Regulation of phosphoenolpyruvate carboxykinase gene transcription by thyroid hormone involves two distinct binding sites in the promoter

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Transcription of the gene for phosphoenolpyruvate carboxykinase (PEPCK) is stimulated by thyroid hormone (T_3), glucagon (via cyclic AMP) and glucocorticoids. A region of the PEPCK promoter between –332 and –308 mediates the induction of transcription by T_3 . To characterize this region further, mutations were introduced into this region of the PEPCK promoter and the modified promoters ligated to the chloramphenicol acetyltransferase (CAT) reporter gene. Using these PEPCK–CAT vectors in transient transfections in HepG2 cells, it was found that T_3 stimulates PEPCK transcription through two direct repeats of the AGGTCA motif located between nucleotides –330 and –319 [PEPCK–thyroid-hormone-responsive element (TRE)]. The β form of the T_3 receptor ($TR\beta$) bound PEPCK–

TRE as a homodimer but bound far more efficiently as a heterodimeric complex with the retinoid X receptor (RXR). An additional region called P3(I) (–250 to –234) is required for T_3 responsiveness and binds members of the CCAAT-enhancer-binding protein (C/EBP) family. P3(I) contains an AGGTCA-like motif that can bind the $TR\beta$ –RXR heterodimer. Mutagenesis of this motif abolished $TR\beta$ –RXR binding without reducing T_3 induction. Mutation of the C/EBP-binding site or insertion of a cyclic AMP-responsive-binding-protein site at P3(I) eliminated the T_3 response. Our results indicate that T_3 stimulation of PEPCK transcription is mediated by $TR\beta$ bound to PEPCK–TRE and requires C/EBP to be bound at the P3(I) site.

INTRODUCTION

The thyroid hormone tri-iodothyronine (T_3) has profound effects on numerous enzymes involved in hepatic metabolism [1]. Phosphoenolpyruvate carboxykinase (PEPCK) controls the initiating and rate-limiting step in the pathway of gluconeogenesis [2]. PEPCK abundance and gluconeogenesis are elevated in the hyperthyroid state [3]. Transcription of the PEPCK gene is increased in hyperthyroid rats and decreased in hypothyroid animals [4]. In isolated hepatocytes, T_3 induces PEPCK expression and greatly potentiates the induction by cyclic AMP (cAMP) [5]. In addition, transcription of the PEPCK gene is stimulated by glucagon (via cAMP), glucocorticoids and retinoic acid, whereas it is inhibited by insulin [6–9]. Sequences in the promoter of the PEPCK gene involved in these hormone responses have been identified. A region in the PEPCK promoter from –332 to –308 was identified which confers T_3 responsiveness to the PEPCK promoter [10]. A consensus cAMP-responsive element (CRE) (–90 to –82) has been defined that can bind the cAMP-responsive-element-binding protein (CREB) and members of the CCAAT-enhancer-binding protein (C/EBP) family [11–13]. An upstream region called P3(I) (–250 to –234) is required for cAMP and T_3 responsiveness as well as for directing expression of PEPCK to the liver [10,11,14]. This element binds C/EBPs [12,13]. Members of the C/EBP family are involved in directing expression of liver-specific genes and adipocyte differentiation [15–17]. In addition, C/EBP α has been proposed to be a regulator of genes encoding proteins involved in energy metabolism [18].

T_3 mediates its effects through a nuclear T_3 receptor (TR) which has two isoforms, α and β [19]. The liver contains primarily the β -isoform [19]. An idealized T_3 -responsive element (TRE) has been defined which consists of two direct repeats of an AGGTCA motif separated by 4 bp (DR4) [20]. This element comprises part of the 3–4–5 rule in which direct repeats of the AGGTCA sequence separated by 3, 4 and 5 bp form hormone-response elements for vitamin D, T_3 and retinoic acid respectively [20]. However, TR can bind other motifs including palindromes and single repeats [21,22]. In the natural TREs that have been described, the AGGTCA sequence has been loosely conserved [23]. In addition, the nucleotides flanking the AGGTCA motif may contribute to TR binding [22].

Although TR can bind to its DNA-recognition site as a monomer or a homodimer, it binds with far greater affinity as a heterodimer with the retinoid X receptor (RXR) [24]. RXRs are composed of several family members which are present in most tissues and cell lines [19]. RXR α is expressed most abundantly in the liver [24]. RXR forms high-affinity DNA-binding heterodimers with several additional receptors, including the vitamin D receptor, the retinoic acid receptor (RAR) and the peroxisomal proliferator receptor (PPAR) [19].

The goal of our studies was to characterize further the sequences and proteins involved in T_3 stimulation of PEPCK transcription. Our results indicate that T_3 stimulates PEPCK transcription through a direct repeat of the AGGTCA motif between –330 and –319 and that $TR\beta$ can bind this sequence either alone or as a heterodimer with RXR α . PEPCK–thyroid-hormone-responsive element (TRE) does not conform to the

Abbreviations used: T_3 , 3,5,3'-tri-iodothyronine; PEPCK, phosphoenolpyruvate carboxykinase; $TR\beta$, thyroid hormone receptor β ; TRE, thyroid-hormone-responsive element; RXR, retinoid X receptor; RAR, retinoic acid receptor; PPAR, peroxisomal proliferator receptor; DR4, direct repeat separated by 4 nucleotides; CAT, chloramphenicol acetyltransferase; AF1, accessory factor 1; C/EBP, CCAAT-enhancer-binding protein; CRE, cyclic AMP-responsive element; CREB, cyclic AMP-responsive-element-binding protein; cAMP, cyclic AMP.

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MATERIALS AND METHODS

The full-length cDNA of rat TR β [25] was ligated into the Flag-Mac expression vector, which contains the Flag recognition peptide at the N-terminus (IBI). This protein was expressed in the M15pREP *E. coli* strain. After induction for 1.5 h with 1.0 mM isopropyl β -D-thiogalactopyranoside, the *E. coli* were harvested and lysed by repeated freeze-thawing. The particulate material was removed by centrifugation at 175000 *g* (50000 rev./min) in a Ti80 rotor. The FI-TR β was bound to Flag antibody linked to resin in buffer containing 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 % glycerol and protease inhibitors. After extensive washing of the resin, the FI-TR β was eluted with a competitor peptide as described by the manufacturer (IBI). The cDNA of RXR α [24] was ligated into the vector, pQE9, which contains a six-amino acid histidine tag at the N-terminus (Quiagen). An *Nco*I site at amino acid 27 and a *Hind*III site in the 3' untranslated region of RXR α were used to subclone into pQE9. The His-RXR α expression vector was transformed into M15pREP cells. After induction with 1.0 mM isopropyl β -D-thiogalactopyranoside, the *E. coli* were lysed by freeze-thawing in 10 mM Tris/HCl, pH 7.5, containing 400 mM KCl, 1 mM 2-mercaptoethanol, 20 % glycerol and protease inhibitors. Further purification was performed by binding the RXR α to a nickel-affinity resin (Quiagen) in the lysis buffer. After washing of the resin with lysis buffer containing 1, 10 or 40 mM imidazole, the His-RXR α vector was eluted from the resin with the same buffer containing 200 mM imidazole [26]. Proteins were isolated from rat liver nuclei as described previously [27].

Gel mobility assays

Construction of chloramphenicol acetyltransferase (CAT) vectors

nucleotides and overlapped in the 20 bp region from -330 to -310. The DNA segments were purified from the primers, mixed and amplified again using the outside primers. The DNA product contained the entire PEPCK promoter from -490 to +73 and was ligated in front of the CAT reporter gene in *Xba*I and *Bgl*II sites. The promoter containing each mutation was sequenced with Sequenase II (USB) to confirm its identity. The sequences of the top strand of the oligomers used to create the vectors with mutations in the -330 to -310 region are given: PTREM329-326 cccctactattgacccccacctgac, PTREM323-320 cctgtccttactaccacctgac, PTREM317-314 cctgtccttgacccccactatgac, PTREM328-320 cccggtgaaccataccccacctgac, PTREM320-311 cctgttccttgacataggttaacc. The nucleotides in the P3(I) region were altered by the PCR-based strategy. The sequences of the primers on the top strand are given: P3M235-32 aaacgttgtgtaactgatacactatg and P3M248-7/42-1 tcttagctcatgtaaggactc.

HepG2 cells were transfected by calcium phosphate precipitation as described previously [10,12]. Each transfection mixture contained 5.0 μg of PEPCK-CAT vector, 5.0 μg of an expression vector for Rous sarcoma virus (RSV)-TR β and 2.0 μg simian virus 40 (SV40)- β -galactosidase as a transfection control. CAT assays were conducted with [^3H]chloramphenicol and n-butyryl-CoA using the xylene-phase extraction method [29]. All transfections and CAT assays were performed in duplicate.

Identification of the T₃-responsive nucleotides in the PEPCK promoter

Transcription from the wild-type PEPCK-CAT vector was increased threefold by the addition of T_3 in the transient-transfection experiments (Figure 1b). This stimulation corresponds to the previously reported effects of T_3 on PEPCK transcription *in vivo* [4]. The PEPCK-CAT vector, PTREM329-326, in which the 5'-most repeat was disrupted, was unable to respond to T_3 . Likewise, PTREM323-320 was not induced by T_3 . However, T_3 stimulated transcription of the PEPCK-CAT vector, PTREM317-314, in which the 3'-most repeat was altered, indicating that this sequence was not involved in the T_3 response. Two broad mutations were also tested. PTREM328-320 did not respond to T_3 , but PTREM320-311 was induced by T_3 . These data indicate that the two direct repeats

Figure 1 Sequences involved in the induction of PEPCK transcription by T_3

(a) A model outlining the protein-binding domains in the PEPCK promoter. (b) HepG2 cells were transiently transfected with 5 μ g of PEPCK-CAT vectors that contained -490 to +73 bp of the PEPCK promoter, 5 μ g of RSV-TR β and 5 μ g of SV40- β gal as described in the Materials and methods section. HepG2 cells were exposed to 100 nM T_3 for 40 h before being harvested for CAT assays. On the left are the sequences of the PEPCK promoter between -333 and -309. The mutations introduced by site-directed mutagenesis of the full-length promoter are indicated by underlined italics. The arrow containing the X indicates the disrupted repeat. Results are presented as fold induction by T_3 . (c) The PEPCK-CAT vectors which contained mutations in either the P3(II) or P4(I) protein-binding sites in the PEPCK promoter were used in transient-transfection assays as described above. The nucleotides altered are indicated by underlined italics. (d) PEPCK-CAT vectors containing mutations in the P3(I) region were transfected as indicated above. On the left are the sequences of the PEPCK promoter between -250 and -230. The arrows above the wild-type (WT) sequence indicate the TR-binding site and the hatched bar outlines the C/EBP-binding site in the P3(I) region. Values are presented as means $\pm$ S.E.M. of three to nine transfections. All transfections were performed in duplicate.

contained within nucleotides -330 and -319 are required for T_3 stimulation of PEPCK transcription.

Binding of TR β to PEPCK-TRE

As the effects of T_3 are mediated by the binding of the liganded TR to response elements, the next experiments examined the interaction of TR β with PEPCK-TRE. The β form of the TR was used as this is the primary form expressed in the liver [25]. A TR β which contained an eight-amino acid Flag recognition peptide at the N-terminus (F1-TR β) and a histidine-tagged RXR α (His-RXR α) were obtained by overexpression in *E. coli*. These receptors were further purified by affinity chromatography as outlined in the Materials and methods section.

Gel mobility assays were used to determine if TR β could bind PEPCK-TRE. To the binding reaction mixture was added a 32 P-labelled oligomer representing PEPCK-TRE (-332 to -308) and either the F1-TR β or His-RXR α . As shown in Figure 2, TR β formed a complex with PEPCK-TRE. This complex was supershifted by the addition of a monoclonal antibody (IBI) which recognizes the Flag peptide. The supershift indicates that

Figure 2 Binding of TR and RXR to PEPCK-TRE

TR β s containing the Flag antibody recognition epitope (F1-TR β) and histidine-tagged RXR α (His-RXR α) were purified as described in the Materials and methods section. The binding reaction mixtures contained 25000 c.p.m. of 32 P-labelled oligomer representing the sequence from -332 to -308 in the PEPCK promoter and TR β or RXR α as indicated by +. The monoclonal antibody to the Flag epitope (FI-Ab) was added to the binding reaction mixtures as shown. Binding reactions were conducted for 20 min at room temperature. The complexes were resolved on a 5% non-denaturing acrylamide gel in Tris/glycine buffer and visualized by autoradiography.

TR β interacted with PEPCK-TRE. TR β is probably bound as a homodimer as it preferentially binds in this form whereas TR α binds as a monomer [19]. Because RXR enhances the binding of several nuclear receptors to their cognate elements [24], we examined whether RXR α would improve the binding of TR β to PEPCK-TRE. The addition of RXR α greatly increased the binding of TR β to PEPCK-TRE. This complex could be

Figure 3 Identification of nucleotides involved in TR β binding to PEPCK-TRE by oligonucleotide competition

Gel mobility assays were conducted exactly as described in Figure 2. Each binding reaction mixture contained 25000 c.p.m. of 32 P-labelled oligomer representing the sequence from -332 to -308 in the PEPCK promoter and purified TR β and RXR α . As indicated above each lane, an excess of unlabelled oligomer was added to each binding reaction mixture. The competitor oligomers represent either the wild-type PEPCK-TRE (WT-PEP-TRE) sequence or the PEPCK-TRE sequence (Mut) containing the base-pair changes outlined in Figure 1(b). The CRE oligomer represents the cAMP-responsive element of the PEPCK promoter (-94 to -77) and TREpal is the palindromic AGGTCA motif (AGGTCATGACCT).

Figure 4 Binding of proteins from rat liver nuclei to PEPCK-TRE

(a) Gel mobility assays were conducted exactly as described in Figure 2. Each binding reaction mixture contained labelled oligomer representing the sequence from -332 to -308 in the PEPCK promoter and purified TRβ, RXRα or proteins from rat liver nuclei extract (RLNE) as indicated. RLNE (1.0 μg) was added to the binding reaction mixture as indicated by + + +. (b) Gel mobility assays were conducted exactly as in (a) except that 0.1 μg of RLNE was added to the binding reaction mixture as indicated by +. An antibody to TRβ (TR-Ab) was added to two of the binding reaction mixtures.

supershifted by the addition of the Flag peptide antibody indicating that the complex contained TRβ. RXRα alone did not bind to PEPCK-TRE. These results demonstrate that TRβ can bind to PEPCK-TRE and suggest that *in vivo* TRβ binds preferentially to TRE as a heterodimeric complex containing RXRα.

To determine which nucleotides are required for binding of TRβ to PEPCK-TRE, a competition experiment was conducted in which unlabelled oligomers were allowed to compete for the binding of the TRβ-RXRα heterodimer to the ³²P-labelled PEPCK-TRE oligomer (Figure 3). Both TRβ and RXRα were added because the heterodimer represents the high-affinity binding form of TRβ. The competitor oligomers were either the wild-type PEPCK-TRE (-332 to -308) or PEPCK-TREs containing the nucleotide changes shown in Figure 1. The unlabelled wild-type oligomer eliminated the binding of the TRβ-RXRα heterodimer to the labelled TRE, whereas the oligomer Mut 329-326 did not compete effectively for binding of TRβ-RXRα (Figure 3). The oligomers Mut 323-320 and Mut 317-314 reduced the binding of the receptors to the labelled TRE. The oligomer representing the CRE did not affect binding of TRβ-RXRα to PEPCK-TRE, whereas an oligomer containing the palindromic TRE was an effective competitor. These data indicate that the AGGACA motif between -330 and -325 is required for the high-affinity binding of the heterodimeric complex of TRβ and RXRα.

The next experiments examined whether other proteins in rat liver nuclear extract could bind to PEPCK-TRE. A gel mobility assay was conducted using the ³²P-labelled PEPCK-TRE oligomer and rat liver nuclear extract (Figure 4a). Two complexes were formed between rat liver nuclear extract and PEPCK-TRE, one of which migrated in a similar fashion to the TRβ-RXRα heterodimer. Several groups have demonstrated that nuclear extract will enhance the binding of TR to TREs [21,25]. Therefore we tested whether rat liver nuclear extract would improve the binding of TRβ to PEPCK-TRE (Figure 4b). In these binding reactions, concentrations of TRβ and rat liver nuclear extract

Figure 5 Identification by oligonucleotide competition of nucleotides involved in the binding of proteins from rat liver nuclei to PEPCK-TRE

Gel mobility assays were conducted essentially as described in Figure 3. Each binding reaction mixture contained 25 000 c.p.m. of ³²P-labelled oligomer representing the sequence from -332 to -308 in the PEPCK promoter and 1.0 μg of rat liver nuclear extract (RLNE). As indicated, an excess of unlabelled oligomer was added to each binding reaction. The oligomers are described in the legend to Figure 3.

were used that did not form detectable complexes in gel mobility assays. When both TRβ and rat liver nuclear extract were added to the reaction mixture, two additional complexes were formed. These complexes could be supershifted or disrupted by the addition of an antibody to TRβ. No complexes were formed when TRβ antibody and rat liver nuclear extract were added to the reaction mixture. These results demonstrate that a protein, which is probably the RXR in rat liver nuclear extract, enhances the binding of TRβ to PEPCK-TRE.

To characterize further the binding of proteins from rat liver nuclei to PEPCK-TRE, a competition analysis was conducted using rat liver nuclear extract, PEPCK-TRE and unlabelled competitor oligomers (Figure 5). The oligomer, Mut 329-326, competed for the binding of rat liver nuclear extract to PEPCK-TRE, whereas Mut 323-320 was not an effective competitor, indicating that these nucleotides were involved in the binding of this protein. The protein(s) in rat liver nuclear extract had a different binding specificity for PEPCK-TRE from that of the TRβ-RXRα heterodimer. These data indicate that, in addition to TR and RXR, there is another protein present in rat liver nuclei which will bind to PEPCK-TRE.

Identification of additional sites required for T₃ action

In addition to the CRE, several adjacent elements including P3(I) (-250/234), P3(II) (-260 to -250) and P4 (-285 to -270) are required for the full induction of PEPCK transcription cAMP [11]. These elements contain binding sites for C/EBP and Fos/Jun [12,30]. The P3(I) site is also involved in the stimulation of PEPCK transcription by T₃ [10]. To determine whether P3(II) or P4 were required for the induction of PEPCK transcription by T₃, transient transfections with PEPCK-CAT vectors containing mutations in these elements of the promoter were conducted. Disruption of the P3(II) or P4(I) binding sites did not block the T₃ effect (Figure 1c). These results suggest that there is a specific interaction between the proteins bound to the P3(I) element and the TRE.

As the P3(I) site is required for T₃ induction, the possibility that TRβ could bind to P3(I) was examined. In gel mobility

Figure 6 Binding of TR and RXR to the P3(I) element

(a) The sequence of the wild-type P3(I) element (–250 to –230) is presented as P3WT. The oligomers containing mutations in specific nucleotides are presented as P3M. The arrows above the wild-type sequence outline the TR-binding site and the hatched box underneath indicates the C/EBP-binding site. The mutated nucleotides are underlined. (b) Gel mobility assays were conducted as described in Figure 2. Each binding reaction mixture contained 25 000 c.p.m. of 32 P-labelled P3(I) oligomer and purified TRβ and RXRα as indicated. (c) The binding of the TRβ–RXRα heterodimer to the P3(I) oligomer was examined by competition with unlabelled oligomers. Each binding reaction mixture contained the labelled wild-type P3(I) oligomer and the TRβ and RXRα. An excess of unlabelled oligomer containing the sequences shown in (a) was added to each binding reaction as indicated. CRE is the oligomer that contains the cAMP-responsive element in the PEPCK promoter (–94 to –77).

assays, an oligomer containing sequences in the PEPCK gene from –250 to –230 [P3(I)] bound the TRβ–RXRα heterodimer (Figure 6b). This observation suggested that the requirement for the P3(I) element in the T_3 response might involve binding of TRβ. The C/EBP-binding site (TTTGTGAAG) is contained within nucleotides –243 to –235. It appeared likely that the TRβ would bind to a sequence from –246 to –231 which contains two direct repeats separated by four nucleotides although the 5' repeat has a low similarity (3 of 6) to the optimal AGGTCA motif. To determine which nucleotides were involved in the binding of TRβ–RXRα to P3(I), gel mobility assays were conducted in which mutated oligomers competed for the binding of the proteins to a labelled P3(I) oligomer (Figure 6c). Unlabelled P3(I) oligomer eliminated binding of TRβ–RXRα to P3(I). The oligomer, M235–232, in which the 3'-AGGTCA-like motif is disrupted, did not compete for binding of TRβ–RXRα to labelled P3(I), indicating that this motif is essential for binding of TRβ to P3(I). The unlabelled oligomer M248–242 reduced the binding of TRβ–RXRα. This mutation eliminated the binding of C/EBP (results not shown). The oligomer, M248–247/242–241, which introduced the PEPCK–CRE sequence (TTACGTCA) into the P3(I) site, was an effective competitor for TRβ–RXRα binding. The nucleotide changes in M248–247/242–241 allow CREB to bind but reduce the affinity of C/EBP for this site (results not shown).

To determine whether the binding of a C/EBP or TRβ at the P3(I) site was required for T_3 stimulation of PEPCK transcription, the mutations contained in the oligomers used in Figure 6 were introduced into the PEPCK–CAT vector, and these constructs transiently transfected into HepG2 cells (Figure 1d). T_3 stimulated transcription of the vector P3M235–232, in which the TRβ-binding site was disrupted. This result indicates that T_3 induction does not require TRβ to be bound to P3(I). The P3M248–242 vector in which the C/EBP site is disrupted does not respond to T_3 . Introduction of a CREB-binding site (P3M248–247/242–241) also eliminated T_3 responsiveness. These observations suggest that the T_3 response involves specific interactions between a C/EBP bound to the P3(I) site and TRβ bound to PEPCK–TRE.

DISCUSSION

T_3 affects the hepatic pathways of glucose and lipid metabolism by increasing the activity of enzymes such as glucokinase, fatty acid synthetase and carnitine palmitoyltransferase-I as well as gluconeogenic enzymes such as PEPCK [1,4,31,32]. In this report, we demonstrate that T_3 induction of PEPCK transcription involves interactions between TR bound to TRE and a C/EBP bound to the P3(I) site in the promoter. The TRE in the PEPCK promoter does not conform to the 3–4–5 rule. Our data indicate

that PEPCK-TRE consists of a direct repeat. Direct repeats of the AGGTCA motif will function as a TRE, as a single copy of a direct repeat conferred a 4-fold stimulation of transcription on a neutral promoter in transient-transfection assays in HeLa cells [28]. The TR can bind a variety of arrangements of the AGGTCA sequence other than a DR4, including palindromes [21], inverted palindromes [28], direct repeats [28] and single sites [22]. Interestingly, the retinoic acid response element in the PEPCK promoter does not follow the 3-4-5 rule and consists of a DR1 [8]. To our knowledge, PEPCK-TRE is the first natural TRE to be described consisting of direct repeats which mediates a T_3 response and further emphasizes that a variety of elements are able to confer T_3 responsiveness.

It was reported that TR will protect a 30 bp region of the PEPCK promoter from -324 to -294 in DNase I footprinting assays [33]. This region partially overlaps the functional TRE (-330 to -319) defined in this study. In our gel mobility assays, none of the 4 bp mutations in the -330 to -313 region completely eliminated the ability of TR to bind to PEPCK-TRE in gel mobility assays (results not shown). Because the -330 to -294 region contains several AGGTCA-like motifs, TR may be able to bind in several different conformations in the *in vitro* binding assays. However, only the nucleotides between -330 and -319 are essential for T_3 stimulation of transcription.

Previous studies using a variety of TREs have shown that TR binds far more effectively in the presence of rat liver nuclear extract [21,25]. This enhancement is probably due to the heterodimerization of TR with RXR present in the nuclear extract, although the possibility that other proteins might be involved cannot be excluded. Our experiments demonstrate directly that TR β will bind to PEPCK-TRE more effectively when present as a heterodimer with RXR (Figure 2) and that TR β will form a heterodimer with a protein present in rat liver nuclei (Figure 4b). Using the -332 to -308 oligomer in gel mobility assays with subsequent Scatchard analysis, we determined a K_d of 2 nM for the binding of TR β and RXR α (results not shown). Affinity determinations of the binding of TR alone and in the presence of rat liver nuclear extract to the PEPCK promoter were made using the avidin-biotin complex DNA assay and a similar K_d of 4 nM was obtained for the combination of TR and rat liver nuclear extract [33]. Schmidt et al. [33] also reported that the affinity of TR and of the heterodimer of TR and rat liver nuclear extract was identical but that the capacity of TR binding was far greater in the presence of rat liver nuclear extract. Our results in combination with these previous observations suggest that *in vivo* TR β will probably bind to PEPCK-TRE as a heterodimer with RXR. We determined that another protein present in rat liver nuclei will also bind to PEPCK-TRE although not to the key nucleotides for TR β binding. As might be expected given the complex regulation of PEPCK gene expression, many elements in the PEPCK promoter such as CRE or the accessory factor 1 (AF1) sites have the ability to bind several proteins present in the nuclear extract [13,34].

TR has been shown to bind to three sites within the PEPCK promoter including the AF1 site (-450 to -435) [35], P3(I) and TRE. The AF1 site is essential for the induction of PEPCK transcription by glucocorticoids and retinoic acid [7,8], but does not contribute to T_3 stimulation of PEPCK transcription, as serial deletions of the PEPCK promoter that remove this sequence did not eliminate T_3 induction [10]. However, the AF1 site binds RAR, RXR, hepatic nuclear factor 4, PPAR and other unidentified proteins present in rat liver nuclear extract [8,34,36]. Since TR binds poorly to a direct repeat spaced by one nucleotide, it is more likely that this site will be bound by the high-affinity RAR-RXR or PPAR-RXR heterodimers or one of the other

proteins present in the liver nuclei. Although TR β can also bind to the P3(I) site, our experiments clearly show that the binding of TR to the P3(I) element is not necessary for T_3 response (Figure 6). Therefore only the binding of TR to TRE (-330 to -319) contributes to T_3 induction.

The P3(I) site plays a central role in liver-specific expression and cAMP responsiveness of the PEPCK gene [11,4]. Transgenic mice containing a chimaeric gene with the PEPCK promoter driving bovine growth hormone gene express high levels of the transgene in the liver, but mice containing this chimaeric gene with a mutated P3(I) site have reduced hepatic expression [14]. Only members of the C/EBP family and D-site-binding protein can bind to this site [13,37]. The P3(I) site also has a critical role in T_3 responsiveness. There have been previous reports of TR interacting with proteins bound to other sites in the promoter. Schaufele et al. [38] found that there was synergistic activation of transcription from the rat growth hormone promoter by TR and Pit-1 transcription factor [38]. Interestingly, this synergism was independent of T_3 . Voz et al. [39] demonstrated interactions between TR and Pit-1 on the human placental lactogen B promoter. These binding sites were immediately adjacent and the interaction diminished in a distance-dependent manner. In addition, the replacement of the Pit-1 recognition sequence with that of other nuclear factors did not eliminate the synergistic interaction. The interaction between the proteins bound to TRE and the P3(I) sites in the PEPCK promoter is unique in that it is ligand-dependent and that the binding sites are separated by 80 bp. It has been shown that C/EBP α can stimulate transcription through both the CRE and P3(I) sites [11]. However, C/EBP β enhances PEPCK transcription only through the CRE even though it binds the P3(I) site [13]. These observations suggest that TR may interact with C/EBP α or another member of the C/EBP family rather than C/EBP β .

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