mediate regulation.

a. Glucagon via cAMP

In the liver, acute changes in the level of PEPCK are regulated primarily by alterations in the concentrations of insulin and glucagon. The concentration of these two hormones responds to diet (Muller et al.,
1971) and are altered during development (DiMarco et al., 1978). The
effect of glucagon is mediated by cAMP which exerts its effects on
PEPCK gene transcription and mRNA stability (Hod and Hanson, 1988).
The administration of glucagon (Shrago et al., 1963) or cAMP (Lamers
et al., 1982) to rats, resulted in an increase in PEPCK synthesis in 90
minutes. Glucose fed animals that were administered Bt2cAMP had a 6
fold elevation in PEPCK mRNA levels within 60 minutes in the liver
(lynedjian et al., 1977). Previous studies indicated that cAMP acts to
stimulate the rate of transcription from the PEPCK gene and also to
stabilize the PEPCK mRNA in the liver.

Studies utilizing 5' deletions of the PEPCK promoter first identified
a cAMP responsive element (CRE) (-95 to -74) (Short et al., 1986;
Wynshaw-Boris et al., 1986). Many cAMP regulated genes have a CRE
positioned relatively close to the transcription start site. The CRE region
has been shown to be required for the cAMP induction of PEPCK gene
transcription (Short et al., 1986; Quinn and Granner, 1989). A 43 kD
protein, cAMP Regulatory Element Binding Protein (CREB) was isolated
from either brain or placenta nuclei by affinity chromatography using the
CRE sequence from the somatostatin gene (Montminy et al., 1986). The
CRE of the PEPCK promoter also binds a 43 kD protein present in rat
liver nuclei (Roesler et al., 1989). The CRE in the PEPCK promoter is
the only site within the -460/+73 bp segment of the PEPCK promoter
that binds CREB (Roesler et al., 1989). CREB belongs to a family of
transcription factors that contain a leucine zipper motif, which allows for
dimerization of members of the family (Gonzalez et al., 1989). The leucine zipper proteins require dimerization in order to bind to DNA. Although CREB binds to the CRE of the PEPCK promoter, phosphorylation of CREB by a cAMP dependent protein kinase (PKA) is required to stimulate its transcriptional efficiency (Liu et al., 1991).

Although the CRE is required for cAMP-mediated induction of PEPCK gene transcription, transfection studies using vectors which contained chimeric PEPCK-CAT genes with specific base pair substitutions in putative protein binding regions demonstrated that multiple regions are required for cAMP stimulation from the PEPCK promoter (Liu et al., 1991). These studies demonstrated that the CRE, P3(I) and P4 regions were required for cAMP induction of PEPCK gene transcription in hepatoma cells (Liu et al., 1991). As stated previously, the P3(I) region as well as the CRE and P4 regions bind members of the C/EBP family of proteins. The level of hepatic C/EBPβ mRNA increased within 90 minutes upon administration of cAMP to rats (Park et al., 1993). C/EBPβ is a cAMP-responsive transcription factor. The cAMP-mediated induction of PEPCK gene transcription in the liver may involve the interaction of multiple sites on the PEPCK promoter which bind either CREB or C/EBP.

b. Insulin

Insulin inhibits gluconeogenesis and PEPCK synthesis in the liver (Young et al., 1964). The effect of insulin is rapid, acting at the level of
gene transcription (Granner et al., 1983). Within 90 minutes after the administration of insulin to diabetic animals, PEPCK mRNA levels are decreased by half in the liver (Tilghman et al., 1974). Feeding glucose to starved animals resulted in a rapid decrease in PEPCK gene transcription in the liver as determined by nuclear run off transcription assays (Lamers et al., 1982). The mechanism by which insulin decreases PEPCK gene transcription has not been as well characterized as the cAMP-mediated regulation of PEPCK gene transcription. It is possible that insulin acts to directly effect gene transcription or it may act to block the stimulatory effect of factors that induce PEPCK gene transcription. Studies in hepatoma cells demonstrated that insulin could block the inductive effect of cAMP and dexamethasone on PEPCK transcription (Granner et al., 1983).

Recently, O'Brien et al. (1990) reported an insulin responsive sequence (IRS) at position -416 to -402 in the PEPCK promoter. The IRS accounted for 60% of the inhibitory effect of insulin on PEPCK gene transcription in transient transfection studies where the IRS was deleted from a PEPCK-CAT gene (O'Brien et al., 1990). These studies also demonstrated that the IRS of the PEPCK promoter was orientation-independent (O'Brien et al., 1990). The IRS of the PEPCK promoter conferred insulin mediated inhibition when linked to a heterologous promoter and transiently transfected into H4IIE cells (O'Brien et al., 1990). Several other insulin responsive genes contain an IRS in their promoter regions (Alemany et al., 1990; Min and Spiegelman, 1986;
Magnuson et al., 1989). Three proteins that bind to the IRS have been partially purified by affinity chromatography (Lucas et al., 1993). These IRS proteins have been shown to dimerize with members of the C/EBP family of proteins in vitro (Lucas et al., 1993). The IRS of the PEPCK promoter is part of a larger regulatory region, the glucocorticoid response unit (GRU), (-355 to -460), which also contains a glucocorticoid response element, (GRE).

c. Glucocorticoids

In the liver, glucocorticoids have only a marginal effect on the transcription rate of the PEPCK gene. Adrenalectomy did not affect the rate of PEPCK gene transcription in the livers of rats (Gunn et al., 1974). When adrenalectomized rats were administered dexamethasone, a synthetic glucocorticoid, there was only a marginal increase in PEPCK gene transcription. Glucocorticoid mediated induction of PEPCK gene transcription is linked to insulin. PEPCK gene transcription was induced in diabetic rats while animals were both adrenalectomized and diabetic, the rate of PEPCK gene transcription was not increased in the liver (Gunn et al., 1974).

The PEPCK promoter contains two glucocorticoid responsive sequences, located at -389 to -353. The GRE were characterized by 5' deletion analysis of the PEPCK promoter and sequence homology to the GRE consensus sequences (Short et al., 1986). The GRE of the PEPCK promoter is part of a larger regulatory region, the GRU (-455 to -353)
which contains two accessory factor binding regions, one of which maps at the IRS. Consequently these protein binding regions interact to produce glucocorticoid stimulated transcription from the PEPCK gene in hepatoma cells (Imai et al., 1990) and may also be involved in the insulin-mediated inhibition of PEPCK gene transcription (Friedman et al., 1993).

5. Nutritional Regulation of PEPCK

The influence of nutritional factors on the regulation of hepatic PEPCK is well documented (Peret et al., 1975; Peret et al., 1976; Peret et al., 1981). Factors which stimulate gluconeogenesis such as starvation, high protein/low carbohydrate diets and glucagon administration also stimulate PEPCK gene transcription. Nutritional factors have many physiological effects one of which is altering circulating hormone concentrations. Two hormones that are particularly sensitive to alterations in circulating glucose concentrations are insulin and glucagon. The circulating plasma levels of insulin and glucagon can be altered by changing the composition of the diet (Peret et al., 1981). The response of PEPCK synthesis due to alterations in the levels of carbohydrate and protein in diets has been extensively characterized (Peret et al., 1971; Peret et al., 1976; Peret et al., 1981).

Feeding animals diets high in carbohydrate increased the concentration of plasma insulin and decreased the levels of circulating
glucagon (Peret et al., 1981). This hormonal condition results in the inhibition of PEPCK synthesis and thus gluconeogenesis in the liver. Feeding studies demonstrated that PEPCK levels in the liver decreased with increasing levels of carbohydrate in the diet (Peret et al., 1975). The carbohydrate content of the diet ranged from 10% to 80% by weight of the diet (Peret et al., 1975). These studies indicated that the percentage of carbohydrate in the diet altered the level of PEPCK in the liver.

Alternatively, diets high in protein but devoid of carbohydrate result in elevated levels of plasma glucagon and decreased levels of plasma insulin (Eisenstein et al., 1971; Peret et al., 1981). This hormonal state leads to the stimulation of PEPCK and gluconeogenesis. Previous studies indicated that the induction of PEPCK synthesis was due to the percentage of protein (by weight) in the diet not the source of the protein (Peret et al., 1975). Casein, wheat gluten and egg white protein produced similar levels of induction in PEPCK synthesis (Peret et al., 1975). The only factor which altered the level of induction was the percentage of dietary protein (by weight), regardless of its source. This led to the conclusion that dietary protein was altering the concentrations of insulin and/or glucagon and thus effecting PEPCK synthesis. Yet as the percentage of dietary protein was increased, the percentage of dietary carbohydrate decreased in the experimental diets. It is thus possible that alterations in PEPCK synthesis result primarily from the carbohydrate content in the diet.
The effects of altering the plasma insulin/glucagon ratio results in alterations in PEPCK transcription in the liver. In studies where both the insulin and glucagon concentrations were increased or decreased yet maintaining the same insulin/glucagon ratio, PEPCK synthesis was not altered (Parrilla et al., 1974).

6. Developmental Regulation of PEPCK

Before birth, the rat fetus is supplied with glucose from the maternal circulation. This continuous supply of glucose results in high levels of plasma insulin and low levels of plasma glucagon in the fetal circulation (DiMarco et al., 1978). PEPCK is the only enzyme in the gluconeogenic pathway that is present at negligible levels in the fetal liver (Ballard et al., 1967). The three other gluconeogenic enzymes are present before birth in the liver (Fig. 4). Due primarily to the hormonal state in the fetus, hepatic PEPCK expression and thus gluconeogenesis is suppressed (Fig. 4). At birth, the supply of maternal glucose is severed, requiring the newborn to adapt to a period of fasting. As the level of plasma glucose decreases, there is a decrease in the concentration of plasma insulin and a rise in plasma glucagon and epinephrine levels leading to an increase in the concentration of hepatic cAMP (DiMarco et al., 1978). These changes result in a dramatic increase in the synthesis of PEPCK and signal the onset of hepatic gluconeogenesis.
Figure 4. Alterations in the expression of gluconeogenic enzymes, gluconeogenesis, the insulin/glucagon and cAMP in the liver

Relative activity of hepatic gluconeogenesis and the four gluconeogenic enzymes (A), and changes in the insulin/glucagon ratio and cAMP concentrations in the liver (B). Redrawn from Tilghman et al., 1976.
A. Relative change in activity

- Glucose-6-phosphatase
- Fuctose-1,6-diphosphatase
- Pyruvate carboxylase
- PEPCK
- Glucagon

B. Relative change in activity

- cAMP
- Insulin/glucagon
Previous studies have shown that administration of Bt2cAMP or glucagon to 17 day old rat fetuses resulted in the premature expression of PEPCK in the liver (Reshef et al., 1973). This suggests that the regulatory factors necessary for the induction of the PEPCK gene are present as early as day 17 of gestation. The pattern of PEPCK expression during development appears to involve many factors. Alternatively, insulin or glucose given at birth, suppressed PEPCK expression in the liver (Yeung and Oliver, 1968). The evidence suggests that the hormonal status of the animal is the primary determinant of PEPCK expression and gluconeogenesis in the liver during development. These studies along with the inverse relationship of the plasma insulin and glucagon concentrations noted during the perinatal period suggest that the ratio of insulin and glucagon is the primary factor determining the extent of PEPCK expression in the liver.

7. Analysis of PEPCK gene regulation utilizing transgenic mice

Studies utilizing cells in culture as models for gene regulation, have assigned putative regulatory elements and transacting factors to characterize the regulation of PEPCK gene transcription. Although cultured cells provide a system in which isolated effects can be analyzed, aspects such as tissue specificity, developmental and dietary regulation can only be examined in animal models. Animals provide an
accurate physiological environment in which to study regulation. The advent of transgenic animals provided an opportunity to investigate gene regulation in vivo. Transgenic animals are produced by introducing foreign genes into the germline of recipient host animals by microinjection into fertilized eggs. PEPCK gene regulation has been investigated in several laboratories using transgenic mice (McGrane et al., 1988; McGrane et al., 1990; Short et al., 1992; Eisenberger et al., 1992; Friedman et al., 1993).

Studies by McGrane et al (1988) first reported that nearly all the transcriptional regulation attributed to the PEPCK promoter regulatory region is contained within a -460/+73 bp segment of the PEPCK gene. This segment of the PEPCK promoter contains the necessary sequences to direct expression of a chimeric PEPCK-bovine growth hormone (bGH) gene to the liver and kidney (McGrane et al., 1988). Although the transgene was expressed in the livers and kidneys of transgenic mice, the levels of bGH mRNA in the kidney were low when compared to the levels of bGH mRNA in the livers of transgenic mice. The level of endogenous PEPCK mRNA is the same in the liver and kidney (McGrane et al., 1990). Therefore the low levels of bGH mRNA in the kidneys of transgenic mice may be due to the choice of marker gene or may require sequences further upstream of -460 in the PEPCK promoter. Transgenic mice containing a -2000/+73 bp segment of the PEPCK promoter linked to bGH still had low levels of bGH mRNA in the kidneys of transgenic mice (McGrane et al., 1990). These low levels of
renal transgene expression were also reported by Short et al (1992) in transgenic mice containing a chimeric PEPCK(2000)-human growth hormone (hGH) gene. In contrast, transgenic mice containing a chimeric PEPCK gene (rat PEPCK gene with a 3' chicken PEPCK insert) had equal levels of transgene expression in the liver and kidney (Eisenberger et al., 1992). This finding indicated that there may be post-transcriptional regulation of the PEPCK gene in the kidney.

Preliminary studies using transgenic mice with a chimeric PEPCK(460)-bGH gene indicated that the regulatory elements responsible for the cAMP induction of PEPCK transcription are within the -460/+73 bp segment of the PEPCK promoter (McGrane et al., 1992). The induction from the PEPCK promoter of a PEPCK(460)-bGH gene in the liver due to feeding animals diets high in protein but devoid of carbohydrate or the deinduction of transgene expression by feeding mice diets high in carbohydrate, was also present in transgenic mice.

To further characterize sequences required for the complex regulation of the PEPCK gene, the expression of a series of 5' deletions of the PEPCK promoter were examined using transgenic mice (McGrane et al., 1990; Short et al., 1992; Eisenberger et al., 1992). These studies concluded that sequences necessary for liver specific expression of the PEPCK gene are downstream of -460 from the transcription start site. These studies also demonstrated that sequences required for kidney specific expression of the PEPCK gene are within a -355/+73 bp segment of the PEPCK promoter. Transgenic mice
containing a PEPCK(174)-bGH gene did not express bGH mRNA in any tissue (McGrane et al., 1990). Therefore the sequences required for tissue specific expression of the PEPCK gene are within -460 bp for liver and -355 bp for kidney.

To determine the elements in the PEPCK promoter that are responsible for the dietary regulation of the PEPCK gene, transgenic mice were fed either diets high in carbohydrate (80% by weight) or high in protein (64% by weight) and devoid of carbohydrate (McGrane et al., 1988). Hepatic expression of either a chimeric PEPCK(460)-bGH gene or a PEPCK(402)-hGH in transgenic mice was decreased when mice were fed diets high in carbohydrate (McGrane et al., 1988; Short et al., 1992) and increased when mice were fed a diet high in protein but devoid of carbohydrate. Several putative cAMP (CRE and P3(l)) and insulin (IRS) responsive regions have been identified within the -460/+73 bp fragment of the PEPCK promoter. The deletion of IRS did not affect the ability of the high carbohydrate diet to inhibit transcription from the PEPCK promoter in a PEPCK(402)-hGH gene in the livers of transgenic mice (Short et al., 1992). The effect of high carbohydrate diets on PEPCK gene transcription appears to involve more than just the IRS.

Developmental regulation of PEPCK gene expression, which involves the alterations of glucagon and insulin concentrations was investigated in transgenic mice. Mice containing a PEPCK(460)-bGH gene displayed the same pattern of expression during development as the endogenous PEPCK gene in the liver (McGrane et al., 1990).
Expression of the transgene was suppressed in the fetal liver until day 19 of gestation at which time low levels of bGH mRNA were detected. At birth there was a dramatic increase in the level of bGH mRNA in the livers of newborn mice. Whether the suppression during fetal life or the induction after birth of the PEPCK gene is regulated by the putative cAMP and Insulin responsive regions remains to be determined.

Previous studies using 5' deletions of the PEPCK promoter in transgenic mice provided an initial indication of the regions that conferred specific aspects of transcriptional regulation from the PEPCK promoter. To delineate the specific sequences that confer transcriptional regulation to the PEPCK gene requires a systematic analysis of putative regulatory elements in transgenic mice.

B. Rationale of Thesis Research

The goals of my thesis were to characterize the roles specific regulatory elements in the PEPCK promoter play in the transcriptional regulation of the PEPCK gene. The specific aims of my research were:

1. to determine if multiple elements are required for transcriptional regulation of the PEPCK gene

2. to determine if acute and chronic hormonal alterations regulate transcription of the PEPCK gene via the same elements, and
3. to propose a model of transcriptional regulation for tissue specific expression.

To determine the mechanism of the complex pattern of transcriptional regulation of the PEPCK gene, both the transacting factors that mediate the expression and the DNA sequences that bind these factors must be identified. In this thesis I have identified the role specific regulatory regions confer to the expression and regulation of the PEPCK gene. My approach was to construct chimeric genes which contained a -460/+73 bp fragment of the PEPCK promoter with various block mutations in putative protein binding regions, fused to the bovine growth hormone structural gene. These chimeric genes were microinjected into fertilized mouse eggs to produce transgenic mice. The tissue-specific, developmental, hormonal and dietary regulation were determined in the transgenic mice containing various chimeric genes.
II MATERIALS AND METHODS

A. Materials

All DNA modifying enzymes and a random primed labelling kit were purchased from Boehringer Mannheim (Indianapolis, IN) and used with the buffers recommended by the manufacturer. All chemicals used were of the highest quality commercially available. [α-32P]dCTP (3000 Ci/mmol), and Gene Screen Plus were purchased from DuPont-New England Nuclear (Boston, MA). Bt2cAMP was obtained from Sigma and theophylline from Calbiochem. The pBSU322 plasmid (pBR322 vector containing the entire bGH structural gene) was a generous gift from Dr. Fritz Rottman (Case Western Reserve University, Cleveland, OH). The bGH primary antibody and purified recombinant bGH protein were also a gift from Dr. Rottman, (CWRU). The peroxidase conjugated goat anti-rat IgG antibody was purchased from Kirkaagard and Perry, INC. ECL (enhanced chemiluminence kit) was purchased from Amersham (Arlington Heights, IL). Immobilon PVDF was purchased from Millipore.

Experimental Diets- Three synthetic diets were purchased from Nutritional Biochemical Corp. (Cleveland, OH). The high carbohydrate diet consisted of 80% sucrose, 12.2% casein, 0.3% DL-methionine, 4% cottonseed oil and a 3.5% mineral mix plus vitamins. The high protein diet contained 64% casein, 0.5% DL-methionine, 22% alphacel non-nutritive bulk, 8% cottonseed oil, 2% brewers' yeast, and a
3.5% mineral mix with vitamins. The high fat diet consisted of 17.4% casein, 0.5% DL-methionine, 14.3% cottonseed oil, 14.3% butter, 48.6% alphacel non-nutritive bulk, 5% mineral mix with vitamins. The mice were fed the diets *ad libitum* with continuous access to water

B. METHODS

1. Construction of chimeric PEPCK-bGH genes containing mutations in the PEPCK promoter

Mutations were introduced into specific protein binding domains of the PEPCK promoter (-460 to +73) using a variation of the Kunkel method (Kunkel, 1985), as described by Liu et al (1990). These mutations replaced the entire sequence of the CRE (-87 to -74), P1 (-123 to -87), P2 (-200 to -164), P3(l) (-248 to -230) and a combination of the CRE and P3(l) regions of the PEPCK promoter; these mutations eliminated protein binding at these sites as assayed by footprinting analysis (Liu et al., 1990). A pTZ18R vector containing this region of the PEPCK promoter with the block mutations (Table 2), described above ligated to the chloramphenicol acetyltransferase (CAT) structural gene was first digested with Pst I to linearize the plasmid. T4 DNA polymerase and nucleotides were used to produce flushed ends. The plasmid was then digested with Bgl II to remove the CAT gene (Fig. 5). The pTZ18R vector (Mead et al., 1986) containing the PEPCK promoter was
Table 2. Oligonucleotide primers used to produce block mutations in regulatory elements of the PEPCK promoter

The regulatory elements that were mutated are indicated in the left margin and the specific bases that were altered are indicated by bold-faced letters. The data is taken from Liu et al., 1990.
<table>
<thead>
<tr>
<th>Mutants</th>
<th>Oligonucleotide Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRE</td>
<td>GGCCCCCTGCATGCAGGGCGAGCCT</td>
</tr>
<tr>
<td>P1</td>
<td>TGCTGACCATTCTCGAGGTTCACCAAGGCGGCCCT</td>
</tr>
<tr>
<td>P2</td>
<td>TCCCAACATTCTCGAGGTTCACCTAAGTTCATCATT</td>
</tr>
<tr>
<td>P3(I)</td>
<td>GTCAATCGGTTACCGCTGAAGGAC</td>
</tr>
</tbody>
</table>
Figure 5. Construction of the PEPCK-bGH genes

A pTZPCCAT plasmid which contained a -490/+73 bp fragment of the PEPCK promoter was ligated to the CAT gene was digested with PstI to linearize the plasmid. The PstI ends were made flush by T4 DNA polymerase treatment. The linearized plasmid was then digested with BglII to remove the CAT gene. The linearized plasmid containing the PEPCK promoter was gel purified. The pBSUBa plasmid is a pBR322 based vector containing the entire structural bGH gene was digested with EcoRI to linearize the plasmid. The plasmid was then Klenow treated to produce flushed ends. The plasmid was digested with BamHI to separate the bGH gene from plasmid sequence. The bGH gene was gel purified and ligated into the BglII-blunt PstI site of the PEPCK promoter containg plasmid. The PEPCK-bGH genes were digested with EcoRI and BglII to seperate the transgene from plasmid sequence.
separated from the CAT gene by gel electrophoresis and then extracted from agarose by electroelution. A pBR322-based vector which contained the entire bGH structural gene; 1.7 kb of 5' sequence which included all 5 exons and 4 introns of the bGH gene and 500 bp of 3' flanking sequence (Woychick et al., 1982). This plasmid was digested with EcoRI and then Klenow enzyme was used to make the ends flush. The plasmid was then digested with BamHI to remove plasmid sequences (Fig. 5). The fragments were separated by gel electrophoresis and the bGH gene was extracted from agarose by electroelution. The BamHI-EcoRI fragment of the bGH gene was ligated into the the BgIII-PstI site of the pTZ18R vector containing the PEPCK promoter (Fig. 5). This ligation procedure was done for all five chimeric genes.

2. Microinjection of DNA into fertilized eggs

Recombinant DNA used for microinjection was separated from plasmid sequences by digestion with EcoRI and BgII followed by electrophoresis in a 1% agarose gel. DNA was then extracted from agarose by electroelution and phenol-chloroform purified for microinjection. The PEPCK-bGH genes were resuspended in TE buffer (10mM Tris-1 mM EDTA). The five PEPCK-bGH genes that were microinjected are shown in Fig. 6. The procedure for microinjection has been described previously by Wagner et al, (1981). Briefly, fertilized mouse eggs were flushed from the oviducts of superovulated C57BL/6 x SJL mice. Two pl of DNA solution (2 ng/μl) were injected into the male
Figure 6. Schematic representation of the PEPCK-bGH genes

Location of block mutations in the PEPCK promoter of the PEPCK-bGH genes that were used to produce transgenic mice. The line represents PEPCK sequence. The stripped boxes represent the site of block mutations in the PEPCK promoter. The site of each block mutation is indicated above each stripped box. The solid boxes represent the exons of the bGH gene and the open boxes represent the introns of the bGH gene.
pronuclei of fertilized eggs (Fig. 7). The viable embryos were reimplanted into the oviducts of pseudopregnant mice as described previously. The pregnancies were allowed to come to term. Four weeks after birth, mice were assayed for the presence of the transgene by dot blot analysis of tail DNA samples. Microinjection was performed by Dr. Jeung Yun at the Edison Animal Biotechnology Center, Ohio University, Athens, Ohio. Heterozygote F1 transgenic mice were used for the cAMP, dietary and developmental studies.

3. Probes

The following segments of DNA were used as hybridization probes for DNA and RNA: \textit{bGH}, the entire 1.0 kb bGH cDNA (Woychick et al., 1982); \textit{PEPCK}, a 1.1-kb PstI-PstI fragment from the 3' end of the PEPCK cDNA (Yoo-Warren et al., 1983); and \textit{actin}, a 1.2 kb PstI-PstI fragment of the mouse a-actin cDNA (Minty et al., 1981). All DNA fragments were labeled with [$\alpha^{32}$P]dCTP using a random primed labeling kit (Boehringer Mannheim) and the conditions specified by the manufacturer. Unincorporated isotope was separated from incorporated isotope by a G-50 Sephadex spun column.

4. DNA isolation

Genomic DNA was isolated from mouse tail samples (approximately 1 cm) according to a modified version of the procedure described by Davis et al., (1980). Tail samples were crushed to a powder
Figure 7. Generation of transgenic mice that contain the PEPCK-bGH gene

PEPCK-bGH genes containing various block mutations in the PEPCK promoter regulatory region were microinjected into the male pronucleus of a fertilized egg. The eggs were reimplanted into pseudopregnant females and brought to term. DNA was extracted from tail biopsies from mice produced from microinjection and assayed for the presence of the transgene.
Female rat

Eggs

Pronuclei

PEPCK-bGH gene

Wash fertilized eggs out of oviduct

Fertilized egg

Suction pipette holding egg

Impiant eggs into pseudopregnant mouse

Isolate DNA from tail

Assay for the presence of PEPCK-bGH gene

Male rat

Sperm
in liquid nitrogen. The dry powder was incubated overnight at 55°C in an extraction buffer containing, STE (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4), 0.5% SDS and 100 mg/ml proteinase K. Samples were then RNase treated (10 units/ml) for one hour at 37°C with constant rocking. DNA was extracted by a mixture containing an equal volume of phenol and chloroform, followed by chloroform extraction and ethanol precipitation. DNA samples were resuspended in water by rocking at 4°C.

5. DNA dot blot analysis

Mice produced from microinjection were screened for the presence of the transgene by dot blot analysis. Genomic DNA isolated from mouse tail samples was denatured in a 0.1 M NaOH - 2.0 M NaCl solution and blotted onto Gene Screen Plus filters using a Schleicher and Schuell dot blot manifold apparatus. Each sample was blotted at 3 different concentrations, 10, 5 and 2.5 μg of DNA. Genomic DNA from a nontransgenic mouse was also blotted at the three different concentrations, as a negative control. A pPCbGH plasmid (a pBR322 based vector containing a -460/+73 bp fragment of the PEPCK promoter ligated to the bGH structural gene) was blotted as a positive control and also a standard (at a copy number of 1, 5, 10, 25, 50 and 100) to estimate the copy number of integrated copies of the PEPCK-bGH gene in the mouse genome. Filter was baked in a vacuum oven at 80°C for 2 hours and prehybridized in 50% deionized formamide, 0.25 M NaCl, 1
mM EDTA, 0.25 M NaH₂PO₄, 1% SDS, 0.1% BLOTTO and 0.25 mg/ml
denatured salmon sperm DNA at 42°C for 2 hours and hybridized in this
same solution plus ³²P-random primed labelled probe (2 x 10⁶ cpm/ml).
A bGH cDNA probe was used to hybridize the dot blot filters to detect
the presence of the transgene in transgenic mice. Filters were washed
with 0.1 x SSC (0.015 M NaCl and 1.5 mM Na₃Citrate)- 0.1% SDS at
room temperature for 1 minute and then in 0.1 x SSC - 0.1% SDS at
52°C for 15 minutes, blotted dry and scanned by a Phosphor Imager
(Molecular Dynamics, CA).

6. Southern Blot analysis

Southern blot analysis was carried out as described by Maniatis
et al, (1982). Twenty µg of genomic DNA was digested with Kpn I (a
restriction enzyme that has only one site in the PEPCK-bGH gene) using
the conditions specified by the manufacturer. Digested DNA was then
fractionated on a 0.8% agarose gel by electrophoresis. Gels were
denatured in a 1.5 M NaCl-0.5 N NaOH solution for 40 minutes and
neutralized in a NaOH-NH₄CH₃COH solution for 1 hour. The DNA was
then transferred in a NaOH-NH₄CH₃COH solution onto Gene Screen
Plus filters overnight. The filters were then baked for 2 hours at 80°C in
a vacuum oven.

7. Serum bGH protein analysis
The relative concentration of serum bGH was determined by Western blot analysis. Mice were bled from a tail vein. Approximately 200 μl of blood was taken and centrifuged for 10 minutes at 4°C. The samples were frozen until further analysis. Serum samples (2 μl) were added to a sample buffer (18 μl) (0.125 M tris-Cl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heated at 90°C for 5 minutes. The sample was then subjected to SDS-PAGE in 10% acrylamide, 2.7% bisacrylamide (Laemelli, 1970) and a Tris-glycine pH buffer system containing 0.1% SDS. Proteins were transferred to an Immobilon membrane electrophoretically. The filters were blocked with 1 x PBS pH 7.4, 0.03% polyoxyethylenesorbitan monolaurate (Tween 20) and 10% dry skim milk for 1 hours at room temperature. Filters were then incubated with a rabbit anti-bGH IgG antibody (1:250 dilution) for 2 hour at room temperature. Filters were washed 3 times in 1 x PBS pH 7.4 and 0.03% Tween 20 and then incubated with a goat anti-rabbit IgG horseradish peroxidase linked antibody (1:500 dilution) for 1 hour at room temperature. Filters were washed 4 times 1 x PBS pH 7.4 and 0.03% Tween 20. An ECL kit was used to detect deposited HRP. A standard curve was established using 1, 5, 10, 25, 50, and 100 μg of recombinant bGH resuspended in mouse sera from a nontransgenic mouse.

8. RNA isolation
Total RNA was isolated from mouse tissues by a modified acid-phenol guanidinium thiocyanate (GTC) procedure (Chomczynski et al., 1987). Tissue samples were homogenized in 4 M GTC β-mercaptoethanol solution for 1 minute. 400 μl of 2 M NaAcetate pH 4.0 was added to the sample, mixed and followed by 4 ml of water saturated phenol and 800 μl of chloroform. The samples were vortexed for 15 seconds and placed on ice for 15 minutes. Samples were centrifuged at 3000 rpm for 20 minutes at 4°C. The aqueous phase was added to 4 ml of isopropanol, mixed and placed at -20°C for 1 hour. The samples were then centrifuged at 3000 rpm for 20 minutes at 4°C. The supernatant was decanted and the RNA pellet was resuspended in 500 μl of a 4 M GTC-β-mercaptoethanol solution and precipitated with 1 ml of ethanol at -20°C for 1 hour. Samples were then centrifuged at top speed in a microfuge (12,000 rpm) for 15 minutes at 4°C. The supernatant was decanted and the pellet washed 3 times with 80% ethanol (made with (DEPC) diethylypyrocarbonate-treated water). DEPC-treated water (0.1%) was prepared by adding 500 ml of DEPC solution to 500 μl of water. The pellet was dried and resuspended in DEPC water.

9. Northern blot analysis

Northern blot analysis was carried out as described by Davis et al. (1986). Twenty μg of total RNA was fractionated in a 1% agarose gel in a 5% formaldehyde-1 x borate buffer by electrophoresis (Maniatis et al., 1982). RNA was transferred to Gene Screen Plus filters in a 10 x
SSC-4% formaldehyde buffer overnight. The membrane was UV-cross-linked for 3 minutes and then baked in a vacuum oven for 2 hours at 80°C, prehybridized and hybridized as described previously. Northern blot analysis was used to determine the size of the mRNA transcribed from the transgene and also to determine the level of bGH mRNA by normalizing to actin mRNA values.

10. RNA slot blot analysis

RNA samples for slot blot analysis were prepared with 50% deionized formamide, 6% formaldehyde, 10 μg of RNA and brought to 200 μl with DEPC water. Dilutions of total RNA (1, 5, 10, 20 μg) isolated from the liver, give a linear hybridization pattern to a 32P-labeled cDNA probe (Fig. 8). Samples were heated at 52°C for 1 hr and then blotted onto a Gene Screen Plus filter using a 48 well slot blot apparatus (Bio-Rad, Richmond, Ca). RNA was UV crosslinked to the filters. Filters were then hybridized overnight, washed at 55°C (as previously described) and the relative radioactivity in the individual bands was determined using a Phosphor Imager. The levels of bGH mRNA and PEPCK mRNA were expressed relative to actin mRNA levels in a specific tissue in order to control for differences in loading the RNA onto the slot blot apparatus.
Figure 8. Analysis of linear hybridization of hepatic RNA

Hepatic RNA from a transgenic mouse containing a chimeric PEPCK WT(460)-bGH gene was blotted onto filters at concentrations of 1, 5, 10, and 20 µg (lanes 1-4, respectively) and hybridized with a \(^{32}\)P-labeled bGH cDNA. The radioactivity in the hybridized complex was determined by scanning with a Molecular Graphics Phosphor Imager.
11. Dietary treatments

In order to assess the responsiveness of the PEPCK-bGH chimeric genes to diet, transgenic mice were fed a high carbohydrate (80% by weight) diet for one week followed by 1 week of a high protein (65% by weight)-carbohydrate free diet or a high fat (30% by weight)-carbohydrate free diet. The effects of these diets on PEPCK regulation in the liver have been extensively characterized (Peret et al., 1981). Mice were fed ad libitum with continuous access to water. Three mice were assigned to each group and body weight and food intake were recorded at the beginning and end of each dietary treatment. Mice were killed between 9:00 and 10:00 A.M. and tissues taken and frozen in liquid nitrogen for further analysis.

12. cAMP treatment

To determine the effect of cAMP on hepatic PEPCK-bGH gene expression in transgenic mice. Twelve week old mice were fed a high carbohydrate diet for one week followed by cAMP treatments. The Bt₂cAMP was dissolved in saline at a concentration of 30 mg/kg of body weight. One intraperitoneal injection of Bt₂cAMP (30 mg/kg) was given every 30 minutes for a total of 90 minutes at which time mice were killed by cervical dislocation. A single injection of theophylline (30 mg/kg of body weight) was given intraperitoneally at the beginning of the experiment. Three mice were assigned to each treatment group and all
experiments began between 9:00 and 10:00 A.M. Tissues were taken and frozen at -80°C until further analysis.

13. Fetal experiments

To determine the pattern of PEPCK-bGH gene expression in the liver during development fetal liver samples were taken at day 17 of gestation and one day after birth. Liver samples from all the fetuses (6-13 fetuses/litter) were taken and frozen at -80°C for further analysis. Since the transgenic mice used in these studies are heterozygous for the transgene, not all the offspring possess the transgene. Therefore tail samples from each fetus or pup were assayed for the presence of the transgene. Only mice that contained the PEPCK-bGH gene were used so that the absence or presence of expression of the transgene during development was due to developmental regulation of positive transgenic mice and not the inclusion of nontransgenic mice.
III RESULTS

A. Characterization of mice with PEPCK-bGH genes containing block mutations in the PEPCK promoter

1. Characterization of PEPCK-bGH genes

Five PEPCK-bGH chimeric genes contained various block mutations within putative regulatory regions in a -460/+73 bp fragment of the PEPCK promoter ligated to the bGH structural gene (Fig. 6). The regulatory elements which were replaced were chosen by cell transfection assays and footprinting analysis. Four sites were chosen, CRE; PEPCK(CRE)-bGH, P1; PEPCK(P1)-bGH, P2; PEPCK(P2)-bGH, P3(l); PEPCK(P3(l))-bGH. One PEPCK-bGH chimeric gene contained base pair substitutions in two putative protein binding regions of the DNA, CRE and P3(l); PEPCK(CRE/P3(l))-bGH. These five PEPCK-bGH genes containing block mutations in the PEPCK promoter (Table 2) were used to produce transgenic mice. The number of mice produced from microinjection of each transgene is given in Table 3.

2. Identification of founder mice

Mice produced from microinjection were assayed for the presence of the PEPCK-bGH gene by DNA dot blot analysis. Genomic DNA samples (extracted from tail biopsies) were blotted onto filters at a concentration of 10, 5 and 2.5 μg. A DNA sample from a nontransgenic
Table 3. Frequency and expression of PEPCK-bGH genes in transgenie mice

The number of mice produced by microinjection of each PEPCK-bGH gene is presented. Mice that integrated the transgene was determined by DNA dot blot analysis. Transgenic mice that expressed serum bGH was determined by Western blot analysis. The ratio of integration to expression is presented.
<table>
<thead>
<tr>
<th>MUTATION</th>
<th># of offspring</th>
<th># of integrants</th>
<th># of expressors</th>
<th>PERCENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRE</td>
<td>220</td>
<td>13</td>
<td>8</td>
<td>63</td>
</tr>
<tr>
<td>P1</td>
<td>153</td>
<td>6</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>P2</td>
<td>169</td>
<td>6</td>
<td>4</td>
<td>67</td>
</tr>
<tr>
<td>P3(I)</td>
<td>80</td>
<td>8</td>
<td>6</td>
<td>75</td>
</tr>
<tr>
<td>CRE/ P3(I)</td>
<td>71</td>
<td>8</td>
<td>5</td>
<td>63</td>
</tr>
</tbody>
</table>
mouse was also blotted as a negative control. The filters were hybridized with a $^{32}$P-random prime labeled bGH cDNA probe (Fig. 9) Mice that had incorporated the PEPCK-bGH gene into their genome are referred to as founder mice. This assay was conducted on DNA samples from every mouse produced by microinjection. The number of founder mice produced for each PEPCK-bGH gene is given in Table 3.

3. Determination of orientation and copy number of the transgenes

In order to determine the relative orientation of the transgenes to each other in the mouse genome, Southern blot analysis was performed. Twenty μg of genomic DNA from founder mice was digested with KpnI (a restriction enzyme with only one site in the PEPCK-bGH gene), separated by electrophoresis on a 0.8% agarose gel, transferred to a filter and hybridized with a $^{32}$P-labeled bGH cDNA probe. The restriction pattern of the digested DNA should indicate the orientation of the PEPCK-bGH genes (Fig. 10). The size of the PEPCK-bGH gene was 2.7 Kb. A 2.7 Kb band was detected, indicating that the PEPCK-bGH gene integrated in a tandem head-to-tail repeat at a single chromosomal loci (Fig. 11). Analysis of genomic DNA from each founder mouse indicated integration of the transgene at a single chromosomal loci and a head-to-tail tandem repeat of the PEPCK-bGH gene (data not shown).
Figure 9. Analysis of DNA from mice produced by microinjection of the PEPCK-bGH gene

Genomic DNA from tail biopsies was isolated and blotted onto Gene Screen Plus filters at 10, 5 and 2.5 μg. Filters were hybridized with a bGH cDNA probe. Samples (1-9) are from mice produced from microinjection of the PEPCK(P1)-bGH gene and samples (10-12) are from nontransgenic control mice.
Figure 10. Possible orientations of the PEPCK-bGH gene in the genomes of transgenic mice

Hatched boxes represent PEPCK sequences and the solid boxes represent the entire bGH structural gene. Solid lines represent genomic sequences. The fragment sizes of a Kpn I digest are indicated below the two orientations of the PEPCK-bGH genes. (A) A head-to-tail tandem repeat orientation integrated into the genome. (B) A head-to-head and tail-to-tail orientation of an integrated PEPCK-bGH gene in the mouse genome.
Figure 11. Southern blot analysis of DNA from transgenic mice containing a PEPCK-bGH gene

Genomic DNA (20 µg) isolated from tail samples of transgenic mice microinjected with a PEPCK(CRE/P3(II))-bGH gene, was digested with KpnI, separated by electrophoresis and transferred to a Gene Screen filter. Also included on the blot was a pPCbGH plasmid at 1, 5, 10, 25, and 100 copies (lanes 2-6) that was used as a standard and lanes (1) is a DNA sample from a mouse containing a PEPCK (P3)-bGH gene. Filters were hybridized with a random prime labeled bGH cDNA probe. The size of the hybridized fragment is indicated.
In order to determine the number of copies of the transgene that had integrated into the mouse genome, a standard curve using a pPCbGH plasmid (a pBR322 based plasmid containing a -550/+73 bp fragment of the PEPCK promoter ligated to the entire bGH structural gene) at 1, 5, 10, 25, 50 and 100 copies was included on the Southern blot (Fig. 11). The copy number was quantitated using a Phosphor Imager. The copy number of specific lines of transgenic mice is given in Table 4.

4. Expression of the PEPCK-bGH gene in transgenic mice

To determine if the integrated transgene was expressed, total RNA from various tissues was isolated and subjected to Northern blot analysis. Filters were hybridized sequentially with a $^{32}$P random prime labeled bGH cDNA and a Pst I-Pst I fragment of the 3' end of the PEPCK cDNA. A representative Northern blot of RNA from liver, kidney, adipose tissue, jejunum and spleen of a mouse containing the PEPCK(P3(I))-bGH gene is shown in Fig. 12. The bGH cDNA probe hybridized to RNA from liver, kidney and jejunum but not adipose tissue and spleen. Since PEPCK is not expressed in the spleen, it was used as a negative control. The bGH cDNA probe hybridized to a single band of the predicted length (1.0 Kb), which indicated that the bGH cDNA probe is specific for the transgene and the correct processing of the transgene occurred. The bands which hybridized to the mouse $\alpha$-actin probe and the 3' end of the PEPCK probe were 2.0
Table 4. Properties of specific lines of transgenic mice containing PEPCK-bGH genes

Copy number was determined by Southern blot analysis and serum bGH concentration was determined by Western blot analysis as described in Methods. The block mutation contained within the PEPCK-bGH gene and the specific line of transgenic mice are indicated.
<table>
<thead>
<tr>
<th>MUTATION</th>
<th>COPY NUMBER (copies/haploid genome)</th>
<th>SERUM bGH (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(460) #36</td>
<td>25</td>
<td>9.5</td>
</tr>
<tr>
<td>CRE #4</td>
<td>15</td>
<td>75.0</td>
</tr>
<tr>
<td>P1 #21</td>
<td>2</td>
<td>5.0</td>
</tr>
<tr>
<td>P2 #120</td>
<td>20</td>
<td>20.0</td>
</tr>
<tr>
<td>P3(I) #56</td>
<td>20</td>
<td>10.0</td>
</tr>
<tr>
<td>CRE/P3(I) #24</td>
<td>8</td>
<td>9.5</td>
</tr>
</tbody>
</table>
Figure 12. Northern blot analysis of total RNA from different tissues of mice containing PEPCK-bGH genes

Twenty μg of total RNA was isolated from skin, adipose tissue, jejunum, heart, spleen, kidney and liver (lanes 1-7) and separated by electrophoresis on a 1% denaturing agarose gel. RNA was then transferred to a Gene Screen Plus filter, UV cross-linked to the filter and hybridized with a random prime labeled bGH cDNA probe and a PstI-PstI fragment of the mouse α-actin cDNA probe. The size of the hybridized fragments are indicated.
Actin 2.0 Kb
bGH 1.0 Kb
and 2.8 Kb, respectively (Fig.12). Each probe detected only a single band of the correct size which indicated that the cDNA probes were specific for one mRNA species. Therefore, all further studies of PEPCK-bGH gene expression were analyzed by slot blot analysis (Fig. 13), as described in the Methods section.

5. Determination of bGH in the serum of transgenic mice

Mice that had integrated the PEPCK-bGH gene were analyzed for the presence of bGH in their serum. Mice were bled from a tail vein and serum samples (2 µl) were subjected to Western blot analysis. The concentration of serum bGH from various lines of mice containing PEPCK-bGH genes is given in Table 4. The level of serum bGH ranged from 5-75 µg/ml in the mice and resulted in a growth rate approximately twice that observed for nontransgenic mice (measured by weight) (Fig.14). This observation is similar to that noted by McGrane et. al. (1988). The percentage of founder mice which were positive for both integration and expression for each transgene was determined by dividing the number of transgenic mice expressing bGH by the number of mice that had incorporated the transgene into their genome. The PEPCK-bGH gene was 67% for PEPCK(CRE)-bGH, 50% for PEPCK(P1)-bGH, 67% for PEPCK(P2)-bGH, 75% for PEPCK(P3(I))-bGH, and 63% for PEPCK(CRE/P3(I))-bGH. This ratio of integration to expression of the transgene is similar to that noted by McGrane et al,
Figure 13. Slot blot analysis of RNA from livers of mice containing a PEPCK-bGH gene

Ten µg of RNA from liver was blotted onto a Gene Screen Plus filter, UV cross-linked and hybridized sequentially with a random prime labeled Pst I-Pst I 3' fragment of the PEPCK cDNA, bGH cDNA and a PstI-PstI fragment of the mouse α-actin cDNA. Lane 1, WT(460), Lanes (2 and 4), nontransgenic control mice, Lanes (3,5,6), mice containing a PEPCK(CRE)-bGH gene.
Figure 14. Growth rate of mice containing PEPCK-bGH genes

The growth pattern of mice containing a PEPCK-bGH gene with block mutations in the CRE, P1, P2, P3(I) and CRE/P3(I) regions of the PEPCK promoter is compared to the change in weight of a nontransgenic mouse.
The level of expression of the PEPCK-bGH gene was independent of the number of copies of the transgene that integrated into the genome (Table 4). Mice that were both positive for integration and expression of the PEPCK-bGH gene were used to establish individual transgenic mouse lines. To determine germline transmission of the PEPCK-bGH gene, founder mice were bred to nontransgenic mice and offspring were assayed for the presence of the transgene by DNA dot blot analysis.

B. The effects of specific mutations in the PEPCK promoter on the tissue-specific expression of the PEPCK-bGH gene

The level of bGH mRNA was determined in various tissues from mice containing the PEPCK-bGH genes. Total RNA was isolated from various tissues and subjected to slot blot analysis. Blots were hybridized with $^{32}$P labeled probes of bGH cDNA and a PstI-PstI fragment of the mouse $\alpha$-actin cDNA. Actin values were used to normalize the bGH mRNA values.

1. CRE mutation

Five lines of mice with the PEPCK(CRE)-bGH gene were generated, all of which expressed considerable bGH mRNA in the liver and had high concentrations of bGH in the blood (20 to 100 $\mu$g/ml of serum). Mice with a PEPCK(CRE)-bGH gene expressed bGH mRNA in
liver and kidney with variable expression in the jejunum (4 lines assayed). The level of hepatic bGH mRNA varied in different lines of mice (founder mice for CRE #53, #64, #119 and #123 and F1 mice for CRE #4) (Table 5). The amount of bGH mRNA in the kidney also varied between lines of transgenic mice but was always less than the levels of bGH mRNA found in the liver. Mice containing the PEPCK(CRE)-bGH gene had a similar pattern of expression as mice containing a PEPCK(460)-bGH gene in the tissues analyzed. Mice containing a PEPCK(CRE)-bGH gene had a marked elevation (average 4 fold) in the basal level of hepatic bGH mRNA compared to values from transgenic mice with the intact PEPCK promoter (Table 5, Fig. 15). Also, there was a marked elevation (20 fold) in the expression of the transgene in the kidneys of mice with a mutation in the CRE of the PEPCK promoter (Table 5, Fig 15). The data suggest that the CRE has little effect on the tissue-specific pattern of PEPCK gene expression but did influence the extent of basal expression of the PEPCK-bGH gene in the liver and kidney.

2. P1 mutation

Mice containing the PEPCK(P1)-bGH chimeric gene had bGH mRNA in the liver and kidney (3 lines assayed) but not in the jejunum, lung, skeletal muscle or adipose tissue (data not shown). The level of expression varied in the liver of different lines but was always
Table 5. Relative expression of the transgene containing block mutations in the PEPCK promoter in the livers and kidneys of transgenic mice

The level of hepatic and renal bGH mRNA was determined by hybridization to a radiolabeled bGH cDNA probe using a slot blot apparatus. Each value is the average of 3 F1 mice. The values for CRE (#53, #64, #119 and #123) and P2 (#65 and #138) were determined from only one sample, obtained from the founder mouse for each line. The radioactivity in the hybridized complex was determined by scanning with a Molecular Graphics Phosphor Imager and the values were normalized to the level of actin mRNA determined in the tissue sample using the same method. The number in parenthesis is the designation for a specific line of transgenic mouse and the average corrected bGH mRNA values for all lines constructed from a specific transgene are presented as the mean and standard deviation for the number of animals in each group.
<table>
<thead>
<tr>
<th></th>
<th>WT(460)</th>
<th>CRE</th>
<th>P1</th>
<th>P2</th>
<th>P3(I)</th>
<th>CRE/P3 (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIVER</td>
<td>(#36) 8.0</td>
<td>(#4) 69.9</td>
<td>(#1) 65.9</td>
<td>(#19) 2.8</td>
<td>(#56) 3.5</td>
<td>(#7) 15.9</td>
</tr>
<tr>
<td></td>
<td>(#40) 13.9</td>
<td>(#53) 34.2</td>
<td>(#4) 1.5</td>
<td>(#65) 4.8</td>
<td>(#58) 4.5</td>
<td>(#19) 8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(#64) 25.7</td>
<td>(#21) 5.7</td>
<td>(#120) 27.9</td>
<td>(#138) 8.2</td>
<td>(#24) 4.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(#119) 43.2</td>
<td></td>
<td></td>
<td></td>
<td>(#64) 19.0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>11.0 ± 4.2</td>
<td>43.0 ± 16.6</td>
<td>24.4 ± 36.0</td>
<td>10.9 ± 11.5</td>
<td>4.0 ± 0.7</td>
<td>12.1 ± 6.5</td>
</tr>
<tr>
<td>KIDNEY</td>
<td>(#36) 0.25</td>
<td>(#4) 7.0</td>
<td>(#1) 1.0</td>
<td>(#19) 0.05</td>
<td>(#56) 1.4</td>
<td>(#7) 107.8</td>
</tr>
<tr>
<td></td>
<td>(#40) 0.10</td>
<td>(#53) 0.4</td>
<td>(#4) 0.6</td>
<td>(#65) 0.06</td>
<td>(#58) 2.3</td>
<td>(#19) 6.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(#64) 0.5</td>
<td>(#21) 0.7</td>
<td>(#120) 0.05</td>
<td></td>
<td>(#24) 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(#119) 7.2</td>
<td></td>
<td></td>
<td></td>
<td>(#64) 54.3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.18 ± 0.11</td>
<td>3.5 ± 3.4</td>
<td>0.77 ± 0.21</td>
<td>0.05 ± 0.01</td>
<td>1.9 ± 0.6</td>
<td>42.5 ± 49.7</td>
</tr>
</tbody>
</table>
Figure 15. The effect of mutations in regulatory elements in the PEPCK promoter on the level of expression of the PEPCK-bGH gene in the livers and kidneys of transgenic mice

RNA was isolated from the liver and kidney of mice containing mutations in regulatory elements in the PEPCK promoter and the level of bGH mRNA was determined as outlined in the legend to Table 5. The results are expressed as the mean ± standard deviation for the number of animals in each line presented in Table 5. The mean was determined as described in Table 5 and the level of bGH mRNA from mice containing a PEPCK(460)-bGH gene was set to a value of 1 and the data are expressed as a fold change from WT(460) values. Note that in Panel B (Kidney), the ordinate for CRE/P3(I) is 10 times higher than the values for the other PEPCK-bGH genes tested.
significantly higher than the concentration of bGH mRNA in the kidney (Fig. 15). This pattern of expression is similar to mice with the PEPCK(460)-bGH gene. Thus the loss of the P1 site did not effect the pattern of tissue-specific expression or the levels of transgene expression in liver or kidney.

3. P2 mutation

The P2 region of the PEPCK promoter has been shown to bind a protein(s) present in the nuclei from the kidney (Roesler et al., 1989) and with a different pattern to protein(s) present in the liver. The P2 region is protected in DNase1 footprint assays by HNF-1 (Gurney and Hanson unpublished observations). Mice containing the PEPCK(P2)-bGH gene had similar hepatic bGH mRNA levels as mice with a PEPCK(460)-bGH gene (Fig. 15), but there was no detectable bGH mRNA in the kidneys of these animals (4 lines assayed) (Fig. 15). Expression of bGH mRNA was not detected in the jejunum or adipose tissue of mice with a PEPCK(P2)-bGH gene (data not shown).

4. P3(1) mutation

The P3(1) region of the PEPCK promoter binds to protein(s) present in hepatic and not renal nuclei (Roesler et al., 1989) and footprints with members of the C/EBP family of proteins which are present in the liver (Roesler et al., 1992; Park et al., 1993). C/EBPα
(Park et al., 1990), C/EBPβ (Park et al., 1993) and DBP (Roesler et al., 1992) transactivate transcription from the PEPCK promoter of a PEPCK(460)-CAT gene which was transfected into hepatoma cells. In mice containing the PEPCK(P3)-bGH fusion gene (three lines assayed), bGH mRNA was detected in the liver, kidney and jejunum but not in adipose tissue and skeletal muscle. However, the concentration of hepatic bGH mRNA in these mice was approximately 40% of the level noted in the livers of animals with a PEPCK(460)-bGH gene (Fig. 15). and was remarkably consistent between different lines of mice. Surprisingly, the level of renal bGH mRNA in mice with a PEPCK(P3)-bGH gene was approximately 10-fold higher than renal bGH mRNA levels present in mice with a PEPCK(460)-bGH gene (Fig. 15). The levels of bGH mRNA in the jejunum were variable in the different lines (data not shown).

5. CRE/P3(I) mutation

Bovine growth hormone mRNA was detected in the liver, kidney and jejunum of mice containing the PEPCK(CRE/P3(I))-bGH chimeric gene but not in skeletal muscle or adipose tissue. The concentration of bGH mRNA in the livers of mice with a PEPCK(CRE/P3(I))-bGH gene was the same as that noted with mice containing the PEPCK(460)-bGH gene (4 lines assayed) (Fig. 15). The level of renal bGH mRNA expression was variable but significantly higher than that noted with mice containing a PEPCK(460)-bGH
chimeric gene (Fig. 15). The increase in bGH mRNA levels in the kidneys of mice with a PEPCK (CRE-I/P3(I))-bGH gene was much greater than was noted with mice containing a mutation in the CRE or P3(I) elements alone (Fig. 15).

The level of bGH mRNA in the liver and kidney of transgenic mice containing various block mutations in the PEPCK promoter is given in Table 5. We selected for detailed analysis of dietary and hormonal regulation of transcription from the PEPCK promoter, lines 36 (intact PEPCK promoter), 4 (CRE mutation), 21 (P1 mutation), 120 (P2 mutation), 56 (P3(1) mutation) and 24 (CRE/P3(1) double mutation) since these lines are generally representative of the other lines of transgenic mice which express the PEPCK-bGH gene. In general, we noted the highest level of PEPCK-bGH gene expression in the liver (except in case of a mutation in P3(1)), with less in the kidney and jejunum of the small intestine; adipose tissue and spleen had only marginal levels of bGH mRNA (Fig. 16).

C. Regulatory elements in the PEPCK promoter responsible for the transcriptional response of the PEPCK-bGH gene to diet
Figure 16. Tissue specific expression of bGH mRNA in mice containing a PEPCK-bGH gene with specific block mutations in the PEPCK promoter

RNA was isolated from the liver, kidney, jejunum, adipose tissue and spleen. Ten µg of RNA was blotted onto a filter using a slot blot apparatus. The RNA was UV cross-linked to the filter. The filter was then hybridized sequentially with a random prime labeled bGH cDNA probe and then a PstI-PstI fragment of the mouse α-actin cDNA probe. The bGH mRNA was normalized to actin mRNA values. (A) liver, (B) kidney, jejunum, adipose tissue and spleen. Representative lines for each transgene are presented: WT(460), (□); CRE # 4, (□); P1 #21, (■); P2 #120, (□); P3 # 56, (□) and CRE/P3(1) # 24, (□).
Transcription of the PEPCK gene is dramatically reduced in animals fed a diet high in carbohydrate and increased in animals fed a high protein/carbohydrate-free diet (Friedman et al., 1993). Regardless of the mutation in the PEPCK promoter, feeding the animals a diet high in carbohydrate for one week resulted in a 70-90% decrease in the concentration of bGH mRNA in the livers (Fig. 17). In contrast, a diet high in protein but devoid of carbohydrate induced the levels of bGH mRNA in the livers of all transgenic mice (Fig. 17). Unexpectedly, mice with a PEPCK-bGH gene containing a mutation in P3(1) showed the most marked response to the high protein/carbohydrate free-diet (a 40-fold induction when compared to mice fed a high carbohydrate diet). This effect of a mutation in the P3(1) element of the PEPCK promoter was also reflected in the response of mice with a PEPCK-bGH gene containing a CRE/P3(1) mutation to the high protein diets (Fig. 17). In all cases the concentration of the endogenous PEPCK mRNA in the various lines of transgenic mice responded to the dietary alterations in manner similar to that noted in the livers of mice with the PEPCK(460)-bGH gene or to non-transgenic control animals (data not shown). PEPCK mRNA levels were decreased by feeding mice a diet high in carbohydrate and increased by feeding mice diets high in protein and devoid of carbohydrate.

The effect of diet on transcription of the PEPCK-bGH gene in the kidneys of transgenic mice was also determined (Fig. 17, insert) Feeding the mice a high carbohydrate diet had only a slight effect on
Figure 17. Dietary regulation of hepatic and renal bGH mRNA in mice containing a PEPCK-bGH gene with block mutations in the PEPCK promoter.

Mice were fed a high carbohydrate diet (80% CHO) for one week (■), followed by one week of a high protein (65% protein)-carbohydrate free diet (■). Bovine GH mRNA in the liver was determined as outlined in the legend to Table 5. The level of bGH mRNA in chow-fed controls (■) was set to a value of 1 and the data are expressed as a fold change from control values. The results are expressed as the mean ± standard deviation for 3 animals per treatment from one line of representative mice for each transgene. The insert contains the relative concentration of bGH mRNA in the kidneys of mice fed the diets indicated in the legend.
transcription from the intact PEPCK promoter or any of the other mutated forms of the promoter used in this study, while a high-protein-carbohydrate-free diet caused an induction in the level of PEPCK mRNA in the kidney (data shown only for PEPCK-460 promoter). A mutation in the P2 element in the PEPCK promoter caused a marked reduction in basal transcription of the PEPCK(P2)-bGH gene (Fig. 15) and also abolished the effects of diet on transcription from the PEPCK promoter (Fig. 17, insert).

To determine if the induction of hepatic PEPCK gene expression resulting from the high protein diet was due to the high levels of dietary protein or the lack of carbohydrate in the diet, transgenic mice containing the PEPCK(460)-bGH, PEPCK(CRE)-bGH or the PEPCK(P3(I))-bGH genes were fed a high carbohydrate diet for 1 week followed by 1 week of a diet high in fat and devoid of carbohydrate (22% kcal protein and 78% kcal fat) (Table 6). Comparison of food intake, weight gain and energy intake of transgenic mice consuming the chow-fed diet and the three experimental diets is given in Table 7. The high fat diet resulted in a 5, 23 and 17-fold induction of hepatic bGH mRNA respectively, over the values noted in the same lines of mice fed the high carbohydrate diet (Fig. 18). The endogenous PEPCK gene in the livers of the transgenic mice discussed above was also induced 5-fold by one week on the same high fat/carbohydrate-free diet (data not shown). Thus, the induction of hepatic PEPCK gene transcription is primarily responsive to levels of dietary carbohydrate
Table 6. Composition of experimental diets

Caloric density is expressed as kcal per g diet. Protein, carbohydrate and fat were calculated by using the physiological fuel values of 4 kcal/g protein, 4 kcal/g carbohydrate and 9 kcal/g fat.
<table>
<thead>
<tr>
<th>Composition</th>
<th>Lab Chow</th>
<th>High-CHO</th>
<th>High-Protein</th>
<th>High-Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>(g/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>230.7</td>
<td>122.0</td>
<td>640.0</td>
<td>174.4</td>
</tr>
<tr>
<td>DL Methionine</td>
<td>4.3</td>
<td>3.0</td>
<td>5.0</td>
<td>4.3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>494.0</td>
<td>800.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cottonseed oil</td>
<td>65.0</td>
<td>40.0</td>
<td>80.0</td>
<td>143.0</td>
</tr>
<tr>
<td>Butter</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>143.0</td>
</tr>
<tr>
<td>Alphacel Non-nutritive Bulk</td>
<td>10.1</td>
<td>0.0</td>
<td>220.0</td>
<td>485.3</td>
</tr>
<tr>
<td>Brewers' Yeast</td>
<td>0.0</td>
<td>0.0</td>
<td>20.0</td>
<td>0.0</td>
</tr>
<tr>
<td>AIN Vitamin and Mineral Mix</td>
<td>10.5</td>
<td>35.0</td>
<td>35.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Caloric Density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(kcal/g)</td>
<td>3.50</td>
<td>4.06</td>
<td>3.30</td>
<td>3.29</td>
</tr>
<tr>
<td>Protein (% kcal)</td>
<td>26.8</td>
<td>12.3</td>
<td>78.2</td>
<td>21.7</td>
</tr>
<tr>
<td>Carbohydrate (% kcal)</td>
<td>56.4</td>
<td>78.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Fat (% kcal)</td>
<td>16.8</td>
<td>8.9</td>
<td>21.8</td>
<td>78.3</td>
</tr>
</tbody>
</table>
Table 7. Body weight, weight gain and energy intake of transgenic mice that were fed lab chow or experimental diets

Energy intake is expressed as kcal and weight gain and body weight are expressed as grams. Mice were weighed before and after each dietary treatment. Energy intake was determined by weighing the food at the beginning and end of each dietary treatment and then expressed as kcal.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Lab Chow</th>
<th>High-CHO</th>
<th>High-Protein</th>
<th>High-Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>41 ± 4</td>
<td>40 ± 4</td>
<td>39 ± 5</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>1.9 ± 0.9</td>
<td>0.8 ± 0.4</td>
<td>1.8 ± 1.1</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>Energy intake (kcal)</td>
<td>212.9 ± 10.5</td>
<td>154.2 ± 18.0</td>
<td>111.9 ± 27.4</td>
<td>356.4 ± 52.9</td>
</tr>
</tbody>
</table>
Figure 18. The effect of a high fat, carbohydrate-free
diet on the level of expression of the PEPCK-
bGH gene in the livers of transgenic mice

Mice were fed a high carbohydrate diet (80% CHO) for one
week (■) followed by a high fat (12% fat)-carbohydrate free diet for
one week (□). Hepatic RNA was isolated as described in
Experimental Procedures. Bovine GH mRNA in the livers of transgenic
mice was determined as outlined in the legend to Table 5. The data
are expressed relative to the level of bGH mRNA in the livers of chow-
fed mice (■) which were arbitrarily set to a value of 1. The results are
the mean ± standard deviation for 3 animals per treatment.
and not to the elevated concentration of protein in the diets. Also, the elements that are necessary for induction of PEPCK gene transcription in the absence of dietary carbohydrate are located between -460 and +73 of the PEPCK promoter. The response to a high carbohydrate diet does not require either of the cAMP responsive sites (CRE or P3(I)) identified in the PEPCK promoter (see Fig. 18).

D. Regulation of PEPCK-bGH expression by cAMP

Administration of Bt2cAMP to carbohydrate fed rats caused a marked induction in PEPCK mRNA in the liver (Lamers et al., 1982). This pattern of rapid induction was also noted with transgenic mice containing a fragment of the PEPCK promoter from -460/+73 (McGrane et al., 1986). All five PEPCK-bGH chimeric genes were assayed for cAMP responsiveness. Transgenic mice were fed a high carbohydrate (80%) diet for one week and then given an intraperitoneal injection of Bt2cAMP (30 mg/kg) every 30 minutes for 90 minutes and one injection of theophylline (30 mg/kg) at the beginning of the experiment. Mice were killed after 90 minutes and RNA was isolated from livers. To determine which DNA elements are involved in cAMP responsiveness, PEPCK and bGH mRNAs were assayed in the livers of treated animals. In all mice hepatic PEPCK mRNA increased 5 fold within 90 minutes of Bt2cAMP administration. Mice containing a PEPCK(460)-bGH gene also had a 5 fold induction in hepatic bGH
mRNA concentrations within 90 minutes of cAMP administration (Fig. 19), as did mice containing a PEPCK(P2)-bGH chimeric gene (data not shown). Administration of Bt2cAMP did not induce the expression of bGH mRNA in livers of mice containing either the PEPCK(CRE)-bGH, PEPCK(P1)-bGH, PEPCK(P3(I))-bGH or the PEPCK(CRE/P3(I))-bGH chimeric gene (2 lines tested for each transgene) (Fig. 19). This finding confirms studies using HepG2 cells which identified both the CRE and P3(I) sites as being involved in cAMP responsiveness of the PEPCK promoter (Liu et al., 1991). In addition, Park et al (1993) demonstrated that the P1 site is required for full induction of transcription from the PEPCK promoter by C/EBP even though P1 does not bind C/EBP. The interaction between the CRE and the P1 domains seems to be required for cAMP induction of the PEPCK gene in the liver.

E. Analysis of the insulin response element

It was previously shown that regulatory elements necessary for dietary inhibition of PEPCK gene transcription by feeding a high carbohydrate diet are contained within a -460/+73 bp fragment of the PEPCK promoter. A diet high in carbohydrate inhibited transcription from all five of the PEPCK-bGH transgenes containing promoter block mutations. Therefore the regions of the promoter involved in the inhibition of PEPCK gene transcription due to high carbohydrate diets
Figure 19. The effect of cAMP on bGH mRNA in the livers of mice containing a PEPCK-bGH gene with block mutations in regulatory elements of the PEPCK promoter.

Mice were fed a high carbohydrate (80% CHO) diet for one week, ( ) followed by an intraperitoneal injections of Bt2cAMP (30 mg/kg of body weight) every 30 minutes for a total of 90 minutes and one intraperitoneal injection of theophylline at the initiation of the experiment (30 mg/kg of body weight) ( ). Mice were killed after 90 minutes and RNA was isolated from the liver. Bovine GH mRNA was determined as outlined in legend to Table 5. The data are expressed, relative to chow-fed controls ( ), as the mean ± standard deviation.
are still unknown. Previous studies using cell transfection assays (O'Brian et al., 1990) identified a putative insulin response sequence (IRS) at position -407 to -416 in the PEPCK promoter. To determine the role of the putative IRE in the dietary regulation of PEPCK gene transcription, mice containing a (-355 to +73 bp) PEPCK promoter fragment ligated to bGH were analyzed. Mice containing a PEPCK(355)-bGH gene were fed a high carbohydrate diet for 1 week followed by 1 week of a high protein/carbohydrate free diet. Feeding a high carbohydrate diet decreased the level of hepatic bGH mRNA to 40\% that of chow fed levels in transgenic mice with a PEPCK(355)-bGH (Fig. 20). Feeding a high protein diet increased the bGH mRNA 3.5 fold over high carbohydrate levels in the livers of transgenic mice with a PEPCK(355)-bGH (Fig. 20).

F. Regulatory elements in the PEPCK promoter responsible for the developmental expression of the PEPCK-bGH gene in liver

Previous studies have shown that a fragment of the PEPCK promoter from -460 to +73 directed appropriate developmental expression of the PEPCK-bGH gene in the livers of transgenic mice (McGrane et al., 1990). To determine the regulatory elements which are responsible for the pattern of development of PEPCK gene
Figure 20. Dietary regulation of hepatic bGH mRNA in mice containing a PEPCK(355)-bGH gene

Mice were fed a high carbohydrate diet for one week, followed by one week of a high protein-carbohydrate free diet. Bovine growth hormone mRNA in the liver was determined as outlined in the methods section. The level of bGH mRNA in chow-fed controls was set to a value of 1 and the data are expressed as a fold change from control values. The results are expressed as the mean ± standard deviation for 3 animals per treatment.
expression in the liver, PEPCK promoter mutations were analyzed at two time points during development; both PEPCK and bGH mRNAs were assayed in the livers of transgenic mice at day 17 of gestation and 1 day after birth. Bovine GH mRNA was not detectable in the livers of mice with either the PEPCK(P2)-bGH or PEPCK(P3)-bGH genes at day 17 of gestation but was detectable at day 1 after birth (Fig. 21). The pattern of transgene expression in these mice was similar to that of the endogenous PEPCK gene.

In the livers of mice containing the PEPCK(P1)-bGH chimeric gene, bGH mRNA was detectable at day 17 of gestation at a relatively low level, (2 lines assayed) (Fig. 21). The concentration of bGH mRNA increased significantly in the livers of mice with a PEPCK(P1)-bGH gene 1 day after birth, in a pattern similar to the endogenous gene (Fig. 21). In mice containing either the PEPCK(CRE)-bGH or PEPCK(CRE/P3)-bGH genes, (2 lines assayed for each transgene) a low level of bGH mRNA was detectable in the liver at day 17 of gestation (Fig. 21, A). Expression of bGH mRNA increased significantly in the livers of 1 day old newborn mice containing either PEPCK(CRE)-bGH or PEPCK(CRE/P3)-bGH chimeric genes (Fig. 21, B). There was a significant increase in transgene expression in the liver at birth in all of the mice containing the various PEPCK-bGH genes analyzed in this study. The pattern of PEPCK mRNA expression during development was the same in all transgenic mice and paralleled that of nontransgenic mice, indicating that the presence of bGH in the blood of the animals
Figure 21. The expression of bGH mRNA in the livers of transgenic mice containing block mutations in the promoter of the PEPCK-bGH gene during fetal development.

RNA was isolated from the livers of (A) 17 day old fetuses and (B) 1 day old mice. The level of bGH mRNA in the livers of mice containing the PEPCK(460)-bGH, PEPCK(CRE)-bGH, PEPCK(P1)-bGH, PEPCK(P2)-bGH, PEPCK(P3(l))-bGH or PEPCK(CRE/P3)-bGH genes was determined as described in the legend to Table 5. The results are expressed as the mean ± standard deviation.
did not interfere with the development of PEPCK gene expression. Thus both the CRE and P1 regions of the PEPCK promoter are involved in the proper timing of development of hepatic PEPCK gene transcription.
IV DISCUSSION

The studies presented in this thesis demonstrate the importance of animal models to investigate the requirements of specific sequences necessary for the transcriptional regulation of a gene as complex as the PEPCK gene. Three major points are presented in this thesis. One, there are specific sequences that are required for the expression of the PEPCK gene in the liver and kidney. In this thesis I have identified that the P2 and P3(I) regions are responsible for PEPCK gene expression in the kidney and liver, respectively. The transcription factors associated with the P2 and P3(I) regions are HNF-1 and the C/EBP family of proteins, respectively. This study emphasizes the importance of C/EBP in the regulation of PEPCK gene transcription in the liver.

The second major finding indicated that acute and chronic regulation of PEPCK gene transcription requires different elements in the PEPCK promoter. Acute hormonal regulation (cAMP administration) most probably affects the phosphorylation state of nuclear proteins which can then alter transcription. In contrast, chronic hormonal regulation (diet, diabetes, development) affects the concentration of hormone-responsive transcription factors (C/EBP) in the liver. Multiple C/EBP sites on the PEPCK promoter may ensure increased transcription of the PEPCK gene.

The third major finding in this study is that suppression represents a mechanism by which basal levels of PEPCK gene
expression are regulated in both the liver and kidney. Loss of the CRE region in the liver and the CRE and P3(1) regions in the kidney resulted in increased basal levels of bGH mRNA in transgenic mice. This finding is not completely unexpected due to the fact that PEPCK is maintained at low levels in the absence of hormonal stimulation in the liver. A mutation in the CRE resulted in an increase in the basal level of bGH mRNA in the liver. The CRE may not only be involved in the cAMP induction of PEPCK gene transcription, but also in the inhibition of PEPCK gene transcription in the absence of cAMP stimulation. The theme of suppression of basal expression as a method of transcriptional control can also be suggested in the regulation of PEPCK gene expression in the kidney. In transgenic mice, the loss of either the CRE or the P3(1) regions in the PEPCK-bGH gene resulted in increased levels of bGH mRNA in the kidney.

A. System

Transgenic animals provide a powerful tool to investigate the cis-acting sequences involved in the tissue-specific, developmental and dietary regulation of genes encoding metabolic enzymes. The advantages of utilizing an in vivo model system are: (i) it provides the full array of developmental stages of cell differentiation, (ii) the full range of cell types, (iii) the physiological context in which to study
responses to complex stimuli, such as diet and diabetes and (iv) it provides the appropriate concentration and compartmentation of transcription factors that regulate gene transcription.

Although transgenic animals provide a physiological context in which to investigate gene regulation, the expression and regulation of the transgene can be influenced by genomic DNA surrounding the integration site of the transgene. To eliminate the possibility of integration site effects, all the experiments presented in this study were conducted on at least two different lines of transgenic mice for each mutation.

In these studies bGH was used as a phenotypic marker to identify transgenic animals. The presence of bGH did not alter the expected pattern of expression of the PEPCK gene (McGrane et al., 1988) in response to a variety of hormonal and dietary factors used in this study. There are two advantages in using bGH as a marker, one, it is a secreted protein and is therefore easily assayed in the blood, and two, mice expressing bGH are twice the size of their nontransgenic littermates, which permits rapid screening for mice which express the transgene.

Although characterization of tissue-specific sequences for many genes have been determined using cells in culture, transgenic animals provide the most rigorous test for tissue specific expression. There are examples in which the sequences necessary for tissue-specific expression differed in the two systems. One such example is
the albumin gene, which requires approximately 12 kb of 5' flanking sequence to direct tissue-specific expression in transgenic mice (Pinkert et al., 1987) as compared to only a 150 bp segment of the 5' region of the albumin gene to direct expression when introduced into hepatoma cells in culture (Herbomel et al., 1989). A similar discrepancy is observed in the case of the PEPCK promoter. In cultured adipocytes, only a 362 bp segment of the PEPCK promoter was required for transcription from the promoter (Benvenisty et al., 1989) while studies with transgenic mice indicated that sequences between -540 and -2000 bp were required for expression of the transgene in adipose tissue (McGrane et al., 1990; Short et al., 1992; Eisenberger et al., 1992). Thus transgenic animals can conclusively identify the sequences that are necessary for gene expression and regulation.

B. Tissue specific regulatory elements in the PEPCK promoter

In all the lines of transgenic mice examined in the study, expression of the transgene was restricted to tissues that expressed the endogenous PEPCK gene. The transgene was not expressed in tissues that did not express the PEPCK gene. The data suggest that the tissue-specific expression of the PEPCK gene is regulated by the positive interaction between transcription factors and regulatory
elements within a -460/+73 bp fragment of the PEPCK promoter. It
does not appear that there are factors which mediate the suppression
of PEPCK gene expression in tissues that do not express PEPCK,
rather, these tissues lack the necessary factors for transcription of the
gene. Previous studies have demonstrated that transcription factors are
expressed in a tissue specific pattern (such as C/EBP and HNF-1). This
study has conclusively identified two regions of the PEPCK promoter,
P2 and P3(I) that are required to direct expression of the transgene to
the kidney and the liver, respectively. Previously, it had been shown by
DNase I footprint analysis, that the P3(I) region bound proteins only
from liver nuclear extracts and that the P2 region binds proteins from
kidney nuclei.

1. LIVER

In mice containing a PEPCK(P3(I))-bGH gene, the level of basal
expression of the transgene in the liver was reduced to 40% that of the
level of mice containing a PEPCK(460)-bGH gene. The P3(I) region
has been shown to bind C/EBPα, C/EBPβ and DBP. All three of the
transcription factors have been shown to bind and transactivate
transcription when cotransfected with a PEPCK-CAT gene into HepG2
cells (Park et al., 1990; Park et al., 1993; Roesler et al., 1992). All three
proteins are present in the liver at significant concentrations. C/EBPα is
expressed in a gradient from the pericentral to periportal region of the
liver, unlike PEPCK gene expression which is highest in the periportal
region of the liver. Therefore C/EBPα is expressed at lower concentrations in the region of the liver where PEPCK mRNA is expressed and is unlikely to be involved in transcriptional regulation of the PEPCK gene in the liver. However, C/EBPβ mRNA is expressed in a similar gradient across the liver as PEPCK mRNA and has a greater affinity than does C/EBPα for sites in the PEPCK promoter (Park et al., 1993).

It was shown previously, that transgenic mice containing a PEPCK(355)-bGH gene had hepatic bGH mRNA levels comparable to mice with a PEPCK(460)-bGH gene. Therefore the regulatory elements directing expression of the PEPCK gene to the liver are within a -355/+73 bp fragment of the PEPCK promoter. This study identified the P3(l) region as one of the regions necessary to direct expression of the PEPCK gene to the liver. This suggests that there are other regions in the promoter that are involved in directing expression of the PEPCK gene to the liver. There is strong evidence that the involvement of other C/EBP sites in directing liver specific expression of the PEPCK gene in cultured cells (Liu et al., 1992). The P4 region of the PEPCK promoter is the most likely candidate for directing liver specific expression because it binds members of the C/EBP family of proteins, and it is within the -355/+73 region of the promoter. Previous studies have shown that mice containing a PEPCK(174)-bGH gene did not express bGH mRNA (McGrane et al., 1990). Also mice containing a -207/+69 bp fragment of the PEPCK promoter in a PEPCK-human GH
(hGH) chimeric gene had extremely low levels of hGH mRNA in the liver (Short et al., 1992). Both the P3(I) and P4 regions are contained within the -355 to -207 bp region of the PEPCK promoter. The data suggest that transgenic mice containing a PEPCK-bGH gene with block mutations in both the P3(I) and P4 regions would not express bGH mRNA in the liver. This supports the observation that C/EBP is expressed in highly differentiated cell types and is involved in the liver specific expression of many genes.

2. KIDNEY

The level of PEPCK is equal in the liver and kidney, but in transgenic mice, bGH mRNA levels are much lower in the kidney than the livers of mice containing a PEPCK(460)-bGH gene (McGrane et al., 1988). This relatively low level of GH expression is also present in the kidneys of mice containing a PEPCK-hGH chimeric gene. Yet, Eisenberger et al (1992) indicated that in transgenic mice containing a chimeric rat-chicken PEPCK transgene, the level of transgene expression is equal in the liver and the kidney. This may be due to the post-transcriptional control of renal PEPCK mRNA (Kaiser et al., 1992) via mRNA stabilization. Mice containing either a PEPCK-bGH (McGrane et al., 1988) or PEPCK-hGH (Short et al., 1992) gene expressed higher levels of GH in the liver compared to the kidney. Yet mice containing a chimeric PEPCK gene (a rat PEPCK promoter and gene with a 3' chicken PEPCK insert) expressed equal levels of the
chimeric PEPCK gene in the liver and kidney (Eisenberger et al., 1992). This may be due to the sequences in the 3' untranslated region of the PEPCK gene that stabilize the PEPCK mRNA in the kidney and thus increase its concentration. Kaiser et al (1992) has shown that acidosis, which induces PEPCK gene expression in the kidney, increases the half-life of PEPCK mRNA from 3 hours to 9 hours in cell culture.

Although there is regulation of PEPCK at the level of message stability, there is also transcriptional regulation of the PEPCK gene in the kidney (Hwang et al., 1991). Mice containing a PEPCK(P2)-bGH gene had undetectable levels of bGH mRNA in their kidneys. In DNase I footprinting analysis, the P2 region of the PEPCK promoter was protected by proteins present in renal nuclei. The P2 region contains a consensus sequence for the transcription factor HNF-I. HNF-I is present at high concentrations in the liver, kidney and small intestine. HNF-I has been shown to bind the P2 region of the PEPCK promoter. Unlike the liver, where more than one region is directing expression of the transgene, the kidney requires only the P2 region, to direct expression of the transgene. The data suggest that regulation of transcriptional control centers around the P2 region of the PEPCK promoter.

C. Dietary Regulation
Diet is a powerful regulator of PEPCK gene expression. Previous studies have shown that diet regulated expression of a PEPCK(460)-bGH gene in a similar manner as the endogenous PEPCK gene. All of the transgenes examined in this study were regulated by diet in a similar manner as the PEPCK(460)-bGH gene in the liver. A diet high in carbohydrate decreased expression and a diet devoid of carbohydrate and high in either protein or fat induced expression of the transgene as well as the endogenous PEPCK gene. Therefore induction of PEPCK gene expression is regulated by the level of carbohydrate in the diet, not the level of protein.

The induction of PEPCK gene transcription due to feeding mice a carbohydrate-free diet is not mediated through the two known cAMP responsive elements (CRE and P3(l)) of the PEPCK promoter. This implies that the induction of PEPCK gene expression cannot be solely due to the increase in the level of hepatic cAMP, since intraperitoneal injections of Bt2cAMP to mice containing either a PEPCK-bGH gene with mutations in the CRE or the P3(l) regions singly or in combination did not induce expression of the transgene in the livers of mice fed a diet high in carbohydrates. Alternatively, there may be other cAMP regulatory regions in the PEPCK promoter between -460 and +73, however this seems unlikely. The difference between the two experiments (cAMP and carbohydrate-free diets) may be the length of the treatment. The Bt2cAMP was administered over a 90 minute period, while the mice were fed the diets for one week. The cAMP induction of
PEPCK gene transcription may be mediated by phosphorylating protein(s), such as (CREB, C/EBP), that are already present in the nucleus. Another possibility involves the cAMP-mediated recruitment of transcription factors to the nucleus (Metz et al., 1993) to effect PEPCK gene transcription. Previous studies have demonstrated that upon administration of cAMP to cells, C/EBPβ translocated to the nucleus (Metz et al., 1993) and could thus induce transcription from the PEPCK promoter.

The carbohydrate levels in the diets may alter the concentration of hormone responsive transcription factors (C/EBP). C/EBPβ mRNA is increased in the livers of mice treated with cAMP for 90 minutes (Park et al., 1993). An increase in the concentration of C/EBPβ in the livers of animals may take longer to be achieved. An increase in C/EBP concentration may enable binding of the protein to other sites on the PEPCK promoter that would not be bound at lower concentrations of the protein such as the P4 region. The P4 region has a relatively lower affinity for C/EBPβ than does the P3(I) region (Park et al., 1993).

Deinduction of mRNA levels of the transgene by feeding diets high in carbohydrates was still possible in the livers of mice containing a PEPCK-bGH gene with various block mutations in the PEPCK promoter as well as a PEPCK(355)-bGH gene. This PEPCK(355)-bGH transgene does not have the IRS that was implicated in insulin regulation in studies using cultured cells (O'Brien et al., 1990). Also Short et al (1992) reported that a PEPCK(402)-human growth hormone
chimeric gene was deinduced in the livers of mice fed high carbohydrate diets. It appears unlikely that carbohydrate feeding, which increases insulin levels alters transcription of the PEPCK gene by acting at the IRS, or by lowering the cAMP concentration in the liver and influencing transcription through the CRE or the P3(I) regions. Lucas et al. (1993) reported that one of the factors that bind to the putative IRS can also dimerize with C/EBPβ. This finding suggests that insulin may mediate inhibition of PEPCK gene transcription, (via transcription factors) that interact with transcription factors (C/EBP) that stimulate transcription. This provides further evidence that C/EBP regulates PEPCK gene transcription in the liver, now with a possible role for both induction and deinduction.

The necessity of PEPCK gene expression during long term glucose deprivation may require a redundancy of liver-specific and hormone-inducible regions on the promoter to insure transcription of the PEPCK gene in stressful conditions. There may be a regulatory domain in the PEPCK promoter which is influenced directly by glucose, (independent of insulin). Studies of other genes that are expressed in the liver and responsive to diet have identified a conserved sequence that may be responsible for the regulation of gene transcription by glucose. The promoter regions of both the L-type pyruvate kinase (L-PK) (Thompson et al., 1991) and S14 genes contain this sequence. Insertion of this element into a heterologous promoter resulted in the regulation of transcription by glucose (Bergot et al., 1992). Studies
using transgenic mice containing a L-PK transgene, indicated that a region between -171 and -124 was involved in the glucose induction of the L-PK gene. The core motif of the carbohydrate regulatory element, CACGTG bind the transcription factor LF-A1. This transcription factor is required for the transcription of the human a1-antitrypsin (Tripodi et al., 1991) and apolipoprotein A1 genes (Hardon et al., 1988). The PEPCK promoter contains a 5 out of 6 base pair match to the sequence of the carbohydrate responsive element, at position -217 to -212. This site maps at a hypersensitive region in the PEPCK promoter in DNase I footprinting analysis. This site was not investigated in these studies, but may be a putative carbohydrate regulatory element in the PEPCK promoter.

D. Diabetes

Hepatic gluconeogenesis is induced during diabetes. Friedman et al (1993) analyzed the sequences responsible for the regulation of hepatic PEPCK gene transcription during liver streptozotocin-induced diabetes, using transgenic mice containing the PEPCK-bGH genes with various mutations in the PEPCK promoter. Expression of a PEPCK(355)-bGH gene was not induced in the liver of diabetic transgenic mice (Friedman et al., 1993). and expression of the transgene did not decrease when insulin was administered to these
mice. This contrasts with the effects of high carbohydrate and carbohydrate-free diets which did alter transcription of a PEPCK(355)-bGH gene in the livers of mice.

The difference appears to be the effect of glucocorticoids. Previous studies demonstrated that mice that were diabetic had increased levels of PEPCK gene transcription, but mice that were both adrenalectomized and diabetic were unable to induce transcription of the PEPCK gene in the liver. If dexamethasone was administered to these animals PEPCK mRNA levels increased in the liver. The difference between the induction of the PEPCK gene in the liver resulting from diabetes and that of dietary carbohydrate levels appears to be the level of glucocorticoids.

Like the feeding studies, diabetes involves a long term alteration in the glucagon and insulin concentrations in the animal. A PEPCK-bGH gene containing block mutations in both the CRE and the P3(I) regions of the PEPCK promoter was able to induce expression in the livers of transgenic mice that were made diabetic by streptozotocin treatment and deinduced expression from the transgene when insulin was administered (Friedman et al., 1993). This finding along with the deinduction of expression by feeding mice a high carbohydrate diet suggest that insulin does not act by altering the level of cAMP in the liver.

E. Developmental Expression
The developmental pattern of PEPCK gene expression in the liver is thought to be primarily controlled by the alterations in the insulin/glucagon ratio present at birth in the neonate. At birth, when the maternal glucose supply is severed, there is a decrease in plasma insulin concentration and a concomitant rise in the plasma glucagon level of the newborn. This alteration in the insulin/glucagon ratio results in a rise in hepatic cAMP which causes the induction of PEPCK gene transcription in the liver and the newborn is able to produce glucose via gluconeogenesis.

It has been shown that PEPCK activity can be induced as early as the 14th day of gestation if cAMP or glucagon is administered to the fetus. Therefore the pathway necessary for glucagon-mediated induction of PEPCK gene transcription is present as early as day 14 of gestation. The pathway involves the presence of adenylate cyclase. The transcription factors that are required for cAMP responsiveness are also present at day 14 of gestation. It is suggested that the hormonal conditions present in the fetus are the primary components regulating PEPCK gene transcription. PEPCK gene transcription in the liver can also be delayed at birth by administration of glucose or insulin in utero. This finding further suggests that PEPCK transcription is regulated by the insulin/glucagon ratio in the fetus and the newborn.

Previous studies indicated that the sequences required for the correct pattern of hepatic PEPCK gene expression during development
are within a -460/+73 bp fragment of the PEPCK promoter (McGrane et al., 1990). In this study, the induction of hepatic PEPCK gene expression seen at birth was present for all PEPCK-bGH genes examined in this study regardless of the block mutation present. This finding was unexpected since the two cAMP responsive regions of the PEPCK promoter, (CRE and P3(I)) which conferred cAMP responsiveness to the PEPCK gene in the liver, had no effect on the inducibility of either the PEPCK(CRE)-bGH or PEPCK(P3(I))-bGH gene expression in the liver at birth. This result is similar to the dietary studies in which the CRE and the P3(I) regions either alone or in combination were not required for the induction of the transgene in the liver due to feeding mice a carbohydrate-free diet.

Only mice containing a block mutation in either the CRE or P1 sites had a low level of bGH expression in the livers of 17 day old fetuses. This finding suggested that the CRE and the P1 regions may be involved in the suppression of PEPCK gene transcription before birth in the liver. The low levels of bGH expression from either a PEPCK(CRE)-bGH or PEPCK(P1)-bGH gene in the livers of transgenic mice are probably due to the lack of hormonal stimulation (high level of insulin and the low level of glucagon) in the fetus. This idea of transcriptional suppression of PEPCK gene transcription will be discussed later.
F. Models representing the tissue specific regulation of
PEPCK gene transcription in the liver and kidney

The information presented in this study in combination with
previous work from this and other laboratories, allows the proposal of a
model to explain the mechanism of transcriptional regulation of the
PEPCK gene. Specific regulatory elements have been identified and
the transacting factors that bind to these regions and effect transcription
of the PEPCK gene have been determined. Figure 22 is a schematic
representation of a -460/+73 bp fragment of the PEPCK promoter.
Specific protein binding sites and the transacting factors that bind to
these sites are indicated. In the model of the PEPCK promoter, a
protein X is proposed as a linking protein by which transcription factors
bound to upstream regions of the promoter can affect transcription by
interacting with TATA box binding proteins (TBP). The model does not
rely on the presence of the linking protein, since it is possible that
proteins bound to different regions of the promoter can interact directly
with the TBP, without a linking protein.

The figure represents a-460/+73 base pair segment of the
PEPCK promoter and its binding proteins that are applicable for
transcriptional regulation for both the liver and the kidney. A
mechanism of transcriptional regulation of the PEPCK gene in the liver
and kidney based on findings from transgenic animals is proposed.
Figure 22. Proposed model of the PEPCK promoter

The figure presents the putative interactions between cis-acting DNA elements within a -460/+73 bp fragment of the PEPCK promoter and specific transacting factors. The proteins bound to the regulatory elements are: TATA box, TATA box binding protein (TBP) and RNA polymerase II; CRE, CREB or C/EBP; P1, NF-1; P2, HNF-1; P3(1), C/EBP; P4, C/EBP; TRE, thyroid hormone receptor (TR); GRE, glucocorticoid receptor (GR); IRS. Protein X is proposed to act as a linking protein, linking upstream elements with the TBP.
1. Liver

The model presents several points about transcriptional regulation of PEPCK gene transcription in the liver and the kidney:

(1) the interaction and cooperation between transcription factors

(2) the need for redundant sites on the promoter

(3) suppression of basal transcription

Figure 23 is a representation of the effect specific block mutations had on various PEPCK-bGH genes containing block mutations in the PEPCK promoter. The graph in the figure is redrawn from figure 15.

The model suggests that transcription factors that bind to regulatory elements, must interact either directly or indirectly (through a linking protein) to effect PEPCK gene transcription. The cAMP stimulation of PEPCK gene transcription requires both the CRE and the P3(l) regions. Cyclic AMP did not stimulate transcription from a PEPCK-CAT gene containing mutations in either the CRE or the P3(l) regions of the PEPCK promoter in HepG2 cells (Lui et al., 1991) or transgenic mice. In the liver, the CRE and the P3(l) regions have opposing effects on basal transcription. In mice containing a PEPCK(CRE)-bGH gene, the basal level of bGH mRNA was 4 fold that of levels in mice containing a PEPCK(460)-bGH gene. Since the CRE has been shown to bind proteins (CREB, CEBP) that stimulate transcription it is
Figure 23. Proposed model of transcriptional regulation from the PEPCK promoter in the liver

The figure presents models of the PEPCK promoter and the effect of a block mutation in a specific regulatory element on transcription. The mutation in the PEPCK promoter in each model is presented in the left margin. The bar graph is redrawn from figure 15 and indicates the effect of specific mutations in the PEPCK promoter on the level of basal transcription of the PEPCK-bGH gene in the liver. Values in the bar graph are the fold induction of bGH mRNA relative to the level of bGH mRNA values in the livers of mice with a PEPCK(WT460)-bGH gene.
surprising that the CRE has a suppressive effect on PEPCK gene transcription. Transcription from a PEPCK(CRE)-CAT gene was not inhibited in studies performed in hepatoma cells. We propose that the CRE region of the PEPCK promoter acts to suppress the transcription of the PEPCK gene in the absence of cAMP stimulation. The CRE region is constitutively bound in in vivo footprinting assays (Faber et al., 1989), indicating that whether or not PEPCK is expressed, the CRE region is bound. Therefore, it is plausible that if the CRE is bound, the transcription is somewhat suppressed in the absence of hormonal stimulation. Loss of the CRE region, resulted in an increase in the basal level of hepatic expression as well as loss of cAMP induction.

The second idea presented is redundancy of regulatory elements. The loss of the P3(I) region reduced hepatic levels of bGH mRNA, suggesting that the P3(I) region is directing expression of the PEPCK gene to the liver. This finding was predicted from footprinting assays that indicated that only proteins present in nuclei from liver extracts bound to this region. The data also suggest that the P3(I) region is not the only site involved in liver specific expression. The model suggests that the P4 region (which also binds C/EBP) is involved in liver specific expression of the PEPCK gene. Since PEPCK is the rate limiting enzyme in gluconeogenesis, its expression is crucial not only to gluconeogenesis but also to glucose homeostasis.

In mice containing a PEPCK(CRE/P3(I))-bGH gene, the accumulation of bGH mRNA was similar to that noted in mice
containing a PEPCK(460)-bGH gene. It would appear the inductive effect of the CRE is compensated for by the P3(l) region resulting in a promoter with a similar transcriptional activity as an intact PEPCK promoter. In this scenario, upstream regions may play an important role in transcriptional activity. The P4 region is one such region, because it bound members of the C/EBP family of proteins. It is possible that both the P3(l) and P4 regions of the PEPCK promoter are required to direct expression of the PEPCK gene to the liver. This redundancy of protein binding regions to direct expression of the gene to a specific tissue, may be the result of evolution as a mechanism of protection, to ensure expression of a critical gene, the PEPCK gene. Redundancy of protein binding sites may also ensure stimulation of PEPCK gene transcription during long periods of glucose deprivation. All the transgenes examined in this study, were regulated by carbohydrate levels in the diets in a similar manner as the endogenous PEPCK gene, regardless of the block mutation in the PEPCK promoter.

The induction of PEPCK gene transcription at birth was noted for all the PEPCK-bGH genes examined in this study, regardless of the mutation in the PEPCK promoter. It is possible that multiple C/EBP sites on the promoter ensure stimulation of transcription of the PEPCK gene at birth. The developmental expression of C/EBPα parallels that of the PEPCK gene. C/EBPα is expressed at low levels beginning at day 18 of gestation and increases at birth, a similar pattern as the PEPCK gene. There is compelling evidence that members of the C/EBP family
proteins activate transcription from the PEPCK promoter in the liver and that it is involved in the cAMP stimulation of the PEPCK gene in the liver.

2. Kidney

In contrast to the liver, PEPCK gene regulation in the kidney uses the interaction of multiple sites to ensure suppression of transcription as a mechanism of regulation. Figure 24 is a representation of the transcriptional regulation of the PEPCK gene in the kidney based on the finding from this and other studies. In the kidney, the P2 region appears to be the critical site directing expression of the transgene to the kidney. In mice containing the PEPCK(P2)-bGH gene there were undetectable levels of bGH mRNA in the kidney and the levels in the liver were unaffected. Unlike the liver which had redundant protein binding regions directing expression of the transgene to the liver, it appears that the kidney requires only the P2 region. The P2 region has been shown to bind HNF-1, a protein which is abundant in the kidney (Gurney and Hanson, unpublished observations). The importance of the P2 region is illustrated in the dietary regulation of the transgene in the kidney. In the kidney, a diet high in carbohydrate did not affect transcription of the PEPCK gene, but carbohydrate-free diets induce expression of the PEPCK gene in the kidney. Dietary regulation of all the PEPCK-bGH genes in the kidneys of transgenic mice was similar to that of the endogenous PEPCK gene,
Figure 24. Proposed model of transcriptional regulation from the PEPCK promoter in the kidney

The figure presents models of the PEPCK promoter and the effect of a block mutation in a specific regulatory element on transcription. The mutation in the PEPCK promoter in each model is presented in the left margin. The bar graph is redrawn from figure 15 and indicates the effect of specific mutations in the PEPCK promoter on the level of basal transcription of the PEPCK-bGH gene in the kidney. Values in the bar graph are the fold induction of bGH mRNA relative to the level of bGH mRNA values in the kidneys of mice with a PEPCK(WT460)-bGH gene.
except the PEPCK(P2)-bGH gene. Mice fed a carbohydrate-free diet were not able to induce expression from a PEPCK(P2)-bGH gene in the kidney but were able to induce expression in the liver. The P2 region of the PEPCK promoter is not only necessary to direct expression of the PEPCK gene to the kidney but is also involved in the dietary regulation of PEPCK gene transcription in the kidney.

The data suggest that PEPCK gene transcription utilizes two sites, CRE and P3(I) to suppress basal transcription in the kidney and that these two sites interact. In mice containing a PEPCK(CRE)-bGH gene the level of bGH mRNA in the kidney increased 20 fold over levels in the kidneys of mice containing a PEPCK(460)-bGH gene. This induction of basal transcription is similar to that found in the liver where the CRE region was mutated. The CRE region appears to be involved in the suppression of transcription from the PEPCK gene in both the liver and the kidney. Yet unlike the liver, mice containing a PEPCK(P3(I))-bGH gene had a 10 fold increase in the level of bGH mRNA levels compared to levels in mice containing a PEPCK(460)-bGH gene. In mice containing a PEPCK(CRE/P3(I))-bGH gene the level of bGH mRNA in the kidney was approximately 200 fold that of the levels in the kidneys of mice containing a PEPCK(460)-bGH gene. This is in contrast to the liver in which the two regions (CRE and P3(I)) have opposite effects on transcription. In the kidney, block mutations in both the CRE and P3(I) regions had a synergistic effect on transcription of the PEPCK-bGH gene. It appears that both the CRE region and the
P3(l) region are involved in the suppression of transcription of the PEPCK gene in the kidney. The model suggests a way that the CRE and the P3(l) regions may interfere with the interaction between the proteins associated with the P2 region and the linking protein. Although the P3(l) region did not bind proteins present in nuclei from kidney extracts, C/EBP is present in the kidney at low levels.

These 3 regions of the PEPCK promoter (CRE, P2 and P3(l)) appear to modulate the level of basal PEPCK gene transcription in the kidney. The level of PEPCK gene transcription has the potential to be comparable to that of the liver but the kidney has another mechanism to regulate the levels of PEPCK mRNA in the kidney, message stability.

G. Future Studies

Based on findings presented in this thesis, a number of future studies will be pursued. The studies presented in this thesis have shown the utility of identifying specific sequences that are involved in tissue specific expression of a chimeric gene in transgenic animals. Future studies will examine which other elements are required to direct the expression of the PEPCK gene to the liver. Since the P3(l) site binds C/EBP, the other C/EBP binding region (P4) of the PEPCK promoter will be examined. The possibility that the P4 region is involved in directing expression of the PEPCK gene to the liver will be
examined by constructing transgenes which contain either a block mutation in the P4 region alone or a transgene containing block mutations in both the P3(I) and P4 regions of the PEPCK promoter. This will address two points, (1) that there are multiple binding sites on the PEPCK promoter that direct high levels of transcription of the PEPCK gene in the liver and (2) that the C/EBP family of proteins is responsible for directing expression of the PEPCK gene to the liver.

A major advantage of the using transgenic animals as a model system is that it provide the full range of cell types. As I outlined in the introduction, PEPCK gene expression is not only tissue specific, but also cell-type specific. The PEPCK gene is expressed predominantly in the periportal region of the liver and the proximal convoluted tubules of the kidney cortex. Based on the findings presented here, the CRE and P3(I) regions have profound effects on the level of expression of the PEPCK-bGH gene in the livers and kidneys of transgenic mice. The level of expression from the PEPCK(CRE)-bGH gene was increased in both the livers and kidneys of transgenic mice compared to levels in mice with a PEPCK(460)-bGH gene. To determine if the increase was due to increased expression in specific cell types or if the pattern of expression had been altered to other regions of the liver and kidney, in situ hybridization assays will be performed. This type of information is only possible using transgenic animals as a model, since they possess all the necessary cell types in a physiological context.
Other studies that will be pursued involve the use of knockout mutations in animal models to investigate the role of specific transcription factors. The candidate genes to be investigated are the transcription factors HNF-1 and the family of C/EBP family of proteins. These studies would provide the link between the cis-acting elements and specific transcription factors and the properties they confer to gene expression. Recent studies using knockout mutations of some transcription factors in mice have suggested that there may be redundancy in transcription factors to ensure regulation of critical genes.

Another avenue of studies that will be investigated is the use of specifically constructed promoter regions to direct the expression of genes. The identification of tissue specific and regulatory elements may allow us to construct transgenes that direct expression only to specific tissues and that can be precisely regulated for pharmaceutical purposes and gene therapy.
V REFERENCES


137


Giralt, M., Park, E. A., Gurney, A. L., Liu, J., Hakimi, P., and


Gunn, J., Hanson, R., Meyuhas, O., Reshef, L., and Ballard, F. (1975) Biochem. J. 150, 195-203


Iynedjian, P., Ballard, F., and Hanson, R. (1975) J. Biol. Chem. 250, 5596-5603

Iynedjian, P., and Hanson, R. (1977) J. Biol. Chem. 252, 8398-8403


Lim, I., Dumenco, L.L., Hatzoglou, M., Hanson, R. W., and Gerson, S. L. (1990) Carcinogenesis 11, 737-743

Liu, J., Roesler, W. J., and Hanson, R. (1990) BioTechniques 9, 738-742


Roesler, W., Vandenbark, G., and Hanson, R. (1989) J. Biol. Chem. 264, 9657-9664


Short, M. K., Clouthier, D. E., Schaefer, I. M., Hammer, R. E.,


Tilghman, S., Ballard, F., and Hanson, R. (1976) in Gluconeogenesis: Its Regulation in Mammalian Species (Hanson, R. and Mehlman, M., eds) pp.47-87, John Wiley and Sons, New York


Yeung, D. and Oliver, I. T. (1968) Biochemistry 7, 3231-3239
