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REGULATION OF THE PYRUVATE DEHYDROGENASE COMPLEX

by

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

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May, 1995
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REGULATION OF THE PYRUVATE DEHYDROGENASE COMPLEX

ABSTRACT

BY

SHARON S NAIK

The mammalian pyruvate dehydrogenase complex (PDC) is subjected to both short-term (product inhibition and covalent modification) and long term (increases in total activity and protein mass) regulation mediated by dietary and hormonal treatments. Initial research efforts were focused on the isolation and characterization of the human E1β gene. The 2.3 kb E1β promoter region lacked a TATA box homology but contained initiator sequences (two) and Sp1 sites (three) frequently found in TATA-less promoters that may play a role in defining the start site of transcription. Transient expression of chloramphenicol acetyltransferase (CAT) activity of nested deletions of the E1β 5'-flanking region (-2316 to +32 base pairs) measured in human hepatoma (HepG2) cells, suggested the possible presence of repressor elements located between sequences -2316 to -930 base pairs. Deletion constructs containing sequences from -929 to +32 and -98 to +32 base pair showed approximately 7- and 20-fold increases in CAT over that containing -2316 to +32 base pairs of the E1β promoter region. The presence of a repressor element between -2316 to -929 base pairs was further confirmed by measuring CAT activity from a heterologous thymidine kinase promoter.

Similar studies on the measurement of promoter activity of a 796 base pair E1α promoter fragment (-763 to +33 bp) generated by polymerase chain reaction (PCR) from normal human skin fibroblasts was measured in HepG2 cells. Several sequences showing consensus with known functional elements have been identified within the 5'-flanking region of the human E1α gene. They include the following: activator protein
AP-1, cAMP-responsive element (CRE), two activator proteins 2 (AP-2), CCAAT/enhancer binding protein (C/EBP) and an insulin-responsive sequence (IRS). It was observed that a construct containing the 796 base pair fragment (-763 to +33) resulted in very high CAT expression comparable to that derived from pSV2CAT (positive control). The smallest construct, containing only 102 base pairs of the promoter region resulted in a significant decrease in CAT activity, but was still capable of efficiently promoting CAT expression. The E1α promoter (-763 to +33) resembles facultative promoters in having appropriately positioned "TATA" and "CAAT" boxes with several upstream potential enhancer sequences which allows for efficient expression of CAT in HepG2 cells.

Diet-induced long-term regulation of PDC appears to involve coordinated modulation of all the component proteins of the complex. The present study was carried out to evaluate the long-term effects of high-sucrose and high-fat diets on PDC activity in the liver. Rats fed a high-sucrose diet for two and three weeks resulted in a 2-fold increase in total activity compared to chow-fed rats. Changes in total PDC activity closely correlated with alterations in the amount of proteins quantified by immunoblotting, suggesting that increased enzyme content is the predominant mechanism underlying the adaptive response to high-sucrose feeding. There was no significant change in the total PDC activity in rats fed a high fat diet for two weeks as compared to animals fed the chow diet. The levels of RNA determined by Northern analysis showed an increase of approximately 1.5 fold in the high-sucrose group as compared to the chow fed animals. This indicates that the increase in the amount of PDC proteins observed in the liver upon feeding a high-sucrose diet may be mediated by transcriptional and/or translational mechanisms.
This thesis is dedicated to my parents, Suvas and Mamatha Naik and my husband Anup Shetty.
Acknowledgements

I wish to express my love and gratitude to my husband and friend Anup Shetty. His contribution to this thesis is immeasurable. He has supported me throughout this long protracted journey and continues to enrich and enlighten my life. I sincerely thank my advisor Dr. Mulchand S. Patel who guided me patiently through my program. His faith in me has made it possible for me to have attained my goals. I have learned a great deal and I am grateful for the opportunity that has been provided to me.

I have had the good fortune to work in not one but two superior laboratories that has given me the opportunity to interact with many talented people. I wish to thank Dr. Richard Hanson and everyone in his laboratory for making me a part of their team.

I was fortunate to have excellent committee members and I am very grateful to Dr. Kerr, Dr. Eckert and Dr. Samols for their support and understanding. I especially like to thank Dr. Samols for allowing me to be a part of his laboratory journal club. It was entertaining and stimulating and I will miss our Monday meetings. I am also grateful to him for his time and valuable suggestions on proof-reading this manuscript. I thank Dr. Kerr and Marilyn Lusk who helped me with the PDC assays.

All of the members of Dr. Patel's laboratory have contributed either directly or indirectly to this project. In particular, I would like to acknowledge Dr. Gary Johanning for teaching me how to screen a genomic library, Dr. Mei Chang and Dr. Madhusudhan for their collaborations on the E1α and E1β projects, Drs. Gregory Grossman, Jed Friedman and Steve Nizielski for helping me on the animal project.

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<th>Description</th>
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<tbody>
<tr>
<td>PDC</td>
<td>pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>BCKADC</td>
<td>branched chain a-keto acid dehydrogenase complex</td>
</tr>
<tr>
<td>α-KGDC</td>
<td>α-ketoglutarate dehydrogenase complex</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>TPP</td>
<td>thiamine pyrophosphate</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>LipS2</td>
<td>lipoic acid</td>
</tr>
<tr>
<td>HE=TPP</td>
<td>hydroxyethylidene thiamine pyrophosphate</td>
</tr>
<tr>
<td>HepG2</td>
<td>human hepatoma</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>E1</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>E2</td>
<td>dihydrolipoamide acetyltransferase</td>
</tr>
<tr>
<td>E3</td>
<td>dihydrolipoamide dehydrogenase</td>
</tr>
</tbody>
</table>
CHAPTER I. THE MAMMALIAN PYRUVATE DEHYDROGENASE

COMPLEX

I. Introduction

Glucose is a major fuel for oxidative metabolism in mammalian tissues and in the developing fetus. The initial metabolism of glucose to pyruvate occurs via the glycolytic pathway in the cytosol. Further oxidation of pyruvate to CO₂ takes place via the tricarboxylic acid cycle (TCA) in the mitochondria. These two pathways are functionally linked by the pyruvate dehydrogenase complex (PDC) in the mitochondria. PDC, a member of the α-keto acid dehydrogenase complex family, is a classic example of a multienzyme complex wherein the multiple catalytic components are held together by noncovalent bonds. PDC plays a pivotal role in intermediary metabolism (Figure 1). PDC catalyzes an irreversible step in glucose and amino-acid metabolism. It catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA with the following overall reaction:

\[ \text{Pyruvate} + \text{CoA} + \text{NAD}^+ \longrightarrow \text{Acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \]

The acetyl-CoA generated may be directed either for production of energy via the TCA or in lipogenic tissues it has an additional role, the provision of acetyl-CoA for the synthesis of fatty acids and cholesterol. Over the past three decades, much of the
Figure 1. Location of the pyruvate dehydrogenase complex in the intermediary metabolism of carbohydrate, fat and amino acids. PDC, pyruvate dehydrogenase complex; BCKADC, branched-chain α-keto acid dehydrogenase complex; α-KGDC, α-ketoglutarate dehydrogenase complex; TCA cycle, tricarboxylic acid cycle; BCAAs, branched-chain amino acids; AAs, amino acids; FAs, long chain fatty acids. (Patel and Harris, 1995)
II. Structure and function of the complex

The α-ketoacid dehydrogenase complexes, namely, PDC, α-ketoglutarate dehydrogenase and branched-chain α-ketoacid dehydrogenase complexes catalyze the decarboxylations of pyruvate, α-ketoglutarate and branched-chain amino acids, respectively. Each complex consists of dehydrogenase (E1), dihydrolipoamide acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3). E3 is a common component of all three α-ketoacid dehydrogenase complexes.

PDC contains multiple copies of three catalytic and three regulatory components (reviewed by Reed, 1974; Patel and Roche, 1990). Five coenzymes or cofactors are associated with the enzyme complex: thiamine pyrophosphate (TPP), lipoic acid (LipS₂), coenzyme A (CoA), flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD⁺) (Reed, 1974). The regulatory components are pyruvate dehydrogenase kinase (E1-kinase) (Linn et al., 1969b; Wieland, 1983), phospho-pyruvate dehydrogenase phosphatase (phospho-E1 phosphatase) (Linn et al., 1969b; Wieland, 1983) and a component currently referred to as protein X or E3 binding protein (Gopalakrishnan et al., 1989). E1 catalyzes the decarboxylation of pyruvate and the subsequent reductive acetylation of the lipoyl moiety of E2. E2, in turn,
catalyzes the transfer of the acetyl group to free CoA. The reduced lipoil moieties of E2 are reoxidized by E3, with NAD$^+$ as the final electron acceptor (Figure 2).

PDC has been purified from numerous plant, microbial and mammalian sources. PDC from prokaryotes and eukaryotes have molecular weights in the millions. The subunit composition of mammalian PDC is shown in Table I. The complex is organized around a central core composed of multiple copies of the E2 subunit; in *Escherichia coli* and other Gram negative bacteria, the core consists of 24 copies of E2, arranged as a hollow cube of 8 trimers; in yeast and in mammalian systems, the core consists of 60 copies of E2 arranged in an icosahedral dodecahedron with 532 symmetry (Reed *et al.*, 1989). Plants are unique in containing two distinct, spatially separated types of PDCs, one within the mitochondrial matrix and the other in the plastid stroma. Each type of PDC has characteristic structural, catalytic and regulatory properties (Miernyk *et al.*, 1985). The mitochondrial PDC serves as a primary entry point for carbon into the citric acid cycle. The plastid PDC provides both acetyl-CoA and NADH for fatty acid and isoprenoid synthesis. In contrast to mammalian cells and microbes, all *de novo* fatty acid biosynthesis by plant cells occurs within the plastids (Ohlrogge *et al.*, 1979). Plant cells also contain two glycolytic pathways, one in the cytoplasm and the other in the plastids. It was a search for the connecting link between the pyruvate produced by the plastid glycolysis and the acetyl-CoA required for fatty acid synthesis which led to the discovery of the PDC within the leucoplasts of the developing castor oil seeds (Reid *et al.*, 1975; Reid *et al.*, 1977).
Figure 2. Schematic representation of the sequence of partial reactions catalyzed by the PDC component enzymes. E2 forms the core of the enzyme complex around which E1 and E3 are arranged. E1, pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoamide dehydrogenase; TPP, thiamine pyrophosphate; Lip, lipoic acid.
Table I. Subunit composition of mammalian pyruvate dehydrogenase complex.
<table>
<thead>
<tr>
<th>Subunit</th>
<th>Mr</th>
<th>Subunits</th>
<th>Subunits per molecule of complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native complex</td>
<td>8,500,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1 tetramer</td>
<td>154,000</td>
<td>2</td>
<td>41,000</td>
</tr>
<tr>
<td>E1α</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2 core</td>
<td>3,100,000</td>
<td>2</td>
<td>52,000</td>
</tr>
<tr>
<td>E3 dimer</td>
<td>110,000</td>
<td>2</td>
<td>55,000</td>
</tr>
<tr>
<td>E3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>X</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinase</td>
<td>100,000</td>
<td>1</td>
<td>48,000</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>150,000</td>
<td>1</td>
<td>97,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>
A. Structural Aspects of PDC-E1

This component catalyzes the rate-limiting step in the overall PDC reaction (Barrera et al., 1972). E1 differs in structure between prokaryotes and eukaryotes. In E.coli, E1 exists as a single polypeptide with an estimated molecular mass of 100,000 daltons (Guest et al., 1984), whereas in yeast and mammalian systems, functional E1 is a tetramer (α2β2) containing a 41-kDa α subunit and 36 kDa β subunit (Barrera et al., 1972). In the yeast and mammalian E1 systems, 30 E1 tetramers are arrayed about the surface of the E2 core (Reed, 1974).

The E1 component of the mammalian PDC catalyzes the oxidative decarboxylation of pyruvate in a two-step process. In the first step, it forms CO2 and hydroxyethylidene-thiamine pyrophosphate (HE=TPP) intermediate. The HE=TPP intermediate is then oxidized to an acetyl group with the concomitant reduction and acetylation of the lipoyl group covalently linked to the E2 component.

\[
\text{CH}_3\text{COCOOH} + \text{E1-TPP} \rightarrow \text{CH}_3\text{C(OH)=TPP-E1} + \text{CO}_2 \quad (i)
\]

\[
\text{CH}_3\text{C(OH)=TPP-E1} + [\text{lipS2}]-\text{E2} \rightarrow [\text{CH}_3\text{CO-S-lipSH}]-\text{E2} + \text{E1-TPP} \quad (ii)
\]

The E1 component is classified into two types; one type is mitochondrial and in Gram positive bacteria and consists of α and β subunits, another type is in Gram-negative bacteria and is a large polypeptide of Mr = 100,000 daltons. Although the E1 component catalyzes the rate-limiting step in the overall reaction, we know very little
about the individual roles played by the $\alpha$ and $\beta$ subunits of E1 in catalyzing the first two partial reactions. This is because attempts to isolate functionally active E1$\alpha$ and E1$\beta$ separately have been unsuccessful. E1 binds two molecules of TPP with different affinity and has two active centers of equal catalytic efficiency (Khailova et al., 1989). From chemical modification studies, the amino-acid residues which play important roles in catalysis have been characterized in pigeon breast muscle E1 (Khailova et al., 1989) and in *Escherichia coli* E1 (Schwartz and Reed, 1970). For example, a tryptophan residue was shown to interact with TPP by forming a charge transfer complex between the indole group of trp as a donor and the thiazolium ring of TPP as an acceptor (Korotchkina et al., 1984; Ali et al., 1995). By differential peptide mapping in the presence or absence of TPP following modification with N-bromosuccinimide, tryptophan residue 135 in the $\beta$ subunit of human E1 was identified in the active site of E1 which is highly conserved in E1$\beta$ from several species. Using the sulfhydryl-specific reagent N-ethylmaleimide, the critical cysteiny1 residue of bovine E1$\alpha$ which was found to be equivalent to Cys-62 of human E1$\alpha$ was identified at or near the active site of E1 (Ali et al., 1993). Hawkins and his colleagues (Hawkins et al., 1989) have identified a sequence motif of approximately 30 residues in length in several TPP-dependent enzymes. This motif begins with the sequence G-D-G and ends with D-D.

Mammalian E1, but not the bacterial enzyme, is phosphorylated and dephosphorylated by a specific kinase and a specific phospho-E1 phosphatase,
respectively (Linn et al., 1969b). The α subunit of E1 is phosphorylated leading to inactivation of the complex (Linn et al., 1969b). Three phosphoserine residues designated as sites 1, 2 and 3, on the E1α subunit have been identified (Yeaman et al., 1978; Sugden et al., 1978). Phosphorylation of site 1 is correlated with the initial and major inactivation (60-70%) of E1. Further inactivation is accompanied by continued phosphorylation of site 1 as well as sites 2 and 3 (Yeaman et al., 1978). A role for phosphorylation of sites 2 and 3 in attenuating the rate of E1 reactivation has been suggested in one study (Sugden et al., 1978) but not in another (Teague et al., 1979).

With the exception of Saccharomyces cerevisiae, E1 from all other eukaryotic sources including avian, plant and Neurospora crassa, is regulated by phosphorylation (Reed et al., 1974). Yeast E1 can be phosphorylated and dephosphorylated in vitro in the presence of purified bovine E1-kinase and phospho-E1 phosphatase, respectively (Uhlinger et al., 1986) but there is no evidence that the yeast E1 can be phosphorylated in vivo which may indicate that the E1-kinase is either not expressed or maybe present at very low levels (Uhlinger et al., 1986). Inactivation of E1 by phosphorylation is correlated with the inability to form HE=TPP from TPP and pyruvate (Walsh et al., 1976). However, the ability of E1 to utilize synthetic HE=TPP in the reductive acetyltransfer reaction is unaffected by phosphorylation (Roche and Reed, 1972; Walsh et al., 1976). Phosphorylation does not affect the number of TPP binding sites but decreases the affinity of the enzyme for TPP by 2-3 fold. However, the two to three fold change in the affinity for TPP is considered insufficient to
account for the total inactivation of E1 in response to phosphorylation (Walsh et al., 1976; Butler et al., 1977). The affinity of E1 for pyruvate was minimally affected by phosphorylation since inactivation of E1 was not reversed by increasing the concentrations of TPP and pyruvate (Butler et al., 1977). Thus, it is likely that phosphorylation of E1 may affect the formation of HE=TPP from enzyme bound TPP and pyruvate.

The primary structure of both α and β subunits of E1 from several species have been deduced from their cDNA sequences. Although numerous reports have been published of the cDNA structures of E1 subunits of the human PDC, production of a functionally active enzyme has been reported only recently. A co-expression plasmid containing the coding sequence of both the human liver pyruvate dehydrogenase E1α and E1β subunits was introduced into the host *Escherichia coli* to generate functionally active E1 (Jeng et al., 1994). In contrast, production of E1α alone resulted in a catalytically inactive protein, suggesting an important role for the E1β subunit in catalysis. The recombinant E1 protein produced in *E.coli* was capable of being phosphorylated by PDH-specific kinase.

The E1α and the E1β subunits of the E1 component of *Bacillus stearothermophilus* was overexpressed separately in *E.coli*. (Lessard and Perham, 1994). A functional E1 enzyme was generated from disrupted mixtures of cells containing the separately overexpressed E1α and E1β genes. Mixing equimolar amounts of the pure recombinant E1α and E1β subunits *in vitro* resulted in a functional active enzyme
suggesting that no chaperonins were needed *in vitro* to promote assembly of the separate subunits to form an intact E1 component.

Recently, overexpression and purification of human E1 and its individual subunits, E1α and E1β, using polyhistidine-Ni-nitrilotriacetic-agarose chromatography was performed (Korotchkina et al., 1995). Recombinant human E1 catalyzed the partial reactions of the oxidative and nonoxidative conversion of pyruvic acid with the same efficiency as E1 purified from bovine kidney. However, when expressed individually, the E1α and E1β subunits lacked any catalytic activity and demonstrated no TPP binding to either subunit. The phosphorylation of E1α alone was only 12% of that observed with the tetrameric E1. These results suggest that both the subunits are indispensable for formation of the active center and for catalysis. A reasonable hypothesis is that some contribution to catalysis is made by both subunits, and that the active site may exist in the interface between the subunits.

The cDNAs encoding the human E1α have been cloned and sequenced by several laboratories (Dahl et al., 1987; De Meireir et al., 1991; Ho et al., 1989; Koike et al., 1988). The first 29 amino acids corresponds to a typical mitochondrial targeting leader sequence. The remaining 361 amino acids represents the mature mitochondrial E1α peptide. The cDNA has 43 base pairs in the 5' untranslated region and 210 base pairs in the 3' untranslated region, including a polyadenylation signal and a short poly(A) tract (Figure 3). In Northern analysis of human tissue RNA, two hybridizable E1α mRNA species have been identified (a more abundant 1.6 kb species and a less
Figure 3. Size and partial restriction maps of the human PDC E1α, E1β, E2 and E3 cDNA clones. The hatched and solid boxes represent coding regions for the leader peptide and mature polypeptide respectively, and the solid lines represent the 5'- and the 3'-untranslated regions of the clones. bp, basepairs. (Patel et al., 1990).
abundant species of 3.3 kb). These two E1α mRNA species appear to have identical 5’ untranslated and coding regions, but differ in the length of the 3’ untranslated region (Dahl et al., 1987). The human liver E1β cDNA encodes the entire mature E1β, including 329 amino acids, as well as a portion (26 amino acids) of the leader peptide (Ho and Patel, 1990) (Figure 3). Blot analysis of poly(A)⁺ RNA from Hela cells identified a single mRNA of 1.7 kb for E1β (Koike et al., 1989).

The cDNAs for the α and β subunits of rat E1 have been characterized and consists of 2367 and 1401 base pairs, respectively (Matuda et al., 1991). The predicted amino acid sequences for α and β subunits of rat PDC are 98% and 90% identical to that of human α and β subunits. The size of mRNAs for α and β subunits of rat PDC are 2.5 kb and 1.6 kb, respectively (Matuda et al., 1991). The mRNA for the rat α subunit apparently was longer than the mRNA of 1.8 kb for the human α subunit. In contrast, the mRNAs for the human and rat β subunits are the same length. The gene encoding the β subunit of PDC from Saccharomyces cerevisiae has been cloned, sequenced and expressed (Miran et al., 1993). Yeast and human E1β cDNAs exhibit 62% sequence identity.

B. The testis-isoform of the PDC-E1α subunit

In situ hybridization of a human E1α cDNA to human metaphase chromosomes revealed the presence of two loci (Brown et al., 1989). The gene for the E1α subunit of this complex, expressed in somatic tissues, is located on band p22.1 of the human X
chromosome. This gene, PDHa1, contains 10 introns and spans approximately 17 kb. A testis-specific form, PDHa2, showing significant cross-hybridization with an E1α cDNA probe, was detected on chromosome 4 in the region q22-23 (Dahl et al., 1990). The similarity with the X-linked cDNA coding sequence at the nucleotide level is 84%. The transcribed region of PDHa2 gene is highly homologous to the somatic form of E1α. However, the testis-specific form lacks introns and possesses characteristics of a functional processed gene and is believed to have originated by gene duplication via retroposition (Dahl et al., 1990).

Chromosome mapping in mouse has also revealed the presence of two E1α loci, one maps to the X-chromosome and the other to chromosome 19 (Brown et al., 1990). The cDNA clones encoding the testis-specific form of the rat E1α subunit have also been isolated (Cullingford et al., 1993). Figure 4 shows an alignment of the deduced amino acid sequences for the somatic and testis-specific forms of the E1α subunit of the PDC of mouse, rat and man. Of note is the degree of conservation (98 to 99% identity) of the somatic E1α subunits of mouse, rat and man. Comparison of the predicted amino acid sequences for rat and mouse testis-specific forms show that they exhibit 92% identity, whereas comparison between rat and human and between mouse and human testis-specific forms yields 78% and 75% identities, respectively (Cullingford et al., 1993).

The existence of a second, autosomal gene encoding the E1α subunit of PDC may have been selected during the evolution of the gamete cells to compensate for the loss
Figure 4. Alignment of the deduced amino acid sequences for the somatic (a1) and the testis-specific (a2) forms of the E1α subunit of the PDC of mouse (mou), rat (rat) and man (hum) based on the corresponding cDNAs. At each alignment position, amino acid residues identical in four or more of the six forms are shaded. P1, P2 and P3 indicate the three serine residues which coincide with the phosphorylation sites found in studies on bovine and porcine E1α subunit protein (Cullingford et al., 1993).
of expression of the somatic form due to loss of the X chromosome in Y-containing sperm and X inactivation in X-containing sperm. An analogous situation exists for the glycotic enzyme phosphoglycerate kinase (PGK), which is also encoded by an X-linked gene expressed in somatic tissues and an autosomal form which is intronless and expressed only in the testis (McCarrey et al., 1987).

The mouse PDHa2 gene is one of a number of testis-specific genes that are expressed initially at the meiotic prophase stage of spermatogenesis (Iannello and Dahl, 1992). However, although PDHa2 is strongly upregulated in the testis, its transcription is repressed in somatic tissues (Iannello and Dahl, 1992). Recently, the mouse PDHa2 promoter region was isolated and characterized (Iannello et al., 1993). In vitro, the core promoter displayed high constitutive activity in somatic cell lines. The 5'-flanking region of the testis-specific mouse E1α gene harbors a transcriptionally active core region between nucleotides -187 to +22. The core promoter lacked both TATA and CAAT boxes, although potential Sp1 and ARF/CREB binding sites were identified. DNase I footprinting of this region revealed four regions of protection (Figure 5). One of these contains the Sp1 binding site and the other the ATF/CREB cis element. The sequences of the other two protected regions (designated MEP-2 and MEP-3; Mouse E1α Promoter site) showed no apparent homology to any known consensus element. The factor which recognized MEP-3 appeared to be ubiquitous, whereas the MEP-2 protein complexes were tissue specific involving the formation of a complex between MEP-2 and a putative testis-
**Figure 5.** Diagrammatic representation of the proximal promoters of the (a) mouse Pdha-1, (b) human PGK-2 and (c) mouse Pgk-2 genes. Regions represented as enhancers and repressors are shown upstream of the proximal promoters. Regulatory cis-elements within the proximal promoters shown to either bind to testis-specific factors or required for transcriptional activity are indicated (Iannello et al., 1994).
specific binding factor (τ-MEP-2BF), first observed in the testis of 2 week old mice (Iannello et al., 1993).

Transgenic mice containing 323 base pairs of the 5'-flanking region of the Pgk2 gene fused to either chloramphenicol acetyltransferase (CAT) or luciferase was found to contain information essential for testis-specific expression of the transgene. Transgene RNA was first detected at day-15 and increased to a high level by day-60 which was similar to the pattern seen with the expression of the endogenous mouse PGK-2 RNA. (Robinson et al., 1989). The mouse testis-specific Pgk-2 promoter has been analysed in some detail. By deletion analysis and cell-free transcription assays, a cis-element was identified within the proximal promoter that functioned positively to mediate transcription (Goto et al., 1993). The nucleotide sequence that comprises this element bears homology to an Ets-binding motif and appears to interact with an Ets-related factor (TAP-1). Such a consensus binding site for this factor is present in the proximal region of the Pdha-2 promoter, however it has not yet been analysed in any detail. It is of interest to note that Sp1 is also differentially regulated during spermatogenesis (Saffer et al., 1991). In testis, there was an age-associated increase in Sp1 protein which was localized to the spermatids. Interestingly, in both the mouse Pdha-2 and Pgk-2 promoters, the Sp1 binding site lies just downstream of the Ets consensus motif. An attractive role for Sp1 binding maybe to mediate the effects of testis-specificity in synergy with other effectors.
C. Structural Aspects of PDC-E2

The E2 subunits from most organisms assemble into either a 24-subunit cubic structure or a 60-subunit structure with the appearance of a dodecahedron. Limited proteolysis of mammalian PDC-E2 results in the formation of two large fragments, a compact domain (also referred to as the catalytic domain) at the carboxy terminus and a lipoyl-bearing domain at the N-terminus linked by a subunit-binding region (Bleile et al., 1981). Examination of the lipoyl-bearing domain of human PDC-E2 revealed the presence of two similar, but not identical (55% homology) repeating units of 127 amino acids. Each repeating unit encompasses a lipoyl prosthetic group attached to a specific lysine. Between each of these domains are sequences rich in alanine, proline and charged amino acids. Richard Perham’s laboratory established the highly mobile nature of the interdomain segments using [\(^1\)H]NMR (Perham and Packman, 1989; Texter et al., 1988). A family of sharp resonances, not expected from the large oligomeric structure, revealed the conformational mobility of these segments (referred to as hinge regions).

The number of lipoyl domains in E2 from different sources ranges from three (gram negative bacteria) to one (gram positive bacteria and yeast). The human E2 revealed a total structure of four domains with two lipoyl containing domains. The subunit binding domain of the mammalian complex binds the E1 component. In the bacterial complexes, the homologous domain binds only the E3 component or the E3 and the E1 component. The inner domains catalyze the transacylation reaction and self
associate to form the dodecahedral structure. Comparison of the domain structures of the E2 components of PDC form various sources and to another lipooyl bearing subunit of PDC, protein X are depicted in figure 6.

The lipooyl domains of E2 are required for regulatory effects on kinase and phosphatase activities. The E1 kinase and the phospho-E1 phosphatase associate with specific regions in the outer lipooyl domains of E2. Both regulatory enzymes undergo substantial enhancement in their activities as a result of these associations. The assembled E2 oligomer can bind at least 15 kinase molecules. The lipooyl-lysine moiety functions as a flexible structure that promotes intermediate transfer. Shuttling of acetyl groups and electrons between lipooyl moieties is suggested by some studies. The availability of several lipooyl groups to each active site in the E1, E2 or E3 components apparently aids the catalytic process. Removal of more than 40% of the lipooyl domains of mammalian E2 is not correlated with loss in the capacity to support the overall reaction suggesting that the presence of an array of lipooyl domains with a reserve potential to support rapid intermediate transfer (Rahmatullah et al., 1990).

PDC-E2 cDNA encodes a mature protein of 561 amino acids with a leader sequence of 54 residues (Thekkumkara et al., 1987). The protein has a calculated molecular mass of 59,927 daltons when two molecules of lipoic acid are covalently linked to mature PDC-E2. Northern blot analysis of RNA isolated from human heart and rat kidney revealed the presence of three hybridizing species of approximately 2.3, 2.9 and 4.0 kb in size (Thekkumkara et al., 1987). The E2 cDNA sequence was
Domains of the E2 and protein X subunits. Lipoyl domains are designated by an L, subunit binding domains by a B, and the inner domains by an I. The connecting hinge regions are shown as a wiggle. A vin. is Azotobacter vinelandii and B.ste. is Bacillus steathermophilus (Patel and Roche, 1990).
determined from rat heart and appears to be most closely related to the human liver sequence (Matuda et al., 1992). Recently, a cDNA encoding the E2 subunit of PDC from *Arabidopsis thaliana* was isolated (Guan et al., 1995). The amino acid sequence and organization and number of functional domains within the *Arabidopsis* protein are identical to those of the human E2.

D. Structural Aspects of PDC-E3

E3 catalyzes the reoxidation of dihydrolipoamide in the presence of FAD with NAD$^+$ as the final electron acceptor. E3, is representative of a class of ubiquitous flavin-containing dehydrogenases known as the pyridine nucleotide-disulphide oxidoreductases (Carothers et al., 1989). In addition to PDC-E3, members of this family also include glutathione reductase, mercury (II) reductase and thioredoxin reductase. Comparison of the primary amino acid sequences of E3s from several species demonstrates considerable homology in their sequences (Carothers et al., 1989). Comparison of the primary amino acid structure of human E3 revealed a high degree of homology (33%) with the human glutathione reductase (Pons et al., 1988). A 3-dimensional structural model for the human E3 was constructed based on the 3-dimensional structure of glutathione reductase (Jentoft et al., 1992). E3 has four different domains: FAD, NAD$^+$, the central and the interface domains.

In PDC, E3 exists as a dimer of Mr 110,000, six of which are bound to the surface of the E2 core (Reed, 1974). The homodimeric enzyme contains one noncovalently
attached molecule of FAD per subunit (Reed and Yeaman, 1987). E3 is shared among all three α-ketoacid dehydrogenase complexes which, in addition to PDC include the α-ketoglutarate dehydrogenase and the branched-chain α-ketoacid dehydrogenase complex (Sakurai et al., 1970; Heffelfinger et al., 1983). E3 is also a component (referred to as L protein) in the glycine cleavage system (Carothers et al., 1989).

Our nucleotide sequence of the E3 cDNA consists of 2082 base-pairs and an open reading frame of 1527 base-pairs encoding a precursor E3 polypeptide of 509 amino acids (Pons et al., 1988). The first 35 amino acids of this precursor protein represents a typical mitochondrial import leader sequence (Figure 4). The mature E3 of 474 amino acids with an FAD molecule added has a calculated molecular mass of 50,919 daltons. Two hybridizable E3 mRNA species (2.2 and 2.4 kb) are detectable in human tissues, consistent with the presence of two polyadenylation signals whereas only a single 2.4 kb band was seen in rat tissue samples (Pons et al., 1988).

The gene for E3 has been assigned to human chromosome 7 and consists of 14 exons ranging in size from 69 to 780 bp contained within a 20 kb genomic region (Feigenbaum and Robinson, 1993). Approximately 2 kb of the 5'-flanking region of this gene was sequenced and appears to have characteristics of a housekeeping promoter as it lacks a canonical TATA box and contains a CpG-rich region near the transcription start site. Transient expression assays with a series of 5'-nested deletions of the E3 promoter in HepG2 cells indicated that a region between -769 to -1223 bp
suppresses basal transcription and a very small fragment of the 5’-region from -19 to +47 maintains a significant level of expression (Johanning et al., 1993)(Figure 7).

E. Structural Aspects of Protein X

The mammalian PDC has a structural core composed of two lipoyl-bearing components-E2 and protein X (DeMarcucci and Lindsay, 1985; Rahmatullah et al., 1989a; Rahmatullah et al., 1989b). The molecular mass of protein X is estimated to be 50,000 daltons and approximately six copies of protein X are associated tightly with the E2 core (Jilka et al., 1986). Proteolytic digestion of protein X releases a 35,000 dalton inner domain and a 15,000 dalton outer domain which contains lipoic acid (Rahmatullah et al., 1989b) (Figure 6). The inner domain of protein X binds to the E2 inner domain and together these form an oligomer apparently composed of about 60 E2 and about 6 X domains.

Evidence indicates that protein X is required for optimal PDC activity (Gopalakrishnan et al., 1989). Selective protease cleavage of protein X was associated with a loss of the overall PDC activity and this cleavage was protected by the presence of E3. Binding of E3 to the E2 oligomer increased two-fold in the presence of intact protein X. Evidence indicates that protein X is involved in the binding of E3 to the complex and/or it might also mediate electron transfer from E2 to E3 (Gopalakrishnan et al., 1989).
Figure 7. Schematic representation of cis-elements and promoter activity of the human E3 gene in HepG2 cells. The coding region of the CAT gene was fused to the E3 promoter region to generate a series of pE3CAT constructs. CAT activity is represented as fold increase over that of the 1.8 kb pE3CAT construct and normalized to the β-galactosidase activity. CCAAT, CCAAT binding protein; CRE, cyclic AMP response element; FSE-2, fat specific element2 (Johanning et al., 1992).
Protein X apparently plays a structural role in PDC. It binds and positions E3 to the E2 core and this binding is essential for a functional PDC. It was observed that removal of most of the lipoyl domain had little or no effect on the overall activity of PDC. However, deletion of the putative subunit binding domain of protein X resulted in loss of E3 binding and the concomitant loss of overall activity of PDC (Lawson et al., 1991). At present, neither the cDNA nor the gene has been cloned for the mammalian protein X, or more recently termed the E3 binding protein, E3BP. The sequence of the E3BP cDNA from Saccharomyces cerevisiae seems to indicate a common ancestry with the E2 gene as determined by similarity of the amino terminal lipoyl domains. The open reading frame within the 1.5 kb cDNA indicates a mature protein of 380 amino acids with a 30 residue leader peptide (Behal et al., 1989).

F. Structural Aspects of E1-kinase

The E1-specific kinase catalyzes the phosphorylation of all three E1α serine residues. The rate of phosphorylation being site1 > site 2 > site3 (Kerby et al., 1979; Sale and Randle, 1982). The enzyme is specific for PDC; it was inactive against other substrates tested, namely, glycogen synthase, histones and casein (Reed and Yeaman, 1987). The E1 kinase was first successfully purified from bovine kidney mitochondria (Stepp et al., 1983). Resolution of the kinase purified from bovine kidney or heart demonstrated that the kinase comprises of a catalytic subunit (designated Kc; Mr = 46,000) and another subunit (Mr = 43,000) which has a high isoelectric point and
referred to as the basic subunit, K₀ (Roche et al., 1989). The Kₑ subunit of the kinase binds to the E₂ oligomer in the absence of protein X. Binding does not require the Kₐ subunit. Proteolytic cleavage of the Kₐ subunit does not cause loss of any known regulatory effects on kinase activity.

The bound kinase has a greatly enhanced rate of phosphorylating the E₂-bound E₁ tetramers versus the dissociated E₁. This E₂-activated kinase function was completely prevented by selective alkylation of reduced lipooyl groups (Radke et al., 1993). Selective release of the lipooyl domains of E₂ also released the kinase suggesting that the kinase is tightly bound to PDC by the lipooyl domain region of E₂ (Li et al., 1992). One interesting hypothesis is that the lipooyl group plays a role in bringing the kinase and the E₁ substrate in close proximity. Lipooyl-mediated stimulation of kinase activity by NADH and acetyl-CoA is effectively achieved by the lipooyl domain of E₂ even in the absence of protein X (Roche et al., 1989).

Similar to the kinase from bovine kidney, rat heart kinase appears to consist of two subunits that differ in electrophoretic mobility (Mr 48 kDa (p₄₈) and 45 kDa (p₄₅), respectively). Recently cDNAs encoding the p₄₈ and p₄₅ subunits were isolated from rat heart (Popov et al., 1993; Popov et al., 1994). The protein sequence of p₄₅ has 70% identity to the protein sequence of p₄₈. The deduced protein sequence revealed high homology to another mitochondrial protein kinase, the branched-chain α-ketoacid dehydrogenase (BCKADC) kinase. It lacked sequence similarity with all eukaryotic
Ser/Thr protein kinases other than BCKADC kinase. Both the kinases have considerable homology to the prokaryotic histidine protein kinases.

The E1-kinase reaction requires the presence of either Mg$^{2+}$ or Mn$^{2+}$ (Reed and Yeaman, 1987). The apparent Km for either divalent cation is 20 mM (Weiland, 1983). The E1-kinase is stimulated by acetyl-CoA and NADH, both of which are the products of the oxidative decarboxylation (Randle, 1981). Pyruvate, as well as the cofactors NAD$^+$ and TPP, inhibit E1-kinase. Inhibition by TPP involves the induction of conformational changes in E1α upon binding of TPP, thus rendering the phosphoseryl groups inaccessible to the E1-kinase (Butler et al., 1977). The E1-kinase is inhibited by ADP via competition with ATP (Reed and Yeaman, 1987). A variety of acids (acetate, propionate and butyrate), halogenated carboxylic acids (dichloroacetate [DCA]) and divalent cations (Ni$^{2+}$, Ca$^{2+}$, Co$^{2+}$ and Mn$^{2+}$) also inhibit E1 phosphorylation (Whitehouse et al., 1974; Weiland, 1983). The mechanism by which divalent cations inhibit E1-kinase appears to be due to the competition with Mg$^{2+}$ATP complex formation (Weiland, 1983).

G. Structural Aspects of phospho-E1-phosphatase

The phospho-E1-phosphatase catalyzes the dephosphorylation of phosphoryl groups from all three sites in the E1α subunit. The relative rates of dephosphorylation are site2 > site3 > site1 (Randle, 1981). This enzyme is loosely associated with PDC. The bovine enzyme is a heterodimer composed of a 50 kDa catalytic subunit (P$_c$) and a
90 kDa subunit (P₁) with a tightly bound FAD (Roche et al., 1989). Recently, the cDNA encoding the catalytic subunit of bovine PDC phosphatase was cloned and coexpressed in E.coli with the chaperonin proteins groEL and groES (Lawson et al., 1993). The cDNA is 1900 base pairs in length, encoding a putative presequence of 71 amino acids and a mature protein of 467 residues with a calculated Mr of 52,625.

The activity of the phosphatase is increased by more than four fold by the E2-X subcomplex (requires Ca²⁺) (Pettit et al., 1972). The lipoyl domains of E2 are critical for efficient function and regulation of the phosphatase. Positive regulators of the phosphatase include divalent cations and polyamines. Divalent cations, Mg²⁺ or Mn²⁺ are also required for phosphatase activity (Wieland, 1983). Among polyamines, spermine is the most effective activator of the phosphatase (Damuni et al., 1984). Spermine (and other polyamines) activates the phosphatase by greatly lowering its Km for Mg²⁺ and by increasing its Vₘₐₓ (Roche et al., 1989). The phosphatase is inhibited by NADH, the product of the PDC reaction. Fluoride ion at 0.6 mM concentration also inhibits phospho-E1-phosphatase (Wieland, 1983).

III. Regulation of PDC

The mammalian PDC is associated with the mitochondrial inner membrane and it irreversibly commits three-carbon intermediates derived from the catabolism of carbohydrate or certain amino acids to conversion of acetyl-CoA. There is no alternative pathway allowing acetyl-CoA, the product of the reaction, to be converted
to pyruvate. Thus we are unable to utilize fatty acids as precursors for gluconeogenesis. It is a “point of no return” in the metabolism of carbohydrate. Dr. Philip Randle, who has conducted a thorough analysis of the utilization of various fuels, concluded that “The activity of the PDH complex is the major determinant of glucose oxidation in well-oxygenated tissues in vivo.” (Randle, 1986).

There are two alternative metabolic fates for acetyl-CoA generated via the PDC reaction. In most tissues, the metabolic fate of acetyl-CoA is predominantly its complete oxidation to CO₂ via the TCA cycle. In lipogenic tissues (e.g., liver, adipose tissue and lactating mammary gland), acetyl-CoA entering the TCA cycle via PDC can additionally be utilized for fatty acid synthesis via the condensation of acetyl-CoA with oxaloacetate to form citrate, efflux of citrate to the cytoplasm and cleavage of citrate by ATP-citrate lyase. This regenerates oxaloacetate and acetyl-CoA in the cytoplasm, where acetyl-CoA enters the lipogenic pathway via acetyl-CoA carboxylase.

Because PDC plays a pivotal role in intermediary metabolism, it is an ideal candidate as a regulatory enzyme. For example, in the liver the activity of PDC must be decreased under gluconeogenic conditions when pyruvate is channeled to the pyruvate carboxylase reaction to synthesize oxaloacetate. During lipogenesis, increased PDC activity is needed to supply acetyl-CoA for fatty acid or sterol synthesis. In cardiac tissue, PDC needs to be able to react rapidly to increased work load. In the presence of alternative fuels such as fatty acids or ketone bodies, suppression of PDC activity is vital to allow “sparing” of carbohydrate for tissues such as the brain that are dependent
upon glucose for energy metabolism. PDC is subjected to both short-and long-term regulation. Short-term regulation of PDC is accompanied by a combination of end-product inhibition and reversible covalent phosphorylation (reviewed by Patel and Roche, 1990). Long-term regulation of PDC involves changes in the level of the enzyme in the mitochondria.

A. Regulation by product inhibition

End-product inhibition of PDC is imparted by two metabolites of pyruvate, namely, acetyl-CoA and NADH. Both inhibitors act in a competitive manner against CoASH and NAD+, respectively (Furuta et al., 1977). Hansen and Henning reported similar results with the E.coli complex, although the bacterial complex appeared to be much more sensitive to NADH than to acetyl-CoA (Hansen and Henning, 1966). It has been proposed that inhibition is mediated by the lipoyl group of E2 (Wieland, 1983). Increases in the concentration of acetyl-CoA and/or NADH would maintain a larger proportion of E2 lipoyl groups in the reduced state, thus impeding the regeneration of E1-TPP.

B. Regulation by phosphorylation and dephosphorylation

Reversible covalent phosphorylation is catalyzed by the E1-kinase and the phospho-E1 phosphatase. The phosphorylation-dephosphorylation cycle that regulates mammalian PDC is tightly regulated (Fig.8). Both intrinsic (metabolites) and extrinsic
Figure 8. Phosphorylation-dephosphorylation cycle of the E1 of mammalian PDC. Abbreviations used are: E1, dephosphorylated E1, $\text{P}^{-}\text{E1}$, phosphorylated E1; activator (+) or inhibitor (−), of both E1 kinase and phospho-E1 phosphatase.
(hormones) effectors regulate the active state of PDC. The intrinsic factors namely, increase in the intramitochondrial ratios of NADH/NAD⁺, acetyl-CoA/CoA and ATP/ADP ratios stimulate kinase activity. Some aspects of the mechanism of effector control of PDC have been established. ADP and pyruvate inhibit by directly binding to the kinase, imparting synergistic inhibition (Hucho et al., 1972). The feedback products NADH and acetyl-CoA stimulate kinase activity through the reduction and acetylation of lipoyl moieties in the complex (Cate and Roche, 1978; Roche et al., 1989). Acylation of E1 led to enhanced inactivation of the enzyme by kinase-mediated phosphorylation of the E1 component.

Mg²⁺ is a required cofactor of phosphatase. Ca²⁺ enhances phosphatase activity both by facilitating the association of the phosphatase with the complex and by decreasing the Km for its substrate, namely the phosphorylated E1 component (Pettit et al., 1972). The discovery that dehydrogenase enzymes may be activated by Ca²⁺ ions in the range of concentrations found within cells suggested that this activation may provide a mechanism whereby mitochondrial oxidations are enhanced during periods of elevated tissue work load, especially in excitable tissues. By increasing the amount of catalytically active PDC, activation by Ca²⁺ ions leads to more active reduction of NAD⁺ and permits the mitochondria in activated tissues to maintain higher NADH/NAD⁺ ratios than would otherwise be possible (Hansford, 1980; Katz et al., 1987).
C. Nutritional and hormonal regulation of PDC.

The activity of PDC in most tissues is maintained at constant levels, however the levels of “active” PDC (dephosphorylated) in most tissues is influenced by nutritional and hormonal modifications (Patel and Roche, 1990; Behal et al., 1993; Sugden and Holness, 1994). Inactivation of PDC via phosphorylation by the PDC kinase is crucial for glucose conservation in states of carbohydrate deprivation, such as starvation, as the activity of the complex is the major determinant of the rate of irretrievable loss of glucose from the body. Under these conditions, E1 kinase is activated by increases in the mitochondrial acetyl-CoA/CoA and NADH/NAD+ concentration ratios occasioned by high rates of fatty acid oxidation (Sugden and Holness, 1990).

Heart and kidney tissue in fed rats normally contain about 70% active PDC (Wieland et al., 1971). However, fasting or induction of experimental diabetes decreases active enzyme to about 15%. Brain tissue contains about 70% active enzyme, but starvation has no effect on the enzyme in the brain. In contrast, liver and adipose tissue normally contain only 20% active enzyme (Wieland 1983). The fraction of PDC present in the “active” state varies from tissue to tissue and is determined by the activites of PDC kinase and E1-phosphate phosphatase and the susceptibility of the E1 to either enzyme.

Starvation is associated with net phosphorylation of the PDC (Wieland, 1983, Randle, 1986, Sugden and Holness, 1989). In progressive starvation, the short term effects of oxidation of fatty acids and ketone bodies to decrease the percent active
PDC in heart are supplemented by a more long term mechanism, involving a stable increase in the specific activity of PDC kinase which is observed after 24 to 48 h (Kerbey and Randle, 1982). The increase in the PDC kinase specific activity may be mediated by fatty acids and cAMP. Culture of cells from fed rats with agents that increase cAMP or free fatty acids increased kinase activity 2-3 fold (Stace et al., 1992). In contrast, fatty acids failed to inhibit pyruvate decarboxylation and the activity of PDC from mitochondria isolated from livers of adrenalectomized rats (Cipres, et al., 1994). Thus, the diminished effect of fatty acids in stimulating gluconeogenesis in livers from adrenalectomized rats was the result of a limited pyruvate availability for the carboxylase reaction due to a lack of inhibition of flux through the PDC. It is also plausible that adrenalectomy prevented the full activation of PDC kinase.

In both liver and heart, the reactivation of PDC after prolonged starvation has been correlated with a reversal of starvation induced increases in kinase. Similarly, the provision of a high fat diet (47% of calories as fat) for 28 days evoked a decline in cardiac “active” PDC activity together with increases in the activity of kinase from adult rats (Orfali et al., 1993). In contrast to the enzyme in other tissues, PDC activity in the brain is not reduced by starvation and remains about 70%. The insensitivity of the brain PDC to starvation is thought to be due to the ability of brain mitochondria to accumulate a high concentration of pyruvate and hence maintain the enzyme in an active form and ensure a continuous supply of acetyl-CoA to the TCA cycle. Increased
PDC activity is believed to be an important regulatory step leading to enhancement of de novo hepatic lipogenesis associated with high-fructose feeding. This activation is achieved via stable and long-term downregulation of the kinase as well as by acute activation of the phosphatase (Sugden and Holness, 1994).

Activation of PDC by insulin has been demonstrated in various cell types, namely, in isolated rat hepatocytes (Clot et al., 1988), fat cells (Denton et al., 1989), human fibroblasts (Buffington et al., 1984), and circulating mononuclear cells (Clot et al., 1992). Exposure of rat epididymal adipose tissue or isolated fat cells in vitro to insulin leads to a two-to-threefold increase in the activity of PDC within a few minutes (Jungas, 1971; Weiss et al., 1971). This is an important mechanism whereby insulin stimulates the conversion of glucose into fat in adipose tissue, a pathway where PDC plays a pivotal rate-controlling role (Wieland et al., 1973). It is now evident that insulin increases PDC activity in other tissues which are important sites of fatty acid synthesis, namely, liver (Topping et al., 1977), lactating mammary gland (Baxter et al., 1979), and brown adipose tissue (McCormack and Denton, 1977).

Insulin activates PDC via phospho-E1 phosphatase activation (Wieland et al., 1989). This activation persists even in mitochondria isolated from insulin treated fat cells. It has been suggested that inositol phosphate glycans may act as secondary messengers in insulin signalling of PDC (Larner et al., 1989). Denton's group demonstrated a decreased $K_m$ of the phosphatase for $\text{Mg}^{2+}$ upon insulin treatment. Recent findings suggested that insulin may retain its ability to stimulate PDC activity in
CHO cells expressing the insulin receptor with an inactivated tyrosine kinase domain (Gottschalk, 1991).

The signal transduction pathways associated with insulin function are only partly understood. Insulin stimulates PDC through the production of a inositolglycan/inositol phosphoglycan mediator which has been shown to stimulate the most abundant phosphatase, the divalent cation dependent phosphatase by decreasing the magnesium requirement (Lilly, et al., 1992). A metal independent PDC phosphatase found in bovine heart mitochondria was also stimulated by the purified insulin mediator. The mechanisms by which mediators regulate phosphatase activity is not known at present. A direct interaction between the inositolglycan mediators and the phosphatase has not been demonstrated.

Recent observations also indicate that the signal transduction pathway whereby insulin activates PDC in several cell-lines involves a protein kinase C step. The protein kinase C (PKC) activator 4β-phorbol 12β-myristate 13α-acetate (PMA) has been reported to mimic the effects of insulin on PDC activity in cultured BC3H-1 myocytes (Farese et al., 1985). In Zajdela hepatoma cells (ZHC), PMA activates PDC and the insulin-induced PDC activation was almost completely blocked in PKC-depleted cells (Benelli et al., 1994). These results indicated that the signal transduction pathway whereby insulin activates PDC in hepatoma cells involves a PKC-dependent step. It may be speculated that PKC-stimulated activation of the PDC phosphatase may be involved in the role of insulin in PDC activation.
Pyruvate carboxylase and PDC catalyze the initial steps of the two major routes of pyruvate metabolism, namely, gluconeogenesis and pyruvate oxidation, respectively. Thyroid hormones can alter the flux of gluconeogenesis from pyruvate or lactate in the liver (Freedland and Krebs, 1976). Livers from hyperthyroid rats had twice the pyruvate carboxylase activity of normal rats (Weinberg and Utter, 1979). This increase was due to the presence of increased amounts of protein and further, that the increase was due to an increased rate of synthesis. Similar investigations of PDC demonstrated that the activity of the complex was not affected by hyperthyroidism. However, total PDC activity in rat liver was decreased by 33% in the hypothyroid state (Weinberg and Utter, 1979). Immunological experiments suggested that the decreased activity of PDC activity in the hypothyroid state was accompanied by decreased amounts of E1. These studies provided no evidence concerning the effects of thyroid status on the rates of synthesis or breakdown of other enzymes associated with the complex, although it is likely that they are coordinately regulated in a similar fashion in the hypothyroid state.

Modulation by insulin of expression of several genes involved in glucose metabolism appears to be dependent on the presence of glucose. Recent evidence indicates that aerobic glycolysis is required for glucose-induced insulin secretion (Gottschalk, 1991). Pyruvate carboxylase and E1α mRNA levels were significantly altered by glucose in primary pancreatic islets (MacDonald et al., 1991). The carboxylation via pyruvate
carboxylase and decarboxylation via E1 may play an important role in the signal transduction for insulin release in the β-cells of the pancreas.

The exact biochemical nature of the signals that link glucose metabolism and other nutrient stimuli to insulin secretion is not well defined. Leucine, a potent physiological insulin secretagogue stimulates insulin release in the absence of extracellular glucose (Milner, 1969). The mRNA of the E1α subunit of branched-chain α-ketoacid dehydrogenase complex (BCKADC) and its activity was decreased by 90% in islets cultured at 20 mM glucose (MacDonald et al., 1991). Conversely, at 20 mM glucose concentration the glucose-induced insulin release was preserved with increase in the mRNA of PDH E1α and pyruvate carboxylase. The E1 component of PDC and BCKADC are very similar. Increase in the level of their mRNAs in response to glucose implies regulation occurring at the gene expression level via “glucose response elements”. Recently a putative carbohydrate response element was determined in the L-type pyruvate kinase gene whose expression is induced after feeding a diet rich in carbohydrate (Thompson and Towle, 1991). Similar glucose response elements have been identified in the insulin gene, whose expression increased in primary cultures of rat islet cells with increasing concentrations of glucose (Docherty and Clark, 1994).

The differentiation of 3T3-L1 preadipocytes to adipocytes is accompanied by dramatic increases in the activities of enzymes associated with de novo lipogenesis and triacylglycerol mobilization (Hu et al., 1983). Adipocyte differentiation was accompanied by a 6-8 fold increase in the specific activity of PDC, a lipogenic enzyme.
This was accompanied by increased synthesis of E1α, E1β and E3 peptides (Hu et al., 1983; Carothers et al., 1988). Specific activity of BCKADC increased 10-fold in 3T3-L1 adipocytes compared to preadipocytes (Chuang et al., 1983). This suggests that a common mechanism may be involved in the hormone-dependent increase in the activities of these two complexes during adipocyte differentiation. The Fat Specific Elements (FSE-1 and FSE-2) present in the promoter-regulatory regions of genes transcriptionally activated in adipocytes may play a role in such tissue-specific regulation (Hunt et al., 1986).

Glucose is the major fuel for oxidative metabolism in the developing fetus. PDC activity, like that of other mitochondrial enzymes, is low in most fetal tissues and increases gradually during the postnatal period. Total PDC activity is low in fetal rat liver and increases slowly to the adult level over 3-4 postnatal weeks (Knowles and Ballard, 1974, Wilbur and Patel, 1974). The change in activity of the developing rat brain was paralleled by a coordinate increase in the abundance of several of the component proteins and in the message levels between the perinatal period and 10 days postpartum to reach near-adult levels (Cullingford et al., 1994). However, the lack of temporal coordination between the changes in the mRNA levels (2.5 fold increase), determined by RNase protection assays, and protein levels (approximately 6 fold increase), suggests control at translational and/or posttranslational level(s).

Most of the PDC in developing rat liver is present in the inactive form due to the presence of low levels of insulin, which in turn is due to the consumption of a high fat-
low carbohydrate milk during the suckling period. However, the “active” and “total” PDC activities in rat liver increase significantly during the first 2 and 6 postnatal hours, respectively (Chitra et al., 1985). This is due to an increased content of all the PDC catalytic component proteins (Serrano et al., 1989). The transient increase in active PDC during the first two postnatal hours plays an important role in the rapid clearance of the lactate pool through oxidative metabolism.

IV. Genetic defects of the pyruvate dehydrogenase complex.

Human PDC deficiency is a potentially severe inborn error of oxidative metabolism. PDC deficiency is the most common cause of congenital lactic acidosis, although it accounts for less than 15% of the cases of this large group of disorders. There have been more than a 100 reported cases of PDC deficiency identified by measurements of PDC activity (Table II). PDC deficiency is extremely heterogeneous in its clinical manifestations and are primarily limited to the central nervous system. This is due to the dependence of the brain on glucose oxidation for energy production. Symptoms vary from mild ataxia to devastating congenital neuroanatomic defects often typical of Leigh’s encephalomyelopathy. There is a male preponderance among severely affected patients while females tend to have milder metabolic and neurologic dysfunction (Robinson, 1989, Ho et al., 1989).

Due to the multisubunit structure of PDC, a variety of genetic defects are possible (Table II). Approximately 90% of cases of PDC deficiency involve defects of the E1
Table II: Human pyruvate dehydrogenase complex deficiency: chromosomal localization and identification of the affected subunits.
Table II. Human pyruvate dehydrogenase complex deficiency: chromosomal localization and identification of the affected subunits.

<table>
<thead>
<tr>
<th>PDC components</th>
<th>Human cDNA cloned</th>
<th>Human gene cloned</th>
<th>Human chromosome location</th>
<th>Number of reported cases</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1α</td>
<td>yes</td>
<td>yes</td>
<td>X, 4</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>E1β</td>
<td>yes</td>
<td>yes</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>yes</td>
<td>no</td>
<td>11</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>yes</td>
<td>yes</td>
<td>7</td>
<td>7</td>
<td>1</td>
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<tr>
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<td>no</td>
<td>3</td>
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</tr>
<tr>
<td>Kinase</td>
<td>no</td>
<td>no</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phosphatase</td>
<td>no</td>
<td>no</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
component and thus far all of the defects identified are in the coding region of the E1α subunit. No defect in the E1β subunit has been reported. There have been a small number of deficiencies involving E3 (Liu et al., 1993). In addition, there have been reports of both E1 phosphatase, E2 and protein X defects (Table II).

Sequence analysis of patient-specific cDNAs of E1α and E1β has facilitated the identification of specific mutations in nearly 40 E1 deficient patients. All the identified mutations are in the coding region of the E1α(X) gene. Most E1α mutations are localized to the 3' end of the cDNA and occur primarily in exons X and XI. The effects of these mutations are pleomorphic with some mutations affecting the synthesis or stability of E1 while others disrupt the catalytic function of the component (Chun, et al., 1993; Dahl et al., 1992). Recently, two substitution mutations have been identified in an E3-deficient patient with compound heterozygosity (Liu et al., 1993). This patient had very low level of E3 activity with reduced levels of E3 protein.

Alternative strategies have been tested to treat PDC deficiency. Unfortunately, none have been tested in a systemic fashion and to date there is no clear evidence of an effective therapy for the treatment of this disorder. As a means of providing sufficient metabolizable energy especially during brain development, ketotic diets provide an alternative source of acetyl-CoA to maximize ketosis and avoid lactic acidosis. Thiamine therapy may be effective in cases where mutations may affect cofactor binding. DL-lipoic acid has been given to patients with E3 deficiency. Treatment with dichloroacetate (DCA), an E1-kinase inhibitor, has shown some benefits in terms of
reduced lactic acidosis (Stacpoole, 1989). Hence molecular characterization of this complex is vital in understanding the genetic basis of PDC deficiency and in the long term it may be possible to develop an effective therapeutic approach(s) in treatment of this disorder.

Primary biliary cirrhosis is an autoimmune disease characterized by the production of antibodies against the PDC component proteins. The autoantigen in most cases appeared to be the E2 subunit (Surh et al., 1989). Antibodies targeted against E1α was also detected (Fregeau et al., 1990). Defects in E1 and other TPP-containing enzymes have also been reported in brain and peripheral tissues from patients suffering from Alzheimer’s disease (Gibson et al., 1988).

V. Research objectives

The goals of the research contained within this thesis are concentrated in three areas, namely (i) the isolation of the human E1β genomic clone and characterization of its promoter-regulatory region, (ii) the function of various cis-elements identified in the E1α promoter which may control expression of the human E1α gene and (iii) the investigation of long-term consequences of various dietary manipulations on the regulation of PDC in animals. Knowledge of the promoter-regulatory regions and their transcriptional analysis is prerequisite for our understanding of possible coordinate regulation of the PDC genes as well as identification of mutations which may be localized in these regions.
The initial focus of my research was concentrated in screening and isolation of the human E1β genomic clone described in chapter three. Approximately 2 kb of the 5'-regulatory region was sequenced and it appeared to have characteristics of a housekeeping-type promoter as it lacks a canonical TATA box and contains a CpG rich region near the transcription start site. To begin to define functional domains in the promoter-regulatory region, transient expression assays were performed using a series of 5' nested deletions ligated to the reporter gene, chloramphenicol acetyltransferase (CAT). Similar analysis was performed on the E1α promoter region (-763 to +33 bp) generated by Dr. Mei Chang using PCR of genomic DNA isolated from human skin fibroblasts. Since E1α and E1β proteins are present as a heterotetramer in E1 of PDC, it was of interest to pursue promoter analysis of both components. The work discussed in chapter four leads you through detailed analysis of the E1α promoter.

PDC activity responds to changes in nutritional and hormonal factors such as fasting, refeeding and experimental diabetes. More recently, evidence suggests that PDC activation is important for the increased rates of hepatic lipogenesis promoted by high-sucrose or fructose diets (Carmona and Freedland, 1989). Limited information is available concerning the effects of high-carbohydrate or high-fat diets on the long-term regulation of the enzyme. Earlier studies had suggested that significant effects of such dietary manipulations were mainly observed in liver and adipose tissue. The present study was carried out to evaluate the long-term effects of high-sucrose and high-fat
diets on PDC activity in the liver. Chapter five discusses dietary manipulations subjected on rats and the consequences measured by "total" PDC activity, immunological analysis of the PDC catalytic proteins and measurement of mRNA levels by Northern analysis of the PDC components in liver. The present study was undertaken to elucidate the mechanisms involved in the control of the levels of PDC in rat liver mitochondria.
I. MATERIALS

All restriction and modification enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN), Bethesda Research Laboratories, and New England Biolabs (Beverly, MA). Radioisotopes such as (α-\(^{32}\)P)dCTP, (γ-\(^{32}\)P)ATP, (\(^{3}H\))chloramphenicol and (\(^{14}\)C)pyruvate were from New England Nuclear, Dupont (Boston, MA). All other chemicals were of analytical reagent grade. Two plasmid vectors, BSKCAT and pSV2CAT, were a gift from Dr. John Nilson, Department of Pharmacology, and RSVβgal was kindly provided by Dr. Richard W. Hanson of this department. Thymidine kinase CAT (TKCAT) vector was a gift from Dr. Richard Eckert, Department of Physiology and Biophysics of this medical school. Purified bovine PDC was obtained from Sigma. Antibodies raised against the components of bovine PDC were reported earlier (Ho et al., J. Clin. Invest. 1986). The PDC primary antibody was obtained from our laboratory and the peroxidase conjugated goat anti-rabbit IgG antibody was purchased from Sigma. ECL (enhanced chemiluminesence kit) was purchased from Amersham (Arlington Heights, IL).

Experimental Diets: Two custom-made diets were purchased from ICN Biomedicals, Inc. (Aurora, OH). The high fat diet contained 45% corn starch, 25% casein, and 22% corn oil. The custom high sucrose fat free diet contained 2% corn
starch, 63.9% sucrose, 25% casein, 0.9% linoleic acid (65%), and 0.2% linoleic acid. All diets contained 3% alphacel non nutritive bulk, 0.3% DL methionine, 3.5% AIN 76 vitamin mixture, 1% AIN vitamin mixture, 0.2% choline chloride and 0.05% DL alpha tocopherol acetate (250U/gm). The rats were fed the diets ad libitum with continuous access to water.

II. METHODS

A. Screening of human genomic placental library

A human placental genomic library constructed in EMBL3 (Clonetech laboratories, Inc., Palo Alto, Ca) having a range of insert size from 8 to 21 kb was screened for the isolation of the E1β gene of PDC with a 500 bp labeled cDNA fragment containing the leader sequence and nucleotides coding up to 148 amino acids of the human E1β subunit (Ho and Patel, 1990). The library was plated at a density of 1x10^5 plaques per 150 mm plate and about 15x10^5 plaques were screened. Nylon (Nytran; Schleicher & Schuell) filter lifts were prepared from the plates; the filters were alternatively denatured and neutralized in 0.5 M NaOH/1.5 M NaCl and 0.5 M Tris-HCl, pH 8/1.5 M NaCl, respectively. The filters were rinsed in 2 X SSC (1X SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), air-dried and heated at 80° C in a vacuum oven for 30 min. The filters were prehybridized for 4 h in a solution containing 50% deionized formamide, 5 X SSC, 3 X Denhardt’s solution (1 X Denhardt’s solution =
0.02% Ficoll / 0.02% polyvinylpyrrolidone / 0.02% bovine serum albumin), 0.5% sodium dodecyl sulphate (SDS) and 200μg of denatured fragmented salmon sperm DNA per ml. The 500 bp EcoRI probe prepared by the random priming method using [α-32P]dCTP, was then added and hybridization was continued for 16-20 hr at 42°C. The filters were rinsed with 2XSSC/0.2% SDS, washed twice with 2XSSC/0.2% SDS at 65°C for 30 min, washed with 1XSSC/0.1% SDS at 65°C for 30 min and subjected to autoradiography. One positive plaque was obtained upon screening the library and the clone was plaque purified and a large scale preparation of the phage was made using a glycerol step gradient (Sambrook, Fritsch and Maniatis, 1989).

B. Southern blot analysis and DNA sequencing of the E1β genomic clone

DNA from the positive phage clone was digested with Sac I which released the 19 kb insert from the phage arms and EcoRI which cut at multiple sites within the insert. Southern blot analysis was carried out as described by Sambrook et al, (1989). Twenty μg of genomic DNA was digested with Sac I and EcoRI and the resulting fragments were resolved by agarose gel electrophoresis. Gels were denatured in a 1.5 M NaCl/0.5 N NaOH solution for 40 minutes and then 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 by capillary action. The filters were then baked for 2 hours at 80°C in a vacuum oven. The Southern filters were hybridized with the 0.5 kb E1β cDNA probe described above or
the 90 bp E1β fragment encoding the leader sequence. The filters were prehybridized in a solution containing 50% formamide, 7% SDS, 0.1% nonfat dried milk, 0.25 M Na₂HPO₄/NaH₂PO₄ (pH 7.2), 0.25 M NaCl, 1 mM EDTA and 250 mg denatured fragmented salmon sperm DNA per ml for 4 hr at 42°C, after which time the ³²P-labeled probe was added. The filters were hybridized for 16-20 hr at 42°C and washed with 0.1 X SSC/1% SDS at 55°C for 15-20 min. Positive fragments were subcloned into the plasmid vector pBluescript (KS; Stratagene). DNA sequencing was performed by the dideoxy chain-termination method (Sanger et al., 1977) using the bluescript forward and reverse primers, human E1β cDNA primers and a modified T7 DNA polymerase (Sequenase)(United States Biochemical Corporation; Solon, OH). The reaction products were separated on 8 M urea/6% acrylamide sequencing gels.

C. Molecular cloning of the human E1β promoter regulatory region

The insert from the positive phage was excised and digested with EcoRI. A 4 kb (EcoRI) DNA fragment containing the first exon and intron plus a part of the second exon (1.7 kb) together with the 2.3 kb promoter region was cloned into the EcoRI site of pUC19 (pUC4E1β). To study the promoter region, subclones were constructed by digesting pUC4E1β containing the 4 kb insert with Nae I/Sma I. A 2.3 kb DNA fragment containing the E1β promoter and the 5' non-coding region was cloned into the Sma I site of pUC19 (pMP6). To prepare deletion clones, pMP6 was
digested with EcoRI/BamHI releasing the entire insert and the insert was recloned into the EcoRI/BamHI sites of pBluescript (SK+; pMP14).

The 2348 bp DNA fragment (-2316 to +32) containing the promoter-regulatory region of the E1β gene (pMP6) and its deletion constructs were cloned at the EcoRV site in the BSKC4T vector. The BSKC4T vector (4850 bp) had the CAT gene and the SV40 polyadenylation signal subcloned at the SmaI site in the polylinker region of pBluescript (Kennedy et al., 1990). The pSV2CAT vector containing the SV40 early promoter/enhancer to drive CAT expression (Gorman et al., 1982) was used as a positive control in all experiments. The deletion clones of the E1β gene were also cloned into the thymidine kinase (TKC4AT) vector either in the forward or the reverse orientation by using convenient restriction sites. The TKC4AT vector had the TK promoter, CAT stuctural gene and the SV40 polyadenylation signal in pBR322 (Jacoby et al., 1989).

D. Construction of E1α-CAT fusion constructs

A 796 bp DNA fragment containing 763 bp of the proximal promoter and 33 bp of the 5'-untranslated region of the E1α gene was generated by PCR amplification using isolated genomic DNA from cultured normal human skin fibroblasts (Chang et al., 1993). This fragment was cloned into the EcoRV site of the BSKC4T vector containing the coding region of the E.coli CAT gene and the SV40 polyadenylation signal subcloned at the SmaI site of pBluescript (Kennedy et al., 1990). Four
subsequent deletions from the 5' end of the clone were generated from the above E1α-promoter-CAT construct (pαCAT1) using restriction sites at -506 bp (AccI; pαCAT2), -333 bp (PstI; pαCAT3), -221 bp (ApaI; pαCAT4) and -102 bp (StuI; pαCAT5). All the constructs were confirmed by restriction enzyme analysis and by sequence analysis using the Bluescript forward and reverse primers.

E. Preparation of plasmid DNA

Plasmid DNA was prepared according to Maniatis et al. (1982). Small scale preparation was performed using the alkaline lysis method from 5-10 ml overnight cultures of bacteria. All plasmids used in transfection studies or sequencing analysis were isolated by the Qiagen (Qiagen Inc. Chatsworth, CA) method and were judged to be free of both RNA and genomic DNA by ethidium bromide staining of nucleic acid after electrophoresis in 1% agarose.

F. Cell culture and transfection conditions

Human hepatoma cells (HepG2) were grown in modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM glutamine at 37°C under 5% CO2. Cells from a single plate were washed with sterile phosphate-buffered saline, trypsinized and cotransfected with 5 μg of the test plasmid and 3 μg of RSVβgal control plasmid containing the β-galactosidase gene driven by the Rous sarcoma virus promoter.
Transfection was carried out by the calcium phosphate coprecipitation procedure (Sambrook et al., 1989). Cells were harvested after 48 hr and lysed by freeze-thawing. Aliquots of the supernatant were assayed for the β-galactosidase activity (Sambrook et al., 1989) and protein content (Bradford, 1976). The remainder of the supernatent was heated at 65°C for 5 min to inactivate deacetylase, and the soluble lysate was stored at -20°C prior to determination of CAT activity.

G. CAT assays

Analysis of CAT activity was performed by the phase extraction method (Seed and Sheen, 1988) using [3H]chloramphenicol as substrate. The reaction conditions were chosen so that substrate in the presence of butyryl-CoA was converted into product within the linear range of the assay (0.01%-50%). After the incubation at 37°C, butyrylated chloramphenicol was extracted with xylenes, the xylenes were back extracted twice with water to decrease the background and radioactive [3H] was determined by scintillation spectroscopy. The CAT activity was normalized to the β-galactosidase activity and expressed as fold increase in activity over that of the full length promoter.

H. Dietary treatments

Male Sprague-Dawley rats (initial weight 80-100 g) were purchased from Zivic-Miller. In order to assess the responsiveness of the endogenous PDC components to
the various custom-made diets, rats were fed the high sucrose, high fat and standard laboratory chow for up to three weeks respectively. Rats were fed *ad libitum* with continuous access to water. Weight gain was recorded every 2 days. Final body weights varied between 200-250 g in all groups. Animals were decapitated with a guillotine and then free bleeding encouraged with cold running water. The liver, heart, adipose tissue and skeletal muscle were isolated from each animal and quickly frozen in liquid Nitrogen.

I. Western analysis

**Preparation of tissue extracts:** Approximately 200 mg of frozen liver tissue was homogenized in 2 ml Pilch's buffer (25 mM Hepes, 4 mM EDTA, 25 mM benzamidine, pH 7.4). The following inhibitors were added just prior to use namely, 1000 μM pepstatin, 1000 μM leupeptin, 100 μM aprotinin, and 2 mM phenylmethane sulfonyl fluoride. The samples were homogenized on ice and sonicated three times at 30 second pulses. After sonication, 40 μl 10% Triton was added to each sample and sonicated again for 30 seconds. Samples were kept on ice for 1.5 hr and then spun in a microcentrifuge for 15 minutes. Supernatents were collected and then frozen at -70°C.

**SDS polyacrylamide gel electrophoresis and immunoblotting:** Samples were denatured by boiling for 5 min in SDS-PAGE loading buffer. The samples were then subjected to SDS-PAGE (Laemelli, 1970) in 10% acrylamide, 2.7% bisacrylamide and
a Tris-glycine pH buffer system containing 0.1% SDS. Proteins were transferred to an immobilin membrane electrophoretically using a Bio-rad Western blot apparatus at room temperature at 100 mA for 1 hr. The transfer buffer contained 25 mM Tris, 192 mM glycine, pH 8.3. The filters were blocked with TTBS [0.9% NaCl, 0.01 M Tris pH 7.4 and 0.05% polyoxyethylenesorbitan monolaurate (Tween 20)] and 5% dry skim milk for 1 hr at room temperature or overnight at 4°C. Filters were then incubated with rabbit anti-PDC antibody (1:100 dilution) for 1 hr at room temperature. Filters were washed 3 times with TTBS and then incubated with a goat anti-rabbit IgG conjugated horseradish peroxidase (1:2000 dilution) for 1 hr at room temperature. Filters were washed 4 X TTBS. An ECL kit was used to detect the immunoreactive proteins. Densitometric scanning was performed on the HRP-conjugated proteins using SciScan 5000 (United States Biomedical Corporation, Solon, OH).

J. Assay for pyruvate dehydrogenase complex

Principle of the PDC assay:

\[ [1^{-14}C] \text{pyruvate} + \text{NAD}^+ + \text{CoASH} \rightarrow \text{Acetyl-CoA} + \text{NADH} + [^{14}C] \text{CO}_2 \]

PDC activity was measured as thiamine pyrophosphate and coenzyme A-dependent decarboxylation of [1^{-14}C]pyruvate, using a minor modification (Kerr et al., 1987) of
the method of Sheu et al., (1981). Radioactive CO$_2$ formed during the pyruvate decarboxylation was trapped in hyamine hydroxide, a strong base. The amount of trapped CO$_2$ is then determined by liquid scintillation spectrometry.

**Preparation of rat liver homogenates:** Twenty-five to thirty mg frozen tissue was homogenized in 20 volumes of KCL-MOPS buffer, pH 7.4 (80 mM KCl, 50 mM morpholinopropane sulphonyc acid(MOPS), 2 mM MgCl$_2$, 0.5 mM EDTA). An aliquot of 150 µl of the homogenate was used for the assay. PDC assay on the treated frozen liver homogenate was performed within 24 h. The rest of the homogenate was quick frozen at -70°C and stored at this temperature until needed.

Total PDC activity was assayed as follows: 150 µl liver homogenate (25-30 mg) was preincubated for 15 min at 37°C in the presence of 5 mM dichloroacetate and 0.7 U/ml of purified phospho-E1 phosphatase, (kindly provided by Dr. Thomas Roche, Kansas State University, Manhattan, KS) freeze-thawed twice and incubated in 0.1 ml final volume of the assay mixture containing potassium phosphate, 50 mM, pH 8.0; MgCl$_2$, 2 mM; thiamine pyrophosphate, 0.1 mM; NAD$^+$, 2.5 mM; dithiothreitol, 1 mM; coenzyme A, 0.5 mM; phosphotransacetylase, 1 µg/ml, fetal calf serum, 2.5%. Exactly 1-1/2 minutes after adding the 10 and 20 µl of cells, the reaction was started by addition of [1-14C]pyruvate to a concentration of 50 nm. The incubation tube was sealed with a serum stopper from which a plastic cup was suspended, containing filter paper impregnated with hyamine hydroxide. Incubations were carried out at 37°C for 5 and 10 min respectively. The assay was terminated with 20 ul of 20%
trichloroacetate (TCA). The samples were incubated for an additional 45 minutes after adding the TCA to allow time to collect the evolved CO₂ in the hyamine. After trapping the evolved ¹⁴CO₂, the entire cup and the hyamine saturated filter paper was transferred to a vial containing liquid scintillation for counting. Thiamine pyrophosphate and coenzyme A were omitted from the blank tubes. Each assay included quadruplicate reaction tubes and blanks.

Calculations:

\[
\text{cpm in sample} - \text{cpm (blank mean)}
\]

\[
\frac{\text{assay time (minutes) x Sp. Act. of pyr-C¹⁴ (cpm/nm) x mg protein in sample}}{= \text{nmoles CO}_2 / \text{mg protein} / \text{minute}}
\]

K. Citrate synthase assay

Citrate synthase activity depends on oxaloacetate dependent formation of the product, free coenzyme-A, measured by the reduction of dithionitrobenzoic (DTNB) which reacts with thiols, such as coenzyme-A to form a yellow color with maximum absorbance at 412 nm (Srere, 1969). Frozen liver homogenates prepared for PDC assays were thawed at 37°C and diluted 1:4 with the KCl-MOPS buffer. 10 and 20 µl of the diluted extracts was assayed for citrate synthase. The reaction mixture for the citrate synthase assay contained 100 nM Tris-HCl, pH 7.8, 10 nM dithionitrobenzoic acid, 0.1 mM acetyl-CoA, 0.5 mM oxaloacetate and 0.05 % Triton X-100 in a total volume of 1 ml. The reaction was started by the addition of oxaloacetate. Blank
reactions contained no added oxaloacetate. The reaction was followed for 5 minutes on a recording spectrophotometer at 412 nm, at 37°C.

Calculations:

\[
\text{nm/mg protein/minute} = \frac{\text{change in OD}}{0.0136 \times \text{mg protein} \times \text{time}}
\]

L. Protein determination by the Lowry method

Protein concentration was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard. To each of 0.1 ml sample was added 3 ml of solution containing 2% Na₂CO₃ in 0.1 N NaOH, 0.01% CuSO₄ and 0.02% Na-K tartarate. The solution was incubated at room temperature for 10 min. After incubation, 0.1 ml Folin and Ciocalteu’s phenol reagent from Sigma (St. Louis, MO) was added and incubated at room temperature for 30 min. The optical density was read at 750 nm on a Gilford spectrophotometer.

M. RNA isolation

Total RNA was isolated from rat tissue by a modified acid-phenol guanidinium thiocyanate procedure. Tissue samples were homogenized in 4 M guanidinium thiocyanate β-mercaptoethanol solution for 30 sec to 1 min. Four hundred μl of 2 M sodium acetate pH 4.0 was added to the sample, mixed and followed by 4 ml water saturated phenol and 800 μl chloroform. The samples were vortexed for 15 seconds
and placed on ice for 15 min. Samples were centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was decanted and the RNA pellet was resuspended in 500 μl of 4M GTC-β-mercaptoethanol solution and precipitated with 1 ml of ethanol at -20°C for 1 hr. Samples were then centrifuged at top speed in a microfuge (12,000 rpm) for 15 min at 4°C. The supernatant was then decanted and the pellet washed 3 times with 80% ethanol. Diethylpyrocarbonate-treated water (0.1%) was prepared by adding 500 μl of diethylpyrocarbonate solution to 500 ml water. The pellet was dried and resuspended in diethylpyrocarbonate water.

N. Northern blot analysis

Northern blot analysis was carried out as described by Davis et al, (1986). Forty μg of total RNA was fractionated in a 1% agarose gel/1XMOPS/5% formaldehyde in 1X MOPS 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 membranes were UV-cross-linked for 3 min and then baked in a vacuum oven for 2 hr at 80°C, prehybridized and hybridized as described previously except, that the hybridization was carried out in Church’s buffer (1% BSA, 1mM EDTA, 0.5 M NaPO₄, 7% SDS) at 65°C. Filters were washed at room temperature with 2XSSC followed by washes at 55°C for 20 min with 2XSSC/0.2% SDS. The filters were finally washed with 1XSSC/0.1% SDS at 55°C for 15 min. Northern blot analysis was used to determine the level of the PDC components mRNAs (E1α, E1β, E2 and E3)
by normalizing to actin mRNA values. The relative radioactivity in the individual band was determined using a PhosphorImager. The levels of PDC mRNAs were expressed relative to actin in order to control for differences in loading efficiency. Statistical analysis was performed on all samples using the StatView program.

O. Labeled probe

The cDNA fragments for human E1α (1.4 kb), E1β (1.1 kb), E3 (1.7 kb), rat PEPCK (1.1 kb), mouse actin (1.8 kb) and glucokinase (2.3 kb) were purified from the plasmid vector sequence by restriction enzyme digestion followed by electroelution of the fragments. The purified cDNAs were denatured by heating in boiling water for 10 min and then rapidly cooled on ice. Labeled probes were prepared from denatured template using DNA polymerase I (Klenow fragment) and (α-32P)-dCTP in a random priming reaction. All the reagents were provided by the Random Primed DNA Labeling Kit from Boehringer Mannheim Biochemicals (Indianapolis, IN). At the end of the reaction, labeled oligonucleotides were purified from unincorporated nucleotides by fine Sephadex G50 spin-column chromatography. Before using, the labeled probe was denatured by heating in boiling water bath for 10 min followed by rapidly cooling on ice.
I. Overview

The E1 component of PDC is composed of two nonidentical α and β subunits that form a heterotetramer (α2β2) to catalyze the decarboxylation and dehydrogenation reactions. The cloning and cDNA sequences of the α and β subunits of human PDC have been reported (Ho et al., 1989; Ho and Patel, 1990). The DNA sequence of the human E1α gene has also been reported (Maragos et al., 1989). At the time this project was initiated, the human E1β gene had not been cloned. Since E1 is the major site of PDC regulation, it is of interest to investigate the gene expression and possible coordinate expression of the α and β subunits of the E1 component. The emphasis of this project was to isolate the promoter-regulatory region of the E1β gene and study its expression in cell lines.

II. Molecular cloning of the human E1β gene

A 500 base pair (bp) labeled cDNA fragment containing the leader sequence and the nucleotides coding up to 148 amino acids of the human E1β subunit (Ho and Patel, 1990) was used to screen a human placental DNA genomic library. One positive plaque was isolated from a total of $1.5 \times 10^6$ plaque-forming units of recombinant phage. The human genomic DNA insert released from the phage arms by XhoI digestion was about 19 kb in length. The insert was partially mapped with restriction enzymes and subjected to
the human E1β cDNA. A photograph of a gel containing DNA from this E1β clone, cut separately with SacI and EcoRI is shown in figure 9A. When this clone was digested with SacI and used for Southern analysis, two smaller fragments of 1.2 and 3.2 kb hybridized to the 500 bp E1β cDNA probe derived from the coding region for the leader sequence and a 4 kb EcoRI fragment showed strong hybridization as well (Figure 9B), suggesting that these three fragments harbor sequences for the E1β exonic regions. These fragments were further subcloned and the presence of exons and introns in the clone was confirmed by dideoxy sequencing using oligonucleotide primers (18-29 mers) that hybridize to the DNA in the exon regions. The sequence and the partial map of the clone was identical to the published sequence (Koike et al., 1990). The human E1β gene is localized on chromosome 3 and is approximately 8 kb long and contains 10 exons ranging from 36 to 550 bp (Koike et al., 1990).

The phage insert contained four EcoRI fragments, and two of which used for cloning the 4 kb fragment are shown in Figure 10. This 4 kb fragment harbored 2.5 kb of the 5′-flanking region, the first exon, the first intron, the second exon and a part of the second intron. The transcriptional start site of the E1β gene of human PDC was confirmed by S1 nuclease mapping by Dr. Madhusudhan which corresponded to the same adenine residue as +1 site reported by Koike et al, (1990). The partial nucleotide sequence was determined by Dr. Madhusudhan for 1.2 kb of the 5′ flanking region of the E1β gene. When the nucleotide sequence was compared to the published sequence of the E1β gene by Koike et al (1990), 18 differences were observed which may be attributed to the differences in methodologies and reagents (that would resolve GC compressions) rather than to
Figure 2: Southern blot analysis of E1β phage DNA. Purified phage DNA was digested with SacI and EcoRI and DNA fragments were resolved on an agarose gel (A) and then transferred onto a Genescreen membrane. The membrane was hybridized with a $^{32}$P-labeled 500 bp E1β cDNA probe (B). Lane1: Molecular weight markers. Lane2: Undigested phage DNA. Lane 3 and 4: Phage DNA digested with SacI and EcoRI.
Physical map and subcloning of the promoter-regulatory region of the E1β gene of human PDC. The 19-kb insert in λEMBL3 was digested with EcoRI, and the 4-kb insert containing the promoter-regulatory region, exon 1, intron 1 and exon 2 was subcloned into puc19. Appropriate restriction sites of the multiple cloning sites (MCS) of the vector and insert are shown. The insert is shown with respect to the direction of lacZ transcription.
MCS

MCS ⊨
polymorphism. Six direct repeats and seven inverted repeats were identified, the longest direct repeat being 11 bp in the 5'-untranslated region (Figure 11). The G+C contents of the promoter region and the cDNA sequence was approximately 50 and 47% respectively.

The nucleotide sequence of the promoter region is important in terms of identifying the putative protein binding sites that may be involved in the regulation of the E1β gene. Both strands of the nucleotide sequence of the E1β promoter region was analyzed for consensus sequences using the VAX/VMS computer program tfsites, version 7.30. The E1β promoter lacks a TATA box but shows a CAAT box at -81 to -78 bp and multiple potential Sp1 sites around the transcription start site. The absence of a TATA box and the presence of multiple GC boxes have been reported for promoters of several genes that encode enzymes involved in vital metabolic reactions (Valerio et al., 1985; Melton et al., 1984).

II. Functional analysis of the E1β promoter

The protein-binding regions of the E1β 5'-flanking region from +32 to -1245 bp was mapped by DNaseI footprinting using crude rat liver nuclear extracts by Dr. Madhusudhan (Madhusudhan et al., 1995). The entire promoter-regulatory region showed seven protein-binding domains termed P1-P7 (Figure 12). The CAAT box and the Sp1 site was protected in P1. The E1β promoter showed multiple GC boxes between +1 and -200 nucleotides. It has been observed that the GC-rich regions of many promoters contain unusual numbers of CG dinucleotides, relative to the bulk of the genome that can be
Nucleotide sequences of the promoter-regulatory region of the E1b gene of human PDC. The transcriptional start site (+1) of the mRNA is shown by a star. The nucleotide numbering is relative to the transcriptional start site. The differences between our DNA sequence and the published sequence (Koike et al., 1990) are also identified. The addition and deletion of bases are indicated as up and down arrows, respectively, compared to the published sequence. Base substitutions are shown by lowercase letters, the top nucleotide being the base reported in the previous report (Koike et al., 1990). Direct and inverted repeats in the DNA sequence are shown by double and single underlines, respectively. (Madhusudhan et al., 1995).
Figure 12: DNase I footprinting analysis of the promoter-regulatory region of the E1β gene. DNase I footprinting was performed using 25-90 ug of rat liver nuclear extract (indicated by a plus sign). The DNA fragments 200-700 bp long were labeled at the 3' end. The footprint patterns of coding and noncoding strands are shown separately. The protected regions are outlined and denoted as P1-P7. The nucleotides protected by DNase I are shown in the margin for each region and the nucleotide number is relative to the transcriptional start site (Madhusudhan et al., 1995).
undermethylated. It is possible that a transcription factor such as Sp1 which hinders methylation may play a role in at least some instances (Dyanan, 1986).

E1α and E1β are present as a heterotetramer in the complex in a 1:1 molar ratio. This raises the possibility that these two genes may be coordinately expressed. Comparing the DNase footprinting patterns of the 5'-flanking region of the E1β gene to that of the E1α gene (Chang et al., 1993) revealed several differences. The promoter region of the E1α gene contains consensus sequences to TATA box, AP-1, AP-2, CAAT and CACCC within 200 bp of the transcriptional start site. However, comparison of the two promoter regions indicated no obvious sites for similar transcriptional regulation.

To define the regulatory elements required for the E1β gene expression, nested deletions of the E1β 5'-flanking region (-2316 to +32 bp) were created by exonuclease III and S1 nuclease, and inserted into the BSKCAT vector. The constructs were transfected into human hepatoma cells (HepG2) and the transient expression of CAT activity was measured after normalization for transfection efficiency with β-galactosidase. The full-length E1β constructs (pβCAT1 and pβCAT1-R)(-2316 to +32) resulted in a very low level of CAT activity in either orientation (Figure 13). However, upon deletion of the 5'-sequences from -2316 to -929, the CAT activity of pβCAT2 (-929 to +32) increased approximately 7-fold compared to pβCAT1. This suggested the presence of a putative repressor element within this region of the E1β promoter. Several protein binding domains (P5-P7) are deleted in the pβCAT2 construct. There were no marked changes in the CAT activity of pβCAT3 (-564 to +32) and pβCAT4 (-397 to +32) as compared to pβCAT2. However, when the deletion was extended to -283 bp (pβCAT5), CAT activity increased
Scheme 4. Schematic representation of the protein-binding domains and transient expression of CAT activity by the human E1β promoter in HepG2 cells. (A) The protected regions identified by DNaseI footprint analysis are outlined by boxes and denoted as P1 to P7. The location of the different cis-elements are also shown. (B) The full-length E1β promoter (-2316 to +32 bp) and the deletion constructs were cloned into the BSKCAT vector and transfected into human hepatoma cells (HepG2). The cells were cotransfected with RSVβgal and the β-galactosidase activity of the cell extracts was used to correct for the transfection efficiency. The CAT activity (mean ± S.E.; n=6) shown for each construct is the fold change compared to the activity of pβCAT1. Percentage conversion represents the relative CAT activity as a fraction of the total $[^3H]$-chloramphenicol added to the assay.
apparently required for repression. The pβCAT6 construct lacked the protein-binding domains from P2-P7. Further deletion to -98 bp (pβCAT6) resulted in nearly a 20-fold increase in CAT activity compared to pβCAT1. This maximum activity was achieved from a minimal promoter containing a potential Sp1 site (-102 bp), a CAAT box (-78 bp) and a P1 footprinted region. Two additional potential Sp1 sites (+8 and +16 bp) are present in the 5'-untranslated region of the E1β gene which may contribute to the high activity of pβCAT6. These results strongly suggest the presence of one or more negative regulatory elements in the regions from -2316 to -929 and from -397 to -98 bp of the E1β gene.

To further localize the repressor elements in the E1β promoter, DNA fragments containing the repressor were cloned in front of the thymidine kinase (TK) promoter to determine whether this may affect the expression of a heterologous promoter. The DNA fragments were cloned in both orientations in the TKCAT vector at the XbaI site in the polylinker region. Constructs pβTKCAT1 and pβTKCAT2 containing the sequence from -2316 to -929 bp in both orientations, repressed CAT activity from pTKCAT by 50% (Figure 14). The E1β promoter region from -397 to -98 bp was also cloned in both orientations, yielding four constructs (pβTKCAT3 to pβTKCAT6). Surprisingly, the CAT expression of pβTKCAT3 and pβTKCAT5 was approximately 50% higher than pTKCAT. pβTKCAT4 and pβTKCAT6 were mildly inhibitory, showing 40% and 74% of pTKCAT activity, respectively. This suggests the presence of cis-elements which act in an orientation-dependent manner on a heterologous promoter. Further deletion analysis
Effect of the human E1β promoter fragments on the transcriptional activity of the thymidine kinase (TK) promoter. The DNA fragments of the human E1β promoter were ligated in both orientations into the XbaI site at the 5' end of the TK promoter in the TKCAT plasmid (pTKCAT). These constructs were assayed for CAT expression in HepG2 cells and CAT activity was expressed as a percentage of the activity of pTKCAT.
and mutagenesis of the E1β promoter should define more precisely the repressor regions and the factors involved in regulation of the E1β gene.

Initiation of transcription from all class II promoters is believed to be mediated by the assembly of a common set of general factors on the core promoter. For promoters that lack a TATA box, an activator protein such as Sp1 may direct site-specific initiation by tethering TFIID to the DNA and by stabilizing the preinitiation complex via protein-protein interactions. In the carbamoyl-phosphate synthase (glutamine-hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase gene promoter, the Sp1 site at -49 substitutes for the missing TATA box and plays a major role in start site selection (Kollmar et al., 1994).

In other instances, a factor called an initiator (Inr) has been found to mediate the same functions as TATA elements (Smale and Baltimore, 1989). An initiator may play an important role in localizing the start site of transcription by mediating recognition of the RNA polymerase II. Inr elements can also cooperate with a TATA box if both elements are found in the same core promoter. The protein that recognizes the Inr consensus sequence has not yet been reported. Almost every Inr that has been described functions with an upstream Sp1 site(s).

The E1β promoter lacks a TATA box but contains two initiator consensus sequences (TCAGCCCG/TCACCCGG) at -24 and -2 bp respectively of the core promoter. These sequences are nearly 87% homologous to the consensus initiator sequence (KCABHYBY; K = G or T; B = C, G or T; H = A, C or T; Y = C or T; Buchner, 1990). The E1β promoter also contains two Sp1 sites (+8 and +16) which may allow for efficient CAT
initiator sequences and Sp1 sites) should shed light on the minimal sequence requirement for sufficient and accurate initiation of transcription of the human E1β gene.

IV. Summary

A genomic clone (19 kb) harboring the intron-exon sequences and the promoter-regulatory region of the E1β gene of human PDC was isolated by screening a placental genomic library. The E1β promoter lacked a TATA box homology but contained initiator sequences and Sp1 sites which are frequently found in TATA-less promoters. Transient expression of CAT activity in HepG2 cells, suggested the possible presence of negative cis-elements located within the sequence from -2316 to -930, whereas deletion constructs containing -929 to +32 and -98 to +32 DNA sequences showed approximately 7- and 20-fold increases in CAT activity over the basal CAT activity. The presence of repressor elements was further confirmed by additional studies measuring the effect of the E1β DNA fragments from -2316 to -930 on CAT activity driven by a heterologous thymidine kinase (TK) promoter. Hence the E1β promoter exhibits low, basal level of expression due to the presence of repressor elements and is made up of a unique combination of initiator sequences and Sp1 sites within 100 bp upstream of the transcription start site.
I. Overview

The E1α subunit of PDC exists as two isozymes encoded by two different genes. One isoform, Pdha1, is located on chromosome X (Xp. 22.1-p22.2) in humans (Brown et al., 1989) and is expressed in somatic tissues. The second variant, Pdha2, maps to an autosome (chromosome 4 in humans and chromosome 19 in mice) and is testis-specific (Dahl et al., 1990). In addition, Pdha2 is intronless suggesting that it may be a functional processed retroposon. In contrast, a single Pdha1 homologous gene was isolated in marsupials. The gene is autosomal and maps to a region that contains other human Xp genes (Fitzgerald et al., 1993). This suggests that the translocation of Pdha1 to the X chromosome in eutherian mammals, which is inactivated during spermatogenesis, led to the evolution of a second testis-specific, intronless Pdha variant by retroposition to an autosome after the marsupial/eutherian divergence. The X-linked gene for the E1α subunit is approximately 17 kb long, containing 11 exons ranging from 61 to 174 base pairs and introns ranging from 600 base pairs to 5.7 kilobase pairs (Maragos et al., 1989; Koike et al., 1990).

The activity of mammalian E1α is regulated posttranslationally by the phosphorylation/dephosphorylation of the E1α peptide (Reed, 1974). The level of E1α mRNA is regulated during differentiation of 3T3-L1 preadipocytes to adipocytes (Huh et al., 1993) and by the glucose concentration in cultured pancreatic islets. The genomic
structure and the 5'-flanking sequence of the human E1α gene have been reported (Maragos et al., 1989) (Fig. 15). This information provided the basis for further investigation of the mechanisms underlying regulated expression of this gene.

II. Characterization of the E1α promoter

To understand how specific protein-DNA interactions in the promoter region may regulate human E1α expression and thus identify the cis- and trans-acting elements, the isolation and characterization of the E1α promoter region was undertaken. Using the polymerase chain reaction (PCR), Dr. Mei Chang amplified a 796 base pair DNA fragment (-763 to +33) of the E1α promoter from genomic DNA isolated from cultured normal skin fibroblasts (Chang et al., 1993). The sequence was confirmed from at least two independent PCR amplifications and found identical to the published sequence of the E1α gene (Maragos et al., 1989).

The G+C content of the 763 bp 5'-flanking region is high, with an average of 57%, compared to 40% for the entire human genome. The G+C content was even higher (71%) for the 300 bp proximal to the putative transcription start site. Several consensus sequences were identified in the 5'-flanking region of the E1α gene. They include the following: one activator protein 1(AP-1)(-610 to -603), one cAMP-responsive element (CRE), two activator protein 2 (AP-2) (-106 to -99 and -172 to -166), one CCAAT/enhancer-binding protein (C/EBP) (-527 to -519); one insulin responsive sequence (IRS) (-646 to -637) (O’Brien et al., 1990) (Figure 15).
Figure 13: Nucleotide sequence of the promoter region of the gene for the human E1α subunit. The promoter region of the gene is numbered from -767 to -1. +1 to +13 denotes part of the first exon. The boxed areas denote the following elements: TATA box, CAAT box, Sp1 binding sites, glucocorticoid responsive element (GRE) and AP-2 sites, respectively. The brackets with the intervening broken lines marked IR-1 to -6 denote inverted repeats. Paired arrows DR-1 and DR-2 denote direct repeats (Maragos et al., 1989).
DNase I footprinting analysis of the E1α 5'-flanking region performed by Dr. Chang, using rat liver nuclear extracts identified seven major protein-binding domains, termed P1 through P7 between -763 to +33 bp of the E1α promoter region (Chang et al., 1993) (Fig. 16). These protein-binding domains overlap several consensus sequences such as the TATA box, CCAAT box, Sp1 and CRE all of which have been implicated in basal transcription of several genes (Figure 17). To further identify the proteins which may bind to the E1α promoter, binding studies using well characterized and purified transcription factors or footprint competition studies were conducted by Dr. Chang. It was observed that purified Fos and Jun bound to the CRE site within the P1 region (these proteins were the gifts of Dr. Tom Curran, Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology), AP-2 (Promega) bound to its proximal site within P2 and P3 elements and a distal site within P4, C/EBPα protein (this was a gift from Dr. Steven McKnight, Department of Embryology, Carnegie Institute of Washington), bound to its consensus site within P6 and Sp1 bound to its site within P1 and P4. Oligonucleotide competition studies indicated that CTF/NF-1 and Sp1 displaced the nuclear proteins bound to the CAAT box within P3 and an Sp1 site within P4, respectively.

To measure promoter activity, the -769 bp of the E1α gene was ligated to the reporter gene CAT and transiently transfected into HepG2 cells. The 796 bp fragment of the E1α promoter was subcloned at the EcoRV site of the BSKCAT vector containing the coding region of the E.coli CAT gene and the SV40 polyadenylation signal subcloned at the Smal site of pBluescript (Kennedy et al., 1990). Four subsequent deletions were generated from the above E1α promoter-CAT construct (pαCAT1) using convenient restriction sites at
Figure 4B. DNase I footprint analysis of the E1α promoter. DNase I footprinting was performed using 50 μg of rat liver nuclear extract. The E1α promoter fragments were end-labeled using T4 polynucleotide kinase and [γ-32P]ATP or Klenow and [α-32P]dNTP, respectively, in the footprinting analysis. (A) Noncoding strand; (B) coding strand. The protected regions are outlined by boxes and denoted as elements P1-P7 (Chang et al., 1993).
-306 bp (Ace1; pøCAT12), -333 bp (Pst1; pøCAT13), -221 bp (Apa1; pøCAT14), and -102 bp (Stu1; pøCAT5). All of the above constructs were sequenced to confirm their identity and sizes.

The E1α promoter-CAT constructs (pøCAT1-pøCAT5) were transiently transfected into human hepatoma cells (HepG2) using the calcium phosphate precipitation procedure (Sambrook et al., 1989). Each transfection contained 5 µg of the test vector and 3 µg of RSV-βgal which contains the β-galactosidase gene driven by the Rous Sarcoma Virus promoter to determine the transfection efficiency. The pSV2CAT vector containing the SV40 promoter/enhancer to drive the CAT expression was used as a positive control in all the assays. As a negative control cells were transfected with the BSKCAT vector alone. CAT assays were performed by the phase extraction method (Seed and Sheen, 1988).

Each of the deletion constructs successively deleted the protein binding domains, P3 to P7 (Figure 17). The full-length E1α promoter (pøCAT1), resulted in very high CAT activity, nearly as high as the level observed in cells transfected with the pSV2CAT vector. When the sequence between -763 to -333 bp was successively deleted, there was a gradual decrease in CAT activity (pøCAT1 vs pøCAT2, 18% reduction; pøCAT2 vs pøCAT3, 38% reduction). The region deleted in pøCAT2 (-763 to -506), contained the P7 and P6 domains and a putative “enhancer” reported previously in three mitochondrial energy-transducing genes including the E1α gene (Tomura et al., 1990). Interestingly, no protein binding was detected over this region by DNaseI footprinting with liver nuclear proteins. This element (TGGTGTTTGTCTCT: -646 to -637) also shares 100% identity to an insulin response element (IRE) present in the promoter regulatory regions of several
Figure 17. Schematic representation of the cis-elements and protein-binding domains of the E1α promoter and transient expression of CAT activity in HepG2 cells. (A) The DNaseI protected regions are outlined by boxes and denoted as elements P1-P7. The location of the different cis-elements are also shown. These include: TATA box, CAAT box, Sp1 sites, AP-1, AP-2, glucocorticoid response element (GRE), cAMP responsive element (CRE), insulin-response sequence (IRS), fat-specific elements 1 and 2 (FSE-1 and FSE-2) and CCAAT/enhancer-binding protein (C/EBP). (B) The diagram depicts the human E1α promoter (from bp -763 to +33 bp) ligated to the CAT structural gene (pαCAT1). Deletions were constructed from pαCAT1 using restriction sites at -506 bp (AccI; pαCAT2), -333 bp (PstI; pαCAT3), -221 bp (ApaI; pαCAT4) and -102 bp (StuI; pαCAT5). The BSKCAT vector was devoid of the E1α promoter sequence. HepG2 cells were cotransfected with 5 μg of the E1αCAT constructs or pSV2CAT (positive control) and 3 μg RSVβgal. The results shown are corrected for transfection efficiency using the β-galactosidase activity and expressed as fold change compared to pαCAT1. Percentage conversion represents the relative CAT activity as a fraction of the total [3H] chloramphenicol present in the assay. The results are the means ± SE of eight independent transfections.
genes including phosphocholeolpyruvate carboxylase (O'Brien et al., 1990). The -506 to -333 bp region deleted in pCAT3 contained the F5 domain.

When the deletion was extended to -221 bp, there was a 53% increase in expression of pCAT4, implying the presence of a negative regulatory element within this region (Figure 17). However, no protection was observed in this region from DNase I footprinting with liver nuclear proteins. Progressive deletion to -102 bp (pCAT5) resulted in a decrease in CAT activity (pCAT4 vs pCAT5, 47% reduction, p < 0.05), suggesting the presence of a possible enhancer in the F3 and F4 domains which are deleted from pCAT5. In the -221/-102 region, the CAAT box is part of P3 and Sp1 is part of P4. Both of these elements have been implicated in playing a critical role in directing efficient transcription in a variety of eukaryotic and viral promoters (Dyman and Tjian, 1983). Moreover, the two sites were effectively competed by oligonucleotide competition studies.

The plasmid pCAT5, which included only 102 bp of the promoter region and 33 bp of exon 1 of the Elα gene was still capable of efficiently promoting CAT expression. This region of the promoter contained TATA box, CRE, Sp1 and AP-2 sites and was almost completely protected by liver nuclear proteins as observed from DNase I footprint analysis.

In order to determine whether the transcription factors that were shown to bind to the Elα promoter region by DNase I analysis could transactivate the Elα promoter, cotransfection studies were performed in HepG2 cells. The constructs CMV-Fos and CMV-Jun which expressed the Fos and Jun proteins from the cytomegalovirus promoter, transfected either alone or in combination showed no marked changes in CAT activity as
compared to pαCAT1 (-763 to +33 bp) transfected alone (Figure 18). Also, the expression vectors for C/EBP(α) and C/EBP(β)[LAP] did not transactivate the E1α promoter with the same construct. The mutant forms of the above transcription factors, namely, Fos BR, Jun BR and 12V C/EBP which carry mutations in their DNA binding domains, had no effect on the expression of the CAT gene driven by the -796 bp E1α promoter. The E1α promoter showed very high CAT expression in HepG2 cells, comparable to that of the SV40 promoter. Hep-G2 cells contain most of the transcription factors present in normal liver albeit not at the same concentration. McKnight and his colleagues have shown that the C/EBP concentration in HepG2 cells is only 5% that of normal liver (Landshulz et al., 1988). However the limiting amounts of these transcription factors present in HepG2 cells may be sufficient to transactivate the E1α promoter and allow for such high expression of CAT. The other possibility may be that these factors may not be involved in basal expression of the gene and the redundancy of transcription factors that bind to similar cis-elements may allow for more than one such protein to bind to any given consensus sequence.

III. Summary

Universal expression of PDC suggests constitutive expression of all its gene products. The E1α 5’-flanking region bears structural similarities to both facultative and housekeeping gene promoters. Similar to the former group that includes genes that are tissue- or temporal-specific in their expression, the E1α gene contains an appropriately positioned “TATA” box, “CAAT” box and a number of upstream putative control
Figure 13. Cotransfection of Fos, Jun and C/EBP with the E1α promoter in HepG2 cells. Transient transfections with 5 μg of pαCAT1 alone, or with 5 μg each of c-fos, c-jun, c-fos and c-jun, C/EBP and 12V C/EBP, a mutant form of the C/EBP protein (mutation in the DNA binding domain) was measured in HepG2 cells. The results shown are expressed as fold increase of CAT activity compared to pαCAT1 transfected alone. The results are the means ± SE of six independent transfections.
Additions

- None
- c-Fos + c-Jun
- c-Fos
- c-Jun
- C/EBP(α)
- C/EBP(β)[LAP]
- 12V C/EBP

Relative CAT activity
(fold change)
(hormonal) cis-elements. The 796 bp E1α promoter exhibited strong promoter activity in HepG2 cells. Crucial elements(s) for promoter activity and complex DNA-protein interactions are confined within a region spanning -221/+33 which retained more than 75% of the promoter activity of the 796 bp sequence. The protein-binding domains of the E1α promoter contain several known consensus sequences such as TATA box, CAAT box, Sp1 and CRE, all of which have been implicated in the constitutive expression of several genes.
L. Overview

PDC activity responds to changes in nutritional and hormonal factors such as fasting, re-feeding and experimental diabetes. Under most conditions the proportion of the active (dephosphorylated) form of the enzyme varies without significant changes in total activity (Weiland, 1983). Estimates of the relative PDC activity state in vivo using measurements of enzymatic activity in extracts of frozen or freeze-clamped tissue both before and after incubation with purified phosphatase have been made. In this method, the first assay measures the amount of “active” enzyme (PDCa), whereas the second assay measures the amount of “total” enzyme (PDCb) or the amount expressed only after complete activation by dephosphorylation. The active/total ratio (percentage active form) reflects the percent activity in vivo. As such, the PDCa/PDCb ratio is postulated to play a major role in the ability of various tissues to switch from carbohydrate to lipid utilization and vice versa according to the shifts in fuel availability which takes place during a number of metabolic and nutritional conditions such as, fasting or diabetes.

It has been demonstrated that the liver PDC is inducible by feeding a high carbohydrate diet. High carbohydrate intake usually leads to hypertriglyceridemia in man. Triglyceridemia is influenced by the type of diet, with sucrose or fructose having
a larger effect than starch or glucose (Carmona and Freedland, 1989). The differential effect of fructose has been attributed among other factors to increased triacylglycerol synthesis and secretion. Dietary fructose has been shown to be preferentially used, as compared to glucose, as a carbon source for hepatic lipogenesis. In hepatocytes from rats fed a 60% sucrose or fructose diet, the rate of lipogenesis was two times higher than in cells from rats fed a 60% glucose diet and three times higher than in cells from rats fed a commercial diet (Carmona and Freedland, 1989). This effect is due to the induction of several key enzymes involved in fructose metabolism.

PDC activation is important for the increased rates of hepatic lipogenesis promoted by high-sucrose or fructose diets. In isolated hepatocytes, low concentrations of fructose elicit an acute increase in the concentration of fructose-2-6 bisphosphate (an important activator of 6-phosphofructose-1-kinase). As well as providing carbon for net pyruvate production, fructose promotes flux from glucose toward pyruvate. In vivo, hepatic E1 kinase activity is downregulated by long-term administration of a diet high in fructose (Park et al., 1992). The suppression of E1 kinase activity is associated with increased hepatic “active” E1 activity (Park et al., 1992). The percent active form of hepatic PDC in rats fed 70% fructose for 3-5 weeks was 144% of the value in glucose-fed rats (15.4 ± 1.2% versus 10.7 ± 0.5%; p < 0.002), whereas cardiac muscle PDC activity was not different (45.5 ± 6.6% versus 41.0 ± 7.8%) (Park et al., 1992). Thus, activation of hepatic PDC can be achieved via stable and long-term down-regulation of E1 kinase as well as by acute activation of phospho-E1 phosphatase.
Soling and Bernhard have reported that intravenous injection of fructose leads within a few minutes to a significant increase in the activity of PDC in rat liver without affecting the total activity of the complex (Soling and Bernhard, 1971). Limited information is available regarding the effects of high-carbohydrate and high-fat diets on the long-term regulation of the enzyme. Feeding a high sucrose diet (63%) for 3 weeks resulted in a significant increase in total activity when compared to values obtained in animals fed on the standard chow (Chicco et al., 1986). Begum et al. (1982) reported increases in PDCa and total PDC activity in adipose tissue and liver of rats fed with 67% glucose for 5 days. Hepatic total PDC was approximately 50% higher in rats fed a high-sucrose diet for 15 days (9.8 ± 1.0 mU/mg protein [sucrose] versus 5.5 ± 0.5 mU/mg protein[chow]) (DaSilva et al., 1992). In contrast, feeding a diet high in fat (22% corn oil) for 15 days resulted in about 40% reduction in total PDC activity in liver (3.8 ± 0.2 mU/mg protein [fat] versus 5.5 ± 0.5 mU/mg protein [chow]) (DaSilva et al., 1993). Dietary saturated fat (butter) had no inhibitory effect on the sucrose-induced increase in total hepatic PDC activity whereas feeding of polyunsaturated fats (corn oil or fish oil) resulted in marked reductions in total PDC activity (Da Silva et al., 1993) and these changes in total PDC activity were closely correlated with the changes in the content of the component proteins of PDC. No effects on total and active PDC was observed in heart and skeletal muscle (DaSilva et al., 1992).
As shown in Table III, liver PDCa and total activity obtained after 7 days of high-sucrose, fat free diet were significantly higher than those from the chow-fed rats, but lower than in animals maintained on the fat-free diet for 15 days, without appreciable changes on percentage of active complex (Da Silva et al., 1993). Thus the increase in PDCa observed after 15 days of feeding the sucrose diet could be due to an increase in total PDC activity. As suggested previously (Stansbie et al., 1976), adaptive changes of PDC activity may be rather slower than those of other enzymes of the lipogenic pathway. After 6 days of feeding a diet containing 40% fat, the initial decrease of PDC in rat epididymal fat pads was largely the result of a diminution in the active form without appreciable changes in total amount of the enzyme (Stansbie et al., 1976). In contrast, after 14-26 days on the high-fat diet the decrease in activity was largely accounted for by a marked diminution in the total amount of PDC, with very little evidence of any change in the proportion of enzyme in the active form. Diet-induced long-term regulation of PDC appears to involve coordinated modulation of all the component proteins of the complex and may involve either transcriptional or posttranscriptional regulation or both. The aim of this project was to investigate the long-term regulation of PDC under various dietary conditions at the level of enzyme activity, protein concentration and RNA measurements.
Table III. Hepatic PDC activity in rats fed sucrose-based diets. Animals were fed *ad libitum* as indicated on high-sucrose (fat free) diets. PDCa, total PDC and citrate synthase (CS) activities were determined in liver extracts spectrophotometrically according to Coore et al., 1971. Results represent the mean ± S.E., n = 5-10. Values in the same column with different superscript letters are significantly different (p < 0.05) N.D., not determined (Da Silva et al., 1993).
### Table III. Hepatic PDC activity in rats fed high-sucrose diets.

<table>
<thead>
<tr>
<th>Type of diet</th>
<th>PDCa mU/g wet wt.</th>
<th>Total PDC activity mU/mg protein</th>
<th>PDCa mU/U CS</th>
<th>PDCa (%) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sucrose (7 d)</td>
<td>113 ± 14°</td>
<td>12.4 ± 1.2°</td>
<td>137 ± 18°</td>
<td>5.9 ± 0.6°</td>
</tr>
<tr>
<td>sucrose (15 d)</td>
<td>111 ± 19°</td>
<td>14.4 ± 1.4°</td>
<td>N.D.</td>
<td>4.6 ± 0.7°</td>
</tr>
<tr>
<td>chow</td>
<td>63 ± 7°</td>
<td>7.8 ± 0.5°</td>
<td>62 ± 6°</td>
<td>6.0 ± 1.0°</td>
</tr>
</tbody>
</table>
I. Measurement of total PDC activity in rat liver

The present study was carried out to evaluate the long-term effects on total PDC activity in the liver of animals chronically fed a high-sucrose and high-fat diet, respectively. The objective of this work was to establish a possible correlation between the changes in total PDC activity to that occurring at the level of the protein or the mRNA of the genes involved in the complex. Male Sprague Dawley rats (initial weights 90-100 g) were fed ad libitum for two and three weeks the following diets: 65% sucrose, fat free (high sucrose), 45% corn starch plus 22% corn oil (high-fat) and standard laboratory chow. Detailed composition of the diets are listed in Table IV. Rats from the three groups were killed by decapitation and the livers were quickly removed and frozen in liquid nitrogen. For the assay of total PDC activity, liver homogenates were incubated with purified phosphatase and dichloroacetate (which inhibits the kinase) to allow maximal stimulation of the enzyme complex and then assayed for the release of $^{14}$CO$_2$ from [1-14C]pyruvate (Kerr et al., 1988).

Table V summarizes the effects of the diets on total PDC activity of the liver of rats fed for 15 days. Significant differences were observed only between the high-sucrose and the chow animals. There was nearly a two-fold increase in PDC activity in the high-sucrose group as compared to the chow group. Surprisingly, there was no significant effect of the high-fat on the total PDC activity. Citrate synthase (CS) is a mitochondrial enzyme of the TCA cycle which catalyzes the formation of citrate from acetyl-CoA and oxaloacetate. Activity of CS is assayed as an internal reference for
<table>
<thead>
<tr>
<th>Component</th>
<th>High-fat</th>
<th>High-sucrose</th>
<th>Chow</th>
</tr>
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<tbody>
<tr>
<td>Corn starch</td>
<td>45</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
<td>63.9</td>
<td>2.57</td>
</tr>
<tr>
<td>Casein</td>
<td>25</td>
<td>25</td>
<td>23.5</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.43</td>
</tr>
<tr>
<td>Cellulose</td>
<td>3</td>
<td>3</td>
<td>3.8</td>
</tr>
<tr>
<td>AIN mineral mix</td>
<td>3.5</td>
<td>3.5</td>
<td>6.8</td>
</tr>
<tr>
<td>AIN Vitamin mix</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0</td>
<td>0.9</td>
<td>1.37</td>
</tr>
<tr>
<td>65%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>0</td>
<td>0.2</td>
<td>0.09</td>
</tr>
<tr>
<td>Corn oil</td>
<td>22</td>
<td>0</td>
<td>6.5</td>
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</table>
Table V: Dietary regulation of endogenous total PDC and citrate synthase activity in rat liver. Animals were fed either a high sucrose diet or chow for 15 days. Liver extracts were assayed for endogenous PDC and citrate synthase activities. The mean represents +/- SEM of 6-9 animals. The * denotes p < 0.05.
Table V: Total PDC and citrate synthase activity in rat liver.

<table>
<thead>
<tr>
<th></th>
<th>Total PDC activity</th>
<th></th>
<th>Citrate Synthase activity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/min/mg protein</td>
<td></td>
<td>nmoles/min/mg protein</td>
<td></td>
</tr>
<tr>
<td>Chow</td>
<td>4.43</td>
<td>9.10</td>
<td>3.74</td>
<td>83.05</td>
</tr>
<tr>
<td></td>
<td>5.42</td>
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<td></td>
<td>5.32</td>
<td>6.27</td>
<td>6.5</td>
<td>68.52</td>
</tr>
<tr>
<td></td>
<td>3.75</td>
<td>8.20</td>
<td>5.74</td>
<td>82.07</td>
</tr>
<tr>
<td></td>
<td>4.69</td>
<td>7.89</td>
<td>6.7</td>
<td>83.78</td>
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<tr>
<td></td>
<td>4.06</td>
<td>8.87</td>
<td></td>
<td>79.69</td>
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<td></td>
<td>5.81</td>
<td>9.80</td>
<td></td>
<td>75.56</td>
</tr>
<tr>
<td></td>
<td>5.81</td>
<td>9.43</td>
<td></td>
<td>69.89</td>
</tr>
</tbody>
</table>

Mean:  
4.85 ± 0.3  8.53 ± 0.4*  5.30 ± 0.5  78 ± 1.8  69 ± 3.1  81 ± 5.4

The * denotes p < 0.05.
mitochondrial protein content and viability of the samples. Considering that the CS activity, an exclusively mitochondrial enzyme, did not significantly vary in any of the groups studied, the observed changes in total PDC activity possibly result from alterations in PDC enzyme levels per mitochondrion.

II. Effects of diets on immunodetectable hepatic PDC levels

Immunodetection using specific antibodies directed against all the catalytic subunits of PDC was used as a probe to establish whether the changes observed in total PDC activity were due to alterations in the content of the hepatic complex. Whole liver extracts were subjected to SDS-PAGE and immunoblotted with anti-PDC sera. A large increase in hepatic PDC levels was observed when animals were transferred from standard chow to high-sucrose for 15 days (Figure 19). This was observed in gels loaded with equal amount of protein for each sample (50 μg). The color intensity increased linearly with protein concentration over a range of 25-150 μg (Figure 20). This was observed for all the subunits including E1α, E1β, E3 and E2 proteins. Densitometric scanning of the blots under all the peaks indicated a 2-2.5 fold increase in PDC levels in the liver of animals fed the high-sucrose diet as compared to chow. This pattern of differences was confirmed in three different determinations. However, there was no change in the level of PDC in the high-fat animals as compared to the chow fed group. This was expected since no change was observed in the total PDC activities between the two groups.
Figure 19. Immunological detection of hepatic PDC from rats fed a high-sucrose and chow diets for 15 days. Whole liver extracts from rat liver was prepared as described in Methods and Materials. After electrophoresis on 10% polyacrylamide gels, proteins were transferred to nitrocellulose and probed with anti-PDC serum (diluted 1:150). Part A (upper panel): Whole liver extracts were loaded at different concentrations from one animal each in the high-sucrose and chow-fed group. Lanes 1 and 6, 25 μg protein; lanes 2 and 7, 50μg; lanes 3 and 8, 75 μg; lanes 4 and 9, 100 μg; lanes 5 and 10, 150 μg. Part B (lower panel): Extracts were prepared from four different animals in each group and 50 μg of protein was loaded in each lane. Lanes 2 to 5, high-sucrose extracts; lanes 6 to 9, chow extracts. The PDC standard (lane 1) was obtained from Sigma. The pattern of subunits was similar to that of the PDC standard although the E2 component from the rat complex migrated as a protein of a smaller Mr (Da Silva et al., 1993).
Figure 20. Linear relationship between protein concentration and relative intensity of the E1β and E3 proteins from rat liver. Whole liver extracts from the high-sucrose and chow fed animals were subjected to Western analysis at concentrations ranging from 25-150 μg (see Figure 19, part A). After immunoblotting with anti-PDC serum and horse radish peroxidase labelled secondary antibody, the bands were densitometrically scanned using a SciScan 5000 (USB). The results are the mean of three individual experiments. Similar analysis was performed on the E2 subunit (data not shown).
III. Dietary effects on the level of mRNA of the PDC subunits

Dietary treatments did not cause a preferential effect on a particular PDC component, but had a similar influence on the levels of all of the PDC polypeptides. Thus, long-term regulation (15 days) by sucrose (or its metabolites) appears to involve coordinated changes in the turnover rate of all the components of the complex in rat liver. The changes in the level of protein may reflect control of gene expression of PDC polypeptides by dietary factors.

Total liver RNA was isolated from animals fed the sucrose-rich and chow diets for 15 and 21 days. Forty micrograms was loaded in each lane and subjected to Northern analysis. The filter was probed with human cDNAs for E1α, E1β, E2 and E3. The mouse α-actin was used as an internal control to normalize for the amount of RNA loaded on the gels (Figure 21). Each probe was subjected to PhosphorImage analysis to compare the relative mRNA levels between the two groups. At day 15 the level of E1α and E3 increased by nearly 1.5 fold in the high-sucrose group as compared to chow (Table VI).

The same filter when probed with the glucokinase cDNA (positive control), there was nearly a 2-3 fold increase in the high-sucrose group (Figure 21). The glucokinase gene is highly inducible in animals fed a high-carbohydrate diet (Lemaigre and Rousseau, 1994). Glucokinase (hexokinase D or IV), the predominant isoenzyme form of hexokinase in the liver, catalyzes the phosphorylation of glucose to glucose 6-phosphate and controls the rate of glycolytic flux. Using a rat phosphoenolpyruvate
carboxykinase (PEPCK) cDNA probe (negative control), the level of mRNA for the high-sucrose group decreased to one-fourth the levels in the control group (Figures 21 and Table VI). The PEPCK gene, being involved in gluconeogenesis, is repressed under high-carbohydrate conditions (McGrane et al., 1989). This implies that the high-sucrose and chow diets worked as expected.

To determine whether a linear relationship exists between RNA concentration loaded on the gel and the relative intensity of the probes measured by phosphorimage analysis, RNA from the 15- and 21-day fed animals were loaded at three different concentrations (10, 25, 40 µg) and probed with the human E1α cDNA. There was a linear correlation between the intensity of the probe and the amount of RNA loaded on the gel (Figure 22).

V. Summary

A lot of information is available regarding short-term regulation of PDC that responds to changes in nutritional and hormonal conditions. Under most circumstances, the proportion of the active form varies without significant changes in total activity. Administration of a diet containing 64% sucrose for 15-21 days, substantially increased the total activity in rat liver. Total PDC activity is a measure of an increase in the number of enzyme molecules and is measured in the presence of dichloroacetate, an inhibitor of the E1 kinase and purified phosphatase. These conditions allow for maximal activation of the complex.
Figure 21. Northern blot analysis of total RNA from the liver of rats fed the high-sucrose and chow diets for 15 and 21 days respectively. Forty μg of total RNA was fractionated by electrophoresis on a denaturing formaldehyde/0.8% agarose gel. RNAs were transferred from the gel onto a nylon membrane. The membrane was hybridized sequentially with $^{32}$P-labeled human E1α, E1β, E3, mouse glucokinase, rat PEPCK and mouse actin cDNAs. The filter was stripped with 1% glycerol at 80°C for 10 min after each probe was analyzed.
**Table VI.** PhosphorImage scanning analysis of RNAs for E1α, E1β, E3 and PEPCK in rat liver. Total liver RNA was isolated from the high-sucrose and chow fed animals after initiation of dietary treatment for 15 and 21 days, respectively. Forty micrograms of RNA was loaded for Northern analysis and probed with human cDNAs for E1α, E1β, E3, rat PEPCK, and mouse actin. Values were corrected for loading efficiency with actin as a control. Each result is the mean +/- SEM of 4-6 animals. The * denotes p < 0.05. Arbitrary units are values obtained by phosphorimage scanning of the mRNA bands which are corrected for actin and expressed as percentage of chow in brackets.
Table VI. Phosphorimage scanning analysis of RNA for PDC and PEPCK from rat liver

<table>
<thead>
<tr>
<th></th>
<th>HIGH SUCROSE (% of chow)</th>
<th>CHOW (100%)</th>
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<tbody>
<tr>
<td></td>
<td>arbitrary units</td>
<td>arbitrary units</td>
</tr>
<tr>
<td><strong>15 days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1α</td>
<td>0.44 ± 0.042 (144)</td>
<td>0.31 ± 0.072</td>
</tr>
<tr>
<td>E1β</td>
<td>0.45 ± 0.171 (113)</td>
<td>0.4 ± 0.072</td>
</tr>
<tr>
<td>E3</td>
<td>0.2 ± 0.028 (142)</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>PEPCK</td>
<td>0.10 ± 0.046 (18)*</td>
<td>0.54 ± 0.063</td>
</tr>
<tr>
<td><strong>21 days</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1α</td>
<td>0.379 ± 0.022 (122)</td>
<td>0.31 ± 0.013</td>
</tr>
<tr>
<td>E1β</td>
<td>0.541 ± 0.198 (136)</td>
<td>0.4 ± 0.044</td>
</tr>
<tr>
<td>E3</td>
<td>0.204 ± 0.006 (104)</td>
<td>0.2 ± 0.016</td>
</tr>
<tr>
<td>PEPCK</td>
<td>0.23 ± 0.043 (46)*</td>
<td>0.51 ± 0.046</td>
</tr>
</tbody>
</table>
Figure 22. Linear relationship between RNA concentration and relative intensity of the E1α subunit of PDC. Total liver RNA was isolated from animals fed high-sucrose and chow diets for 15 days (upper panel) and 21 days (lower panel). Ten, twenty-five and forty micrograms of RNA were loaded on the gel for Northern analysis. The filter was probed with a 32P-labeled human E1α cDNA and mouse actin. The labeled filter was scanned by a Phosphorimage and the relative intensity of E1α, after correction for loading efficiency with actin, was plotted. Each data point represents mean +/- SE of 3 animals each.
Changes in total PDC activity closely correlated with modifications in the content of enzyme quantified by immunoblotting, indicating that increased enzyme content and not activation was the predominant mechanism underlying the adaptive response. These results indicate that PDC activity was modified in a similar way to that reported for other lipogenic enzymes, but the response seems to be slower. This slower response may be explained by the relatively long half-life of hepatic PDC (8.1 days) (Weinberg and Utter, 1979) as compared to other lipogenic enzymes (1-3 days) (Numa and Yamashita, 1979).

Changes in total PDC activity could result from alterations in enzyme content per mitochondrion which may suggest that gene expression of the PDC components may play a role in the regulation by dietary factors. Measurement of the levels of RNA in rat liver for the E1α, E1β and E2 subunits in animals fed the high-sucrose diet for 15 and 21 days showed modest changes as compared to the chow fed group. Expression of E1α showed a slight increase of 1.5 fold throughout the feeding period. One possibility is that the changes in the rate of transcription may be low enough to allow for increased total PDC activity over a long period. The controls used for Northern analysis which include glucokinase and PEPCK were appropriately regulated at the RNA level by the high-sucrose diets.

Since PDC polypeptides are encoded in the nucleus and imported into the mitochondria, PDC levels may be regulated by mechanisms that modify the rates of synthesis and/or degradation of the polypeptides as well as the rates of import and
assembly of the complex inside the organelles. Elucidation of the mechanisms involved in the control of PDC in rat liver mitochondria as well as the interactions of signals derived from nutrients and/or hormones with these mechanisms needs further investigation.
CHAPTER VI. DISCUSSION

I. Discussion

The multienzyme, PDC has long been considered and studied as the archetypical "housekeeping" enzyme that is present in all cells, where it plays a key role in cellular metabolism. The importance of the housekeeping function of the enzyme is best exemplified by the PDC deficiency syndrome, a disease manifested by lactic acidosis and cerebral neuropathy (Patel et al., 1992). Short-term regulation involves both the supply of metabolic intermediates and the control of the catalytic properties of the enzyme through covalent modification (phosphorylation/dephosphorylation). Long-term regulation in liver may involve changes in protein turnover and/or stabilization. While the genes can be considered to be constitutively expressed "housekeeping" genes in many tissues, there are several other tissues (liver, adipose, mammary glands and pancreatic islets) (reviewed in the introduction) wherein modulation of cellular PDC activity represents an important component of the integrated response to external stimuli (insulin, nutrients, differentiation). Under most circumstances, adaptive regulation of PDC has been found to be exerted mainly at the level of phosphorylation of the enzyme.

Since PDC is made up of different regulatory and catalytic components, much of the research in this field thus far has involved cloning and identification of cDNAs and genes of the subunits. This has allowed us to perform structure-function studies and
aided in the understanding of mutations that occur in PDC-deficient patients. Among patients with reduced levels of both E1 peptides, two cases of simultaneous reduction of both E1α and E1β proteins had decreases in the amounts of E1α mRNA only. Mutations in these cases may impair the transcription, nuclear processing or stability of E1α mRNA. Knowledge of the promoter-regulatory regions and identification of key cis-elements involved in transcriptional expression is prerequisite for our understanding of possible coordinate regulation of the PDC genes as well as identification of mutations which may be localized in these regions.

As a first step towards this goal, the isolation and characterization of the E1β promoter region was performed. The E1β promoter is very typical of "housekeeping" promoters which lack a TATA box and upstream "inducible" cis-elements. The 2.3 kb 5′-flanking region had very low promoter activity possibly due to the presence of repressor elements that may allow for low constitutive expression of the gene in vivo. Such "represssor" elements have been identified in many genes to play an important role in either basal low level expression of several genes (Savagner et al., 1990) or in tissue specific expression of the insulin gene in the pancreatic islets (Boam et al., 1990) or in the sp-2 gene (Hunt et al., 1986) that specifically allows expression in the adipocytes.

On the other hand, the E1α promoter (-763 to +33) exhibits very strong CAT activity in HepG2 cells. Unlike the E1β promoter, E1α contains a TATA box and many putative upstream response elements which may play a role in efficient
transcription of the gene. Crucial elements for promoter activity and DNA protein interactions were confined within a region spanning -221/+33 bp which includes Sp1, CCAAT box, AP-2 and CRE. This region also retained more than 75% of the promoter activity of the 796 bp promoter sequence. Deletion of an Sp-1, AP-2 and CCAAT box (-102/+33 bp) elements in the E1α promoter region resulted in approximately a 50% decline in CAT activity which suggests that one or more of these cis-elements may play a role in high level basal expression. E1α and E1β are found in cells as a heterotetramer and are present in a 1:1 molar ratio. This raises the possibility that these two genes may be coordinately expressed. Comparison of their promoter analysis and the description of consensus sequences within the E1α and E1β promoters reveals no apparent relationship which would indicate obvious sites for similar transcriptional regulation.

Comparison of the E1α and E1β promoters suggests that the promoters are not likely to be controlled by a common set of transcription factors. There are multiple ways to attain similar mRNA levels which include regulation at the transcriptional level or factors affecting stability of the mRNA levels. It may be possible that the E1α and E1β genes may not be coordinately regulated in the sense of using a single mechanism. Preliminary evidence of transgenic mice bearing the 796 bp region of the E1α promoter ligated to CAT, indicated barely detectable levels of CAT activity in adult liver while relatively high levels were detected in brain (Dey et al., unpublished data). The adipose tissue, spleen, heart and kidney expressed intermediate levels of CAT in
reducing order. Specificity of expression, however was maintained in the testis and was temporally regulated similar to the endogenous gene in mice. Interestingly, in HepG2 cells, a human hepatoma derived cell-line, expression of the 796 bp E1α promoter was extremely efficient unlike CAT expression in vivo. The composition of transcript factors present in the nuclear milieu of a transformed hepatic cell line such as HepG2 which may not represent the endogenous environment within the liver may play an important role in regulating expression from this promoter region of the E1α gene. Additionally, key regulatory elements of the gene which are required for in vivo expression of E1α may be absent within the -796 bp E1α promoter.

The half-life of rat liver pyruvate dehydrogenase is 8.1 days which is considerably longer than that of the average mitochondrial fraction which is about 3.8 days (Weinberg and Utter, 1979). Several implications of such a slow rate are apparent. First, regulation by changing the intracellular levels may require at least a week to show measurable effects. This suggests that regulation at the catalytic level may be more important under acute metabolic changes, but chronic metabolic management may be regulated by changes in the enzyme level, as well as a superimposition of both types of control. Second, measurement of the total activity and turnover of these proteins should take into account the time necessary for an induced enzyme to reach a new steady state. In the present studies, dietary treatment of rats for 15 days was sufficient in "inducing" PDC activity from control levels.
Animals fed a high-sucrose diet for two weeks resulted in a two-fold increase in total PDC activity which corresponded with concomitant increases in all the subunits of the complex, determined by immunological methods. An increase in the level of all the components involved in the complex may suggest coordinate regulation which may be due to an increase in the rate of de novo synthesis or decreased rate of degradation of the enzymes.

Measurement of RNA levels of the E1α, E1β and E3 components of PDC detected no significant changes in the high-sucrose group as compared to the chow-fed. However, one may speculate that to increase the level of proteins and hence activity of the complex by two fold over a 15 day period, the rates of transcription may be altered at a subtle pace which may remain undetectable using the method of Northern analysis. On the other hand, translational regulation or stability of the proteins may be altered under such a metabolic condition. For example, over short periods of time, insulin synthesis is controlled principally through translation of pre-existing mRNA (Welsh et al., 1986). Glucose stimulates the rate of initiation of translation as demonstrated by an increased transfer of cytoplasmic insulin mRNA to subcellular fractions containing ribosomes and larger polysomes. This redistribution of insulin mRNA may occur as a result of glucose stimulating the interaction of the signal-recognition particle (SRP) initiation complex with the SRP receptor on the endoplasmic reticulum (ER) or it may reflect the effect of glucose on the interaction of proteins with RNA stem loop structures within the 5’ untranslated region of the insulin mRNA.
Mammalian PDC is a mitochondrial enzyme containing multiple copies of six different components. These component proteins are synthesized as precursors in the cytoplasm, imported into the mitochondria and processed to their mature forms prior to their involvement in the formation of the complex. Furthermore, chaperonins appear to be involved in promoting the assembly of the active complex. Thus increase in the steady state of the individual components upon feeding a high-sucrose diet may involve regulation at any one or more of these processing steps. The importance of and the need to clarify the mechanism(s) which regulate PDC under chronic metabolic conditions must await further studies.

II. Future directions

The long term goals of the study of the promoter regions of the PDC subunits should include new insights into whether all the genes that comprise PDC are coordinately regulated. Thus in vivo nuclear run-on assays may provide useful information in this regard. Comparison of the 2.3 kb upstream promoter region of \( E1 \beta \) to the 796 bp region of the \( E1 \alpha \) promoter suggested significant dissimilarity in the promoter activities in HepG2 cells. Additionally, transgenic experiments with the 796 bp fragment of the \( E1 \alpha \) promoter suggested that key regulatory elements of the gene which are required for in vivo expression of the endogenous gene product in various tissues may be absent from this truncated promoter region. Thus to understand
regulation of the E1α gene in its entirety, one may have to isolate an upstream region of the promoter from a human genomic library. The deletion analysis and DNaseI footprinting data have provided important information on the nature of regulation by the E1α and E1β promoter regions in HepG2 cells. However what needs to be determined are the cis-elements that play a key role in basal high level expression from the E1α promoter and whether initiator sequences (Inr) and/or Sp1 cis-elements are required for accurate start site selection in the E1β promoter. Mutagenesis studies of the various cis-elements will provide a better understanding and knowledge of the identity of transcription factors that facilitate transcription of the E1α and E1β genes.

The liver plays a central role in maintaining blood glucose homeostasis. In the liver, glycolysis functions at a low rate, except in anoxia or when encountering a large excess of glucose. The pyruvate formed in this tissue is used mainly for the synthesis of fatty acids. In contrast, gluconeogenesis, which consumes energy and replenishes blood glucose during fasting, exercise and a low carbohydrate diet, functions at a high rate. Flux through the enzymes of these cycles is modulated by short-term (phosphorylation and/or allosteric effectors) and long-term (changes in enzyme activity) regulatory mechanisms. Hormones are involved in these regulatory mechanisms, the key ones being glucagon and insulin. They act at different levels (pre- and posttranslational) and in opposite directions on the activities of key regulatory enzymes. In vivo, variations in the insulin and glucagon secretion rates are primarily under dietary control. Recently, glucose has been shown to be able to act on the
transcription of glycolytic and lipogenic genes. DNA elements responsible for the response of these genes to carbohydrates and insulin have been identified only recently in the L-type pyruvate kinase gene (Thompson and Towle, 1991), spot 14 (Shih and Towle, 1992), fatty acid synthase and insulin (German, et al., 1992) genes. The exact nature of the protein complexes bound to these “carbohydrate response elements” and the mechanism of their activation by glucose derived metabolites/insulin is not known as yet.

Transcriptional regulation of E1α has been observed in pancreatic islets incubated at high glucose concentrations. Such an induction has been speculated to play a role in insulin signalling. Many genes are regulated by glucose concentration via the presence of “carbohydrate response elements” in the regulatory regions of these genes. E1α, in addition to the TATA box, Sp1 and CAAT elements, also contain putative hormone response elements such as insulin response element (IRE) and the cAMP response element (CRE). Whether these cis-elements play a functional role in E1α regulation needs to be addressed. During differentiation of preadipocytes to adipocytes, a number of lipogenic genes are induced including all the components of PDC. Progress towards understanding the mechanisms of coordinate regulation will require the development of new techniques to resolve the identity of factors interacting with various subunits in vivo.

Dietary regulation of PDC plays an important role in the metabolic adaptation of animals. Short-term regulation of PDC involving product inhibition and covalent
modification has been very well characterized. Long-term regulation involving a change in total activity of PDC and in the levels of the catalytic proteins continues to be a formidable task due to the multienzyme nature and the mitochondrial localization of PDC. A complex number of regulatory steps are involved in the final assembly of the complex and one can only begin to speculate on the regulatory processes that occur in vivo which the cell performs with such precision.
Bibliography


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