The role of CCAAT Enhancer-binding Protein β in the Thyroid Hormone and cAMP Induction of Phosphoenolpyruvate Carboxykinase Gene Transcription*

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Transcription of the gene for phosphoenolpyruvate carboxykinase (PEPCK) is stimulated by thyroid hormone (T₃) and cAMP. Two DNA elements in the PEPCK promoter are required for T₃ responsiveness including a thyroid hormone response element and a binding site called P3(I) for the CCAAT enhancer-binding protein (C/EBP). Both the α and β isoforms of C/EBP are highly expressed in the liver. C/EBPβ contributes to the liver-specific expression and cAMP responsiveness of the PEPCK gene. In this study, we examined the ability of C/EBPβ when bound to the P3(I) site to regulate PEPCK gene expression. We report that C/EBPβ can stimulate basal expression and participate in the induction of PEPCK gene transcription by T₃ and cAMP. The cAMP-responsive element-binding protein and AP1 proteins that contribute to the induction by cAMP are not involved in the stimulation by T₃. A small region of the transactivation domain of C/EBPβ is sufficient for the stimulation of basal expression and cAMP responsiveness. Our results suggest that C/EBPα and C/EBPβ are functionally interchangeable when bound to the P3(I) site of the PEPCK promoter.

Expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene is controlled by a variety of hormones including glucagon (via cAMP), thyroid hormone (T₃), glucocorticoids, retinoic acid (RA), and insulin (1). Our studies have been directed at elucidating the mechanisms by which T₃ and cAMP stimulate the expression of the gene for PEPCK in the liver. Regulation of gene transcription by T₃ is mediated through the binding of liganded T₃ receptors (TR) to T₃ response elements (TRE) in the promoters of genes (2, 3). The TR binds to DNA primarily as a heterodimer with the retinoid X receptor (RXR) (3, 4). The ligand binding, dimerization, and transactivation domains of TR are contained within a broad region between amino acids 160 and 456 (3, 5). Binding of T₃ by the TR results in conformational changes in the receptor and alteration of its transactivation properties (5). There are two isoforms α and β of the TR, and the TRβ is highly expressed in the liver (2, 6). Two elements in the PEPCK promoter are required for the full induction of PEPCK transcription by T₃ (see Fig. 1A). The first is the PEPCK-T₃-responsive element (PTRE) located within nucleotides −330 to −319, and the second is a binding site for the CCAAT enhancer-binding protein (C/EBP) called P3(I) between −250 and −234 (7, 8). The PTRE has an unusual architecture consisting of two direct repeats separated by zero nucleotides (7). Mutation of either the PTRE or the P3(I) site eliminates the T₃ response (8).

Several elements in the promoter are involved in the T₃ induction of PEPCK transcription including a cAMP-responsive element (CRE) at −90 to −82, the P3(I) site, and an AP-1 binding site at −260 to −250 (Fig. 1A) (9, 10). Three families of transcription factors that contribute to the cAMP stimulation include the cAMP-responsive element-binding protein (CREB), AP-1 proteins, and C/EBP (11). The P3(I) site is central to both the T₃ and cAMP induction of PEPCK transcription. In vivo studies have confirmed the importance of the P3(I) site in regulation of PEPCK gene expression. Transgenic mice carrying a PEPCK-bovine growth hormone fusion gene with a mutation in the P3(I) site have reduced hepatic expression of the bovine growth hormone transgene and reduced cAMP responsiveness of the PEPCK-bovine growth hormone reporter vector (12).

C/EBP proteins constitute a family of transcription factors of which the α and β isoforms are highly expressed in the liver (13–15). C/EBPα is expressed primarily in liver and adipose tissue and regulates the expression of hepatic genes involved in energy metabolism (16, 17). In addition to participating in hormonal responsiveness, C/EBP proteins play an important role in PEPCK gene transcription by contributing to the induction of the PEPCK gene at birth and directing liver-specific expression (11, 17, 18). Our early studies showed that C/EBPα and β could bind to the CRE, P3(I), and P4(I) elements in the PEPCK promoter (15, 19). C/EBPα can participate in the T₃ and cAMP induction of PEPCK transcription, and recent studies have shown that C/EBPα can mediate a cAMP induction without CREB being present (8, 11, 20).

Since C/EBPβ is highly expressed in the liver and binds to identical sites in the PEPCK promoter, we examined whether C/EBPβ when bound to the P3(I) site could participate in the hormonal induction of PEPCK transcription. Our data demon-
strate that C/EBPβ can stimulate PEPPCK gene expression through the P3(I) site and modulate hormone responsiveness. The results indicate that the α or β isoforms of C/EBP have similar functions when bound to this element.

MATERIALS AND METHODS

**Gel Mobility Assays**—Gel mobility assays were conducted on 5% non-denaturing polyacrylamide gels (80:1 acrylamide:bisacrylamide) in 22 mM Tris and 190 mM glycine at 4 °C (8). Double-stranded oligomers were labeled with Klenow enzyme and [α-32P]dCTP (8). The binding reactions were performed at room temperature in 10% glycerol, 80 mM KCl, 25 mM Tris (pH 7.4), 1 mM EDTA, and 1 mM dithiothreitol. Each binding reaction contained 1.0 μg of poly(dI-dC) as nonspecific competitor and proteins as indicated. Nuclear proteins were prepared from HepG2 cells by the method of Shapiro et al. (21). Antibodies to C/EBPα and RXRα were obtained from Santa Cruz Biochemicals. The antibody to C/EBPβ was a generous gift from Anna May Diehl.

**Construction of CAT and Luciferase Vectors**—The ligation of the PEPPCK promoter from −490 to +73 to the CAT reporter gene (−490-PCAT) has been described (7). To introduce the Gal4 binding site into the P3(I) site of the PEPPCK promoter (−490-PS4G), the −490-PCAT vector containing a BstII site in the P3(I) element (−490PS4(I)-PCAT) (8) was digested with BstII and ligated with the double-stranded oligomer containing the sequence gattcaggtgctgtctgcct. The amplified promoter fragment was digested with XbaI and BglII and ligated into the polylinker of pGL3 basic (Promega). The Gal4 site was introduced into the TRE region of the PEPPCK promoter from −108 to +66 by PCR amplification of the 5′ primer, tctccgtctgac, containing the altered nucleotides and a 3′ primer, ttagatct-9, using the PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotides representing amino acids 108–102 (ggtcgc-gatcagctgctgtctgtctgcctggatcggcttttttc, was used in PCR amplification. Additionally, two reverse primers containing PaiI sites and the nucleotide...
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HepG2 cells were transfected with 5 μg of a PEPCK-luciferase gene (−490-PLuc), 1 μg of an expression vector for the catalytic subunit of protein kinase A (PKA), 1 μg of a dominant negative expression vector for either C/EBP (A-C/EBP), C/EBP (A-CREB), or Jun (A-Fos), and 1 μg of SV40-βgal. The cells were harvested 36 h after transfection. The luciferase activity was corrected for protein content of the cell extract and transfection efficiency. All transfections were performed three to five times in duplicate. The inductions by PKA are presented as fold induction ± S.E. To assess basal transcription, the 490-PLuc vector in the absence of a dominant negative vector was assigned a value of 1.0, and the effect of the A-vectors was determined by comparison with this activity.

| Reporter vector | Dominant negative vector | Induction by PKA | Basal transcription |
|-----------------|--------------------------|------------------|---------------------|
| −490-PLuc       | A-C/EBP                  | 6.2 ± 0.9        | 1.0                 |
| −490-PLuc       | A-CREB                   | 1.9 ± 0.2        | 0.7 ± 0.1           |
| −490-PLuc       | A-Fos                    | 3.0 ± 0.3        | 1.5 ± 0.3           |
| −490-PLuc       |                          | 2.5 ± 0.1        | 0.4 ± 0.1           |

These observations indicate that the role of C/EBP in T₃ responsiveness is specific to the PEPCK gene and that the inhibition of PEPCK-CAT by A-C/EBP is not an inhibition of the TR. We have used both the CAT and luciferase reporter genes in these studies. Our results are similar with either reporter gene. Generally, we have used the CAT gene for our studies with T₃ and PEPCK promoter so that we can easily compare our results with our previous work. Our recent experiments with cAMP and the PEPCK promoter have been conducted with the luciferase gene.

Previous studies had shown that C/EBP, CREB, and AP-1 proteins were involved in the stimulation of PEPCK transcription by cAMP (9). To determine whether dominant negative vectors for C/EBP, CREB, or Jun would affect the cAMP response, HepG2 cells were cotransfected with a mammalian expression vector for the catalytic subunit of protein kinase A (PKA), −490 PEPCK-luciferase (−490-PLuc), and the dominant negative vectors. Overexpression of PKA increased the luciferase activity 6-fold, and this stimulation was reduced by each dominant negative protein (Table II). These results confirm that the cAMP induction requires all three proteins and support the mutational studies of the PEPCK gene in which disruption of CRE, P3(I), P3(II), or P4(I) decreased the induction by cAMP (9, 10). The A-C/EBP and A-Fos vectors decreased the basal expression of the PEPCK gene, while A-CREB did not reduce basal expression.

To test whether the DNA binding domain of the TRβ was required for the T₃ induction of PEPCK transcription, the PTFE was converted into a Gal4 binding site to create −330 PTFE/G4-PCAT (see Fig. 1A). This CAT vector was cotransfected with a Gal4-TRβ mammalian expression vector, which has the ligand binding/transactivation domain of the TRβ (amino acids 168–456) fused to the Gal4 DNA binding domain (25). The Gal4-TRβ will bind to the Gal4 site in the PEPCK-CAT vector. An 80-fold stimulation of PEPCK-CAT activity was obtained (Table III). Even with the robust T₃ induction obtained with the Gal4-TRβ vector, mutation of the P3(I) site (−330 PTFE/G4-P3 m PCAT) eliminated the T₃ response. Co-transfection of −330 PTFE/G4-PCAT with A-C/EBP also severely decreased the T₃ induction (data not shown).

To determine if the T₃ effect could be mediated by TR-XXR heterodimers, we used a Gal4-RRα mammalian expression vector (Gal4-RRα) and a mammalian expression vector for the ligand binding domain of TRβ (CMV-ΔΔTRβ) (26). ΔΔTRβ cannot bind DNA. When cotransfected with Gal4-RRα, ΔΔTRβ associates with the promoter through heterodimerization with Gal4-RRα. Co-transfection of −330 PTFE/G4 PCAT with the combination of Gal4-RRα and ΔΔTRβ stimulated transcription 56-fold in the presence of T₃ (Table III). ΔΔTRβ alone did not mediate a T₃ response, and mutation of the P3(I) site eliminated the T₃ induction. These observations indicate that the TRβ ligand binding domain is sufficient for the interaction with C/EBP proteins if it is tethered to the PEPCK promoter. In addition, these results suggest that the T₃ effect could be mediated by either TR homodimers or TR-XXR heterodimers.

The next experiments examined whether sequences adjacent to the C/EBP binding site were required for T₃ and cAMP responsiveness. A series of mutations was introduced through the P3(I) binding region in the context of the −490-PLuc and −490-PCAT vectors. The C/EBP binding site (TTGTTTGGAG) is contained within nucleotides −243 to −235. The mutations that were introduced into the −490-PCAT vector of nucleotides −248 to −245, −243 to −238, and −237 to −235 are shown in Fig. 1B. Alteration of nucleotides −243/−238 and −237/−235 eliminated C/EBP binding, whereas mutation of nucleotides −248/−245 did not affect C/EBP binding (data not shown). Mutation of nucleotides −243/−238 and −237/−235 diminished PKA responsiveness and basal expression (Table IV). The mutation of nucleotides −248/45 caused a slight reduction in the PKA responsiveness and a small increase in basal transcription. Alteration of the base pairs −243/−238 and −237/−235 eliminated the T₃ response. Mutation of nucleotides −248 to −245 reduced the T₃ stimulation modestly. In conjunction with the data from the dominant negative vectors, these results indicate that the protein bound to the P3(I) site that contributes to basal expression and hormone responsiveness is a member of the C/EBP family.

Both the α and β isoforms of C/EBP are present in the liver (13, 15). To evaluate the relative abundance of C/EBP isoforms binding to P3(I) in rat liver or HepG2 cell nuclei, supershift gel mobility assays were conducted using nuclear proteins isolated from rat liver or HepG2 cells, a labeled P3(I) oligomer and antibodies to C/EBPα or C/EBPβ. Antibodies to C/EBPα and C/EBPβ generated supershifted complexes, indicating that both C/EBP isoforms are present in rat liver nuclear extract and can bind P3(I) (data not shown). Since our transfection experiments were conducted in HepG2 cells, the binding of proteins from HepG2 cell nuclear extract to the P3(I) element was examined (Fig. 2). The pattern of binding of HepG2 cell nuclear extract to P3(I) was similar to that observed with rat liver nuclear extract, but one significant difference was observed. The antibody to C/EBPα caused only a slight disruption of binding, while the antibody to C/EBPβ supershifted most of the binding activity. C/EBPα has been shown to be present in HepG2 cells (13), but C/EBPβ is the predominant isoform. Adding competitor nucleotides with mutations in −248/−245 did not affect the gel shift pattern, but adding those with mutations in −243/−238 did disrupt most of the protein DNA interactions. These data suggest that C/EBPβ is the major protein binding to this site in HepG2 cells.

Previous studies had established that C/EBPα could restore cAMP and T₃ responsiveness to the PEPCK promoter (8, 11). Since C/EBPβ is the major C/EBP isoform in HepG2 cells, our next experiments examined the ability of the C/EBPβ to modulate the basal transcription and cAMP responsiveness of the PEPCK promoter. To conduct these experiments, a series of Gal4-C/EBPβ mammalian expression vectors was created (shown in Fig. 1C). A Gal4 DNA binding site was introduced into the P3(I) site in the −490 PEPCK-CAT vector to generate −490-P3G4-PLuc (see Fig. 1A). The −490 P3G4-PLuc vectors were cotransfected with the expression vectors Gal4-C/EBPβ into HepG2 cells. The results from these experiments are
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Fig. 1. Models of PEPCK reporter genes and Gal4-C/EBPβ expression vectors. A, the names of the various vectors used in these studies is indicated at left. The -490 PEPCK vectors contain -490 bp of the PEPCK promoter ligated to either the CAT reporter gene (CAT) or the luciferase reporter gene (Luc). The binding sites indicated above the promoter include the PEPCK T₃ response element (PTRE), the C/EBP binding site called P3(I), the cAMP response element (CRE), the AP-1 site called P3(II), the P4 site, and the Gal4 binding site (Gal4). The DR4 × 2 SV40 vector contains two copies of a direct repeat or the AGGTCA motif separated by 4 nucleotides ligated to the SV40 enhancerless promoter. B, the sequence of the P3(I) region between nucleotides -254 and -230 (P3(I) WT) is shown. The core C/EBP binding motif is underlined. Various mutations in the P3(I) region are shown below, and the mutated nucleotides are underlined. The P3G4 sequence contains the Gal4 DNA binding element. C, the Gal4-C/EBPβ expression vectors are shown. At the top is a model of the C/EBPβ protein with the numbers underneath indicating amino acids. BR and LZ represent the basic region and the leucine zipper, respectively. The numbers on the right indicate the amino acids of C/EBPβ included in each expression vector.

Table III

T₃ responsiveness of the PEPCK promoter can be mediated by TR homodimers or heterodimers

HepG2 cells were transfected with 5 μg of the PEPCK-CAT vector and either 0.5 μg of Gal4-TRβ, 0.5 μg of ΔTRβ, 0.5 μg of Gal-RXRα, or 5 μg of RSV-TRβ. All transfections contained 2 μg of SV40-β-gal. Following transfection, the cells were placed in medium containing 10% charcoal-stripped fetal bovine serum with 100 nM T₃. Cells were harvested after 48 h and assayed for CAT activity. All transfections were done in duplicate and repeated at least three times. The data are presented as -fold induction ± S.E.

| Reporter vector | Receptor expression vector | Induction by T₃ |
|-----------------|---------------------------|----------------|
| -330 TRE/G4-PCAT | None                      | 1.2 ± 0.1      |
| -330 TRE/G4-PCAT | Gal4-TRβ                  | 80.7 ± 20.7    |
| -330 TRE/G4-PCAT | Gal4-ΔTRβ                 | 2.1 ± 0.2      |
| -330 TRE/G4-PCAT | Gal4-RXRα                  | 1.1 ± 0.1      |
| -330 TRE/G4-PCAT | Gal4-RXRα/ΔTR              | 1.3 ± 0.3      |
| -330 TRE/G4-PCAT | RSV-TRβ                   | 56.0 ± 16.9    |
| -330 TRE/G4-PCAT | RSV-TRβ                   | 0.8 ± 0.1      |
| -330 TRE/G4-PCAT | Gal4-RXRα/RSV-TRβ         | 3.9 ± 0.3      |
| -330 TRE/G4-PCAT | Gal4-RXRα/ΔTR             | 2.5 ± 0.3      |

The results are presented as -fold induction ± S.E.

Table IV

Effect of various mutations within P3(I) region on responsiveness to T₃, overexpression of PKA, and basal expression

Mutations were introduced into the P3(I) region (PMut) of the -490PCAT and -490PLuc vectors. The sequence of the nucleotide changes is shown in Fig. 1B. To assess basal activity and cAMP inducibility, HepG2 cells were transfected with 3 μg of -490PLuc, 1.0 μg of PKA, and 1.0 μg of SV40-β-gal as described in the legend to Table II. To measure the effects of T₃, HepG2 cells were transfected with 5 μg of PCAT vector, 5 μg of RSV-TRβ, and 2.5 μg of SV40-β-gal as described in the legend to Table I. T₃ at a concentration of 100 nM was added for 48 h. All transfections were performed three to five times in duplicate. The results are presented as -fold induction ± S.E.

| Reporter vector | Basal transcription | Induction by PKA | Induction by T₃ |
|-----------------|---------------------|-----------------|----------------|
| -490-PLuc/CAT   | 1.0                 | 9.4 ± 2.0       | 3.5 ± 0.8      |
| 490-PMut248/45  | 1.35 ± 0.3          | 7.9 ± 0.9       | 2.8 ± 0.3      |
| 490-PMut243/38  | 0.43 ± 0.1          | 5.9 ± 0.4       | 1.2 ± 0.1      |
| 490-PMut237/35  | 0.71 ± 0.3          | 3.1 ± 0.9       | 0.9 ± 0.3      |

Table V

Effects of Gal4-C/EBPβ on transcription

Transfected with Gal4-C/EBPβ 1–264, expression of -490P3G4-PLuc was not stimulated. Others have reported that the leucine zipper and basic regions of C/EBPβ are inhibitory when expressed in the context of a Gal4 fusion protein (27). The Gal4-C/EBPβ vectors expressing amino acids 1–138 and 1–108...
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Table VI

| Reporter vector | Gal4 expression vector | Induction by T₃ |
|-----------------|------------------------|----------------|
| −490-CAT       | Gal4                   | 4.3 ± 0.5      |
| −490 P3G4-CAT  | −490 P3G4-CAT          | 1.4 ± 0.2      |
| −490 P3G4-CAT  | Gal4-C/EBPβ 1–264      | 1.9 ± 0.2      |
| −490 P3G4-CAT  | Gal4-C/EBPβ 1–138      | 2.5 ± 0.3      |
| −490 P3G4-CAT  | Gal4-C/EBPβ 1–108      | 2.8 ± 0.3      |
| −490 P3G4-CAT  | Gal4-C/EBPβ 1–66       | 2.0 ± 0.4      |
| −490 P3G4-CAT  | Gal4-C/EBPβ 1–25       | 1.4 ± 0.2      |

FIG. 2. Binding of proteins HepG2 nuclei to the P3(I) region of the PEPCK gene. Gel mobility assays were conducted as described under “Materials and Methods.” Each binding reaction contained 25,000 cpm of probe representing the sequence from −254 to −230 in the PEPCK promoter and proteins isolated from HepG2 nuclei (HepG2 NE). Oligomers to the P3(I) region containing the mutated nucleotides 248–245 (M248), 243–238 (M243), and 237–235 (M237) were also used, as is indicated above each lane. The sequence of the mutant oligomers is given in Fig. 1B. To some binding reactions were added antibodies to either C/EBPα (C/EA), C/EBPβ (C/EB), RXRa, or the IgG fraction, as is indicated above the lane. The autoradiograms were scanned and printed using Adobe Photoshop.

TABLE V

| Oligo | wt | wt | M248 | M243 | M237 | wt | wt | wt | wt |
|-------|----|----|------|------|------|----|----|----|----|
| Ab    | −  | −  | −    | −    | −    | −  | −  | −  | −  |
| HepG2 | +  | +  | +    | +    | +    | +  | +  | +  | +  |

were able to stimulate the basal expression 8–10-fold above the −490-P3G4-PLuc vector (Table V). Smaller regions of C/EBPβ were unable to increase the basal expression of the PEPCK-Luc gene. These results indicate that peptides within the first 108 amino acids of the transactivation domain of C/EBPβ will stimulate basal transcription.

To evaluate the ability of these constructs to mediate a cAMP induction, the Gal4-C/EBPβ expression vectors were cotransfected with the catalytic subunit of PKA and −490-PLuc (Table V). The −490-PLuc vector was stimulated 9.4-fold by the overexpression of PKA, while mutation of the P3(I) site decreased this induction to 2.3-fold. The Gal4-C/EBPβ 1–264 was not able to increase the cAMP responsiveness of the PEPCK promoter. Previously, it had been shown that a Gal4-C/EBPβ vector with full-length C/EBPβ was not able to restore cAMP responsiveness (11). However, cotransfection with Gal4-C/EBPβ 1–108 and 1–108 completely restored cAMP responsiveness. The Gal4-C/EBPβ 1–66 increased the cAMP stimulation from 2.2–7-fold, and the Gal4-C/EBPβ 1–25 vector did not mediate a cAMP response. As a control, the −490-P3G4-PLuc was cotransfected with Gal4-C/EBPβ 6–217, which contains amino acids 6–217 of C/EBPα ligated to the Gal4 DNA binding domain (11). The Gal4-C/EBPβ 6–217 vector stimulated both basal expression and cAMP responsiveness, as had been reported previously (20). These results demonstrate that either isoform of C/EBP can regulate PEPCK gene expression through the P3(I) site.

We tested whether the Gal4-C/EBPβ vectors could restore T₃ responsiveness to the −490-P3G4 reporter gene. The −490 P3G4-PCAT vector was cotransfected with Gal4-C/EBPβ expression vectors and the transfected cells exposed to T₃. As is shown in Table VI, introduction of the Gal4 site into P3(I) eliminated T₃ responsiveness. Cotransfection with Gal4-C/EBPβ 1–138 and 1–108 increased the T₃ response 2.5- and 2.8-fold, respectively. The Gal4-C/EBPβ 1–66 vector was less effective in restoring T₃ responsiveness, while the vector with amino acids 1–25 had no effect. These data suggest that additional regions of the protein such as the basic region or leucine zipper are needed for the T₃ induction. Gal4 expression vectors expressing full-length C/EBPα or β were tested, but these vectors did not increase the T₃ response (data not shown). Since the Gal4-C/EBP vectors with full-length C/EBP did not restore T₃ responsiveness to the PEPCK gene, the basic region or the leucine zipper may need to be associated with the P3 site to mediate this response.

Previously, we reported that cotransfection of Gal4-C/EBPβ 6–217 with −490-P3G4-PCAT partially restored T₃ inducibility (8). Since the DNA binding and dimerization domains of C/EBP are on the carboxyl terminus, it is possible that having the Gal4 domain on the amino terminus of the fusion protein was preventing the full restoration of T₃ responsiveness. The Gal4-C/EBPβ 6–217 “flipped” vector, which has the Gal4 DNA binding domain on the carboxyl terminus of the fusion protein rather than the amino terminus, was tested (Table VII). Like Gal4-C/EBPβ 6–217, this vector increased the T₃ response to 2.4-fold. Serial deletions of Gal4-C/EBP were tested, and they were less effective than Gal4-C/EBPα 6–217 in providing a T₃ induction (Table VII). These observations suggest that either the C/EBPα or β isoforms will be able to mediate a T₃ induction of PEPCK gene expression. Importantly, these results demonstrate that different regions of the C/EBP proteins participate in the T₃ and cAMP response. The transactivation domains of C/EBPα or β are sufficient to restore cAMP responsiveness, but other domains of C/EBPα or β appear to be required for the full T₃ response.
Table VII

| Reporter vector | Gal4 expression vector | Induction by T3 |
|-----------------|------------------------|----------------|
| −490-CAT        | −490-P3G4-CAT          | 3.2 ± 0.4      |
| 1–66 vector     | −490-CAT              | 0.9 ± 0.1      |
| 6–217 vector    | −490-CAT-Gal4-C/EBP    | 2.3 ± 0.2      |
| 6–217 vector    | −490-CAT-Gal4-C/EBP    | 2.2 ± 0.3      |
| 1–175 vector    | −490-CAT-Gal4-C/EBP    | 1.6 ± 0.3      |
| 1–135 vector    | −490-CAT-Gal4-C/EBP    | 1.5 ± 0.1      |
| 1–112 vector    | −490-CAT-Gal4-C/EBP    | 1.3 ± 0.1      |
| 1–66 vector     | −490-CAT-Gal4-C/EBP    | 1.2 ± 0.3      |

**DISCUSSION**

Expression of the PEPCk gene is controlled by hormonal signals as well as by developmental and tissue-specific factors. Each hormonal response of the PEPCk gene is mediated through a combination of hormone response elements and accessory factor sites. Our studies have defined various transcription factors involved in regulation by T3 and cAMP. Previous work has focused on the role of C/EBPα in the T3 and cAMP effects because C/EBPα is highly expressed in the liver and clearly contributes to the induction of PEPCk expression at birth (8, 18, 20). In this study, we have examined the contributions of C/EBPβ to the basal expression and hormonal responsiveness of the PEPCk gene. Our results indicate that C/EBPβ, along with the TR, can mediate the T3 induction of PEPCk transcription. C/EBPβ can participate in cAMP responsiveness, and 108 amino acids of the transactivation domain will impart full cAMP responsiveness to the PEPCk promoter.

In several genes, the transcriptional stimulation by T3 is dependent on both the TR and other transcription factors, often called accessory factors. Accessory factors can be positioned in close proximity or widely separated from the TR. In the liver, N-F1 is required for the T3 induction of S14 gene transcription (28). The TREs of the S14 gene are located between −2,700 and −2,500, while the N-FY binding site is near the start site of transcription (28, 29). In the heart, myocarditis-specific enhancer factor 2 (MEF2) is involved in the T3 induction of the α-cardiac myosin heavy chain gene (30). MEF2 and the TR bind to adjacent sites in the α-myosin heavy chain promoter and can physically interact in solution (30). The stimulation of the human placental lactogen B gene by T3 is dependent on the binding of the pituitary factor GHF1 (also called Pit-1) (31). Our studies have identified C/EBP proteins as an additional family of accessory factors involved in T3 action.

C/EBP proteins possess three functional regions including a transactivation domain on the amino terminus, a basic region that binds DNA and leucine zipper that dimerizes with other C/EBP proteins. Within the first 100 amino acids of C/EBPα and β are three conserved peptide sequences, which contribute to the transactivation capabilities of these proteins (27, 32, 33). In C/EBPβ, these peptides regions were called activation domain modules 1, 2, and 3 (AD1, 2, and 3) and removal of any of these peptides reduced the ability of C/EBPβ to stimulate basal transcription (27). The ADM3 (amino acids 83–92) is homologous to the homology box 2 (HOB2) domain found in Fos and Jun (33). The Gal4-C/EBPβ vector expressing the first 108 amino acids of C/EBPβ stimulated PEPCk basal transcription and restored cAMP responsiveness. In the Gal4-C/EBPβ vector expressing amino acids 1–66, the ADM3 is deleted and a portion of ADM2 (amino acids 56–72) is removed. Since the Gal4-C/EBPβ 1–66 vector did not stimulate basal expression of −490-P3G4-Pluc, it suggests that ADM3 is crucial for the stimulation of basal transcription. However, amino acids 1–66 did partially restore the cAMP response, indicating that additional regions of C/EBPβ are involved in mediating the cAMP induction.

The regions of C/EBPα that are involved in cAMP responsiveness have been narrowly defined (20). Amino acids 1–124 were sufficient for a cAMP effect (20). In these studies, three Gal4 sites were ligated in front of a CAT reporter gene driven by a neutral promoter and this reporter vector was cotransfected with Gal4-C/EBPα vectors. Further deletion beyond amino acid 124 from the carboxyl terminus of C/EBPα resulted in a loss of cAMP responsiveness. In addition, the first 50 amino acids were essential for the cAMP induction. Most interestingly, mutation of amino acids 67, 77, and 78 in C/EBPα that interact with TBP did not affect the ability of this Gal4-C/EBPα protein to mediate a cAMP response (20, 32). However, the Gal4-C/EBPα vector with these mutations was unable to stimulate basal transcription. Therefore, different amino acids within C/EBPα are involved in the regulation of basal transcription and cAMP responsiveness. Our results suggest that different domains of C/EBPα and β mediate cAMP responsiveness. Amino acids 96–124 are essential for the cAMP induction by C/EBPα. There are no homologous domains within C/EBPβ. These experiments were conducted in a slightly different manner since the transfections in this study were all conducted with a Gal4 binding site in the context of the PEPCk gene, while those of the previous study were conducted using multimerized Gal4 sites. Nonetheless, there does not appear to be a single homologous peptide region of C/EBPα and β that is responsible for the cAMP induction.

One important issue is why the Gal4-C/EBPα and β vectors do not restore full T3 responsiveness. One possibility is that either the leucine zipper or the basic region of C/EBP is required for the T3 induction, as has been shown in the regulation of other genes. For example, the leucine zipper of C/EBP synergizes with Sp1 in the regulation of the rat CYP2D5 P450 gene (35). We cannot address this question with the Gal4-C/EBP expression vectors because, when the full-length C/EBPα or β protein is attached to the Gal4 DNA binding domain, the resulting protein inhibits basal and hormone-regulated transcription (20, 27). This observation suggests that the DNA binding domain must be in contact with the C/EBP binding site. Another possibility is that the Gal4 DNA binding domain altered the configuration of the C/EBP transactivation domain, making the necessary protein interactions for T3 responsiveness impossible. While both the α and β isoforms of C/EBP can have a role in the regulation of PEPCk expression, in vivo they are not functionally redundant. In C/EBPα knock-out mice, the PEPCk gene was not induced at birth although PEPCk expression did rise after several hours (17, 18). In addition, the PEPCk gene was poorly responsive to cAMP (18). C/EBPα knock-out mice die at birth of hypoglycemia and other complications (17, 18). In the liver of adult mice in which the C/EBPα was conditionally knocked out, PEPCk expression was decreased (36). C/EBPβ knock-out mice displayed a mixed phenotype with 50% of the mice dying at birth and showing reduced PEPCk gene expression (18). The remaining mice were viable and normal with respect to PEPCk expression. Other data indicate that the binding of C/EBPβ to the PEPCk promoter may be increased by cAMP. When animals are injected with cAMP, the abundance of C/EBPβ rises rapidly in the liver and the binding of C/EBPβ to the P3(U) site increases (37). In physiologic situations such as with increased exercise, the abundance of C/EBPβ increases in the liver (37). Our data.
Role of C/EBPβ in the T₃ and cAMP Induction of PEPCK

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