Identification of thyroid hormone response elements in the human fatty acid synthase promoter

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ABSTRACT To investigate the regulation of the human fatty acid synthase gene by the thyroid hormone triiodothyronine, various constructs of the human fatty acid synthase promoter and the luciferase reporter gene were transfected in combination with plasmids expressing the thyroid hormone and the retinoid X receptors in HepG2 cells. The reporter gene was activated 25-fold by the thyroid hormone in the presence of the thyroid hormone receptor. When both the thyroid hormone and the retinoid X receptors were expressed in HepG2 cells, there was about a 100-fold increase in reporter gene expression. 5'-Deletion analysis disclosed two thyroid hormone response elements, TRE1 (nucleotides −870 to −650) and TRE2 (nucleotides −272 to −40), in the human fatty acid synthase promoter. The presence of thyroid hormone response elements in these two regions of the promoter was confirmed by cloning various fragments of these two regions in the minimal thymidine kinase promoter—luciferase reporter gene plasmid construct and determining reporter gene expression. The results of this cloning procedure and those of electrophoretic mobility shift assays indicated that the sequence GGGTACGTCGTCA (nucleotides −716 to −731) represents TRE1 and that the sequence GGTGCC (nucleotides −117 to −112) represents TRE2. The sequence of TRE1 is very similar to the consensus sequence of the thyroid hormone response element, whereas the sequence of TRE2 contains only a half-site of the thyroid hormone response element consensus motif because it lacks the direct repeat. The sequences on either side of TRE2 seem to influence its response to the thyroid hormone and retinoid X receptors.

In animal tissues, the multifunctional enzymes acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthase (FAS; EC 2.3.1.85) are required for the de novo synthesis of fatty acids (1, 2). Acetyl-CoA carboxylase catalyzes the synthesis of malonyl-CoA from acetyl-CoA and HCO₃⁻ in the presence of ATP. FAS catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH (1, 2). Recently, we cloned the cDNA coding for the human FAS and determined its sequence (3). Analysis of this sequence showed that FAS protein sequences are conserved across species, suggesting that multifunctional eukaryotic FASs evolved from the monofunctional enzymes of FAS complexes of prokaryotic origin.

To understand the regulation of human FAS, we measured the FAS mRNA levels in various normal human tissues (3). The results showed that FAS mRNA exists at very low levels in most tissues and at very high levels in lipogenic tissues such as liver, lung, and brain (3). The regulation of animal FAS by diet and hormones is well established (1, 4). Feeding a carbohydrate-rich diet for 2 days to rats or chickens after livers of these animals before the enzyme is isolated (1, 2). Insulin and the thyroid hormone triiodothyronine (T₃) increase FAS transcription, whereas glucagon decreases it (4–8). T₃-induced gene expression involves activation of the thyroid hormone receptor (TR) to form a heterodimer with the retinoid X receptor (RXR). This heterodimer (TR/RXR) binds to the well-characterized thyroid hormone response elements (TREs) and promotes transcription (9–12). The consensus TRE sequence (A/G)GGTCANNN(A/G)GGTCA, called DR4, contains a direct repeat that is separated by four nucleotides (9). There are other sequence motifs that are known to function as well as TREs (10–12). These motifs include ER6 (TGCCITNNNNNNAGGCA) (10), a palindrome called IR0 (AGGTGATGAGCCT) (11), and an RII enhancer, specifically a DR4 half-site that lacks the direct repeat (12). Although regulation of gene expression by T₃ can be demonstrated in primary cell cultures of target tissues (4, 6), most of the cultured cell lines do not respond to T₃ because they lack TR. To achieve such a response, the cells must be transfected with the expression plasmids of these receptors (4).

We reported earlier that the human FAS gene is regulated by two promoters (13). Promoter I is 15 times stronger than promoter II, which is located in intron I (13). Herein, we show that only human FAS promoter I is regulated by T₃ and that two TREs are involved in this regulation.

MATERIALS AND METHODS

Materials. Cell culture media and Lipofectamine were purchased from Life Technologies (Grand Island, NY). Charcoal-treated fetal bovine serum was purchased from Cocalico Biologicals (Reamstown, PA). Ming-Jer Tsai (Department of Cell Biology, Baylor College of Medicine) provided the prSV-hTRβ and pRSV-mRXRβ plasmids for expressing human TR and mouse RXR, respectively, in animal cell lines under the control of the Rous sarcoma virus (RSV) promoter and the pT7/βhTRβ and pT7/βmRXRβ plasmids for in vitro transcription and translation of the same receptor proteins (14, 15). The minimal thymidine kinase promoter—luciferase reporter gene plasmid (TK-Luc) was a gift from Anne Gambel (Department of Cell Biology, Baylor College of Medicine). The mouse TRβ monoclonal antibodies, polyclonal RXRβ antibodies, and the double-stranded consensus TRE oligonucleotide DR4 were purchased from Santa Cruz Biotechnology. The sources of all the other chemicals, radioactive materials, and DNA-modifying enzymes were as described before (13).

Abbreviations: T₃, triiodothyronine; TRE, thyroid hormone response element; RXR, retinoid X receptor β; TR, thyroid hormone receptor β; TK-Luc, minimal thymidine kinase promoter—luciferase reporter gene plasmid; RSV, Rous sarcoma virus; DR4, direct repeat separated by four nucleotides; EMSA, electrophoretic mobility-shift assay.

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**Construction of Reporter Gene Plasmids.** The human FAS-luciferase reporter gene plasmids LC9, LC9-1, LC9-2, LC9-3, LC11, and LC204 were constructed as described previously (13). Plasmid LC9-6 was generated by cloning a PCR-derived fragment of human FAS (nucleotides –116 to +56) in the luciferase vector pXP-1. Plasmids TK–272/–40, TK–272/–67, TK–272/–116, and TK–870/–650, which contained fragments of human FAS promoter I in TK-Luc, were generated either by cloning appropriate PCR fragments from human FAS promoter I or by using available restriction sites. Plasmids TK-DR4, TK-TRE1, TK–121/–87, TK–128/–103, TK–133/–87, and TK—133/–107 were generated by cloning the appropriate synthetic oligonucleotides in TK-Luc by using blunt-end ligation.

**Cell Culture and Transfection.** HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. For the transfection experiments, the cells were cultured for 16 hr in DMEM containing 10% charcoal-treated serum and maintained in the same medium during and after transfection. The transfection procedures were performed according to the manufacturer’s recommendations by using 5 μl of Lipofectamine and the amounts of plasmid DNAs indicated below. Cells (2 × 10⁶) were seeded in each well of 6-well culture plates and transfected with Lipofectamine complexes containing 0.5 μg each of plasmids pRSV-hTRβ and pRSV-mRXRβ and the luciferase reporter gene constructs and 0.2 μg of pCMV-β-gal (β-galactosidase under the control of a cytomegalovirus promoter). The medium was changed after 16 hr, and T₃ was added to a final concentration of 10⁻⁶ M. The cells were harvested after 48 hr, and the β-galactosidase and luciferase activities were determined as described previously (13). For normalizing the transfection efficiencies, the luciferase activity was divided by the β-galactosidase activity and the reporter gene expression was expressed as relative luciferase units.

**In Vitro Transcription and Translation.** The pT7hTRβ and pT7 mRXRβ plasmids (2 μg each in a total volume of 20 μl) were used as templates to synthesize the TR and RXR proteins by using a reticulocyte transcription and translation kit (Promega). To ensure that nonradioactive receptor proteins have been synthesized, parallel transcription and translation reactions were performed with [³⁵S]methionine, and the radioactive proteins were analyzed by SDS/PAGE and detected by autoradiography. The reticulocyte lysates incubated in the absence of template DNA were used as controls in electrophoretic mobility-shift assays (EMSAs).

**EMSA.** The in vitro-synthesized TR and RXR (1 μl each) proteins were incubated for 15 min at room temperature with ³²P-labeled DNA templates (20,000 cpm) and 2 μg of poly(dI-dC) in 15 μl of a reaction mixture containing 10 mM Hepes (pH 7.9), 50 mM KCl, 1 mM MgCl₂, 1 mM DTT, 10% glycerol, and 10⁻⁶ M T₃. In competition assays, nonradioactive probes or DR4 oligonucleotides were added to the reaction mixture in 5- to 50-fold excess of the radioactive probe. To perform supershift analysis, either anti-TR or anti-RXR antibodies were added to the reaction mixture and incubated for an additional 10 min. The DNA–protein complexes that were formed were analyzed on a 5% polyacrylamide gel by using Tris-borate-EDTA buffer (45 mM Tris/44.5 mM borate/1 mM EDTA, pH 8.0) (16) at room temperature and were detected by autoradiography.

**RESULTS AND DISCUSSION**

**Human FAS Promoter I Responds to T₃.** We reported earlier that human FAS is regulated by two promoters (13). To determine which of the two promoters of the human FAS gene responds to T₃ in HepG2 cells, we performed transient transfection assays with the promoter I plasmid LC9 and the promoter II plasmid LC208 (13). As shown in Fig. 1, the

**Fig. 1.** Activation of human FAS promoter I by T₃. The luciferase reporter gene constructs LC9 (promoter I) and LC208 (promoter II) were constructed as described earlier (13) and were tested for their response to T₃ as described in Materials and Methods. HepG2 cells were transfected with LC9 or LC208 together with receptor plasmids pRSV-hTRβ and pRSV-mRXRβ alone or in combination. The pCMV-β-gal plasmid was cotransfected in all experiments, and the β-galactosidase activity obtained was used to normalize transfection efficiencies. To serve as a control, the pRSV plasmid that lacked the receptor coding sequences was transfected instead of the pRSV-hTRβ and pRSV-mRXRβ plasmids (represented as “none”).
luciferase reporter gene activity of neither promoter I nor promoter II was affected by T3, suggesting that HepG2 cells either do not have the T3 receptor or have it in limited amounts. When LC9 was cotransfected with pRSV-hTRb, the reporter gene response to T3 increased by 25-fold. On the other hand, when LC208 was cotransfected with pRSV-hTRb, the reporter gene response to T3 did not significantly increase (Fig. 1). Although LC9 did not respond to T3 when cotransfected with pRSV-mRXRb alone, its response to T3 increased by ~100-fold when it was cotransfected with both pRSV-hTRb and pRSV-mRXRb (Fig. 1). LC208 did not respond significantly to T3, even when the plasmid was cotransfected with both pRSV-hTRb and pRSV-mRXRb (Fig. 1).

These results indicated that human FAS promoter I, but not promoter II, is regulated by T3 and that this regulation is mediated by means of the TR/RXR heterodimer. Furthermore, in the absence of TR, reporter gene expression was reduced more in the cells transfected with LC9 and the TR-expression plasmid than in the cells transfected with LC9 alone (Fig. 1). This reduced expression probably results from the repression by TR of the basal promoter activity of TRE-containing promoters in the absence of T3 (17, 18). The 25-fold increase in reporter gene expression in the presence of T3 suggests that either RXR or some other TR partner present in HepG2 cells is responsible for this induction.

Localization of TRE Elements in Human FAS Promoter I

To identify the location of TREs in human FAS promoter I, several 5′-deletion constructs of the promoter I-luciferase reporter gene construct were tested for their expression in HepG2 cells. As shown in Table 1, all of the reporter gene plasmids except LC9-6 (−116) responded to T3 in the presence of TR or TR/RXR. In the absence of T3, TR reduced the basal promoter activity of all the reporter plasmids except LC9-6 (−116) (data not shown). These results suggested that LC9-6 (−116) lacks a functional TRE (17, 18). The 5′-deletion constructs, however, varied in their response to T3 in the presence of TR alone and in the presence of both TR and RXR. The reporter gene LC9-1 (−870) showed a response to T3 that was 4 times higher than that of LC11 (−1900) in the presence of TR (Table 1). The T3 response of LC11 (−1900) was stimulated another 7-fold when both TR and RXR were expressed in HepG2 cells, whereas LC9-1 (−870) showed only an additional 4-fold increase. These results suggested that the sequence upstream of nucleotide −870 influenced the TRE function of human FAS promoter I. Deletion of sequences between nucleotides −870 and −650 resulted in a drastic difference in the relative T3 response of the reporter gene activity in the presence of TR and TR/RXR (Table 1, LC9 and LC9-1). The reporter gene LC9-1 (−650), LC9-2 (−361), and LC9-3 (−272) showed only a 2- to 3-fold increase in T3 response in the presence of TR, as compared with the 21-fold increase in response observed with LC9, and an additional 10-fold increase in T3 response in the presence of TR/RXR (Table 1). The LC9-6 (−116) reporter gene showed no significant increase in T3 response in the presence of TR but showed a 4-fold increase in reporter gene expression when both TR and RXR were expressed in the HepG2 cells. These results suggested that various sequences in human FAS promoter I influence the binding of TR/RXR to TRE. Despite the differences in the way the 5′-deletion constructs respond to T3 in the presence of TR alone or in the presence of both TR and RXR, it appears that there are two TREs in promoter I. One of these TREs (TRE1) is located in the sequence between nucleotides −870 and −650, and the second one (TRE2) is located in the sequence between nucleotides −272 and +56.

The response of LC9-3 (−272), which contains TRE2, to T3 in the presence of both TR and RXR was ~20–30% of that of LC9 (−870), which contains both TRE1 and TRE2. Therefore, based on the 5′-deletion analysis, TRE1 is 2- to 3-fold more efficient than TRE2 in eliciting a T3 response in the presence of both TR and TXR.

To further confirm the existence of the two TREs in human FAS, we cloned the human FAS promoter segments that contain TRE1 (nucleotides −870 through −650) and TRE2 (nucleotides −272 through −40 and −272 through −67) in TK-Luc, thereby generating TK-Luc−870/−650, TK-Luc−272/−40, and TK-Luc−272/−67, respectively. As shown in Fig. 2, TK-Luc−870/−650 showed an increase in T3 response of about 25-fold in the presence of TR and of 75-fold in the presence of both TR and RXR. The T3 response of TK-Luc−272/−40 and TK-Luc−272/−67 increased 2-fold and 4-fold, respectively, in the presence of TR and 28-fold and 53-fold, respectively, in the presence of TR/RXR (Fig. 2). These results confirmed those obtained in the 5′-deletion analysis, namely that there are two T3 response elements in human FAS promoter I (Table 1).

The basal promoter activity of TK−272/−40 in the absence of receptor expression plasmids was found to be severalfold higher than that of TK−272/−67 (Fig. 2). The 27-bp sequence between nucleotides −67 and −40 is identical to the sequence that has been shown to contain the insulin response element in the rat FAS promoter (8, 13). However, we did not observe insulin-mediated promotion of the T3 response when we transfected HepG2 cells with any of the reporter gene constructs we used (Table 1) or with TK-Luc−272/−40 (data not shown). The insulin response element present in the 27-bp sequence is an E-box motif (CAGTGGA at nucleotide −57) and binds trans-activating factors (USF1 and USF2) that contains the basic helix-loop-helix motif (19). It is possible, however, that some other trans-activating factor that binds to this sequence enhances the basal promoter activity of the TK−272/−40 construct (Fig. 2).

EMSAs Using TR and RXR Proteins. These assays were performed with in vitro-synthesized TR and RXR proteins as described under Materials and Methods. The reticulocyte lysate or the lysate containing only TR did not retard the electro-

Table 1. Identification of TREs in human FAS promoter I

<table>
<thead>
<tr>
<th>Construct</th>
<th>RLU +T3</th>
<th>TR +T3</th>
<th>TR + RXR + T3</th>
<th>Relative T3 response</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC11 (−1900)</td>
<td>3,298 (±9%)</td>
<td>13,276 (±5%)</td>
<td>99,569 (±9%)</td>
<td>25</td>
</tr>
<tr>
<td>LC9 (−870)</td>
<td>2,496 (±5%)</td>
<td>52,902 (±15%)</td>
<td>221,837 (±8%)</td>
<td>40</td>
</tr>
<tr>
<td>LC9-1 (−650)</td>
<td>6,375 (±12%)</td>
<td>14,889 (±2%)</td>
<td>134,999 (±5%)</td>
<td>23</td>
</tr>
<tr>
<td>LC9-2 (−361)</td>
<td>5,263 (±11%)</td>
<td>18,839 (±20%)</td>
<td>130,694 (±12%)</td>
<td>28</td>
</tr>
<tr>
<td>LC9-3 (−272)</td>
<td>7,356 (±1%)</td>
<td>13,359 (±7%)</td>
<td>140,490 (±1%)</td>
<td>36</td>
</tr>
<tr>
<td>LC9-6 (−116)</td>
<td>282 (±13%)</td>
<td>289 (±19%)</td>
<td>1,409 (±1%)</td>
<td>12</td>
</tr>
</tbody>
</table>

A series of 5′ deletions were tested for their response to T3. The experimental conditions are as described in the legend to Fig. 1. The relative luciferase activities (RLU) obtained in the presence of T3 are shown. The numbers in parentheses indicate the standard deviation. In HepG2 cells not transfected with the reporter plasmids, all of these constructs showed similar relative luciferase units in the presence or absence of T3.
phoretic mobility of the 32P-labeled 220-bp human FAS promoter fragment (−870 through −650) containing TRE1 (Fig. 3). Similar results were obtained by using RXR alone (data not shown). When the DNA probe was incubated with a mixture of lysates containing TR and RXR, however, a slow-moving band (Fig. 3) was observed. The unlabeled probe fragment competed for this complex formation (slow-moving band) and essentially eliminated it at a 50-fold excess of the radioactive probe (Fig. 3). The electrophoretic mobility of the slow-moving band was further retarded when anti-hTR antibodies were added to the reaction mixture after the receptor protein-DNA complexes (supershifted bands, Fig. 3) had been formed. This supershifting of the TRE-receptor complexes confirmed that these complexes do contain TR.

To determine the location of TRE1, we used several synthetic double-stranded oligonucleotides (≈30 bp each), which covered the entire nucleotide sequence of −870 through −650, as unlabeled competitors for the 32P-labeled 220-bp human FAS promoter fragment (−870 through −650) in an EMSA. Based on these experiments (data not shown), we found that a double-stranded oligonucleotide with the sequence GGGTCA (nucleotides −747 through −713) competed with the radioactive probe. The complementary strand of this oligonucleotide (CCCGGTTACTGCGTCATCGCAGCGCATCCCC; nucleotides −713 through −747) contained a TRE motif (GGGTTACTGCAGCGCATCCCC) that is similar to the consensus TRE sequence (A/GGTCA(N)NNA/GGTCA) (9), which has a direct repeat separated by four nucleotides (DR4). In EMSAs, the double-stranded oligonucleotide (−747 through −718) that contained TRE1 was found to behave like a TRE (Fig. 4A). TR and RXR are both required for the mobility shift of this fragment (Fig. 4A). In addition, the consensus DR4 double-stranded oligonucleotide A/GGTCA(N)NNA/GGTCA competed effectively with the binding of the probe to the receptor proteins (Fig. 4A). Because the complementary sequence −716 through −731 is very similar to the DR4 consensus sequence we considered this sequence to be TRE1.

When we used a truncated synthetic double-stranded oligonucleotide probe that contained the −747 through −718 sequence instead of the −747 through −713 sequence (Fig. 4A), none of the lanes contained a shifted band (Fig. 4B). We considered the faint band present in all the lanes, except those to which the nonradioactive competitor was added, to be nonspecific because it was present even in the first lane, which contained no receptor proteins (Fig. 4B). These results suggest that in the complementary sequence GGGTACTGCGGTCA (nucleotides −716 through −731) the first two G residues are critical for the binding of the receptor to this TRE.

To further confirm our findings, we constructed TK-TRE1, which contained nucleotides −747 through −713. TK-TRE1 showed a 10-fold response to T3 in the presence of TR and a 40-fold response in the presence of both TR and RXR (Fig. 5). A TK-DR4 construct, which contained the consensus DR4 sequence, showed about a 4-fold response to T3 in the presence of TR and a 25-fold response in the presence of TR and RXR. These results confirmed that this sequence functions in vivo as TRE and that its activity is comparable with that of the consensus TRE construct TK-DR4 (Fig. 5).
appreciable T3 response, we concluded that the half-site 2 of both T3 and TR and only an additional 2.5-fold increase in downstream of this TRE in TK-Luc vector. As shown in Table 1, we determined the location of TRE2 to be between nucleotides 121 and 117 and the other between nucleotides 117 and 112. Because the 5’-deletion construct LC9-172, which contained sequences from nucleotides −172 through +56, showed an appreciable T3 response, we concluded that the half-site sequence at nucleotide −117 may function as TRE2 and not the one located at nucleotide −174 (data not shown). To confirm the functionality of the TRE half-site located at nucleotide −117, we cloned several fragments upstream and downstream of this TRE in TK-Luc vector. As shown in Table 2, all the TK constructs that contained TRE2 exhibited a 60- to 80-fold increase in reporter gene expression in the presence of both T3 and TR and only an additional 2.5-fold increase in the presence of both TR and RXR. The constructs TK−121/−87 and TK−133/−107 showed a similar T3 response, suggesting that TRE2 is located between nucleotides −121 and −107 (Table 2). The TRE2 motif located in this region at nucleotide −117 is similar to the R1 enhancer, a half-site TRE of the major histocompatibility complex I genes that activates gene expression in response to retinoic acid (12). The results shown in Table 2 and in Fig. 2 also suggest that sequences upstream of nucleotide −133 on the 5′ side and downstream of nucleotide −87 could influence the way TRE2 responds to T3 and its receptors.

To determine whether this half-site TRE sequence binds the TR/RXR heterodimer and whether the neighboring sequences influence the binding of TR/RXR, we performed an EMSA using a double-stranded oligonucleotide (CGCGCGGCGCGCCGCGTCCCGGGGGGGGCGAGCCCGACGC-TCATTGG) as a labeled probe and various mutant oligonucleotides as nonradioactive competitors. The probe fragment was shifted (i.e., retarded) only in the presence of both TR and RXR. Adding anti-hTRβ antibodies caused a supershift. The unlabeled probe oligonucleotides and the DR4 oligonucleotides competed for the binding of the TR/RXR complex to the labeled probe (Fig. 6). Among the mutant oligonucleotides, the one containing AAATCC instead of GGGTCC did not compete for the binding of TR/RXR, suggesting that the first three Gs in the sequence GGGTCC of TRE2 are critical for TRE2 function (Fig. 6). This result is consistent with the observation that LC9-6 (−116), a FAS promoter reporter gene construct that lacks the first G of the GGGTCC sequence, did not respond to T3 (Table 1). When the five nucleotides within the immediate 5′ or 3′ portion of the GGGTCC sequence in

Determining the Location of TRE2. Based on the 5′-deletion analysis (Table 1) and the TK-Luc cloning procedures (Fig. 2), we determined the location of TRE2 to be between nucleotides −272 and −67. Although we could not identify a typical DR4-type TRE sequence in this region, we found two half-site sequences that both contained GGGTCC. One of the half-site sequences is located between nucleotides −174 and −169 and the other between nucleotides −117 and −112. Because the 5’-deletion construct LC9-172, which contained sequences from nucleotides −172 through +56, showed an appreciable T3 response, we concluded that the half-site sequence at nucleotide −117 may function as TRE2 and not the one located at nucleotide −174 (data not shown). To confirm the functionality of the TRE half-site located at nucleotide −117, we cloned several fragments upstream and downstream of this TRE in TK-Luc vector. As shown in Table 2, all the TK constructs that contained TRE2 exhibited a 60- to 80-fold increase in reporter gene expression in the presence of both T3 and TR and only an additional 2.5-fold increase in the presence of both TR and RXR. The constructs TK−121/−87 and TK−133/−107 showed a similar T3 response, suggesting that TRE2 is located between nucleotides −121 and −107 (Table 2). The TRE2 motif located in this region at nucleotide −117 is similar to the R1 enhancer, a half-site TRE of the major histocompatibility complex I genes that activates gene expression in response to retinoic acid (12). The results shown in Table 2 and in Fig. 2 also suggest that sequences upstream of nucleotide −133 on the 5′ side and downstream of nucleotide −87 could influence the way TRE2 responds to T3 and its receptors.

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Fig. 4. EMSA of TRE1 oligonucleotides. (A) [γ-32P]ATP and T4 polynucleotide kinase were used to end-label a synthetic double-stranded oligonucleotide (−747 through −713) containing TRE1. The unlabeled template DNA and a consensus TRE sequence containing an oligonucleotide (DR4) were used as cold competitors. (B) The same as in A, except that a truncated TRE1 containing the sequence −747 through −718 was used as a probe. Lane 1, lysate; lane 2, TR only; lane 3, RXR only; lane 4, TR and RXR; lanes 5, TR, RXR, and anti-TR antibody; lanes 6 and 7, TR, RXR, and a 10- (lane 6) or a 50-fold (lane 7) excess of unlabeled DNA fragments; lanes 8 and 9, TR, RXR, and a 5- (lane 8) or 30-fold (lane 9) excess of unlabeled DR4 oligonucleotides.

Fig. 5. T3 response of TRE1 oligonucleotides cloned in TK-Luc. A double-stranded oligonucleotide containing TRE1 (TK-TRE1) and a consensus TRE sequence (TK-DR4) were cloned in TK-Luc and tested for T3 response as described in Materials and Methods.

Table 2. Localization of TRE2 in human FAS promoter I

<table>
<thead>
<tr>
<th>Construct</th>
<th>RLU</th>
<th>Relative T3 response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+T3</td>
<td>TR + T3</td>
</tr>
<tr>
<td>TK−133/−87</td>
<td>468 (±3.6%)</td>
<td>37,511 (±0.76%)</td>
</tr>
<tr>
<td>TK−121/−87</td>
<td>243 (±6%)</td>
<td>15,477 (±0.7%)</td>
</tr>
<tr>
<td>TK−133/107</td>
<td>397 (±0.75%)</td>
<td>30,999 (±1%)</td>
</tr>
<tr>
<td>TK−128/−102</td>
<td>235 (±0.1%)</td>
<td>17,569 (±10%)</td>
</tr>
</tbody>
</table>

Various regions of the human FAS promoter that contain TRE2 were amplified by using the polymerase chain reaction and were cloned in minimal TK-Luc vector. HepG2 cells were transfected with these plasmids, and their response to T3 was assessed. The experimental conditions used were as described for Table 1 and in the legend to Fig. 1. The relative luciferase activities (RLU) obtained in the presence of T3 are shown. The numbers in parentheses indicate the standard deviation.
porter genes in the presence of T3. The complementary independently activated hFAS-promoter-Luc and TK-Luc re-

spond to TRE1 in human FAS.

Conserved, this sequence in the rat FAS promoter corresponds to TRE1 and that both receptor proteins

influence its binding to TR\(^2\). Consequently, the core motif GGCTTCC functions as TRE2.

In conclusion, promoter I of the human FAS gene is strongly activated by T3 when HepG2 cells are transfected with plasmids expressing TR or RXR. Under the same experimental conditions, promoter II of the human FAS gene does not respond to T3. There are two regions in promoter I (nucleotides 870 to 650 and 272 to 67) that contain TRE and independently activated hFAS-promoter-Luc and TK-Luc reporter genes in the presence of T3. The complementary sequence of the nucleotides between 716 and 731, which contains 5'-GGGTTCC-3' as a half-site TRE sequence, is considered to be TRE1 because it contains five nucleotides on either side of TRE2 do not influence its binding to TR/RXR. Consequently, the core motif GGCTTCC functions as TRE2.

Based on the 5'-deletion analysis and the cloning of various fragments in the TK-Luc reporter gene, TRE2 is located between nucleotides 121 and 102. The EMSAs performed with various mutant oligonucleotides identified a half-site sequence, GGCTTCC, which is located between nucleotides 117 and 112. This half-site TRE is similar to the RII enhancer as described above (12). The TRE2 sequence is also conserved in both human and rat FAS promoters (13, 20). Interestingly, the various TRE2-containing DNA fragments that were cloned in the reporter gene constructs used in these studies responded differently to T3 in the presence of TR alone and in the presence of TR/RXR. It is possible that some trans-activating factors that bind to the sequences upstream and downstream of the TRE2 sequence may influence the way in which TRE2 binds TR and endogenous RXR. These results clearly demonstrate the role of T3 in the regulation of human FAS.

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