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Two uniquely arranged thyroid hormone response elements in the far upstream 5' flanking region confer direct thyroid hormone regulation to the murine cholesterol 7 α hydroxylase gene

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ABSTRACT

Cholesterol 7 α hydroxylase (CYP7A1) is a key enzyme in cholesterol catabolism to bile acids and its activity is important for maintaining appropriate cholesterol levels. The murine CYP7A1 gene is highly inducible by thyroid hormone *in vivo* and there is an inverse relationship between thyroid hormone and serum cholesterol. Eventhough gene expression has been shown to be upregulated, whether the induction was mediated through a direct effect of thyroid hormone on the CYP7A1 promoter has never been established. Using gene targeted mice, we show that either of the two TR isoforms are sufficient to maintain normal hepatic CYP7A1 expression but a loss of both results in a significant decrease in expression. We also identified two new functional thyroid hormone receptor-binding sites in the CYP7A1 5' flanking sequence located 3 kb upstream from the transcription start site. One site is a DR-0, which is an unusual type of TR response element, and the other consists of only a single recognizable half site that is required for TR/retinoid X receptor (RXR) binding. These two independent TR-binding sites are closely spaced and both are required for full induction of the CYP7A1 promoter by thyroid hormone, although the DR-0 site was more crucial.

INTRODUCTION

Cholesterol 7 α hydroxylase (CYP7A1) catalyzes the initial and rate-limiting step in the neutral synthetic pathway of bile acids from cholesterol. Because the bile acid synthetic

pathway is a major route to remove excess cholesterol from the body, CYP7A1 is considered an important enzyme in cholesterol homeostasis (1). CYP7A1 is exclusively expressed in the liver and the gene is subject to metabolic regulation by oxysterols, bile acids, hormones, nutrients and cytokines. In mice and rats, CYP7A1 is activated by cholesterol excess through by product oxysterols that function as ligand agonists for the liver X receptor (LXR)/retinoid X receptor (RXR) heterodimer which binds to direct repeats of half sites separated by 4 nt (DR-4, LXRE) in the CYP7A1 promoter, increasing expression of CYP7A1 (2–4). Conversely, bile acids inhibit CYP7A1 gene expression through a negative feed back mechanism operating through several molecular pathways. In one pathway, bile acids activate the farnesoid X receptor (FXR), which in turn induces expression of the small heterodimer partner (SHP). SHP binds to and interferes with the activity of the α 1-fetoprotein transcription factor (FTF-1, also called LRH-1), leading to an inhibition of CYP7A1 expression (5,6). Hepatocyte nuclear factor 4 (HNF-4) has also been shown to mediate bile acid-induced repression of CYP7A1 (7). In fasted mouse livers and in type I diabetic mice, PPAR- γ -coactivator one alpha (PGC-1 α) plays an important role in activating CYP7A1 gene expression (8). Additionally, CYP7A1 expression is regulated by thyroid hormone through a direct effect on gene transcription (9–11).

Thyroid hormone mediates its action through the thyroid hormone receptor (TR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors (12,13). There are two major isoforms of TR generated by different genes, TR β and TR α . Each isoform exhibits a distinct pattern of tissue and developmental expression and there are multiple transcripts from each TR gene. TR β is the primary isoform in the liver (14). TR binds to specific DNA sequences, TR response elements (TREs), as monomers, homodimers, or with RXR in a heterodimer. Since RXR enhances the binding affinity of TR to TRE, TR/RXR heterodimers have been suggested to be major protein complexes that mediate thyroid

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hormone responses *in vivo* (12,15). Through the use of synthetic DNA and *in vitro* DNA-binding studies, a high affinity consensus element for TR was determined to be the DR-4 motif (16). However, naturally occurring TREs diverge significantly from this consensus and many consist of different orientations and configurations of repeats of the nuclear receptor half site AGGTCA half site. This can vary from a single half site (17) to a DR-0 (18), palindromes (17,19) and multiple separate and variably spaced direct repeats (20,21).

The association of hypothyroidism with hypercholesterolemia was first recognized in 1930 (22,23). This thyroid hormone effect is thought to be through direct regulation of target genes of cholesterol metabolism at the transcriptional level (9–11). Cholesterol homeostasis is maintained through cooperative regulation of cholesterol uptake and *de novo* synthesis together with cholesterol catabolism to bile acids (1). Accordingly, our previous studies have shown that thyroid hormone directly up-regulates expression of sterol regulatory element binding protein-2 (SREBP-2), which in turn increases expression of low density lipoprotein receptor (LDLR), resulting in a decrease in plasma cholesterol levels (24).

Cholesterol catabolism is also modulated by thyroid hormone primarily through changes in CYP7A1 mRNA levels. CYP7A1 mRNA is induced rapidly within 1 h of triiodothyronine (T3) treatment in hypophysectomized rats (10,11) and T3 treatment also increases the rate of CYP7A1 gene transcription (9). This rapid induction suggests that the increase in CYP7A1 mRNA may be directly mediated by thyroid hormone at the transcriptional level. In addition, induction of CYP7A1 expression by T3 was blunted in TR β knockout mice (25) and knock-in mice where a mutant TR β was inserted that has a defect in ligand binding (26). These studies argue strongly for a direct action of TR on CYP7A1. Indeed, protein–DNA-binding studies have shown direct binding of TR/RXR to the proximal DR-4 of the CYP7A1 promoter which has been characterized as an LXRE (26). However, whether this proximal site or any other TR-binding site(s) in the promoter is responsible for TR responsiveness has not been established. In the current study, we have identified two closely spaced TREs in the far upstream region of the CYP7A1 promoter that are responsible for the CYP7A1 stimulation by T3. Both these TREs were required for full induction of T3 and unlike some TREs, these CYP7A1 T3 response elements do not respond to LXR signaling.

MATERIALS AND METHODS

Animal treatments

All animals used in this study were acquired and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. For studies at UC Irvine, the protocols were approved by the campus IACUC committee (approval 97-1545). For studies in Lyon, mice were housed, maintained and sacrificed with approval from the animal experimental committee of the Ecole Normale Supérieure de Lyon and in accordance with the 'Commission de Génie Génétique' (Agreement number 12837).

The 4-week-old B6129 male mice were obtained from Taconic and maintained on a 12 h light/dark cycle with free access to food and water. The mice were allowed to adapt to the new environment for 1 week before experiments. Thyroid hormone deficiency was induced by feeding a low-iodine diet supplemented with 0.15% propylthiouracil (PTU) (Harlan Teklad) for 3 weeks, as described previously (24). For the thyroid hormone-supplemented group, mice were given 1 μ g of T3 (ICN) per gram of body weight on the 18th day of the low-iodine diet with PTU by intraperitoneal injections daily for 4 days. The control group was fed *ad libitum* with normal chow diet. The mice were sacrificed between 8:00 and 10:00 a.m. Livers were removed and frozen in liquid nitrogen and stored at -80°C until RNA was isolated.

For the studies in TR knockout mice, 8- to 10-week-old TR $\alpha^{0/0}$ (27), TR $\beta^{-/-}$ (28), TR $\alpha^{0/0}$ /TR $\beta^{-/-}$ (28) and the respective wild-type control animals were used in this study. They were maintained on a 12 h day/12 h dark schedule (light on at 7 a.m.) and fed standard mouse chow and water *ad libitum*. For the experiments, animals were sacrificed at 2 p.m. after 4 h of starving. The liver was quickly removed and frozen in liquid nitrogen and used for RNA extraction.

RNA isolation and northern blot analysis

Total RNA was isolated from mouse livers using TRIzol (Invitrogen). Total RNA (20 μ g) from two to four individual animals per feeding condition was subjected to northern analysis with ^{32}P -labeled probes as described previously (24). Expression of ribosomal protein L32 was measured as a control to normalize signals from different lanes. The following cDNA probes were used: CYP7A1 (a gift from G. Gil, Virginia Commonwealth University), a 0.7 kb AccI/EcoRI fragment from the pBSK7a; 5'DI, a 0.8 kb BamHI/EcoRI from pCR2.1-mouse 5'DI, and a 80 base HindIII/EcoRI fragment of rat ribosomal protein L32 cDNA (24).

Cell culture and transient transfection assay

HepG2 cells were cultured at 37°C and 5% CO_2 in minimal essential medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Transient transfections were performed by the calcium phosphate co-precipitation method, as described previously (24). Briefly, cells were seeded in 6-well dishes at 350 000 cells/well one day before transfection. The next day (16 h later), cells were transfected with 2 μ g/well of the indicated CYP7A1 luciferase reporter and 2 μ g/well of cytomegalovirus β -galactosidase plasmid as an internal control for transfection efficiency. Expression vectors for CMX-hTR- β (2 μ g), CMX-LXR α (0.5 μ g) and CMX-hRXR α (0.5 μ g) were included as indicated in the figures. Equal amounts of DNA were used for all transfection reactions by adding empty vector DNA. After 4–6 h, cells were treated with 10% glycerol for 2 min, washed three times with phosphate-buffered saline (PBS), and refed with a serum-free medium supplemented with 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenite, 5 μ l/ml defined lipid mix and 0.1% de-lipidated BSA (Sigma) in the presence or absence of 1 μM 3,3'T3. Cells were incubated for additional 40 h and harvested for luciferase and β -galactosidase assays and normalized expression was determined as described

previously (24). Values represent the mean of duplicates \pm SD. Each experiment was repeated at least three times.

Plasmids

The region corresponding to $-7454/+59$ of the rat CYP7A1 promoter was obtained from pR7 α -Cat9 (a gift from G. Gil, Virginia Commonwealth University) by digestion with Sall and XbaI and inserted into the XhoI and NheI sites of pGL3 basic to generate pGL3R7 α -7454. pGL3R7 α -3640 was constructed by digestion of pGL3R7 α -7454 with SacI followed by re-ligation. To construct pGL3R7 α -1667, pGL3R7 α -3640 was digested with SacI and PstI, and blunted by T4 polymerase, and re-ligated. A series of 5'-deletion mutants of pGL3R7 α -3640 was constructed by PCR-based amplification. The following forward primers were used: pGL3R7 α -3382, 5'-GTGAACTTCTGTATGGGT-3'; pGL3R7 α -3132, 5'-TGGTATGCCAGGACTTTGGA-3'; and pGL3R7 α -3008, 5'-ACTTCAGTGCCACCATGCA-3'. The reverse primer for all was 5'-ACAAGTAGACTGCAAGGGGA-3'. The SacI site was added to the forward primer for cloning purpose. Because the PCR fragments include the SmaI site at -2770 bases, they were digested with SacI and SmaI and inserted into the SacI and SmaI sites of pGL3R7 α -3640 to generate pGL3R7 α -3382, pGL3R7 α -3132 and pGL3R7 α -3008. pGL3R7 α -3640mTRE1, pGL3R7 α -3132mTRE1, pGL3R7 α -3132mTRE2 and pGL3R7 α -3132mTRE1/2 were constructed by site-directed mutagenesis (QuikChange, Stratagene). The sequences of one strand of the complementary primers are shown below: mTRE1, 5'-CAATAATAACCCTGTCTTTTCAAAGCATCTATCTGTACTGCTGC-3'; and mTRE2, 5'-CTGCTGCAATAGAAAC-TCCACAGGTCAAAATCACAGCTGTTGTGT-3'.

To generate pGL3R7 α -3132/342, a fragment from -3132 to -3008 was produced by PCR. The forward primer was the same as the one for pGL3R7 α -3132. The reverse primer was 5'-AATCCTGGGACACTGTGTA and included the XbaI site for cloning purpose. The PCR fragment was digested with SacI and XbaI and inserted into the SacI and NheI sites of pGL3R7 α -342. CMX-hTR- α and CMX-hTR- β were from Dr Bruce Blumberg, University of California, Irvine, and CMX-hRXR- α and CMX-LXR α were from Dr Peter Tontonoz (UCLA).

Electrophoretic mobility shift assays

Human TR α , TR β and RXR α proteins were synthesized using the TNT-coupled transcription/translation system (Promega). The sequences of one strand of the complementary oligonucleotide probes are as follows: wild-type TRE1, 5'-AACCTGTCTTTTCAGGGCATCTATCTGTACTGCTGCAATAGAAA-3'; mutant TRE1, 5'-AACCTGTCTTTTCAAAGCA TCTA TCTGTA CTGCTGCAATAGAAA-3'; wild-type TRE2, 5'-AATAGAAACTCCACAGGTCA GGGTCA CAGCTGTTGTGTTTACACA-3'; mutant TRE2, AATAGAAACTCCAC AGGTCAAAAATCACAGCTGTTGTGTTTTACACA-3'; and DR-4, 5'-CTAGAGCTTCAGGT-CACAGGAGGTCAGAGACT-3'. The complementary oligonucleotides were annealed, and where indicated, they were labeled with [γ - 32 P]ATP by T4 polynucleotide kinase. Protein-DNA-binding assays were performed as described previously (24). For competition experiments, unlabeled

probes were included in the binding buffer at the concentration indicated in the legend to Figure 3B and C.

Chromatin immunoprecipitation assays (ChIP)

Rat hepatoma H4IIE cells were grown to 70% confluence in minimum essential medium supplemented with 10% FBS at 37°C and 5% CO₂. Nuclear proteins were crosslinked to DNA by adding formaldehyde to the culture medium to a final concentration of 1% for 5 min at room temperature. Crosslinking was stopped by adding glycine to a final concentration of 0.125 M for 10 min. Cells were washed three times and collected in ice-cold PBS supplemented with protease inhibitors. Cell pellets were resuspended in lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl, 0.5% NP-40 and protease inhibitors) and homogenized with five strokes of a dounce homogenizer with a B pestle to release nuclei. After centrifugation at 313 \times g for 10 min at 4°C, nuclear pellets were resuspended in nuclear lysis buffer (50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1% SDS and protease inhibitors) and sonicated for 5 min with 30 s on/off intervals to reduce the size of chromatin to \sim 500 bases in length (monitored by gel electrophoresis). Lysates were diluted 1:20 in IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 7.6, 167 mM NaCl and protease inhibitors). Crosslinked chromatin samples were precleared by adding salmon sperm DNA-protein G agarose slurry and mouse IgG for 2 h at 4°C with rotation. Supernatants were recovered and incubated with 5 μ l of anti-TR β (Affinity BioReagents, Golden, CO) or mouse IgG overnight at 4°C with rotation. The immune complexes were then mixed with salmon sperm DNA-protein G agarose slurry for 2 h at 4°C with rotation. The beads were collected by microspin column and washed sequentially with the following buffers: buffer B (20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.05% SDS, 1% Triton X-100 and 150 mM NaCl), buffer D (20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 0.05% SDS, 1% Triton X-100 and 500 mM NaCl), buffer 3 (10 mM Tris-HCl, pH 7.6, 250 mM LiCl, 1 mM EDTA, 1% NP-40 and 1% deoxycholate) and IP wash buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA and 1% Triton X-100). The immune complexes were eluted with IP elution buffer (40 mM sodium bicarbonate and 1% SDS). After reversing the protein-DNA crosslinks in the chromatin, DNA was recovered by Qiagen PCR purification kit and used as a template for quantitative PCR with an I-cycler (Bio-Rad). Supernatants from immunoprecipitation with IgG were saved and used to prepare control samples representing the unenriched starting material or 'input' DNA. A series of dilutions of input DNA with known concentrations (OD₂₆₀) was used as a template to monitor the efficiency of quantitative PCR and to quantify DNA crosslinked to TR protein. The following primers were used for quantitative PCR: rCYP7A1 TREs forward 5'-AAACTCAACTTGGTATGCCAGGAC-3'; rCYP7A1 TREs reverse 5'-TCACACACATGCACACAAGCAC-3'; rCYP7A1 DR-4(LXRE) forward 5'-AGCACATGAGGGACAGACCTTCAG-3'; rCYP7A1 DR-4(LXRE) reverse 5'-TGCACAGACCATGATCCAATAAC-3'; rYY1 exon 4 forward 5'-GCTGCACAAAGATGTTTCAGGGATAA-3'; and rYY1 exon 4 reverse 5'-CTGAAAGGGCTTTTCTCCAGTATG-3'.

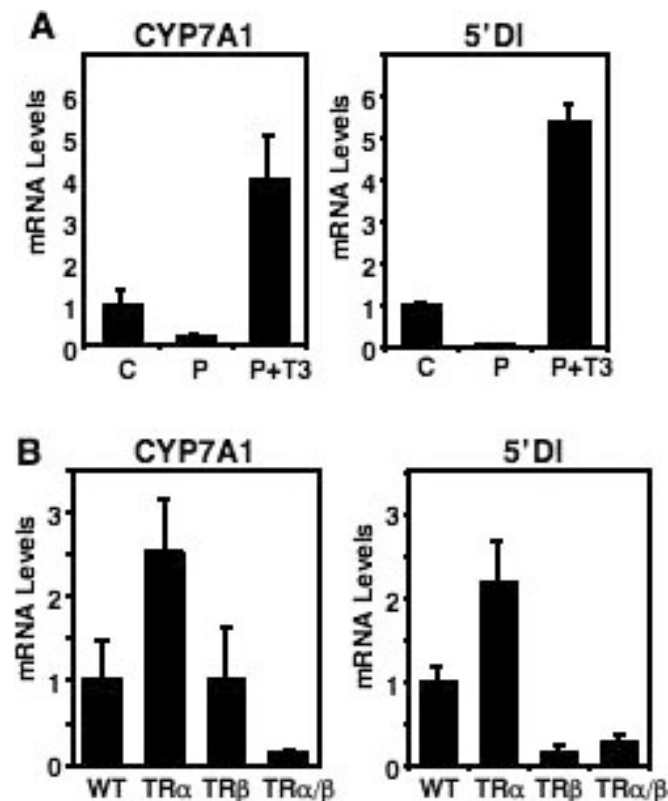


Figure 1. Thyroid hormone regulation of the CYP7A1 gene. (A) Total RNA was isolated from livers of mice that were fed a normal chow (C), an iodine-deficient diet supplemented with PTU (P) or an iodine-deficient diet supplemented with PTU and injected with T3 (P+T3). Equal amounts (20 μ g) of total RNA from individual animals was loaded in separate lanes and analyzed by northern analysis to measure mRNA levels for CYP7A1 and 5'DI. Signals were quantified using Quantity One software from Bio-Rad using densitometric scans from autoradiograms, and the intensities were normalized relative to the control ribosomal protein L32 mRNA for each lane. Results are expressed as a fold change relative to the value from the control chow-fed animals. The mean values obtained from individual measurements from six animals in each group are shown with error bars. (B) Total RNA was isolated from livers of WT, TR α (0/0), TR β (-/-) and TR α (0/0)/TR β (-/-) that were fed a normal chow.

RESULTS

Thyroid hormone induces CYP7A1 mRNA *in vivo*

Expression of the murine CYP7A1 gene fluctuates in response to thyroid hormone status (9–11). This is demonstrated in Figure 1A where CYP7A1 mRNA was barely detectable in livers of thyroid hormone depleted mice but was significantly induced when T3 was added back after the depletion. The 5' deiodinase gene, a well-known target of thyroid hormone (29), displayed a similar response to T3. As the mice were treated with supraphysiological doses of T3 to induce hyperthyroid conditions (30) mRNA levels for CYP7A1 and 5' deiodinase in mice treated with T3 were hyperactivated relative to control mice. To investigate the regulation of CYP7A1 by T3 further we evaluated expression in knockout mice that totally lack each or both of the TR isoforms. When these animals were fed normal chow diets without manipulating the thyroid hormone content, CYP7A1

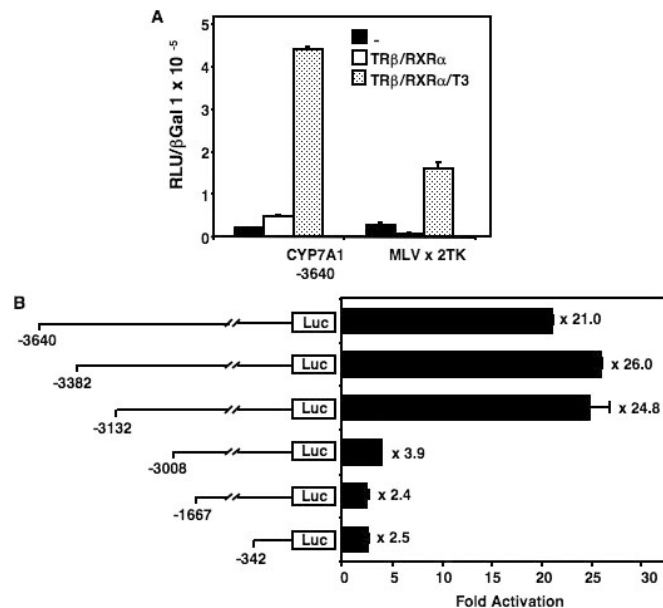


Figure 2. CYP7A1 promoter is activated by TR in response to T3. (A) HepG2 cells were transfected with the indicated promoter–luciferase fusion construct and where indicated expression vectors were added for TR β and RXR α . Cells were cultured in serum-free minimal medium and 1 μ M T3 was added as indicated and described in Materials and Methods. Results are expressed as corrected luciferase light units divided by the internal control signal for β -galactosidase activity. (B) Similar experiments were performed in HepG2 cells with the indicated promoter–luciferase fusion construct along with expression vectors for TR β and RXR α . The fold (x) change in the promoter activity by T3 relative to cells transfected with each luciferase reporter alone is shown beside each bar. The data from (A) and (B) represent the mean of duplicates for three individual experiments and include error bars (SEM). RLU, relative light units.

mRNA was elevated in livers from TR α knockout mice and only when both TR receptors are disrupted was there a notable reduction in basal CYP7A1 mRNA (Figure 1B, $P = 0.026$). In contrast, expression of the 5' deiodinase gene was sensitive to the specific loss of the TR β isoform. These results indicate that either TR isoform can activate CYP7A1 whereas the 5' deiodinase gene is preferentially activated by TR β (Figure 1B).

Taken together with previous studies, these results suggest that CYP7A1 is a direct thyroid hormone target gene; however, a T3 response element had not been located previously. Therefore, a fragment of the 5'-flanking region of the rat CYP7A1 promoter from -3640 to +59 (pGL3R7 α -3640) was fused to the luciferase reporter gene and tested for T3 responsiveness in co-transfection assays in HepG2 cells. The HepG2 cell-based co-transfection assay has been established to evaluate T3 regulation of other genes (24). In this assay, expression vectors for TR β and RXR α were co-transfected with pGL3R7 α -3640 into HepG2 cells that were subsequently cultured in the presence or absence of T3 and the activity of the reporter gene was measured. As shown in Figure 2A, T3 treatment resulted in a 21-fold increase in the activity of pGL3R7 α -3640. In contrast, when T3 was added without the expression constructs or if the TR β and RXR α expression vectors were added without T3, there was only minimal promoter activity. As a control, the activity

of murine leukemia virus luciferase (MLVLuc) reporter, a known T3 responsive promoter (31), was also activated significantly in response to T3 in these studies. These data suggest that the CYP7A1 promoter is T3 responsive and that TRE(s) directly mediating the response are located within the 3640 bases flanking the 5' end of the transcription start site.

To identify the TRE(s), a series of progressive 5' deletions of the promoter were prepared and tested for T3 responsiveness as above. Truncation from -3640 to -3382, or to -3132 caused no significant change in T3 responsiveness (Figure 2B). However, deletion to -3008 resulted in a marked decrease in T3 activation from 24.8- to 3.9-fold. Further deletion down to -1667 showed a modest drop of T3 activation to 2- to 3-fold of the basal promoter activity observed in pGL3R7 α -342. A modest increase in promoter activity by 2- to 3-fold has been shown for the empty pGL3-basic vector in the presence and absence of T3 (24) and was not considered to represent specific T3 response. These results suggest that a T3 responsive region is located at 3000 bp 5' to the CYP7A1 mRNA start site.

Identification of thyroid hormone receptor-binding sites in the CYP7A1 promoter

Using synthetic response and binding elements, it was shown that TR preferentially binds to a site containing two direct repeats of hexameric half sites of AGGTCA spaced by 4 nt. However, naturally occurring TR-binding sites are diverse with respect to the sequence and orientation of the half sites, and the number of nucleotides in the spacer (12,15). When we scanned the -3000 region of the promoter for nuclear receptor half sites, two DR motifs were found: one consisting of a single half site, designated TRE1, and the other consisting of a single half site, designated TRE2 (Figure 3A). Importantly, although the sequence of the mouse and rat promoters diverge significantly in this region of the promoter, the TRE1 is moderately conserved and TRE2 is exactly identical.

To evaluate whether these putative TREs directly bind TR, gel shift assays were performed using ³²P-labeled oligonucleotides containing either TRE1 or TRE2 with *in vitro* translated TR α , TR β and RXR α protein. Heterodimers of both TR α /RXR α or TR β /RXR α formed complexes with

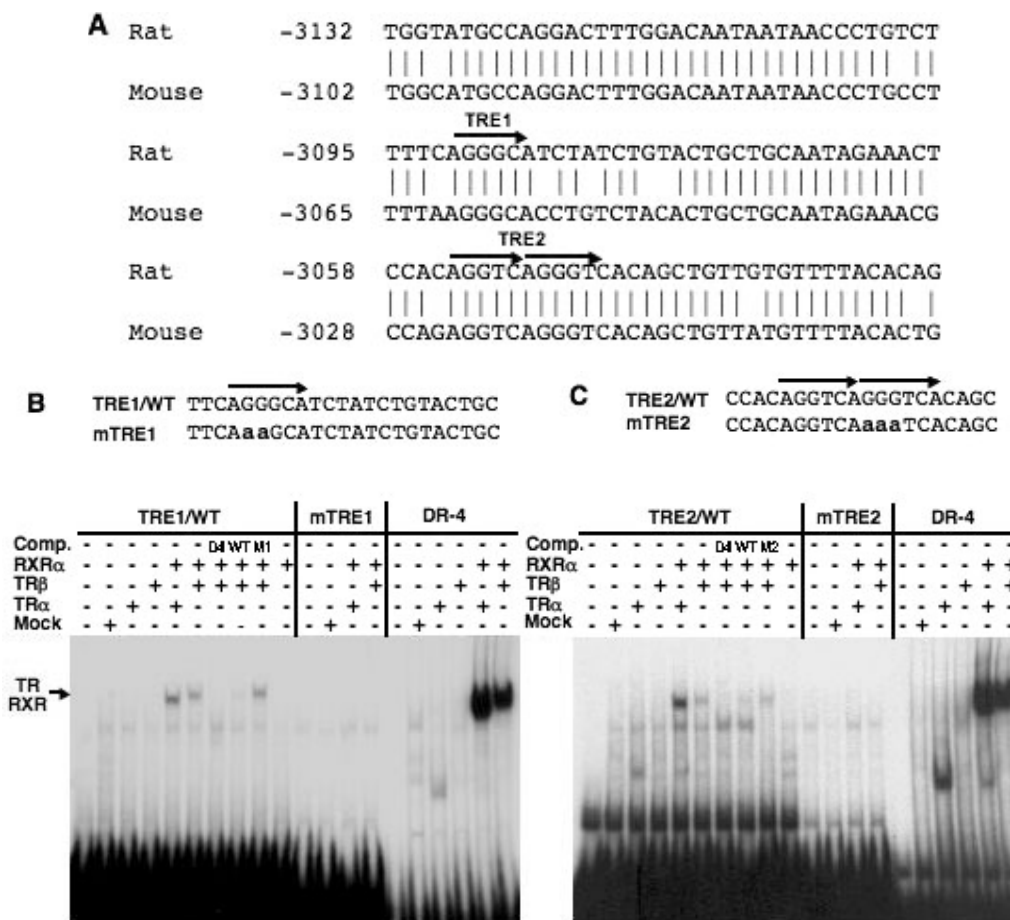


Figure 3. Identification of TR/RXR-binding sites in the CYP7A1 promoter. (A) The 5'-flanking sequence alignment of the rat and mouse CYP7A1 promoter is shown. Arrows indicate putative TREs. (B) In the upper panel, the sequence of the wild-type TRE1 is presented as TRE1/WT. The sequence of mutations in TRE1 is indicated with lower case lettering with mTRE1. The full sequences for the oligonucleotide probes are detailed in Materials and Methods. An autoradiogram from a representative gel shift assay is shown in the lower panel. ³²P-labeled probes were incubated with *in vitro* translated TR α , TR β and RXR α , as indicated. Where indicated, a 100-fold molar excess of the indicated unlabeled probe (Comp.) was included in the binding reactions with the labeled probe. D4 denotes the consensus DR-4. WT and M1 denote the wild-type TRE1 and mutant TRE1, respectively. The arrow denotes the position of specific protein-DNA complexes. (C) Similar experiments were performed for TRE2 as described in (B).

oligonucleotide probes containing either TRE1 and TRE2 (Figure 3B and C, lanes 5 and 6). A 100-fold molar excess of unlabeled wild-type TRE1 or a consensus TRE, DR-4 (32), efficiently competed out TR β /RXR α for binding to TRE1 (lanes 7 and 8). A 2-base mutation in the 5' half site of either TRE disrupted binding of TR β /RXR α with DNA (lanes 13 and 14). Consistently, unlabeled mutant probe (mTRE1 or mTRE2) was not able to compete out TR β /RXR α for the binding to the respective TRE (lane 9) and similar protein–DNA complexes were observed for the consensus DR-4 site that was used as a positive control. Taken together, these results suggest that TR/RXR binds independently to both TRE1 and TRE2.

TRE1 and TRE2 are responsible for the TR response of CYP7A1

To determine whether TRE1 or TRE2 contribute to the T3 stimulation of the CYP7A1 promoter, point mutations identical to the bases mutated in the gel shift assays were introduced into either the TRE1 or TRE2 or both simultaneously in the pGL3R7 α -3640 and pGL3R7 α -3132 reporter plasmids by site-directed mutagenesis. The mutant constructs were then examined for T3 responsiveness as described in Figure 2. A mutation in TRE1 of pGL3R7 α -3640 caused a 61% decrease in T3 response (Figure 4A), compared to its respective wild-type promoter, but it retained a significant amount of the T3 response. Similarly, mutation of TRE1 of pGL3R7 α -3132 reduced T3 mediated-promoter activity by

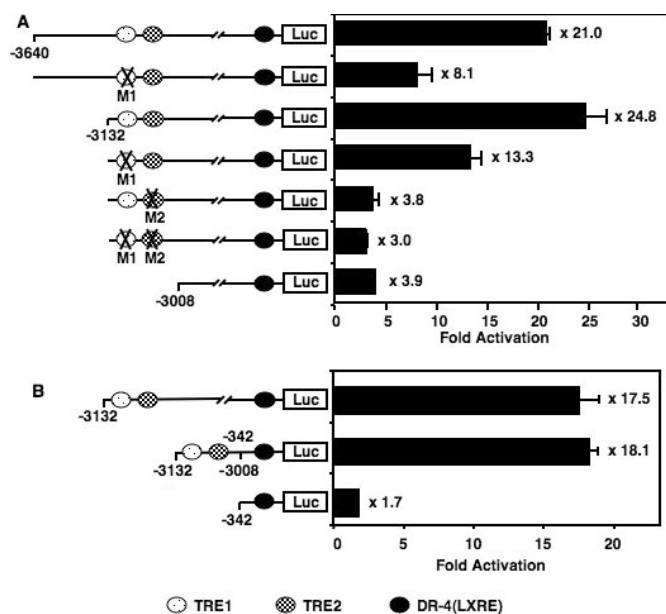


Figure 4. TRE1 and TRE2 are responsible for the TR response of CYP7A1. (A) Transfection assays with wild-type and the indicated mutant luciferase reporter constructs were performed in cultured HepG2 cells as described in the legend to Figure 3. The data represent the mean of duplicate samples for three individual experiments and include error bars. M1 and M2 denote mutants of TRE1 and TRE2, respectively, where the identical nucleotide substitutions used to disrupt TR binding in Figure 3B and C were introduced into TRE1 or/and TRE2 of the luciferase reporter constructs. (B) The sequence from -3132 to -3008 was fused to the -342 sequence of the truncated CYP7A1 promoter reporter construct and compared to the full-length and -342 truncated promoter construct for T3 responsiveness as described in (A).

46%, compared to its respective wild-type promoter. When a mutation was introduced into the TRE2, the mutant construct displayed almost no response to T3. The complete loss of T3 responsiveness was also observed for the TRE1/TRE2 double mutant. These data suggest that both of the TREs contribute to the T3 responsiveness, although TRE2 appears to be more critical.

To test the ability of these specific TREs to confer T3 stimulation to an otherwise non-responsive promoter the short region containing -3132 to -3008 that harbors both TREs was ligated to pGL3R7 α -342 to generate pGL3R7 α -3132/342. As shown in Figure 4B, addition of this small fragment containing both TREs to the -342 promoter resulted in an 18-fold increase in response to T3 which was similar to the full-length -3640 construct. As expected, expression from the simple pGL3R7 α -342 construct was insensitive to T3.

TRE1/TRE2 do not mediate an LXR response

Because TR and LXR both prefer binding to DR-4 elements (33) and because there is a DR-4 in the proximal murine CYP7A1 promoter that is responsive to LXR signaling (3), we evaluated whether these newly identified TREs might also contribute to LXR activation of CYP7A1. pGL3R7 α -3132/WT, pGL3R7 α -3132/mTRE1,2 or pGL3R7 α -3008/WT were co-transfected with expression vectors for LXR α and RXR α in the presence or absence of GW3695, a synthetic agonist for LXR. The results from these experiments were compared with those for T3 responsiveness of the same constructs. As shown in Figure 5, the potent stimulation by LXR was not affected by mutations in or loss of either TRE1 or TRE2.

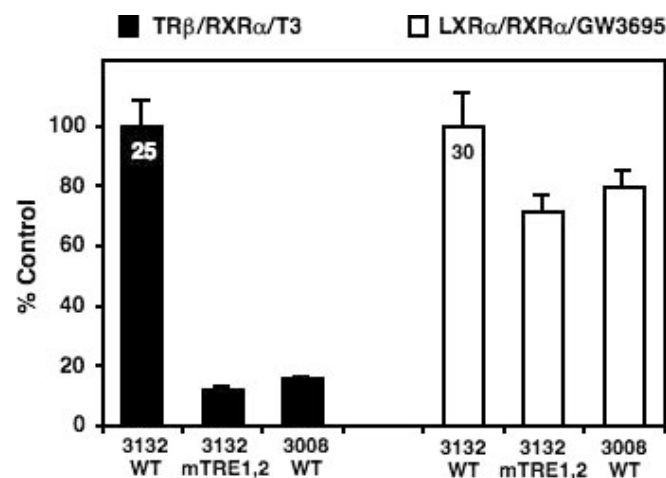


Figure 5. The TRE1/TRE2 do not mediate an LXR response. HepG2 cells were transfected with the indicated promoter–luciferase fusion construct and where indicated expression vectors for TR β /RXR α or LXR α /RXR α were also included. Cells were cultured in serum-free minimal medium in the presence or absence of 1 μ M T3 or 5 μ M GW3695. Results are expressed as normalized luciferase light units divided by β -galactosidase activity. The 25-fold activation by T3 for the pGL3R7 α -3132/WT construct was set to 100%. The magnitude of the T3 response of pGL3R7 α -3132/mTRE1,2 and pGL3R7 α -3008/WT is presented relative to that of pGL3R7 α -3132/WT. The 30-fold activation of pGL3R7 α -3132/WT by GW3695 relative to cells transfected with luciferase reporter alone was set to 100%. The magnitude of the GW3695 response of pGL3R7 α -3132/mTRE1,2 and pGL3R7 α -3008/WT was presented relative to that of pGL3R7 α -3132/WT. The data represent the mean of duplicates for three individual experiments and include error bars.

Recruitment of TR β into the TREs in H4IIE cells

To directly evaluate if TR β is capable of binding the TREs of the CYP7A1 promoter on the endogenous CYP7A1 gene in cellular chromatin, a ChIP study was performed in H4IIE cells. These cells have been used as a useful system to study regulation of gene expression by thyroid hormone (34) and has been used successfully to demonstrate that TR binds to TR target genes using the ChIP technique. Because TR has been shown to bind in chromatin in the presence or absence of its ligand (35), we made chromatin from H4IIE cells cultured under normal conditions without T3. After crosslinking with formaldehyde, aliquots were immunoprecipitated with anti-TR β or a control mouse IgG fraction and binding to specific chromatin sites was analyzed by a quantitative PCR protocol. As shown in Figure 6, TR β was efficiently recruited to the -3000 region of the CYP7A1 promoter (8-fold compared to a non-specific IgG control). Although the proximal DR-4 does not mediate a T3 response, TR was shown to be recruited to this proximal LXR responsive DR-4 in a previous study (26,36). Therefore, we also evaluated TR binding to the proximal promoter as well where a modest 2-fold binding enrichment of TR was observed. As a negative control, TR binding to a non-relevant region of the genome was also

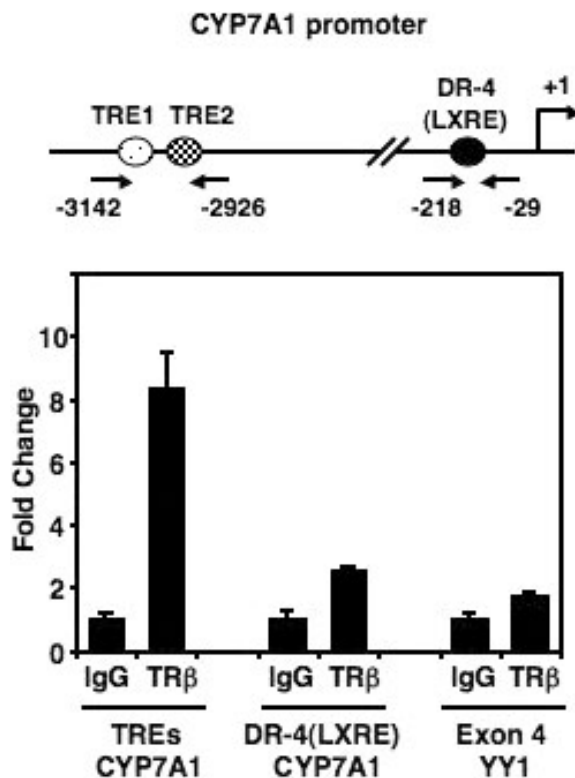


Figure 6. Recruitment of TR β to the CYP7A1 TREs in H4IIE cells. Chromatin was prepared from H4IIE cells grown under normal conditions. The ChIP assays were performed with anti-TR β as described in Materials and Methods. Immunoprecipitates were analyzed by quantitative PCR using primers that flanked the distal TREs or proximal DR-4 (LXRE) as indicated at the top of the figure. Results are expressed as a fold change in comparing the level of DNA amplification specifically precipitated by the TR- β antibody relative to that precipitated by a normal mouse IgG as control. The recruitment of TR β to a non-relevant region of the genome in the YY1 locus is shown as an additional negative control. The data represent the mean of triplicates for two individual experiments and include error bars.

evaluated and shown to be negative (the YY1 locus). These data demonstrate that TR β is specifically recruited to the -3000 TREs of the CYP7A1 promoter in cellular chromatin where the two TREs identified in our study are located.

DISCUSSION

Thyroid hormone influences many aspects of hepatic metabolism, and the rate controlling enzyme of neutral bile acid metabolism, CYP7A1, was shown to be activated at the transcriptional level by thyroid hormone *in vivo* in previous studies (9–11). However, no functional TRE(s) capable of conferring T3 responsiveness to CYP7A1 had been identified previously. Using transient transfection assays and DNA-binding studies, we have identified two TREs that are responsible for the T3 stimulation of the murine CYP7A1 gene. The two TREs are closely spaced and located in a far upstream region of the promoter at 3 kb upstream relative to the transcription start site.

TREs are generally found in the proximal region of promoters, as for the rat growth hormone (17,19), rat malic enzyme (20), myelin basic protein (37) and α -myosin heavy chain genes (38). However, there are some TREs that are located in a far upstream 5' region. For example, the TREs of the rat Spot 14 and chicken malic enzyme genes are located 2.7 and 3.8 kb upstream of the respective transcription start sites, respectively (39,40). These natural TREs are often clustered and arranged with multiple repeats of the half site, functioning synergistically or cooperatively. Similarly, the two closely spaced TREs are located 3 kb upstream from the CYP7A1 mRNA start site and are both necessary for full responsiveness to T3, although the TRE2 plays a more crucial role in T3 stimulation (Figure 4).

We also examined the recruitment of TR β to the TREs and DR-4 (LXRE) of the endogenous CYP7A1 in H4IIE cells. Our finding of greater recruitment of TR β to the -3 kb TREs compared to the proximal DR-4 (LXRE) is consistent with the data from transfection assays where the distal TREs mediate a strong T3 response. This would likely occur when the affinity of TR binding simply reflects the functional augmentation of the T3 response. It is also possible that LXR may be more abundant than TR in H4IIE cells such that the DR-4 (LXRE) may be preferentially occupied by LXR/RXR rather than TR/RXR. Regardless, the presence of the proximal DR-4 is not sufficient to provide a significant response to T3 stimulation.

We have also shown that either TR α or TR β appears sufficient for normal expression of CYP7A1 when animals are euthyroid and maintained on a chow diet (Figure 1). However, when both receptors are gone there is a significant decrease in CYP7A1 mRNA. In contrast, expression of the 5' deiodinase gene specifically requires TR β under these conditions. Thus, either TR isoform appears capable of sustaining basal CYP7A1 expression. It is important to note that earlier reports showed that TR β was key to activating CYP7A1 under dietary and pharmacological manipulation that dramatically alter thyroid hormone levels (25,41). There is a significant increase in CYP7A1 mRNA in the TR α knockout mice suggesting that this receptor may function as a negative regulator of CYP7A1 when TR β is also

present. Alternatively, this result may be due to other secondary effects that result from the loss of TR α .

Using synthetically designed promoter constructs, a consensus DR-4 element was shown to be a potent TRE (16). However, as mentioned above, TREs found in natural promoters are quite diverse in sequence, and the number and orientation of the individual half sites are variable (17–21). TRE1 contains only a mutation sensitive single TR half site. *In vitro* protein–DNA-binding assays in the present studies revealed that both of the TREs bind to TR/RXR heterodimers and no significant binding of monomers or homodimers of TR or RXR was detected (Figure 3). An extensive mutagenesis analysis failed to identify a crucial second half site for TRE1 (data not shown). The TRE2 is a DR0 which is similar to the TRE of the PEPCK (18). Although TR can bind to TRE as a monomer, homodimer or heterodimer with RXR (15,42–46), it is likely that the TR/RXR heterodimer binds to the TRE preferentially to mediate T3 response *in vivo* because both TR and RXR were required for efficient binding and both TR and RXR are required for activation as shown in other studies (18,21).

The two TRE's identified here in the rat CYP7A1 are conserved in the mouse promoter even though the surrounding sequence is not highly conserved, suggesting there is evolutionary pressure to preserve the T3 response in these two species. In an earlier study, expression of the human CYP7A1 gene was not stimulated by T3 (47). Additionally, the distal TR-binding sites we identified in the murine promoters do not seem to be conserved in the human gene and expression of a human transgene containing a large segment of 5' flanking DNA in mice was not regulated by T3 (48). Additionally, the proximal DR-4 (LXRE) is not conserved in the human gene (49) and if anything, the human CYP7A1 may in fact be negatively regulated by T3 (50) through a putative negative TRE at –227 to –247 in the promoter. Taken in total, the data suggest there are significant species differences in the effects of thyroid hormone on lipid metabolism that need to be explored further.

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