Conserved amino acids within CCAAT enhancer binding proteins (C/EBPα and β) regulate phosphoenolpyruvate carboxykinase (PEPCK) gene expression.

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Running Title: C/EBP and CBP modulate T3 induction of PEPCK

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Summary

Thyroid hormone and cAMP stimulate transcription of the gene for phosphoenolpyruvate carboxykinase (PEPCK). CCAAT enhancer binding proteins (C/EBPα and β) are involved in multiple aspects of the nutritional, developmental and hormonal regulation of PEPCK gene expression. Previously, we have identified a thyroid hormone response element in the PEPCK promoter and demonstrated that C/EBP proteins bound to the P3(I) site are participants in the induction of PEPCK gene expression by thyroid hormone and cAMP. Here, we identify several peptide regions within the transactivation domain of C/EBPα that enhance the ability of T3 to stimulate gene transcription. We also demonstrate that several conserved amino acids in the transactivation domain of C/EBPα and C/EBPβ are required for the stimulation of basal gene expression and identify amino acids within C/EBPβ that participate in the cAMP induction of the PEPCK gene. Finally, we show that the CREB binding protein (CBP) enhanced the induction of PEPCK gene transcription by thyroid hormone and that CBP is associated with the PEPCK gene in vivo. Our results indicate that both C/EBP proteins and CBP participate in the regulation of PEPCK gene transcription by thyroid hormone.
Introduction

Transcription of the gene for phosphoenolpyruvate carboxykinase (PEPCK) is stimulated in various nutritional and pathologic states such as high protein diets, fasting, hyperthyroidism and diabetes (1). Multiple hormones including glucagon (via cAMP), thyroid hormone (T3), glucocorticoids and retinoic acid increase PEPCK gene expression (2). The hormonal induction of the PEPCK gene by T3 and glucocorticoids is mediated through the binding of nuclear receptors to weak hormone response elements in the PEPCK promoter (3, 4). The full induction by these hormones requires accessory factors associated with the PEPCK promoter. Our studies and those of others have shown that CCAAT enhancer binding proteins (C/EBP) are accessory factors required for the stimulation by cAMP, T3 and glucocorticoids (5, 6, 7, 8).

We have defined two critical sites in the PEPCK promoter that are required for the stimulation by T3 (6). A thyroid hormone response element (TRE) (−330/−320) binds the thyroid hormone receptor (TR) as a heterodimer with the retinoid X receptor (RXR). In addition, a site called P3(I) (−250/−234) binds C/EBPα and C/EBPβ (7). Both sites are required for T3 to stimulate PEPCK gene expression. The induction of PEPCK transcription by cAMP involves multiple sites in the promoter including a cAMP response element (CRE) (−90/−82) and the P3(I) site (2, 9). The PEPCK CRE can bind both CREB and C/EBP proteins with similar affinity (10). The P3(I) site is involved in both the T3 and cAMP induction of the PEPCK gene. Glucocorticoids induce PEPCK gene expression through two weak glucocorticoid response elements, but multiple accessory factors are involved in the glucocorticoid induction of the PEPCK gene including C/EBPβ bound to the CRE (8, 11). Therefore, C/EBP proteins are centrally involved in regulating multiple hormone responses of PEPCK gene expression. C/EBP proteins also contribute to the tissue specific expression and developmental regulation of the PEPCK gene (12).

The CCAAT enhancer binding proteins consist of a family of bZIP proteins. The transactivation domain is contained in the amino terminus, while the DNA binding domain and leucine zipper are contained within the carboxy terminal region (13). C/EBPα and β have been shown to have important roles in directing the expression of many genes encoding metabolic enzymes in the liver. In addition, C/EBP
isoforms have prominent roles in adipocyte differentiation (14). However, these isoforms are not redundant. C/EBPα is a terminal differentiation factor that is associated with inhibition of cell division (15). C/EBPα stimulates expression of the PEPCK gene at birth. Both C/EBPα and C/EBPβ knockout mice have impaired expression of the PEPCK gene (12, 16).

The T3 induction of gene transcription is mediated through the binding of the liganded thyroid hormone receptor (TR) to hormone response elements (17). The TR binds to TREs primarily as a heterodimer with RXR (18). T3 is not required for DNA binding and in the absence of ligand the TR generally acts as a repressor of gene expression. Without T3, the TR is associated with nuclear corepressors such as NCoR and SMRT (17). When ligand is added, various coactivators may be recruited to the nuclear receptors including steroid receptor coactivator (SRC-1/NcoA-1), CREB binding protein (CBP/p300) and thyroid receptor accessory proteins (TRAP/DRIP/PBP) (17, 19). SRC-1 can interact with many liganded nuclear receptors and with orphan receptors such as HNF-4 and COUP-TF through a conserved LXXLL peptide motif (20). The CREB binding protein (CBP) is associated with SRC proteins and liganded nuclear receptors. CBP was initially described as a coactivator for CREB and enhancer of cAMP responsiveness (21). CBP is able to interact with a variety of proteins and therefore offers the potential for mediating the interactions between receptors and accessory factors (22). In these studies, we have defined specific regions within C/EBP that are involved in the T3 induction of PEPCK transcription. In addition, we provide evidence that CBP can participate in the T3 induction of PEPCK gene transcription.

Materials and Methods

Construction of CAT and Luciferase vectors. The ligation of the PEPCK promoter from -490 to +73 to the CAT reporter gene (-490-PCAT) has been described (3). The introduction of the Gal4 binding site into the P3(I) site of the PEPCK promoter to create -490-P3G4-CAT was described previously (6). The -490 to +73 region of the PEPCK promoter was ligated in front of the Luciferase reporter gene by removing the PEPCK promoter fragment from -490-PCAT by digestion with KpnI and BglII and ligating into the polylinker of pGL3 basic (Promega). The Gal4 site was introduced into the
TRE region of -330-PTRE/G4 CAT by PCR amplification with the 5′ primer, ccctctagatcggaggtactgtcctccgtctgac, containing the altered nucleotides and a 3′ primer, ttagatctcagagcgtctcgcc, (+73 to +52) which included the Bgl II site at +73. The 5′ primer introduced a XbaI site. The amplified promoter fragment was digested with XbaI and Bgl II and ligated in front of the CAT reporter gene. The sequence was confirmed by sequence analysis at the St. Jude Center for Biotechnology (Memphis, TN).

**Construction of Gal4-C/EBP expression vectors.** The Gal4-C/EBPβ vectors with alanine substitutions were constructed by two step PCR amplification. The initial PCR reactions contained the forward primer which contained an EcoRI site and the first 18 nucleotides of the rat C/EBPβ cDNA (tccgaattcatgcaccgccgctgtggcctgggac) and a reverse primer with the alanine switches M27, 28 (ggcagtcggggctcgtaggcggcttgccacttcatg) M57,58,59 (gaagtcgatggcgcgcgcggccgcgaatggccggctc) or M61,62 (ccaggtaggggtcagacgcggccggccgtgtgctc) and reverse primers containing PstI sites and the nucleotides representing amino acids 108 to 102 (gagctgcaggtaaccgtagtcggccggctc). The PCR reactions were conducted using the PCR kit from Clontech and consisted of 20 cycles of 94°C for 30 secs and 68°C for 2 mins. All PCR reactions contained 10% GC melt buffer (Clontech) as this region of mouse C/EBPβ is extremely GC rich. The mouse C/EBPβ cDNA was the template (23). The PCR products were purified from agarose gels. The PCR products encompassing approximately amino acids 1-70 and 60-108 were mixed along with the outside primers and the PCR reactions were repeated. The appropriate 330 base pair DNA fragment was isolated from an agarose gel and subcloned into TOPO-TA vector (Invitrogen) as outlined by the manufacturer. The C/EBPβ fragment was removed from TOPO-TA by digestion with EcoRI and PstI. This DNA fragment was ligated into the mammalian Gal4 DNA expression vector called pM (Clontech).

To create the Gal4-C/EBPβ 1-100 and the Gal4-C/EBPβ 1-100 M86, 87 vectors, PCR reactions were conducted with the forward primer encompassing nucleotides 1-18 and a reverse primer encoding either the wild type sequence (cggctgcaggctcggcttggcgccgtagtcg) or containing mutations in the amino
acids 86,87 (cgctcaggctgctgcggctcggcttggtgcg). PCR conditions and subcloning into TOPO-TA was conducted as described above. Construction of the Gal4-C/EBPα vectors was given elsewhere (5, 24). The sequence of all Gal4-C/EBPβ vectors was confirmed by sequence analysis. To construct the C/EBPα prey vectors for the mammalian two-hybrid assays, the C/EBPα fragment was isolated from the Gal4-C/EBPα vector by digestion with EcoRI and PstI. These C/EBPα fragments were ligated into the VP16 vector (Clontech).

**Cell transfections, Luciferase and CAT assays.** HepG2 cells were transfected by calcium phosphate precipitation as described previously (6). CAT assays were conducted with [3H]-chloramphenicol and n-butyryl-coenzyme A using the xylene phase extraction method (6). All transfections were performed in duplicate and repeated three to six times. Luciferase assays were conducted with the luciferin reagent as outlined by the manufacturer (Promega).

**Chromatin immunoprecipitation assay:** We used a modification of the technique described by Shang et al (25). A 1% solution of formaldehyde prepared in buffer (0.1M NaCl, 1mM EDTA, 0.5mM EGTA, 50mM Hepes, pH 8.0) was added to hepatocytes for 5 min at 4°C to crosslink DNA and its associated proteins. Hepatocytes were prepared as we have described previously (26). The crosslinking reaction was stopped by the addition of glycine. Cross-linked cells were then recovered by centrifugation and washed three times with 5 ml of ice-cold PBS. The cells were resuspended with 1 ml of buffer (20mM Tris-HCl, pH 8.0, 2mM EDTA, 150mM NaCl) plus protease inhibitors (1mM phenylmethylsulfonyl fluoride, 1mM benzamidine, 0.1µg/ml aprotinin, 1µg/ml leupeptin, and 0.1µg/ml pepstatin) and sonicated at 4°C for 10 sec at maximum setting. The sonication was repeated five times after 30 sec intervals at 4°C.

The sonicated cells were centrifuged for 10 min at 4°C to remove cell debris and each supernatant diluted up to a final volume of 1.5 ml with binding buffer (20mM Tris-HCl, pH 8.0, 2mM EDTA, 150mM NaCl, 1% Triton X-100) plus protease inhibitors. Each supernatant was pre-cleared by adding 50µl of BSA-blocked protein A-sepharose (for rabbit antibodies) or protein G-sepharose (for mouse antibodies). These mixtures were incubated overnight at 4°C with constant shaking and pre-cleared supernatant recovered by centrifugation and finally transferred to pre-chilled microcentrifuge tubes.
Immunoprecipitation was performed with specific antisera raised against C/EBPβ, C/EBPα, TRβ1, and CBP (Santa Cruz Biotechnology, CA, USA). As a control, monoclonal anti-polyHistidin antibody (Sigma Chemical Co, MO, USA) was used. The mixtures were incubated at 4°C for 1 hour followed by isolation of antibody-protein-DNA complexes with 50µl of BSA-blocked protein A-sepharose or protein G-sepharose for 1 hour at 4°C. Immunoprecipitates were recovered by centrifugation and the resins washed sequentially three times for 3 min with wash buffer #1 (20mM Tris-HCl, pH 8.0, 150mM NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100), wash buffer #2 (20mM Tris-HCl, pH 8.0, 500mM NaCl, 2mM EDTA, 0.1% SDS, 1% Triton X-100), and wash buffer #3 (10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.25M LiCl, 1% Igepal, 1% deoxycholate). Precipitates were then washed three times with TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA) and extracted two times by incubation for 15 min at room temperature with 250 µl of 1% SDS, 0.1M NaHCO₃. Eluates were pooled, 30µl of 5M NaCl added and heated at 65°C for 3 hours to reverse the formaldehyde cross-linking. Proteinase K (Boehringer-Manheim) was added and the heating continued at 65°C for 3 hours. DNA fragments were purified with pellet paint kit (Novagen, USA). Precipitated DNA was washed with 70% ethanol, air-dry for 10 min, and resuspended in 100 µl sterile water. Bound DNA fragments were analyzed by PCR using AmpliTaq Gold Kit (Applied Biosystems, New Jersey, USA), 2µM of each primer, and 5µl of immunoprecipitated DNA per reaction. Cycling parameters were: 1 cycle of 94 °C for 9 min, 30 cycles of 94 °C for 30 sec, 63 °C for 30 sec, 72 °C for 30 sec and 1 cycle at 72 °C for 7 min. The primers used for amplification of promoter rat PEPCK were: Forward TRE (-479/-459) 5’-caggtctcagctgatcttccttc-3’ and reverse TRE (-290/-314) 5’-actatagctttgtcttaatgtgctc-3’. Amplified PCR products were electrophoresed through a 3% Nusieve/agarose gel in TAE buffer and visualized by ethidium bromide staining.

Results

In previous studies, we found that mutation of the C/EBP binding site called P3(I) in the PEPCK promoter eliminated the T3 induction of the PEPCK gene (6). A model of some of the key regulatory elements in the PEPCK promoter is shown in figure 1A. Our first experiments were designed to demonstrate that the P3(I) site could function as an enhancer of T3 responsiveness out of the context of the
PEPCK gene. One copy of an idealized thyroid hormone response element (DR4) was ligated in front of a minimal PEPCK promoter from –68 to +73 that contains only a TATA box but no hormone response elements to create DR4X1 -68 PLuc. A schematic of the Luciferase vectors used in these studies is shown in figure 1B. Addition of three copies of the P3(I) element (DR4X1 P3WTX3 -68PLuc) greatly enhanced the T3 induction from 0.3 fold to 2.4 fold (Table 1). We added three copies of the P3(I) site because there are three C/EBP binding sites in this region of the PEPCK gene including P3(I), P4(I) and P4(II) (2). A Gal4 site was ligated in front of the enhancerless SV40 promoter driving the Luciferase reporter gene to generate Gal4X1 SV40-Luc. Cotransfection of this vector with Gal4-TRß allowed a strong 8.1 fold induction by T3. Addition of three P3(I) sites in the Gal4X1 P3WTX3 SV40-Luc increased this stimulation to 61 fold. This induction could be reduced either by cotransfection with a dominant negative C/EBP vector (A-C/EBP) or the introduction of mutations into the P3(I) site (P3Mut 243 or P3Mut 247) that eliminated the ability of C/EBP to bind the P3(I) site (27) (Table 1). The A-C/EBP vector will inhibit the binding of all C/EBP isoforms (27). These results indicate that C/EBP can enhance the T3 induction through a TRE and that this enhancement does not require being in the context of the PEPCK promoter.

The next experiments were designed to identify domains within C/EBPα or C/EBPβ that were involved in the enhancement of T3 responsiveness. To test the ability of the TR and C/EBP to synergize, one copy of an idealized TRE and one copy of a Gal4 site were ligated in front of the –68 PEPCK-Luc reporter gene. HepG2 cells were cotransfected with mammalian expression vectors for RSV-TRß and Gal4-C/EBPα as well as the DR4X1 Gal4X1 –68 PLuc reporter gene. In the absence of C/EBP, a single TRE was unable to confer T3 responsiveness to the minimal PEPCK promoter (Fig. 2). The Gal4-C/EBPα vector 6-217 that contains the transactivation domain allowed a 4.1 ± 0.2 fold induction by T3. Deletion of amino acids 175 to 217 diminished the T3 induction. Likewise deletion of the first 50 amino acids in the Gal4-C/EBPα 50-217 reduced the T3 response. Three amino acids, tyrosine, phenylalanine and leucine, at positions 67,77,78 in C/EBPα had been found to be critical for the induction of basal expression (5). As is shown in figure 2, mutation of these amino acids in the vector called Gal4-C/EBPα TM reduced but did not eliminate the induction by T3. We had reported previously that these amino acids were not required for the cAMP induction of PEPCK gene expression (5).
Our next studies examined the possibility that TRß and C/EBPα could physically interact. To conduct these studies, we utilized several approaches including GST pulldowns and mammalian two hybrid assays. We tested whether bacterially expressed GST-C/EBPα could pull-down 35S-labeled TRß in GST pulldown assays. In these experiments, we found that GST-C/EBPα interacted with 35S-TRß although only a small percentage of the input 35S-TRß was retained by the GST-C/EBPα (data not shown). Addition of T3 or His-tagged RXRα did not strengthen the interaction. For the mammalian two-hybrid experiments, the Gal4X4 –68PLuc reporter gene was used. The Gal4-TRß was the bait and C/EBPα-VP16 was the prey. In the absence of T3, coexpression of the C/EBPα-VP16 did not increase Luciferase expression (data not shown). Addition of T3 caused a 26.5 ± 5.8 fold increase in Luciferase activity, while cotransfection of C/EBPα-VP16 increased the T3 induction to 37.5 ± 12.0 (data not shown). Overall, our results suggested that while C/EBPα could interact with TRß, these interactions were quite weak and most likely did not form the basis for C/EBP's enhancement of T3 action.

Since both C/EBPα and C/EBPß are present in rat liver nuclei and can bind to the P3(I) element in the PEPCK promoter, we tested whether Gal4-C/EBPß could enhance the T3 induction of the DR4X1 Gal4X1 –68PLuc (Fig. 2). Cotransfection with Gal4-C/EBPß 3-181 or Gal4-C/EBPß 1-100 increased the T3 induction two fold. However, cotransfection with a Gal4-CREB vector did not enhance the T3 induction indicating that this effect was mediated by C/EBP proteins (Fig. 2). These data indicate that regions within the first 100 amino acids of C/EBPß could increase the T3 response. We examined the first 100 amino acids of the transactivation domains of the rat C/EBPα and mouse C/EBPß and found that several amino acid regions are conserved as is shown in figure 3. In particular, the phenylalanine and leucine (FL) amino acids are highly conserved. We introduced a mutation in the Gal4-C/EBPß 1-100 expression vector in which the FL amino acids 86, 87 were switched to alanine. Interestingly, mutation of amino acids 86 and 87 (C/EBPß M86,87) which are conserved between C/EBPα and C/EBPß did not decrease the induction by T3 (Fig. 2). These results indicate that there is an additional domain within the first 100 amino acids of C/EBPß that participates in the T3 induction of PEPCK transcription.

We tested whether the FL to alanine switch would affect the ability of C/EBPß to stimulate basal expression. The Gal4-C/EBPß vectors were cotransfected with either Gal4X3 –68Pluc (Fig. 4A) or -490
Mutation of amino acids 86/87 decreased the stimulation of basal expression by C/EBPβ from 15.2 ± 3.7 to 2.4 ± 0.3 indicating that these amino acids are critical in both the C/EBPα and C/EBPβ isoforms in the stimulation of basal expression (Fig. 4A). We altered several additional amino acids in the transactivation domain of C/EBPβ. The amino acids 54 to 70 of the mouse C/EBPβ are partially conserved in the rat C/EBPα in amino acids 55-71 as well as a region of homology in the amino terminal regions of these proteins (Fig. 3). Mutation of amino acids 56, 57, 58 or 61, 62 reduced the ability of Gal4-C/EBPβ to stimulate basal transcription of a Gal4X3 –68PLuc reporter gene, but the expression of the –490 P3G4 PLuc was not reduced as compared with the Gal4-C/EBPβ 1-108 (Fig. 4). The difference in the response to these Gal4-C/EBPβ vectors indicates that promoter context as well as transactivation domains contribute to the ability of C/EBPβ to stimulate transcription. The -68PLuc vector has a minimal promoter containing only a TATA box, while the -490 PEPCK promoter has a number of binding sites for other factors. Alteration of amino acids 27, 28 in C/EBPβ did not affect the ability of C/EBPβ to stimulate basal expression.

Previously, we had demonstrated that the P3(I) site was required for the full induction of the PEPCK gene by cAMP (5, 7). We tested the ability of these C/EBPβ vectors to restore protein kinase A (PKA) responsiveness by cotransfecting –490 P3G4 PLuc with the Gal-C/EBPβ vectors. The P3G4 vector has a Gal4 site substituted for the P3(I) site in the –490 PLuc (Fig. 1B). Mutation of amino acids 61, 62 and to a lesser extent 56, 57, 58 in the Gal4-C/EBPβ vectors decreased the PKA response. Amino acids 60-72 of C/EBPα have been implicated in the cAMP induction of the PEPCK gene (28). These results suggest that the conserved amino acids between 55 and 71 in C/EBPα and C/EBPβ may be important in the contribution of these proteins to cAMP responsiveness. C/EBPβ M86, 87 was as effective as the Gal4-C/EBPβ 1-100 in mediating a PKA induction although the basal expression was greatly decreased (Fig. 5). The Gal4-C/EBPβ was not able to mediate a cAMP induction out of the context of the PEPCK gene as overexpression of PKA did not increase the activity of Gal4X3 –68PLuc when cotransfected with Gal4-C/EBPβ (data not shown).

Since we observed only a minimal physical interaction between C/EBPα and TRβ, we next tested whether overexpression of the coactivators SRC-1 or CBP could enhance the T3 induction of the PEPCK
gene. To conduct these experiments, we transfected –490 PCAT with RSV-TRβ and mammalian expression vectors for SRC-1 and/or CBP. Overexpression of SRC-1 enhanced the basal expression of –490 PCAT three fold, and the reporter gene was stimulated an additional four fold by the addition of T3 (Fig. 6). These results indicate that SRC-1 can interact with factors bound to the PEPCK promoter to enhance the basal expression of the gene. Overexpression of CBP did not elevate the basal expression of PEPCK-CAT. However, the T3 response was increased from 4.9 ± 0.7 to 7.1 ± 0.7 fold. This stimulation was significant at a P value of 0.045 using a one-tailed T test. Cotransfection of SRC-1 and CBP did not further increase the effect of T3. These results indicate that CBP can enhance the T3 induction of the PEPCK gene. We tested whether tethering CBP next to a single TRE would restore T3 responsiveness as had the Gal4-C/EBPα 6-217 (Table 2). Full length CBP was ligated to Gal4 to create Gal4-CBP. Both the Gal4-C/EBPα and the Gal4-CBP stimulated the basal expression of the reporter gene (data not shown). The Gal4-CBP was able to restore T3 responsiveness to this vector suggesting that recruitment of CBP to the PEPCK promoter would enhance the induction by T3.

Given that CBP increased the T3 responsiveness of the PEPCK gene, we examined whether we could observe physical interactions between CBP and C/EBPα. To conduct these studies, we initially used a mammalian two-hybrid assay. Overexpression of Gal4-CBP strongly potentiated basal expression of the Gal4 X 4 –68Pluc reporter gene. Cotransfection with C/EBPα 6-217-VP16 increased the expression of the Luciferase reporter gene 9.1 ± 4.4 fold (Fig. 7). Our results indicated that CBP could interact with C/EBPα. These experiments were repeated eight times as there was considerable variability in the extent of the induction although expression of the reporter gene was consistently induced by the C/EBPα-VP16 vector. These results indicate that CBP can interact with C/EBPα. However, we were not able to demonstrate interactions between various GST-CBP proteins and 35S-C/EBPα in pull-down experiments. Our data suggest that the interactions between C/EBP and CBP are not strong and that CBP may need to interact with several factors for stable interaction with the PEPCK promoter. Since C/EBP proteins are involved in the cAMP induction of PEPCK transcription, we tested whether CBP could enhance the induction of PEPCK gene expression by overexpression of the catalytic subunit of protein kinase A
Overexpression of CBP did not enhance the induction by PKA suggesting that CBP alone would not increase the cAMP response (data not shown).

Our final experiments examined whether the TR, C/EBP and CBP were associated with the PEPCK promoter in vivo. To test this question, we utilized the chromatin immunoprecipitation (ChIP) assay. Freshly prepared hepatocytes were treated briefly with 1% formaldehyde to crosslink the proteins to DNA. The cross-linked proteins and DNA were immunoprecipitated with antibodies to either TRβ, C/EBPα, C/EBPβ or CBP. PCR primers were created that amplified regions around the TRE and the P3(I) site in the PEPCK promoter. Our experiments using immunoprecipitated DNA as a template for PCR reactions show that the TRβ is associated with the PEPCK promoter (Fig. 8). In addition, antibodies to C/EBPα, C/EBPβ and CBP immunoprecipitated the PEPCK promoter. Given that the sheared chromatin DNA was up to 500 bp in length, these results do not demonstrate the specific binding of these proteins to any element in the gene. The PCR products may represent proteins bound to the CRE, and C/EBP proteins bind to the P3(II), P4(I) and P4(II) sites in the PEPCK promoter with high affinity. In addition, we found that C/EBP proteins and CBP were also associated with the PEPCK promoter in H4IIE rat hepatoma cells (data not shown). These data indicate that these proteins are associated with the PEPCK gene in vivo.

**Discussion:**

The PEPCK gene has been studied extensively as a model for the multi-hormonal regulation of gene expression (1, 2, 9). PEPCK transcription is regulated through the interactions of nuclear hormone receptors and accessory factors bound to the promoter. The involvement of multiple accessory factors allows for subtle modulation of PEPCK gene expression in response to dietary, developmental and hormonal alterations. Our studies have focused on the role of C/EBP proteins in the cAMP and T3 induction of PEPCK gene expression. Here, we show that C/EBP proteins participate in the T3 and cAMP responsiveness and that the coactivator CBP can enhance the T3 stimulation of the PEPCK gene.

C/EBP proteins have been identified as accessory factors in the thyroid hormone and cAMP induction of several genes. Recently, it was found that C/EBPs participated in the T3 induction of the malic
enzyme gene (29). In addition, it was reported that C/EBPβ was involved in the ability of cAMP to induce the StAR and prolactin genes (30, 31). In the kidney, C/EBPβ rather than CREB mediates the cAMP induction of the PEPCK gene (32). Experiments from the laboratory of Richard Hanson utilizing C/EBPα knock-out mice have shown that C/EBPα is required for the induction of the PEPCK gene by cAMP (12). A TRE has been identified in the promoter of the C/EBPα gene and it has been reported that T3 can stimulate C/EBPα transcription (33). These observations raise the possibility that T3 stimulates PEPCK gene expression by increasing C/EBPα levels. We were not able to observe an increase in C/EBPα or C/EBPβ protein abundance in the livers of hyperthyroid as opposed to euthyroid rats (data not shown). Our data suggest that C/EBP proteins are directly involved in the T3 induction and that T3 does not stimulate the PEPCK gene by increasing C/EBP abundance.

There have been several studies that have identified regions of homology between C/EBPα isoforms from different species (34, 35, 36). A recent report from MacDougal and coworkers outlined four conserved regions in the transactivation domain of C/EBPα which were called CR1, 2, 3 and 4 (37). The CR2 domain of C/EBPα, which contains amino acids 55 to 108, has several conserved amino acids between C/EBPα and C/EBPβ (Fig. 3). Our data highlight several points regarding the relevance of this CR2 region within C/EBP proteins in the regulation of PEPCK gene transcription. The phenylalanine/leucine amino acids (FL) are important for the stimulation of basal expression by both C/EBPβ and C/EBPα as mutation of these two amino acids in this region eliminates the ability of C/EBPβ to stimulate basal expression (Fig. 4). It was reported that the FL amino acids in C/EBPα contacted TBP and were essential for stimulating basal expression (34). Our data suggested that the amino acids 61, 62 were important for the participation of C/EBPβ in the cAMP stimulation of PEPCK gene expression (Fig. 5). Deletional analysis of the C/EBPα transactivation domain identified a short peptide stretch from amino acids 55 to 65 involved in mediating cAMP responsiveness (27). This conserved stretch of amino acids between C/EBPα and β is involved in the cAMP induction of the PEPCK gene.

Previous reports have suggested that CBP might have a role in regulating PEPCK gene expression through its ability to interact with CREB, NF-1 and various steroid receptors (38). In addition, overexpression of E1A reduced the ability of the catalytic subunit of protein kinase A to stimulate PEPCK
gene expression (39). Finally, overexpression of CBP stimulated basal expression of the PEPCK gene (38). Our data supports the concept that CBP has a role in the hormonal regulation of PEPCK gene expression. However, we did not observe that overexpression of CBP enhances either the basal expression or the PKA induction of our PEPCK-Luc vectors in transient transfections. Such an observation does not rule out a role for CBP in the cAMP induction despite the fact that we did not observe any synergism between CBP and PKA in transient transfections. For example, the overexpression of the coactivator SRCAP, which interacts with CBP, greatly enhanced the PKA induction of the PEPCK gene (40).

Our data does suggest a role for CBP in the T3 induction of PEPCK transcription. Our data is compatible with some of the previous observations regarding the interactions of CBP/p300 with C/EBPα. MacDougal et al reported that overexpression of p300 enhanced the ability of C/EBPα to stimulate leptin gene expression (37). The ability of C/EBPα to synergize with p300 was mediated through all the conserved motifs of C/EBPα. In keeping with that observation, our C/EBPα-VP16 vectors were able to interact with Gal4-CBP in our mammalian two-hybrid assays. Previous reports indicated that C/EBPα and CBP did not interact in GST-pulldown assays (37). We also believe as demonstrated by our mammalian two hybrid experiments that the interactions between C/EBPα and CBP are not likely to be strong. CBP may be recruited to the PEPCK promoter through its interaction with multiple proteins including CREB, NF-1, C/EBPα and β as well as others. CBP and C/EBPα have been reported to colocalize in the nucleus using fluorescently tagged proteins (37). C/EBPβ proteins have been shown to interact with p300 through the amino terminus of C/EBPβ and the CH3 domain of p300 (41). Previous studies have demonstrated that multiple regions in the amino terminus of C/EBPβ are involved in the interaction with CBP (41). This region of C/EBPβ contains the CR2 region, which is highly conserved between both C/EBP isoforms, it is likely that this domain will be important for the interaction of CBP and C/EBP isoforms.

Several models have been developed in which the various nuclear coactivators are assembled to form a functional complex. For example, SRC-1/NCoA-1 can be recruited by many liganded nuclear receptors, and CBP can interact with SRC-1 (19, 20). SRC-1 has been shown to interact with several receptors that are part of the PEPCK glucocorticoid response unit including HNF-4, glucocorticoid receptor and COUP-TF (42). When tethered to the PEPCK promoter, SRC-1 can substitute for other
members of the glucocorticoid response unit suggesting that SRC-1 is involved in the glucocorticoid induction (42). However, we did not observe any enhancement of the T3 induction by cotransfection of an expression vector for SRC-1. SRC-1 increased basal expression of our PEPCK reporter genes indicating that SRC-1 was interacting with the PEPCK promoter. It has been shown in primary hepatocytes that glucocorticoids are required for the full induction of PEPCK transcription by glucagon (43). It is possible that SRC-1 and CBP are involved in the synergistic activation of PEPCK gene transcription by glucocorticoids and cAMP. In summary, our data have defined limited domains of the C/EBPα and C/EBPβ transactivation domains that are involved in the stimulation of PEPCK gene transcription by cAMP and T3. In addition, we have determined that CBP can enhance the T3 induction of the PEPCK gene.

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The abbreviations used are: T3, 3,5,3′-triiodothyronine; PEPCK, phosphoenolpyruvate carboxykinase; TRβ, thyroid hormone receptor β; TRE, thyroid hormone responsive element; PTRE, TRE in the PEPCK promoter; P3(I), C/EBP binding site in PEPCK promoter; RXR, retinoid X receptor; DR4, direct repeat separated by 4 nucleotides; CAT, chloramphenicol acetyltransferase; Luc, Luciferase; C/EBP, CCAAT enhancer binding protein; CRE, cAMP responsive element; CREB, cAMP responsive element binding protein; CBP, CREB binding protein; SRC-1, steroid receptor coactivator-1.

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Figure Legends

Figure 1: Model of the PEPCK promoter and Luciferase vectors. A. A model of the PEPCK promoter is shown. The binding sites including the cAMP response element (CRE), T3 response element (TRE), and P3 sites are labeled beneath the promoter. The transcription factors that can bind these elements are shown above. B. The Luciferase vectors are shown in which either one (X1) or three (X3) copies of various elements were ligated in front of a minimal -68/+73 PEPCK promoter. The DR4 is a direct repeat separated by four nucleotides while the Gal4 is a binding site for the Gal4 promoter.

Figure 2: Identification of domains within C/EBPα that enhance thyroid hormone responsiveness. One copy of a T3 responsive element (DR4X1) was ligated with one copy of a Gal4 site in front of –68 PEPCK-Luciferase reporter gene. A model of the reporter vector is shown at the top of the figure. Transfections included 3 µg of the Luciferase reporter gene, 3 µg of RSV-TRβ and 0.5 µg of Gal4 expression vector into HepG2 cells. The numbers of the Gal4-C/EBPα vectors indicate the amino acids of C/EBPα. The Gal4-C/EBPα TM vector has alanines introduced at amino acids 67, 77 and 78. Cells were exposed to 100 nM T3 in serum free media for 16 hours and then harvested. The data are expressed as Luciferase activity corrected for protein and transfection efficiency. All transfections were conducted in duplicate and repeated at least four times.

Figure 3: Amino acids sequences of C/EBPβ and C/EBPα. The peptide sequences of rat C/EBPα from amino acids 55 to 85 and mouse C/EBPβ between 54 and 94 are shown. The dark lines above the amino acid sequences indicate regions of high homology. The underlined alanines (A) are mutated from the wild type sequence. These mutations were introduced into the Gal4-C/EBPβ vectors.

Figure 4: Identification of specific amino acids within C/EBPβ that stimulate basal transcription. A. The Luciferase (Luc) reporter gene, Gal4X3 -68PLuc, was cotransfected with 100 ng of Gal4-C/EBPβ
expression vector and TK-renilla as described in the legend to figure 2. The “M” indicates which amino acids in the transactivation domain of CEBPβ have been switched to alanines. All transfections were repeated four to six times in duplicate. The data are expressed as Luciferase activity corrected for protein content and transfection efficiency. B. The transfections were conducted as above except the -490-P3G4-PLuc vector was used as the reporter gene.

Figure 5: Identification of amino acids in C/EBPβ that participate in cAMP responsiveness. The -490-P3G4-PLuc reporter vectors (3 µg) were cotransfected with 1 µg of Gal4-C/EBPβ expression vector, 1.0 µg of the catalytic subunit of protein kinase A and TK-renilla as described in the legend to figure 2. The “M” indicates which amino acids in the transactivation domain of CEBPβ have been switched to alanines. All transfections were repeated four to ten times in duplicate. The Luciferase activity was corrected for protein content and transfection efficiency. The data are expressed as the induction relative to the induction of wild type -490-PLuc by PKA.

Figure 6: CBP enhances the T3 induction of the PEPCK gene. The -490 PEPCK-CAT vector was cotransfected with RSV-TRβ and 3 µg of mammalian expression vectors for either SRC-1 or CBP. The cotransfected expression vector is indicated on the left side of the figure. Following transfection, the cells were exposed to 100 nM T3 for 40 hours. The data are expressed as CAT activity corrected for protein and transfection efficiency. All experiments were repeated at least four times in duplicate.

Figure 7: C/EBPα can interact with CBP. The Gal4 X 4 -68Pluc was cotransfected with 10 ng of Gal4-CBP and 500 ng of C/EBPα-VP16 vectors as described previously. The data are expressed as Luciferase activity corrected for protein and transfection efficiency. All transfections were conducted in NIH-3T3 cells and were repeated eight times in duplicate.

Figure 8: TRβ, C/EBP and CBP are associated with the PEPCK promoter in vivo. Chromatin immunoprecipitation (ChIP) assays were performed using formaldehyde crosslinked hepatocytes and
antibodies to the C/EBPα, C/EBPβ, TRβ and CBP as indicated above the figures. Immunoprecipitation using antibody to the six histidines tag (His tag) was used as a control for these experiments. The cross-linking was conducted on freshly isolated hepatocytes that were in suspension. The PCR products were resolved on a 3% Nusieve agarose gel. The sequence of the primers around the TRE and the conditions for the ChIP assay are provided in the methods section.
HepG2 cells were transiently transfected with the Luciferase reporter genes. Each transfections contained 3 µg of Luciferase gene, 1 µg of RSV-TRβ or Gal4-TRβ, 0.5 µg of the dominant negative C/EBP vector (A-C/EBP) and 0.5 µg of TK-renilla. Transfected cells were exposed to 100 nM T3 for 16 hrs. The data are expressed as Luciferase activity corrected for protein content and transfection efficiency.

Table 1
The C/EBP binding site in the PEPCK promoter enhances the induction of transcription by thyroid hormone.

| Reporter genes                  | Cotransfected Vectors | Fold Induction by T3 |
|---------------------------------|-----------------------|----------------------|
| DR4X1 –68 PLuc                  | RSV-TRβ               | 0.35 ± 0.02          |
| Gal4X1 –68 PLuc                 | RSV-TRβ               | 0.31 ± 0.04          |
| DR4X1 P3WTX3 –68 PLuc           | RSV-TRβ               | 2.4 ± 0.3            |
| Gal4X1 P3WTX3 –68 PLuc          | RSV-TRβ               | 0.3 ± 0.03           |
| Gal4X1 SV40-Luc                 | Gal4-TRβ              | 8.1 ± 0.9            |
| Gal4X1 SV40-Luc                 | Gal4-TRβ + A-C/EBP    | 7.6 ± 1.06           |
| Gal4X1 P3WT X3 SV40-Luc         | Gal4-TRβ              | 61.2 ±10.5           |
| Gal4X1 P3WT X3 SV40-Luc         | Gal4-TRβ + A-C/EBP    | 12.1 ± 1.5           |
| Gal4X1 P3Mut 243 X3 SV40-Luc    | Gal4-TRβ              | 5.1 ± 0.5            |
| Gal4X1 P3Mut 237 X3 SV40-Luc    | Gal4-TRβ              | 5.3 ± 0.5            |
Table 2
Effect of CBP on the T3 induction of the PEPCK gene.

HepG2 cells were transfected with 3 µg of DR4 X1 Gal4 X 1 –68Pluc, 1 µg RSV-TRß and 100 ng of the Gal4 expression vector. Cells were exposed to 100 nM T3 for 24 hrs. All transfections were conducted at least four times in duplicate. The data are expressed as Luciferase activity corrected for protein content and transfection efficiency.

| Reporter Vector | Expression Vector | Fold Induction by T3 |
|-----------------|-------------------|----------------------|
| DR4 X1 Gal4 X 1 –68Pluc | Gal4-C/EBPα 6-50 | 0.9 ± 0.07           |
|                 | Gal4-C/EBPα 6-217 | 4.7 ± 0.2            |
|                 | Gal4-CBP         | 2.6 ± 0.09           |
A. COUP-TF  
RAR  
TRα  C/EBP  Fos/Jun  C/EBP  
TRE P4(I/II)  P3(I)  CRE  
-330/-318  P3(II)  -250/242  -90/-82  

B. DR4X1  
TRE  
-68/+73 PLuc  

DR4X1 P3(I)X3  
TRE  Gal4  
P3(I)  -68/+73 PLuc  

Gal4X1  
Gal4  
SV40 Luc  

Gal4X1 P3(I)X3  
TRE  Gal4  
P3(I)  SV40 Luc  

DR4X1 Gal4X1  
TRE  Gal4  
-68/+73 PLuc  

Gal4X4  
SV40 Luc  
-68/+73 PLuc  

Figure 1
ICEHETSIDISAYIDPAA---------FNDEFLADLFQHS rC/EBPα aa 55-85
ICEHETSIDISAAIDPAA---------FNDEAALDLFQHS rC/EBPα aa 55-85
mC/EBPβ M 57,58,59
mC/EBPβ M 61,62
mC/EBPβ M 86,87

MEVANFYYEP mC/EBPβ aa 22-31
MEVANAAYEP mC/EBPβ M 27,28

Figure 3
Figure 4

A. Fold Induction of Gal4 X 3 -68-PLuc by C/EBPβ

Gal4-
Gal4-β 1-108
Gal4-β 1-108 M61,62
Gal4-β 1-108 M56,57,58
Gal4-β 1-108 M27, 28
Gal4-β 1-100
Gal4-β 1-100 M86, 87
Gal4-β 1-25

B. Fold Induction of 490-P3G4-PLuc by C/EBPβ

Gal4-
Gal4-β 1-108
Gal4-β 1-108 M61,62
Gal4-β 1-108 M56,57,58
Gal4-β 1-108 M27, 28
Gal4-β 1-100
Gal4-β 1-100 M86, 87
Gal4-β 1-25
Figure 5

-490-PLuc  -490-P3G4-PLuc

Gal4-

Gal4-ß 1-108

Gal4-ß 1-108 M61,62

Gal4-ß 1-108 M56,57,58

Gal4-ß 1-108 M27,28

Gal4-ß 1-100

Gal4-ß 1-100 M86,87

Gal4-ß 1-25

Induction by PKA relative to 490-PLuc

0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
Figure 6
Figure 7

Relative Expression of Gal4 Reporter Gene

VP16 Vector
- VP16
- C/EBPα 6-217
- C/EBPα 6-50
- C/EBPα 6-350

Gal4-
Gal4-CBP

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Figure 8

No DNA
Imput
His tag
C/EBPβ
C/EBPα
TRβ
CBP
PEPCK DNA
TRE
Conserved amino acids within CCAAT enhancer binding proteins (C/EBPα and β) regulate phosphoenolpyruvate carboxykinase (PEPCK) gene expression

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