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CCAAT/Enhancer-binding Protein β Is an Accessory Factor for the Glucocorticoid Response from the cAMP Response Element in the Rat Phosphoenolpyruvate Carboxykinase Gene Promoter*

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The cyclic AMP response element (CRE) of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene promoter is required for a complete glucocorticoid response. Proteins known to bind the PEPCK CRE include the CRE-binding protein (CREB) and members of the CCAAT/enhancer-binding protein (C/EBP) family. We took two different approaches to determine which of these proteins provides the accessory factor activity for the glucocorticoid response from the PEPCK CRE. The first strategy involved replacing the CRE of the PEPCK promoter/chloramphenicol acetyltransferase reporter plasmid (pPL32) with a consensus C/EBP-binding sequence. This construct, termed pΔCREC/EBP, binds C/EBPα and β but not CREB, yet it confers a nearly complete glucocorticoid response when transiently transfected into H4IIE rat hepatoma cells. These results suggest that one of the C/EBP family members may be the accessory factor. The second strategy involved cotransfecting H4IIE cells with a pPL32 mutant, in which the CRE was replaced with a GAL4-binding sequence (pΔCREGAL4), and various GAL4 DNA-binding domain (DBD) fusion protein expression vectors. Although chimeric proteins consisting of the GAL4 DBD fused to either CREB or C/EBPα are able to confer an increase in basal transcription, they do not facilitate the glucocorticoid response. In contrast, a fusion protein consisting of the GAL4 DBD and amino acids 1–118 of C/EBPβ provides a significant glucocorticoid response. Additional GAL4 fusion studies were done to map the minimal domain of C/EBPβ needed for accessory factor activity to the glucocorticoid response. Chimeric proteins containing amino acid regions 1–84, 52–118, or 85–118 of C/EBPβ fused to the GAL4 DBD do not mediate a glucocorticoid response. We conclude that the amino terminus of C/EBPβ contains a multicomponent domain necessary to confer accessory factor activity to the glucocorticoid response from the CRE of the PEPCK gene promoter.

Phosphoenolpyruvate carboxykinase (PEPCK)[1] (EC 4.1.1.32), a rate-controlling gluconeogenic enzyme, is expressed in a tissue-specific manner and is regulated at the transcriptional level by various hormones and nutrients (1, 2). Rat liver PEPCK gene transcription is stimulated by glucocorticoids, glucagon (via cyclic AMP), and retinoic acid and is inhibited by insulin (1–5).

Glucocorticoid induction of the PEPCK gene is mediated through a complex glucocorticoid response unit (GRU). This unit consists of (from 5′ to 3′) two accessory factor elements (AF1 and AF2), two glucocorticoid receptor-binding sites (GR1 and GR2), a third accessory element (AF3), and the cyclic AMP response element (CRE) (see Fig. 1). The glucocorticoid response decreases by 50–60% when any one of the accessory elements is mutated, and it is abolished when any combination of two are disrupted (6, 7). We have identified the proteins that bind to the three accessory elements. Hepatocyte nuclear factor 4 (HNF4) or chicken ovalbumin upstream promoter-transcription factor (COUP-TF) confers accessory factor activity by binding to the AF1 element, and COUP-TF binds to and confers activity from the AF3 element (7–9). Members of the CCAAT/enhancer-binding protein (C/EBP) and hepatocyte nuclear factor 3 (HNF3) families bind to AF2, but only the latter provide accessory factor activity for the glucocorticoid response from this element (10, 11).

An intact CRE is also required for a complete glucocorticoid response, because deletion of this element results in a 50% decrease of the glucocorticoid response (12). The CRE is therefore a multifunctional element, as it is also necessary for basal transcription and the cAMP response (4, 13). Multiple members of the leucine zipper transcription factor family bind to the CRE *in vitro*, including cyclic AMP response element-binding protein (CREB), C/EBP family members, D site-binding protein, and activator protein 1 (14–18). We, and others, have shown previously that CREB and C/EBP family members are involved in the cAMP response of the PEPCK gene (4, 13, 18–20). However, the roles played by these CRE-binding factors in the glucocorticoid response were unclear. We have previously reported that GR physically interacts with CREB *in vitro* and that both C/EBP family members and CREB bind to the PEPCK gene CRE (12, 21, 22).

The aim of the present study was to determine which pro-

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[1] The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; GRU, glucocorticoid response unit; AF, accessory factor; GR, glucocorticoid receptor; CRE, cyclic AMP response element; HRUs, hormone response units; HNF, hepatocyte nuclear factor; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; C/EBP, CCAAT/enhancer-binding protein; CREB, cyclic AMP response element-binding protein; CAT, chloramphenicol acetyltransferase; DBD, DNA-binding domain; EMSA, electrophoretic mobility shift assay; PCR, polymerase chain reaction; bZIP, basic and leucine zipper; PKA, cyclic AMP-dependent protein kinase A; PKI, heat-stable inhibitor of the cyclic AMP-dependent protein kinase; PKI^{mut}, mutant of PKI; CREwt, CRE wild type; USF, upstream stimulatory factor.

tein(s) provides accessory factor activity for the glucocorticoid response through the CRE. A very good glucocorticoid response was retained when the CRE was replaced with a consensus C/EBP-binding site, suggesting that C/EBP family members contribute accessory activity. A PEPCK promoter/chloramphenicol acetyltransferase (CAT) reporter gene construct, in which the CRE was replaced by a GAL4-binding site, was co-transfected with GAL4 DNA-binding domain (DBD)-fusion protein expression vectors to determine which protein(s) potentiates the glucocorticoid response from the CRE. We found that C/EBPβ mediates accessory factor activity through the CRE, whereas C/EBPα and CREB do not. Furthermore, the amino-terminal domain of C/EBPβ is required for accessory factor activity.

MATERIALS AND METHODS

Electrophoretic Mobility Shift Assay (EMSA)—The conditions of the EMSA have been described previously (22). The sequences of the oligonucleotides used in the EMSA are listed in Table I. The TNT T7 Quick Coupled Transcription/Translation System (Promega Corp., Madison, WI) was used for the *in vitro* translation of C/EBPα, C/EBPβ, and CREB using the plasmids pGEM-C/EBPα, pGEM-C/EBPβ, and pET-CREB, respectively (22). The pET-CREB plasmid was constructed by inserting the rat CREB coding sequences into the *Eco*RI/*Nde*I sites of the pET-3a vector (23). Nonspecific, single-stranded DNA-binding proteins were competed away with unlabeled, single-stranded CRE wild type (CREwt) antisense oligonucleotide, added at a 10-fold molar excess of the labeled probe. For supershift analyses, anti-CREB1, anti-C/EBPα, anti-C/EBPβ, and anti-Tyk2 antisera from Santa Cruz Biotechnology, Inc. were used.

Plasmids—Construction of the plasmid pPL32, which contains the PEPCK promoter region from −467 to +69 bp relative to the transcription start site, has been described previously (21). pPL32 was used as a template in polymerase chain reactions (PCRs) to create mutations in the CRE. The nucleotide sequences of all the PCR primers used in this study are listed in Table I. A downstream primer (CAT, Table I) and the CRE mutant primers, C/EBP or GAL4, were used in PCRs to generate two megaprimers (24–26). The megaprimers were gel purified and used with an upstream primer (Table I, PLF) in a second PCR to generate a fragment that, after digestion with*Hin*dIII and *Bgl*II, was ligated into the reporter plasmid pPLF to generate the plasmids pΔCREC/EBP and pΔCREGAL4, respectively.

pRSV-GR, a glucocorticoid receptor expression vector, was obtained from Dr. Keith R. Yamamoto (University of California, San Francisco, CA). pBR322, a standard cloning vector, was obtained from Promega. The plasmid pSG424 expresses the yeast transcription factor GAL4 DBD driven by the SV40 promoter (27).

The plasmid pcDNA1-GAL4-CREBΔbZIP(4–283), which encodes a GAL4·CREB fusion protein that lacks the basic region and leucine zipper (bZIP) domain of CREB, was a gift from Dr. Richard A. Maurer (28). An *Eco*RI/*Xba*I fragment from this vector, which encodes the CREBΔbZIP fusion protein, was subcloned into the *Eco*RI/*Xba*I sites of the pSG424 plasmid to produce the mammalian expression vector pGAL4·CREB 283. Dr. Maurer also provided RSV-PKI and RSV-PKI^{mut} vectors that encode the heat-stable inhibitor of the cyclic AMP-dependent protein kinase and a mutant of the inhibitor, respectively (29).

The GAL4·C/EBPα 358, 317, 108 and GAL4·C/EBPβ 276, 118, 84, GAL4 DBD-C/EBPα and GAL4 DBD-C/EBPβ fusion protein expression vectors were gifts from Dr. Peter F. Johnson (30). To make GAL4·C/EBPβ 52–118 and GAL4·C/EBPβ 85–118, the DNA fragments that encode amino acids 52–118 and 85–118 in the C/EBPβ gene were amplified by the reverse transcription-PCR method. One of two upstream primers, β52 or β85, and a downstream primer, β118, were used in these reactions (Table I). Total RNA was isolated from H4IIE cells using the acid guanidine-phenol-chloroform method (31). Reverse transcription was performed at 37° C for 1 h in a 50-μl reaction mixture that contained 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 35 units of RNase inhibitor, 1 mM dNTPs, 50 units of AMV reverse transcriptase (Promega) in the presence of 1.5 μg of total RNA and 25 pmol of the β118 oligonucleotide. PCR was carried out using the β118 oligonucleotide and either the β52 or the β85 oligonucleotide as primers (Table I). Briefly, a 100-μl reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 2 mM dNTPs, 10 pmol of each primer, 10 μl of the reverse transcription reaction as a template, and 5 units of AmpliTaq DNA polymerase (Perkin-Elmer). The PCR conditions used were as follows: denaturation for 3 min at 98° C; 5 cycles of 1 min at 98° C, 30 s at 55° C, and 30 s at 72° C; and another 25 cycles of 1 min at 98° C, 30 s at 58° C, and 30 s at 72° C. The amplified DNA fragments were digested with *Eco*RI and *Xba*I, purified by agarose gel electrophoresis, and then subcloned into the *Eco*RI/*Xba*I sites of the plasmid pGAL4·C/EBPβ 118.

The DNA sequence of each construct was verified by dideoxy sequencing. All of the plasmids used in this study were purified by the CsCl/ethidium bromide density gradient ultracentrifugation method.

Cell Culture, DNA Transfections, and CAT Assays—H4IIE cells, a rat hepatoma cell line, were cultured as described previously (21). HeLa cells, a human cervical carcinoma cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. For the analysis of the glucocorticoid response in H4IIE cells, 10 μg of reporter plasmid and 5 μg of pRSV-GR, with or without GAL4-fusion protein expression vectors, were co-transfected using the calcium-phosphate method, as described previously (6). Four hours after transfection, the cells were subjected to a 20% Me_2SO shock for 4 min and then maintained in serum-free DMEM for an additional 16 h. This additional incubation was not performed for the transfections involving PKI and PKI^{mut}. The medium was then changed to serum-free DMEM, with or without 0.5 μM dexamethasone, and the cells were cultured for an additional 24 h. A titration using 0.5, 1, or 2.5 μg of the expression vector was performed when the cells were co-transfected with the GAL4-fusion protein expression vectors. The greatest dexamethasone response was employed to calculate the fold induction. The measurement of CAT activity was carried out as described previously (32).

The GAL4-fusion protein expression vectors were transfected into HeLa cells to determine the relative expression of the GAL4-fusion proteins. The medium was changed 16 h after transfection, and after an additional 32 h, nuclear extracts were prepared according to the method of Schreiber *et al*. (33). EMSAs or Western blots were used to measure the relative expression of these proteins (28, 30).

RESULTS

Analysis of CRE Region-substituted Constructs—CREB and C/EBP family members are the only proteins from rat liver nuclear extracts that bind specifically to the PEPCK CRE (22), making them obvious candidates for providing accessory factor activity to the glucocorticoid response. Transient transfections of H4IIE cells were performed using wild type (pPL32) and mutated PEPCK promoter/CAT reporter plasmids (Fig. 1). In these experiments, pPL32 conferred about a 10-fold glucocorticoid response, which is taken as the maximal, or 100%, response (Fig. 1, *top row, right*). In contrast, when the PEPCK CRE was replaced with a GAL4-binding site (pΔCREGAL4) (Fig. 1, *middle row*), the basal transcriptional activity and the glucocorticoid response decreased to 61 and 45% of the wild type activity, respectively. Both responses are equivalent to those observed when either an internal deletion or a block mutation of the CRE is made (Ref. 12 and data not shown). However, when the CRE was replaced with a consensus C/EBP-binding site (pΔCREC/EBP) (Fig. 1, *bottom row*), basal transcription increased 2-fold, and the absolute dexamethasone response was substantially greater than that conferred by the wild type promoter (*cf.* Fig. 1, *top row*). When corrected for the increase in basal expression, however, the dexamethasone response from pΔCREC/EBP was 71% that of wild type (Fig. 1, compare *bottom row* with *top two rows*). These results suggest that a member of the C/EBP family confers accessory factor activity to the glucocorticoid response.

EMSAs were performed to confirm that the wild type PEPCK CRE and the two mutant sequences described above bind the appropriate transcription factors (Fig. 2). As expected, no protein-DNA complex was formed when the wild type CRE (Table I, CREwt) oligonucleotide was used as the ^{32}P-labeled probe and incubated with an unprogrammed reticulocyte lysate (Fig. 2, *lane 1*). However, a protein-DNA complex was formed when this probe was mixed with a reticulocyte lysate programmed to express CREB (Fig. 2*A*, *lane 2*). The addition of a specific antiserum directed against CREB abolished the complex, whereas a nonspecific antiserum directed against Tyk2 did not

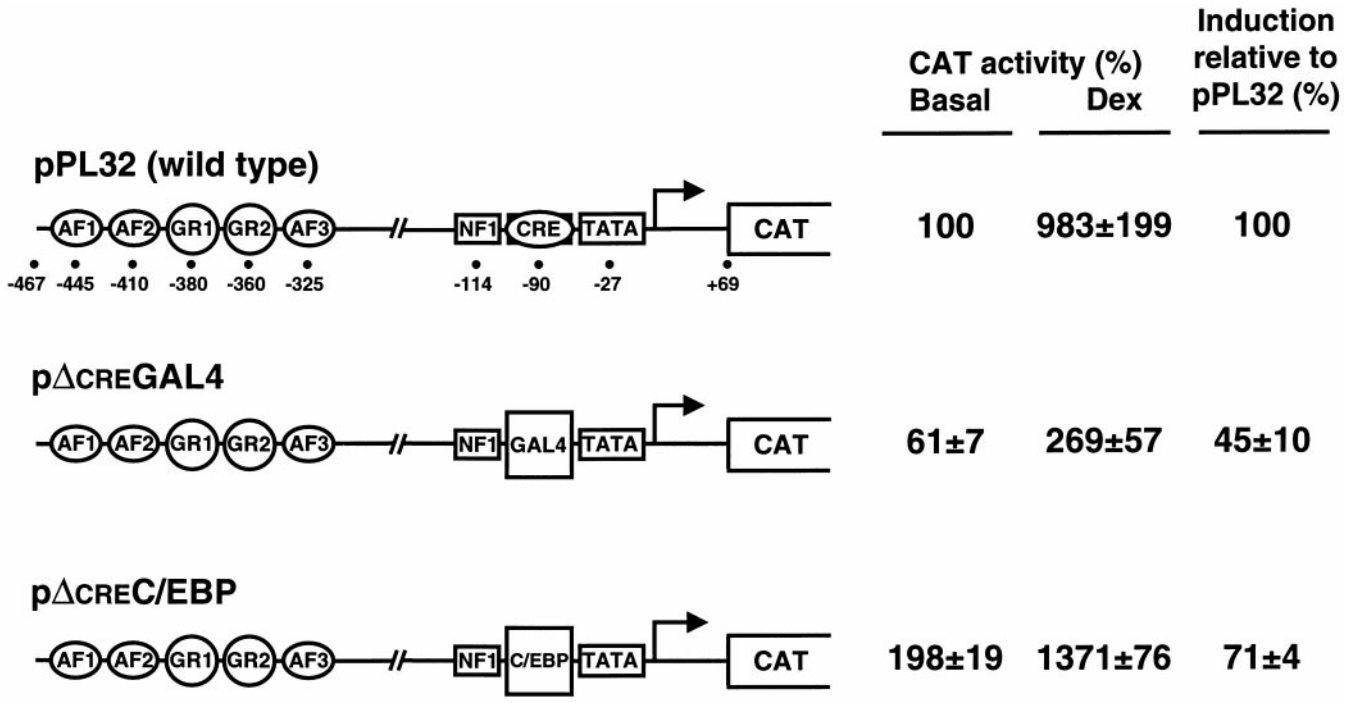


FIG. 1. **Comparison of basal and glucocorticoid-induced PEPCK transcription between wild type and mutant plasmids.** Schematic diagrams of the reporter constructs are depicted on the *left*. pPL32 is the wild type PEPCK-CAT reporter plasmid. In this diagram of pPL32, *boxes* denote those elements required for basal transcription, *circles* denote the glucocorticoid receptor binding sites, and *ovals* denote the accessory elements essential for the glucocorticoid response. Note that the CRE is involved in both processes. pΔCREGAL4 is a mutant of pPL32 in which the CRE is replaced with a GAL4-binding site. pΔCREC/EBP is a mutant of pPL32 in which the CRE is replaced with a C/EBP-consensus binding sequence. Both basal and glucocorticoid (dexamethasone (*Dex*))-induced CAT activities are shown on the *right*. CAT activity from the wild type plasmid (pPL32) was set at 100%. Glucocorticoid induction is defined as the quotient between the dexamethasone response and the basal activity. The level of glucocorticoid induction for the wild type plasmid (pPL32) was set at 100%. All values shown represent the mean ± S.E. of at least six separate experiments.

affect the formation of the complex (Fig. 2*A, lanes 3* and *4*). In competition experiments, the formation of this complex was prevented by the addition of a 100-fold molar excess of an unlabeled CREwt oligonucleotide but not by equivalent amounts of unlabeled oligonucleotides that contained binding sites for C/EBP, GAL4, or USF (Table I; Fig. 2*A, lanes 5–8*). The formation of the complex was not prevented even by the addition of a 500-fold molar excess of the unlabeled C/EBP oligonucleotide (data not shown); furthermore, no shifted complex was observed when the *in vitro* translated CREB protein was incubated with a labeled C/EBP oligonucleotide (Table I; data not shown). Together, these data reaffirm that although CREB binds specifically to the PEPCK CRE, it binds to neither the consensus C/EBP site nor the GAL4 DNA-binding site. Therefore, in the context of pΔCREC/EBP, CREB does not provide accessory factor activity to the glucocorticoid response (*cf.* Fig. 1, *bottom row*).

Shifted protein-DNA complexes were formed when the labeled CREwt oligonucleotide was incubated in binding reactions that contained either *in vitro* translated C/EBPα or C/EBPβ (Fig. 2, *B* and *C*, respectively, *lane 2*). These complexes were supershifted by specific antisera directed against C/EBPα or C/EBPβ, respectively (Fig. 2, *B* and *C, lane 3*). By contrast, nonspecific antiserum directed against Tyk2 did not affect the formation of the C/EBP-DNA complexes (Fig. 2, *B* and *C, lane 4*). Additionally, the C/EBPα antiserum did not cross-react with *in vitro* translated C/EBPβ, nor did the C/EBPβ antiserum cross-react with C/EBPα (data not shown). In competition experiments, the formation of these complexes was prevented by the addition of a 100-fold molar excess of the unlabeled CREwt and C/EBP oligonucleotides, but not an equimolar amount of either the GAL4 or USF E-box oligonucleotides (Table I; Fig. 2, *B* and *C, lanes 5–8*). Together, these results indicate that the CREwt oligonucleotide is bound by CREB, C/EBPα, and C/EBPβ; that the C/EBP oligonucleotide is bound by C/EBPα and C/EBPβ but not CREB; and that the GAL4 oligonucleotide does not bind any of these proteins *in vitro*.

C/EBPβ Is an Accessory Factor for the Glucocorticoid Response—H4IIE cells were co-transfected with the pΔCREGAL4 reporter plasmid and various GAL4 DBD fusion protein expression vectors to determine whether C/EBPα, C/EBPβ, or CREB (Fig. 3*A*) can confer accessory factor activity to the glucocorticoid response. The α and β isoforms of the C/EBP family were tested because both proteins are highly expressed in the liver and bind to the consensus C/EBP-binding site that confers partial accessory activity from the CRE (*cf.* Fig. 1, *bottom row*). The cellular expression of the GAL4 fusion proteins was found to be essentially equivalent, as determined by Western blotting or EMSA (Refs. 28 and 30; data not shown).

When H4IIE cells were co-transfected with pΔCREGAL4 (Fig. 3*B*) and the GAL4 DBD expression vector, the basal and glucocorticoid responses were 78 and 42% of those conferred by the wild type pPL32 construct, respectively (as shown in Fig. 3C, *row 1*). These responses are similar to those obtained when the reporter plasmid alone was transfected (data not shown). It is important to reiterate the fact that a glucocorticoid response of 42% is equivalent to that obtained when mutations are made that abolish accessory factor activity from the CRE (Ref. 12; data not shown).

When GAL4·C/EBPα 358, a vector that expresses the full-length C/EBPα·GAL4-DBD fusion protein, was co-transfected with pΔCREGAL4, basal transcription was restored to that of wild type, but the glucocorticoid response was unaffected at 50% of the control (Fig. 3*C, row 2*). These results were expected because the full-length C/EBPα·GAL4 DBD fusion protein con-

tains two bZIP domains that are known to interfere with transactivation (30). In fact, when this construct was co-transfected with the $(GAL4)_5$E1bCAT heterologous reporter (34), a construct that contains a TATA box and five tandem GAL4-binding sites (Fig. 3*B*), transcription activity was almost the same as that provided by the GAL4 DBD alone (Fig. 3*C, rows 1* and *2, right column*). Therefore, we next tested GAL4·C/EBPα 317, which lacks the leucine zipper domain, and GAL4·C/EBPα 108, which lacks both the basic region and the leucine zipper domain. When these constructs were co-transfected into H4IIE cells with the pΔCREGAL4 reporter, basal transcription increased, but the glucocorticoid response remained at 39 and 45% of wild type, respectively. We also tested the ability of these constructs to confer transcriptional activity to $(GAL4)_5$E1bCAT. In this heterologous system, the constructs that expressed the truncated forms of C/EBPα provided significantly greater transcriptional activity than did the full-length, bZIP-containing construct (Fig. 3*C, rows 3* and *4*). In fact, as the fusion proteins were truncated to leave only the amino-terminal activation domain, a greater level of basal transcription was observed for both the pΔCREGAL4 reporter construct and the $(GAL4)_5$E1bCAT heterologous promoter (Fig. 3*C, row 4*). This is in agreement with previous observations (30). Thus, although these C/EBPα constructs are expressed well in H4IIE cells and are capable of mediating transactivation, C/EBPα does not provide accessory factor activity for the glucocorticoid response.

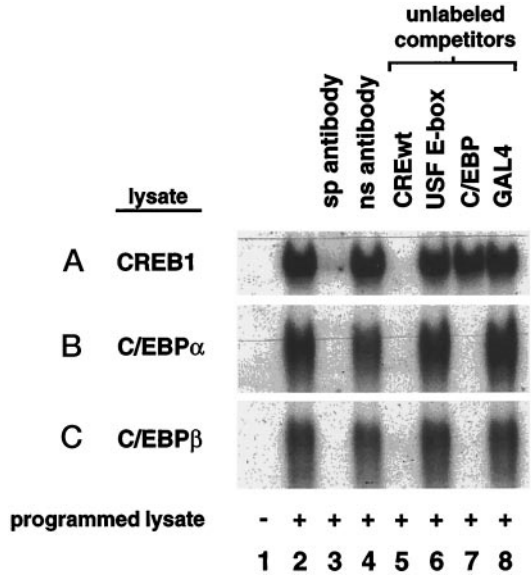


FIG. 2. **Electrophoretic mobility shift assay analysis of binding to the CRE.** In three separate experiments, the ^{32}P-labeled CREwt probe was mixed with *in vitro* translated CREB, C/EBPα, or C/EBPβ (designated lysates are shown on the *left*). *Lane 1,* unprogrammed lysates did not form shifted complexes. *Lane 2,* programmed lysates formed protein-DNA complexes with the radiolabeled probe. *Lanes 3* and *4,* specific (*sp*) antisera (anti-CREB, anti-C/EBPα, or anti-C/EBPβ) supershifted protein-DNA complexes, whereas nonspecific (*ns*) antiserum (anti-Tyk2) did not. *Lanes 5–8,* competition analyses were performed with a 100-fold molar excess of unlabeled oligonucleotides (CREwt, USF E-box, C/EBP, or GAL4) added to the binding reactions as competitor DNA.

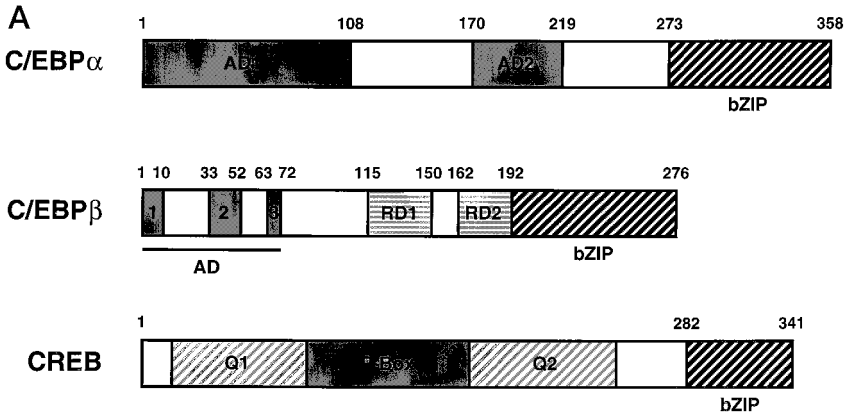
The possible role of C/EBPβ as an accessory factor was examined using the same system. We used GAL4·C/EBPβ 276, a plasmid that encodes the full-length C/EBPβ, and GAL4·C/EBPβ 118, a plasmid that lacks the bZIP region and the two repressor domains, RD1 and RD2, of C/EBPβ (see Fig. 3*A* and Ref. 30). When the full-length bZIP-containing GAL4·C/EBPβ 276 construct was co-transfected with pΔCREGAL4, basal transcription and the glucocorticoid response were almost the same as those seen with the GAL4 DBD alone (Fig. 3*C,* compare *rows 1* and *5*). Additionally, the GAL4·C/EBPβ 276 construct did not confer transcriptional activity in the $(GAL4)_5$E1bCAT heterologous system, an observation that is consistent with previous results (30). As noted before with C/EBPα, this result was anticipated because this fusion protein retains the bZIP domain of C/EBPβ. In contrast, GAL4·C/EBPβ 118 conferred an increase of both basal transcription and the glucocorticoid response, to 100 and 74% of wild type, respectively (Fig. 3*C, row 6*). Therefore, this C/EBPβ construct, containing amino acids 1–118, is able to confer accessory factor activity to the glucocorticoid response.

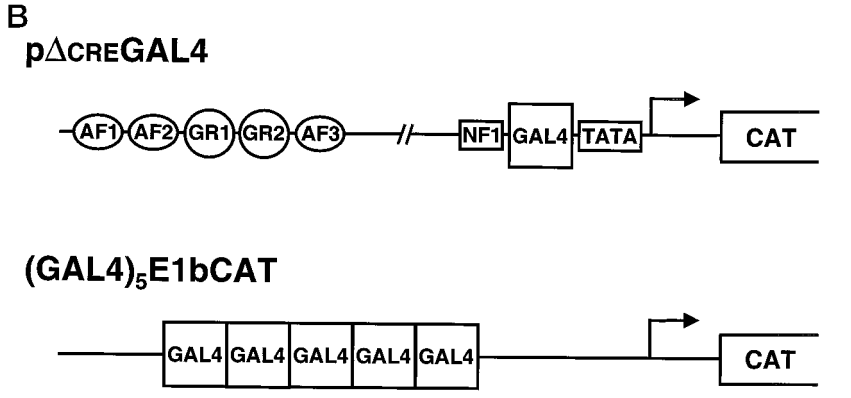
As shown in Fig. 3*A,* the activation domain of C/EBPβ is located in the region spanning amino acids 1–84. To determine whether this activation domain alone is capable of mediating accessory activity, GAL4·C/EBPβ 84 was co-transfected with pΔCREGAL4 into H4IIE cells. As was the case for C/EBPα, truncation of the C/EBPβ construct down to its amino-terminal activation domain increased basal transcription from both the pΔCREGAL4 promoter and the $(GAL4)_5$E1bCAT promoter (Fig. 3*C, rows 5–7*). However, the amino-terminal activation domain of C/EBPβ was not sufficient to confer accessory activity to the glucocorticoid response (Fig. 3*C, row 7*). This suggests that the region between amino acids 85 and 118 plays a role in conferring accessory activity to the glucocorticoid response.

To test whether the amino terminus of C/EBPβ is required for accessory activity, we next constructed the GAL4 DBD fusion proteins GAL4·C/EBPβ 52–118 and GAL4·C/EBPβ 85–118, wherein two segments of the amino-terminal activation

TABLE I
Oligonucleotides used in this study

Oligonucleotide	Nucleotide sequence
For EMSA	
CREwt	5′-GATCCGGCCCCTTACGTCAGAGGCGAG-3′ 3′-GCCGGGGAATGCAGTCTCCGCTCCTAG-5′
C/EBP	5′-GATCCGGCCCCTTGCGCAATAGGCGAG-3′ 3′-GCCGGGGAACGCGTTATCCGCTCCTAG-5′
GAL4	5′-GATCCGGCCGGCGGAGGACTGTCCTCCGGAGCCTCG-3′ 3′-GCCGGCCGCCTCCTGACAGGAGGCCTCGGAGCCTAG-5′
E-box	5′-GATCTCCGGTCACGTGACCGGA-3′ 3′-CTAGAGGCCAGTGCACTGGCCT-5′
For PCR	
CAT	5′-CTCCATTTTAGCTTCCTTAGCTCC-3′
C/EBP	5′-CAAAGGCCGGCCCCTTGCGCAATAGGCGAGCCTCCAGGT-3′
GAL4	5′-TATGATCCAAAGGCCGGCGGAGGACTGTCCTCCGGAGCCTCCAGGTCCAG-3′
PLF	5′-GCCGCATAGTTAAGCCAGCC-3′
β52	5′-CGCGGAATTCGCCGCCGCGGACTTCGC-3′
β85	5′-CGCGGAATTCGACTACGGTTACGTGAGCCT-3′
β118	5′-CGCGTCTAGATTAGAAGCCCGGCTCGGCCT-3′





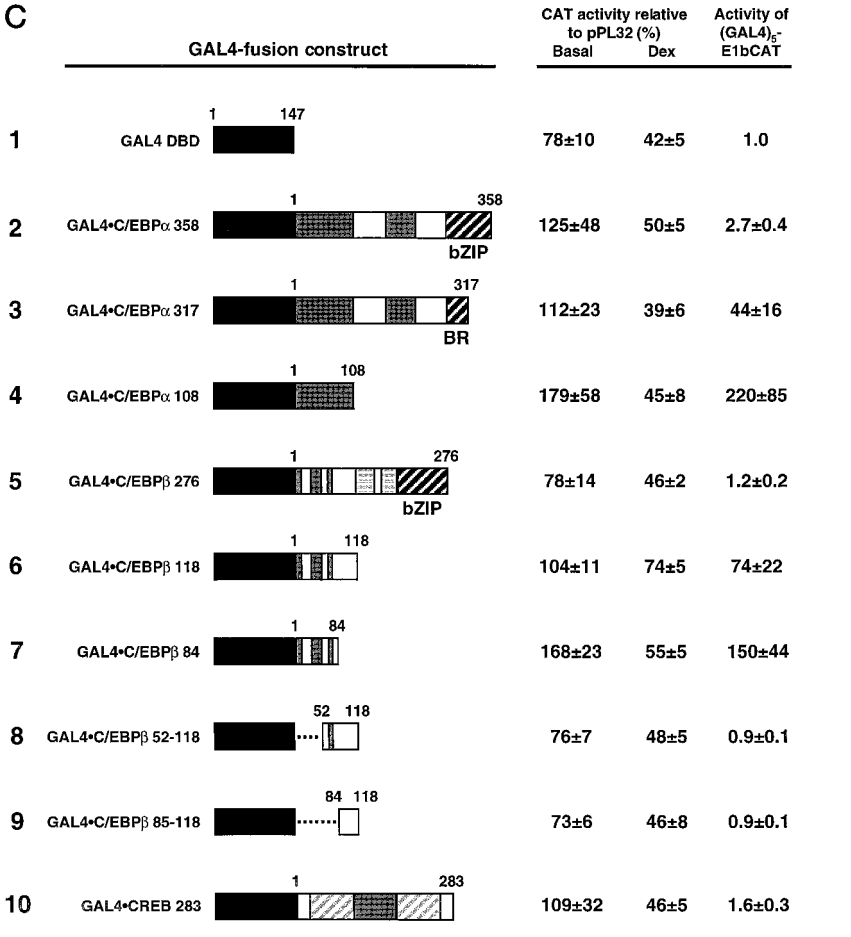


FIG. 3. **Identification of the transcription factor mediating the glucocorticoid response from the PEPCK CRE.** *A,* linear representations of the transcription factors that are able to bind the PEPCK CRE (*i.e.* C/EBPα, C/EBPβ, and CREB) are depicted above. C/EBPα contains two activation domains (*AD1* and *AD2*) and a carboxyl-terminal bZIP domain. C/EBPβ contains an amino-terminal activation domain (*AD*) composed of three subdomains, two repressor domains (*RD1* and *RD2*), and a carboxyl-terminal bZIP domain. CREB contains three activation domains (*Q1, P-box,* and *Q2*) and a carboxyl-terminal bZIP domain. *B,* the reporter constructs used for the GAL4-fusion experiments are pΔCREGAL4 and $(GAL4)_5$-E1bCAT. pΔCREGAL4 is a mutant of pPL32 in which the CRE was replaced with a GAL4-binding sequence. $(GAL4)_5$-E1bCAT is a heterologous CAT reporter plasmid driven by an E1b promoter into which five tandem GAL4-binding sites have been inserted. *C,* pΔCREGAL4 was co-transfected with various GAL4-fusion protein expression vectors into rat hepatoma (H4IIE) cells to determine basal and glucocorticoid-induced PEPCK transcription. The GAL4-fusion constructs are depicted on the *left*. The wild type PEPCK-CAT plasmid (pPL32) was used as an internal control for all experiments, and its CAT activity was set at 100%. The *left data column* shows the basal CAT activity mediated by each construct relative to pPL32. The *middle data column* shows the glucocorticoid-induced response of each construct relative to pPL32. To ascertain basal transcriptional activity mediated by the GAL4-fusion proteins in a heterologous context, 10 μg of the $(GAL4)_5$-E1bCAT reporter construct and 0.2 μg of the GAL4-fusion protein expression vectors were co-transfected into H4IIE cells. CAT activity from the H4IIE cells co-transfected with the $(GAL4)_5$E1bCAT reporter construct and the GAL4 DBD was set at 1. All values shown represent the mean ± S.E. of three or more experiments.

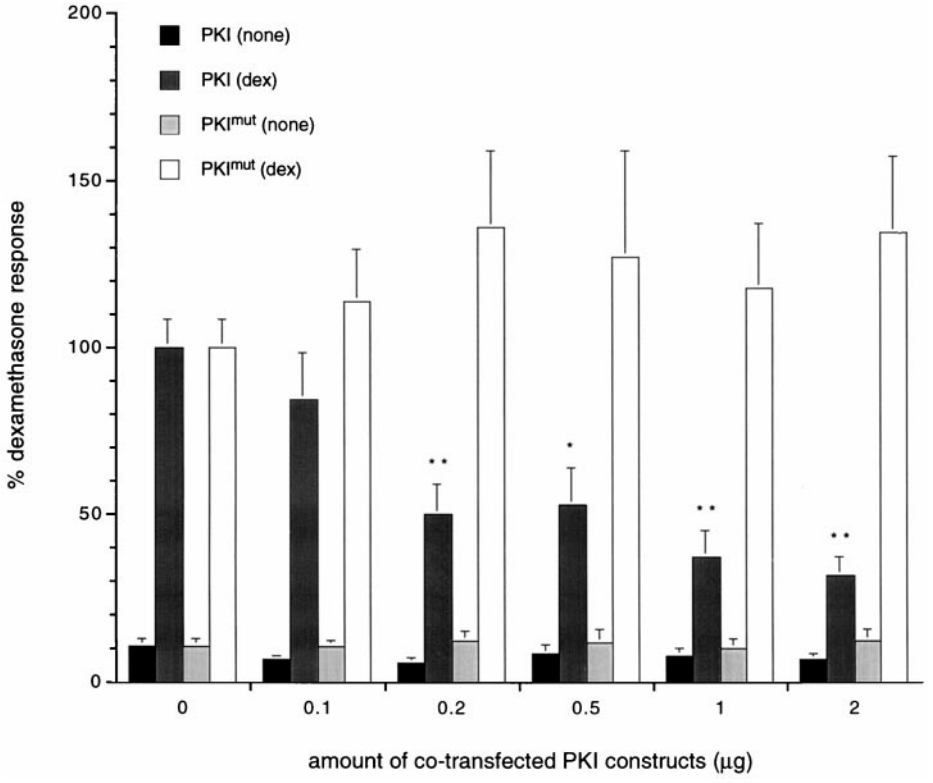


FIG. 4. **Inhibition of the glucocorticoid response by PKI.** The wild type PEPCK reporter plasmid (pPL32) and an expression vector for the glucocorticoid receptor (pRSV-GR) were co-transfected into H4IIE cells with increasing amounts of either PKI or PKI^{mut}. The total amount of DNA transfected into the tissue culture cells in each experiment was equalized using the empty cloning vector pBR322. As indicated in the *key,* the *black bars* denote basal PEPCK transcription levels in the presence of PKI, the *dark gray bars* denote dexamethasone-induced PEPCK transcription in the presence of PKI, the *light gray bars* denote basal PEPCK transcription levels in the presence of PKI^{mut}, and the *white bars* denote dexamethasone-induced PEPCK transcription in the presence of PKI^{mut}. CAT reporter activity is expressed in terms of percentage relative to dexamethasone-induced PEPCK transcription levels in the absence of either PKI or PKI^{mut}. All values shown represent the mean ± S.E. of five or more separate experiments. Statistical significance was calculated using Student's *t* test, so that differences are designated as highly significant for $p < 0.01$ (*) and very highly significant for $p < 0.001$ (**).

domain of C/EBPβ were deleted. Neither of these constructs conferred accessory factor activity to the glucocorticoid response (Fig. 3*C, rows 8* and *9*). Taken together, these results suggest that the accessory factor activity of C/EBPβ requires the 1–84 activation domain but that one or more amino acids in the 85–118 segment are also required. In addition, these amino-terminal truncation constructs were unable to confer transcriptional activation to the $(GAL4)_5$E1bCAT reporter plasmid, a result that was expected because these deletions affected the activation domain (see Fig. 3*A*).

When GAL4·CREB 283, which lacks the bZIP domain of CREB, was co-transfected with pΔCREGAL4, basal transcription was increased, but the glucocorticoid response remained at 46% of wild type. Once again, this is equivalent to the GAL4 DBD alone or a deletion of the CRE (12) (Fig. 3*C,* compare *rows 1* and *10*). Although GAL4·CREB 283 conferred neither accessory activity from the pΔCREGAL4 reporter nor basal activity from the $(GAL4)_5$E1bCAT reporter, co-transfection with a protein kinase A (PKA) expression vector increased transcriptional activity in the latter system (data not shown). This PKA-dependent stimulation is consistent with previous observations (28). Taken together, these results suggest that C/EBPβ, rather than C/EBPα or CREB, provides accessory factor activity for the glucocorticoid response from the PEPCK CRE.

PKI Inhibits the Glucocorticoid Response of PEPCK—To test whether endogenous cAMP-dependent protein kinase (PKA) activity is necessary for glucocorticoid stimulation of PEPCK gene transcription, H4IIE cells were co-transfected with the pPL32 reporter plasmid, pRSV-GR, and increasing amounts of either RSV-PKI or RSV-PKI^{mut}. We utilized a heat-stable inhibitor of PKA (PKI), as well as an inactive mutant of the PKA inhibitor (PKI^{mut}). As depicted in Fig. 4, there is a dose-dependent decrease in dexamethasone-induced PEPCK transcription with increasing amounts of PKI. Interestingly, the dexamethasone response is decreased to a little less than 50% of its original value, reminiscent of what happens when the CRE is mutated in the context of the wild type PEPCK promoter. In contrast to PKI, when the inactive mutant of the heat-stable inhibitor (RSV-PKI^{mut}) is co-transfected, there is no statistically significant difference observed between glucocorticoid induction in the presence or absence of the mutant. These results hold true at higher concentrations of PKI and PKI^{mut} (data not shown).

DISCUSSION

Simple hormone response elements are DNA sites that bind transcription factors that, directly or indirectly, alter the rate of transcription of a specific gene. As classically defined, hormone response elements mediate hormone responses when placed in the context of heterologous promoters, and they function in a manner that is independent of orientation or position. In recent years, it has become apparent that, in natural promoter contexts, this description of hormone response elements is an oversimplification. Indeed, multiple arrays of transcription factors in a very specific, ordered arrangement are required for the proper regulation of genes. The operation of the PEPCK gene promoter in mediating a glucocorticoid response provides a case in point. Four accessory factor elements, including the CRE, and two GR-binding sites are required for a complete glucocorticoid response, and the proper order and placement of these elements is necessary for this effect (35). Schüle *et al.* (36) explored the role accessory factors play in the glucocorticoid response mediated by a simple GRE in a minimal promoter system, and they found that virtually any transcription factor that binds near the GR-binding site augments the glucocorticoid response. By contrast, in the context of the PEPCK promoter, specific transcription factors mediate accessory activity only from particular accessory elements. For example, the AF2 element of the PEPCK gene promoter binds HNF3 and members of the C/EBP family. When the AF2 element is changed to the sequence of the AF1 element, which binds HNF4 or COUP-TF, or to a consensus Sp1-binding site, there is a reduction in the glucocorticoid response equivalent to

that observed when the AF2 element is functionally eliminated (11). These transcription factors, at least, will not replace HNF3. Furthermore, the insertion of a consensus HNF3-binding site in place of the AF2 element provides a complete glucocorticoid response, whereas mutations that prevent the binding of HNF3, but allow C/EBP family members to bind, abolish AF2 accessory factor activity. Hence, HNF3, rather than C/EBP family members, mediates accessory activity from the AF2 element (11). In addition, accessory activity from AF1 and AF3 is mediated by specific sets of transcription factors. For example, HNF3 cannot mediate AF1 or AF3 activity, but HNF4 and/or COUP-TF serve this function (35). In the present study, we demonstrate that C/EBPβ, rather than C/EBPα or CREB, confers accessory activity to the glucocorticoid response from the CRE. This is consistent with the idea that accessory factor activity for native promoters requires specific transcription factors.

C/EBPβ interacts with a variety of transcription factors to mediate the transcriptional activation of many genes. Both the carboxyl-terminal leucine zipper and the amino-terminal activation domains of C/EBPβ are required for the interaction with the glutamine- and serine/threonine-rich activation domains of Sp1, an association that is necessary for transcription of the rat *CYP2D5* gene (37). The leucine zipper domain of nuclear factor-interleukin 6, the human homologue of C/EBPβ, interacts with the glucocorticoid receptor *in vitro,* and these interactions are presumed to result in increased transcription of the α1-acid glycoprotein gene by glucocorticoids in F9 cells (38, 39). In addition, the same domain of C/EBPβ interacts with the Rel-homology domain of nuclear factor-κB to regulate transcription of the interleukin-6 and interleukin-8 genes (40–43). The amino-terminal region of nuclear factor-M, the chicken homologue of C/EBPβ, interacts with the E1A-binding domain of CREB-binding protein/p300 to regulate transcriptional activation of the chicken *mim*-1 gene (44). Our data suggest that amino acids 1–118 of C/EBPβ, a region that includes the CREB-binding protein-binding domain and the amino-terminal activation domain (see Fig. 3*A*), are required for the maximal transcriptional stimulation of the PEPCK gene by glucocorticoids. The activation domain (amino acids 1–84), although necessary, is not by itself sufficient to provide accessory factor activity. This implies that the region from amino acids 84–118 must contain residues that, along with the activation domain, provide accessory factor activity. The idea that a transcription factor, within the context of a hormone response unit, would contain distinct modular domains for basal transcriptional activation and accessory transcriptional activation provides a mechanism for integrating multiple hormonal signals and providing an adaptive response. Just such a separation of activation domains was reported recently by Merika *et al.* (45), who found both a "synergism-specific" domain and a basal transcriptional activation domain within the carboxyl terminus of nuclear factor-κB. In an observation that was strikingly similar to the results that we found with C/EBPβ, they observed that the small region of p65 that is adjacent to yet distinct from the basal activation domain was not required for basal activation alone, but was nevertheless essential for synergistic effects.

A complete restoration of the glucocorticoid response was not observed with the experimental strategies employed in these studies. For example, the glucocorticoid response is only 71% of wild type when the CRE is replaced with a consensus C/EBP-binding site. However, because the expression of C/EBPα and C/EBPβ, the two most abundant C/EBP isoforms in the liver (46), are induced by glucocorticoids to equivalent levels in H4IIE cells (47), and because C/EBPα and C/EBPβ have the same binding affinity for the consensus C/EBP-binding site (22), it might be argued that C/EBPα, which does not confer accessory activity, may act as a competitive antagonist to C/EBPβ at the C/EBP site, and so blunt the glucocorticoid response. Alternatively, the position of C/EBPβ on the consensus C/EBP-binding site in the context of the PEPCK promoter may be a few base pairs out of register from its location on the wild type PEPCK CRE, because the core binding site for C/EBP family members is not the same size as the CRE (48). This could result in a reduced glucocorticoid response, because there is a very stringent requirement for spacing between accessory elements in the GRU (35).

The presence of a cell line in which the glucocorticoid response can be restored by the provision of one or more missing accessory factors would be very helpful. Unfortunately, there is no such cell line. Although not ideal, the GAL4 system affords us the power not only to approximate the functions of various factors but also to manipulate these factors in such a way as to specifically dissect their different activities and assign these activities to different modules within the protein. Similar to that seen with the first approach, the GAL4·C/EBPβ 118 construct provides significant but incomplete restoration of activity to the glucocorticoid response. There are a number of possible explanations for this observation. First, the GAL4-fusion protein may not have the exact conformation of native C/EBPβ *in vivo*, due either to the fusion itself or to allosteric effects mediated by the GAL4-binding site. Another possibility is that the leucine zipper domain, which interferes with transactivation in the GAL4 system (30) and, therefore, was removed in order to unmask accessory activity, may be required by the native protein for complete accessory factor activity.

Yet another possibility is that, *in vivo*, there may be another factor that acts as a better accessory factor for the glucocorticoid response through the CRE than does C/EBPβ. If so, other CRE-binding factors, such as members of the Fos, Jun, CREB modulator, or activating transcription factor families, could confer the activity *in vivo*. However, we find this scenario unlikely, because the major rat liver nuclear extract CRE-binding proteins found by EMSA are all members of the C/EBP family (22). In fact, our conclusions with C/EBPβ concur with several previous studies that independently associated this particular transcription factor with the induction of PEPCK by hormones (*e.g.* cAMP and glucocorticoids) at the CRE. In 1993, Park *et al.* (18) reported a role for C/EBPβ, and not C/EBPα, at the CRE in the induction of PEPCK by cAMP. The following year, in accordance with previous work published by this lab, Angrand *et al.* (49) found that a full glucocorticoid response for PEPCK requires the CRE. Soon afterward, in a set of *in vivo* studies using transgenic mice, Friedman and colleagues (50, 51) correlated the dramatic increase in C/EBPβ levels with the CRE-dependent glucocorticoid induction of PEPCK gene transcription during exercise.

Our results with PKI correlate well with work done by Angrand *et al.* (49) to demonstrate how important endogenous PKA activity is to the glucocorticoid response of the PEPCK gene. Moreover, they suggest that the PEPCK GRU and cAMP response unit are intertwined not only at the level of DNA elements but also at higher signaling levels. Such a cooperative integration of signaling and hormone response elements between hormone response units (HRUs) may provide the complex mechanism through which synergism occurs when two hormones act simultaneously to regulate the transcription of a single gene. Future studies will be directed toward determining whether or not C/EBPβ plays a role in the integration of these two hormonal signals at the PEPCK CRE.

The identification of C/EBPβ as an accessory factor for the glucocorticoid response completes the cataloging of accessory

factors for the complex PEPCK GRU that began a number of years ago (3, 6–8, 9, 11, 12). Several HRUs of the PEPCK gene have been described in some detail, including the GRU, the retinoic acid response unit, the insulin response unit, and the cAMP response unit. These HRUs share a common feature in that they each contain several multifunctional elements able to bind more than one set of proteins and thereby mediate responses to different signals. For example, the retinoic acid response unit is composed of two elements: retinoic acid response elements 1 and 2 (which bind RAR/RXR heterodimers), containing sequences that are coincident with AF1 and AF3 of the GRU (which bind COUP-TF/HNF4 and COUP-TF, respectively). The CRE is also a pleiotropic element that is crucial for basal transcription, the cAMP response, and the glucocorticoid response. CREB binds to the CRE to mediate the cAMP response, whereas C/EBPβ binds to the same sequence to confer accessory activity to the glucocorticoid response. This arrangement of the PEPCK promoter, with its constellation of overlapping HRUs, is termed a metabolic control domain. It is the unique structure of the metabolic control domain that permits the integration of multiple hormonal stimuli, thereby facilitating a multifaceted response of the PEPCK gene to a variety of environmental challenges.

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