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THE ROLE OF C/EBP IN NUTRIENT AND HORMONAL REGULATION OF GENE EXPRESSION

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■ **Abstract** C/EBPs are a family of transcription factors that play important roles in energy metabolism. Although initially thought to be constitutive regulators of transcription, an increasing amount of evidence indicates that their transactivating capacity within the cell can be modulated by nutrients and hormones. There are several mechanisms whereby this occurs. First, hormones/nutrients are known to directly alter the expression of C/EBPs. Second, hormones/nutrients may cause an alteration in the phosphorylation state of C/EBPs, which can affect their DNA-binding activity or transactivating capacity. Third, C/EBPs can function as accessory factors on gene promoters within a hormone response unit, interacting with other transcription factors to enhance the degree of responsiveness to specific hormones. Given their role in regulating genes involved in a wide variety of metabolic events, advancing our understanding of the molecular mechanism of action of C/EBPs will undoubtedly further our appreciation for the role these transcription factors play in both health and disease.

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INTRODUCTION

Nutrients exert a wide variety of biochemical and physiological effects in vivo. They stimulate secretion of hormones and neurotransmitters, act as allosteric regulators of metabolic enzymes, and regulate the expression of certain genes. With regard to the latter, nutrients have been shown to regulate gene expression by both direct and/or indirect mechanisms. Molecules such as fatty acids and their metabolites can directly interact with and alter the activity of transcription factors that control expression of specific genes through *cis*-regulatory elements in the promoter region. Alternatively, most nutrients may regulate genes indirectly by regulating the secretion of one or more hormones, such as insulin, glucagon, glucocorticoids, and thyroid hormone, which themselves go on to alter the expression of specific genes. Furthermore, studies performed in isolated cell cultures have indicated that nutrients such as monosaccharides, amino acids, and fatty acids can indirectly affect gene expression by mechanisms that, although not completely understood, are clearly not hormone dependent. It is not surprising that genes whose expression are regulated by nutrients have been found generally to encode proteins that are somehow involved in the metabolism of those same nutrients.

This review article attempts to provide insight into the mechanisms whereby nutrients alter transcription rates of genes, with the focus on a specific transcription factor family called CCAAT/enhancer binding proteins (C/EBPs). Shortly after its discovery by McKnight and associates, the first member of this family, C/EBPα, was dubbed "a central regulator of energy metabolism" on rather scant evidence (54). This included the observation that highest levels of expression of this factor were found in such tissues as liver, adipose, and lung, which are organs that control energy metabolism and/or actively metabolize lipids (8). However, subsequent analysis of this transcription factor demonstrated its importance, for example, in regulating adipocyte differentiation (reviewed in 20), and in controlling the expression of a number of genes encoding proteins that are involved in energy homeostasis, such as leptin (28, 29), insulin receptor substrate 1 (52), peroxisome proliferator-activated receptor-γ (15), and phosphoenolpyruvate carboxykinase (PEPCK) (71), as well as controlling its own activity through autoregulation (41, 99). These and other observations have validated the bold prediction and underscored the insight McKnight and colleagues had on the potential biological role for this protein. Although this transcription factor was, and perhaps still is, generally thought to be a constitutive regulator, recent studies highlighted in this review emphasize how nutrients and hormones can alter the transactivating capacity of C/EBPs in the cell, thereby using this protein to mediate their effects onto the transcription of specific genes.

A comment about nomenclature is necessary to avoid confusion. As described below, there are several members in the C/EBP family of transcription factors. The nomenclature suggested by Cao et al (12) has been adopted in this review, whereby the original *c/ebp* gene cloned is designated C/EBPα, and the remaining members are assigned Greek letters indicating their chronological order of discovery. Additionally, the *c/ebp*β gene has been cloned from several different species and is referred to in the literature by different names, including LAP (rat) (21), IL6-DBP (rat) (75), NF-IL6 (human) (2), CRP-2 (mouse) (109), AGP/EBP (mouse) (13), and NF-M (chicken) (36). For the sake of clarity, all of these are referred to as C/EBPβ, although where appropriate, the species origin is identified. This becomes important when the role of specific amino acid residues within C/EBPβ are discussed because equivalent positions of a specific serine residue in the human and rat C/EBP homologue, for example, have different numbering assignments due to variations in the total number of residues in the protein.

PROPERTIES OF C/EBPs

The C/EBP transcription factor family consists of over eight isoforms that are encoded by six genes (reviewed in 42). The additional isoforms arise through translation initiation at different in-frame AUG codons, generating full-length and truncated versions of the protein, as well as by differential splicing and use of alternative promoters. All C/EBP family members possess a highly conserved DNA-binding domain known as a basic region–leucine zipper motif (bZIP). The region that is rich in basic amino acids makes direct contact with DNA and determines the sequence-specific binding properties (1, 32) whereas the leucine zipper motif mediates dimerization between C/EBP polypeptides, which is required for DNA binding and for transactivation (39). The implications of the conserved bZIP domain are twofold. First, all C/EBP isoforms with DNA-binding domains are at least potentially capable of binding to a given C/EBP binding site in a promoter, although there is some evidence that phosphorylation of certain residues in the basic region of specific C/EBP isoforms may alter their binding affinity (discussed later in this review). As a result, different C/EBP isoforms may compete for binding to a cognate DNA sequence. Second, the conserved nature of the leucine zippers make them compatible, allowing for the formation of heterodimer species (12). Thus, a tissue that expresses, for example, the α and β isoforms will in effect contain three species of C/EBP transcription factors: the α/α homodimer, the β/β homodimer, and the α/β heterodimer. Dimerization between isoforms, therefore, clearly has the potential to increase the variety of transcriptional responses that can be obtained from these transcription factors.

The domains of C/EBPs that possess the transactivation function lie in the amino terminus (22, 64, 74, 104, 108). C/EBPs typically have been observed to produce transactivation; however, there are a few examples of specific genes whose transcription is inhibited by a particular C/EBP isoform (52, 102). The amino

acid sequences of the transactivation domains are generally unique for each isoform, although some short, conserved domains have been identified in C/EBPα, -β, and -δ that are critical for the transcriptional activity of these isoforms (65). These transactivation domains act more or less independently of the bZIP domain, displaying similar activity when fused to heterologous DNA-binding domains (65, 73, 74, 78, 104, 108). This modularity has been exploited extensively in studies of the function of specific domains and amino acid residues in C/EBP proteins.

It should be noted that several members of the C/EBP family act as dominant negative inhibitors. For example, C/EBPγ is a small, 16-kDa protein consisting primarily of a bZIP domain and lacks a transactivation domain (16). As a result, dimerization of this molecule with other C/EBP isoforms produces inactive heterodimers. Another isoform, C/EBPζ, acts as a dominant negative protein through a different mechanism. It contains a functional leucine zipper domain and thus dimerizes with other C/EBP isoforms. However, the basic region contains two proline residues that effectively disrupt the α-helical nature of this domain that is necessary for its DNA-binding activity (85). Thus, heterodimers containing C/EBPζ are incapable of binding to DNA and as a result are transcriptionally inactive.

The tissue-specific expression profiles of C/EBPs are isoform specific. C/EBPα, -β, and -δ have some commonality in their patterns, being coexpressed at their highest levels in liver, lung, adipose, and intestine tissue, with lower levels of expression in a number of other tissues and cell types (reviewed in 42). C/EBPε is probably the most limited in its expression, attaining significant levels only in cells of myeloid and lymphoid lineages (3, 61). Conversely, the two inhibitor isoforms of C/EBP, γ and ζ, are ubiquitously expressed (reviewed in 42), which suggests that all cell types are to some extent capable of restricting or inhibiting the transactivation potential of C/EBPs. Obviously, the extent of inhibition depends on the relative levels of expression of the dominant negative and full-length, active isoforms. It is clear that in order to obtain a complete understanding of the biological role of C/EBPs, we must gain an understanding of the mechanisms whereby the expression of these two dominant negative forms of C/EBP are regulated.

RELATIVE ROLES OF C/EBP ISOFORMS IN ENERGY METABOLISM

Advances in our understanding of the specific and relative roles of C/EBP isoforms in energy metabolism have been greatly accelerated by the development of a variety of methodologies that allow inhibition of expression of specific genes. These include expression of antisense RNA and deletion of genomic sequences of interest by knockout approaches. Although these methodologies have been performed in both established cell lines and whole animals, observations made in the latter have allowed the most comprehensive evaluation of the roles of C/EBP isoforms in

energy metabolism. The studies described in this section are devoted to a description of the general metabolic alterations that result from inhibition of expression of specific C/EBP isoforms. Some of these studies have focused on the α and β isoforms because they have been identified as regulators of energy metabolism. The details that relate specifically to nutrient regulation of gene expression are elaborated on in the two subsections below.

It should be mentioned that another novel approach developed for inhibiting the activity of C/EBP proteins that has been developed involves the overexpression of natural or synthetic dominant negative proteins (59, 69, 85). Dominant negative versions of C/EBPs inhibit the activity of all C/EBP proteins via heterodimerization with compatible bZIP proteins, and thus offer no information about the role of any specific C/EBP isoform. This limitation, however, in no way lessens the impact this approach has had on advancing our understanding of the biological roles of C/EBPs. Indeed, this approach has been exploited to create a novel animal model of metabolic disease.

Metabolic Effects of Inhibiting C/EBPα Activity

Initial characterization of C/EBPα revealed that this transcription factor is not expressed until about the time of parturition, and that two of the tissues in which it is most highly expressed are liver and adipose (8). The developmental and metabolic characteristics displayed by mice that have the C/EBPα gene disrupted (105) are fully compatible with the expression profile of this gene. The knockout mice are phenotypically normal at birth, with no difference in birth weight and no gross anatomical abnormalities. However, within a few hours of birth, these mice die from hypoglycemia. This hypoglycemia appears to result primarily from two metabolic perturbations in liver. First, the level of liver glycogen, which is normally high at birth, is undetectable in these mice and is most likely the consequence of reduced expression of the gene coding for glycogen synthase. Second, the expression of three liver gluconeogenic enzymes, glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, and tyrosine aminotransferase, is undetectable at birth, which would impair the ability of the neonate to synthesize glucose de novo. Both of these processes, glycogen mobilization and gluconeogenesis, normally provide the neonate with the nutritional buffer it requires to sustain itself until it is able to obtain sufficient amounts of milk from its mother (33). These mice also have reduced lipid deposits in both inguinal white adipose and brown adipose tissues. This reduction in lipid content, in response to an inhibition of C/EBPα expression, has also been observed in cell lines that can be induced to differentiate into adipocytes (44, 87).

One limitation of the C/EBPα knockout mouse model is that the effects of the gene deletion cannot be assessed in adults because the animals die shortly after birth. To address this issue, Lee and coworkers (40) developed a model system whereby the physiological effects of inhibiting C/EBPα expression in the liver of adult mice could be assessed. Development of this model included (*a*) genetic

manipulation of a mouse such that the endogenous *c/ebpα* gene was modified so as to be recognized by a specific enzyme (*Cre* recombinase), which would splice out the *c/ebpα* gene from the genome, and (*b*) generation of an adenovirus vector that would deliver the *Cre* recombinase to the liver. The mice were infected with the recombinant adenovirus via tail vein infusion, and 10 days later the expression of specific genes was analyzed. The efficiency of the gene knockout was demonstrated by analysis of C/EBPα expression, which was reduced by 90% in mice that received the recombinant adenovirus. The deletion of this gene was specific because expression of C/EBPβ was unaltered in these same mice. More important, deletion of the *c/ebpα* gene in adult liver resulted in a significant reduction in expression of the PEPCK and glycogen synthase genes, mirroring the alterations that occur in the neonatal knockout mice described above. This observation is important because it indicates that C/EBPα is involved not only in the establishment of PEPCK and glycogen synthase expression at birth, but also in the maintenance of their expression in adult liver.

Metabolic Effects of Inhibiting C/EBPβ Activity

In comparison with C/EBPα, delineating the metabolic roles of C/EBPβ has been somewhat more difficult owing to the fact that at least three distinct phenotypes of C/EBPβ (−/−) mice have been observed. In the first published studies of these knockout mice, it was observed that the mutant mice were generally healthy and had no obvious histological or anatomical abnormalities (89, 97). However, the number of mutant mice obtained from heterozygous parents was significantly lower than expected based on Mendelian genetics, which suggests that some in utero loss of homozygous mutants had occurred. A second study by Croniger et al (17) also noted two different phenotypes, although in this case the affected phenotype (B phenotype) was characterized by defective glucose homeostasis, similar to C/EBPα knockouts, and the mice died shortly after birth from hypoglycemia. The defects in the glucose homeostatic mechanism in C/EBPβ knockouts appear to be similar but not identical to those in C/EBPα knockouts. Both mouse models show an absence of hepatic PEPCK gene expression at birth, which suggests a reduced capability for gluconeogenesis. However, although C/EBPα knockout mice lack liver glycogen stores, the B phenotype of C/EBPβ knockouts are able to synthesize and store glycogen but unable to effectively mobilize it (17). The net result is that although there are differences in the precise molecular alterations that occur, both of these mouse models display similar defects in overall glucose homeostasis. The basis for the different phenotypes of the C/EBPβ (−/−) mice has not been established, but some evidence suggests that expression of noninbred modifier genes, present in the genetic backgrounds of the mice, is involved (17).

It would not be accurate to leave the impression that the so-called normal C/EBPβ (−/−) mice are physiologically unaffected. Adult females are sterile (93), both sexes display increased susceptibility to infection by certain bacterial

pathogens (97), and the mice have subtle alterations in several metabolic parameters (46). For example, these mice experience hypoglycemia after an 18-h fast, due to impaired hepatic glycogenolysis (46). This is likely related to the reduction in both basal and glucagon-stimulated cAMP concentrations observed in the livers of these mice. A defect in free fatty acid release from adipose in response to epinephrine has also been noted (46), which undoubtedly contributes to the 40% decrease in plasma levels of this lipid in these knockout mice. Whole body glucose disposal is also higher and is one of several observations that suggest that insulin sensitivity is enhanced in these mice, particularly in their skeletal muscle. Finally, these mice have significantly lower white adipose tissue mass compared with wild-type mice, which is due to a reduction in the number of adipocytes (46). This is consistent with other model systems that indicate an important role for C/EBPβ in adipocyte differentiation (98).

To summarize, the studies described above suggest that both of the major C/EBP isoforms play important metabolic roles. The characteristics of the knockout models allows the general conclusion that C/EBPα plays a broader, more general role in establishing energy metabolism in mammals whereas C/EBPβ mediates more subtle responses by regulating the expression of genes that allow organisms to adapt metabolically to environmental (e.g. nutritional) changes. This conclusion is to some extent biased by the inability to analyze in detail the metabolic characteristics of C/EBPα ($-/-$) mice due to their early death. Nor should one forget the observation that in certain genetic backgrounds, C/EBPβ ($-/-$) mice display generalized metabolic abnormalities similar to C/EBPα knockouts (17). Moreover, as is discussed below, it is now well established that C/EBPα mediates the effects of nutrients and/or hormones onto specific genes. Thus, its role clearly extends beyond simply establishing energy metabolism and includes the regulation of energy homeostasis as well.

MECHANISMS WHEREBY C/EBPs MEDIATE THE EFFECTS OF NUTRIENTS AND HORMONES ON GENE EXPRESSION

There are two general mechanisms whereby nutrients and hormones can alter the amount of C/EBP activity in the cell. One is through the alteration of C/EBP gene expression, which would increase or decrease the steady state levels of the protein in the cell and thus affect the total amount of C/EBP transcriptional activity that is available for regulating the expression of target genes. Included in this mechanism is the possibility of altering the relative levels of active and dominant negative forms of C/EBP, which would determine the amount of C/EBP activity in the cell by regulating the ratio of active versus inactive C/EBP dimers. The second mechanism whereby nutrients might accomplish this is by regulating the intrinsic activity of existing C/EBP, which would include regulation of either the transactivation potency or DNA-binding activity.

Nutrient and Hormonal Effects on C/EBP Expression

The regulation of C/EBP gene expression has been examined primarily in adipose and liver tissue or cells, and some tissue-specific differences have been observed. In liver-derived cells, glucocorticoids (or synthetic analogues such as dexamethasone) induce expression of both alpha and beta isoforms at both the level of mRNA and protein (18, 53, 76). Conversely, glucocorticoids inhibit C/EBPα expression in adipocytes (49). It is curious that there have been no reports of the effect of glucocorticoids on C/EBPβ expression in adipose, although in intestinal epithelial IEC-6 cells, glucocorticoids induce expression of this isoform as it does in liver (10). This suggests that the induction of C/EBPβ expression by glucocorticoids may be a ubiquitous regulatory feature.

Cyclic AMP (cAMP) has been shown to induce expression of C/EBPβ in a wide variety of experimental models and cell types. Injection of a hydrophobic analogue of cAMP into rats induced hepatic C/EBPβ mRNA levels within 90 min but had no effect on levels of C/EBPα mRNA (18). In rat hepatoma H4IIE cells, a cAMP analogue was shown to modestly increase C/EBPα mRNA and protein concentrations and more robustly induce expression of C/EBPβ (18). An induction of C/EBPβ expression by cAMP has also been demonstrated in 30A5 preadipocytes (96), and incubation of primary cultured hepatocytes with glucagon, which stimulates cAMP production, also leads to up-regulation of C/EBPβ expression (53). It is interesting that exercise also leads to an increase in C/EBPβ, but not C/EBPα, expression in rat liver, an effect that is likely mediated by cAMP (68). The mechanism of induction of C/EBPβ expression by cAMP appears to be transcriptional, which is supported by the identification of a typical cAMP response element (CRE) in the promoter of the C/EBPβ gene (66). One well-characterized mediator of cAMP responsiveness, CREB (CRE binding protein), has been shown to bind to the CRE in this promoter. Because CREB is phosphorylated and, as a result, activated by protein kinase A (PKA), the presence of a CREB binding site in the promoter likely defines the mechanism whereby cAMP induces expression of C/EBPβ (66).

The effects of insulin on C/EBP expression have generally been shown to be inhibitory or without effect. MacDougald et al (50) reported a 90% drop in C/EBPα in 3T3-L1 adipocytes treated with insulin for 24 h. In this same study, insulin transiently induced expression of C/EBPβ. Wang et al (106) also reported an inhibitory effect of insulin on C/EBPα expression in adipocytes, although this was observable only in the presence of high (24 mM) concentrations of glucose. Bosch et al (9) observed that mice either fed a high-carbohydrate diet or injected with insulin had reduced levels of hepatic C/EBPβ mRNA but unaltered levels of C/EBPα mRNA. In rat hepatoma H4IIE cells, insulin treatment was demonstrated to transiently induce levels of C/EBPβ protein but had little effect on C/EBPα protein even though a modest stimulatory effect on mRNA levels was detected (18). Studies examining the impact of streptozotocin-induced diabetes as well as subsequent insulin treatment offer further support for the conclusion that the effect

of insulin on C/EBP expression is either neutral or inhibitory. Crosson et al (18) showed that C/EBPβ mRNA and protein levels were unaffected in diabetic rat liver whereas C/EBPα expression was inhibited, which could be partially reversed by insulin treatment. At odds with this study is a study by Bosch et al (9), who reported that induction of diabetes had no effect on C/EBPα mRNA levels whereas C/EBPβ mRNA levels decreased, something insulin treatment was able to fully reverse. Whether the changes in mRNA levels were reflected in corresponding changes in protein concentrations was not assessed. The basis for the discrepancies between these two studies is not readily apparent but could presumably be due to differences in the dose of streptozotocin used to induce diabetes, which might generate animals with diabetes of dissimilar severity and different metabolic derangements.

A final aspect of C/EBP expression regulation that should be addressed is the issue of the relative abundance of the C/EBP isoforms that act as dominant negatives. A shift in the relative abundance of these inhibitor isoforms should affect the overall C/EBP activity in the cell and, as a consequence, the expression of target genes. There is some evidence that the expression of inhibiting isoforms can be regulated. GADD153 (also called CHOP) is the C/EBPζ isoform that has a truncated transactivation domain, as well as amino acid substitutions in its basic region that abrogate its DNA-binding activity. Overexpression of GADD153 has been shown to inhibit the ability of C/EBPs to transactivate target genes (85). The expression of this C/EBP isoform is induced both during the acute phase response (95) and under conditions of amino acid limitation (11), indicating that different physiological and metabolic states can determine and/or regulate cellular C/EBP activity. This, in fact, was demonstrated by Batchvarova et al (5), who found that incubation of 3T3-L1 cells in low glucose (2 mM), which represents a metabolic stress, led to the induction of GADD153 expression. More important, it also resulted in the inhibition of adipocytic differentiation, a process known to require the presence and activity of C/EBPα and C/EBPβ. In a separate study, Crosson et al (18) observed that in streptozotocin-induced diabetic rat liver, there was a selective decrease in the concentration of the truncated and less-active 29-kDa isoform of C/EBPα whereas the levels of the full-length, fully active 42-kDa form remained unchanged. It was hypothesized that this could result in elevated C/EBP activity in the cell due to a shift in the equilibrium toward the formation of active homodimers of the full-length protein. It is interesting that insulin treatment of the diabetic rats restored the ratio of 42- and 29-kDa isoforms back to that of controls. Together, these observations strongly suggest that there is hormonal and metabolic influence over the ratio of C/EBP isoforms.

Regulation of the Intrinsic Activity of C/EBPs Via Phosphorylation

The other mechanism whereby the amount of C/EBP activity can be altered in the cell is through regulation of the intrinsic activity of the molecule. A number of studies have demonstrated that the various functional properties of C/EBP are

regulatable, and in many, but not all, instances this involves phosphorylation/dephosphorylation of the protein. As described below, the phosphorylation state of C/EBPs can affect their transactivation capacity and their DNA-binding properties, as well as play a role in controlling translocation of the protein to the nuclear compartment.

Phosphorylation of C/EBPα C/EBPα is a highly phosphorylated molecule (27), and the results of several studies suggest regulatory roles for this phosphorylation, although none of the studies have attributed an unequivocal regulatory function to either a specific protein kinase or a residue in C/EBPα that can be phosphorylated. Mahoney et al (51) were the first to suggest that phosphorylation may play a role in regulating its DNA-binding activity. They observed that C/EBPα was efficiently phosphorylated by protein kinase C (PKC), but not by PKA, on Ser^{248}, Ser^{277}, and Ser^{299}. Although no role for the first two phosphorylation sites could be established, it was observed that phosphorylation of Ser^{299} decreased its sequence-specific DNA-binding affinity by approximately 80%. However, it was not determined whether this phosphorylation event abrogated the ability of C/EBPα to transactivate a target gene, and if so, whether regulation by PKC is lost when Ser^{299} is mutated. It has never been determined whether Ser^{299} is phosphorylated in vivo. Thus, the issue of whether the DNA-binding activity of C/EBPα is regulated through phosphorylation of Ser^{299} needs further assessment.

There is fairly convincing evidence that insulin regulates the phosphorylation state of C/EBPα. Preliminary analysis indicated that insulin had little effect on the overall level of the phosphorylation state of C/EBPα (27). However, a more detailed examination suggested that insulin stimulated the dephosphorylation of at least two sites in C/EBPα (27). The effect of insulin could be blocked by inhibitors of phosphatidylinositol 3-kinase, but not by inhibitors of mitogen-activated protein kinase (MAPK), which identified the signaling pathway through which the insulin effect is exerted. Further evidence supporting these inhibitor data was from Ross et al (86), who determined that C/EBPα was phosphorylated in vivo on Thr^{222}, Thr^{226}, and Ser^{230}. The two threonine residues were present within amino acid sequences that are known substrates for glycogen synthase kinase 3 (GSK3). This kinase is inhibited by insulin through a phosphatidylinositol 3-kinase–dependent pathway and, thus, offered a possible mechanism for the insulin-stimulated dephosphorylation observed. Further analysis indicated that GSK3 could indeed phosphorylate C/EBPα in vitro on Thr^{222} and Thr^{226}. Overexpression of a constitutively active GSK3 mutant in 3T3-L1 adipocytes enhanced the level of phosphorylation of C/EBPα, which could be inhibited by incubation of these cells with lithium, a known inhibitor of GSK3. Lithium also inhibited the differentiation of 3T3-L1 preadipocytes into adipocytes, which suggests one possible role for the phosphorylation of these sites in C/EBPα. The phosphorylated and dephosphorylated forms of C/EBPα appear to have different conformations, which may influence one or more of its biological activities. However, the study of Ross et al (86) was unable to assign any specific functional role to the threonine residues. Neither mutation of

the threonine residues nor overexpression of GSK3 altered the ability of C/EBPα to transactivate a target gene. It is possible that the role for one or more of these phosphorylations manifests itself not in the constitutive activity of C/EBPα but in one of its hormone-regulated activities (discussed later in this review). Thus, although there is compelling evidence that GSK3 is an insulin-regulated C/EBPα kinase, the biological significance of this phosphorylation is not yet apparent.

Phosphorylation of C/EBPβ C/EBPβ is phosphorylated on a number of different residues by several protein kinases, some of which appear to play a role in the regulation of its biological functions. The translocation of C/EBPβ to the nucleus is one of these. Regulation of its translocation has been observed in several cell types, although it is not universally employed (107). For example, treatment of rat pheochromocytoma PC12 cells with forskolin stimulates translocation of C/EBPβ from the cytosol to the nucleus (56). A similar redistribution of this protein occurs in hepatocytes incubated with tumor necrosis factor α (112) and in human colon cancer DKO-1 cells treated with antioxidants (14). The observation that (*a*) inhibition of PKA blocks translocation of C/EBPβ (14), (*b*) mutation of Ser^{299} to an alanine prevents human C/EBPβ (also called NF-IL6) from translocating into the nucleus and activating the c-*fos* gene (56), and (*c*) PKA directly phosphorylates this serine residue in vitro (14) suggests that phosphorylation of Ser^{299} (Ser^{240} is the equivalent position in rat C/EBPβ) by PKA induces translocation of this transcription factor. Because PKC phosphorylates this same site in vitro (103), the possibility exists that extracellular signals that activate this particular kinase may also increase shuttling of C/EBPβ into the nucleus.

The phosphorylation status of C/EBPβ can also modulate its ability to bind to DNA. Trautwein et al (103) showed that phosphorylation of Ser^{240} (in rat C/EBPβ) by PKA or PKC in vitro inhibits its DNA-binding activity. Thus, the data obtained to date suggest that phosphorylation of Ser^{240} stimulates translocation of C/EBPβ into the nucleus but, on its arrival, may not be able to efficiently bind to its cognate DNA sequence. Other studies have reported that C/EBPβ is not phosphorylated by PKA in vitro to any significant extent (107), and elevating cAMP levels in cells does not increase its degree of phosphorylation (101). Thus, some uncertainty remains regarding the role of PKA in regulating the intrinsic activities of this C/EBP isoform. Finally, a study by Liao et al (43) demonstrated that treatment of 3T3-F442 fibroblasts with growth hormone rapidly induces the binding of C/EBPβ to a site in the c-*fos* gene promoter. Some evidence was obtained to suggest that this effect was mediated via dephosphorylation of this transcription factor, although the specific residue involved was not identified.

Phosphorylation has also been demonstrated to alter the intrinsic transactivation ability of C/EBPβ. Wegner et al (107) found that murine C/EBPβ binds to a DNA sequence that had been shown to confer responsiveness to a Ca^{2+}-calmodulin–dependent protein kinase II (CaMKII), which suggests that this C/EBP isoform could mediate the effect of this protein kinase. Subsequent analysis showed that treatment of pituitary G/C cells with an inhibitor of CaMKII resulted in a reduced

phosphorylation state of C/EBPβ, whereas treatment with a calcium ionophore increased phosphorylation (107). Phosphorylation by CaMKII altered neither the DNA-binding activity of C/EBPβ nor the translocation step; instead, it stimulated its transactivation capacity. It is interesting that the site of phosphorylation was determined to be Ser^{276} (equivalent to Ser^{277} in rat C/EBPβ), which lies in the DNA-binding domain, although the stimulatory effect of the phosphorylation required the presence of the amino-terminal transactivation domain of C/EBPβ. The strength of this study was that a causal relationship was observed between the phosphorylation of Ser^{276} by CaMKII and the enhanced transcriptional activity. In a separate study, Trautwein et al (101) showed that activation of the PKC pathway by phorbol esters resulted in enhanced phosphorylation of rat C/EBPβ on residue Ser^{105}, which lies in the transactivation domain. This phosphorylation enhanced the transactivation potential without altering the DNA-binding activity of this transcription factor. Because PKC does not appear to phosphorylate Ser^{105} directly (103), it was hypothesized that PKC activation affects some other signaling pathway that regulates C/EBPβ transactivation capacity. And finally, MAPK has been shown to phosphorylate human C/EBPβ on Thr^{235} (equivalent to Thr^{189} in rat C/EBPβ), which leads to a significant activation of this transcription factor (63). Phosphorylation of the nearby Ser^{231} appears to be a requirement for this MAPK-dependent activation of C/EBPβ. The limitation of this study, and others like it, was that no specific gene target, which is activated through a MAPK-catalyzed phosphorylation of C/EBPβ, was identified. However, because MAPK is a component of a signaling pathway that is activated by a number of growth factors, including insulin, it is intriguing to speculate that C/EBPβ might mediate some of the stimulatory effects that growth factors have on the expression of specific genes.

C/EBPs AS ACCESSORY FACTORS IN NUTRIENT AND HORMONAL RESPONSIVENESS OF GENE TRANSCRIPTION

Our understanding of how hormones and nutrients regulate transcription of genes has advanced steadily over the past 15 years. One of the initial advances in this area came from the identification of short sequences within promoters of genes that could mediate the effect of hormones or nutrients. These sequences, termed enhancers or response elements, can in most cases function independently to some extent. Thus, for example, a cAMP response element (CRE) from one promoter can be artificially linked to a different, normally unresponsive promoter that converts it to one whose activity is now stimulated in the presence of cAMP. Enhancer elements that mediate the transcriptional responses to a variety of nutrients and hormones have been identified, including those for cAMP (84), insulin (94), thyroid hormone (25), steroid hormones (6), glucose (100), and amino acids (34). All these response elements function through their capacity to bind specific transcription

factors that perform the actual job of mediating the hormone/nutrient effect onto the transcriptional apparatus. These concepts have led to the development of simplistic models to describe how hormones alter the transcription of genes.

However, as more and more promoters were characterized, it became increasingly evident that hormone and nutrient responses are often mediated by a set of *cis*-elements, called a response unit (48), rather than by a single sequence. Typically, these response units consist of a recognizable or consensus-like *cis*-element that is known to mediate the response being characterized and one or more accessory elements. Accessory elements usually have no intrinsic ability to mediate hormonal or nutrient response; rather, they either boost the fold responsiveness mediated by the consensus hormone/nutrient response element or act permissively. In the latter case, the accessory element is necessary but not sufficient to mediate the response. A detailed discussion of response units and the regulatory advantages they offer can be found in several reviews (48, 77, 81).

The reason for including a brief description of response units in this review is that in the vast majority of genes where C/EBPs have been shown to participate in mediating the effects of hormones and nutrients onto transcription, C/EBPs play the role of accessory factors that bind to accessory elements. That is, although C/EBPs usually have no intrinsic capacity to mediate a response, they are able to cooperate with other transcription factors to mediate a response to a hormonal or nutritional signal. To date, C/EBPs have been implicated in transcriptional responses to cAMP, glucocorticoids, thyroid hormone, and perhaps insulin and, in each case, are known to function within a response unit (discussed below).

A large portion of the body of information regarding the role C/EBPs play in mediating these responses has come from analyses of the PEPCK gene promoter, where C/EBPα and C/EBPβ play different roles in mediating responses to several hormones. Because of the significant amount of information available on the role of C/EBPs in the regulation of this gene, it is discussed in a separate section below. Ahead of this section, a brief description of other genes that utilize C/EBPs as accessory factors to mediate hormonal responsiveness is provided.

By far the most common type of hormonal response with which C/EBPs are associated is that of glucocorticoids. These steroid hormones interact with a specific receptor in the cytosol, which then causes its translocation to the nucleus, where the receptor-ligand complex binds to specific DNA sequences in select genes, producing either stimulation or repression of their transcription. However, as mentioned above, the full response to glucocorticoids in a number of genes requires the participation of other transcription factors, which frequently include C/EBPs. In their studies examining the acquisition of glucocorticoid responsiveness in chicken embryonic neural retina, Ben-Or & Okret (7) found that induction of glutamine synthetase expression by glucocorticoids was delayed even though the glucocorticoid receptor was expressed early in ontogeny. Detailed analysis of the glutamine synthetase gene promoter demonstrated that a C/EBP-like factor was required for glucocorticoid responsiveness. Although ectopic expression of murine C/EBPα in nonresponsive embryonic day 7 retinal cells enhanced the

responsiveness of the glutamine synthetase promoter to glucocorticoids, other experiments suggested that the actual accessory factor is not C/EBPα. The ability of other C/EBP isoforms to reconstitute responsiveness was not examined, and it is possible that chickens express a species-specific isoform that functions in this system.

A study by Nishio et al (67) examining the glucocorticoid responsiveness of the α1-acid glycoprotein gene promoter not only provided further support for a generalized role for C/EBPs in glucocorticoid responsiveness, it also offered a possible molecular mechanism as to how it participates in this response. In this particular promoter, there are two C/EBP binding sites located just downstream of a glucocorticoid response element that binds the glucocorticoid receptor. Overexpression of C/EBPβ enhanced the activity of the promoter in the presence of glucocorticoids. It is interesting that this synergism was observed even when a point mutant of C/EBPβ, which interfered with its DNA-binding activity, was expressed. Subsequent analysis indicated that C/EBPβ and the glucocorticoid receptor could physically interact in vitro, although whether a causal relationship exists between this interaction and the synergistic activation produced by the two transcription factors was not examined. Because the physical interaction occurred through the bZIP domain of C/EBPβ, which is highly conserved among isoforms, it is possible that other members of the C/EBP family could also substitute as an accessory factor for this response.

C/EBPα appears to be involved in the transcriptional regulation of the prolactin gene by insulin. Jacob & Stanley (31) showed that C/EBPα binds to a multihormonal response element in the promoter, and that overexpression of C/EBPα in GH4 cells increased both the basal and insulin-stimulated activity of the prolactin promoter. It has not been determined whether C/EBPα alone mediates the insulin response, and if so how, or whether it functions within a response unit with other factors. Nor is it clear how such a short sequence (10 bp) can mediate the effects of epidermal growth factor, insulin, and cAMP. Several different transcription factors can bind to this sequence, at least in vitro, although it is unlikely that more than one factor could bind at any one time given the length of the sequence.

HORMONAL REGULATION OF PEPCK GENE TRANSCRIPTION

The PEPCK gene has played a special role in advancing the field of molecular endocrinology and metabolism. This gene codes for a rate-limiting enzyme of gluconeogenesis, and its rate of transcription and mRNA turnover is responsive to a variety of hormones and metabolic conditions (reviewed in 26). Because of these characteristics, this model is frequently used to examine multihormonal regulation of gene expression. Moreover, its expression is both tissue-specific and ontogenetically controlled, making it attractive to investigators working in these areas.

Transcription of this gene in liver is activated by cAMP, glucocorticoids, retinoic acid, and thyroid hormone and is inhibited by insulin (26). The PEPCK gene promoter was the first in which a CRE was defined (91), which ultimately assisted in the discovery of a specific transcription factor that binds to this sequence (60) and which led to an understanding of one molecular mechanism whereby cAMP induces transcription (90). It was also analysis of this promoter that helped to advance the concept of hormone response units because all the hormonal responses this promoter displays are mediated by more than one *cis*-element. The promoter has several binding sites for C/EBPs (71), one or more of which participate in the hormone response units described below and shown schematically in Figure 1. It was the examination of the involvement of C/EBPs in the regulation of the PEPCK promoter that led to the realization that these transcription factors, more than just constitutive regulators, also assist in mediating the effects of nutrients and hormones onto the transcription rate of specific genes.

Cyclic AMP Responsiveness

Transcription of the PEPCK gene in liver responds rapidly and robustly to a rise in the level of cAMP (38). This contrasts with a weak response to this second messenger in other tissues, such as kidney (55). Initial analysis of the PEPCK promoter identified a *cis*-element, known as the cAMP response element (CRE), which mediated a cAMP response (91). However, the magnitude of the response mediated by the CRE alone is weak compared with that of a promoter fragment that extends out to nucleotide −490 (45, 80). Further analysis of the promoter indicated that there was an upstream region extending from −355 to −200, which, when coupled with the CRE, conferred a strong response to cAMP although it had little or no activity on its own (80). Detailed analysis of this region ultimately demonstrated that it contained three binding sites for C/EBP and one site for activator protein-1, all of which were required, along with the CRE, for maximal responsiveness to cAMP (83). Thus, five *cis*-elements make up the cAMP response unit (CRU) in this promoter (Figure 1*B*). The nature of this response unit (and indeed all response units) likely provides the benefit of allowing for an expanded range of responses the promoter can have to the initial stimulus (77).

Attempts to identify the precise transcription factors that mediate the response to cAMP through the five *cis*-elements was not a straightforward task because some of the *cis*-elements were shown to bind multiple proteins. For example, three different transcription factors can bind, at least in vitro, to the CRE, the CRE-binding protein (CREB) (71), and both α- and β-isoforms of C/EBP (71, 73). And both C/EBP isoforms can bind to the three C/EBP binding sites in the distal portion of the promoter (71, 73). However, using a variety of molecular biological approaches, the roles of specific transcription factors, and the *cis*-elements through which they exert their effects, have been assessed (reviewed in 77, 111).

C/EBPα appears to play a particularly critical role in the ability of the PEPCK promoter to respond to cAMP. Mice with the C/EBPα gene knocked out have

an impaired response to cAMP whereas those with a deletion in the C/EBPβ gene do not (17). Moreover, recent studies by Crosson & Roesler (19) showed that inhibition of C/EBPα gene expression by antisense technology completely abrogated the induction of PEPCK gene expression by cAMP in rat hepatoma cells, whereas antisense directed against C/EBPβ had no effect. This does not necessarily rule out a role for C/EBPβ in the CRU; a response to cAMP can be observed when either isoform is bound to the three distal C/EBP binding sites in the promoter (73; PJ McFie, WJ Roesler, unpublished observations).

What remains unclear is the identity of the transcription factor that binds to the CRE, a critical component of the CRU, to confer a robust response to cAMP. The obvious candidate is CREB because it binds in vitro to this site and is a direct substrate of PKA, a cAMP-activated kinase (reviewed in 90). Indeed, tethering of CREB to the CRE reconstitutes a strong cAMP response in hepatoma cells (79). However, both C/EBP isoforms can also bind to this site (71, 73), but with drastically different consequences. When C/EBPα is bound to the CRE, the promoter displays a significant responsivity to cAMP, whereas with C/EBPβ bound, responsivity is lost (82). These data suggest that the degree to which the PEPCK promoter responds to cAMP is governed by the specific combination of transcription factors that are bound to the components of the CRU at any given time. This in turn might be determined by the relative concentrations of the transcription factors involved. Recently, a model that incorporates the above information has been proposed (77) describing how the PEPCK promoter could exist in various states of cAMP responsivity, depending on the physiological and metabolic signals to which it responds.

The precise role C/EBPs play in the CRU is unknown. C/EBPα is not directly phosphorylated by PKA (51), and although C/EBPβ may be a substrate for this kinase (103), there is no evidence that it leads to an increase in its activity (101). Structure/function analysis of both C/EBP isoforms have identified the domains that are required for their ability to mediate cAMP responsiveness (73, 82), and some conserved amino acid sequences within these domains exist (65). In the case of C/EBPα, mutational analysis has led to the conclusion that the mechanism whereby it mediates constitutive transactivation is distinct from that whereby it mediates cAMP-inducible transactivation (82). This is an important observation because it suggests that unique nuclear targets exist with which C/EBPα makes contact, allowing the manifestation of its cAMP-inducible activity. The identity of these nuclear proteins is unknown, although one potential candidate is the coactivator CBP (CREB-binding protein). This protein mediates the effects of a number of transcription factors by bridging them to the preinitiation complex (35). A functional homologue of CBP, p300, has been shown to physically interact with C/EBPβ and to enhance its ability to transactivate target promoters (57). In this same study, p300 increased the transcriptional capacity of C/EBPα, although no physical interaction between the two was reported. Studies examining a possible role for CBP in the cAMP responsiveness of the PEPCK promoter, as

well as experiments attempting to demonstrate a direct interaction between CBP and C/EBPα, have been unsuccessful (WJ Roesler, PJ McFie, unpublished observations).

Thyroid Hormone Responsiveness

A role for thyroid hormone (T_3) in the regulation of the PEPCK gene was first suggested when it was detected that the amount of PEPCK activity and its rate of gene transcription were affected by alterations in the thyroid status of animals (47, 62, 92). Analysis of the promoter indicated that there are two *cis*-elements that mediate the T_3 response: a typical T_3 response element that binds the thyroid hormone receptor, and a binding site for C/EBP (24, 70) (Figure 1*C*). Mutation of either sequence resulted in complete loss of response to the hormone, so the binding of C/EBP to its cognate site is required in order for the promoter to be responsive to T_3 (70). Experiments aimed at determining which C/EBP isoform is involved suggested that either C/EBPα or β can play the role of accessory factor in this response (72, 73). Here again, the mechanism whereby C/EBPs participate in this hormone response is not known, although it has been speculated that by acting together, thyroid hormone receptor and C/EBP may more efficiently recruit a necessary coactivator to the promoter that mediates the T_3 response (73).

Glucocorticoid Responsiveness

The glucocorticoid responsiveness of the PEPCK promoter is mediated by an extremely complex response unit, which employs two glucocorticoid response elements and four accessory elements (Figure 1*D*) (110). Mutation of any one element results in a significant decrease in fold responsiveness (58, 88). It is interesting that one of the accessory elements in this response unit is the CRE (30), which as mentioned above can bind both CREB and C/EBP family members. Recently, Yamada et al (110) examined which of these CRE-binding proteins acted as an accessory factor for the glucocorticoid response. Their first approach was to change the CRE sequences in the PEPCK promoter to a consensus C/EBP binding site, to which CREB did not bind. This altered promoter was shown to retain full responsiveness to glucocorticoids, which suggests that a C/EBP isoform, and not CREB, was the accessory factor. A second approach was to replace the bZIP (DNA-binding) domain of CREB and C/EBP isoforms with the DNA-binding domain of the yeast transcription GAL4, and to test for their ability to reconstitute glucocorticoid responsiveness on a PEPCK promoter variant that had the CRE replaced by a binding site for GAL4. Using this system, these investigators were able to demonstrate that although CREB and C/EBPα had no accessory factor activity, C/EBPβ was able to enhance responsiveness to this hormone. Thus, the CRE appears to be a multifunctional *cis*-element, participating in the cAMP response unit when either CREB or C/EBPα is bound to it and acting

as an accessory element in the glucocorticoid response unit when C/EBPβ is bound. Precisely what determines which transcription factor will be bound to the CRE at any point in time, and thus what hormone response unit it will participate in, is a puzzle yet to be solved.

The CRE is not the only *cis*-element that participates in overlapping hormone response units in the PEPCK promoter. The C/EBP binding site at -230, for example, which can bind either C/EBPα or -β, is a component of both the cAMP and T_3 response units. This complex of overlapping response units, which collectively has been termed a metabolic control domain (110), likely permits the integration of the various hormonal and nutritional stimuli that regulate the expression of the PEPCK gene. Although the individual components of the various hormone response units have, for the most part, been identified, we do not have any clear vision as to how these bound factors functionally interact in order to mediate their responses. Thus, despite the enormous number of studies that have been performed analyzing the various hormonal responses of the PEPCK promoter, many of the molecular details of its regulation remain a mystery.

CONCLUDING REMARKS

The studies cited and summarized in this review article have attempted to document the important role that C/EBPs play in the regulation of metabolism, via their ability to mediate the effects of nutrients and hormones onto gene expression. I hope this article has also identified important issues yet unaddressed regarding the functions and mechanisms of action of this family of proteins. For despite their importance in such critical processes as adipocyte differentiation and glucose homeostasis, our understanding of many aspects of their biochemical function remains incomplete. For example, although there are suggestions that individual gene promoters utilize one specific C/EBP isoform in its regulation, we do not have a good grasp as to how selective recruitment of one isoform can occur, given the conserved nature of the bZIP domain. Does differential phosphorylation of isoforms play a role? Perhaps the unique transactivation domains of C/EBP isoforms provide a puzzle-like "fit" with other bound transcription factors on promoters (23, 37). Alternatively, C/EBPs have been shown to distort DNA on binding, with the extent of the distortion dependent on not only the sequence of the binding site but also which isoform(s) it binds (4). Thus, it appears possible that isoforms could be differentially recruited on the basis of their ability to introduce the "correct" distortion into the promoter region that is required for its regulatory effects. Another area that remains entirely unexplored is whether C/EBPs regulate expression of genes that code for proteins that participate in fatty acid oxidation. Given that C/EBPs play a critical role in energy metabolism and alter the expression of genes that code for enzymes involved in carbohydrate metabolism and fatty acid synthesis, it seems probable that genes involved in fatty acid oxidation have been overlooked as a group of C/EBP-regulated genes. Thus, despite the increasing appreciation of the

important functions that C/EBPs play in regulating the expression of genes linked to critical metabolic events, there remains much to uncover and understand about this class of transcription factors.

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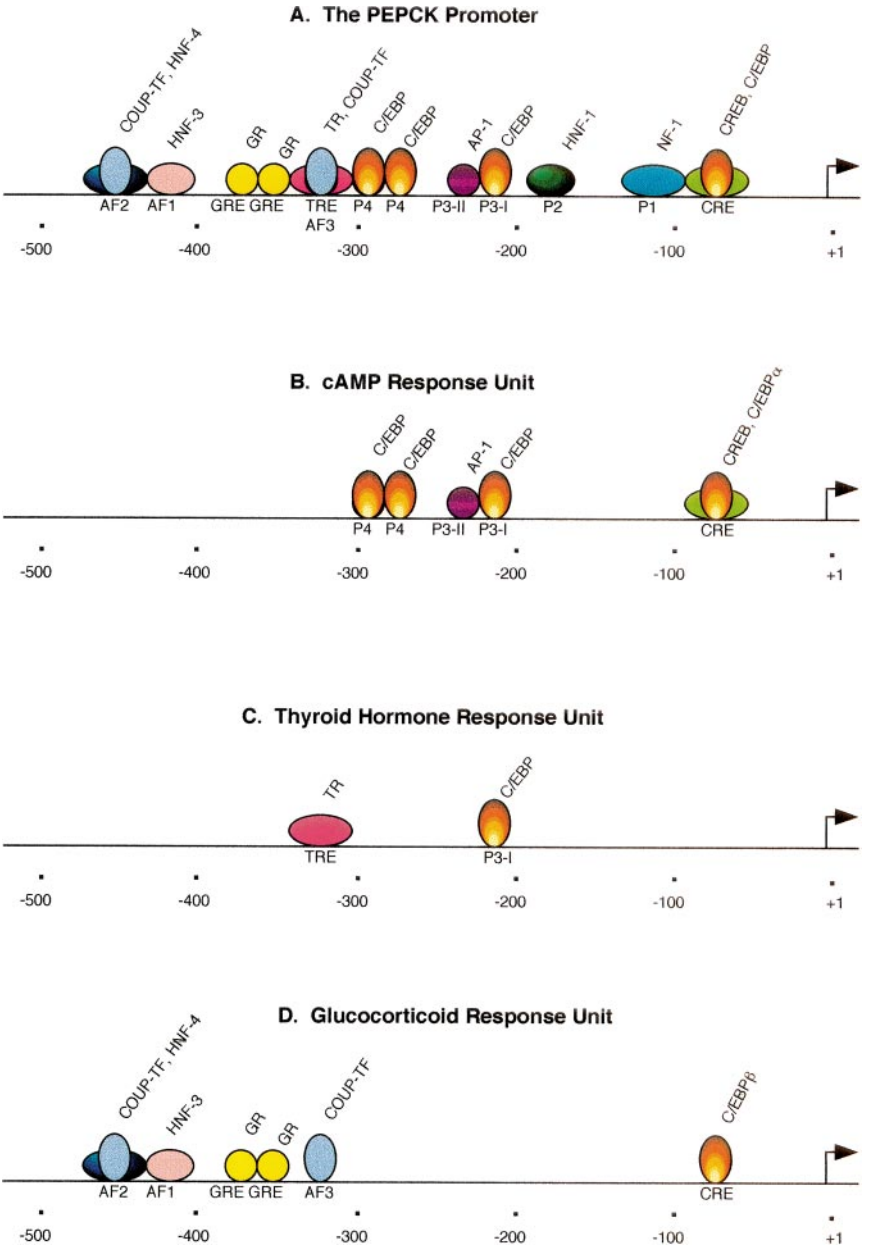

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Figure 1 See figure previous page

The phosphoenolpyruvate carboxykinase (PEPCK) promoter contains a number of hormone response units, some of which share *cis*-elements. (*A*) The various *cis*-elements and their corresponding DNA-binding protein(s). The name of the *cis*-element is shown below the line whereas the name of the transcription factors is placed above the shape depicting the bound protein. It should be noted that for three of the *cis*-elements, more than one potential DNA-binding protein has been identified. The term C/EBP indicates that both alpha and beta isoforms can bind to the site. (*B*) The subset of *cis*-elements and corresponding binding proteins that make up the cyclic AMP (cAMP) response unit. The binding of either cAMP response element binding protein (CREB) or C/EBPa to the cAMP response element (CRE) is permissive for the activity of this response unit. (*C*) The two *cis*-elements and corresponding binding proteins that make up the thyroid hormone response unit. The binding of either C/EBPa or C/EBPb to phosphatidylinositol 3-kinase is permissive for its activity. (*D*) The *cis*-elements and corresponding transcription factors that make up the glucocorticoid response unit. Maximal response to glucocorticoids can be achieved when either chicken ovalbumin upstream promoter transcription factor (COUP-TF) or hepatic nuclear factor 4 (HNF-4) is bound to accessory factor 2 (AF2), whereas there is a specific requirement for the binding of C/EBPb to the CRE. P1–P4, promoter elements 1–4; TR, thyroid hormone receptor; NF-1, nuclear factor 1; GR, glucocorticoid receptor; GRE, glucocorticoid response element.