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Orphan Nuclear Hormone Receptor Rev-erbα Regulates the Human Apolipoprotein CIII Promoter*

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Apolipoprotein CIII (apoCIII) plays an important role in plasma triglyceride and remnant lipoprotein metabolism. Because hypertriglyceridemia is an independent risk factor in coronary artery disease and the presence in plasma of triglyceride-rich remnant lipoproteins is correlated with atherosclerosis, considerable research efforts have been focused on the identification of factors regulating apoCIII gene expression to decrease its production. Here we report that the orphan nuclear hormone receptor Rev-erbα regulates the human apoCIII gene promoter. In apoCIII expressing human hepatic HepG2 cells, transfection of Rev-erbα specifically repressed apoCIII gene promoter activity. We determined by deletion and site-directed mutagenesis experiments that Rev-erbα dependent repression is mainly due to an element present in the proximal promoter of the apoCIII gene. In contrast, we found no functional Rev-erbα response elements in the convergently transcribed human apoAI gene or the common regulatory enhancer. The identified Rev-erbα response element coincides with a RORα1 element, and in the present study we provide evidence that functional cross-talk between these orphan receptors modulates the apoCIII promoter. *In vitro* binding analysis showed that monomers of Rev-erbα bound this element but not another upstream RORα1 response element. In addition, we showed that the closely related nuclear orphan receptor RVR also specifically repressed the human apoCIII gene. These studies underscore a novel physiological role for members of the Rev-erb family of nuclear receptors in the regulation of genes involved in triglyceride metabolism and the pathogenesis of atherosclerosis.

Recent large scale clinical trials indicate that hypertriglyceridemia is an independent risk factor for coronary artery disease (1–4). In addition, triglyceride-rich remnant lipoproteins are positively correlated to the progression of atherosclerosis, which remains one of the leading causes of death in the Western world (5, 6).

Apolipoprotein CIII (apoCIII)[1] is a key protein in plasma triglyceride metabolism. It is well established that the plasma concentration and synthesis rate of apoCIII are positively correlated with plasma triglycerides, both in normal and hypertriglyceridemic subjects (7–9). In fact, apoCIII deficiency in humans results in increased catabolism of very low density lipoprotein particles (10), whereas increased apoCIII synthesis is associated with hypertriglyceridemia (11). Overexpression of human apoCIII in mice results in severe hypertriglyceridemia (12), whereas disruption of the endogenous apoCIII gene protects the mice from postprandial hypertriglyceridemia (13). apoCIII has been shown to delay the catabolism of triglyceride-rich particles by several mechanisms (14–17), including inhibition of lipoprotein binding to the cell surface glycosaminoglycan matrix (17) and lipolysis by lipoprotein lipase (10, 18). Moreover, studies with retinoids, fibrates, and other pharmacological agents show evidence that the modulation of apoCIII expression has an important role in hypertriglyceridemia (19–23). Therefore, the identification of factors capable of decreasing plasma apoCIII concentrations by down-regulation of its expression is of major importance for the treatment of dyslipidemia.

In humans, apoCIII is synthesized in the liver and, to a much lesser extent, in the intestine. The expression of apoCIII is strongly regulated at the transcriptional level, and the regulatory sequences responsible for the tissue-specific expression pattern of the apoCIII gene and for its modulation by signal transduction pathways have been extensively described (reviewed in Ref. 24). The 5′-flanking region of the human apoCIII gene contains a proximal promoter and a distal regulatory enhancer (25). The gene for apoCIII resides in an apolipoprotein gene cluster on chromosome 11q23 between the apoAI and apoAIV genes (26, 27). The product of the convergently transcribed apoAI gene is the major protein component of high density lipoproteins. Plasma levels of apoAI and high density lipoprotein cholesterol correlate with each other, and both correlate inversely with the incidence of coronary heart disease (28, 29). Importantly, the distal regulatory region of apoCIII acts as a common enhancer for the three genes of the cluster (24, 30–32).

The orphan nuclear receptor Rev-erb (NR1D) group contains two members in mammals: Rev-erbα (33–35) and RVR (also known as Rev-erbβ and BD73) (36–39). Rev-erb receptors are widely expressed (33, 37) and have been described as negative transcriptional regulators of the Rev-erbα gene itself (40) as well as N-*myc* (41), enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (42), α-fetoprotein (43), and rat apoAI (44) genes. Rev-erbα mRNA is induced dramatically during adipogenesis (45), and a role has also been proposed for both Rev-erbα and RVR in myogenesis (46). However, the specific biological functions of these receptors in adipogenesis or in other physiological processes are still unknown.

Monomers of Rev-erbα and RVR bind to the six-nucleotide

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[1] The abbreviations used are: apoAI, apoAIV, and apoCIII, apolipoprotein AI, AIV and CIII, respectively; HNF-4, hepatocyte nuclear factor-4; nt, nucleotide(s); PPAR, peroxisome proliferator-activated receptor; ROR, retinoic acid-related orphan receptor; RORE, retinoic acid-related orphan receptor response element; RXR, retinoid X receptor; TK, thymidine kinase.

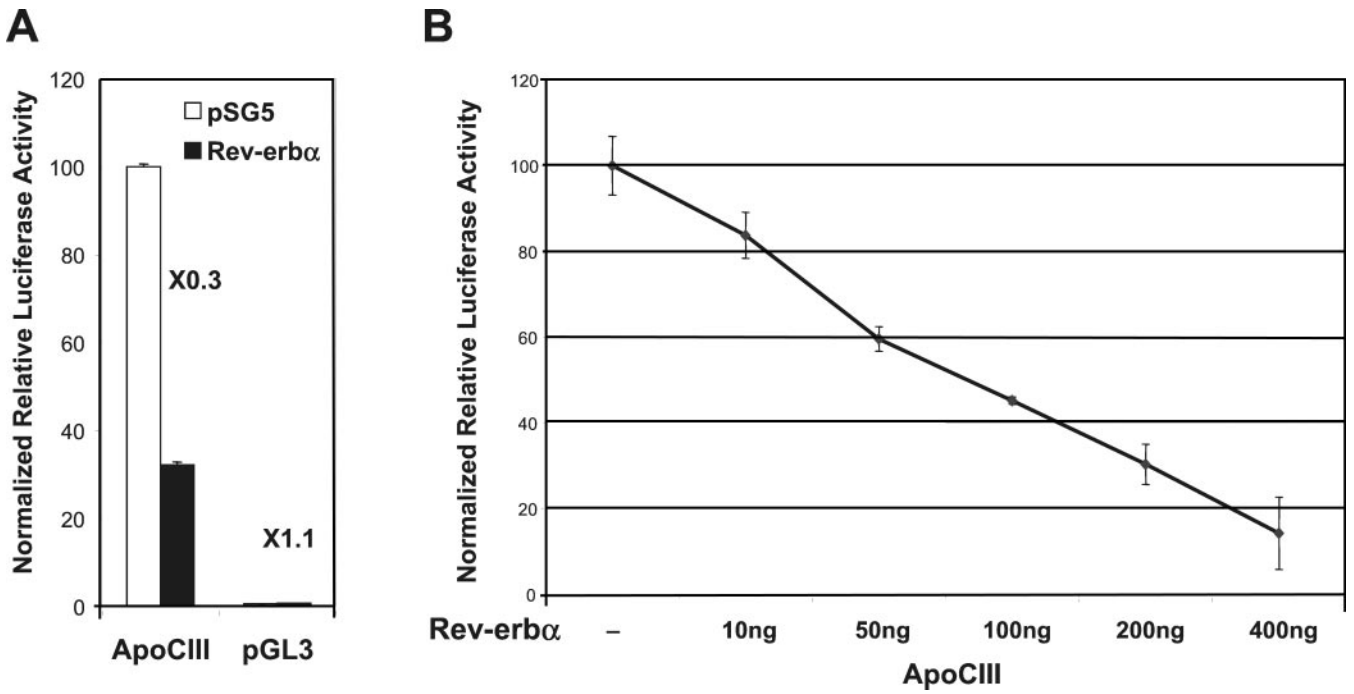

FIG. 1. **Rev-erbα represses the activity of the human apoCIII gene promoter.** *A*, HepG2 cells were transiently transfected with a plasmid containing a luciferase reporter gene driven by the 5′-flanking region (−1408/+24) of the human apoCIII gene or the empty pGL3-basic vector along with a plasmid expressing human Rev-erbα (200 ng) or the empty expression vector as control (pSG5). *B*, the reporter plasmid containing the −1408/+24 region of human apoCIII cloned in front of the luciferase gene was cotransfected with increasing amounts of a Rev-erbα expression plasmid into HepG2 cells. Plasmid dosage was kept constant by the addition of empty expression vector. Luciferase activities were measured and expressed as described under "Experimental Procedures." The fold change relative to control level is shown for each construct.

core motif PuGGTCA preceded by a 6-bp AT-rich region (36–39, 47, 48). In addition, homodimers of Rev-erbα and RVR bind to a direct repeat of the core sequence separated by two nucleotides with an AT-rich 5′ extension (Rev-DR2) (40, 48). Although the core binding site is recognized by the P-box/first helix of the DNA-binding domain, the precise recognition of the sequences 5′ to the core binding site is determined by the C-terminal extension of the zinc finger domains (49). Rev-erbα and RVR share a high degree of sequence similarity with the C-terminal extension/third helix of peroxisome proliferator-activated receptors (PPARs) (NR1C) and retinoic acid-related orphan receptors (RORs, also termed RZRs) (NR1F). As a consequence, Rev-erbα binds to the PPAR response elements of CYP4A6 and the hydratase/dehydrogenase bifunctional enzyme genes and interferes with the PPAR-dependent transactivation of the former gene (42, 49). Conversely, the Rev-DR2 site described in the promoter of the human Rev-erbα gene is also a PPAR response element (50). It should be noted that although the sequence determinants required for Rev-erbα and PPAR bindings are similar, they are not identical as evidenced by the incapacity of Rev-erbα to bind to the fatty acyl-CoA oxidase PPAR response element (42, 49). The gene for N-*myc*, as well as the rat α-fetoprotein and apoAI genes, have been shown to contain elements that bind and are regulated by members of both Rev-erb and RORα groups (41, 43, 44). The sequence requirements for RORα, Rev-erbα, and RVR are closely related (37). Nevertheless, they are not identical (49), so the putative cross-talk between those nuclear receptors should be analyzed in terms of case-by-case studies.

We have learnt from Staels and co-workers (51) that RORα1 regulates apoCIII gene transcription through binding sites located in its proximal promoter. The importance of RORα in apoCIII expression was underscored by studies with the *staggerer* mice, which carry a natural deletion in the RORα gene (52). These mice show decreased plasma triglyceride and apoCIII levels compared with wild type mice (51).

In this report, we demonstrate that Rev-erbα regulates the human apoCIII gene promoter. Rev-erbα represses transcription through one of the functional RORα1 response elements present in the apoCIII gene proximal promoter. Our results suggest a novel role for Rev-erbα in regulating triglyceride metabolism.

EXPERIMENTAL PROCEDURES

Plasmids—The 5′-flanking region of human apoCIII gene (nt −1408 to +24, relative to the transcription start site) was obtained by PCR using human genomic DNA as a template (CLONTECH). Forward primer contained nt −1408 to −1383 and was tailed with a *Nhe*I restriction site. Reverse primer contained nt +24 to +2 and was tailed with a *Hind*III site. The PCR product was digested with *Nhe*I and *Hind*III and cloned into the corresponding sites of the promoter-less firefly (*Photinus pyralis*) luciferase reporter plasmid pGL3-basic (Promega), generating p−1408/+24hC3LUC (also designated wild type). For the construction of p−108/+24hC3LUC, a DNA fragment was generated by PCR using p−1408/+24hC3LUC as a template and a *Nhe*I site tailed forward primer containing nt −108 to −86, with the reverse primer used for the former construction. The amplified promoter fragment was digested with *Nhe*I/*Hind*III enzymes and cloned into pGL3-basic vector. The 5′ region of human apoAI gene (nt −1170 to +235, relative to the transcription start site) was obtained by PCR using human genomic DNA as a template, a *Bgl*II site tailed forward primer containing nt −1170 to −1152 and a *Hind*III site tailed reverse primer containing nt +235 to +215. After digestion with *Bgl*II and *Hind*III, it was cloned into pGL3-basic vector to produce p−1170/+235hAILUC. The 5′-flanking region of rat apoAI gene (nt −823 to +31, relative to the transcription start site) was obtained by PCR using Sprague-Dawley rat genomic DNA as a template (CLONTECH), a *Nhe*I site tailed forward primer containing nt −823 to −804 and a *Hind*III site tailed reverse primer containing nt +31 to +10. The resulting fragment was cut with *Nhe*I and *Hind*III and then cloned in the corresponding sites of pGL3-basic vector to generate p−823/+31rAILUC. Site-directed mutagenesis of the construct p−1408/+24hC3LUC was accomplished using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the recommendations of the manufacturer and two pairs of 34-mer oligonucleotides containing mutations corresponding, respectively, to nt −21(G → A)/−22(G → C) and to nt −78(G → C)/−79(G → A) of human apoCIII promoter. The vector pGL3-TK contains a frag-

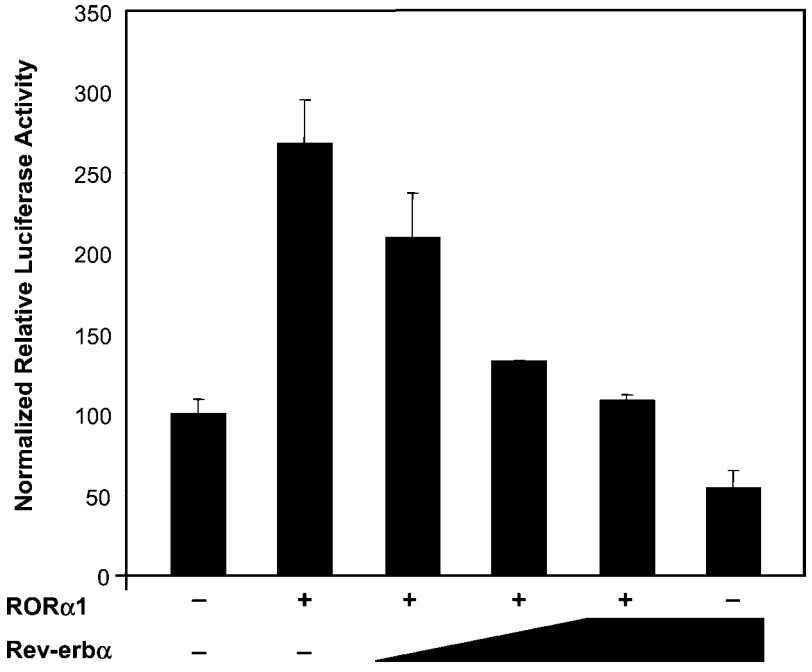

FIG. 2. **Rev-erbα antagonizes RORα1-induced activation of the human apoCIII gene promoter.** HepG2 cells were transfected with a plasmid containing a luciferase reporter gene driven by the 5′-flanking region (−1408/+24) of the human apoCIII gene together with the empty expression vector pSG5 or a constant amount of the expression plasmid encoding human RORα1 (100 ng) and increasing amounts (0, 25, 50, and 100 ng) of the Rev-erbα expression plasmid. Plasmid dosage was kept constant by the addition of empty expression vector. The luciferase activities were measured and expressed as described under "Experimental Procedures."

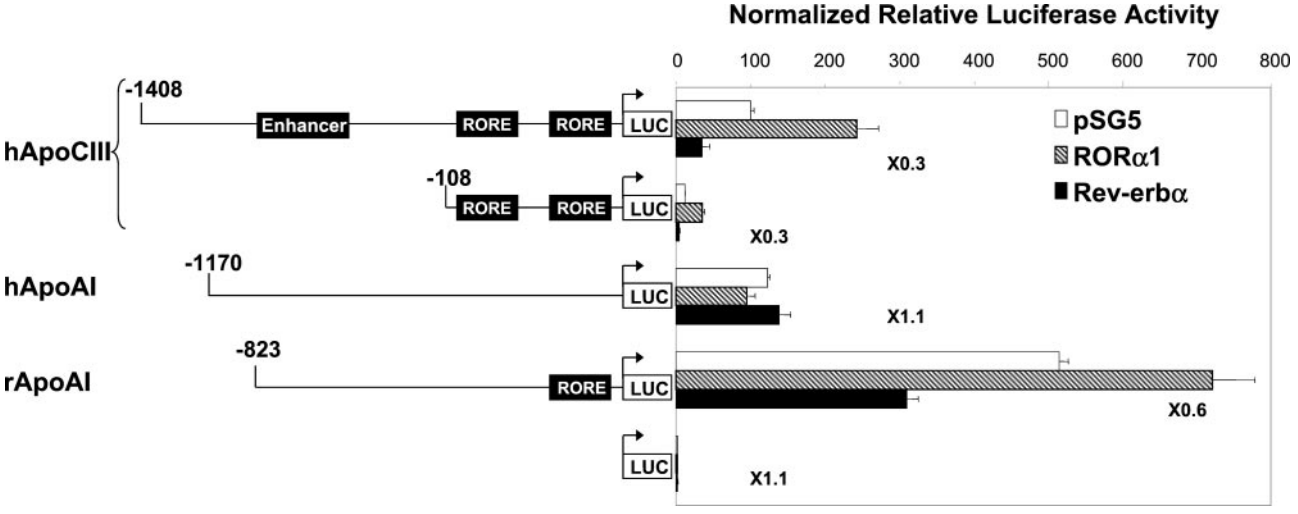

FIG. 3. **Rev-erbα selectively represses the human apoCIII proximal promoter but not the human apoAI promoter.** HepG2 cells were transfected with reporter plasmids containing the firefly luciferase gene driven by nt −1408/+24 or −108/+24 of the 5′-flanking region of the human apoCIII gene, the human apoAI (−1170/+235), or rat apoAI (−823/+31) genes or with the empty pGL3-basic vector as a negative control, along with expression plasmids encoding human RORα1 or human Rev-erbα (200 ng) or the empty pSG5 expression vector as control. The luciferase activities were measured and expressed as described under "Experimental Procedures." The fold change by Rev-erbα relative to control level is shown for each construct. *Enhancer*, distal regulatory enhancer; *LUC*, luciferase.

ment corresponding to nt −109 to +20 of the TK gene promoter of herpes simplex virus (53) subcloned into the *Bgl*II/*Hind*III sites of pGL3-basic vector. The reporter plasmid pREVDR2-TKLUC was generated by insertion of a double-stranded oligonucleotide containing the Rev-DR2 response element of the human Rev-erbα gene promoter (5′-GATCCGGAAAAGTGTGTCACTGGGGCACA-3′) (40) into the *Bgl*II site of pGL3-TK. Similarly, three copies of a double-stranded oligonucleotide containing wild type (5′-GATCCGATATAAAACAGGTCAGAACCCTA-3′) or mutant (5′-GATCCGATATAAAACACATCAGAACCCTA-3′) sequences spanning nt −33 to −11 and wild type (5′-GATCCTCAGCAGGTGACCTTTGCCCAGCA-3′) or mutant (5′-GATCCTCAGCAGGTGATGTTTGCCCAGCA-3′) sequences corresponding to nt −90 to −68 of human apoCIII gene promoter were inserted into the *Bgl*II site of pGL3-TK to generate, respectively, p(wtC3RE1)$_3$-TKLUC, p(mutC3RE1)$_3$-TKLUC, p(wtC3RE2)$_3$-TKLUC, and p(mutC3RE2)$_3$-TKLUC. Plasmids expressing human cDNAs for Rev-erbα, RORα1, and RVR were provided by J. A. Holt and J. T. Moore (GlaxoSmithKline, Research Triangle Park, NC). The backbone of those plasmids was the mammalian expression vector pSG5 with a modified polylinker. Plasmid DNA was prepared using the Qiagen endotoxin-free Maxipreparation method and quantitated spectrophotometrically. The integrities of all of the plasmids were verified by DNA sequencing.

Cell Transfection and Reporter Assays—Human hepatoblastoma HepG2 cells were cultured in basal Eagle medium supplemented with nonessential amino acids and 10% (v/v) fetal calf serum. On day 0, the cells were seeded on 24-well plates at a density of 3.5×10^5 cells/well. On day 1, the cells were transfected with FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Typically, each well of a 24-well plate received 100 ng of firefly luciferase reporter plasmid and, when indicated, 0–400 ng of plasmids expressing human Rev-erbα, RORα1, or RVR. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pSG5. 10 ng/well of a sea pansy (*Renilla reniformis*) luciferase plasmid pRL-SV40 (Promega) was included in all transfections as an internal control for transfection efficiency. On day 3, the cell lysates were prepared by shaking the cells in 200 μl of 1× Promega lysis buffer for 10 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* lucifer-

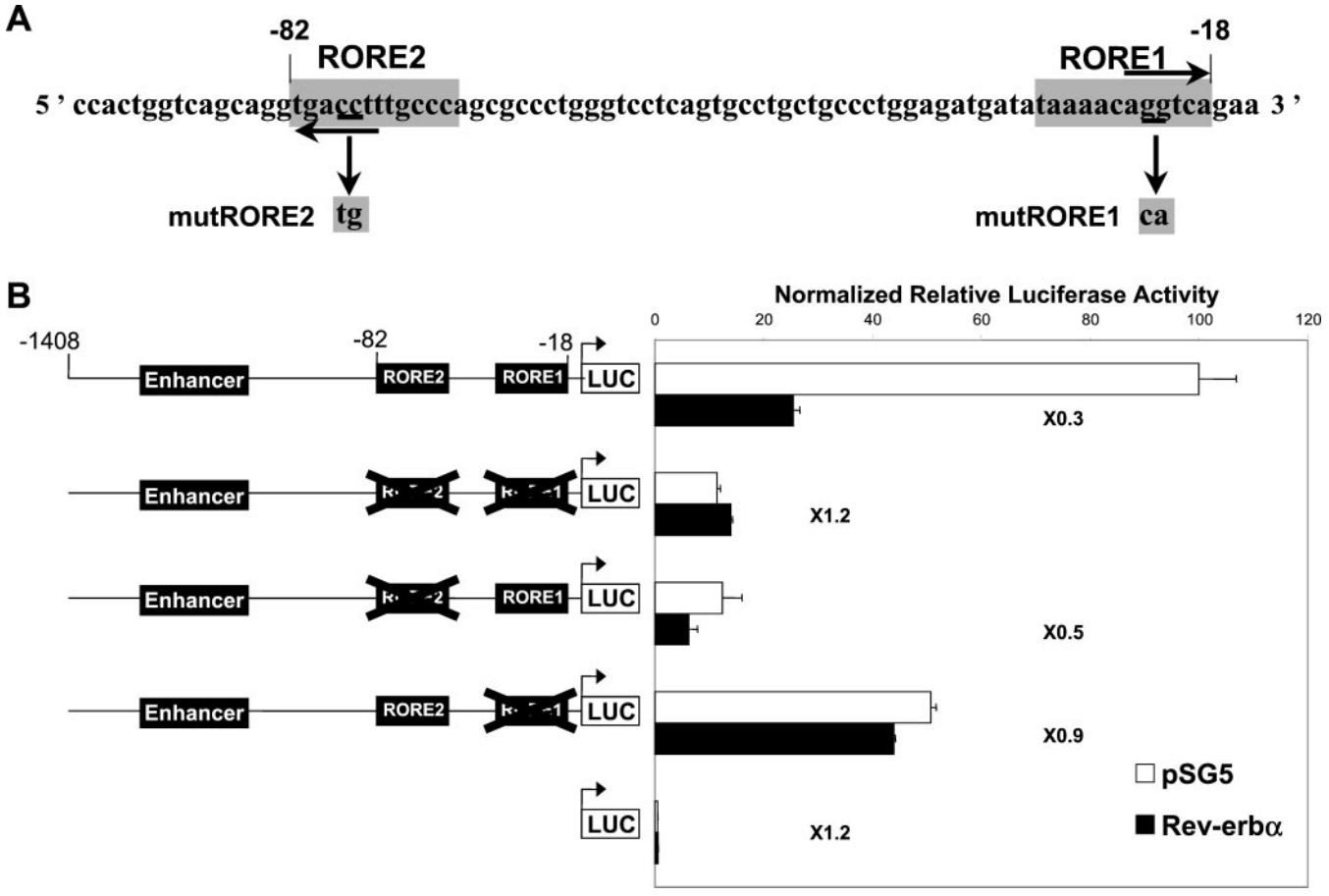

FIG. 4. **Functional effects of mutations in ROREs on the response of the human apoCIII promoter to Rev-erbα.** *A*, human apoCIII gene promoter sequence surrounding the ROREs. The *gray boxes* denote the RORE1 and RORE2 sequences. The AGGTCA half-sites are indicated by *horizontal arrows*. The wild type nucleotides that were modified by site-directed mutagenesis are *underlined*. The corresponding −21(G → A)/−22(G → C) and −78(G → C)/−79(G → A) mutated nucleotides are shown *below* the *vertical arrows* and within the *gray squares*. *B*, HepG2 cells were transfected with a plasmid expressing human Rev-erbα (200 ng) or the empty pSG5 vector as control together with reporter constructs containing the wild type or site-directed mutated 5′-flanking regions (−1408/+24) of the human apoCIII gene or the empty pGL3-basic vector as negative control. The luciferase activities were measured and expressed as described under "Experimental Procedures." The fold change by Rev-erbα relative to control level is shown for each construct. *Crosses* indicate the presence of site-directed mutations in a specific RORE. *Enhancer*, distal regulatory enhancer; *LUC*, luciferase.

ase activity values to obtain normalized luciferase activities. To facilitate comparisons within a given experiment, the activity data were presented as relative luciferase activities. All of the transfection experiments were performed at least three times, with each experimental point done in triplicate. The data are expressed as the means ± S.D.

In Vitro Transcription/Translation and Electrophoretic Mobility Shift Analysis—Human Rev-erbα and human RORα1 proteins were synthesized *in vitro* from the corresponding expression plasmids in rabbit reticulocyte lysate by using TNT® Quick Coupled transcription/translation system (Promega) according to the instructions of the manufacturer. To obtain an unprogrammed lysate as a negative control for electrophoretic mobility shift analysis, a reaction was performed with the empty vector pSG5. Protein-DNA binding assays were performed as described (54). Double-stranded oligonucleotides corresponding to the Rev-DR2 response element of human Rev-erbα gene promoter (RevDR2), wild type nt −33 to −11 (wtC3RE1), wild type nt −90 to −68 (wtC3RE2) of human apoCIII gene promoter (described under "Plasmids"), and a consensus sequence for the binding of monomers of Rev-erbα (47, 48) (consensus sequence) were radiolabeled by fill in with the Klenow fragment of DNA polymerase I. For the competition experiments, 10-, 50-, or 100-fold molar excess of unlabeled probes were included during a 10-min preincubation on ice. mtC3RE1 is a double-stranded oligonucleotide corresponding to a mutant sequence spanning nt −33 to −11 of human apoCIII promoter (described under "Plasmids"). The samples were electrophoresed at 4 °C on a 4.5% polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). The gels were dried and analyzed using a Molecular Dynamics PhosphorImager STORM 860 and ImageQuant software (Amersham Biosciences).

RESULTS

The Orphan Nuclear Receptor Rev-erbα Represses the Activity of the Human apoCIII Gene Promoter—To find factors capable of down-regulating the expression of the human apoCIII gene without affecting apoAI or other human lipid-related genes, we cloned their promoters in front of the firefly luciferase reporter gene and performed cotransfection experiments in the human hepatoblastoma cell line HepG2, which synthesizes apoCIII. Cotransfection of the human orphan nuclear receptor Rev-erbα expression plasmid resulted in a reduction of firefly luciferase activity driven by the 5′-flanking sequence of the human apoCIII gene (nt −1408 to +24) (Fig. 1*A*). The repression by Rev-erbα was promoter-dependent because it was not observed with the promoter-less pGL3-basic vector (Fig. 1*A*) or with constructs containing the promoter of human apoAI (see below) or other lipid-related genes (not shown). Furthermore, increasing amounts of the expression plasmid for Rev-erbα resulted in a dose-dependent inhibition of firefly luciferase activity (Fig. 1*B*). As shown in Fig. 1*B*, the repression by Rev-erbα was already appreciable at low doses.

Rev-erbα Antagonizes Transactivation by RORα1 from Human apoCIII Gene Promoter—Transcription of human apoCIII gene is regulated by the orphan nuclear receptor RORα1 (51). Cross-talk between Rev-erbα and RORα1 has been re-

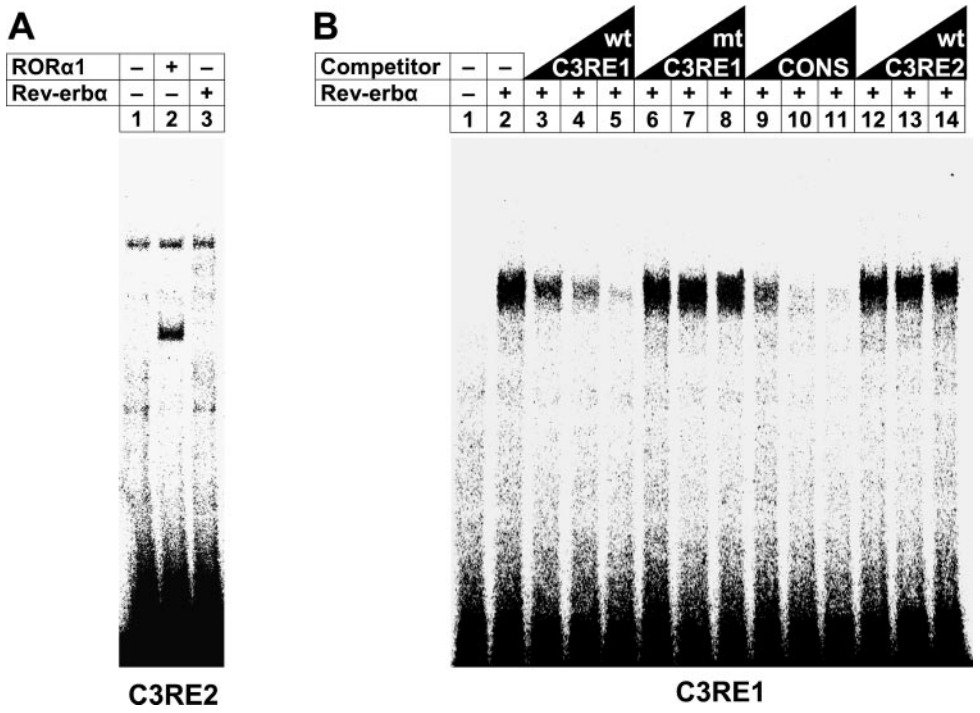

FIG. 5. **Rev-erbα can specifically bind to the C3RE1 site of the human apoCIII promoter.** Electrophoretic mobility shift analyses were performed using *in vitro* transcribed/translated human Rev-erbα (10 μl), human RORα1 (5 μl) or unprogrammed reticulocyte lysate (−), when indicated, and labeled double-stranded oligonucleotides corresponding to nt −90/−68 (C3RE2) (*A*) and nt −33/−11 (C3RE1) (*B*) of the human apoCIII gene promoter as described under "Experimental Procedures." The lysate volumes were kept constant by the addition of unprogrammed lysate. The competition experiments for binding of human Rev-erbα were performed by adding 10-, 50-, and 100-fold molar excess of the indicated unlabeled double-stranded oligonucleotides, whose identities are described under "Experimental Procedures." *mtC3RE1* is identical to *wtC3RE1* but with point mutations in the half-site (−21(G → A), −22(G → C)). *CONS*, consensus sequence for the binding of Rev-erbα; *wt*, wild type.

ported in the rat α-fetoprotein far upstream enhancer (43) and the rat apoAI promoter (44). Both receptors are expressed in HepG2 cells (37, 43, 50). To investigate whether Rev-erbα could repress RORα1-induced transcriptional activity on human apoCIII, we carried out transfection assays using the luciferase reporter plasmid containing the −1408/+24 region of apoCIII along with expression plasmids for human Rev-erbα and RORα1 in HepG2. Cotransfection of human RORα1 led to a 2.5-fold induction over basal levels (Fig. 2). We found that increasing amounts of Rev-erbα expression plasmid with constant levels of RORα1 efficiently decreased transactivation by RORα1 (Fig. 2). These results suggest that the net transcriptional response from apoCIII promoter is influenced by the relative levels of Rev-erbα and RORα1 in the cells.

The Elements Required for Rev-erbα-mediated Repression Are Located in the Proximal Promoter of apoCIII Gene—The 5′-flanking region of human apoCIII gene contains two RORα1 response elements (ROREs), both located in the proximal promoter (51). As expected, the 5′ deletion of 1.3 kilobase pairs led to a notable reduction in basal activity because of the lack of the strong distal regulatory enhancer (Fig. 3). However, the fragment containing the first 108 bp of the apoCIII promoter, which harbors the functional ROREs, is still repressed by Rev-erbα (Fig. 3).

The sequences immediately 5′ to the start of transcription of both apoCIII and apoAI genes are significantly similar (55), and the distal regulatory region of human apoCIII acts as an enhancer for the hepatic and intestinal expression of human apoAI gene expression (30–32). So we investigated whether Rev-erbα could somehow repress human apoAI gene. First, Vu-Dac and colleagues (44) have shown that Rev-erbα is able to repress the rat apoAI promoter but not the first 256 bp of the human apoAI promoter. We confirmed those results by using a reporter construct that contains a longer sequence of the human apoAI gene 5′-flanking region (Fig. 3). We observed that, in contrast to rat apoAI or human apoCIII, neither Rev-erbα nor RORα1 have significant influence on the −1170/+235 human apoAI promoter activity (Fig. 3). Second, we next examined whether, in addition to the proximal promoter, any other Rev-erbα response element could be present in the distal regulatory enhancer of human apoCIII gene. For this purpose, we mutated by site-directed mutagenesis the two 6-bp core motifs AGGTCA present in the proximal promoter, which coincide with the half-sites of ROREs, in the context of the −1408/+24 region of apoCIII gene (Fig. 4*A*). We found that upon mutation of both half-sites (nt −21(G → A)/−22(G → C) and nt −78(G → C)/−79(G → A)), repression by Rev-erbα was lost (compare the 0.3-fold variation of the wild type construct with the 1.2-fold variation of the double mutant in Fig. 4*B*). These results demonstrate that there is no other Rev-erbα response element in the distal enhancer.

Evaluation of the Contribution of Each RORE Present in apoCIII Promoter to Repression by Rev-erbα—The data shown in Fig. 4*B* indicate that each RORE present in apoCIII promoter contributes differently to the Rev-erbα-mediated repression. When the −1408/+24 region of apoCIII gene was mutated at the half-site of the RORE located at position −82/−71 (RORE2), therefore leaving an intact RORE site at −29/−18 (RORE1), the repression by Rev-erbα was still clearly observed. In contrast, mutation of only the half-site present in RORE1 almost completely abrogated Rev-erbα responsiveness, even with an intact RORE2 site (Fig. 4*B*). These results indicate that the response to Rev-erbα is mainly mediated by the RORE site at −29/−18 (hereafter designated C3RE1) and that the contribution of the RORE site at −82/−71 (hereafter designated C3RE2), if any, is minimal.

Specificity of Rev-erbα Binding—Electrophoretic mobility shift analyses were performed to determine whether Rev-erbα

could directly interact to the C3RE1 and C3RE2 sites. Incubation of a labeled double-stranded oligonucleotide containing the −29/−18 site (C3RE1) with *in vitro* translated human Rev-erbα resulted in a retarded complex (Fig. 5*B*, *lane 2*). The binding was specifically competed by the addition of either the cold C3RE1 probe (Fig. 5*B*, *lanes 3–5*) or a cold probe representing a consensus sequence for the binding of Rev-erbα (Fig. 5*B*, *lanes 9–11*). In contrast, a cold double-stranded oligonucleotide (mtC3RE1) that is equivalent to C3RE1 but harbors point mutations in the half-site (nt −21(G → A)/−22(G → C) in Fig. 4*A*) could not compete (Fig. 5*B*, *lanes 6–8*). Interestingly, this binding was not displaced by a cold probe containing the wild type site −82/−71 (C3RE2) (Fig. 5*A*, *lanes 12–14*). The apparent incapacity of C3RE2 to bind Rev-erbα was further assessed by gel mobility shift assays using labeled C3RE2 probe. As shown on Fig. 5*A*, incubation of the C3R2 probe with RORα1 resulted in the formation of a visible retarded complex of C3R2 probe and RORα1 (Fig. 5*B*, *lane 2*). In contrast, no retarded complexes were clearly detected from the incubation with Rev-erbα (Fig. 5*B*, *lane 3*).

Rev-erbα Binds to apoCIII C3RE1 as a Monomer—Rev-erbα is capable of binding DNA as a monomer (47) or as a homodimer (40, 48). To elucidate the binding mode of Rev-erbα to the human apoCIII gene promoter, we performed electrophoretic mobility shift analysis with: 1) labeled C3RE1 and C3RE2, 2) a labeled probe that binds *in vitro* to both monomers and dimers (RevDR2), and 3) a labeled probe that only binds monomers of Rev-erbα (consensus sequence). As shown in Fig. 6, Rev-erbα binds as a monomer to C3RE1. Again, no retarded complexes between C3RE2 and Rev-erbα were clearly detected (Fig. 6, *lane 7*).

apoCIII C3RE1, but Not C3RE2, Confers Rev-erbα Responsiveness to Heterologous Promoters—To study whether either of these two sites could confer Rev-erbα responsiveness to an heterologous promoter, we linked three copies of the human apoCIII C3RE1 and C3RE2 sites upstream of the TK promoter and the luciferase gene. These reporter constructs along with human RORα1 and Rev-erbα expression vectors were then tested by cotransfection in HepG2 cells (Fig. 7). A construct containing the Rev-DR2 element was used as a positive control. As expected, transfection of the RORα1 expression vector resulted in activation of both the wtC3RE1- and wtC3RE2-driven TK promoter constructs. However, reporter constructs with the TK promoter alone or driven by three copies of mutC3RE1 were not induced, which is in agreement with previous observations (51). As demonstrated in Fig. 7, wild type CR3RE1was able to confer Rev-erbα-mediated repression to TK promoter, unlike the mutated CR3RE1 or wild type CR3R2. These data also show that the human apoCIII C3RE1 site was able to mediate functional cross-talk between RORα1 and Rev-erbα in HepG2 cells.

The Nuclear Orphan Receptor RVR Also Represses the Activity of Human apoCIII Gene Promoter—We were interested in determining whether RVR, a nuclear orphan receptor closely related to Rev-erbα that is also expressed in human liver and HepG2 cells (36), could modulate the human apoCIII promoter activity. Cotransfection of a human RVR expression plasmid resulted in a significant reduction of firefly luciferase activity under the control of both the 5′-flanking sequence of human apoCIII gene (nt −1408 to +24) and the apoCIII promoter site C3RE1-driven TK promoter (Fig. 8), as well as the RevDR2-TK. The repression by RVR was specific because it was not observed on the promoter-less pGL3-basic vector or on the TK promoter alone. These results also show that the Rev-erbα response element of human apoCIII gene promoter confers RVR responsiveness to heterologous promoters (Fig. 8).

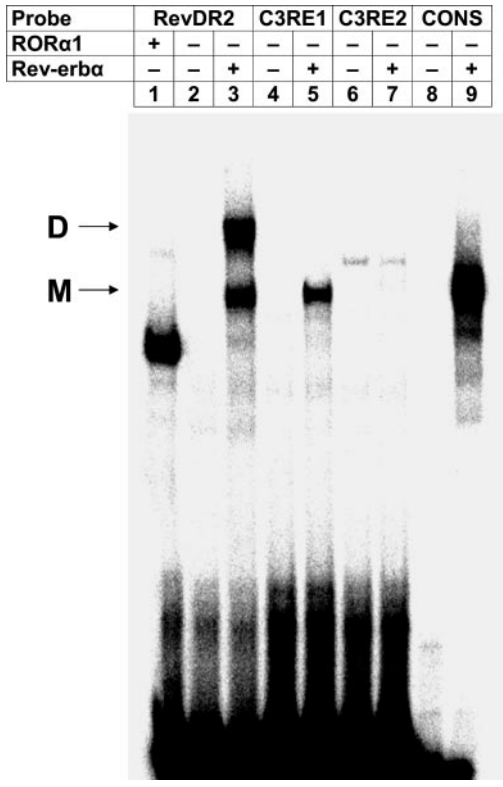

FIG. 6. **Rev-erbα binds to the C3RE1 site of human apoCIII as a monomer.** Electrophoretic mobility shift analyses were performed using *in vitro* transcribed/translated human Rev-erbα (10 μl), human RORα1 (2.5 μl), or unprogrammed reticulocyte lysate (−), when indicated, and labeled double stranded nucleotides containing the Rev-DR2 response element of the human Rev-erbα gene promoter (*RevDR2*), nt −33/−11 (*C3RE1*), and nt −90/−68 (*C3RE2*) of the human apoCIII gene promoter and a consensus sequence for the binding of monomers of Rev-erbα (*CONS*) as described under "Experimental Procedures." The lysate volumes were kept constant by the addition of unprogrammed lysate. Note that the shifted bands of probe C3RE2 (*lanes 6* and *7*) are unspecific. The mobilities of complexes containing dimers (*D*) and monomers (*M*) are indicated.

DISCUSSION

It is well established that the expression of human apoCIII plays a key role in hypertriglyceridemia (10–12, 19–23), whereas the concentration of the convergently transcribed apoAI, the major protein constituent of high density lipoproteins, contributes to the prevention of atherosclerosis (28, 56, 57). Looking for factors capable of reducing the expression of apoCIII without having negative effects on human apoAI or other lipid-related genes, we found that human apoCIII is a selective target gene for orphan nuclear receptor Rev-erbα. Here we show that this transcription factor specifically represses apoCIII through an element present in the promoter, which coincides with one of the two functional RORα1 response elements described previously (51). Our results identify cross-talk between human Rev-erbα and RORα1 that is restricted to apoCIII proximal promoter and is not present in the distal common regulatory enhancer or in the promoter of human apoAI. Interestingly, this Rev-erbα response element (nt −29/−18) overlaps the TATA box of apoCIII promoter (Fig. 4*A*). It remains as an interesting question for future research to determine whether modulation of transcription by Rev-erbα could also involve cross-talk with the TATA-binding protein in this promoter. However, the contribution of this putative cross-talk to the net repression should not play an important part because we have demonstrated that this element can confer Rev-erbα responsiveness when cloned in front of the TK promoter. Strikingly, the Rev-erbα response element of the rat apoAI promoter

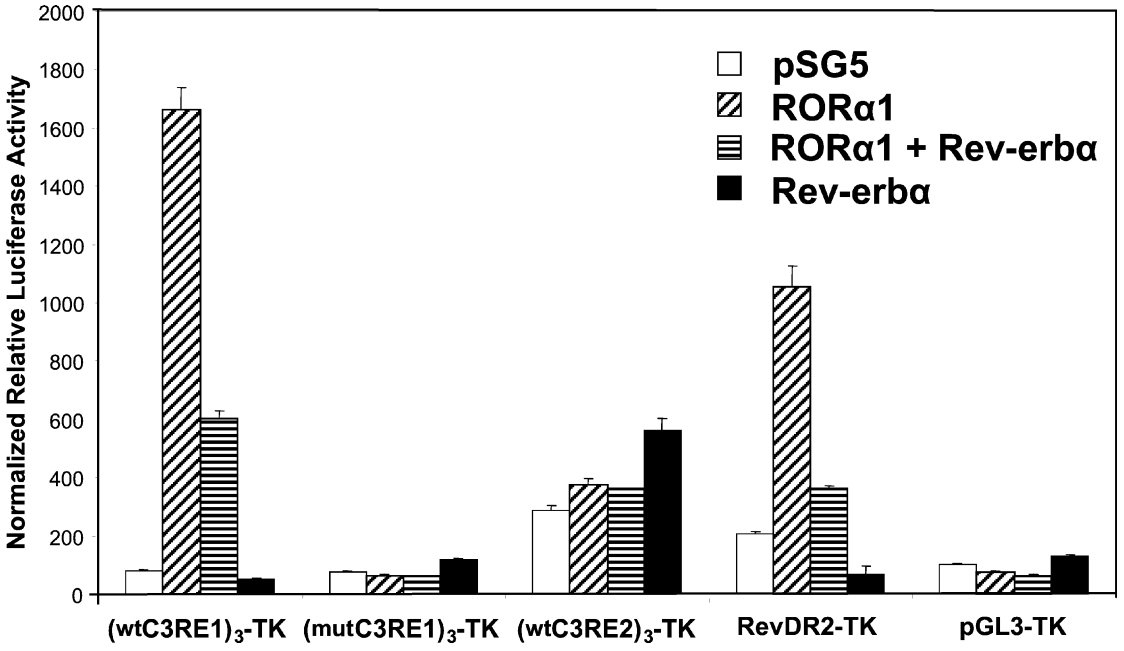

FIG. 7. **The Rev-erbα response element present in the proximal promoter of the human apoCIII gene (C3RE1) confers Rev-erbα responsiveness to heterologous promoters.** HepG2 cells were transiently transfected with plasmids expressing human RORα1 and/or human Rev-erbα or the empty pSG5 vector as control, together with reporter constructs containing three copies of the wild type (*wtC3RE1*) or mutant (*mutC3RE1*) sequence corresponding to nt −33/−11 or the wild type sequence of nt −90/−68 (*wtC3RE2*) of the human apoCIII gene promoter cloned in front of a heterologous TK promoter-driven luciferase. The Rev-DR2 response element of the human Rev-erbα gene promoter (*RevDR2*) in front of TK-luciferase was used as positive control. The empty pGL3-TK reporter vector was used as negative control. The plasmid dosage was kept constant by the addition of empty expression vector. The luciferase activities were measured and expressed as described under "Experimental Procedures."

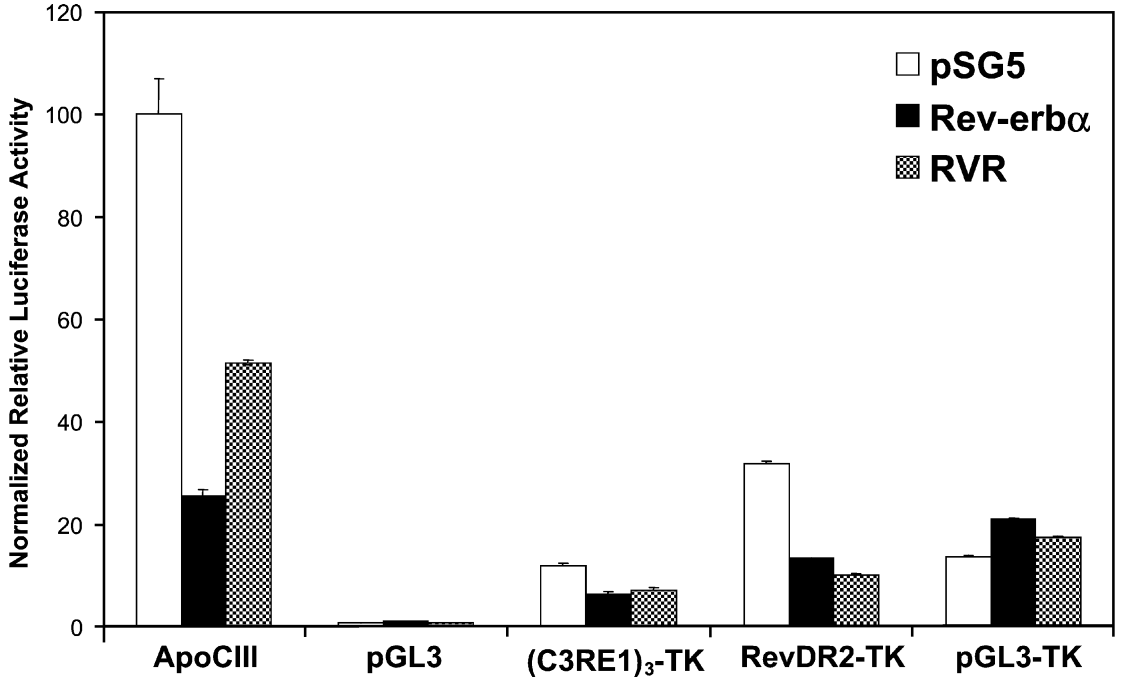

FIG. 8. **RVR also represses the activity of human apoCIII gene promoter.** The Rev-erbα response element in the apoCIII promoter confers RVR responsiveness to heterologous promoters. HepG2 cells were transfected with expression plasmids encoding human Rev-erbα or human RVR (200 ng) or the empty pSG5 vector as control, along with a plasmid containing a luciferase reporter gene driven by the 5′-flanking region (−1408/+24) of human apoCIII gene or the empty pGL3-basic (*pGL3*) vector as control. As well as a reporter construct containing three copies of the sequence corresponding to nt −33/−11 of human apoCIII gene promoter (*C3RE1*) cloned in front of a heterologous TK promoter-driven luciferase or the empty reporter vector pGL3-TK as a negative control. A reporter construct containing the Rev-DR2 response element of human Rev-erbα gene promoter (*RevDR2*) in front of TK-luciferase was used as positive control. The luciferase activities were measured and expressed as described under "Experimental Procedures."

(nt −32/−21) also overlaps the TATA box. This phenomenon gives further insight into the understanding of the process of the evolution of the apoAI and apoCIII genes from a common ancestor (58).

Importantly, our findings are in accord with data showing an increase in the hepatic apoCIII mRNA levels in Rev-erbα-deficient mice (59). Based on the similarity between the sequence of the Rev-erbα-responsive element of the mouse apo-

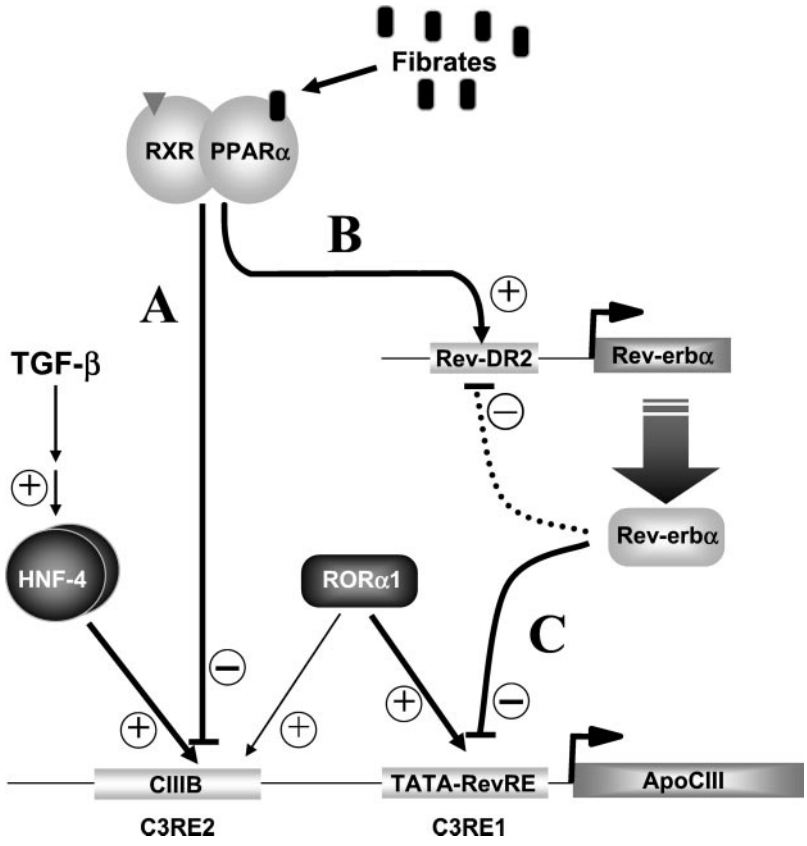

FIG. 9. **Model for negative regulation of apoCIII gene transcription by fibrates in human hepatocytes.** Upon activation by fibrates, PPARα could repress apoCIII promoter by two modes of action. In a direct mechanism (*A*), nonproductive binding of PPAR-RXR heterodimers displaces HNF-4 from the element CIIIB (23) and thereby inhibits transactivation by transforming growth factor-β signal transduction (24). Alternatively, in a multi-step process, PPAR-RXR could bind a Rev-DR2 element in Rev-erbα gene promoter (*B*), and increase its expression (50). Then monomers of Rev-erbα antagonize activation by RORα1 and repress the activity of human apoCIII promoter (*C*). Moreover, upon activation of PPARα, the negative autoregulation of Rev-erbα (40) could be diminished because of a competition between PPAR-RXR and homodimers of Rev-erbα (50).

CIII gene promoter and its human counterpart (55), these results *in vivo* can most likely be extended to the human apoCIII gene. On the other hand, although we have found that RVR also represses the apoCIII gene promoter, the Rev-erbα knockout mice data would indicate that RVR has no complete overlapping function compared with Rev-erbα. Even so, it remains to be determined whether mice carrying at the same time disruptions of both Rev-erbα and RVR genes could have even higher levels of apoCIII expression.

Luciferase reporter assays made in the context of the natural promoter suggest that the other functional RORE, located in nt −82/−71, might have a slight contribution to Rev-erbα-mediated repression of human apoCIII gene. However, in contrast to the site at −29/−18, it does not confer Rev-erbα responsiveness to a heterologous promoter. In addition, gel shift assays show that the binding of Rev-erbα, if any, should be very weak. Conversely, we and others have shown that it confers a weak RORα1 responsiveness to the TK promoter and is capable of binding RORα1 *in vitro* (51). This site discrimination underlines a somewhat different affinity of binding to DNA and suggests the hypothesis that functional ROREs in other promoters could be unresponsive to Rev-erbα.

Our data and those reported previously demonstrate that the Rev-erb subfamily functions as dominant transcriptional repressors (40, 41, 44). Some crystallography studies suggest that Rev-erbα is de facto an orphan receptor because of the small size or absence of a ligand pocket (60). Furthermore, in contrast to most ligand-activated factors, RORα receptors, which also still remain as orphan, and Rev-erb have arthropod homologues. Thus, it has been suggested that such orphans may represent ancestral receptors, before the evolution of ligands and/or ligand binding (61). The lack of ligand have hampered further assessment on the biological role of both receptors. Nevertheless, the convergence of the opposite transcriptional effects played by Rev-erb and RORα on a same gene suggests the existence of an orphan receptor-based signaling pathway. This mechanism should provide a fine tuning required for expression in response to a particular environmental or physiological cue. Because both subfamilies of receptors are expressed in human liver, the expression of apoCIII could be dictated, in part, by the ratio between the Rev-erb and RORα hepatic expression levels. In addition, the regulation of those signals could presumably come from the cellular status of some coregulators that interplay with the orphans (62).

Regulation of Rev-erbα expression has been achieved by treatment with fibrates (44, 50) and glucocorticoids (63). Because fibrates increase the hepatic expression of Rev-erbα through a PPARα-mediated activation of the Rev-erbα gene promoter, it has been pointed out that Rev-erbα could play a functional role in the transcriptional repression of rat apoAI by fibrates (44). As we have demonstrated, apoCIII promoter is repressed by Rev-erbα. Therefore, it is tempting to speculate that Rev-erbα is involved in the previously reported repression of apoCIII gene by fibrates. Studies with PPARα knockout mice have clearly demonstrated that PPARα is required in the negative regulation of apoCIII gene expression by fibrates (64). In

contrast, the involvement of other factors in the transcriptional repression by these agents is still unclear. The hepatocyte nuclear factor-4 (HNF-4) (NR2A1) is a receptor required for the hepatic expression of apoCIII (24, 65). This liver-enriched transcription factor strongly activates the human apoCIII promoter activity through its binding to the previously described proximal element CIIIB (nt −92/−67) and thereby mediates transactivation by the transforming growth factor-β signal transduction pathway (24, 25). Hertz *et al.* (23) showed that the transcriptional suppression of apoCIII exerted by fibrates is due to direct and indirect complementary modes of actions: 1) displacement of HNF-4 from the element CIIIB in the apoCIII promoter mediated by nonproductive PPAR-RXR binding (Fig. 9) and 2) transcriptional suppression of HNF-4 levels. The former mechanism, however, needs further analysis because Vu-Dac *et al.* (44) could not confirm any significant regulation of HNF-4 gene expression in livers of animals treated with fibrates. Here we propose an alternative or an additional mechanism for PPARα-mediated negative regulation of apoCIII promoter by fibrates, namely, repression by an increase in PPARα-induced Rev-erbα levels (Fig. 9). Despite exhaustive efforts we have not been able to demonstrate a direct implication of Rev-erbα in the repression by fibrates. Although studies in animals show a clear suppression of hepatic expression of apoCIII by treatment with fibrates, we, as well as others (66, 67), could not observe these effects in cultured hepatocytes, most likely because the response to fibrates is dependent on the serum used in the culture medium (68). This paradox suggests that other factors, in addition to PPARα and Rev-erbα, should be involved in the negative regulation by fibrates. On the other hand, treatment of rats with dexamethasone results in a decrease of hepatic Rev-erbα mRNA levels. Interestingly, consistent with this observation, dexamethasone increases hepatic apoCIII mRNA in mice (69) and elevates plasma triglycerides in humans (70). It will be of interest to determine whether glucocorticoids also increase human apoCIII expression and to investigate a putative involvement of Rev-erbα.

In conclusion, our results have shown that Rev-erbα specifically inhibits human apoCIII promoter gene activity. These data underscore a physiological role for Rev-erbα in the regulation of genes involved in triglyceride metabolism and, therefore, identify Rev-erbα as a potential pharmacological target for the treatment of hypertriglyceridemia.

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Orphan Nuclear Hormone Receptor Rev-erbα Regulates the Human Apolipoprotein CIII Promoter

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