CCAAT-enhancer-binding protein α (C/EBP α) is required for the thyroid hormone but not the retinoic acid induction of phosphoenolpyruvate carboxykinase (PEPCK) gene transcription

Edwards A. PARK^{*§}, Shulan SONG^{*}, Michelle OLIVE[†] and William J. ROESLER[‡]

^{*}Department of Pharmacology, College of Medicine, University of Tennessee Health Science Center, 874 Union Avenue, Memphis, TN 38163, U.S.A., [‡]Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5E5, Canada and [†]Laboratory of Biochemistry, NCI, National Institutes of Health, Bethesda, MD 20892, U.S.A.

Transcription of the gene for phosphoenolpyruvate carboxykinase (PEPCK) is stimulated by cAMP, the thyroid hormone tri-iodothyronine (T_3) and retinoic acid (RA). Regulation of PEPCK transcription by T_3 involves two sites in the promoter including a thyroid-hormone-response element (TRE) and a CCAAT-enhancer-binding protein (C/EBP) binding site called P3(I). Mutation of either the TRE or P3(I) eliminates the T_3 response. In this study, we examined the role of C/EBPs in the induction of PEPCK transcription by T_3 and RA. PEPCK-CAT vectors were transfected into HepG2 cells. Co-transfection of a dominant negative C/EBP eliminated the T_3 stimulation indicating that a member of the C/EBP family is required. To determine which C/EBP isoform was required, Gal4 fusion proteins were created that contained the Gal4 DNA-binding

domain ligated to the transcriptional activation domain of C/EBP α , C/EBP β or the cAMP-responsive-element-binding protein. A Gal4 DNA-binding site was introduced into the P3(I) site of the PEPCK-CAT vector. Only co-transfection of the Gal4-C/EBP α vector was able to restore T_3 responsiveness to the PEPCK-CAT vector. The T_3 and RA receptors are members of the nuclear receptor superfamily and bind to repeats of the AGGTCA motif. We found that the RA receptor can bind to sequences within the PEPCK-TRE and contribute to RA responsiveness of the PEPCK gene. However, the RA induction of PEPCK transcription was found to be independent of C/EBPs, further demonstrating the specificity of the involvement of C/EBP α in the T_3 effect.

INTRODUCTION

The thyroid hormone tri-iodothyronine (T_3) regulates the expression of many genes involved in hepatic metabolism [1]. The effects of T_3 are mediated by the binding of liganded T_3 receptor (TR) to T_3 -responsive elements (TRE) in genes [2]. The TR has two isoforms, α and β , as well as splice variants of these genes [2]. The TR is part of a family of non-steroid nuclear receptors including the retinoic acid (RAR), peroxisomal proliferator (PPAR) and vitamin D receptors [3]. The binding to DNA-response elements and transactivation capability of each of these receptors is enhanced by heterodimerization with the retinoid X receptor (RXR) [3].

In many genes, the actions of steroid hormones are not mediated solely by the binding of the liganded receptor but also by the interactions of that receptor with other proteins bound to the promoter [4]. The gene for phosphoenolpyruvate carboxykinase (PEPCK) is an excellent model for examining the hormonal regulation of gene expression. Its transcription is acutely stimulated by glucagon (via cAMP), thyroid hormone (T_3), retinoic acid (RA) and glucocorticoids and is inhibited by insulin [5–9]. The modulation of PEPCK expression by each of these hormones involves multiple promoter elements and each element can bind several protein factors present in liver nuclei. Defining the specific factors that participate in each response is essential for a complete understanding of hormone action.

The induction of the PEPCK gene by T_3 is well documented. PEPCK transcription is increased in hyperthyroid rats [6]. In isolated hepatocytes, T_3 stimulates PEPCK gene expression and greatly potentiates the effect of cAMP [10]. Previously, we described a TRE in the PEPCK promoter between nucleotides –330 and –319 [11]. This TRE binds the TR β both as a homodimer and a heterodimer with the RXR [11]. The T_3 induction of PEPCK transcription also involves a liver-specific element called P3(I) (–250/–234) which is a CCAAT-enhancer-binding protein (C/EBP) binding site [12–14]. Both the α and β isoforms of the C/EBP family of proteins can bind to the PEPCK promoter and stimulate transcription [14,15]. Mutation of P3(I) eliminates T_3 responsiveness even though the TR can still bind [11]. Glucagon (via cAMP) stimulates PEPCK expression [16]. Multiple sites on the promoter are also involved in the cAMP stimulation of PEPCK transcription. These elements include the cAMP-responsive element (CRE), P3(I) and an AP-1 binding site which is adjacent to P3(I) [17]. In cAMP induction, the CRE-binding protein (CREB) is bound to the CRE, and C/EBP α participates in the cAMP response through the P3(I) site [17,18].

The goal of these studies was to characterize the role of C/EBPs in the T_3 induction of PEPCK transcription. Our results indicate that the C/EBP α isoform is required for the T_3 stimulation of PEPCK transcription which raises the possibility that similar interactions may occur in the stimulation of other hepatic

Abbreviations used: RA, retinoic acid; T_3 , 3,5,3'-tri-iodothyronine; PEPCK, phosphoenolpyruvate carboxykinase; TR β , thyroid hormone receptor β ; TRE, thyroid hormone-responsive element; RXR, retinoid X receptor; RAR, RA receptor; PPAR, peroxisomal proliferator receptor; DR4, direct repeat separated by four nucleotides; DR5, direct repeat separated by five nucleotides; CAT, chloramphenicol acetyltransferase; AF-1, accessory factor 1; C/EBP, CCAAT-enhancer-binding protein; CRE, cAMP-responsive element; CREB, CRE-binding protein; RARE, RA-responsive element.

§ To whom correspondence should be addressed.

genes by T_3 . We also found that the RAR will bind the PEPCK-TRE and contribute to the RA responsiveness to the PEPCK gene. However, C/EBPs are not involved in the induction by RA.

MATERIALS AND METHODS

Preparation of recombinant proteins in *Escherichia coli*

To generate the histidine-tagged RAR α , the cDNA of the human RAR α [19] was ligated into the *Kpn*I and *Sma*I sites of pQE32 which introduces a histidine tag on the N-terminus (Qiagen). Synthesis of His-rTR β was performed by digestion of the rat TR β cDNA [20] with *Bam*HI and *Pst*I and ligation of this fragment into pQE30 (Qiagen). In this vector, the first 63 amino acids were removed from the N-terminus of rTR β , but the DNA- and ligand-binding domains were intact. Synthesis of His-hRXR α has been described previously [11]. The histidine-tagged receptors were expressed in either the M15pREP or BL21(pLys) *E. coli* strain. After induction for 1.5 h with 1.0 mM isopropyl D- β -thiogalactopyranoside, the *E. coli* were harvested and lysed by repeated freeze-thawing. The particulate material was removed by centrifugation at 175 000 *g* (50 000 rev.min) in a Ti80 rotor. Further purification was performed by binding the histidine-tagged receptors to a nickel-affinity resin (Qiagen) containing 10 mM Tris/HCl, pH 7.5, 400 mM KCl, 1 mM β -mercaptoethanol, 20% glycerol and protease inhibitors. After the resin had been washed with buffer containing 1, 10 or 40 mM imidazole, the histidine-tagged receptors were eluted with 200 mM imidazole [11].

Gel mobility-shift assays

Gel mobility-shift assays were conducted on 5% non-denaturing polyacrylamide gels (80:1, acrylamide/bisacrylamide) in 22 mM Tris and 190 mM glycine at 4 °C [11]. Double-stranded oligomers were labelled with Klenow enzyme and [α - 32 P]dCTP [21]. The binding reactions were performed at room temperature in buffer containing 10% glycerol, 80 mM KCl, 25 mM Tris/HCl, pH 7.4, 1 mM EDTA and 1 mM dithiothreitol. Each binding reaction mixture contained 1.0 μ g of poly(dI-dC) as non-specific competitor and proteins as indicated.

Construction of chloramphenicol acetyltransferase (CAT) vectors

The ligation of the PEPCK promoter from -490 to +73 to the CAT reporter gene has been described [14]. The Gal4-binding site was introduced into the P3(I) site of the PEPCK promoter (-490 P3G4) by using a double-stranded oligomer with *Bst*IIIE and *Sau*I overhangs. The -490PEPCK-CAT vector containing a *Bst*IIIE site in the P3(I) element [-490P3(I)M] [22] was digested with *Bst*IIIE and *Sau*I and the oligomer ligated into this site which introduced the sequence gggtacctcgagtagtctctcggt into nucleotides -248 to -223. The mutations in the TRE region of -350PEPCK-CAT were introduced by PCR amplification using 5' primers containing the altered nucleotides and a 3' primer (+73 to +52) which included the *Bg*/II site at +73. The 5' primer introduced a *Xba*I site. The amplified promoter fragment was digested with *Xba*I and *Bg*/II and ligated in front of the CAT reporter gene. The introduction of a DR4 into -350 CAT altered nucleotides -338 to -322 to aggtcacctgaggtca. The sequences of the other -350-CAT mutations are shown in Table 1. To create the -68/G3 DR4X2 and -68/G3 PTREX2 vectors, the DR4 and PEPCK-TRE oligomers were ligated into the *Ava*I site of -68/G3 [18]. These DR4 and PEPCK-TRE oligomers contained the *Ava*I overhangs and the sequences

aggtcacgttaaggtca (DR4) and cctgtccttgacccccacctgac (PTRE -332/-310) respectively. The sequence of all constructs was confirmed by dideoxy sequencing. Construction of the Gal4-C/EBP α (G α 2) and Gal4-C/EBP β (G β 2) and the Gal4-CREB vectors is given elsewhere [17,18].

Cell transfections and CAT assays

HepG2 cells were transfected by calcium phosphate precipitation as described previously [11]. Each transfection contained 5.0 μ g of PEPCK-CAT vector, 5.0 μ g of an expression vector for either RSV-TR β or RSh-RAR α and 5.0 μ g of SV40- β gal as a transfection control. Total DNA concentration was kept constant by the addition of pBluescript. CAT assays were conducted with [3 H]chloramphenicol and *n*-butyryl-CoA using the xylene phase extraction method [21]. All transfections and CAT assays were performed in duplicate and repeated at least three times.

RESULTS

We previously established that the TRE in the PEPCK promoter was located between nucleotides -330 and -319 and that the PEPCK-TRE bound additional proteins present in rat liver nuclear extract [11]. Earlier studies had identified a RA-responsive element (RARE) (-451/-438) in the PEPCK promoter, but had also suggested that a second RARE was present within the first 350 bp of the PEPCK promoter [23,24]. A model of the PEPCK-CAT vectors with the location of the TRE and RARE is shown in Figure 1(A). To localize the second RARE and determine its relationship to the TRE, serial deletions of the PEPCK promoter ligated to the CAT reporter gene were transiently co-transfected with an expression vector for the RAR α into HepG2 cells (Table 1). The -490-CAT vector was stimulated 5.5-fold by the addition of 1 μ M RA, and the -350-CAT vector retained 55% of the RA responsiveness. The -277-CAT vector was unresponsive to RA indicating that a second RARE was contained within the -355 to -277 region. The -340 to -310 region of the PEPCK promoter contains a number of AGGTCA-like motifs and appeared to have the potential to confer RA responsiveness. Two broad mutations were introduced into this region of -490-CAT. Disruption of nucleotides -328 to -321 in the -490-CAT vector decreased the RA induction to 3.3-fold whereas disruption of -320 to -311 did not reduce the RA response (Table 1). Previous studies had shown that the -490M328-321 vector does not respond to T_3 whereas the -490M320-311 is stimulated by T_3 [11]. These results indicated that the TRE and RARE overlapped.

This region of the promoter contains a DR5 with the two repeats being from -319/324 and -330/335 (shown in Figure 1B). To define the functional nucleotides in the RA responsiveness of this element more precisely, a series of 2 bp mutations was introduced into the -335 to -320 region of the -350 PEPCK-CAT vector. The PEPCK-TRE (-330/-319) consists of two direct repeats of the AGGTCA motif [11]. Mutation of nucleotides -332 and -331 reduced the induction by RA from 3.3- to 2.3-fold (Table 1). Alteration of nucleotides -327/26, -323/22 or -321/20 decreased the fold stimulation by RA to 2.0 ± 0.2 , 1.4 ± 0.2 and 1.9 ± 0.1 respectively. These mutations suggested that, although each of the AGGTCA-like sequences contributed to the RA induction, the GGGTCA motif between -324 and -319 was the most critical for the RA response.

The P3(I) site which binds members of the C/EBP family of transcription factors is essential for the T_3 and cAMP induction of PEPCK transcription [11,17]. The next experiments examined whether the P3(I) site is involved in the RA response. Mutation

Figure 1 Models of the CAT reporter vectors

(A) The names of the various vectors used in these studies are indicated on the left. The -490 -CAT vector contains -490 bp of the PEPCK promoter ligated to the CAT reporter gene (CAT). Various binding sites are indicated above the boxes including RARE, PEPCK TRE (PTRE), CRE, Gal4-binding site (Gal4) and optimal TRE (DR4). The $-68/G3$ vector contains three Gal4 sites ligated 5' to -68 PEPCK-CAT vector. (B) The sequence of the PEPCK-TRE and the optimal TRE (DR4) are shown. The solid arrows indicate the direct repeats in the TREs, and the hatched arrows indicate the DR5 in the PEPCK-TRE.

Table 1 RA stimulates PEPCK transcription through the TRE

HepG2 cells were transfected with $5 \mu\text{g}$ of PEPCK-CAT vector, $5 \mu\text{g}$ of RSh-RAR α and $5 \mu\text{g}$ of SV40- β gal. After transfection, the cells were placed in medium containing 10% stripped fetal calf serum. RA was added at a concentration of $1 \mu\text{M}$ for 48 h before harvesting for CAT assays. All transfections were repeated three to five times in duplicate. The sequence which was mutated in the TRE region of the PEPCK promoter is shown in the final column. The underlined nucleotides indicate the altered nucleotides. The results are presented as the fold induction by RA (means $\pm$ S.E.M.).

CAT reporter plasmid	Induction by RA (fold)	PEPCK sequence from -335 to -311
-490 -CAT	5.5 ± 0.4	AGCCCTGTCCTTGACCCACCTGA
-350 -CAT	3.2 ± 0.2	
-277 -CAT	1.1 ± 0.2	
-490 M328-321	3.3 ± 0.4	AGCCCGGTAACCATACCCACCTGA
-490 M320-311	6.6 ± 1.5	AGCCCTGTCCTTGACATAGGTAACC
-350 M332/31	2.3 ± 0.3	AGCATTGTCCTTGACCCACCTGA
-350 M327/26	2.0 ± 0.2	AGCCCTGTAATTGACCCACCTGA
-350 M323/22	1.4 ± 0.2	AGCCCTGTCCTTAGCCACCTGA
-350 M321/20	1.9 ± 0.1	AGCCCTGTCCTTGATACCCACCTGA
-490 P3G4	3.0 ± 0.1	
-490 CC3	3.6 ± 0.4	

of the P3(I) site in -490 P3G4 decreased the fold induction by RA from 5.5 to 3.0 (Table 1). In the vector CC3-CAT, the CRE sequence of the PEPCK gene is introduced into the P3(I) site of

the -490 -CAT vector [11]. This mutation also decreased the ability of RA to stimulate the PEPCK gene to 3.6-fold. The P3(I) mutation in the -350 -CAT vector also decreased RA responsiveness from 3- to 2-fold (results not shown). Although these decreases are far more modest than were observed with T_3 in which mutation of the P3(I) site completely eliminated the T_3 response, the results raised the possibility that C/EBPs might contribute to the RA induction.

To determine whether the RAR α could bind to the PEPCK-TRE, histidine tagged RAR α was used in gel mobility-shift assays with a PEPCK-TRE probe containing nucleotides -336 to -310 (Figure 2). RAR α alone bound weakly to the PEPCK-TRE, but when RXR α was added a strong complex was formed indicating that RAR α binds as a heterodimer with RXR α . In contrast with the RAR, His-rTR β bound either as a homodimer or as a heterodimer with RXR (Figure 2). Unlabelled oligonucleotide representing an optimal RARE (DR5), a CRE or an optimal TRE (DR4) was added to the binding reactions. Only the DR5 oligomer was able to compete for the RAR-RXR heterodimer, whereas both the DR5 and DR4 oligomers competed for the binding of the TR-RXR complex. These experiments show that both the RAR and the TR can bind to the PEPCK-TRE.

To examine the relative affinity of the RAR-RXR heterodimer for the PEPCK-TRE, a competition experiment was conducted in which the binding reactions were conducted in the presence of

Figure 2 Binding of the RA and T₃ receptors to the PEPCK-TRE
Gel mobility-shift assays were conducted as described in the Materials and methods section. Each binding reaction mixture contained 25000 c.p.m. of probe representing the sequence from -336 to -310 in the PEPCK promoter. To the binding reaction was added RAR, TR β or RXR as indicated above each lane. To some reactions was added an excess of unlabelled competitor representing the idealized RARE (DR5), CRE or the idealized TRE (DR4) as indicated above the lane. The binding reactions were conducted for 20 min at room temperature. The complexes were resolved on a 5% non-denaturing polyacrylamide gel in Tris/glycine buffer and visualized by autoradiography.

Figure 3 RAR binds with greater affinity to a DR5 than the PEPCK-TRE
Binding reactions were conducted as described in the legend to Figure 2. Each reaction contained the PEPCK probe representing the sequences from -336/-310 and the histidine-tagged RAR and RXR proteins. Unlabelled competitor oligomer was added at a 5-, 50- or 250-fold excess as indicated above each lane. The competitors represent the idealized RARE (DR5), the PEPCK-TRE (-336/-310) or the PEPCK CRE.

a 5-, 50- or 250-fold excess of unlabelled oligomer representing the DR5, the PEPCK-TRE or the CRE (Figure 3). In these gel mobility-shift assays, the DR5 competed for RAR-RXR binding at the lowest concentration of unlabelled oligomer. PEPCK-TRE was only able to disrupt binding completely at a 250-fold excess whereas CRE did not compete. These results indicate that the RAR binds with a greater affinity to an idealized DR5 than the PEPCK-TRE. The relatively lower affinity of RAR for the PEPCK-TRE is probably due to the fact that this element consists of an imperfect DR5.
To determine if a C/EBP was involved in the T₃ and RA responses, -490-CAT was co-transfected with a dominant negative expression vector for C/EBPs called GBF-F [25]. GBF-F is a chimaeric protein containing a basic DNA-binding region from the plant bZIP protein (GBF-1) and C/EBP leucine zipper

Table 2 Repression of T₃ and cAMP responsiveness of the PEPCK promoter by the dominant negative C/EBP, GBF-F
HepG2 cells were transfected with 5 μ g of the CAT vector indicated and either 5 μ g of RSV-TR β or RSh-RAR α as well as 5 μ g of SV40- β gal and 5 μ g of GBF-F. After transfection, the cells were placed in medium containing 10% charcoal-stripped fetal bovine serum and either 100 nM T₃ or 0.5 mM Bt₂cAMP and 1 mM theophylline or 1 μ M RA. Cells were harvested after 48 h and assayed for CAT activity. The CAT activity was corrected for the protein in the extract. All transfections were repeated at least three times in duplicate. The data are presented as fold induction (mean $\pm$ S.E.M.).

CAT reporter plasmid	Hormone added	Activation (fold)	Activation with GBF-F (fold)
-490-CAT	T ₃	4.0 $\pm$ 0.5	1.1 $\pm$ 0.2
-490-CAT	cAMP	4.6 $\pm$ 1.0	3.2 $\pm$ 0.8
-490-CAT	T ₃ /cAMP	13.9 $\pm$ 1.9	3.1 $\pm$ 0.5
-490-CAT	RA	5.4 $\pm$ 0.4	5.2 $\pm$ 0.2
-68/G3 DR4X2	T ₃	3.8 $\pm$ 0.2	7.1 $\pm$ 0.4

which forms heterodimers with all C/EBPs, suppressing their transactivation ability [18,25]. -490-CAT was transfected into HepG2 cells and T₃, Bt₂cAMP or RA was added. CAT activity was stimulated 4.0- and 4.6-fold by the addition of T₃ or Bt₂cAMP respectively and 14-fold when both hormones were present (Table 2). Co-transfection of GBF-F with -490-CAT eliminated the 4-fold induction of PEPCK transcription by T₃ (Table 2). GBF-F also reduced the cAMP responsiveness and eliminated the synergistic stimulation of cAMP and T₃. These results were identical with those in which a mutation was introduced in the P3(I) site [12] and indicated that a C/EBP participated in the T₃ response. Co-transfection of -490-CAT with GBF-F did not reduce the RA induction which was 5.2 $\pm$ 0.2. These results indicate that the stimulation by RA does not require the C/EBPs. However, since the mutations in the P3(I) site did decrease the RA response, it raised the possibility that an additional protein might be able to bind to the P3(I) site. As a control, the vector -68/G3 DR4X2 was tested. This vector has two optimal TREs and no C/EBP-binding sites. This vector was stimulated 3.8 $\pm$ 0.2-fold by T₃ and 7.1 $\pm$ 0.4-fold in the presence of GBF-F. These results demonstrate that GBF-F does not inhibit the TR β and that C/EBP is not generally required for T₃ to stimulate transcription in HepG2 cells.
The next experiments addressed the question of which member of the C/EBP family was required for the T₃ induction of PEPCK transcription. In the liver, three C/EBP isoforms α , β and δ are expressed [26]. Since the δ isoform is expressed to a significant extent only during the acute-phase response, our experiments concentrated on the α and β forms [27]. To determine whether C/EBP α or C/EBP β was involved in the T₃ response, we utilized a Gal4 fusion protein expression system in which only one isoform was capable of binding to the P3(I) site. Mammalian expression vectors were created that contained the Gal4 DNA-binding and dimerization domains fused to the transactivation region of either C/EBP α (G α 2) or C/EBP β (G β 2) [18]. A Gal4-CREB vector was tested as a control [17]. A Gal4-binding site was introduced into the P3(I)-binding site in the -490-CAT vector (-490 P3G4) (Figure 1). -490 P3G4 was co-transfected with expression vectors for G α 2, G β 2 or Gal4-CREB. As shown in Table 3, -490-CAT was induced 3.3-fold by T₃, and introduction of the Gal4 site into the P3(I) site eliminated the T₃ response. When co-transfected with G α 2, -490-P3G4 was induced 2.3-fold by T₃, but neither G β 2 nor Gal-CREB were

Table 3 Gal4-C/EBP α restores T₃ responsiveness to the PEPCK promoter

HepG2 cells were transfected with 5 μ g of CAT reporter plasmid, 5 μ g of RSV-TR β , 5 μ g of Gal4 fusion plasmid and 5 μ g of SV40- β gal as described in Table 1. The cells were treated with 100 nM T₃ for 48 h. The CAT activities are presented as the fold activation by T₃ and are means $\pm$ S.E.M. for four experiments.

CAT reporter plasmid	Gal4 fusion protein	Activation by T ₃ (fold)
— 490-CAT	None	3.3 $\pm$ 0.5
— 490 P3G4	None	0.8 $\pm$ 0.1
— 490 P3G4	G α 2	2.3 $\pm$ 0.3
— 490 P3G4	G β 2	1.2 $\pm$ 0.3
— 490 P3G4	Gal4-CREB	1.1 $\pm$ 0.1

Table 4 Gal4-C/EBP α restores T₃ responsiveness to a PEPCK promoter with an idealized TRE

HepG2 cells were transfected with 5 μ g of CAT reporter plasmid, 5 μ g of RSV-TR β , 5 μ g of Gal4 fusion plasmid and 5 μ g of SV40- β gal as described in Table 1. Cells were treated with 100 nM T₃ for 48 h. The CAT activities are presented as the fold activation by T₃ and are means $\pm$ S.E.M. for four determinations.

CAT reporter plasmid	Gal4 fusion protein	Activation by T ₃ (fold)
— 350DR4	None	3.7 $\pm$ 0.4
— 350DR4 P3G4	None	1.3 $\pm$ 0.2
— 350DR4 P3G4	G α 2	2.8 $\pm$ 0.5
— 350DR4 P3G4	G β 2	0.7 $\pm$ 0.2
— 350DR4 P3G4	Gal4-CREB	1.6 $\pm$ 0.2

able to restore the T₃ response (Table 3). In the presence of G α 2, the T₃ induction of — 490 P3G4 was not as great as the wild-type CAT vector suggesting that an additional portion of C/EBP α such as the leucine zipper or DNA-binding domain may be required for the full responsiveness. These experiments can only assess the role of C/EBP homodimers and cannot measure the contribution of heterodimers to the T₃ response. These results indicate that C/EBP α participates in the T₃ induction of PEPCK transcription.

An idealized TRE contains two direct repeats of the AGGTCA motif separated by four nucleotides (DR4) [28]. The PEPCK-TRE has an unusual architecture consisting of two direct repeats of AGGTCA motif (DR0) on the opposite strand [11]. To determine if the interactions between the TR and C/EBP α were dependent on the configuration of the PEPCK-TRE, a DR4 was introduced into the position of the PEPCK-TRE in a — 350PEPCK-CAT vector (— 350DR4) (see Figure 1). When — 350DR4 was transfected into HepG2 cells, it was induced 3.7-fold by T₃ (Table 4). Mutation of the P3(I) site in — 350DR4 also resulted in a loss of T₃ responsiveness indicating that the interactions between the TRE and P3(I) site were not specifically confined to the PEPCK-TRE. The Gal4-binding site was introduced into the P3(I) site of — 350DR4 (— 350DR4, P3G4), and this vector was co-transfected with Gal4 fusion proteins. G α 2 restored T₃ responsiveness to 2.8-fold but G β 2 was unable to increase the T₃ response. The Gal4-CREB vector allowed a modest T₃ response. These results showed that the requirement for C/EBP α was independent of the configuration of the TRE.

The next experiments examined whether C/EBP α could enhance the T₃ response outside the context of the PEPCK promoter. Two copies of either the PEPCK-TRE or the DR4

Table 5 Effect of C/EBPs on the T₃ responsiveness of isolated TREs

HepG2 cells were transfected with 5 μ g of — 68/G3, 5 μ g of RSV-TR β , 5 μ g of Gal4 fusion protein and 5 μ g of SV40- β gal as described in the legend to Table 1. T₃ was added for 48 h. All transfections were performed three to five times in duplicate, and the results are means $\pm$ S.E.M.

CAT reporter plasmid	Gal4 fusion protein	Activation by T ₃ (fold)
— 68/G3	None	0.4 $\pm$ 0.1
— 68/G3	G α 2	0.4 $\pm$ 0.1
— 68/G3 PTREX2	None	1.9 $\pm$ 0.3
— 68/G3 PTREX2	G α 2	2.2 $\pm$ 0.1
— 68/G3 PTREX2	G β 2	0.9 $\pm$ 0.2
— 68/G3 PTREX2	Gal4-CREB	1.2 $\pm$ 0.1
— 68/G3 DR4X2	None	3.9 $\pm$ 0.1
— 68/G3 DR4X2	G α 2	3.4 $\pm$ 0.4
— 68/G3 DR4X2	G β 2	1.3 $\pm$ 0.1
— 68/G3 DR4X2	Gal4-CREB	4.0 $\pm$ 0.7

were introduced 5' to a — 68 PEPCK-CAT vector that contained three Gal4-binding sites (— 68/G3) (see Figure 1). This vector with three Gal4 sites was chosen because the PEPCK promoter contains three C/EBP sites around P3(I) [14]. — 68/G3, which does not contain a TRE, was not stimulated by T₃ in the presence or absence of G α 2 (Table 5). As had been reported previously, co-transfection of G α 2 with the — 68/G3 vector stimulated the basal level of transcription (results not shown) [18]. T₃ stimulated the — 68/G3 PTREX2 and DR4X2 vectors 1.9 $\pm$ 0.3 and 3.9 $\pm$ 0.1-fold respectively in the absence of a Gal4 vector. These results showed that the idealized TRE mediates the T₃ stimulation more effectively than the PEPCK-TRE. Also, these results indicate that there is not a requirement for C/EBP α for T₃ to function outside the context of the PEPCK gene. Co-transfection with G α 2 did not enhance the T₃ response, and G β 2 decreased the stimulation by T₃ (Table 5). The Gal4-CREB vector did not affect the T₃ induction of the DR4 oligomer but reduced the responsiveness of the PTRE. These data indicate that the PEPCK promoter context is essential for establishing the cross-talk between the TRE and P3(I).

DISCUSSION

In the present study, we report that a single element can mediate the T₃ and RA induction of the PEPCK gene transcription. In addition, we describe the novel finding that C/EBP α is a participant in the T₃ but not the RA responsiveness of the PEPCK promoter. The requirement for a C/EBP-like protein was suggested by previous studies which showed that mutation of P3(I), a promoter element that binds C/EBP *in vivo* and *in vitro*, abolished T₃ responsiveness of the PEPCK promoter [12,14]. We extended this observation by showing that expression of a dominant repressor of C/EBPs (GBF-F) in HepG2 cells eliminated the induction by T₃. The specificity of this repressor effect was evident from the absence of any change in the RA responsiveness of the PEPCK promoter when GBF-F was expressed. Furthermore, we showed that expression of a Gal4-C/EBP α fusion protein can reconstitute T₃ responsiveness to a PEPCK promoter derivative which has the P3(I) site replaced by a Gal4-binding site. This observation not only provided further functional evidence for our hypothesis that C/EBPs are involved in the T₃ response but also identified a specific isoform of C/EBP which participates in the response. Although the N-terminal domain of C/EBP α (amino acid residues 6–217) was identified as

being sufficient for mediating the T_3 response along with the TR bound to the upstream TRE, other aspects of C/EBP α may be required for complete restoration of the T_3 induction. It should be noted that our experiments did not address the issue of whether C/EBP heterodimers are capable of mediating the response, but only whether a C/EBP α or β homodimer could produce a response. Also, we cannot rule out a role for C/EBP δ . However, since the expression of this isoform is low in the liver except under acute-phase conditions and the transactivation domains of C/EBP α and δ show no apparent homologies, we feel it is unlikely that C/EBP δ is involved in the T_3 response [26,27].

The results of this study emphasize the importance of promoter context in defining the function of a specific element. Our studies suggest that the PEPCK promoter context creates an environment whereby the T_3 response becomes reliant on the presence of a bound C/EBP α . This requirement is not a function of the unusual architecture of the PEPCK-TRE since replacing it with an idealized TRE did not diminish the dependence on C/EBP α . When taken out of the context of the PEPCK promoter, both the native TRE and the DR4 were capable of mediating T_3 responsiveness independently of C/EBP α . Thus some aspect of the promoter design such as the precise spacing and/or positioning of the *cis* elements allows further fine-tuning of the hormone response. It is conceivable that T_3 responsiveness of the PEPCK promoter would be sensitive to ambient concentration of C/EBP α within the nucleus. Since the expression of C/EBP α is hormonally regulated, this suggests that the T_3 responsiveness of the PEPCK promoter could be impacted by the metabolic state of the liver [15].

We had shown previously that in addition to the TR other proteins present in rat liver nuclear extract could bind to the PEPCK-TRE [11]. While this manuscript was in preparation, Scott et al. [29] reported that the RAR can bind to the PEPCK-TRE through a DR5 extending from nucleotides -319 to -335. Similar results were obtained in our laboratory using competition with mutated oligomers (results not shown). Our site-directed mutagenesis of this region gave similar reductions in the RA responsiveness except that in our experiments mutation of nucleotides -326/327 also reduced the RA induction (Table 1). Although the reason for the reduction with this mutation is uncertain, the nucleotides between the direct repeats may contribute to the transactivation ability of the RAR. It has been reported that the nucleotides flanking the repeats can contribute to the binding and transactivation by the TR and RAR [30,31]. In these studies, the PEPCK-CAT vectors were co-transfected with the RAR α expression vector. The liver contains other isoforms of the RAR. It is not known if these isoforms can stimulate PEPCK transcription or if their transactivation capability is dependent on an intact P3(I) site. The RA response was reduced by mutation of the P3(I) site but not by co-transfection of GBF-F, suggesting that proteins other than C/EBP may be able to bind the P3(I) site and affect the hormone responsiveness of the PEPCK gene.

There is a complex interplay of factors integrating the regulation of PEPCK transcription by steroid hormones. The glucocorticoid induction of PEPCK transcription involves two glucocorticoid response elements, the more 5' RARE (called AF-1) and the CRE [32,33]. AF-1 binds the RAR, HNF-4, COUP-TF and PPAR [24,32,33]. Mutation of AF-1 resulted in a loss of glucocorticoid responsiveness and it is believed that either HNF-4 or COUP-TF modulates the glucocorticoid induction [32]. It was suggested that proteins binding to the PEPCK-TRE may be required for the full glucocorticoid response although the identity of these proteins has yet to be determined [29]. Other hepatic genes have been shown to have multiple

elements involved in regulation by T_3 [34]. For example, the very T_3 -responsive S14 gene is induced by three functionally synergistic TREs. The full induction by T_3 was dependent on additional sequences in the promoter, and the induction was greater in hepatocytes than non-hepatoma cells [34]. Additional proteins bound to the PEPCK promoter may be required for the cross-talk between the TR β and C/EBP α . The AP1 site which is located immediately 5' to the P3(I) site is essential for full cAMP responsiveness [18,22]. However, we reported previously that mutation of the AP-1 site does not reduce the T_3 response [11]. Disruption of the P4(I) site (-285/-270), which is a low-affinity C/EBP site, reduced the T_3 induction from 3.1- to 2.2-fold suggesting that the full complement of C/EBPs is required [11].

Synergy between factors is an important concept in transcriptional regulation generally and for the TR specifically. For example, the TR synergizes with Pit1 in the regulation of the rat growth hormone gene and with the pituitary factor GHF1 in the regulation of the human placental lactogen B promoter [35,36]. The interactions between the TR and GHF1 decrease in a distance-dependent manner [36]. We have yet to determine how C/EBP α and TR synergistically mediate T_3 responsiveness. It is possible that they undergo protein-protein interactions as has been shown for other examples of synergism [37]. Interestingly, C/EBP α has been shown to synergistically interact with HNF-4, an orphan steroid hormone receptor, in the regulation of the liver-specific ApoB gene promoter [38]. These transcription factors bind non-co-operatively to overlapping sites. Alternatively C/EBP α and TR β may contact a co-activator. Although no co-activators have been described for C/EBP α , several have been found for TR [3,39]. It is unlikely that the synergistic effect is mediated through an increase in the binding affinity of the TR since the TRE is functional outside of the context of the promoter.

Recently, it was reported that C/EBP α plays a critical role in mediating the cAMP responsiveness of the PEPCK promoter, and its involvement probably explains the liver-specific nature of the cAMP response [18]. Thus C/EBP α is a common factor in the T_3 and cAMP responsiveness of the PEPCK promoter. It is interesting to note that this promoter is synergistically activated by T_3 and cAMP. We speculate that C/EBP α may serve as a central 'integration' factor in this promoter, integrating the cAMP and T_3 signals and thereby eliciting a unique response. Future studies will examine the mechanism whereby C/EBP α participates in each hormone response separately and it is hoped clarify its role in integrating the two signals in a unified response.

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