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Factors That Control the Tissue-Specific Transcription of the Gene for Phosphoenolpyruvate Carboxykinase-C

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ABSTRACT Transcription of the gene for PEPCK-C occurs in a number of mammalian tissues, with highest expression occurring in the liver, kidney cortex, and white and brown adipose tissue. Several hormones and other factors, including glucagon, epinephrine, insulin, glucocorticoids and metabolic acidosis, control this process in three responsive tissues, liver, adipose tissue, and kidney cortex. Expression of the gene in these three tissues in regulated in a different manner, responding to the specific physiological role of the tissue. The PEPCK-C gene promoter has been extensively studied and a number of regulatory regions identified that bind key transcription factors and render the gene responsive to hormonal and dietary stimuli. This review will focus on the control of transcription for the gene, with special emphasis on our current understanding of the transcription factors that are involved in the response of PEPCK-C gene in specific tissues. We have also reviewed the biological function of PEPCK-C in each of the tissues discussed in this review, in order to place the control of PEPCK-C gene transcription in the appropriate physiological context. Because of its extraordinary importance in mammalian metabolism and its broad pattern of tissue-specific expression, the PEPCK-C gene has become a model for studying the biological basis of the control of gene transcription

KEYWORDS gluconeogenesis, insulin, glucocorticoids, glyceroneogenesis, cAMP, PEPCK-C gene promoter, cataplerosis, metabolic acidosis, transcription factors, synergism, tissue-specific gene transcription

METABOLIC ROLE OF PEPCK

The cytosolic form of PEPCK (PEPCK-C) is generally considered to be the pace-setting enzyme in hepatic and renal gluconeogenesis (Rognstad, 1979). In this role, alterations in PEPCK-C mRNA in the liver in response to a wide variety of metabolic perturbations have been reported in numerous publications. (reviewed in Hanson & Patel, 1994). In fact, the gene for PEPCK-C has become a marker gene for *hepatic* glucose output during fasting or in diabetes, or where the mechanism of action of hormones, transcription factors, or drugs of virtually every category is being assessed. However, the gene for PEPCK-C is expressed in a variety of tissues other than the liver, and its broader biological

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role in metabolic processes and in diseases, such as obesity and diabetes, is seldom discussed. This is dramatically underscored by the fact that the ablation of the gene for PEPCK-C in the livers of mice does not alter the concentration of blood glucose (She *et al.*, 2000); the mice can also be made diabetic in the absence of this enzyme (She *et al.*, 2003). Unpredictably, these animals develop profound fatty livers. This finding does not fit our current picture of PEPCK as solely involved in gluconeogenesis. Furthermore, PEPCK gene expression occurs in tissues that do not make glucose, such as white and brown adipose tissue, and tissues where its role is unknown, such as: lung, mammary gland during lactation, skeletal muscle and the pancreas, to name just a few (Hanson & Patel, 1994). Despite the general lack of attention to the metabolic role of PEPCK in these tissues, there is a growing body of information regarding the alternative biological role for PEPCK in tissues other than the liver. In white (Reshef *et al.*, 1970) and brown adipose tissue (Brito *et al.*, 1999), for example, PEPCK-C has been shown to participate in an abbreviate version of gluconeogenesis, termed *glyceroneogenesis*, a process that generates 3-phosphoglycerol from pyruvate and its precursors. It is also likely that PEPCK is involved in *cataplerosis* (i.e., the removal of citric acid cycle anions generated from sources such as the degradation of amino acids) (Figure 1). This is especially relevant for glutamine metabolism in the kidney cortex in response to alterations in acid/base balance and in the small intestine, where the metabolism of glutamine to carbon dioxide requires the cataplerotic function of PEPCK. A more detailed discussion of the metabolic role of cataplerosis can be found in a review by Owen *et al.* (2002).

To further complicate the biology of PEPCK, there are two isoforms of this enzyme, a mitochondrial (PEPCK-M) and a cytosolic form (PEPCK-C). Interestingly, diet and hormones such as glucocorticoids, glucagon and insulin acutely regulate only PEPCK-C gene transcription. Virtually nothing is known about the factors that control the transcription of the gene for PEPCK-M and not much more about its metabolic role

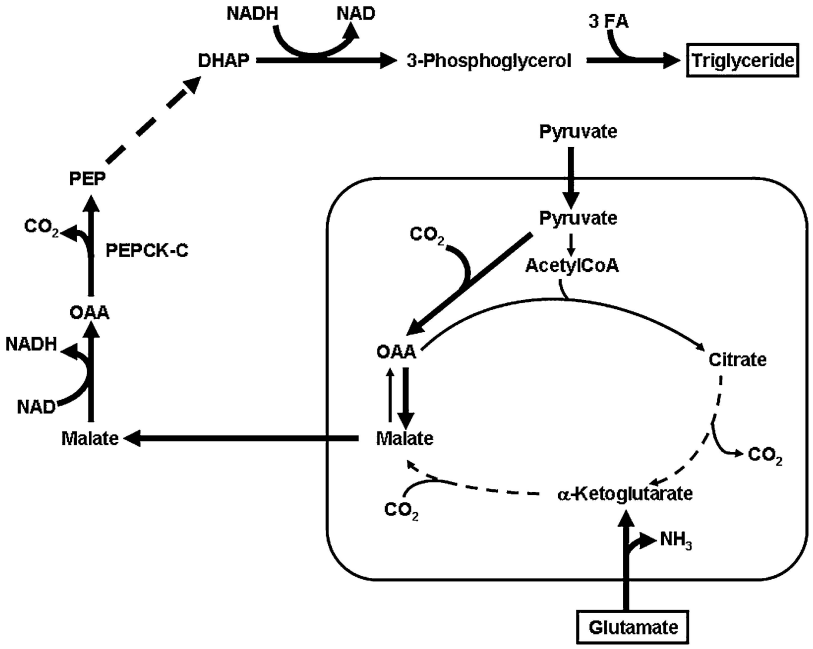

FIGURE 1 **The interrelationship of anaplerosis, cataplerosis and glyceroneogenesis in adipose tissue. The pathway of glyceroneogenesis from pyruvate in adipose tissue is shown. The heavy arrows show the pathway of carbon flow from pyruvate to 3-phosphoglycerol and then into triglycerides. Amino acids, such as glutamate, can contribute to the synthesis of 3-phosphoglycerol by entering the citric acid cycle (*anaplerosis*) and being converted to malate. The carbon skeletons of these amino acids exit the cycle by leaving the mitochondria as malate (*cataplerosis*), which is oxidized in the cytosol to oxalacetate. PEPCK-C decarboxylates oxalacetate to PEP, which is subsequently converted to 3-phosphoglycerol for triglyceride synthesis via glyceroneogenesis. The NADH generated in the cytosol by NAD-malate dehydrogenase is used to reduce dihydroxyacetone phosphate to 3-phosphoglycerol balancing the redox state in the cytosol. PEPCK-C is thus the major catabolic enzyme and glyceroneogenesis is itself a cataplerotic process in adipose tissue and liver. See the review by Owen and colleagues (2002) for details of the role of anaplerosis and cataplerosis in metabolism.**

(Hanson & Patel, 1994) for a review of this subject). It is the rare textbook of biochemistry that even mentions the fact that there are two isoforms of PEPCK or attempts to discuss the physiological significance of this pattern of gene expression. Mammalian species vary in the percentage of each form of PEPCK that is present in tissues (Nordlie & Lardy, 1963; Hanson & Garber, 1972). This difference is especially important, since in the livers of the most widely studied laboratory animals, the rat and the mouse, 90% to 95% of the activity is PEPCK-C. In contrast, in most other mammalian species, including humans, there is an equal activity of the two isoforms of PEPCK (Hanson & Patel, 1994). Adult birds, on the other hand, have only hepatic PEPCK-M (Soling & Kleineke, 1976; Shen & Mistry, 1978; Hod *et al.*, 1982; Weldon *et al.*, 1990). Thus, regardless of the level of transcription of PEPCK-C in tissues such as the liver, many animal species have a very substantial activity of PEPCK-M. The biological roles of the two forms of PEPCK remain to be fully resolved. However, it has been suggested that PEPCK-M is involved in gluconeogenesis from lactate, since there is no need for the reducing equivalent transport from mitochondria to cytosol when lactate is the substrate for glucose synthesis (Soling & Kleineke, 1976; Watford *et al.*, 1981). The interested reader is directed to a review of this subject (Hanson & Patel, 1994); since an in-depth discussion is clearly beyond the scope of this review.

Most discussions of the metabolic role of PEPCK are limited to its gluconeogenic function. However, as mentioned above, PEPCK-C is also involved in lipid metabolism as part of *glyceroneogenesis*, which controls the rate of fatty acid re-esterification in the adipose tissue and liver (Reshef *et al.*, 2003). This pathway has implication for Type 2 diabetes, where the rate of delivery of fatty acids to the muscle, especially in obese individuals, has been proposed to be an important factor in the etiology of this disease (McGarry, 1992; Forest *et al.*, 2003). In general, fatty acids block glucose transport into skeletal muscle and also inhibit its oxidation to carbon dioxide via glycolysis and the citric acid cycle (see Roden [2004] for a review of the literature in this area). The resulting increase in the concentration of glucose in the blood causes the secretion of excess insulin; a state of *insulin resistance* develops, which, if not controlled effectively, can ultimately lead to Type 2 diabetes. The regulation of fatty acid mobilization from the triglyceride stored in white adipose tissue to skeletal muscle is thus a critical element in controlling the onset of this disease. In all mammals, there is extensive triglyceride/fatty acid cycling that controls the overall level of fatty acid re-esterification to triglyceride (Reshef *et al.*, 2003) and thus its availability for oxidation in muscle. As an example, human subjects that had been fasted for 84 hours, re-esterify approximately 70% of the free fatty acids released by adipose tissue back to triglyceride, which is then re-deposited in this tissue (Jensen *et al.*, 2001).

The Role of PEPCK-C in the Triglyceride/Fatty Acid Cycle in the Adipose Tissue and Liver

During starvation, glucose is at a premium for metabolism by tissues such as the brain and red blood cells, so that *glyceroneogenesis* in the liver and adipose tissue becomes a major pathway for 3-phosphoglycerol synthesis. Kalhan and colleagues (2001) reported that up to 65% of the glyceride-glycerol in the triglyceride isolated from very low density lipoprotein (VLDL) in humans fasted overnight is derived from hepatic glyceroneogenesis; only 6% was from glycerol itself. An analysis of the rate of fatty acid (FFA) re-esterification via glyceroneogenesis in adipose tissue of rats fed various diets has demonstrated that extensive glyceroneogenesis occurs in that tissue (Botion *et al.*, 1998; Botion *et al.*, 1995). The control of PEPCK-C gene transcription must therefore be integrated with the metabolic requirement for glyceroneogenesis in specific tissues. The hormonal control of PEPCK-C in the liver and adipose tissue reflect the metabolic response of gene transcription to the physiological status of the animal. In the liver, glucagon and glucocorticoids increase the transcription of the gene for PEPCK-C, thus enhancing the rate of hepatic gluconeogenesis as well as glyceroneogenesis in the fasted state. The result is an increase in the rates of hepatic glucose output and triglyceride recycling via glyceroneogenesis. In adipose tissue, glucocorticoids decrease PEPCK-C gene expression (Reshef *et al.*, 1969a), which reduces the activity of glyceroneogenesis in the tissue and increases the rate of FFA release. This provides fuel for tissues like muscle to support energy requirements during fasting. It is well established from the early work of Bernardo Houssay (1942) and Long and Leukins (1936) that the development of the symptoms of diabetes (i.e., hyperglycemia and ketonemia) requires the presence of glucocorticoids. In their absence, there is a diminished rate of both

gluconeogenesis in the liver and kidney and FFA release from adipose tissue. We have proposed that this is due in part to the differential effect of glucocorticoids on transcription of the gene for PEPCK-C in the liver and adipose tissue (Reshef *et al.*, 2003). This point will be discussed in detail in a later section of this review.

PEPCK-C Is Involved in the Response of the Kidney to Metabolic Acidosis

The kidney cortex is a major source of blood glucose during prolonged fasting. The rate of renal gluconeogenesis is induced by metabolic acidosis, which is greatly increased during starvation and diabetes, due to the large increase in the concentration of β-hydroxybutyrate and acetoacetate (both weak acids) in the blood (Sapir & Owen, 1975). The metabolism of glutamine plays a key role in controlling metabolic acidosis in mammals and forms a link between gluconeogenesis and ammoniagenesis in that tissue (reveiwed in Taylor & Curthoys, 2004). Glutamine is the most abundant amino acid in the blood and is the major source of ammonia for the kidney, where its catabolism is of special significance during acidosis. The kidney extracts more than 30% of the total plasma glutamine for the generation of ammonia during chronic metabolic acidosis, such as occurs during diabetes. This significant catabolism of glutamine greatly increases the quantity of α-ketoglutarate that enters the citric acid cycle (*anaplerosis*). Since the cycle does not completely oxidize four and five carbon compounds to carbon dioxide, it is critical that the anions that enter the cycle are removed (*cataplerosis*). The carbon skeleton of glutamine (α-ketoglutarate) leaves the mitochondria as malate, which is ultimately converted to glucose via gluconeogenesis. The kidney thus contributes significantly to fasting gluconeogenesis and plays a key role in the hyperglycemic characteristic of diabetes (Owen *et al.*, 1976).

Metabolic acidosis induces the levels of glutamate dehydrogenase (Wright & Knepper, 1990) and glutaminase (Curthoys & Lowry, 1973) (both mitochondrial in location) and PEPCK-C in the kidney cortex (Burch *et al.*, 1978); metabolic alkalosis reverses this effect. This adaptive response to metabolic acidosis increases the levels of glutaminase and glutamate dehydrogenase in the kidney cortex and insures the removal of glutamine into the citric acid cycle. The increased activity of these two enzymes is due to a pH-induced stabilization of their mRNA; in contrast, the lowered pH induces PEPCK-C gene transcription (Curthoys & Gstraunthaler, 2001).

Glucocorticoids also stimulate renal PEPCK-C gene transcription (Kaiser & Curthoys, 1991; Pollock & Long, 1989). However, insulin is not a major factor in the control of this process; PEPCK-C gene expression in the kidney is less responsive to insulin than is the gene in the liver (Meisner *et al.*, 1985). Pollock (1989) has reported that the administration of high levels of glucose to rats (which increases insulin secretion) lowers the concentration of PEPCK-C mRNA in the kidney of normal rats but not in the kidneys of acidotic animals. Taken together the available data indicates that metabolic acidosis and glucocorticoids, not cAMP and insulin, are the major regulators of renal PEPCK-C gene transcription in mammals.

THE ANATOMY OF THE PEPCK-C GENE PROMOTER

The sequence of the PEPCK-C gene promoter from three mammalian species, the rat (Beale *et al.*, 1985), human (Ting *et al.*, 1993), and mouse (Williams *et al.*, 1996) have been reported, and the sequence of PEPCK-C from the dog is available from the NCBT. The sequence similarity between them is remarkable; there is virtually no difference in the sequence of the gene promoters from the mouse and the rat from the start site of transcription to −1500. DNase I footprinting analysis of the rat PEPCK-C gene promoter from +73 to −650, using nuclear proteins extracted from rat liver, originally identified 8 binding sites, termed P1 through P6, and a cAMP response element, CRE-1, within this segment of the promoter (Roesler *et al.*, 1989). The originally described P3 site comprises two distinct elements P3(I) and P3(II). The regulatory elements responsible for the dietary and hormonal control of PEPCK-C gene transcription in the liver that have been studied to date occur within a region between −1500 and +73 of the PEPCK-C gene promoter. A number of DNase I hypersensitive sites have been described in the promoter and in the structural gene itself. For example, Ip *et al.*, (1990) and Williams *et al.* (1999) identified an element in the PEPCK-C gene promoter that maps at −4800, which they suggested is involved in the tissue specific expression of the PEPCK-C gene in the liver. This element binds a nuclear protein, tentatively identified as

HNF-3β. They also noted hypersensitive regions in the PEPCK-C gene at about –1400 in the promoter and in intron H (+4650) of the structural gene. Within this hypersensitive region is a site at −1360 that also binds HNF3β (Cassuto, Hanson, & Reshef, unpublished results) and a site at −1000, which binds PPARγ2 and PPARα, (dAF1). This site is critical for expression of the PEPCK-C gene in adipose tissue (Devine *et al.*, 1999). Homozygous progeny of mice generated from ES cells with a targeted mutation of the dAF1 site have an ablated expression of the gene for PEPCK-C in adipose tissue (Olswang *et al.*, 2002). These mice lose white adipose tissue and 25% of the mutant animals develop lipodystrophy due, in part, to the absence of *glyceroneogenesis* in this tissue. The significance of these specific regulatory sites in the PEPCK-C gene promoter will be discussed in a subsequent section of this review.

The PEPCK gene promoter (−1500 to +73) can be arbitrarily divided into three regions based on the clustering of regulatory elements (Figure 2) and functional role of the binding sites in these regions.

Region 1 contains the CRE and an adjacent NF-1 site (P1). Mutation of the CRE markedly reduces the cAMP responsiveness of the PEPCK gene promoter in the livers of transgenic mice (Liu *et al.*, 1991), whereas a mutation of the NF-1 site causes a marked increase in the level of basal gene transcription (Crawford *et al.*, 1998) (Leahy *et al.*, 1999). The CRE from the PEPCK-C gene promoter binds members of the leucine zipper family of transcription factors, including CREB (Quinn, 1993), CREM (Goraya *et al.*, 1995), C/EBPα (Roesler *et al.*, 1989), C/EBPβ (Park *et al.*, 1993), D-Binding Protein (DBP) (Roesler *et al.*, 1992), ATF2 (Cheong *et al.*, 1998), ATF3 (Allen-Jennings *et al.*, 2002) and Fos/Jun (Gurney *et al.*, 1992).

Region 2 of the PEPCK-C gene promoter contains an HNF-1 site (P2), which has been shown to be critical for transcription of the PEPCK gene in the kidney and to be required for the response of the PEPCK-C gene in the kidney to acidosis (Cassuto *et al.*, 1997) and to nuclear receptors (Cassuto *et al.*, 2003). In addition, this region contains a C/EBP binding domain, termed P3(I) (Roesler *et al.*, 1989; Trus *et al.*, 1990). The C/EBP

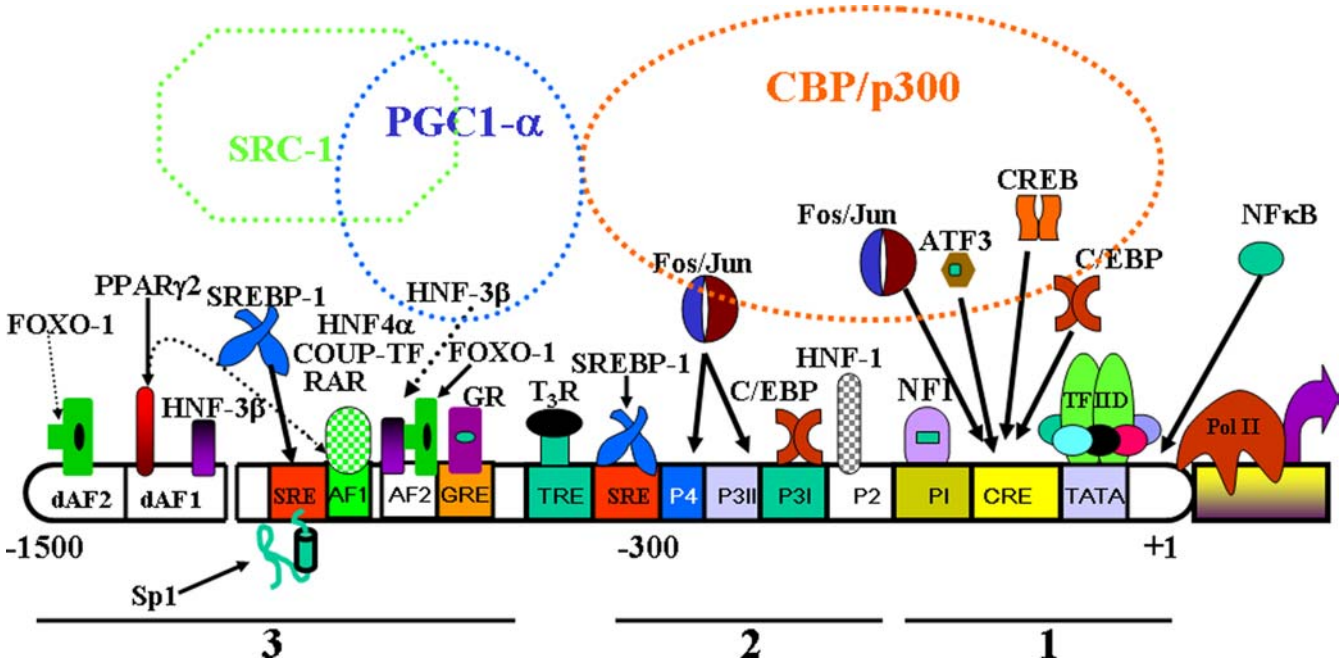

FIGURE 2 **A diagram of the PEPCK-C gene promoter showing the transcription factors and co-activators that regulate the transcription of the gene. CREB = cAMP Regulatory Element Binding Protein; ATF3 = Activating Transcription Factor 3; NFκB = Nuclear Factor κB; COUP-TF = Chicken Ovalbumin Upstream Promoter-Transcription Factor; PPARγ2 = Peroxisome Proliferator-Activated Receptorγ2; SREBP-1 = Sterol Regulatory Element Binding Protein; HNF = Hepatic Nuclear Factor; GRU = Glucocorticoid Regulatory Unit; T_3R, Thyroid Hormone Receptor; C/EBP = CAAT/Enhance Binding Protein; NF1 = Nuclear Factor 1; CREM = cAMP Regulatory Element Modifier; CBP/p300 = CREB Binding Protein/p300; PGC-1α = Peroxisome Proliferator-Activated Receptor Coactivator 1α; SRC-1 = Sterol Receptor Coactivator-1; RAR = Retinoic Acid Receptor; AF1 = Accessory Factor 1; AF2 = Accessory Factor 2; dAF1 = distal Accessory Factor 1; dAF2 = distal Accessory Factor 2; TRE = Thyroid Hormone Regulatory Element; Pol II = RNA polymerase II; GRE = Glucocorticoid Regulatory Element; CRE = cAMP Regulatory Element; P1, P2, P3(I). P3(II) and P4 are protein binding sites identified by DNase 1 footprinting (Roesler *et al.*, 1989). Modified from a figure published by Croniger *et al.* (2002).**

binding site at P3(I) is required for the full transcriptional responsiveness of the promoter to cAMP (Liu *et al.*, 1991) and for the liver-specific expression of the PEPCK-C gene (Patel *et al.*, 1994).

Region 3 contains the GRU, which extends from −320 to −1500 of the promoter. The GRU is composed of two GREs, five Accessory Factor (AF) binding sites and a CRE. The GREs act only in the context of the PEPCK-C gene; they do not confer glucocorticoid responsiveness on a heterologous promoter. The GRU was originally mapped between −321 to −455 of the PEPCK gene promoter (Imai *et al.*, 1990, 1993) but recent studies from our laboratories have demonstrated that it extends to −1500 (Cassuto, Reshef, & Hanson, unpublished work). The AF1 site binds the hepatic-enriched orphan receptors HNF-4β, (Hall *et al.*, 1995), COUP-TFII (Scott *et al.*, 1996), PPARγ2 (Tontonoz *et al.*, 1995), the retinoic acid receptorgα (RARα) (Hall *et al.*, 1992) and retinoid X receptor α (RXRα) (Mitchell *et al.*, 1994). The AF2 site binds members of the Forkhead family including HNF-3β (Foxo2a) and the phosphorylated form of Foxo1 (a.k.a. FKHR) (Puigserver *et al.*, 2003). The deletion of this site results in the inhibition of the diabetes-induced increase of PEPCK gene transcription in the livers of transgenic mice and renders the PEPCK gene promoter refractory to induction by glucocorticoids (Lechner *et al.*, 2001). There is also evidence that C/EBPβ is involved in regulation of PEPCK-C gene transcription by insulin by interacting, as part of a nucleoprotein complex, with regulatory proteins that bind at the AF2 site (Ghosh *et al.*, 2001) (Duong *et al.*, 2002). ChIP analysis has demonstrated that C/EBPβ in rat hepatocytes is part of a complex bound to the AF2 region in the presence of insulin or dexamethasone (see Figure 2). The AF3 site binds the thyroid hormone receptor (Giralt *et al.*, 1991; Park *et al.*, 1995) and overlaps a SREBP-1c binding domain on the PEPCK-C gene promoter (Chakravarty *et al.*, 2004). There are two additional upstream binding sites in the extended GRU that we have termed distal AF1 (dAF1) and distal AF2 (dAF2) that are required for optimal response of the hepatic gene promoter to glucocorticoids (Cassuto, Hanson, & Reshef, unpublished results). The dAF1 (a PPARγ2 binding site) binds PPAR/RXR (Tontonoz *et al.*, 1995) and HNF4α (Cassuto, Hanson, & Reshef, unpublished results) and is required for the expression of the gene for PEPCK-C in adipose tissue (Olswang *et al.*, 2002). HNF-3β and Foxo1 bind to dAF2 (see Figure 1). Finally, there are also two SREBP-1c binding domains in this region of the PEPCK-C gene promoter, at −322 and −590, that are both *potentially* involved in the insulin inhibition of PEPCK-C gene transcription. The significance of these binding sites will be discussed later in this article.

CO-ACTIVATORS AND (PERHAPS) CO-REPRESSORS ARE INVOLVED IN THE CONTROL OF PEPCK-C GENE TRANSCRIPTION

A number of transcriptional co-activators control PEPCK-C gene transcription; these include CREB Binding Protein (CBP) (Leahy *et al.*, 1999), sterol receptor co-activator 1 (SRC-1) (Stafford *et al.*, 2001) and PPARγ co-activator-1a (PGC-1α) (Puigserver *et al.*, 2003). CBP interacts with NF-1, (Leahy *et al.*, 1999) C/EBPβ and with SREBP-1c (Naar *et al.*, 1998) to inhibit PEPCK-C gene transcription. Stafford and coworkers (2001) have suggested that SRC-1 interacts with HNF-4α, COUP-TFII and with HNF-3β, all of which are required for the maximal induction of PEPCK-C gene transcription by glucocorticoids and that the recruitment of SRC-1 is part of a transcriptional complex with CBP. PGC-1α coordinates the control of transcription of genes in the gluconeogenic pathway (PEPCK-C and G-6-Pase) (Yoon *et al.*, 2001). Glucagon induces the levels of PGC-1α mRNA, and over-expression of PGC-1α stimulates transcription of the gene for PEPCK-C (Yoon *et al.*, 2001). However, transcription of the PEPCK-C gene can occur in the absence of PGC-1α (but at a lower level), suggesting that it serves as a "transcription amplifier" for this gene (Herzog *et al.*, 2004). PGC-1α promotes transcription by the assembly of a transcription factor complex that includes SRC-1 and CBP/p300. This interaction has been proposed to cause a conformational change in PGC-1α, which permits the binding of SRC-1 and CBP/p300 (Puigserver *et al.*, 1999). It is thus likely that PGC-1α is involved in coordinating the transcriptional response of hepatic PEPCK-C to inductive stimuli, such as occurs during fasting. PGC-1α has also been proposed to be involved, together with Foxo-1 and HNF-4α, in the insulin-regulated inhibition of PEPCK-C gene transcription (Puigserver *et al.*, 2003). This will be discussed in more detail in a subsequent section of this review.

Motta & colleagus (2004) have shown that mammalian SIRT, an NAD-dependent histone deacetylase, represses the activity of Foxo1 in human embryonic

kidney cells and have suggested a connection with carbohydrate metabolism during diabetes. The implication of these initial studies it that *co-repressors* play a key role in the control of PEPCK-C gene transcription. Finally, both SREBP-1 and Sp1 have been shown to interact, with chromatin, TFIID and a multi-protein co-activator complex, which includes CBP, in a highly purified human transcription system (Naar *et al.*, 1998). The multiprotein co-activator binds to the activation domain of SREBP-1 and mediates synergistic activation of transcription by SREBP-1 and Sp1. Since both SREBP-1 and Sp1 have been shown to be involved in the control of PEPCK-C gene transcription (Chakravarty *et al.*, 2001, 2004; Yamamoto *et al.*, 2004), this finding is of special interest.

CONTROL OF HEPATIC PEPCK-C GENE EXPRESSION BY INSULIN

Background Research Establishing the Basic Aspects of Insulin Regulation

The mechanisms responsible for the regulation of PEPCK-C gene transcription by insulin have been a major area of interest almost since the discovery of the enzyme in 1953 (Utter & Kurahashi, 1953; Kurahashi, 1986) and the realization that it was a critical step in hepatic glucose output in mammals. In 1963, Shrago and colleagues (Shrago *et al.*, 1963) published the first systematic analysis of the regulation of PEPCK-C gene expression by hormones, including insulin. They demonstrated that the activity of PEPCK-C was induced in the livers of rats by diabetes and that the administration of actinomycin D or puromycin to the animals blocked this induction. The injection of insulin into diabetic rats caused a decrease in the activity of hepatic PEPCK-C to below basal levels. These studies were prescient in their prediction that diabetes stimulated PEPCK-C gene transcription, presumably by raising the levels of hepatic cAMP, and that insulin blocked this process. This prediction is especially impressive since research in this era of biochemistry relied mainly on inhibitors of gene transcription and mRNA translation to delineate the control of gene expression.

Additional important information obtained from measuring the rate of synthesis of PEPCK-C demonstrated that intra-gastric administration of glucose to rats fasted for 24 hours rapidly ablated the synthesis of hepatic PEPCK-C (Tilghman *et al.*, 1974; Meyuhas *et al.*, 1976) but not the synthesis of the enzyme in the kidney cortex or adipose tissue. In recent years, the molecular basis of this liver-specific regulation of PEPCK-C gene expression by glucose and insulin has become better understood.

The isolation and characterization of the gene for PEPCK-C provided the opportunity to directly study the mechanisms involved in the effect of insulin on PEPCK-C gene transcription. Soon after the isolation of the gene, it was demonstrated, using "run off" transcription assays, that insulin decreased the rate of PEPCK-C gene transcription in hepatoma cells (Granner *et al.*, 1983) and in the livers of diabetic rats (Lamers *et al.*, 1982); the half-time of this effect was about 30 minutes. Magnuson and coworkers (1987) reported that insulin blocked the induction of transcription from the PEPCK-C gene promoter (+69 to −2100) by dexamethasone and cAMP in H4IIE hepatoma cells. An insulin response element (IRE) was later mapped at −416 to −407 in the PEPCK-C gene promoter. When this putative IRE was mutated, transcription from the PEPCK-C gene promoter in hepatoma cells was reduced by 50% (O'Brien *et al.*, 1990). This IRE lies within the glucocorticoid regulatory unit (GRU), overlapping an Accessory Factor binding region (AF2) that is required for glucocorticoid stimulation of PEPCK-C gene transcription (see Figure 2). When a transgene containing the PEPCK-C gene promoter in which the IRE is mutated was introduced into transgenic mice, the transgene in the liver was not induced by diabetes and the administration of insulin did not inhibit the transcription of the transgene gene (Lechner *et al.*, 2001). Since the mutation of the IRE removes the AF2 site, it is likely that the lack of induction of transcription from the modified transgene is due in part to the lack of stimulation by glucocorticoids.

Potential Mechanisms of Insulin Action of PEPCK-C Gene Transcription

Insulin regulates such a broad variety of cellular process that its effect on PEPCK-C gene transcription, while critical for metabolic control, is only a small part of its overall action on cellular processes. It is likely that PEPCK-C gene transcription is the target of a number of specific pathways that are initiated by insulin, and that this process involves a number of transcription factors that bind to a subset of sites in the gene promoter to

control its expression. The following section will review the possible pathways of insulin action and relates this to specific transcription factors that respond to insulin to inhibit PEPCK-C gene transcription.

Insulin Activation of the PI 3-Kinase Pathway

This pathway has been shown to be a major route of insulin action on the transcription of a variety of genes, including PEPCK-C. The insulin receptor is a tyrosine kinase which, when activated by insulin, phosphorylates members of the family of insulin receptor substrates (IRS). This leads to the recruitment of PI 3-kinase to the plasma membrane, where it is involved in the phosphorylation of PI (4,5) bisphosphate to PI (3,4,5)-P_3, the second messenger of the insulin receptor, as well as a variety of growth factor receptors (Barthel & Schmoll, 2003). Numerous studies have demonstrated that blocking the action of PI 3-kinase, using compounds such as Wortmannin and LY-294002, alters the inhibitory effect of insulin of PEPCK-C gene transcription (Sutherland *et al.*, 1995, 1998). A dominant negative mutant of PI-3 kinase, when introduced into cells via an adenovirus vector, also induces expression of the gene for PEPCK-C *in vivo* (Miyake *et al.*, 2002), suggesting that the PI 3-kinase pathway is a vital element in the inhibition of hepatic PEPCK-C gene transcription by insulin. PI(3,4,5)P_3 indirectly activates several members of the ACG super family of kinases. Of special interest is protein kinase B (PKB/Akt). Activation of PKB in hepatoma cells is sufficient to repress the glucocorticoid and cAMP induction of PEPCK-C gene transcription (Liao *et al.*, 1998; Agati *et al.*, 1998) and the over-expression of the gene for PKB in cultured hepatocytes decreases the levels of mRNA for PEPCK-C and G6Pase (Schmoll *et al.*, 2000). This suggests that the down stream targets of PKB, transcription factors such as Foxo-1, HNF-3β and CREB, are involved in the effect of insulin on the transcription of the genes for these two gluconeogenic enzymes. A number of studies support the importance of PKB in this process. For example, a deletion of the gene for PKBβ in mice results in insulin resistance and hyperglycemia due to an increased rate of hepatic gluconeogenesis, since the normal down-regulation of PEPCK-C and G6Pase by insulin is impaired (Cho *et al.*, 2001). However, Kotani, and coworkers (1999) have shown that a dominant negative form of PKB does not prevent insulin's inhibition of transcription for the PEPCK-C gene promoter in hepatoma cells. These authors concluded that PI-3 kinase is important for the insulin-induced inhibition of PEPCK-C gene transcription but that there are other down-stream factors that control this process that are distinct from PKB.

A major down-stream target of PKB is the transcription factor Foxo1. A homolog of Foxo1 was first identified in *C. elegans* as an insulin sensitive factor that was responsive to the PI-3 kinase analogue, AGE1 (Ogg *et al.*, 1997). The introduction of a dominant-negative variant of Foxo1 into the livers of mice, using an adenoviral vector, caused a decrease in PEPCK-C mRNA and hypoglycemia in the animals (Schmoll *et al.*, 2000). Foxo1 has been shown to bind to a putative insulin response element (AF2) in the PEPCK-C gene promoter and the over-expression of this transcription factor in hepatoma cells stimulated transcription from the promoter. Insulin blocks the activation of transcription from the PEPCK-C gene promoter by Foxo1 in hepatoma cells (Hall *et al.*, 2000). However, some caution would seem in order, since Hall and colleagues (2000) have demonstrated that the effects of insulin on Foxo1 inhibition of PEPCK-C gene transcription depend on an excess of Foxo1 over the concentration of the transcription factor normally found in cells. When Foxo1 is present at a normal cellular concentration, insulin repression of PEPCK-C gene transcription occurs in a Foxo1-independent fashion. It has been proposed that PKB phosphorylates Foxo1, repressing its stimulatory effect on PEPCK-C gene transcription, perhaps by facilitating its export from the nucleus. We have noted, using chromatin immunoprecipitation analysis of primary hepatocytes, that 15 minutes after insulin addition to the cells, the phosphorylated form of Foxo1 is bound to the AF2 and dAF2 sites in the PEPCK-C gene promoter (Cassuto, Chakravarty, Reshef, & Hanson, unpublished data). As noted above, there is evidence that the response to insulin is independent of the wild type levels of Foxo1 found in cells; the effect was noted only when the transcription factor was greatly over-expressed in hepatoma cells However, these studies were carried out with a shorter segment of the PEPCK-C gene promoter (−600 to +68). It is worth noting that, in other insulin regulated genes, such as G6Pase (Ayala *et al.*, 1999), more than one Foxo1 site was found to be required for an optimal transcriptional response to Foxo1 . The short form of the PEPCK-C gene promoter used by Hall and colleagues (2000) contains just a single Foxo1 site. In

our analysis of the promoter, we have noted that insulin addition to hepatocytes *in vitro* causes the binding of the phosphorylated form of Foxo1 to the AF2, as well as dAF2 sites (at −1350) (Cassuto, Chakravarti, Hanson, & Reshef, unpublished results). The potential importance of Foxo1 in the insulin-induced inhibition of PEPCK-C gene transcription will be discussed in more detail subsequently.

PGC-1α has been implicated in the control of transcription of genes involved in gluconeogenesis. It has been proposed that full transcriptional activation of the PEPCK-C gene promoter requires the interaction of PGC-1α with the glucocorticoid receptor and HNF-4α (Yoon *et al.*, 2001). Expression of the gene for PEPCK-C in hepatocytes prepared from mice that lack the gene for HNF-4α did not respond to PGC-1α that was introduced via a retroviral vector (Rhee *et al.*, 2003). PGC-1α binds to Foxo1; this interaction is also required for the stimulatory effect of PGC-1α on PEPCK-C gene transcription (Puigserver *et al.*, 2003). However, the effect of insulin on the interaction of these two proteins and its physiological consequences is not clear at the present time. It has been established that transcription of the gene for PGC-1α itself is stimulated by glucagon and glucocorticoids (Yoon *et al.*, 2001). Insulin does not alter expression of the gene for PGC-1α in the liver and does not block its rapid induction by glucagon. Thus the direct, repressive effect of insulin on PEPCK-C gene transcription is down-stream of PGC-1α.

Glycogen Synthase Kinase-3 (GSK-3)

Glycogen synthase kinase regulates glycogen metabolism by phosphorylating and thus inhibiting glycogen synthase. A major effect of insulin on the synthesis of glycogen is due to its effect on GSK-3. Insulin activates PKB, which in turn phosphorylates and inactivates GSK-3; this increases glycogen synthesis. A number of inhibitors of GSK-3 have been described that have the expected effect of increasing glycogen synthesis, but they also decrease hepatic glucose output in mice (Cohen & Frame, 2001). Inhibition of GSK-3 suppresses transcription of the genes for PEPCK-C and G6Pase in hepatoma cells (Lochhead *et al.*, 2001) however, the over-expression of the gene for GSK-3 in mice does not prevent the regulation of PEPCK-C gene transcription by insulin. Thus the exact pathway by which GSK-3 inhibits PEPCK-C gene expression is not clear and its possible action as an intermediate of insulin action on this gene remains to be determined.

AMP-Activated Kinase

The induction of AMP-activated kinase (AMPK) in hepatoma cells, using 5-Aminoimidazole-4-Carboximide Riboside (AICAR), decreases transcription from the PEPCK-C and G6Pase gene promoters in a manner similar to insulin (Lochhead *et al.*, 2000). AMPK has been shown to repress transcription of the gene for G6Pase in hepatoma cells by reducing the concentration of Foxo1. However, AMPK is sensitive to the energy status of the cell and is not known to be activated by insulin, making it likely that AMPK and insulin control PEPCK-C gene transcription by different, but perhaps converging pathways.

Insulin Control of the Translational Expression of Specific Isoforms of C/EBPβ

The transcription factor C/EBPβ plays an important role in the control of PEPCK-C gene transcription in the liver (Croniger *et al.*, 1998; Lechner *et al.*, 2001). Park and coworkers (1990) first demonstrated that the expression of the gene for C/EBPβ is induced by PKA in hepatoma cells. Two CREB-binding sites that appear to be involved in mediating a cAMP response via the PKA pathway have been identified in the C/EBPβ gene promoter (Niehof, 1997). C/EBPβ binds to several critical regulatory elements in the PEPCK-C gene promoter (see Figure 2) and is involved in the response of the gene to insulin (Duong *et al.*, 2002), cAMP (Park *et al.*, 1990) and glucocorticoids in the liver and the glucocorticoids in adipose tissue (Olswang *et al.*, 2003). Once bound to the DNA, C/EBPβ then binds to a specific domain in CBP, facilitating its interaction with RNA polymerase II.

C/EBPβ mRNA has five in-frame AUG codons within the coding sequence, which leads to the expression of at least three isoforms of C/EBPβ, (as detected in Western blotting), which are designated as A, B1, and C (Calkhoven *et al.*, 2000), corresponding to the products translated from the first three in-frame AUG codons. The major isoform, C/EBPβ B1 (a.k.a. LAP) is 34 kD, C/EBPβ C (a.k.a. LIP) is 20 kD and a minor isoform, C/EBPβ A, is 35 kD. C/EBPβ C (LIP) is a transcriptional inhibitor (Descombes & Schibler, 1991). Since these unique isoforms of C/EBPβ arise from the differential translation of C/EBPβ mRNA, it has been suggested that recruitment of the translation initiation machinery is one of the factors that alters the ratio of LIP (C) to LAP (B1) (C/B1) isoforms for

C/EBPβ (Calkhoven *et al.*, 2000). An elevated level of translation initiation, created by over-expressing a S51A mutant form of eIF2α (this mutant cannot be phosphorylated) caused an increase in C/B1 ratio (LIP/LAP) of C/EBPβ in 3T3-L1 cells (Calkhoven *et al.*, 2000). This finding suggests that the relative ratio of each isoform for C/EBPβ could be regulated by external stimuli, such as nutrients and hormones (for example, insulin) that have been shown to influence the phosphorylation of eIF2α in yeast (Dever *et al.*, 1992; Wek *et al.*, 1995; Rolfes & Hinnebusch, 1993) and mammals (Scheuner *et al.*, 2001).

Duong and colleagues (2002) have reported that insulin treatment of hepatoma cells caused an increase in the ratio of C/B1 isoforms for C/EBPβ; this occurred in a PI-3kinase-dependent manner (i.e., the effect of insulin could be blocked by the PI-3 kinase inhibitor, LY 294002). The ratio of the two isoform of C/EBPβ changed relatively rapidly after the addition of insulin to the cells, reaching a new steady state level after 90 minutes. They also showed that the C isoform of C/EBPβ abrogated the recruitment of CBP and polymerase II, once it bound to the PEPCK-C gene promoter. Studies in our laboratory (Yang & Hanson, unpublished work) have noted a dramatic increase in the C/B1 ratio for C/EBPβ in the livers of mice fed a high carbohydrate diet or in animals that were injected with insulin. In addition, the phosphorylated form of eIF2α decreased as the dose of insulin increased, while the total eIF2α did not change. These findings demonstrate that the administration of insulin induces an increase in the C/B1 ratio for C/EBPβ and also increases the availability of translation initiation machinery in mouse liver, as judged by the alteration in one component of the translation machinery, i.e., phosphorylated eIF2α. This effect is likely part of the adaptive response of PEPCK-C gene transcription to insulin. However, the effect is not rapid enough to account for the immediate response of the PEPCK-C gene to insulin; i.e., insulin causes a 50% reduction in the rate of PEPCK-C gene transcription within 30 minutes of hormone administration (Granner *et al.*, 1983), but the effect is intriguing for its physiological elegance and it is likely to be an important element (if not the dominant factor) in the insulin control of transcription of the gene in the liver.

SREBP-1

The transcription factor SREBP-1 and its isoform, SREBP-2, are critical for the control the expression of genes involved in lipogenesis (SREBP-1c) and cholesterol synthesis (SREBP-2). Recent evidence supports a role for SREBP-1c in the inhibition of transcription of genes involved in carbohydrate metabolism (Becard *et al.*, 2001; Chakravarty *et al.*, 2001, 2004; Yamamoto *et al.*, 2004). SREBPs are members of the basic helix-loop-helix leucine zipper family of transcriptional regulatory proteins, having a unique dual binding specificity. They can bind to SREBP regulatory elements (SRE) (Briggs *et al.*, 1993) or to an E-box motif (Kim *et al.*, 1995). There are two isoforms of SREBP-1, SREBP-1a and SREBP-1c, which are produced from a single gene on human chromosome 17p11.2, and differ only in their first exon (Brown & Goldstein, 1997). SREBP-2 is produced from a separate gene on human chromosome 22q13 (reviewed in Horton *et al.*, 2002). SREBPs are synthesized as precursors and then inserted into the membranes of the endoplasmic reticulum and nuclear envelope through a two-pass trans-membrane segment, resulting in their amino and carboxyl tails facing the cytoplasm (Brown & Goldstein, 1999). The mature form of SREBP-1 is generated by proteolytic cleavage within the plane of the bilayer; this cleavage responds to the concentration of sterols or to insulin (Brown & Goldstein, 1997; Foretz *et al.*, 1999) and results in the nuclear translocation of the mature transcription factor. The two isoforms of SREBP-1 are produced from two promoters on the SREBP-1 gene, each of which generates an mRNA with a different first exon, which codes for one of the specific amino termini of SREBP-1a or SREBP-1c. These alternate exons are attached to the rest of the mRNA during splicing, yielding the two isoforms of SREBP-1.

SREBP-1c is acutely activated by insulin, suggesting that it plays a critical role in coordinating the control of genes involved in carbohydrate, as well as lipid metabolism. The demonstration that SREBP-1c, when introduced into hepatoma cells (Chakravarty *et al.*, 2001), hepatocytes (Chakravarty *et al.*, 2001) or the livers of mice with an adenoviral vector (Becard *et al.*, 2001), inhibits transcription of the gene for PEPCK-C, further supports this concept of reciprocal regulation of the two opposing metabolic pathways by SREBP-1c. However, deletion of the individual genes for SREBP in mice has not provided a clear picture of the role that individual members of this family of transcription factors play in the control of carbohydrate metabolism (if any). For example, over-expression of the gene for SREBP-1 in the livers of mice, using the PEPCK-C gene

promoter, *does not* result in the predicted negative feedback on transcription from the promoter by SREBP-1 (Shimano *et al.*, 1997). However, the deletion of the gene for SREBP-1c resulted in a 2.9-fold increase in the concentration of PEPCK-C mRNA in the livers of these mice (Liang *et al.*, 2002). Yamamato and coworkers (2004) have recently reported that over-expressing the gene for SREBP-1a and SREBP-1c in the livers of mice causes a dramatic reduction of both PEPCK-C and G-6-Pase mRNA in the liver. Thus, there is evidence derived from the use of genetically modified mice that both supports and negates a role for SREBP-1 in the control of PEPCK-C gene transcription.

Our recent studies (Chakravarty *et al.*, 2004) on the control of PEPCK-C gene transcription, using hepatoma cells, strongly support a key role for SREBP-1c in the regulation of PEPCK-C gene transcription by insulin. We have shown that there are two SREBP regulatory elements (SREs) in the PEPCK-C gene promoter (−322 to −313 and −590 to −581) (Chakravarty *et al.*, 2004). The SRE at −590 overlaps an Sp1 site on the opposite strand of the DNA. These SREs bind SREBP-1a and SREBP-1c with low affinity, but the addition of purified upstream stimulatory activity (USA) enhanced the binding of SREBP-1 to both of these sites. Mutating either of these SREs increased PKA-stimulated transcription from the PEPCK-C gene promoter by about 20-fold. The SRE at −590 differs by a single base pair from the SRE in the LDL receptor gene (T in the PEPCK-C gene promoter at −582, compared to an A in the SRE of the gene for the LDL receptor promoter). Introduction of the LDL receptor SRE into the PEPCK-C gene promoter increased SREBP-1c binding and caused a 10-fold enhancement of basal transcription from the promoter, rather than an inhibition as observed with the SRE in the PEPCK-C gene promoter. The T/A change did not alter the binding of Sp1 to its site on the opposite strand of the DNA. Sp1 bound to the promoter in a manner that was independent of SREBP-1c but competed with SREBP-1c for binding. Sp1 does not bind to the SRE at −322. Chromatin immunoprecipitation analysis (ChIP), using rat hepatocytes, demonstrated that SREBP-1 and Sp1 were associated *in vivo* with putative regulatory regions corresponding to the SREs in the PEPCK-C gene promoter (Chakravarty *et al.*, 2004). These findings strongly suggest that insulin represses transcription of the gene for PEPCK-C by inducing SREBP-1c production in the liver, which interferes with the stimulatory effect of Sp1 at −590 of the PEPCK-C gene promoter.

In summary, it seems established that the response of the PEPCK-C gene transcription to insulin involves several transcription factors that bind to multiple sites on the PEPCK-C gene promoter and interact with co-activators (and perhaps co-repressors) to control transcription. Efficient repression of PEPCK-C gene transcription by insulin *in vivo*, involves all or several of binding sites, transcription factors and co-activators discussed in detail above. This is especially true for the liver, where insulin rapidly and totally represses PEPCK-C gene transcription. In contrast, insulin is only marginally effective in altering transcription of the gene for PEPCK-C in the kidney cortex. However, the liver, the kidney cortex, and the adipose tissue are all responsive to glucocorticoids. The molecular basis for the control of PEPCK-C gene transcription by glucocorticoids and its physiological consequences will be considered next.

PHYSIOLOGICAL CONSEQUENCES OF THE TISSUE SPECIFIC RESPONSE OF THE GENE FOR PEPCK-C TO GLUCOCORTICOIDS

Glucocorticoids and the Liver

Glucocorticoids play a fundamental role in the maintenance of energy homeostasis in mammals. The removal of the adrenals severely compromises the ability of animals to withstand fasting and greatly diminishes the symptoms that are characteristic of diabetes, such as an elevated concentration of blood glucose. A key aspect of this response is the control of PEPCK-C gene transcription by glucocorticoids in several target tissues. Glucocorticoids stimulate transcription of genes in the liver that leads to the elevated level of hepatic glucose output noted during fasting and diabetes. Adrenalectomy greatly blunts the effects of diabetes, in part by diminishing the normal induction of hepatic PEPCK-C gene transcription that is characteristic of this disease. Newborn rodents provide an interesting example of the critical role of glucocorticoids in the development of PEPCK-C gene transcription. At birth, rodents, like all mammals, have very low circulating levels of both insulin and glucocorticoids. It is possible to induce diabetes during the neonatal period in rats by injecting streptozotocin or anti-insulin

antibody, which results in hyperglycemia and glucosuria. Surprisingly, despite the clear diabetic phenotype that this treatment induces, the concentration of hepatic PEPCK-C mRNA does not increase. Thus, during the perinatal period the induction of transcription of gene for PEPCK-C in the liver is refractory to the factors that would normally induce diabetes in adults. However, the gene for PEPCK-C will respond to insulin, which, when administered together with glucose to newborn rats decreases the concentration of hepatic PEPCK-C mRNA to below basal levels (Benvenisty *et al.*, 1983). Transcription of gene for PEPCK-C in the livers of neonatal rats also responds normally to glucocorticoids (Benvenisty *et al.*, 1983). We can conclude from these studies that the induction of transcription of the PEPCK-C gene, which is characteristic of diabetes, requires glucocorticoids and that the low levels of glucocorticoids present in blood of newborn mammals results in a general resistance to the induction of diabetes at this critical stage in mammalian development.

Glucocorticoids and the Kidney

Glucocorticoids also control PEPCK-C gene transcription in the kidney cortex and in adipose tissue. In the kidney, glucocorticoids stimulate PEPCK-C gene transcription in coordination with metabolic acidosis (Hwang & Curthoys, 1991; Kaiser & Curthoys, 1991; Meisner *et al.*, 1985), whereas in the adipose tissue, glucocorticoids inhibit transcription of the gene (Nechushtan *et al.*, 1987; Olswang *et al.*, 2003). Renal gluconeogenesis is a major factor in long-term glucose homeostasis in mammals. Targeted ablation of the gene for PEPCK-C in the liver reduces its expression and the consequent hepatic gluconeogenesis to about 10% of control levels but it does not result in hypoglycemia in fasted mice and does not prevent the animals from developing diabetes (She *et al.*, 2000). This suggests either that 10% of normal hepatic PEPCK-C gene expression is sufficient to maintain glucose homeostasis or that renal gluconeogenesis can substitute for hepatic gluconeogenesis when PEPCK-C activity in the liver is insufficient. Owen and colleagues (1969 and 1967) have reported that in humans after prolonged fasting (greater than 3 days) the kidney contributes about half of the total glucose output. Clearly, renal gluconeogenesis is a very important factor in the maintenance of glucose homeostasis in mammals.

A Comparison of the Effect of Glucocorticoids in the Liver and the Adipose Tissue

The physiological significance of the differential control of PEPCK-C gene transcription in the liver and the adipose tissue is related to the key role of the enzyme in glyceroneogenesis in both tissues and in gluconeogenesis in the liver. PEPCK-C activity catalyzes the first committed step of both processes and is generally considered a key regulatory step in both pathways. The fact that glyceroneogenesis occurs in the liver as well as the adipose tissue, was appreciated relatively recently. Botion and colleagues (1998) first reported significant rates of glyceroneogenesis in the liver by studying rats that had been fed a high-protein diet. Later, Kalhan, and coworkers (2001) established the importance of glyceroneogenesis in the livers of fasted humans. They noted that after an over night fast, as much as 60% of the glyceride-glycerol found in the triglyceride of the plasma VLDL in the subjects was derived from glyceroneogenesis. The triglyceride/fatty acid cycle reflects the considerable rate of recycling of fatty acids that occurs in mammalian metabolism, due most likely to the inability of the lipolysis to provide the exact amount of fatty acid required for the use of tissues (such as skeletal and cardiac muscle) during fasting. The excess fatty acid that is not used for energy is reconverted to triglyceride and stored again in the adipose tissue. The energetic cost of such a seemingly "futile cycle" is from 3% to 6% of the total energy in the fatty acids that were mobilized during fasting. The liver thus plays a central role in this cycle; during fasting, the lipid that is not used by the peripheral tissues ends up in the liver, where it can be oxidized to carbon dioxide, used for the synthesis of ketone bodies metabolized by the brain and muscle, or converted back to triglyceride and released into the circulation as VLDL. It is important to note that glucose metabolism by the liver is negligible during fasting, so that the synthesis of the triglyceride requires an alternative source of 3-phosphoglycerol. Surprisingly, glycerol itself is not a major source of the 3-phosphoglycerol in the liver. Kalhan and colleagues (2001) noted that only 6% of glyceride-glycerol moiety of triglyceride in VLDL is derived from the glycerol released from adipose tissue; most of the glycerol taken up by the liver is converted to glucose via gluconeogenesis.

Glyceroneogenesis is important for triglyceride synthesis in both brown and white adipose tissue. White

adipose tissue does not express the gene for glycerol kinase, so it cannot make glyceride-glycerol from glycerol. As fasting progresses, the rate of glucose utilization by the adipose tissue drops markedly, due to a decrease in insulin levels in the blood, so that glucose is not a major source of 3-phosphoglycerol required to support fatty acid re-esterification (Vaughan, 1962; Reshef *et al.*, 1970). This underscores the importance of glyceroneogenesis in providing the 3-phosphoglycerol in adipose tissue during starvation. Support for the importance of this pathway has been provided by a number of recent studies in which the gene for PEPCK-C was either mutated or overexpressed in adipose tissue. A targeted mutation of the adipose tissue-specific enhancer (PPARE) of PEPCK-C gene promoter (a PPARγ2 binding site that maps at −1000 in the gene promoter), which selectively ablates gene expression in adipose tissue, caused a marked diminution of glyceroneogenesis in that tissue (Olswang *et al.*, 2002). Mice homozygous for this mutation lost lipid from adipose tissue even to the extent of lipodystrophy, attesting to the metabolic significance of PEPCK-C and glyceroneogenesis in the adipose tissue (Olswang *et al.*, 2002). Franckhauser and coworkers (2002) over-expressed a chimeric gene containing the PEPCK-C structural gene, linked to the aP2 promoter, causing obesity in adult mice. In brown adipose tissue, PEPCK-C has been shown to play a key role in the regulation of glyceroneogenesis, which is extensive in that tissue (Brito *et al.*, 1999). The metabolic significance of glyceroneogenesis in brown adipose tissue has not been fully established, but it is likely to control the rate of fatty acid availability for mitochondrial oxidation in the tissue during cold exposure.

Glyceroneogenesis and the key enzyme in the pathway, PEPCK-C, thus play an important role in maintaining lipid homeostasis in both liver and adipose tissue underlining the significance of the co-ordinate regulation of PEPCK-C gene expression in both tissues. Glyceroneogenesis affects lipid metabolism in opposite ways in the two tissues; it restrains fat release from adipose tissue (Reshef *et al.*, 1970) and enhances it from the liver (Martin-Sanz *et al.*, 1990). It has been shown that adrenalectomy enhances glyceroneogenesis in rats and diminishes FFA release from epididymal adipose tissue *in vitro* (Reshef *et al.*, 1969b, 1970). The addition of dexamethasone to cultured hepatocytes stimulated the synthesis of triglycerides and apolipoproteins E and Bind, as well as stimulating the release of VLDL (Martin-Sanz *et al.*, 1990). We have previously suggested that the reciprocal regulation of PEPCK-C gene transcription by glucocorticoids provides a mechanism for such coordination, because it represses PEPCK-C gene transcription in the adipose tissue and simultaneously enhances it in the liver. The result is an increase in the rate of hepatic glucose output and a decrease in the rate of fatty acid re-esterification to triglyceride in the adipose tissue, providing fuel for the metabolism of the brain, red blood cells and muscle.

MODELS FOR THE METABOLIC CONTROL OF PEPCK-C GENE TRANSCRIPTION IN THE LIVER, ADIPOSE TISSUE AND KIDNEY

Transcription of the gene for PEPCK-C gene is characterized by its differential hormonal and nutritional control in the liver, kidney, and adipose tissue. A number of models have been proposed to explain this differential regulation. These models integrate the effect of several regulatory elements in the PEPCK-C gene promoter areas (often referred to as regulatory units). In this review, we will present a general schema for the transcriptional control of the gene for PEPCK-C in all three of these tissues, focusing in the role of major hormones that induce transcription (glucocorticoid and cAMP in the liver) and inhibit (insulin) gene transcription. We will also discuss the role of glucocorticoids in repressing PEPCK-C gene transcription in adipose tissue and factors such as glucocorticoids and metabolic alkalosis/acidosis that controls transcription of this gene in the kidney. The positive regulation of transcription of the gene for PEPCK-C in the liver involves the CRU and the GRU and the dominant and rapid repression caused by insulin in the presence of glucose. The information available on the mechanism of the hormonal control of PEPCK-C gene transcription in the kidney cortex and adipose tissue, while extensive, is not at the same level as our knowledge of the control of transcription of the gene in the liver. Despite this, we will summarize the findings from several laboratories on the control of PEPCK-C gene transcription in these three tissues.

Basis for the Proposed Model for the Regulation of PEPCK-C Gene Transcription in the Liver

The tissue-specific basis of control of transcription of the gene for PEPCK-C and its metabolic role have been

best studied in the liver, where the enzyme is involved in gluconeogenesis and glyceroneogenesis. The following section reviews the current information on the transcription factors that regulate hepatic PEPCK-C gene transcription under the control of specific hormones and the organization of the PEPCK-C gene promoter that permits its response to these factors; this information forms the basis for the proposed model for the regulation of transcription of the gene.

The cAMP Regulatory Unit (CRU)

The stimulation of PEPCK-C gene transcription by cAMP is a critical factor in the response of the gene in the liver to starvation, since a lowered concentration of insulin results in elevation in the cAMP levels and a subsequent activation of PKA. Roesler and colleagues (2000) have developed a model for the cAMP control of PEPCK-C gene transcription that involves a cAMP regulatory unit (CRU), which encompasses five CREs that have been described in the PEPCK-C gene promoter. Three of these CREs map in *Region 3* of the gene promoter and interact with the CRE in *Region 1*, a distance of several hundred base pair. Since a number of transcription factors can bind to these sites in the CRU (see above for details), and all are present in the liver, their specific interaction on the promoter is considered as a critical element in determining the response of the PEPCK-C gene to changes in the concentration of hepatic cAMP. Moreover, it has long been documented (Meisner *et al.*, 1983, 1985) that the response of the PEPCK-C gene to cAMP in the liver is tenfold higher than the response of the gene in the kidney. As noted previously, CREB, C/EBPα and C/EBPβ, have all been shown to regulate the response of the PEPCK-C gene promoter to cAMP. The Fos/Jun hetero dimer also binds to the CRE (Gurney *et al.*, 1992). However, the overall cAMP responsiveness of the gene depends on which transcription factor occupies specific *cis*-elements within the CRU, as well as in the interaction of these transcription factors with co-activator proteins that coordinate the transcriptional response. The synergistic response of the hepatic PEPCK-C gene is based on cooperation between C/EBPβ bound to P3(I) site (see Figure 2) and other factors that also bind to the CRE site. It is unlikely that such synergism occurs during transcription of the gene for PEPCK-C in the kidney cortex, a tissue that contains little C/EBP (Berkenmeier *et al.*, 1989). It has also been suggested that the hepatic response to cAMP is controlled by the phosphorylation status of the individual transcription factors, altering their affinity for co-activators such as CBP and PGC-1α (Yoon *et al.*, 2001).

The Glucocorticoid Regulatory Unit (GRU)

The concept of a large and complex GRU, rather than a single GRE in the PEPCK-C gene promoter, was introduced by Granner and colleagues (Imai *et al.*, 1990; Scott *et al.*, 1998; Wang *et al.*, 1996). They first described the existence of a GRU in the PEPCK-C gene promoter, using rat hepatoma cells, and then characterized this unit using a relatively short segment of the PEPCK-C gene promoter (−600 to +68) (Imai *et al.*, 1990). The GRU co-ordinates the hormonal response of the PEPCK-C gene to glucocorticoids and insulin and interacts with the CRU to mediate the cAMP control of gene transcription. The complexity of this regulation is underscored by the suggestion that the AF1 and AF3 sites in the GRU are also part of the CRU and are required for cAMP stimulation of transcription from the PEPCK-C gene promoter (Yamada *et al.*, 1999). Thus, members of the C/EBP family and/or CREB could bind to the AF3 site in the GRU (as well as to the down-stream CREs) and stimulate gene transcription. The various accessory sites function in a context-specific manner and cannot substitute for each other at different positions in the PEPCK-C gene promoter. However, in HepG2 hepatoma cells only a segment of the PEPCK-C gene promoter from −2,000 to +73 responds transcriptionally to glucocorticoids, whereas the shorter form of the gene promoter (−600 to +73) is not induced by these hormones (Cassuto, Hanson, & Reshef, unpublished results). The requirement of additional up-stream sequences (from −660) in the PEPCK-C gene promoter for the full response to glucocorticoids is due to additional accessory factor binding sites (dAF1 and dAF2) in the gene promoter that are involved in the control of PEPCK-C gene transcription by glucocorticoids and insulin (Cassuto, Hanson, & Reshef, unpublished results). In this model, glucocorticoids regulate hepatic PEPCK-C gene transcription via the extended GRU, which includes specific interactions between the GR, C/EBPβ, NF-3β, Foxo1, and co-activators; the accessory factor sites dAF2 and AF2 and dAF1, AF1 and AF3 are all required for the assembly of this multi-protein complex.

Insulin Regulation

As noted above, insulin regulation of PEPCK-C gene transcription involves several regulatory elements in the gene promoter. The IRE that was originally described by O'Brien *et al.* (1990) is within AF2, and is repeated in the dAF2; both of these elements bind HNF3β and Foxo1 and the activity of both is thus potentially regulated by PI3-kinase via PKB. The ratio of the isoforms of C/EBPβ (LIP/LAP ratios) is also controlled in a PI3 kinase dependent manner. However, it is important to note that there is no evidence demonstrating that C/EBPβ binds directly to the AF2 site in the PEPCK-C gene promoter; an interaction probably exists between C/EBPβ and other transcription factor that bind to the DNA. Finally, SREBP-1c inhibits PEPCK-C gene transcription by binding to the SRE at −590 and competing with Sp1 (Sp1 stimulates of PEPCK-C gene promoter), which also binds at the SRE. Each of the above sites responds independently to insulin adding to the complexity of the control of transcription of this gene.

Model for the Hormonal Control of Hepatic PEPCK-C Gene Transcription

The following model is based on the information on PEPCK-C gene transcription that is reviewed above and on recent work from our laboratories (Cassuto, Chakravarty, Reshef, & Hanson, unpublished results), which is shown in Figure 3. A major feature of the model is the proposed extension of the GRU to −1500 in the PEPCK-C gene promoter to include a regulatory site at −1365, which lies within a DNase I hypersensitive region. We term this site dAF2, based on its structural similarity to the AF2 site in the GRU; the two sites bind HNF-3β. The PPARα/RXR binding site at −1000 (which we term dAF1) is also included in the GRU. Mutation of the dAF1 and dAF2 sites (or in any of the previously described accessory factor binding sites) markedly reduces the response of the PEPCK-C gene promoter to glucocorticoids. This indicates that the GRU is a more global regulatory element in which all of the sites act in a synergistic manner to control transcription from the PEPCK-C gene promoter in the liver in response to hormones. In addition, the GRU and the CRU work together to control PEPCK-C gene transcription in the liver. The proposed model covers the following physiological states and the pattern of transcription factor binding.

Basal State

In the absence of added hormones, the CRE (−80) on the PEPCK-C gene promoter is not occupied by the transcription factors C/EBPα or C/EBPβ, while C/EBPβ is bound to the P3(I) site. The AF2 site binds HNF4α, while the SREBP-1 site at −590 is occupied by Sp1.

cAMP

The addition of Bt_2cAMP to hepatocytes results in the occupation of the CRE (−80) with C/EBPα the P3(I) site with C/EBPβ and the AF2 site in the GRU with a complex with Foxo1 and the GR. The effect of Bt_2cAMP on transcription factor binding to the other upstream sites has not yet been determined. Interestingly, the addition of Bt_2cAMP completely ablates the weak binding of both P-Foxo1 and HNF-4α to the AF2 site.

Glucocorticoids

Dexamethasone addition to hepatocytes increases the extent of C/EBPα binding to the CRE (−80) over that noted in the basal state and causes a significant induction of GR, PPARα and P-Foxo1 binding to the AF2 site in the GRU. The detection of PPARα, which does not bind to the AF2 site, by ChIP analysis is most likely due to an interaction with a transcriptional complex, which contains factors that bind to the AF2 site. In addition, there is strong induction of binding of C/EBPβ to the P3(I) site in the PEPCK-C gene promoter. It should be noted that the ChIP analysis, shown in Figure 3, cannot distinguish between C/EBPβ binding to the P3(I) site or an interaction between C/EBPβ with the transcription factors and co-activators present as a complex at the AF2 site. Since there is considerable evidence that C/EBPβ binds to the P3(I) site, the model shown in Figure 4 indicates binding to this site in response to glucocorticoids. Glucocorticoid treatment of hepatocytes totally ablates HNF-4α binding to both the AF2 and dAF2, but it induces HNF-4α binding to the dAF1 site in the PEPCK-C gene promoter (binding to the dAF2 site is not shown in Figure 3). As expected, the binding of GR to the GRE is greatly increased by dexamethasone. Weak binding of Foxo1 to the AF2 and the dAF2 sites in the GRU is detectable after dexamethasone addition. P-Foxo1 binds to the AF2 site in the presence of glucocorticoids, at about the level noted with untreated hepatocytes.

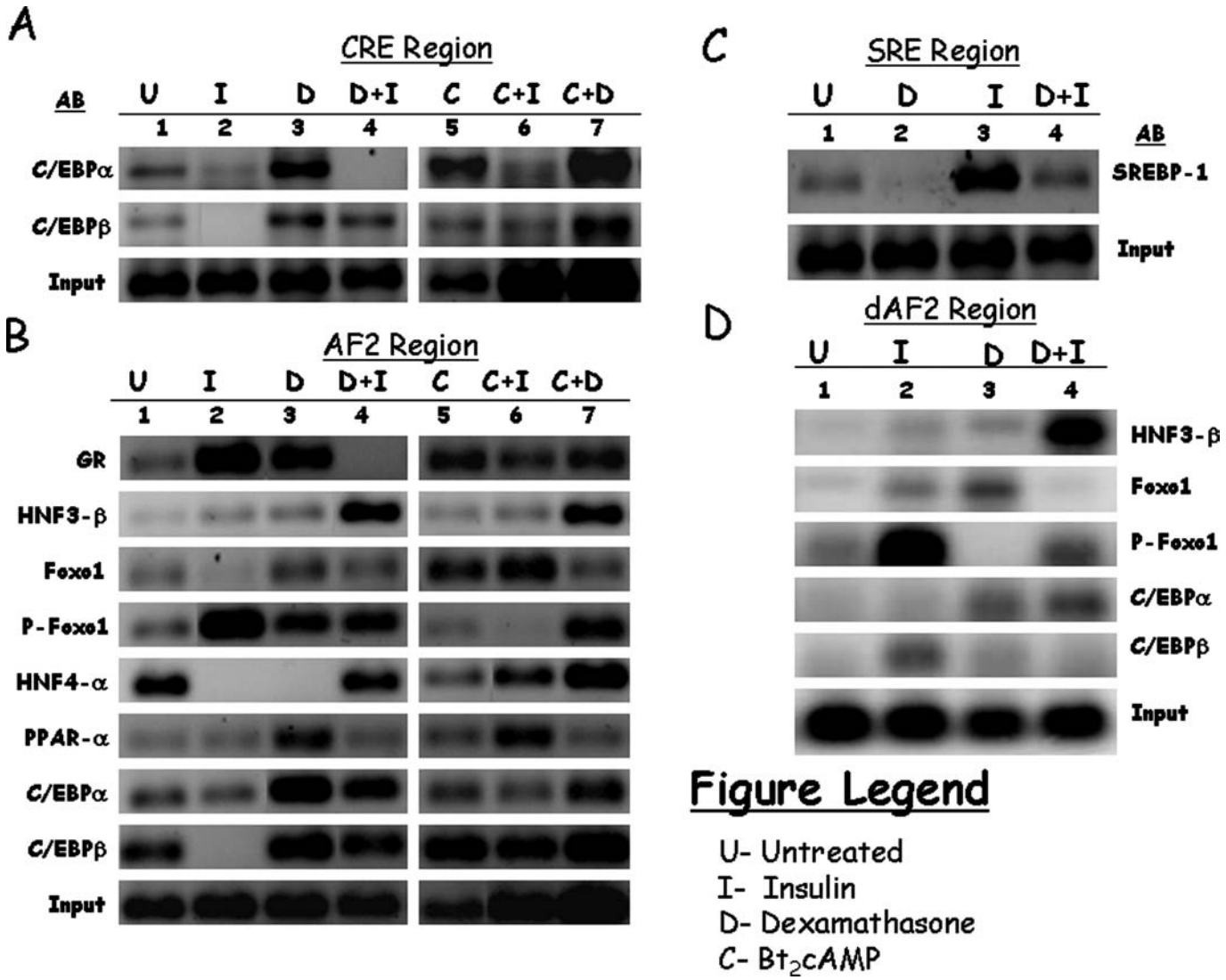

FIGURE 3 **Chromatin Immunoprecipitation (ChIP) analysis of transcription factor binding to the PEPCK-C gene promoter in rat hepatocytes treated with various hormones. Hepatocytes were isolated from rats fasted for 24 h and incubated for 15 minutes with insulin, dexamethasone, insulin, wither alone or in combination; control cells were untreated. ChIP analysis was performed as detailed by Chrakravarty and coworkers (2004), using antibodies against the transcription factors indicated in the figure. The specific regions of the PEPCK-C gene promoter that were amplified by PCR are the CRE (−80), the AF2 (−425), the SRE (−590) and the dAF2 (−1200 to −1400).**

Bt_2 cAMP Plus Glucocorticoids

The combination of Bt_2cAMP and dexamethasone strongly stimulates PEPCK-C gene expression in hepatocytes (Salavert & Iynedjian, 1982). This is caused by a greatly increased binding of C/EBPα and C/EBPβ to the CRE (−80) and P3(I). HNF-4α also binds to the AF2 region (the possible role of the dAF2 was not analyzed as yet by ChIP assay). The GR is bound to the GRE in the AF2 region of the PEPCK-C gene promoter, although we noted binding of the GR in the presence of Bt_2 cAMP alone, the addition of dexamethasone did not increase the binding. It is likely that under these conditions, the presence of glucocorticoids both increases the rate of transcription over the basal state and renders the promoter "competent" for a synergistically increased response of gene transcription in the presence of Bt_2 cAMP plus glucocorticoids, as was suggested by Salavert and Iynedjain (1982).

Insulin

Insulin strongly inhibits PEPCK-C gene transcription and, as expected, has a very dramatic effect on the pattern of transcription factor binding to the gene promoter. In the presence of added insulin (without dexamethasone) the binding of C/EBPα and C/EBPβ to the CRE (−80) and to the P3(I) region is totally abolished. There is an increase (over basal) in GR binding to the GRE and of the binding of P-Foxo1 to the

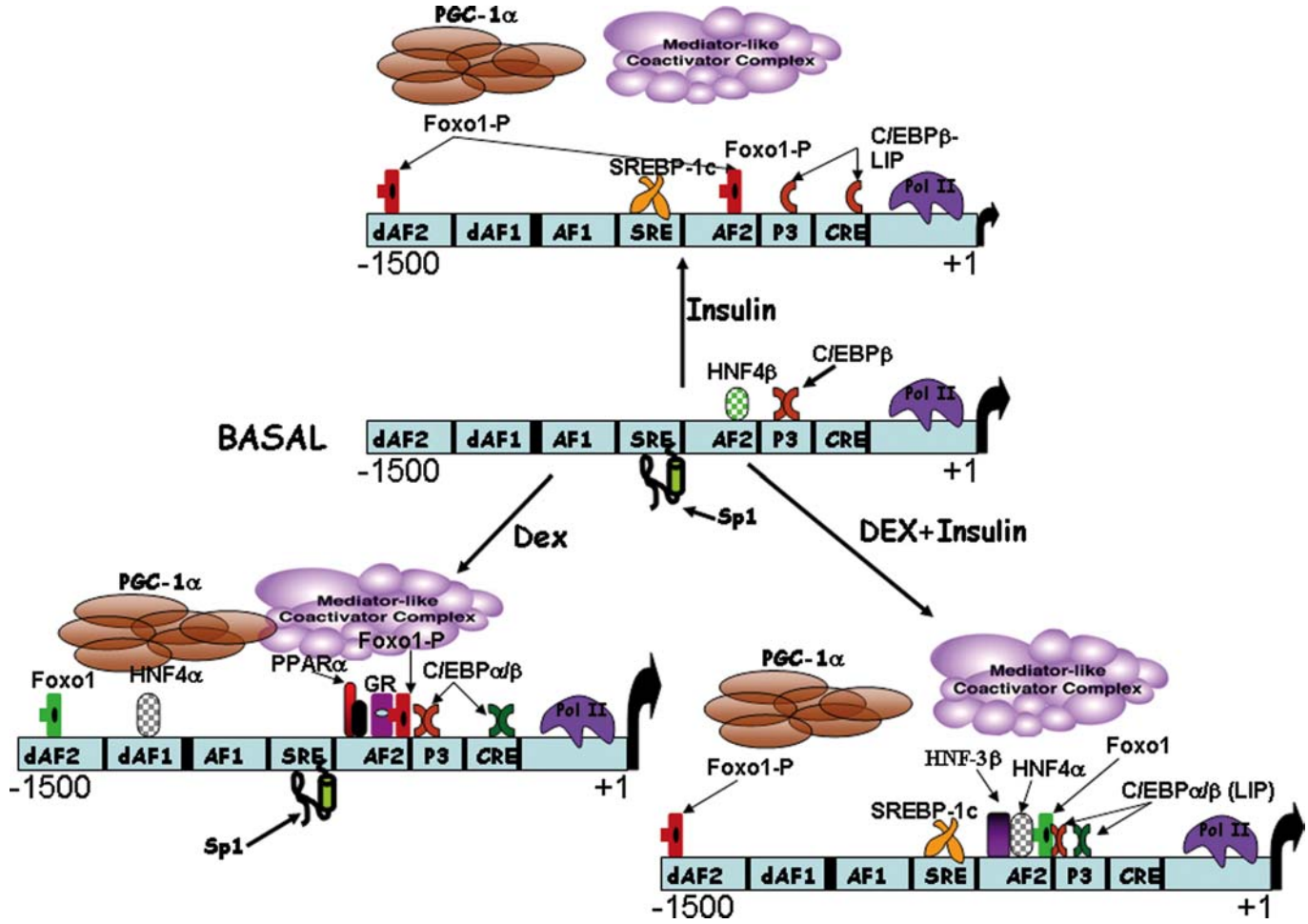

FIGURE 4 **Model for the control of hepatic PEPCK-C gene transcription by dexamethasone and insulin. The figure shows a segment of the PEPCK-C gene promoter from +1 to −1500 that has been implicated in the control of PEPCK-C gene transcription by dexamethasone and insulin, and is based on the ChIP analysis in Figure 3, as well as work by us and others that is described in detail in this article. The size of the arrow at the 3′-end of the promoter segment indicates the overall rate of PEPCK-C gene transcription.**

In the *basal state* (no hormonal additions), the C/EBP isoforms (C/EBPα and β) were not bound to the CRE at −80 and a weak binding of C/EBPβ to the P3(I) site and HNF-4α to the AF2 region was observed. No co-activators are shown associated with the PEPCK-C gene promoter, since the rate of transcription of the gene is at a low basal level. *Dexamethasone* addition markedly increases PEPCK-C gene transcription. It also alters the binding of transcription factors to the CRE at −80 and the AF2, dAF2 and dAF1 regions of the PEPCK-C gene promoter. These include GR, P-Foxo1, PPARα, C/EBPα and C/EBPβ all of which bind to the AF2, HNF-4α which binds to the dAF1 and Foxo1 to the dAF2. In the presence of dexamethasone, the co-activator PGC-1α and other co-activators involved on coordinating gene transcription are shown associated with the transcription factor complex. We detected C/EBPα binding to the CRE at −80 in response to dexamethasone addition. *Insulin* addition markedly inhibits PEPCK-C gene transcription. There is strong binding of P-Foxo-1 to the AF2 and dAF2 sites and binding of SREBP-1c to the SRE on the PEPCK-C gene promoter 15 minutes after insulin addition. There is also a loss of C/EBPβ binding to the P3(I) site in the AF2 region. This is likely due to the insulin-induced increase in the LIP/LAP ratio, which prevents C/EBPβ binding to the transcription complex at the AF2 site (Duong *et al.*, 2002). PGC-1α and other co-activators are dissociated from the transcription factor complex. *Dexamethasone plus insulin* addition to the hepatocytes reduces the induction of PEPCK-C gene transcription by dexamethasone. Insulin totally ablates the binding of C/EBPα to the Cre at −80 noted when dexamethasone was present alone. We also noted that with both insulin and dexamethasone present, P-Foxo1 binds to the dAF2 and dAF1 sites on the PEPCK-C gene promoter. HNF-4α, HNF-3β, Foxo1 and C/EBPβ were all found to be associated with the complex of transcription factors that bind to AF2 DNA site in association with Foxo1. There was no detectable binding of SREBP-1 to the SRE at −590. Since insulin inhibits PEPCK-C gene transcription, even in the presence of dexamethasone, we show the co-activator complexes as being dissociated from the transcription factors on the gene promoter.

AF2 dAF1 and dAF2 sites of the PEPCK-C gene promoter. Insulin addition to the hepatocytes also results in strong of binding of SREBP-1c to the SRE at −590 in the PEPCK-C gene promoter. Conversely, HNF-4α binding to the AF2 is ablated in the presence of insulin (Figure 2). It has been shown by others that insulin induces the phosphorylation of Foxo1 and disrupts its binding to the co-activator PGC-1α (Yoon *et al.*, 2001), contributing to the inhibition of hepatic PEPCK-C gene transcription. Insulin also increases the nuclear

content of SREBP-1c, resulting in the observed increase in the binding of SREBP-1 to the SRE at −590 (and displacement of Sp1 from the gene promoter), which inhibits PEPCK-C gene transcription.In our model, *both* SREBP-1c and P-Foxo1 are involved in the insulin-induced inhibition of PEPCK-C gene transcription. Insulin also clearly prevents the binding of C/EBPα and C/EBPβ to their respective sites on the PEPCK-C gene promoter. The effect of insulin of C/EBPβ binding to the AF2 site is most likely due to an increase in the ratio of C/EBPβ C/B1 (LIP/LAP) caused by insulin (Duong *et al.*, 2002), as discussed in detail above.

In the ChIP analysis presented in Figure 3, we noted transcription factor binding in response to hormones that needs further experimental verification for explanation. For example, the GR is bound to the GRE of the PEPCK-C gene promoter in the presence of insulin. This analysis should be extended to other segments of the gene promoter to test for the presence of co-activators and co-repressors that may also affect the control of transcription. The kinetics and the order of addition of the various transcription factors to the PEPCK-C gene promoter in the liver also remains to be determined. Finally, the role of histone modification and the activity of co-activators and co-repressors in this system have not been established in any detail. The co-activators CBP/p300, SRC-1 and PCG-1α have all been implicated in the control of PEPCK-C gene transcription (see Figure 1) and other factors are surely to be identified in future studies.

Model for the Glucocorticoid Regulation of PEPCK-C Gene Transcription in Adipose Tissue

PEPCK-C is a single copy gene, with a single promoter that is responsible for the control of transcription of the gene in the various tissues in which it is expressed (Hanson & Reshef, 1997). The reciprocal control of PEPCK-C gene transcription by glucocorticoids in adipose tissue and liver requires a tissue-specific interplay of transcription factors with differential effects on gene expression in each tissue. The combination of transcription factors that control the glucocorticoid inhibition of PEPCK-C gene transcription in adipose tissue are C/EBPα and/or C/EBPβ, the GR, PPARγ2 and COUP-TFII. It is well established that C/EBPα is required for the differentiation of adipose tissue (Christy *et al.*, 1991; Samuelsson *et al.*, 1991; Yeh *et al.*, 1995) and activates transcription from the PEPCK-C gene promoter (Roesler *et al.*, 1998; Benvenisty & Reshef, 1991). The possibility that one isoform of C/EBP could substitute for another was tested by Lee and colleagues (1997) who inserted the C/EBPβ structural gene into C/EBPα gene locus (its transcription was driven by the C/EBPα gene promoter) in mice. The animals had normal hepatic function but lacked white adipose tissue, supporting the key role of C/EBPα in adipose tissue differentiation. A deletion of the gene for C/EBPβ in mice caused a rapid loss of triglyceride during starvation and a blunted effect of glucocorticoids on PEPCK-C gene transcription in adipose tissue (Croniger & Hanson, unpublished results). This strongly suggests that C/EBPβ is involved in the response of the gene to glucocorticoids *in vivo*.

Mutating any of the known regulatory elements in the GRU of the PEPCK-C gene promoter (AF1, AF2, GRE1, or GRE2) enhances the induction of transcription from the gene promoter by C/EBPα by three- to fourfold. Despite these mutations in the gene promoter, transcription was repressed by GR in a manner similar to the native gene promoter (Olswang *et al.*, 2003). It thus appears that the GR can exert its negative effect on PEPCK-C gene transcription without binding to the gene promoter; a mutated form of the GR (mutated at its DNA binding domain) is equally effective in repressing C/EBPα-induced gene transcription. However, a mutation of AF1 blocked the inhibitory effect of the mutated GR (Olswang *et al.*, 2003). This underlines the unique role of the AF1 site in the glucocorticoid inhibition of PEPCK-C gene transcription in adipose tissue. This site also binds the nuclear receptor COUP-TFII which, if overexpressed in fibroblasts, can block the stimulation of transcription for the PEPCK-C gene promoter by the PPARγ2/RXR heterodimer (Eubank *et al.*, 2001). Eubank *et al.* (2001) proposed that COUP-TFII inhibits the premature induction of PEPCK-C gene transcription in preadipocytes by binding to the AF1 site in the GRU.

There are several possible mechanisms by which the GR could exert its effect on PEPCK-C gene transcription in adipocytes. It most likely interacts with members of the C/EBP family of transcription factors to repress PEPCK-C gene transcription, either by reducing the concentration of C/EBPα in adipocyte nuclei (Olswang *et al.*, 2003) or by interfering with the binding of nuclear proteins to the C/EBP recognition sites in the DNA (Figure 5). Since an intact AF1 site in the

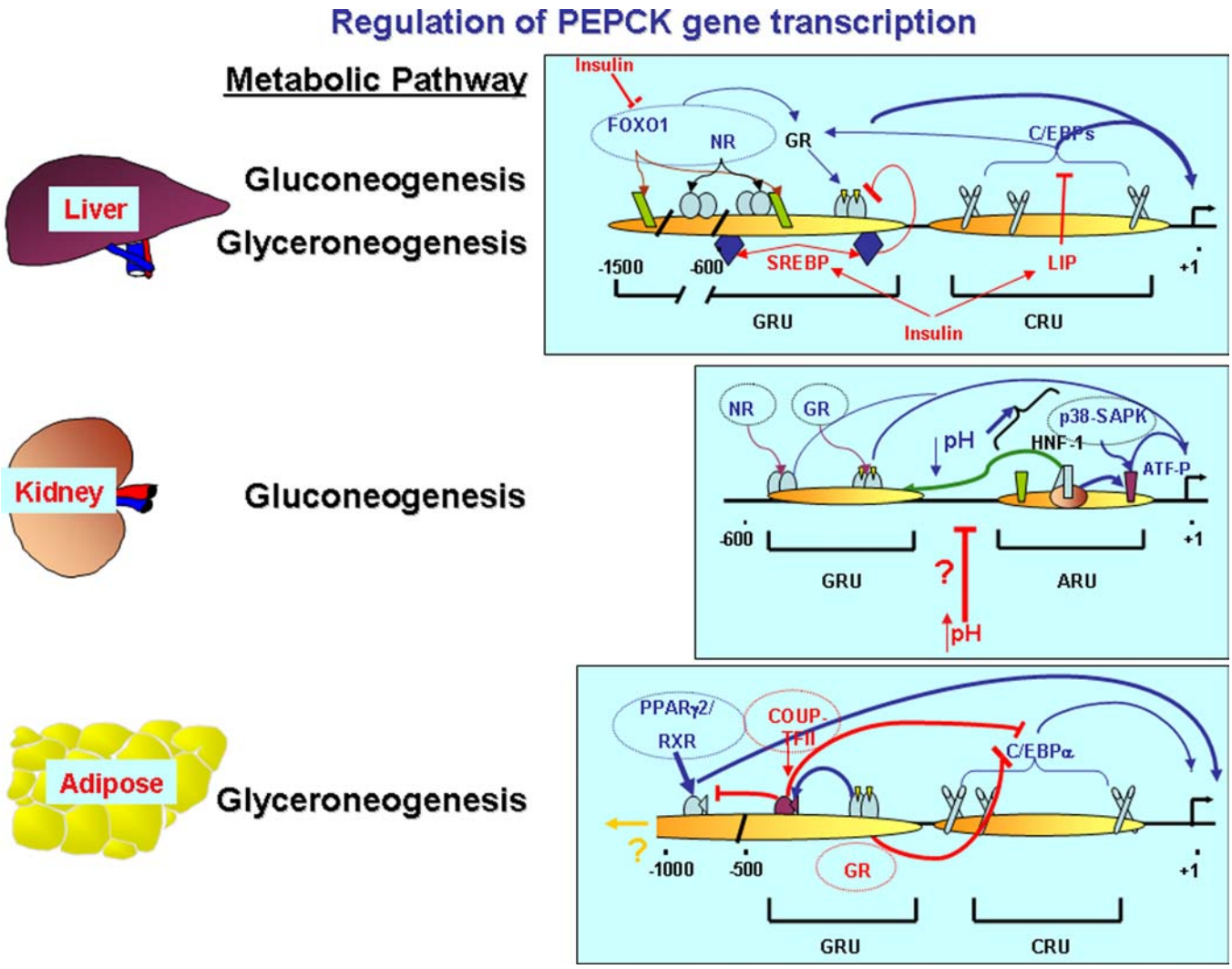

FIGURE 5 **Model for the tissue-specific control of PEPCK-C gene transcription by dexamethasone and insulin in the liver, adipose tissue and kidney. *Hepatic* PEPCK-C (*upper panel*) participates in both gluconeogenesis and glyceroneogenesis. PEPCK-C contains an extended GRU that extends from positions −360 to −1500 upstream of the transcription start site of the PEPCK-C gene. It contains two copies of the components of the "classic" GRU; two low affinity adjacent GRE, two Foxo1 binding sites (termed AF2 (at −410) and dAF2 at −1365 and two nuclear receptors (NR) binding sites (AF1 at −425 and dAF1 at −1000). The extended GRU requires both AF1 and AF2 sites for the optimal, synergistic response of the PEPCK-C gene promoter to GR and NR nucleus receptors in the liver. Insulin affects many target sites within the gene promoter. It interferes with the Foxo1 transcription factors themselves and enhances the phosphorylation of Foxo1 and facilitates the binding of the transcriptional inhibitors SREBP1c (at −590 and −322) and increases the LIP/LAP ratio. This leads to insulin inhibiting the hepatic gene transcription of the gene for PEPCK-C.**

In the *kidney cortex*, the transcription of the gene for PEPCK-C is controlled primarily by the "classic" GRU and by a proximal ARU (acidosis regulator unit) that responds to metabolic acidosis. Each of the two regulatory units are associated with the occupied HNF-1 binding site at position −190 of the gene promoter. Two additional sites participate in the ARU, harboring basic leucine zipper type of proteins (Cassuto *et al.*, 2003; Taylor & Curthoys, 2004). The gene for PEPCK-C in the kidney is expressed in the proximal tubules, and is marginally responsive to insulin. There is considerable evidence that alkalosis inhibits transcription of the gene for PEPCK-C in the kidney, but the molecular mechanism for the effect is as yet unknown.

PEPCK-C in *adipose tissue* participates in glyceroneogenesis. As mentioned above, the PEPCK-C gene promoter contains an extended GRU, and each of its components are involved in the glucocorticoid inhibition of gene transcription, as well as stimulation of gene transcription by C/EBP. The GR inhibits C/EBP stimulated PEPCK-C gene transcription, by reducing the nuclear content of C/EBP, as well as by inhibiting its binding to the DNA (Olswang *et al.*, 2003). The PPAR/RXR binding site (dAF1) is required for PEPCK-C gene transcription in adipose tissue, but it is not yet clear whether the trans-activation of the gene promoter by these factors is inhibited by GR.

GRU of the PEPCK-C gene promoter is required for the inhibitory effect of glucocorticoids on PEPCK-C gene transcription, this site may be involved in the docking of C/EBPα prior to its interaction with a co-repressor that has been recruited by the GR. Alternatively, the GR could interfere with the binding of C/EBPα to a co-activator that is required for PEPCK-C gene transcription in the adipose tissue. Finally, since

COUP-TFII binds to the AF1 site and can inhibit PPARγ2-stimulated PEPCK-C gene transcription, it is possible that C/EBPα could displace COUP-TFII by a process that is impeded by the presence of the GR. All of these possibilities are directly testable.

Model for the Regulation of PEPCK-C Gene Transcription in the Kidney

A major response of mammals to diabetes is an increase in metabolic acidosis. This condition leads to an increase in the excretion of ammonium ions into the urine to titrate the acidity of the tubular urine. As mentioned in detail above, the source of the ammonium ions is the glutamine that is generated by muscle and transported in the blood to the kidney. The glutamine carbon skeleton (α-ketoglutarate) is disposed of by an increase in renal gluconeogenesis, due in part to an induction of transcription of the gene for PEPCK-C by metabolic acidosis. Transcription of gene for PEPCK-C in the kidney cortex is stimulated by acidosis and inhibited by alkalosis (Taylor & Curthoys, 2004). This response of the gene is unique to the kidney; transcription of the gene for PEPCK-C in the liver or adipose tissue does not respond to changes in pH. The gene is also induced in the kidney cortex by glucocorticoids (Meisner *et al.*, 1985), which increase both hepatic and renal gluconeogenesis during starvation and diabetes.

The concentration of PEPCK-C mRNA in the kidney cortex is rapidly induced by chronic acidosis in rats; by 7 hours there is a six-fold increase in specific PEPCK-C mRNA (Hwang & Curthoys, 1991). The effect of acidosis can also be observed in LLC-PK_1 kidney cells by lowering the pH of the culture medium (Kaiser & Curthoys, 1991). The PEPCK-C gene promoter has been extensively analyzed, both in cells and in transgenic mice, to determine the factors that are involved in this response to acidosis and to glucocorticoids (reviewed in Taylor & Curthoys, [2004] for a review). Transgenes containing the intact PEPCK-C gene promoter from −2,000 to +72 or from −460 to +73, linked to a reporter containing either the human or bovine growth hormone structural gene were found to be active in liver but were poorly expressed in the kidney of mice (Eisenberger *et al.*, 1992). A segment of the PEPCK-C gene promoter from –363 to +1, linked to the entire PEPCK-C structural gene, was expressed at normal levels in the kidney of transgenic mice and responds well to acidosis (Eisenberger *et al.*, 1992). This raises the possibility that an acidosis-induced change occurs in the stabilization of renal PEPCK-C mRNA, such as occurs with the mRNA for glutaminase (Hansen *et al.*, 1996) or that a regulatory site is responsive to acidosis with in the structural gene.

The HNF-1 binding site (P2) in the PEPCK-C gene promoter is a critical element for the kidney-specific transcription of the gene for PEPCK-C (Cassuto *et al.*, 1997). Deletion of the this site in the PEPCK-C gene promoter resulted in a markedly diminished expression of a chimeric transgene in the kidney of mice (Patel *et al.*, 1994). When this site is mutated, basal transcription from the promoter was markedly decreased but it still responded to acidosis. Individual mutations in the CRE or in the P3(II) site in the PEPCK-C gene promoter reduced the induction of transcription by acidosis by 40% in transfection assays (Cassuto *et al.*, 1997), suggesting that multiple sites in the promoter are responsible for the inhibitory effect of acidosis (Figure 5). Feifel and colleagues (2002) reported that the transfer of the LLC-PK_1-F^+ kidney cells to an acidic medium resulted in the induction of p38 stress activated kinase (SAPK). This leads to the phosphorylation (and activation) of the transcription factor ATF-2, which then binds to the CRE of the PEPCK-C gene promoter, a site needed for the full transcriptional response of the promoter to acidosis (Feifel *et al.*, 2002), and stimulates gene transcription. SB-203580, an inhibitor of p38 activation, blocked the induction of transcription from the PEPCK-C gene promoter in the LLC-PK_1 kidney cells (Feifel *et al.*, 2002).

The mechanism of glucocorticoid stimulation of PEPCK-C gene transcription in the kidney has also been studied. The GR, HNF-4 and PPARα all stimulate transcription from the PEPCK-C gene promoter (−2000 to +73) when transfected into LLC-PK_1 kidney cells (Cassuto *et al.*, 2003). Unlike the liver or adipose tissue, a short segment (600 bp) of the PEPCK-C gene promoter exhibited a full response to the hormonal stimuli. A mutation in the AF1 site in the GRU of the PEPCK-C gene promoter blocked the induction of transcription by both GR and PPARα (Cassuto *et al.*, 2003). Most significantly, a mutation in the HNF-1 binding site markedly decreased the induction of gene transcription from the PEPCK-C gene promoter by PPARα and by GR. Interestingly, the HNF-1 binding site is necessary for the cAMP-mediated stimulation of transcription from the G6Pase gene promoter in LLC-PK_1 kidney cells, but not in hepatoma cells (Streeper *et al.*,

2000). Moreover, a null mutation in the HNF-1a gene in mice caused severe dysfunction in the kidney proximal tubules but had no overt effect on the liver (Pontoglio *et al.*, 1996). A model for the regulation of renal PEPCK-C gene transcription by acidosis and glucocorticoids, based on the results discussed above is shown in Figure 5.

CONCLUSIONS

The tissue-specific regulation of transcription for the gene for PEPCK-C is directly related to its physiological role in diverse metabolic processes. PEPCK-C is traditionally described as a *gluconeogenic enzyme* and research on the hormonal and dietary control of gene transcription invariably focus on its role in hepatic gluconeogenesis. However, as we have emphasized in this review, the gene for PEPCK-C is expressed in a number of tissues other than the liver, and many of these tissues do not make glucose. The most extensively studied of these non-hepatic tissues is the adipose tissue and the kidney. In addition, the metabolic processes in which PEPCK-C participates are diverse. When the gene for PEPCK-C was deleted in the liver (She *et al.*, 2003, 2000), the mice maintained normal glucose homeostasis and could be made diabetic, supporting the importance of renal gluconeogenesis in the control of blood glucose. However, the mice lacking hepatic PEPCK-C develop fatty livers after a fast, due to the role of PEPCK-C in *cataplerosis* (the removal of citric acid cycle anions). In adipose tissue, PEPCK-C is involved in glyceroneogenesis; overexpressing the gene in adipose tissue results in obesity (Franckhauser *et al.*, 2002).

In all three of the tissues discussed in this review, the control of PEPCK-C gene transcription is responsive to physiological factors that integrate the function of the enzyme with the metabolic response of the animal. Nowhere is this clearer than in diabetes, where a deficiency of insulin results in a variety of metabolic responses, all of which involve the induction of PEPCK-C gene transcription in the three tissues under consideration. In the liver, there is an increase in gluconeogenesis and in the triglyceride/fatty acid cycle, in part due to an elevated level of PEPCK-C. Renal PEPCK-C is induced by the acidosis that is caused by the ketosis (and subsequent acidosis) characteristic of diabetes; as a result, the rate of gluconeogenesis in the kidney increases. In adipose tissue, the elevated levels of glucocorticoid that accompanies diabetes decrease PEPCK-C gene transcription, thereby reducing the rate of fatty acid re-esterification in that tissue. *The result is a decrease in glyceroneogenesis and a concomitant increase in free fatty acids release from adipose tissue*; this results in an elevation in the concentration of fatty acids and subsequently of ketone bodies in the blood. The detailed models for the control of PEPCK-C gene transcription outlined in this review provide a start in understanding the complexity of the transcriptional response of this gene to metabolic stimuli. They also argue for the importance of integrating the regulation of gene transcription with the physiological status of the animal being studied, and of studying the control of gene transcription based on knowledge of its tissue-specific function.

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