THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 30, Issue of July 29, pp. 27533–27543, 2005

Printed in U.S.A.

Thyroid Hormone Regulates the Hypotriglyceridemic Gene *APOA5**

Received for publication, March 22, 2005, and in revised form, May 31, 2005
Published, JBC Papers in Press, June 7, 2005, DOI 10.1074/jbc.M503139200

Xavier Prieur‡, Thierry Huby§, Hervé Coste‡, Frank G. Schaap¶, M. John Chapman§, and Joan C. Rodríguez‡‖

From ‡GlaxoSmithKline, 25 Avenue du Québec, 91951 Les Ulis cedex, France, ¶AMC Liver Center, Meibergdreef 69-71, 1105 BK Amsterdam, The Netherlands, and the §Dyslipoproteinemia and Atherosclerosis Research Unit (U551), National Institute for Health and Medical Research, Hôpital de la Pitié, Paris Cedex 13, France

The apolipoprotein AV gene (*APOA5*) is a key determinant of plasma triglyceride levels, a major risk factor for coronary artery disease and a biomarker for the metabolic syndrome. Since thyroid hormones influence very low density lipoprotein triglyceride metabolism and clinical studies have demonstrated an inverse correlation between thyroid status and plasma triglyceride levels, we examined whether *APOA5* is regulated by thyroid hormone. Here we report that 3,5,3′-triiodo-L-thyronine (T_3) and a synthetic thyroid receptor β (TRβ) ligand increase *APOA5* mRNA and protein levels in hepatocytes. Our data revealed that T_3-activated TR directly regulates *APOA5* promoter through a functional direct repeat separated by four nucleotides (DR4). Interestingly, we show that upstream stimulatory factor 1, a transcription factor associated with familial combined hyperlipidemia and elevated triglyceride levels in humans, and upstream stimulatory factor 2 cooperate with TR, resulting in a synergistic activation of *APOA5* promoter in a ligand-dependent manner via an adjacent E-box motif. In rats, we observed that apoAV levels declines with thyroid hormone depletion but returned to normal levels upon T_3 administration. In addition, treatments with a TRβ-selective agonist increased apoAV and diminished triglyceride levels. The identification of *APOA5* as a T_3 target gene provides a new potential mechanism whereby thyroid hormones can influence triglyceride homeostasis. Additionally, these data suggest that TRβ may be a potential pharmacological target for the treatment of hypertriglyceridemia.

A recent survey estimated that ~30% of the United States adult population exhibits hypertriglyceridemia (1), an independent risk factor for coronary artery disease (2) and a key feature of the highly prevalent metabolic syndrome (1, 3). Therefore, understanding the factors that regulate triglyceride (TG)[1] levels is of major interest and may provide new opportunities for therapeutic intervention in atherogenic dyslipidemia.

Elevation of plasma TG is associated with hypothyroidism (4–8). Indeed, hypertriglyceridemia is clearly associated with hypothyroidism in obese patients, who are characterized by attenuated rates of clearance of very low density lipoprotein (VLDL)-TG, relative to those in obese euthyroid subjects (6). Such elevation in TG levels has been attributed to low lipoprotein lipase (LPL) (9) or low hepatic triglyceride lipase activities (6, 10, 11). In contrast, hyperthyroid patients exhibit elevated rates of clearance of VLDL and normal or decreased circulating TG levels (6), whereas treatment with thyroid hormones (TH) is associated with elevation in both LPL and hepatic triglyceride lipase activities (9, 10, 11) and concomitantly with a tendency to TG lowering (6, 9, 11). At present, the molecular mechanisms by which TH may regulate lipase activities and circulating TG levels in humans remain to be defined.

3,5,3′-Triiodo-L-thyronine (T_3) exerts its biological actions through binding to specific nuclear receptors that modulate gene expression (12). Typically, the T_3 receptor (TR) complexes with the retinoid X receptor (RXR) to form a heterodimer that binds to specific DNA sequence elements known as TREs, composed of two half-core PuGGTCA motifs with specific nucleotide spacing and orientation (13). There are several TRs, which are encoded by two distinct genes, TRα (NR1A1) and TRβ (NR1A2). The TRα gene gives rise to the ligand-binding protein TRα1 and splice variants that do not bind T_3. Several amino-terminal protein variants are produced from the TRβ gene: TRβ1; TRβ2, which is largely restricted to anterior pituitary and hypothalamus (12); and the two recently identified, low level expressed, TRβ3 and TRΔβ3 (14).

The widely expressed isoforms TRα1 and TRβ1 have divergent N-terminal regions but display remarkably sequence homology throughout the rest, especially in their DNA binding domains (12, 15). As a consequence, both isoforms can bind T_3 with similar affinities, and heterodimers of RXR and either TR isoform recognize the same motifs on DNA (12). Despite their structural similarities, distinct patterns of expression of TRs may account for isoform-specific phenotypic functional differences observed in *in vivo* investigations (12). In the liver, TRβ1 is the predominant receptor isoform, representing 80% of T_3-binding activity (16, 17). T_3 influences lipid metabolism through the hepatic regulation of some key TRE-bearing genes, including the lipogenic fatty acid synthase and malic enzyme (18, 19), the mitochondrial fatty acid oxidation rate-controlling enzyme carnitine palmitoyltransferase-Iα (CPT-Iα) (20, 21), the rate-limiting enzyme in bile acid synthesis *CYP7A1* (22), and the sterol regulatory element-binding protein-2, which in turn activates the low density lipoprotein receptor (LDLr) and other genes directly involved in cholesterol homeostasis (23). The administration of thyroid hormones lowers plasma cholesterol in hypothyroid patients. Unfortunately, the natural TH

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. E-mail: jcrodrig@freesurf.fr.

[1] The abbreviations used are: TG, triglyceride(s); VLDL, very low density lipoprotein; LPL, lipoprotein lipase; TH, thyroid hormone(s); T_3, 3,5,3′-triiodo-L-thyronine; TR, thyroid receptor; RXR, retinoid X receptor; CPT-Iα, carnitine palmitoyltransferase-Iα; LDLr, low density lipoprotein receptor; DR, direct repeat; apo, apolipoprotein; nt, nucleotide(s); TK, thymidine kinase; RT, reverse transcriptase; TRE, thyroid receptor response element; PTU, 6-*n*-propyl-2-thiouracil; USF, upstream transcription factor; T_4, 3,5,3′,5′-tetraiodo-L-thyronine; CGS-23425, *N*-[3,5-dimethyl-4-(4′-hydroxy-3′-isopropylphenoxy)-phenyl]-oxamic acid; EMSA, electrophoretic mobility shift assay.

cannot be used therapeutically to treat hypercholesterolemia in euthyroid individuals because they have undesirable effects in the heart, where TRα1 is the predominant isoform (12). These differences in TR isoform expression, together with the fact that TRα1 and TRβ1 isoforms can bind TH analogs with subtle differences in affinity, have spawned attempts to develop thyromimetics with higher selectivity toward TRβ1 *versus* TRα1 that may have cholesterol-lowering effects but minimal cardiac toxicity (12).

Apolipoproteins (apo) play a determinant role in lipid homeostasis. Alterations in the levels of these functionally specialized proteins dramatically influence plasma lipid concentrations. Recently, the gene coding for a new apolipoprotein family member, *APOA5*, was identified 30 kb proximal to the *APOA1/C3/A4* gene cluster and shown to be a major determinant of plasma TG levels (24, 25). Mice expressing a human *APOA5* transgene (24) or injected with adenoviral vectors overexpressing mouse *APOA5* (26) exhibit reduction in plasma TG concentrations to one-third the levels of control mice. Conversely, in knock-out mice lacking *APOA5*, plasma TG levels were 4-fold elevated compared with their wild-type littermates (24). In humans, common polymorphisms across the *APOA5* locus have been associated with elevated plasma TG concentrations (24, 27), familial combined hypertriglyceridemia (28), and increased risk of cardiovascular disease (29, 30). Furthermore, inherited apoAV deficiency is associated with severe hypertriglyceridemia in humans (31). ApoAV is a highly hydrophobic protein mainly expressed in liver that circulates at low concentrations associated with high density lipoprotein (24, 25); in addition, apoAV appears to reduce plasma TG by inhibiting hepatic VLDL-TG production and stimulating LPL-mediated VLDL-TG hydrolysis (32).

Inasmuch as apoAV concentration is a key determinant of plasma TG levels and since the apolipoprotein gene family is highly regulated at the transcriptional level (33), recent research has been oriented to the identification of factors that control *APOA5* expression. Little is known regarding the transcriptional regulation of this newly discovered gene, although it has been shown to date that *APOA5* is regulated by peroxisome proliferator-activated receptor-α (NR1C1), farnesoid X-activated receptor (NR1H4), and sterol regulatory element-binding protein-1c, all of which are transcription factors directly implicated in triglyceride metabolism (34–36).

Since T_3 influences VLDL-TG metabolism, we investigated whether T_3 might regulate *APOA5* expression. In this study, we provide evidence that T_3 induces *APOA5* expression. Furthermore, our findings reveal that TR directly regulates *APOA5* in a ligand-dependent manner via a functional TRE within the promoter. In addition, in rats *in vivo*, apoAV content correlated with thyroid status, and moreover a TRβ ligand increased apoAV and simultaneously diminished TG levels. Therefore, our findings provide evidence for molecular crosstalk between thyroid status and intravascular TG metabolism.

EXPERIMENTAL PROCEDURES

Plasmids—Constructs p-1422/+18hAvLUC, p-617/+18hAvLUC, p-437/+18hAvLUC, p-242/+18hAvLUC, and p-82/+18hAvLUC containing the corresponding sequences of the 5′-flanking region of the human *APOA5* gene cloned in front of the promoterless firefly (*Photinus pyralis*) luciferase gene have been previously described (35). Site-directed mutagenesis of the construct p-617/+18hAvLUC was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the recommendations of the manufacturer and two pairs of oligonucleotides containing mutations corresponding, respectively, to nt −109C→T/−108A→T/−101G→A/−100T→A and to nt −80A→C/−77T→C of the human *APOA5* promoter. The vector pGL3-TK contains a fragment corresponding to nt −109 to +20 of the thymidine kinase (TK) gene promoter of herpes simplex virus (36) subcloned into the BglII/HindIII sites of pGL3-basic vector. The reporter plasmids p(AVDR4)$_n$-TK (n = 1–5) were generated by insertion of 1–5 copies of a double-stranded oligonucleotide containing wild type (5′-GAT CCT GGG AGG CAG CTG AGG TCA ACT TA-3′) or mutant (5′-GAT CCT GGG AAG TTG CTG AGA ACA ACT TA-3′) sequences spanning nt −117 to −94 of human apoAV promoter into the BglII site of pGL3-TK. Plasmids expressing human cDNAs for TRα1 and TRβ1 were provided by J. A. Holt (GlaxoSmithKline, Research Triangle Park, NC). Plasmid DNA was prepared using the Qiagen endotoxin-free maxipreparation method and quantified spectrophotometrically. The integrities of all plasmids were verified by DNA sequencing.

Cell Transfection and Reporter Assays—Human hepatoblastoma HepG2 cells were cultured in Eagle's basal medium supplemented with nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate (medium A), and 10% (v/v) fetal calf serum. On day 0, cells were seeded on 24-well plates at a density of 3.5×10^5 cells/well. On day 1, cells were transfected with FuGENE 6 reagent (Roche Applied Science) according to the manufacturer's instructions. Typically, each well of a 24-well plate received 200 ng of firefly luciferase reporter plasmid and 100 ng of a plasmid expressing human TRα1, TRβ1, and/or USF. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pSG5. 100 ng/well of a sea pansy (*Renilla reniformis*) luciferase plasmid pRL-null (Promega) was included in all transfections as an internal control for transfection efficiency. On day 2, cells were switched to Dulbecco's modified Eagle's medium/F-12 medium (Invitrogen) supplemented with nonessential amino acids, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (medium B) and 1% (v/v) delipidated calf serum (Sigma) and containing, when indicated, T_3 (Sigma), *N*-[3,5-dimethyl-4-(4′-hydroxy-3′-isopropylphenoxy)-phenyl]-oxamic acid (CGS-23425) (Novartis), or vehicle (water or Me_2SO, respectively). On day 3, cell lysates were prepared by shaking the cells in 200 μl of 1× Promega lysis buffer for 15 min at room temperature. Firefly and *Renilla* luciferase activities were measured using a Dual-Luciferase® reporter assay system (Promega) and a Lumistar luminometer (BMG Lab Technologies). Firefly luciferase activity values were divided by *Renilla* luciferase activity values to obtain normalized luciferase activities. To facilitate comparisons within a given experiment, activity data were presented either as relative luciferase activities or as -fold induction over the normalized activity of the reporter plasmid in the absence of nuclear receptor cotransfection and agonist supplementation. The data are expressed as the means ± S.D. Statistic significance analyses were done with Student's t test.

Cell Treatments—On day 0, human hepatoblastoma HepG2 cells or rat hepatoma McArdle-RH7777 (ATCC, Manassas, VA) were plated on 24-well plates at 5×10^5 or 10^5 cells/well, respectively, in medium A and 10% (v/v) fetal calf serum or in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter glucose, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 10% (v/v) fetal bovine serum, and 10% (v/v) horse serum, respectively. On day 2, cells were refed with medium B supplemented with 1% (v/v) delipidated calf serum (Sigma), and T_3 (Sigma), CGS-23425 (Novartis) or vehicle (water or Me_2SO, respectively). On day 3, cells were washed twice with PBS and harvested for isolation of RNA or Western analysis. Human primary hepatocytes in 24-well plates fed with Williams E medium supplemented with 100 nM dexamethasone, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 4 μg/ml insulin (medium C), and 1% (v/v) fetal calf serum were provided on day 0 by Biopredict (batch Hep220069 MW24). On day 1, cells were refed with medium C supplemented with 1% (v/v) delipidated calf serum and containing 50 nM T_3, 10 nM CGS-23425, or vehicle (water or Me_2SO, respectively). After 24 h, the medium was replaced by 500 μl/well fresh medium C containing 50 nM T_3, 10 nM CGS-23425, or vehicle (water or Me_2SO, respectively). After 6 h, media were collected, and the cells were washed twice with PBS and harvested for isolation of RNA or Western analysis.

Western Blot Analysis—For determination of secreted apoAV, media from triplicate wells were pooled, and proteins were precipitated by the addition of 180 μl of trichloroacetic acid (Sigma) and agitation overnight at 4 °C. After centrifugation at 2×10^4 g at 4 °C for 15 min, protein pellets were washed twice in 500 μl of cold acetone, dried at room temperature, and resuspended in 100 μl of 1× NuPage LDS sample buffer (Invitrogen). For determination of cellular apoAV, whole cell lysates were prepared by shaking the cells on 24-well plates in 100 μl/well lysis buffer A (PBS, 1% Triton, 50 mM NaF, 5 mM sodium pyrophosphate, 10 μl/ml protease inhibitor mixture from Sigma) for 30 min at 4 °C. The lysates were clarified by centrifugation at $10^4 \times g$ at 4 °C for 5 min.

60-μl aliquots of resuspended samples from the incubation media and 40 μg of proteins from whole cell lysates were boiled at 100 °C for

5 min, electrophoresed on 10% polyacrylamide MOPS NuPAGE® Novex gels (Invitrogen), and transferred onto nitrocellulose membranes in NuPAGE® transfer buffer (Invitrogen). Membranes were preincubated for 1 h at room temperature in blocking buffer, 5% nonfat dry milk in PBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween 20). Subsequently, blots were incubated overnight at 4 °C with rabbit anti-human apoAV, rabbit anti-rat apoAV (25), or mouse anti-β-actin (A5441; Sigma) in blocking buffer. After washing five times in PBST for 5 min, blots were incubated with IRDye™ infrared dye 38 (800 nm; Rockland Immunochemicals) and Alexa Fluor® 680 (700 nm; Molecular Probes, Inc., Eugene, OR) labeled goat anti-mouse and anti-rabbit secondary antibodies diluted at 1:5000 in PBST for 1 h at room temperature. Signals were detected by using Odyssey™ Infrared Imaging System (LI-COR).

Real-time PCR Quantification of mRNAs—Total RNA was prepared from human primary hepatocytes, HepG2, and rat McArdle cells with the RNeasy™ Mini kit, the QIAshredder™ homogenizers, and the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. Human normal liver total RNA was purchased from BD Biosciences (reference number 64099-1, batch 4120140; Caucasian 51-year-old man, sudden death). A 1-μg aliquot was used as a template for cDNA synthesis employing the TaqMan™ Reverse Transcription Reagent kit (Applied Biosystems). Primers were designed with Primer Express Software (PerkinElmer Life Sciences). The sequences of forward and reverse primers used for the amplifications are as follows: 18 S, 5′-GGG AGC CTG AGA AAC GGC-3′ and 5′-GGG TCG GGA GTG GGT AAT TT-3′; hTRα1, 5′-CAG CCG CTT CCT CCA CAT-3′ and 5′-CCG CCT GAG GCT TTA GAC TT-3′; hTRβ1, 5′-CTG CAC ATG AAG GTG GAA TG-3′ and 5′-TCG AAC ACT TCC AGG AAC AA-3′; cyclophilin, 5′-CAT CTG CAC TGC CAA GAC TGA-3′ and 5′-CCA CAA TAT TCA TGC CTT CTT TCA-3′; h*APOC3*, 5′-CTT CTC AGC TTC ATG CAG GGT TA-3′ and 5′-ACG CTG CTC AGT GCA TCC TT-3′; h*LDLr*, 5′-GTT GCT GGC AGA GGA AAT GAG AAG-3′ and 5′-CAA AGG AAG ACG AGG AGC ACG AT-3′; h*APOA5*, 5′-AGC TGG TGG GCT GGA ATT T-3′ and 5′-GGC CAC CTG CTC CAT CAG-3′; and rAPOA5, 5′-ACA CGG TCG AGC TGA TGG-3′ and 5′-GGC CTT GGT GCC TTT TCC-3′, respectively. The specificity and efficiency of the primers were validated as previously described (35). The reactions contained 4 μl of diluted (1:10) cDNA, a 300 nM concentration of the forward and reverse primers, and 2× SYBR™ Green PCR Master Mix (Applied Biosystems) in a final volume of 20 μl. Real-time PCRs were carried out in 384-well plates by using the ABI PRISM™ 7900 sequence detection system (Applied Biosystems). Levels of TRα1 and TRβ1 were normalized by 18 S to compensate for variations in input RNA amounts. Levels of *APOA5*, *APOC3*, and *LDLr* were normalized to cyclophilin to compensate for variations in input RNA amounts (cyclophilin levels were unaffected by the treatments). The amounts of mRNAs were calculated using the comparative C_T method as described in Ref. 38. All assays were performed in triplicate during two independent experiments, and the reverse transcriptase (RT)-PCRs were carried out in duplicate for each sample.

In Vitro Transcription/Translation and EMSAs—TRα, RXRα, USF1, and USF2 proteins were synthesized *in vitro* from the expression plasmid by using TnT® Quick Coupled transcription/translation system (Promega) according to the instructions of the manufacturer. In order to obtain an unprogrammed lysate as a negative control for EMSA, a reaction was performed with the empty vector pSG5. Double-stranded oligonucleotides corresponding to the sequence spanning nt −117 to −94 and nt −88 to −69 of human *APOA5* promoter (AVDR4 and AVEbox, respectively) or modified versions harboring mutations in the DR4 hexamers and E-box described under "Plasmids" (mutAVDR4 and mutAVE, respectively) were radiolabeled by fill in with the Klenow fragment of DNA polymerase I and used as probes. The control probes contain the DR4 sequence of the human TRβ proximal TR response element (39) and the E-box of the rat CPT-Iα first intron (40), respectively. Protein-DNA binding assays and electrophoreses of samples were performed as described (41). Gels were dried and analyzed using a PhosphorImager STORM 860 and ImageQuant software (Amersham Biosciences).

Hypothyroid Rats—All experimental protocols were performed in accordance with the policies of the institutional Animal Care and Use Committee. 12-week-old male Wistar rats (Charles River France) were housed under controlled conditions (22 °C, 12 h/12 h dark/light cycle) with food and water *ad libitum*. Twelve rats were divided into a control and an experimental group. The experimental group was rendered hypothyroid by gavage with a daily single dose of 10 mg/kg 6-*n*-propyl-2-thiouracil (PTU) (Sigma) aqueous solution for 3 weeks. Seventeen days after the initiation of the PTU treatment, the experimental group received an intraperitoneal injection of saline (PTU, $n = 4$) or 300 μg/kg T_3 (PTU + T_3, $n = 4$) daily for 4 days. Control rats ($n = 4$) received daily gavage and intraperitoneal injection of only the solvents for the same periods of time. Six hours after the final administration, the animals were killed, and livers were excised. Liver sections of 300 mg were placed in 4 ml of lysis buffer A and homogenized for 20–40 s using a conventional rotor-stator. The lysates were clarified by centrifugation at $10^4 \times g$ at 4 °C for 5 min. 40 μg of protein were used for Western analysis.

CGS-23425 Treatment of Fat-fed Rats—Six-week-old male Sprague-Dawley rats (Charles River France) were maintained on a chow diet supplemented with 1.5% cholesterol and 0.5% cholic acid (fat-fed) for 14 days before experiments. Fat-fed rats were treated orally by gavage with a daily single dose of 100 μg/kg/day CGS-23425 (Novartis) or vehicle (0.5% hydroxypropyl methylcellulose, 1% Tween 80) for 1 week. Four hours after the last dose, the animals were killed, and blood and livers were collected. Lysates from liver sections (300 mg) were prepared as described above. Total plasma triglyceride levels were measured enzymatically using the TG-PAP150 kit (Biomerieux, France).

RESULTS

T_3 and the Thyromimetic CGS-23425 Increase APOA5 Expression in Hepatocytes—APOA5 is expressed in human hepatoma HepG2 cells with levels comparable with human primary hepatocytes (see Ref. 36 for a comparison). Although translational or posttranslational factors may ultimately regulate receptor content in cultured cells, we verified by quantitative real-time RT-PCR that both TRα1 and TRβ1 isoforms are expressed in HepG2 and human primary hepatocytes, suggesting that both isoforms may be readily available for T_3-dependent regulation studies. More specifically, TRα1 mRNA levels in HepG2 are 4-fold higher than in human primary hepatocytes and 5-fold higher than in human liver ($2^{-\Delta Ct} \times 10^4$ values were 7.00 ± 0.53, 1.84 ± 0.35, and 1.37 ± 0.06, respectively), whereas TRβ1 levels are similar in the three cases (2.33 ± 0.12 for HepG2, 2.08 ± 0.36 for human primary hepatocytes, and 2.91 ± 0.05 for human liver). To determine whether T_3 can modulate *APOA5* gene expression, we incubated HepG2 cells in the presence or absence of T_3. Treatment with T_3 significantly increased *APOA5* mRNA levels at 6 h, and a 2-fold induction was achieved after 24 h of T_3 addition (Fig. 1*A*). Furthermore, increasing concentrations of T_3 resulted in a dose-dependent induction of *APOA5* expression (Fig. 1*B*). In addition, we aimed to know whether *APOA5* expression might be increased by TH analogs with potential therapeutic interest (12). CGS-23425 is a synthetic thyromimetic with cholesterol-lowering effects but minimal cardiac toxicity in rats that has been reported to be more selective toward TRβ1 *versus* TRα1 and to show a higher binding affinity to intact hepatic nucleic than T_3 (42). As shown in Fig. 1*B*, CGS-23425 increased *APOA5* mRNA levels, attaining a 2.2-fold increase at 5 nM.

Similarly, human primary hepatocytes and rat hepatoma McArdle cells were incubated for 24 h in medium containing T_3, CGS-23425, or vehicle. As shown in Fig. 1, *C–F*, treatment with T_3 or CGS-23425 increased *APOA5* mRNA levels. These effects were specific, since the expressions of *LDLr* (Fig. 1, *C* and *D*) and *CPT-Iα* (Fig. 1, *E* and *F*) were also increased, in accordance with previous studies (40, 43), whereas cyclophilin, used as internal control, remained unaffected by T_3 or CGS-23425 treatments, and in addition, no significant effect on *APOC3* mRNA levels was observed (Fig. 1, *C* and *D*).

T_3 and the Thyromimetic CGS-23425 Increase ApoAV Protein Levels in Human Hepatocytes—Western blot analyses performed on whole cell lysates from HepG2 (data not shown) and human primary hepatocytes (Fig. 2) incubated for 24 h with T_3, CGS-23425, or vehicle revealed that the quantity of cellular apoAV protein was markedly increased by both TR ligands (Fig. 2). Moreover, treatments with T_3 or the thyromimetic CGS-23425 led to a significant increase in levels of apoAV protein secreted into the medium by human primary hepatocytes (Fig. 2).

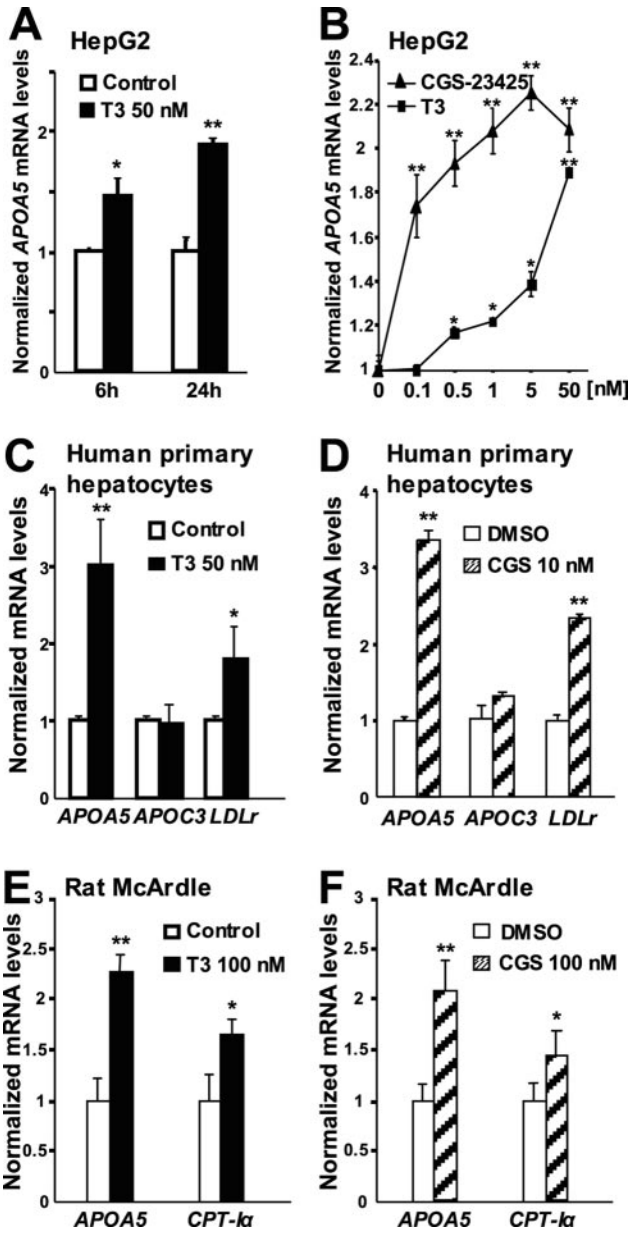

FIG. 1. **T_3 and the thyromimetic CGS-23425 increase *APOA5* mRNA levels.** *A*, human hepatoma HepG2 cells were incubated in the absence (*control*) or the presence of 50 nM T_3 for the indicated periods of time (*A*) or in the presence of increasing concentrations of T_3, CGS-23425 or vehicle (water or Me_2SO, respectively) for 24 h (*B*). Primary hepatocytes isolated from adult human liver were treated for 24 h with 50 nM T_3 (*C*), 10 nM CGS-23425 (*D*), or vehicle (*Control* or *DMSO*, respectively). Rat hepatoma McArdle cells were treated for 24 h with 100 nM T_3 (*E*), 100 nM CGS-23425 (*F*), or vehicle (*Control* or *DMSO*, respectively). Total RNA was extracted for analysis by real-time RT-PCR as described under "Experimental Procedures." Specific *APOA5*, *APOC3*, *LDLr*, and *CPT-Iα* mRNA levels normalized to cyclophilin content are expressed as $2^{-\Delta\Delta Ct}$ and relative to untreated cells set as 1 (mean ± S.D.). Significant differences compared with the corresponding untreated controls are as follows: *, $p < 0.005$; **, $p < 0.001$. The results are representative of two or three independent experiments.

T_3 and CGS-23425 Increase Human APOA5 Expression at the Transcriptional Level via the Nuclear Receptor TRs—To determine whether *APOA5* was directly responsive to T_3-activated TRs, we performed functional analysis of the human *APOA5* promoter. In transient transfection assays in HepG2 cells, treatment with 50 nM T_3 increased the activity of the firefly luciferase reporter gene driven by the −617/+18 sequence of the human *APOA5* promoter (Fig. 3*A*). The effect of

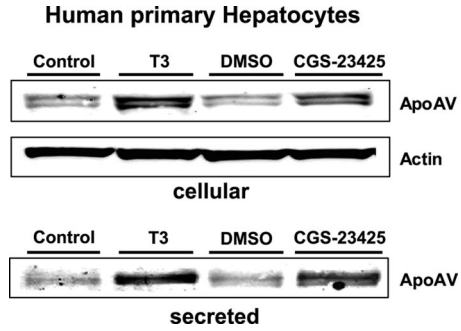

FIG. 2. **T_3 and the thyromimetic CGS-23425 increase apoAV protein secretion in primary human hepatocytes.** On day 1, primary hepatocytes isolated from adult human liver were incubated in the presence of 50 nM T_3, 10 nM CGS-23425, or vehicle (*Control* or *DMSO*, respectively). On day 2, cells received fresh media containing the same treatments, respectively. After 6 h, media and cells were collected, and protein content was analyzed by Western blot as described under "Experimental Procedures." Experiments were performed two times, and a representative result is shown.

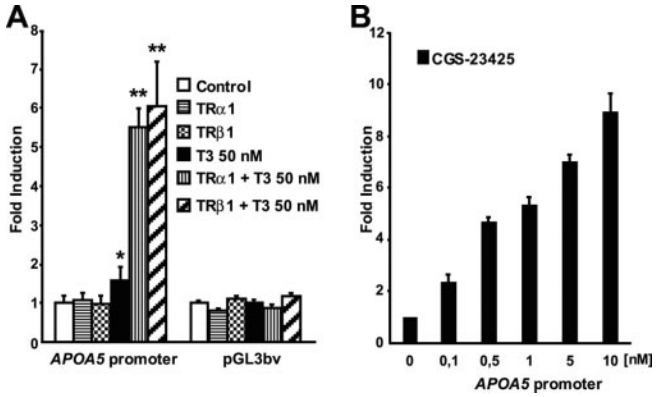

FIG. 3. **Transactivation of the human *APOA5* gene promoter by TRβ and T_3.** *A*, HepG2 cells were transfected with a plasmid containing a luciferase reporter gene driven by the 5′-flanking region (−617/+18) of the human *APOA5* gene or the empty pGL3-basic vector along with a plasmid expressing human TRα1, TRβ1, or the empty vector pSG5 as control. Cells were incubated in the absence (*Control*) or the presence of 50 nM T_3 for 24 h, and luciferase activities were measured as described under "Experimental Procedures." Results are expressed as -fold induction over control. *, $p < 0.005$; **, $p < 0.001$ *versus* control. The results are representative of three independent experiments. *B*, HepG2 cells were transfected with a plasmid containing a luciferase reporter gene driven by the 5′-flanking region (−617/+18) of the human *APOA5* gene along with a plasmid expressing human TRβ1. Cells were incubated with vehicle (*0*) or increasing concentrations of CGS-23425 for 24 h, and luciferase activities were measured as described under "Experimental Procedures." Results are expressed as -fold induction over vehicle. The results are representative of three independent experiments.

T_3 was promoter-dependent, because it was not observed with the promoterless pGL3-basic vector. Cotransfection of human TRα1 or TRβ1 expression plasmids robustly enhanced T_3-induced promoter activity. In contrast, *APOA5* promoter activity was not affected significantly by cotransfection of TRα1 or TRβ1 in the absence of T_3 (Fig. 3*A*). No statistically significant differences on T_3 induction of *APOA5* promoter were observed between TRα1 and TRβ1 transactivations. In order to confirm that the lipid-lowering thyromimetic CGS-23425 induces the *APOA5* gene at the transcriptional level, similar transient transfection assays were performed in HepG2 cells with the human *APOA5* promoter along with a human TRβ1 expression plasmid. As shown in Fig. 3*B*, increasing concentrations of CGS-23425 resulted in a dose-dependent induction of the luciferase activity. The promoterless pGL3-basic vector was unaffected by CGS-23425 (data not shown).

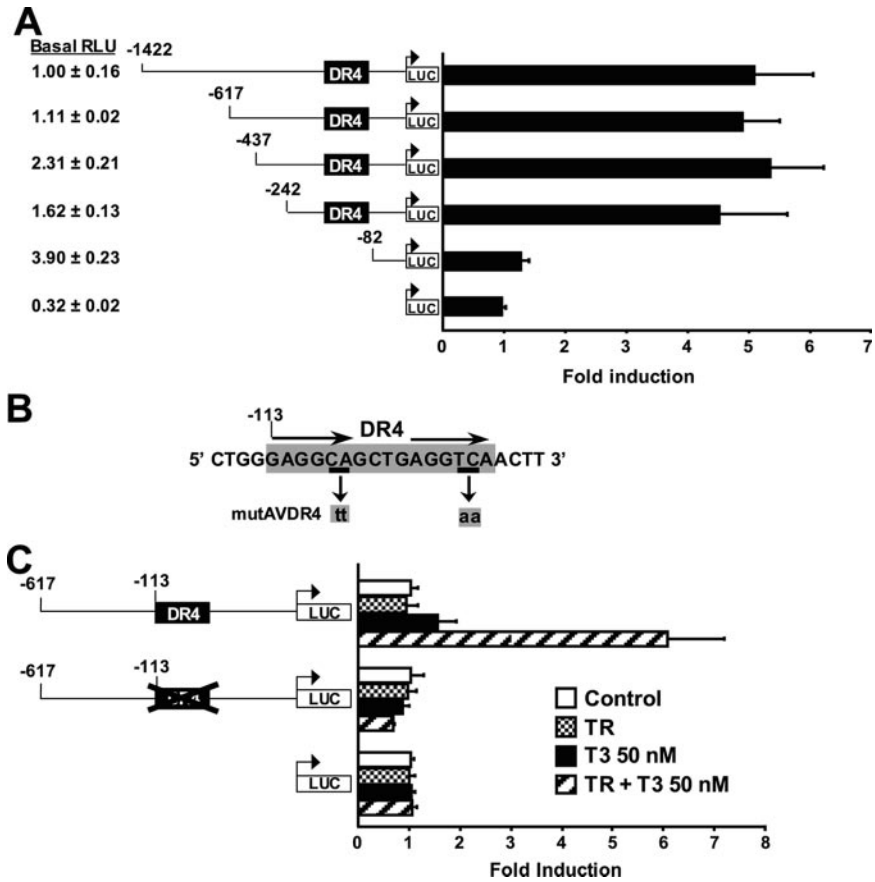

FIG. 4. **Identification and characterization of a TR response element in the human *APOA5* promoter.** *A*, localization of a TR response region by progressive deletion analysis. HepG2 cells were cotransfected with reporter plasmids containing the firefly luciferase gene driven by progressively 5′-shortened fragments of the *APOA5* promoter as indicated, together with the empty vector pSG5 or a plasmid expressing human TRβ1. Cells were incubated in the absence or the presence of 50 nM T_3 for 24 h, and luciferase activities were measured as described under "Experimental Procedures." Results in the histogram are expressed as -fold induction over control. Values of normalized relative luciferase activities (*RLU*) for the different constructs in basal conditions are shown in the *column* to the *left* of the histogram and are expressed as the mean ± S.D. The results are representative of three independent experiments. *B*, human *APOA5* promoter sequence surrounding the DR4 element. The *gray box* denotes the DR4 sequence. The AGGTCA half-sites are indicated by *horizontal arrows*. The wild-type nucleotides that were modified by site-directed mutagenesis are *underlined*. The corresponding −109C→T/−108A→T/−101G→A/−100T→A mutated nucleotides are shown *below* the *vertical arrow* and *within* the *gray squares*. *C*, mutation of the DR4 element on the human *APOA5* promoter eliminates the response to T_3–activated TRβ1. Experiments were performed as in *A* with reporter constructs containing the wild-type or site-directed mutated *APOA5* promoter or the empty pGL3-basic vector as negative control. The *cross* depicts the presence of site-directed mutations in the DR4 element. *LUC*, luciferase. The results are representative of three independent experiments.

A DR4 Element in the Human APOA5 Promoter Is Required for Transcriptional Activation by TR—To localize the region within APOA5 that confers transcriptional responsiveness to T_3-activated TR, a series of constructs containing sequential 5′-deletions from nt −1422 to +18 of the human *APOA5* promoter in front of the firefly luciferase reporter gene were transiently transfected into HepG2 cells together with a human TRβ1 expression plasmid in the presence or the absence of 50 nM T_3. As shown in Fig. 4*A*, the sequence upstream to position −242 could be removed without preventing strong activation of the reporter gene by T_3-activated TR. In contrast, deletion of the fragment between nt −242 and −82 completely abolished the induction of *APOA5* promoter activity by T_3-activated TR, indicating that this region mediates the effects of T_3. Analysis of the sequence in the −242/−82 fragment revealed a direct repeat of the hexanucleotide core motif PuGGTCA with a low degree of degeneration separated by 4 nucleotides between nt −113 and −98 (Fig. 4*B*), thereby conforming to the DR4 response element for TR (TRE).

To unequivocally characterize this DR4 as the functional TRE required for T_3 induction of *APOA5*, HepG2 cells were cotransfected with a human TRβ1 expression vector and an *APOA5* promoter-luciferase reporter plasmid in which the DR4 sequence was mutated (Fig. 4*B*). In contrast to the wild-type promoter construct, T_3 and T_3-activated TRβ1 failed to induce the activity of the construct bearing the mutated DR4 (Fig. 4*C*).

The RXR-TR Heterodimer Binds Specifically to the APOA5 DR4 Element—Direct binding of RXR-TR heterodimers to the *APOA5* DR4 element was examined. For this purpose, gel shift assays were performed using *in vitro* translated human RXRα and TRα and radiolabeled double-stranded oligonucleotides containing the wild-type or a mutated version of the *APOA5* DR4 element. The addition of TRα1 resulted in the appearance of a weak protein-DNA complex band (Fig. 5, *lane 7*). This phenomenon most likely corresponds to the binding of TRα1 monomers and is also perceived with a control probe containing the DR4 sequence of the human TRβ proximal TRE (Fig. 5, *lane 3*) (34). However, when both RXRα and TRα1 were present, this faint band disappeared, and a robust and more shifted band emerged (Fig. 5, *lane 8*). In contrast, a labeled probe that is equivalent to *APOA5* DR4 but harbors point mutations in the half-sites could not form the faster mobility band with TRα1 or the strong RXRα-TRα binding complex (Fig. 5, *lanes 11* and *12*). Furthermore, the specificity of the RXRα-TRα1-*APOA5* DR4 interaction was confirmed by competition analysis using an excess of cold double-stranded oligonucleotides correspond-

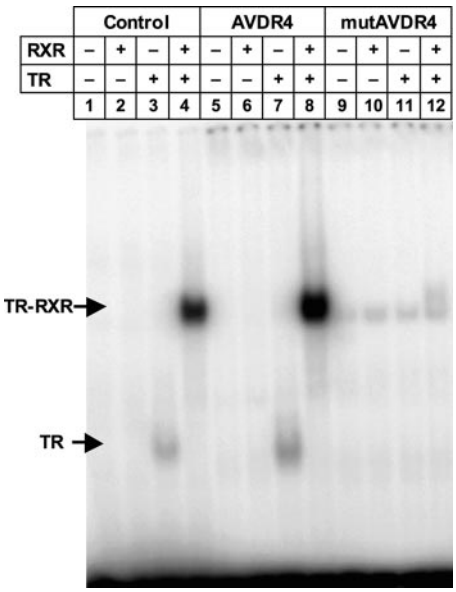

FIG. 5. **TR-RXR heterodimers can bind specifically to the DR4 element of the *APOA5* promoter.** EMSAs were performed using *in vitro* transcribed/translated human TRα1 (2.5 μl), human RXRα (2.5 μl), or unprogrammed reticulocyte lysate (−), when indicated, and labeled double-stranded oligonucleotides corresponding to the sequence spanning nt −117 to −94 of human *APOA5* promoter (*AVDR4*) or a modified version harboring mutations in the DR4 hexamers corresponding to nt −109C→T/−108A→T/−101G→A/−100T→A (*mutAVDR4*) as described under "Experimental Procedures." *Control* corresponds to the DR4 sequence of the human TRβ proximal TRE (34). The lysate volumes were kept constant by the addition of unprogrammed lysate. TRα1-DR4 and TRα1/RXRα-DR4 complexes are indicated by *arrows*. Note that the faint retarded bands of probe mutAVDR4 (*lanes 9–12*) are nonspecific. Experiments were performed two times, and a representative result is shown.

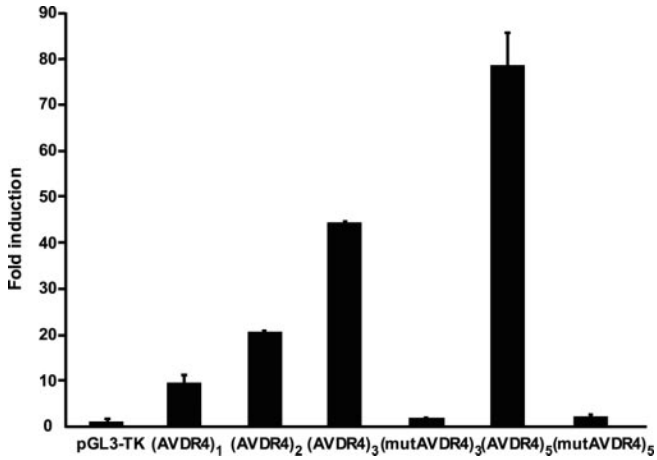

FIG. 6. **The DR4 element present in the *APOA5* promoter confers TR responsiveness to heterologous promoters.** HepG2 cells were transiently transfected with plasmids expressing human TRβ1 or the empty pSG5 vector as control, together with reporter constructs containing 1–5 copies of the wild-type (*AVDR4*) and three or five copies of mutant (*mutAVDR4*) sequence corresponding to nt −117 to −94 of the *APOA5* promoter cloned in front of a heterologous TK promoter-driven luciferase. The empty pGL3-TK reporter vector was used as negative control. Cells were incubated in the absence or the presence of 50 nM T_3 for 24 h, and luciferase activities were measured as described under "Experimental Procedures." Results are expressed as -fold induction over control. The results are representative of three independent experiments.

ing to the control DR4 probe, which inhibited the retarded complex, and the mutated *APOA5* DR4, which failed to displace the labeled wild-type element (data not shown). Additional EMSAs showed that no TRα1 monomers or RXRα-TRα1 heterodimers could bind when the downstream half-site was mutated, whereas mutation of the upstream hexamer markedly diminished heterodimer binding but enhanced the binding of TR monomers (data not shown), thereby suggesting that RXRα binds to the upstream and TRα1 to the downstream hexamer, respectively, of *APOA5* DR4, as a classical direct repeat TRE (44).

The APOA5 DR4 Element Confers TR Responsiveness to Heterologous Promoters—To evaluate whether this DR4 element could confer T_3-activated TR responsiveness to a heterologous promoter, we linked the *APOA5* DR4 site upstream of the TK promoter and the luciferase gene. Reporter constructs containing one, two, three, and five copies of this motif were transiently transfected into HepG2 cells along with a human TRβ1 expression plasmid in the presence or the absence of T_3. As demonstrated in Fig. 6, T_3-activated TRβ1 enhanced the activity of *APOA5* DR4-driven promoter constructs, whereas the reporter constructs with the TK promoter alone or driven by several copies of mutated *APOA5* DR4 were not stimulated. Indeed, an 80-fold induction was attained with five copies, and the response was manifested in a copy number-dependent manner. In addition, we observed that the *APOA5* DR4 site conferred 2-fold more T_3-activated TRβ1 responsiveness to the TK promoter than the CPT-Iα DR4 TRE (data not shown). Taken together, these results show that this *APOA5* DR4 motif is a genuine TRE.

The USF Transcription Factors Cooperate with TR in the T_3-mediated Induction of APOA5—As shown in Fig. 7*A*, there is a nearby sequence 5′-CACGTG-3′ downstream of this DR4 element that constitutes a canonical E-box motif, a binding site for basic helix-loop-helix/leucine zipper proteins. Recently, it was reported that this E-box might make a minor contribution to the sterol regulatory element-binding protein-1c-mediated down-regulation of *APOA5* (36). Inasmuch as USF transcription factors appear to be the predominant basic helix-loop-helix/leucine zipper proteins in liver nuclear extracts (45) and because they can physically interact with TR (40), we set out to investigate whether USF might be involved in the T_3 induction of *APOA5* through this E-box.

EMSAs were conducted using double-stranded oligonucleotides that corresponded to the wild-type or a mutated version of the −88/−69 sequence in human *APOA5*. USF1 (data not shown) and USF2 bound to the wild-type *APOA5* E-box containing probe (Fig. 7*B*, *lane 2*) but not to the equivalent version that harbors −80A→C/−77T→C point mutations (Fig. 7*B*, *lane 4*). Furthermore, competition analysis showed that the retarded complex was inhibited by an excess of an unlabeled control probe (Fig. 7*B*, *lanes 8–10*), containing the E-box of CPT-Iα (40), but not by the mutated *APOA5* E-box probe (data not shown).

Transient transfection assays in HepG2 cells revealed that USF activates at least 2-fold the luciferase reporter gene expression vector driven by the −617/+18 sequence of the human *APOA5* promoter (Fig. 7*C*). In the absence of T_3, cotransfection of TRβ1 produced no effect. However, in the presence of T_3, TR and USF synergistically activate *APOA5*, attaining a 20-fold induction (Fig. 7*C*). Furthermore, as shown in Fig. 8*A*, an internal deletion USF mutant lacking the basic region required for DNA binding (UΔB), which forms dimers and sequesters both endogenous USF1 and USF2 (46), down-regulated *APOA5* promoter activity both in the absence and in the presence of T_3 and transfected TRβ1. Likewise, an N-terminal truncated USF mutant (UΔN), composed only of the basic region and basic helix-loop-helix/leucine zipper domains and which binds DNA and forms dimers but is totally inactive (47), also reduced *APOA5* promoter activity. Therefore, both the DNA binding

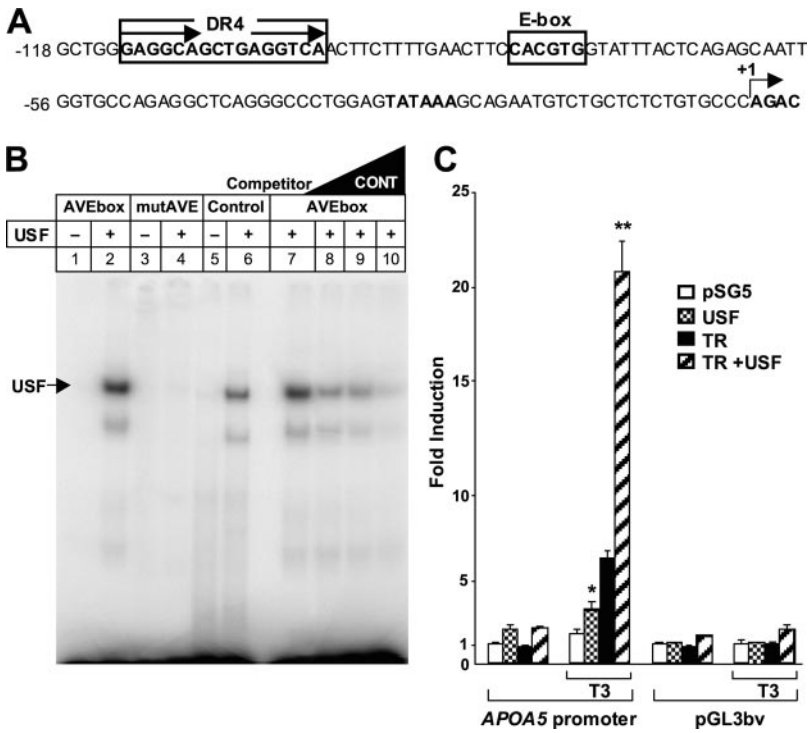

FIG. 7. **USF can bind to the E-box present in the *APOA5* promoter and synergistically activate the T_3-activated TR induction of *APOA5*.** *A*, schematic representation showing the TRE (*DR4*) and the USF binding element (*E-box*) in the nucleotide sequence corresponding to the 5′ region of the human *APOA5* gene. *Numbers* are relative to the transcription start site (+1). The hexameric half-sites in the DR4 element are indicated by *horizontal arrows*. *B*, EMSAs were performed using *in vitro* transcribed/translated USF2 (+) or unprogrammed reticulocyte lysate (−), when indicated, and labeled double-stranded oligonucleotides corresponding to the sequence spanning nt −88 to −69 of human *APOA5* promoter (*AVEbox*) or a modified version harboring −80A→C/−77T→C point mutations (*mutAVE*) as described under "Experimental Procedures." *Control* corresponds to the E-box sequence of rat CPT-Iα (35). The lysate volumes were kept constant by the addition of unprogrammed lysate. USF-E-box complexes are indicated by an *arrow*. The competition experiments for binding of USF2 to the labeled probe *AVEbox* were performed by adding a 10-, 50-, and 250-fold molar excess of the unlabeled CPT-Iα E-box oligonucleotides (*CONT*). EMSAs were performed two times, and a representative result is shown. *C*, HepG2 cells were transfected with a plasmid containing a luciferase reporter gene driven by the 5′-flanking region (−617/+18) of the human *APOA5* gene or the empty pGL3-basic vector together with plasmids expressing USF2, TRβ1, or the empty vector pSG5 as control. Cells were incubated in the absence or the presence of 50 nM T_3 for 24 h, and luciferase activities were measured as described under "Experimental Procedures." Results are expressed as -fold induction over control. *, $p < 0.005$; **, $p < 0.001$ *versus* control. Similar results were obtained with USF1. The *histograms* are average values from three independent transfections, with each point conducted in triplicate.

and the transcriptional activation domains of USF are required for full synergistic activation with T_3-activated TRβ1. Nevertheless, it is worth noting that, in the presence of both TR and UΔN, the magnitude of -fold induction by T_3 was similar to that achieved in the presence of TR and the wild-type USF (Fig. 8*A*, compare data of *T_3/Cont*). These findings suggest that despite the fact that this USF mutant lacking the N-terminal activation domain represses *APOA5*, probably due to competition with the endogenous active USF (47), the enhanced response to T_3 is somehow linked to the ability of USF to bind to DNA.

In order to better assess the importance of the binding of USF on the enhancement of the T_3 induction of *APOA5*, we introduced the mutations that had been shown to disrupt USF binding to the E-box in gel shift assays (Fig. 7*B*) in the −617/+18 construct, leaving an intact DR4, and performed transfection assays. As expected, USF failed to activate the construct bearing the mutated E-box (Fig. 8*B*). This finding indicates that the E-box at −81/−76 is required for the USF response and also that another E-box present at +10 alone is not enough to confer USF responsiveness. Accordingly, the mutation of the USF binding site abolished the synergism between T_3-activated TRβ1 and USF, thereby confirming that the binding of USF to DNA is required for the action of USF on T_3 induction (Fig. 8*B*).

ApoAV Content Correlates with Thyroid Status in Rats—In order to extend our analyses to animal models, we chemically induced hypothyroidism in rats by the well described treatment with PTU, which inhibits the 5′-deiodinase enzyme required to convert the 3,5,3′,5′-tetraiodo-L-thyronine (T_4) form of TH into the more bioactive T_3 isoform (48). Under these conditions, plasma T_4 levels fall significantly by 75–90%, and there is also an ~60% reduction of plasma T_3 (49–51), whereas plasma thyroid-stimulating hormone concentrations rise (52). As shown in Fig. 9, apoAV protein levels were dramatically diminished in the hypothyroid rats, whereas actin levels remained unaltered. However, administration of T_3 restored apoAV protein abundance (Fig. 9). Together, these data demonstrated that apoAV strongly correlates with thyroid status.

The Thyromimetic CGS-23425 Increases ApoAV Protein Levels in Rats—The TRβ-selective agonist CGS-23425 has been found to exert lipid lowering actions in fat-fed rats (42). Thus, we examined the effects of this thyromimetic on hepatic apoAV levels in fat-fed rats treated with a daily single dose of the drug for 1 week. As expected, CGS-23425 increased the levels of apoAV protein, whereas actin content was unaffected (Fig. 10*A*). Furthermore, we observed that this TRβ-selective agonist induced a dramatic decrease both in VLDL-TG (44% of controls, data not shown) and in total plasma TG concentrations (Fig. 10*B*).

DISCUSSION

Clinical observations showing inverse correlation between the degree of triglyceridemia and thyroid status (4–8, 11) coupled with the fact that *APOA5* is a major determinant of TG homeostasis (24–32) prompted us to explore the potential regulation of this recently identified gene by TH. Our present

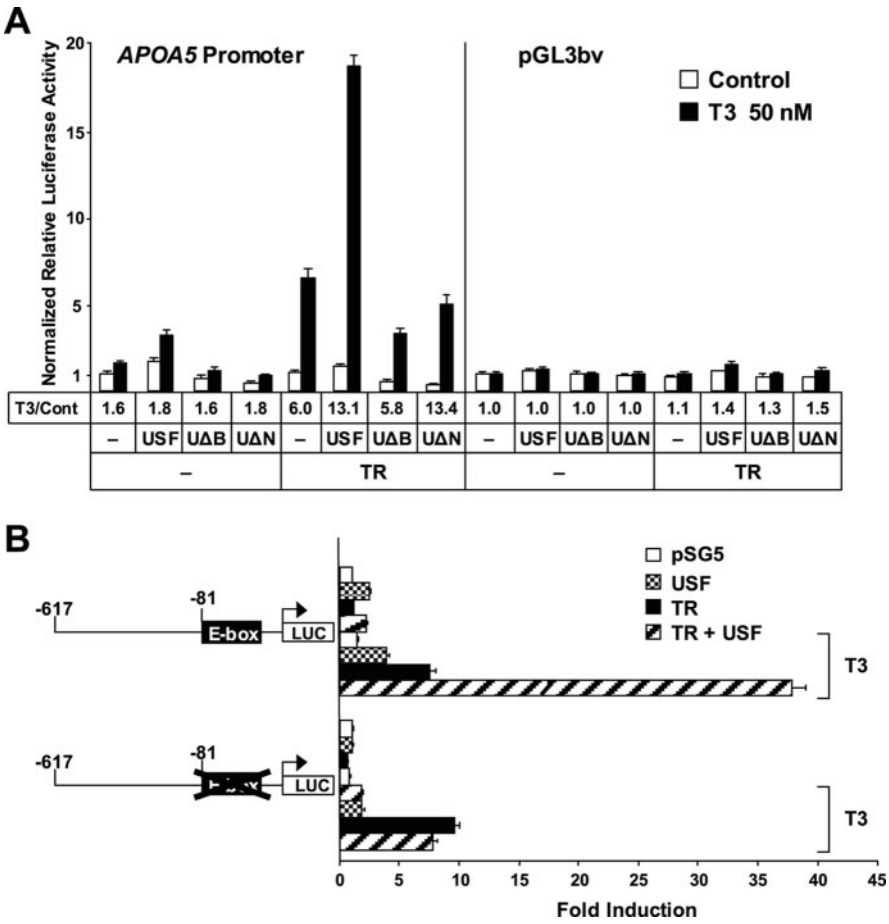

FIG. 8. **Functional evaluation of the contribution of USF transcriptional activation and DNA-binding domains, and the *APOA5* E-box to the T_3-dependent synergism between USF and TR.** *A*, HepG2 cells were transfected with a plasmid containing a luciferase reporter gene driven by the 5′-flanking region (−617/+18) of the human *APOA5* gene or the empty pGL3-basic vector together with plasmids expressing full-length USF2 (*USF*), an internal-deletion mutant of USF2 lacking the basic region required for DNA binding (*UΔB*), an N-terminal truncated USF2 lacking the activation domain (*UΔN*), TRβ1, or the empty vector pSG5 as control (−). Cells were incubated in the absence (*Control*) or the presence of 50 nM T_3 for 24 h, and luciferase activities were measured and expressed as described under "Experimental Procedures." Control levels for each reporter plasmid are set as 1. -Fold inductions by T_3 are indicated (*T3*/*Cont*). *B*, the E-box at −81 is required for the synergism between USF and T_3-activated TRβ1. Experiments were performed as in *A* with reporter constructs containing the wild-type or site-directed mutated *APOA5* promoter. The *cross* depicts the presence of −80A→C/−77T→C point mutations in the E-box site. *LUC*, luciferase. Results are expressed as -fold induction over control. The histograms are average values from two independent transfections, with each point conducted in triplicate.

findings demonstrate that T_3 directly up-regulates expression of the hypotriglyceridemic gene *APOA5*. Our experiments in hepatocytes revealed an increase in *APOA5* mRNA levels and in both cellular and secreted apoAV protein by T_3 and a lipid-lowering synthetic thyromimetic. Transient transfection experiments indicated that T_3 may increase *APOA5* expression at the transcriptional level via both TRα1 and TRβ1 isoforms. As demonstrated by EMSA and mutation analyses, activation by T_3 may be attributed to a DR4 element located within the proximal *APOA5* promoter. Our data from transfection assays using the isolated *APOA5* DR4 showed that this TRE is capable of conferring positive T_3 responsiveness to a heterologous promoter, further confirming that this DR4 motif corresponds to a genuine TRE. Taken together, our results demonstrate that *APOA5* is a direct target of TH.

We have ascertained that USF and TR synergistically activate *APOA5* in a ligand-dependent manner. We do not know the exact mechanisms implicated in this ligand-dependent synergism at this time. Interestingly, it has been shown that TR can physically interact with USF1 and USF2 (40). In addition, although T_3 induction of *APOA2* mRNA or protein abundance is yet to be reported, Kardassis and co-workers (53) have shown a synergistic interaction between TR and USF in the *APOA2* promoter. These authors hypothesized that USF might regulate the TR transcriptional activity by DNA binding-dependent and -independent modes; thus, it might regulate promoters that contain TREs but not necessarily USF binding sites (53). Two evidences suggest that this is not the case of *APOA5*. First, the DNA binding domain of USF is required for the synergism with TR in the T_3 induction of *APOA5*. Second, when the USF binding site in *APOA5* was mutated, USF had no effect in the presence of TR and T_3, thereby indicating that a DNA-binding mechanism is necessary for the synergistic action of USF on T_3-activated TR induction of *APOA5*.

On the other hand, although USF greatly enhances the T_3 stimulation of *APOA5*, several observations suggest that the dependence on USF is not straightforward. First, the *APOA5* TRE alone is sufficient to support a robust response to T_3 when linked to a basal heterologous promoter. Second, cotransfection of a mutant USF that lacks the DNA-binding domain diminishes the *APOA5* promoter activity, probably due to the sequestration of endogenous USF (46), but it produces no significant decrease in the -fold induction by T_3 in the presence of TR (5.8 *versus* 6.0 in Fig. 8*A*). Furthermore, mutational analyses of the E-box show that the induction by T3-activated TR is similar in both wild-type and mutant constructs. Hence, this E-box is absolutely required for the ligand-dependent synergism between TR and USF, but it appears not to be necessary for the sustenance of T_3 response. A similar situation has been described between sterol regulatory element-binding protein and the ubiquitous factor Sp1 in the *FAS* promoter (54). Whereas sterol regulatory element-binding protein and Sp1 synergistically activate the *FAS* promoter, mutational analyses revealed that the Sp1 site is dispensable for sterol regulation (54).

While this manuscript was in preparation, Nowak *et al.* (55) reported that insulin down-regulates *APOA5* expression via USF. Interestingly, in agreement with our study, these authors show by chromatin immunoprecipitation assays that the E-box at −81/−76 may bind USF. Even more interestingly, the combination of their report and our study raises the possibility of a cross-talk between insulin and thyroid signals on the same element in *APOA5*.

Strikingly, USF1 has been recently identified as the gene on human 1q21–23 that is associated with familial combined hyperlipidemia and especially with high triglycerides in men (56). Therefore, additional studies are warranted to address the rele-

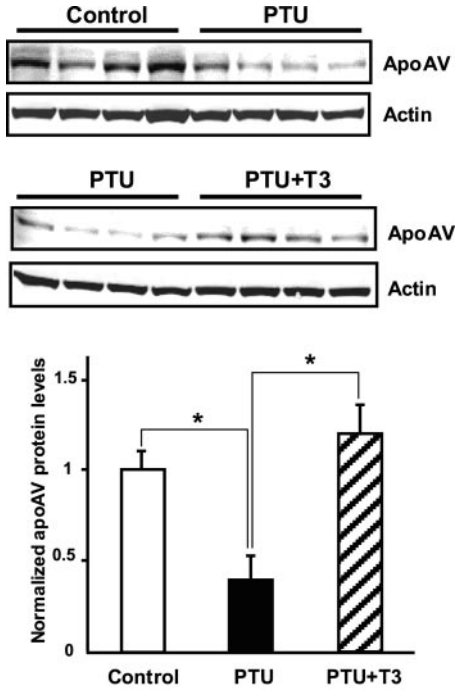

FIG. 9. **Hypothyroidism diminishes and T_3 treatment restores apoAV levels in rat.** Adult male rats were rendered hypothyroid by administration of PTU for 3 weeks. For the last 4 days, rats were treated with vehicle (*PTU*) or 300 μg/kg/day T_3 (*PTU+T3*). Control rats received only vehicles for the same periods of time (*Control*). 40 μg of protein from liver lysates were analyzed by Western blot as described under "Experimental Procedures." Signals were quantified by using Odyssey™ software. ApoAV levels normalized to β-actin content are expressed relative to untreated animals set as 1 and represent the mean ± S.D. of four animals/group. *, $p < 0.005$ *versus* vehicle PTU-treated rats.

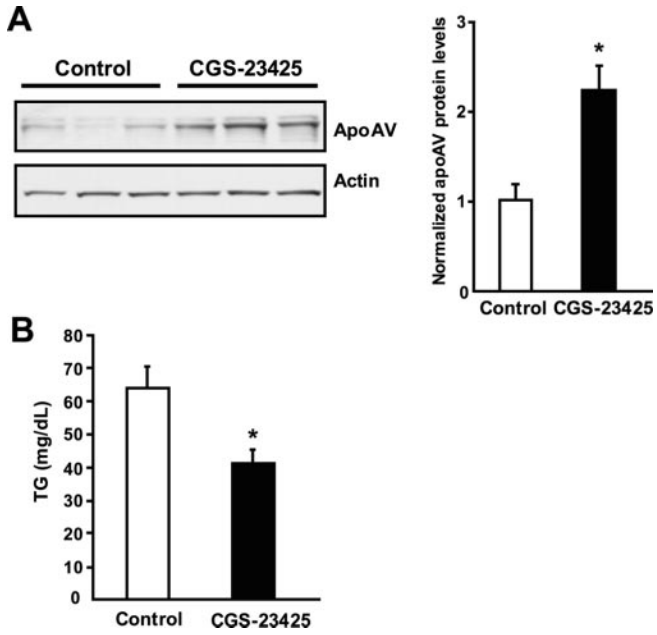

FIG. 10. **Treatment with the TRβ agonist CGS-23425 increases apoAV and reduces total TG in fat-fed rats.** Fat-fed rats were treated with solvent (*Control*) or 300 μg/kg/day CGS-23425 for 7 days. *A*, 40 μg of protein from liver lysates were analyzed by Western blot as described under "Experimental Procedures." A representative blot is shown. Protein signals were quantified by using Odyssey™ software, and apoAV levels were normalized to β-actin content. *B*, total plasma TG levels were determined by enzymatic methods. The data are expressed relative to untreated animals set as 1 and represent the mean ± S.D. of six animals/group. *, $p < 0.005$ *versus* control. The results are representative of two independent experiments.

vance of USF-mediated regulation of *APOA5* and other lipid-related genes in familial combined hyperlipidemia phenotype.

We sought to extend our analyses of TH-mediated regulation to animal models *in vivo*. However, the rat and mouse, commonly used animals for T_3 and TR studies, differ from humans in their response to T_3. Whereas LPL activity positively correlates with TH levels in humans (9, 11), hypothyroidism in rats increases both adipose and heart LPL mass and activity via a translational mechanism involving its 3′-untranslated region (57), whereas T_3 reverses these effects (58). Accordingly, PTU-induced hypothyroidism in rats has been associated with reductions in plasma TG (49, 50, 59, 60). Nevertheless, despite these overall effects, a number of indirect observations suggest the existence of an underlying hypotriglyceridemic response to T_3 in rodents. First, liver TG content decreases in hyperthyroid rats (61) and is increased in congenitally hypothyroid mice (62). Second, there is a lower secretion rate of VLDL by perfused livers from hyperthyroid rats, but the reverse occurs in hypothyroidism (63). Finally, serum TG clearance was significantly hastened by treatment with the TRβ ligand CGS-23425 in fat-fed rats (64). Our results show that PTU-induced hypothyroidism reduced apoAV content, whereas T_3 treatment restored normal levels, thereby indicating that thyroid status strongly correlates with apoAV levels in rats. Consistent with previous reports, we observed that TG levels were reduced in PTU-treated rats (data not shown). This paradox probably has functional implications, since it suggests that a decrease in apoAV does not necessarily impede an overall reduction of TG levels in hypothyroid rats, which is most likely due to the translational increase in adipose and heart LPL mass reported by others (57). On the other hand, we observed that treatment with CGS-23425 at a heart-sparing dose (42) increased apoAV content and decreased plasma TG levels in fat fed rats. This finding is consistent with the TG-lowering effects exhibited by the hepatoselective thyromimetic SK&F L-94901 in euthyroid rats (65) and by the TRβ-selective agonist GC-1 in hypothyroid mice (66). Although these data warrant further functional exploration, we and others (65, 66) hypothesize that the hypotriglyceridemic effects of these thyromimetics are most likely due to TR subtype-specific and organ-selective causes. Indeed, SK&F L-94901 does not discriminate TR subtype in terms of binding affinity, but it is more effectively transported to the nucleus in hepatic cells (67); GC-1, which has a 10-fold reduced affinity for TRα1, distributes primarily to the liver rather than to the heart or the adipose tissue (66); and the TRβ-selective ligand CGS-23425 is also markedly hepatoselective (42). Since TRα1 is the predominant isoform in adipose tissue (68), it is tempting to speculate that the tissue-selective properties of these thyromimetics contribute to a loss of effect on adipose LPL translation, whereas they induce the expression of hepatic genes such as *APOA5*. Given the two hypotriglyceridemic mechanisms suggested for apoAV (namely reduction in hepatic VLDL-TG secretion rate (32, 69) and elevation in the efficiency of LPL-mediated TG hydrolysis (32, 70)), the increase in apoAV that we observed in rats is consistent with studies showing lower secretion rates of VLDL in hyperthyroid rats (63), accelerated TG clearance in CGS-23425-treated rats (64), and lower plasma TG concentrations in thyromimetic-treated rodents (65, 66) (this study). On the other hand, GC-1 has been reported to suppress thyroid-stimulating hormone by 40% and to reduce plasma T_4 levels by 35% at therapeutic doses, but this was not accompanied by a statistically significant reduction in plasma T3 levels in rats (71). Whereas the administered GC-1 would compensate in terms of TRβ stimulation, it may not appreciably activate TRα1, which could therefore cause a relative TRα hypothyroidism. It is not known whether CGS-23425 modifies T_3, T_4, and thyroid-stimulating hormone plasma levels in rats; future work may address

the significance of these potential influences.

The molecular mechanisms implicated in the TG-lowering effects of TH in humans are currently unclear. Although the proximal promoter of the human *APOC2*, which encodes an LPL cofactor, could be transactivated by RXR-TR heterodimers (72), there are no data to indicate stimulation of *APOC2* expression by TH. Similarly, it has been shown that RXR-TR heterodimers bind with low affinity to the distal regulatory region of the human *APOC3* gene (73), an inhibitor of LPL-mediated lipolysis, although regulation of human *APOC3* expression by T_3 has not been previously reported. Indeed, our results in human hepatocytes suggest that the effect of T_3 on *APOC3*, if any, should be minimal. On the other hand, TH regulates hepatic apoB mRNA editing in neonatal mice (62) and rats (64), an effect that has been linked to increased hepatic TG secretion (62). However, since apobec-1 is not expressed in human liver, these findings may not be extended to humans.

The current results may provide a potential explanation for the low VLDL-TG clearance rates and low LPL activity observed in hypothyroid patients (6, 9) and for the increase in LPL activity observed in TH therapies (9, 11).

In conclusion, our data reveal that treatment with T_3 and pharmacological activation of TRβ up-regulates *APOA5* expression, thereby establishing a novel molecular pathway of T_3 action. These results underscore a physiological role of TR in the regulation of genes involved in triglyceride metabolism and, therefore, identify TRβ as a potential pharmacological target for the treatment of hypertriglyceridemia.

Acknowledgments—We thank Dr. Jorge Kirilovsky for encouragement and critical review of the manuscript; Dr. Edwige Nicodeme for scientific discussions; Boucif Djemai and Cyril Girault for excellent technical assistance with animals; and Dr. A. Brewster for manuscript corrections.

REFERENCES

1. Ford, E. S., Giles, W. H., and Dietz, W. H. (2002) *J. Am. Med. Assoc.* **287,** 356–359
2. Cullen, P. (2000) *Am. J. Cardiol.* **86,** 943–949
3. Grundy, S. M. (1998) *Am. J. Cardiol.* **81,** 18B–25B
4. O'Hara, D. D., Porte, D., Jr., and Williams, R. H. (1966) *Metabolism* **15,** 123–134
5. Nikkila, E. A., and Kekki, M. (1972) *J. Clin. Invest.* **51,** 2103–2114
6. Abrams, J. J., Grundy, S. M., and Ginsberg, H. (1981) *J. Lipid Res.* **22,** 307–322
7. O'Brien, T., Dinneen, S. F., O'Brien, P. C., and Palumbo, P. J. (1993) *Mayo Clin. Proc.* **68,** 860–866
8. Pykalisto, O., Goldberg, A. P., and Brunzell, J. D. (1976) *J. Clin. Endocrinol. Metab.* **43,** 591–600
9. Porte, D. Jr., O'Hara, D. D., and Williams, R. H. (1966) *Metabolism* **15,** 107–113
10. Tan, K. C., Shiu, S. W., and Kung, A. W. (1998) *J. Clin. Endocrinol. Metab.* **83,** 2921–2924
11. Ito, M., Takamatsu, J., Matsuo, T., Kameoka, K., Kubota, S., Fukata, S., Tamai, H., Miyauchi, A., Kuma, K., and Hanafusa, T. (2003) *Clin. Endocrinol.* **58,** 621–626
12. Yen, P. M. (2001) *Physiol. Rev.* **81,** 1097–1142
13. Glass, C. K. (1994) *Endocr. Rev.* **15,** 391–407
14. Williams, G. R. (2000) *Mol. Cell. Biol.* **20,** 8329–8342
15. Berkenstam, A., Farnegardh, M., and Gustafsson, J. A. (2004) *Mech. Ageing Dev.* **125,** 707–717
16. Schwartz, H. L., Strait, K. A., Ling, N. C., and Oppenheimer, J. H. (1992) *J. Biol. Chem.* **267,** 11794–11799
17. Weiss, R. E., Murata, Y., Cua, K., Hayashi, Y., Seo, H., and Refetoff, S. (1998) *Endocrinology* **139,** 4945–4952
18. Xiong, S., Chirala, S. S., Hsu, M. H., and Wakil, S. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12260–12265
19. Petty, K. J., Desvergne, B., Mitsuhashi, T, and Nikodem, V. M. (1990) *J. Biol. Chem.* **265,** 7395–7400
20. Barrero, M. J., Marrero, P. F., and Haro, D. (2000) *Biochem. Biophys. Res. Commun.* **279,** 81–88
21. Jansen, M. S., Cook, G. A., Song, S., and Park, E. A. (2000) *J. Biol. Chem.* **275,** 34989–34997
22. Drover, V. A., Wong, N. C., and Agellon, L. B. (2002) *J. Mol. Endocrinol.* **16,** 14–23
23. Shin, D. J., and Osborne, T. F. (2003) *J. Biol. Chem.* **278,** 34114–34118
24. Pennacchio, L. A., Olivier, M., Hubacek, J. A., Cohen, J. C., Cox, D. R., Fruchart, J. C., Krauss, R. M., and Rubin, E. M. (2001) *Science* **294,** 169–173
25. van der Vliet, H. N., Sammels, M. G., Leegwater, A. C., Levels, J. H., Reitsma, P. H., Boers, W., and Chamuleau, R. A. (2001) *J. Biol. Chem.* **276,** 44512–44520
26. van der Vliet, H. N., Schaap, F. G., Levels, J. H., Ottenhoff, R., Looije, N., Wesseling, J. G., Groen, A. K., and Chamuleau, R. A. (2002) *Biochem. Biophys. Res. Commun.* **295,** 1156–1159
27. Pennacchio, L. A., Olivier, M., Hubacek, J. A., Krauss, R. M., Rubin, E. M., and Cohen, J. C. (2002) *Hum. Mol. Genet.* **11,** 3031–3038
28. Ribalta, J., Figuera, L., Fernandez-Ballart, J., Vilella, E., Castro Cabezas, M., Masana, L., and Joven, J. (2002) *Clin. Chem.* **48,** 1597–1600
29. Hubacek, J. A., Skodova, Z., Adamkova, V., Lanska, V., and Poledne, R. (2004) *Clin. Genet.* **65,** 126–130
30. Lai, C. Q., Demissie, S., Cupples, L. A., Zhu, Y., Adiconis, X., Parnell, L. D., Corella, D., and Ordovas, J. M. (2004) *J. Lipid Res.* **45,** 2096–2105
31. Oliva, C. P., Pisciotta, L., Li Volti, G., Sambataro, M. P., Cantafora, A., Bellocchio, A., Catapano, A., Tarugi, P., Bertolini, S., and Calandra, S. (2005) *Arterioscler. Thromb. Vasc. Biol.* **25,** 411–417
32. Schaap, F. G., Rensen, P. C., Voshol, P. J., Vrins, C., van der Vliet, H. N., Chamuleau, R. A., Havekes, L. M., Groen, A. K., and van Dijk, K. W. (2004) *J. Biol. Chem.* **279,** 27941–27947
33. Zannis, V. I., Kan, H. Y., Kritis, A., Zanni, E. E., and Kardassis, D. (2001) *Curr. Opin. Lipidol.* **12,** 181–207
34. Vu-Dac, N., Gervois, P., Jakel, H., Nowak, M., Bauge, E., Dehondt, H., Staels, B., Pennacchio, L. A., Rubin, E. M., Fruchart-Najib, J., and Fruchart, J. C. (2003) *J. Biol. Chem.* **278,** 17982–17985
35. Prieur, X., Coste, H., and Rodríguez, J. C. (2003) *J. Biol. Chem.* **278,** 25468–25480
36. Jakel, H., Nowak, M., Moitrot, E., Dehondt, H., Hum, D. W., Pennacchio, L. A., Fruchart-Najib, J., and Fruchart, J. C. (2004) *J. Biol. Chem.* **279,** 45462–45469
37. McKnight, S. L. (1982) *Cell* **31,** 355–365
38. Applied Biosystems (2001) *User Bulletin* 2, Applied Biosystems, Foster City, CA
39. Suzuki, S., Miyamoto, T., Opsahl, A., Sakurai, A., and DeGroot, L. J. (1994) *Mol. Endocrinol.* **8,** 305–314
40. Jackson-Hayes, L., Song, S., Lavrentyev, E. N., Jansen, M. S., Hillgartner, F. B., Tian, L., Wood, P. A., Cook, G. A., and Park, E. A. (2003) *J. Biol. Chem.* **278,** 7964–7972
41. Coste, H., and Rodríguez, J. C. (2002) *J. Biol. Chem.* **277,** 27120–27129
42. Taylor, A. H., Stephan, Z. F., Steele, R. E., and Wong, N. C. (1997) *Mol. Pharmacol.* **52,** 542–547
43. Bakker, O., Hudig, F., Meijssen, S., and Wiersinga, W. M. (1998) *Biochem. Biophys. Res. Commun.* **249,** 517–521
44. Perlmann, T., Rangarajan, P. N., Umesono, K., and Evans, R. M. (1993) *Genes Dev.* **7,** 1411–1422
45. Shih, H. M., Liu, Z., and Towle, H. C. (1995) *J. Biol. Chem.* **270,** 21991–21997
46. Meier, J. L., Luo, X., Sawadogo, M., and Straus, S. E. (1994) *Mol. Cell. Biol.* **14,** 6896–6906
47. Luo, X., and Sawadogo, M. (1996) *Mol. Cell. Biol.* **16,** 1367–1375
48. Yamada, T., Chopra, I. J., and Kaplowitz, N. (1981) *J. Endocrinol. Invest.* **4,** 379–387
49. Wilcox, H. G., Keyes, W. G., Hale, T. A., Frank, R., Morgan, D. W., and Heimberg, M. (1982) *J. Lipid Res.* **23,** 1159–1166
50. Meyer, B. J., Ha, Y. C., and Barter, P. J. (1989) *Biochim. Biophys. Acta* **1004,** 73–79
51. Vacca, R. A., Moro, L., Caraccio, G., Guerrieri, F., Marra, E., and Greco, M. (2003) *Endocrinology* **144,** 3783–3788
52. Van Herle, A. J., Klandorf, H., and Uller, R. P. (1975) *J. Clin. Invest.* **56,** 1073–1081
53. Hatzivassiliou, E., Koukos, G., Ribeiro, A., Zannis, V., and Kardassis, D. (2003) *Biochem. J.* **376,** 423–431
54. Magana, M. M., Koo, S. H., Towle, H. C., and Osborne, T. F. (2000) *J. Biol. Chem.* **275,** 4726–4733
55. Nowak, M., Helleboid-Chapman, A., Jakel, H., Martin, G., Duran-Sandoval, D., Staels, B., Rubin, E. M., Pennacchio, L. A., Taskinen, M. R., Fruchart-Najib, J., and Fruchart, J. C. (2005) *Mol. Cell. Biol.* **25,** 1537–1548
56. Pajukanta, P., Lilja, H. E., Sinsheimer, J. S., Cantor, R. M., Lusis, A. J., Gentile, M., Duan, X. J., Soro-Paavonen, A., Naukkarinen, J., Saarela, J., Laakso, M., Ehnholm, C., Taskinen, M. R., and Peltonen, L. (2004) *Nat. Genet.* **36,** 371–376
57. Kern, P. A., Ranganathan, G., Yukht, A., Ong, J. M., and Davis, R. C. (1996) *J. Lipid Res.* **37,** 2332–2340
58. Saffari, B., Ong, J. M., and Kern, P. A. (1992) *J. Lipid Res.* **33,** 241–249
59. Dory, L., and Roheim, P. S. (1981) *J. Lipid Res.* **22,** 287–296
60. Popovic, L., Oreskovic, K., Zupancic, B., Petrunic, M., and Kunec-Vajic, E. (1998) *Horm. Metab. Res.* **30,** 137–140
61. Varas, S. M., Oliveros, L. B., and Giménez, M. S. (1999) *Horm. Metab. Res.* **31,** 514–518
62. Mukhopadhyay, D., Plateroti, M., Anant, S., Nassir, F., Samarut, J., and Davidson, N. O. (2003) *Endocrinology* **144,** 711–719
63. Keyes, W. G., and Heimberg, M. (1979) *J. Clin. Invest.* **64,** 182–190
64. Wada, Y., Matsubara, S., Dufresne, J., Hargrove, G. M., Stephan, Z. F., Steele, R. E., and Wong, N. C. (2000) *J. Mol. Endocrinol.* **25,** 299–308
65. Ness, G. C., Lopez, D., Chambers, C. M., Newsome, W. P., Cornelius, P., Long, C. A., and Harwood, H. J., Jr. (1998) *Biochem. Pharmacol.* **56,** 121–129
66. Trost, S. U., Swanson, E., Gloss, B., Wang-Iverson, D. B., Zhang, H., Volodarsky, T., Grover, G. J., Baxter, J. D., Chiellini, G., Scanlan, T. S., and Dillmann, W. H. (2000) *Endocrinology* **141,** 3057–3064
67. Ichikawa, K., Miyamoto, T., Kakizawa, T., Suzuki, S., Kaneko, A., Mori, J., Hara, M., Kumagai, M., Takeda, T., and Hashizume, K. (2000) *J. Endocrinol.* **165,** 391–397
68. Jiang, W., Miyamoto, T., Kakizawa, T., Sakuma, T., Nishio, S., Takeda, T., Suzuki, S., and Hashizume, K. (2004) *J. Endocrinol.* **182,** 295–302

69. Weinberg, R. B., Cook, V. R., Beckstead, J. A., Martin, D. D., Gallagher, J. W., Shelness, G. S., and Ryan, R. O. (2003) *J. Biol. Chem.* **278,** 34438–34444
70. Fruchart-Najib, J., Baugé, E., Niculescu, L. S., Pham, T., Thomas, B., Rommens, C., Majd, Z., Brewer, B., Pennacchio, L. A., and Fruchart, J. C. (2004) *Biochem. Biophys. Res. Commun.* **319,** 397–404
71. Grover, G. J., Egan, D. M., Sleph, P. G., Beehler, B. C., Chiellini, G., Nguyen, N. H., Baxter, J. D., and Scanlan, T. S. (2004) *Endocrinology* **145,** 1656–1661
72. Kardassis, D., Sacharidou, E., and Zannis, V. I. (1998) *J. Biol. Chem.* **273,** 17810–17816
73. Lavrentiadou, S. N., Hadzopoulou-Cladaras, M., Kardassis, D., and Zannis, V. I. (1999) *Biochemistry* **38,** 964–975