

## Disruption of TR4 Orphan Nuclear Receptor Reduces the Expression of Liver Apolipoprotein E/C-I/C-II Gene Cluster\*

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**Apolipoprotein E (apoE) is synthesized in many tissues, and the liver is the primary site from which apoE redistributes cholesterol and other lipids to peripheral tissues. Here we demonstrate that the TR4 orphan nuclear receptor (TR4) can induce apoE expression in HepG2 cells. This TR4-mediated regulation of apoE gene expression was further confirmed *in vivo* using TR4 knockout mice. Both serum apoE protein and liver apoE mRNA levels were significantly reduced in TR4 knockout mice. Gel shift and luciferase reporter gene assays further demonstrated that TR4 can induce apoE gene expression via a TR4 response element located in the hepatic control region that is 15 kb downstream of the apoE gene. Furthermore our *in vivo* data from TR4 knockout mice prove that TR4 can also regulate apolipoprotein C-I and C-II gene expression via the TR4 response element within the hepatic control region. Together our data show that loss of TR4 down-regulates expression of the apoE/C-I/C-II gene cluster in liver cells, demonstrating important roles of TR4 in the modulation of lipoprotein metabolism.**

Apolipoprotein E (apoE)<sup>1</sup> is primarily synthesized in the liver, although it is widely expressed in various tissues (1, 2). ApoE is an important constituent of plasma lipoproteins, such as very low density lipoprotein and chylomicrons, and serves as a ligand for the receptor-mediated uptake of these lipoproteins by the liver (3). ApoE is involved in the pathogenesis of atherosclerosis through the modulation of cholesterol efflux from macrophages (4), and liver-derived apoE also has access to arterial intima and induces regression of atherosclerotic lesions (5).

ApoE expression has been shown to be modulated by tissue-specific enhancers in different tissues. The nuclear receptor liver X receptor/retinoid X receptor  $\alpha$  (RXR $\alpha$ ) heterodimer has been reported to regulate expression of the apoE/C-I/C-IV/C-II gene cluster in macrophages by binding to direct repeat (DR) 4 sequences in multienhancer domains located within the gene

cluster (6, 7). Previous studies using transgenic mice show that in liver, the major source of plasma apoE, the expression of apoE, apoC-I, apoC-IV, and apoC-II are promoted by liver-specific enhancers called hepatic control regions (HCR-1 and HCR-2) (8–10). However, the molecular mechanism controlling HCR regulation of apoE expression in liver remains unclear. Recently apoC-II, one of the members of the apoE/C-I/C-IV/C-II gene cluster, was shown to be induced by farnesoid X receptor (FXR)/RXR $\alpha$  heterodimer via HCRs (11). However, further study will be needed to determine whether the FXR/RXR $\alpha$  heterodimer also regulates the expression of other apolipoproteins (apoE, apoC-I, and apoC-IV) within the apoE/C-I/C-IV/C-II gene cluster.

Members of the nuclear receptor superfamily are transcription factors that regulate gene expression through binding to specific DNA sequences known as hormone response elements (HREs). These nuclear receptors include those for steroids, thyroid hormones, vitamin D<sub>3</sub>, and retinoids as well as a large number of orphan receptors with no known ligands (12).

A particular member of the nuclear receptor family, the TR4 orphan nuclear receptor (TR4), is able to regulate the expression of target genes through binding to DR AGGTCA core motif sequences with variable numbers of spacer nucleotides (13–17). TR4 has been shown to be highly expressed in rat primary hepatocytes (18). To understand the physiological role of TR4, we sought to identify TR4 target genes based on the response element binding preferences of the receptor. *In vitro* binding assays showed that TR4 has the highest affinity for DR1 elements, and we found a DR1 site in the HCR-1, which represents a potential TR4 response element (TR4RE). We hypothesize that the apoE gene, regulated by HCR-1, is a TR4 target gene.

Transcription factors and their effects on gene expression have largely been studied via *in vitro* binding and transfection assays in cultured cells. However, many genes have multiple response elements for different transcription factors, including nuclear receptors. Nuclear receptors have overlapping binding sites in many genes and often compete with other transcription factors for the same binding site under particular conditions (19–22). In many cases, it is not easy to determine which transcription factors play major roles *in vivo*.

Here we demonstrate that TR4 can regulate apoE expression via a DR1 element in the HCR-1 of the apoE/C-I/C-IV/C-II gene cluster and have further confirmed the role of TR4 *in vivo* through analysis of TR4 knockout (TR4KO) mice. Moreover, consistent with previous transgenic mouse studies showing HCR-based gene regulation, TR4 is able to modulate the expression of apoC-I and apoC-II via binding to a response element within the HCR of the apoE/C-I/C-IV/C-II gene cluster.

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<sup>1</sup> The abbreviations used are: apo, apolipoprotein; HCR, hepatic control region; TR4, TR4 orphan nuclear receptor; TR4RE, TR4 response element; TR4KO, TR4 knockout; RXR, retinoid X receptor; FXR, farnesoid X receptor; DR, direct repeat; IR, inverted repeat; HNF-4, hepatic nuclear factor 4; Luc, luciferase; TK, thymidine kinase; HRE, hormone response element; mt, mutant; RT, reverse transcriptase.

## EXPERIMENTAL PROCEDURES

**Reagents and Plasmids**—The plasmids PCMX-TR4 and pG5-luciferase (Luc) have been described previously (23), and the 11.1-kb human apoE gene was provided by Jan Breslow (Rockefeller University) (24). The plasmid VP16-RXR $\alpha$  was a gift from Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France), and pSG5-FXR and GAL4-FXR (ligand-binding domain of human FXR) were gifts from Dr. Timothy M. Willson (GlaxoSmithKline) (25). The pCMV-VP16-TR4 expression plasmid was constructed by fusion of full-length human TR4 to the transcriptional activator VP16, and GAL4-RXR $\alpha$  (ligand-binding domain of human RXR $\alpha$ ) was a gift from Dr. Jaewoon Lee (POSTECH) (26). pcDNA1-HNF-4 and pCMV4-apoE4 were gifts from Dr. Margarita Hadzopoulou-Cladaras (Boston University) and Dr. Mary Jo LaDu (Evanston Northwestern Healthcare Research Institute), respectively.

The apoE 5' promoter region consisting of -1046 to +872 bp was amplified by PCR from the 11.1-kb human apoE gene, including 5.7 kb of the 5'-flanking sequence and 1.9 kb of the 3'-flanking sequence, and cloned into pGL3-Luc (Promega) to generate pGL-apoE-Luc. We first generated pGL-TK-Luc by cloning the thymidine kinase (TK) promoter (-32 to +48 bp) into pGL3-Luc (Promega). HCR-1 was amplified by PCR from HepG2 genomic DNA and subcloned into pGL-TK-Luc or pGL-apoE-Luc to generate pGL-TK-HCR-1-Luc and pGL-apoE/HCR-1-Luc, respectively. Synthesized DR1 and mutant (mt) DR1 oligonucleotides were subcloned into pGL-TK-Luc to create pGL-TK-(DR1)<sub>3</sub>-Luc and pGL-TK-mt(DR1)<sub>3</sub>-Luc, respectively.

**Immunohistochemistry**—Two-month-old C57BL6J mice under pentobarbital anesthesia were perfused with 4% paraformaldehyde in phosphate-buffered saline. The liver was removed after adequate perfusion, and then it was further fixed in 4% paraformaldehyde in phosphate-buffered saline for 6 h. After processing and embedding in paraffin, tissue blocks were cut for staining. The liver sections were rehydrated, washed in phosphate-buffered saline, treated with 3% hydrogen peroxide in methanol for 30 min, blocked in 10% normal goat serum in phosphate-buffered saline for 30 min, and immunostained using the EnVision+ system (Dako, Carpinteria, CA). The primary antibody was a rabbit anti-TR4 polyclonal antibody (150-fold dilution). Preimmune rabbit serum (150-fold dilution) was used as a negative control in adjacent sections. After staining, the sections were developed using a 3,3'-diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). Nuclear counterstain was performed with Gill's hematoxylin (Thermo-Shandon, Pittsburgh, PA) in all sections.

**Cell Culture and Transfections**—HepG2 and COS-1 cells were maintained in Dulbecco's minimum essential medium containing 10% fetal calf serum. Transfections were performed by using the calcium phosphate precipitation method (14) or SuperFect (Qiagen, Valencia, CA). pRL-TK was used to normalize transfection efficiency in the dual luciferase reporter assay system (Promega).

**Metabolic Labeling of Cells and Immunoprecipitation**—HepG2 cells were plated at  $5 \times 10^5$  cells/60-mm dish and transfected with TR4 expression vector or empty vector (pCMX-TR4 or pCMX). After 16 h of transfection, the medium was changed, and cells were grown for another 12 h for recovery. Cells were incubated with serum- and methionine (Met)-free Dulbecco's minimum essential medium for 1 h and then pulse-labeled for 45 min with [<sup>35</sup>S]Met at 150  $\mu$ Ci/ml in medium. After incubation, the medium was collected, and cells were lysed by addition of 0.5 ml of lysis buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.5, 15 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 10  $\mu$ g/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride). Proteins newly synthesized in cell lysates and secreted into the medium were determined using trichloroacetic acid precipitation, and the required amount of each sample was aliquoted into a new tube. The volume of each sample was adjusted to 1 ml with lysis buffer, and samples were immunoprecipitated with an anti-apoE polyclonal antibody (Calbiochem). The immunoprecipitated proteins were separated by 10% SDS-PAGE, and radiolabeled apoE was quantified using a PhosphorImager (Amersham Biosciences).

**Western Blot Analysis**—Serum samples were tested for the presence of apoE by Western blot analyses. Samples were separated by 10% SDS-PAGE. After electrophoresis, proteins were transferred from the gel to Immobilon P transfer membrane (Millipore). ApoE was resolved using an anti-apoE polyclonal antibody (Chemicon) and an alkaline phosphatase-conjugated secondary antibody (Bio-Rad), and then the relative amount of each sample was quantified using a VersaDoc imaging system (Bio-Rad).

**RT-PCR**—Total RNA was isolated from wild-type and TR4KO mouse livers using TriZol reagent (Invitrogen), and RT-PCR was carried out using the SuperScript™ II (Invitrogen) according to the manufacturer's

protocols. Briefly, after denaturation for 5 min at 65 °C in the presence of 0.5  $\mu$ g of random hexamers, 3  $\mu$ g of total RNA was reverse transcribed for 1 h at 43 °C with 200 units of SuperScript II in a 20- $\mu$ l reaction (containing a 0.5 mM concentration of each dNTP). Two microliters of the cDNA sample were used as template for PCR amplification with a forward primer (5'-CAGCAGTTCATCTAACCAGCCC-3') specific to a region of exon 3 present in the TR4 gene in wild-type mice as well as in the targeting construct present in TR4KO mice and a reverse primer (5'-CTGCTCCGACAGCTGTAGGTC-3') specific to a region of exon 5 replaced by the targeting construct in TR4KO mice. Hypoxanthine phosphoribosyltransferase expression was analyzed (primers: forward, 5'-GCTGGTGAAAAGGACCTCT-3'; reverse, 5'-CACAGGACTA-GAACCTGC-3') as an internal control in the same run.

**Northern Blot Analysis**—Total RNA was isolated using TriZol reagent (Invitrogen), and Northern blots were performed as described previously (16). An apoE probe was prepared from human apoE cDNA by AatII and DraIII digestion. Probes for mouse apoC-I (GenBank™ accession number NM\_007469), apoC-II (GenBank™ accession number NM\_009695),  $\beta$ -actin, and 18 S rRNA were generated by RT-PCR. Membrane-immobilized mRNA was hybridized with radiolabeled cDNA probes, and hybridization signals were quantified using a PhosphorImager (Amersham Biosciences). Ratios of apoE, apoC-I, and apoC-II mRNA levels relative to either 18 S rRNA or  $\beta$ -actin were calculated.

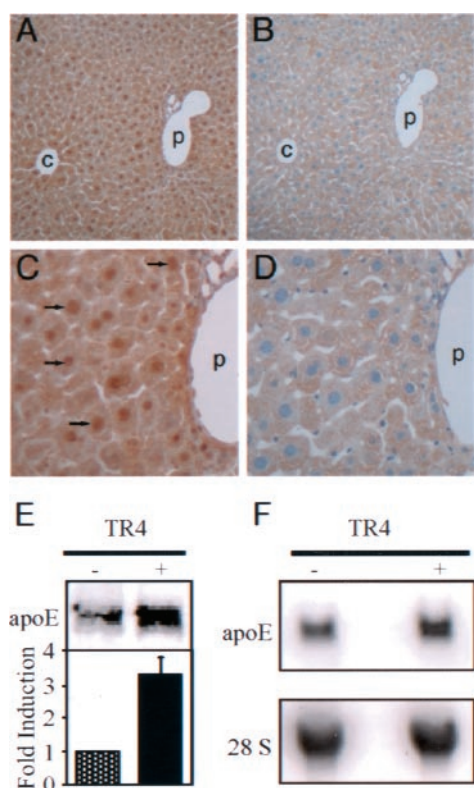
**Gel Shift Assay**—Gel shift assays were performed as described previously (14) with the use of *in vitro* translated proteins or HepG2 nuclear extracts and <sup>32</sup>P-labeled oligonucleotide probes. The following oligonucleotides were used in gel shift assays: TR4RE-DR1-apoE (5'-CTTGGGGCAGAGGTCAGAG-3'), mutated (underlined) TR4RE-DR1-apoE (5'-CTTGGCCGACCTCAGAG-3'), and the DR1/inverted repeat 1 (IR1) element (5'-CTTGGGGCAGAGGTCAGAGACTCTC-3'). For the antibody supershift assays, an anti-TR4 mouse monoclonal antibody was added to the reaction. DNA-protein complexes were resolved on a 5% native gel and analyzed by PhosphorImager (Amersham Biosciences).

## RESULTS

**TR4 Induces ApoE Gene Expression *In Vitro***—TR4 has been shown to be highly expressed in rat primary hepatocytes (18). To verify the physiological relevance for studying the effect of TR4 on hepatic apoE expression, we performed staining of mouse liver tissue with a rabbit polyclonal antibody specific to the N-terminal domain of TR4. In mouse liver sections, the anti-TR4 antibody stained both the cytoplasm and nuclei of hepatocytes with stronger staining in the nuclei compared with the cytoplasm (Fig. 1, A and C, arrows). We also performed staining of adjacent sections with preimmune rabbit serum as a negative control to show whether this staining is TR4-specific. As shown in Fig. 1, B and D, only a weak background appears when staining with preimmune serum. These results supported our interest in further characterizing the potential regulation of hepatic apoE gene expression by TR4.

We then applied a pulse labeling assay in HepG2 cells, transfected with either a TR4 expression vector or an empty vector, to study the effect of TR4 on apoE expression. After transfection and overnight recovery, cells were pulsed with [<sup>35</sup>S]Met for 45 min, and the levels of newly synthesized apoE were determined by immunoprecipitation using an anti-apoE antibody. As shown in Fig. 1E, addition of TR4 can significantly increase apoE protein expression. This induction was further confirmed at the mRNA level using Northern blot analysis. As shown in Fig. 1F, the level of apoE mRNA was higher in HepG2 cells transfected with a TR4 expression vector than in empty vector-transfected HepG2 cells. Together the data from Fig. 1 demonstrate that TR4 can induce apoE expression at both the protein and mRNA levels.

**Hepatic Control Region Contains a TR4 Response Element**—Human hepatic control regions (HCR-1 and HCR-2) were identified in the studies of apoE/C-IV/C-II gene expression using transgenic mice (8–10). HCR-1 and HCR-2 have 85% nucleotide identity and are located 18.4 and 29.4 kb downstream of the apoE transcription initiation site, respectively (Fig. 2A). In studies with transgenic mice, HCRs have been



**FIG. 1. TR4 is expressed in mouse hepatocytes and positively regulates apoE expression in HepG2 cells.** A–D, immunolocalization of TR4 in mouse liver. A and C, liver sections were stained with rabbit anti-TR4 antiserum (1:150 dilution). Brownish 3,3'-diaminobenzidine precipitates were mainly located in hepatocyte nuclei (arrows). B and D, adjacent sections were stained with preimmune rabbit serum as a negative control (1:150 dilution), and only weak background staining was found. Gill's hematoxylin was used as counterstain in all sections. (P, portal vein; C, central vein. A and B,  $\times 40$ . C and D,  $\times 200$ .) E, HepG2 cells were transfected with a TR4 expression vector (pCMX-TR4) or empty vector (pCMX) for 16 h. Medium was then changed, and cells were grown for another 12 h. Cells were pulsed for 45 min with medium containing 150  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]Met and then harvested. After obtaining the required amount of each sample, using trichloroacetic acid precipitation as described under "Experimental Procedures," pulse-labeled medium and cell lysates were immunoprecipitated with anti-apoE antibody. Immunoprecipitated proteins were separated by 10% SDS-PAGE and quantitated by PhosphorImager. F, HepG2 cells were transfected with pCMX-TR4 or pCMX as described above. Total RNA (10  $\mu\text{g}$ ) from transfected cells was used for Northern blot analysis, and 28 S rRNA stained with 0.004% methylene blue was used as a loading control for the RNA.

shown to have critical roles in the regulation of hepatic expression of the apoE/C-I/C-IV/C-II gene cluster. Within the entire HCR-1 774-bp region, the 319 bp at the 5' terminus confer full HCR-1 functional activity (27), and sequence analysis demonstrated that this 319-bp region contains a DR1 element. Of the DR elements recognized by TR4, the receptor binds to DR1 sequences with the highest affinity (13–17). We next performed a gel shift assay to determine that this DR1 element (GGGGCAGAGGTCA named TR4RE-DR1-apoE; core motifs are underlined) functions as a TR4RE. As shown in Fig. 2B, *in vitro* translated TR4 protein formed a specific complex with  $^{32}\text{P}$ -labeled TR4RE-DR1-apoE. In contrast, the mock-translated control lysate was unable to form a complex with the DR1 element (left panel, lane 2 versus lane 1, open arrowhead). The TR4-TR4RE complex could be abolished by unlabeled TR4RE-DR1-apoE but not by mutated, unlabeled TR4RE-DR1-apoE (lane 3 versus lane 4). Moreover a supershift of the specific TR4-DR1 complex was achieved by addition of an anti-TR4 antibody (lane 5, closed arrowhead), further indicating that the TR4RE-DR1-apoE is a specific binding site for TR4.

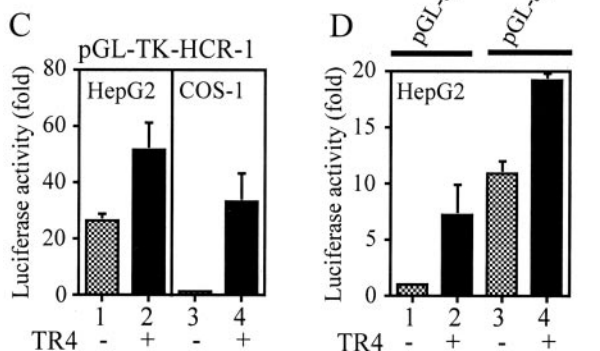
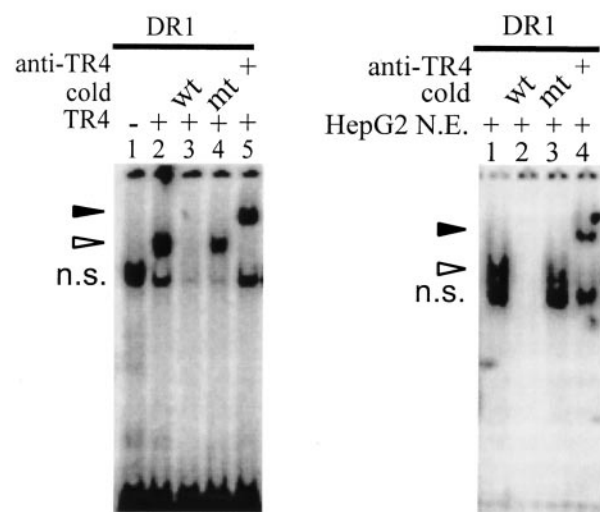
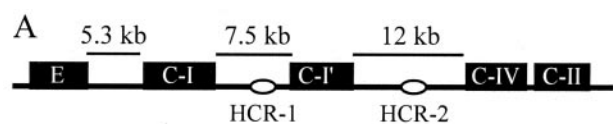
We confirmed the specific TR4-DR1 interaction when we replaced *in vitro* translated TR4 with HepG2 cell nuclear extracts containing endogenous TR4 (Fig. 2B, right panel). This result also confirms that TR4 protein is present in liver cells. We then used luciferase reporter assays to test whether TR4 regulates apoE gene expression through interaction with the HCR-1 region. We first linked HCR-1 to TK-luciferase (pGL-TK-HCR-1-Luc) and tested whether TR4 has any influence on the transcriptional activity of a reporter regulated by HCR-1. This construct shows high basal transcriptional activity in HepG2 cells, and co-transfection of a TR4 expression vector (pCMX-TR4) can induce luciferase activity (Fig. 2C, lane 1 versus lane 2). In contrast, the reporter construct showed very low basal transcriptional activity in COS-1 cells with significantly increased luciferase activity upon addition of the TR4 expression vector (Fig. 2C, lane 3 versus lane 4). The difference in the basal activity of the reporter in HepG2 versus COS-1 cells could be due to variation in the availability of endogenous TR4 and cooperation between endogenous TR4 and other liver-specific transcription factors to modulate HCR-1-regulated gene expression in HepG2 cells.

We next addressed whether the proximal promoter of the apoE gene has any effect on HCR-1 activity. Either pGL-apoE-Luc, containing the apoE gene promoter (–1046/+872 bp) only, or pGL-apoE/HCR-1-Luc, containing the apoE gene promoter fused with the HCR-1 region, were transfected into HepG2 cells in the absence or presence of the TR4 expression vector. TR4 was found to enhance the transcriptional activity of apoE promoter and apoE promoter/HCR-1-driven luciferase reporters (Fig. 2D). However, we were unable to see any further enhancement of the TR4 effect on HCR-1 activity by addition of the apoE proximal promoter (Fig. 2, C versus D). Although TR4 stimulates apoE promoter activity, the induced promoter activity was even lower than the basal activity of pGL-apoE/HCR-1-Luc. This suggests that the apoE promoter may not have an important role in hepatic expression of the apoE gene. Indeed previous reports have indicated that the apoE promoter has no significant role in hepatic apoE expression (8–10). The apoE promoter may have potential TR4 binding sites as suggested by reporter gene assay even though the promoter activity does not have a significant effect on hepatic apoE expression. To define the role of TR4 relative to non-tissue-specific apoE promoter function, further study will be needed.

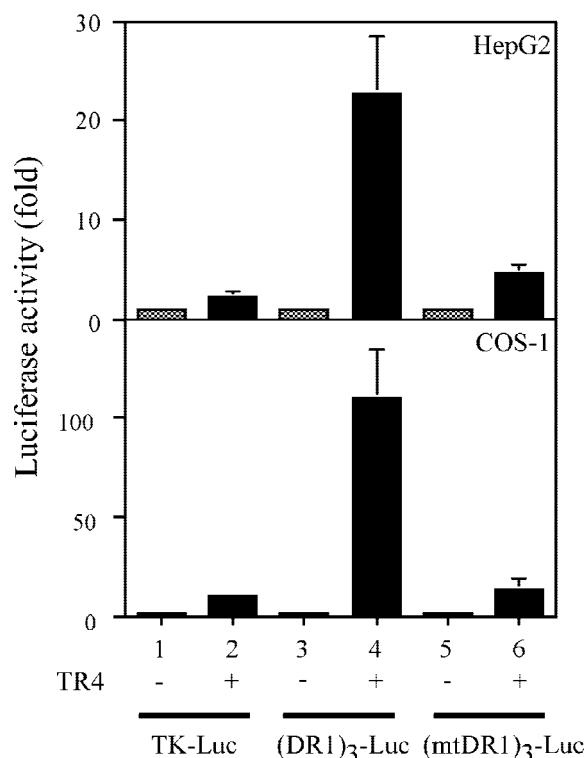
To further confirm that TR4RE-DR1-apoE within the HCR-1 region can mediate TR4 induction of apoE expression in hepatic cells, we co-transfected a reporter with three copies of the TR4-DR1-apoE element (pGL-TK-(DR1)<sub>3</sub>-Luc). Luciferase activity was expressed based on the induction -fold relative to transfection of empty vector (pCMX, set as 1.0-fold) in each reporter gene assay. As demonstrated in Fig. 3, TR4 was able to significantly activate this pGL-TK-(DR1)<sub>3</sub>-Luc reporter in HepG2 and COS-1 cells (lane 3 versus lane 4). In contrast, TR4 only had marginal induction effects when we replaced TR4RE-DR1-apoE with mutated TR4RE-DR1-apoE (pGL-TK-(mtDR1)<sub>3</sub>-Luc) or with parent reporter plasmid (pGL-TK-Luc). Together these data strongly suggest that the DR1 element in HCR-1 is a TR4 response element important for TR4-induced transcriptional activation of the apoE gene.

*ApoE Expression Is Reduced in TR4KO Mouse Liver*—Recently we generated TR4KO mice (in collaboration with Lexicon Genetics Inc.) by the insertion of a LacZ/Neo selection cassette between exons 4 and 5 of the TR4 gene.<sup>2</sup> RT-PCR analysis of total RNAs from wild-type and TR4KO mouse liver

<sup>2</sup> L. L. Collins, Y.-F. Lee, W.-J. Lin, C. A. Heinlein, N.-C. Liu, Y.-Ts. Chen, Y.-T. Chen, C.-R. Shyr, C. K. Meshul, H. Uno, S. M. Chou, K. A. Platt, and C. Chang, manuscript in preparation.



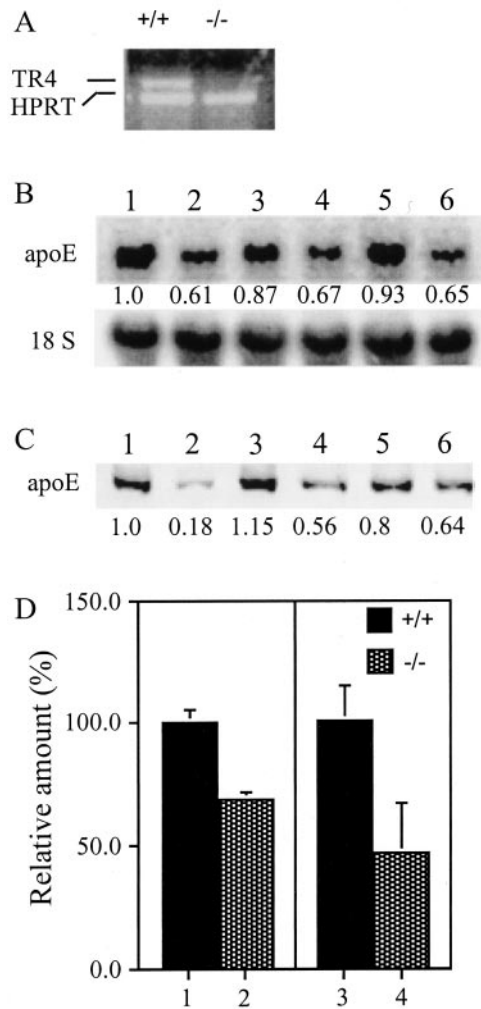
**FIG. 2. TR4 induces the apoE gene through binding to a DR1 element in the HCR-1.** *A*, a schematic of the apoE/C-I/C-IV/C-II gene cluster is shown, and relative locations and distances are indicated (9). *B*, sequence of the putative DR1 TR4 response element (TR4RE-DR1-apoE) in the HCR-1. AGGTC-like sequences are indicated by arrows. The gel shift assays were performed using *in vitro* translated TR4 (*left panel*) or nuclear extracts of HepG2 cells (*right panel*) with <sup>32</sup>P-labeled TR4RE-DR1-apoE. Two hundred (*left panel*) or 20 molar excesses (*right panel*) of unlabeled oligonucleotides (*wt*) or mutated oligonucleotides (*mt*) were added as competitor DNA. For the supershift assay, an anti-TR4 antibody was added as indicated. The retarded complex and the supershifted band are indicated by open and closed arrowheads, respectively. *n.s.*, nonspecific binding. *C*, HepG2 and COS-1 cells were transfected with 3  $\mu$ g of pGL-TK-HCR-1-Luc in the presence or absence of 3  $\mu$ g of pCMX-TR4. Luciferase activity was normalized according to pRL-TK activity, and relative luciferase activity (-fold) was expressed based on the induction -fold relative to transfection of empty vector (pCMX) in COS-1 cells (set as 1.0-fold). *D*, the apoE promoter (300 ng of pGL-apoE-Luc) or apoE promoter/HCR-1 reporter gene construct (300 ng of pGL-apoE/HCR-1-Luc) was co-transfected, with or without 150 ng of pCMX-TR4, into HepG2 cells. Relative luciferase activity (-fold) was expressed based on the induction -fold relative to co-transfection of empty vector (pCMX) with pGL-apoE-Luc (set as 1.0-fold). Data presented in *C* and *D* represent the mean  $\pm$  S.D. of three individual assays.



**FIG. 3. TR4 activates reporter genes under the control of TR4RE-DR1-apoE in HepG2 and COS-1 cells.** Cells were transfected with pGL-TK-Luc, pGL-TK-(DR1)<sub>3</sub>-Luc, or pGL-TK-mt(DR1)<sub>3</sub>-Luc (300 ng of each) in the presence or absence of 100 ng of pCMX-TR4. Luciferase activity was normalized according to pRL-TK activity, and relative luciferase activity (-fold) was expressed based on the induction -fold relative to transfection of empty vector (pCMX) in each reporter gene assay (set as 1.0-fold). Data presented represent the mean  $\pm$  S.D. of three individual assays.

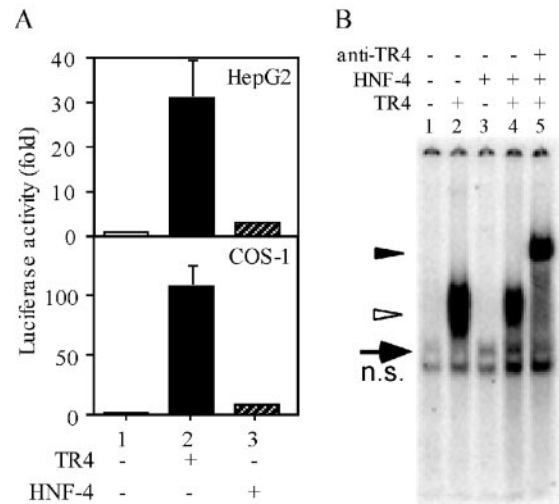
tissues confirmed the deletion of TR4 gene exons 4 and 5 in homozygous TR4KO mouse liver tissues (Fig. 4A). To determine whether TR4 could also regulate apoE expression *in vivo*, we examined apoE expression in wild-type (Fig. 4B, lanes 1, 3, and 5) and TR4KO mice (Fig. 4B, lanes 2, 4, and 6). From Northern blot analysis using three sets of wild-type and TR4KO mouse liver total RNAs, it was found that TR4KO apoE mRNA levels were reduced to 70% of those in wild-type mice (Fig. 4, B and D, left panel). Western blot analysis of serum apoE protein revealed that serum apoE levels of TR4KO mice were decreased by about 50% compared with wild-type serum apoE levels (Fig. 4, C and D, right panel). Considering that most apoE present in the serum is derived from the liver, the low level of serum apoE protein in TR4KO mice could be a result of the reduction of apoE mRNA expression in the liver. *In vivo* data collected from TR4KO mice confirm our *in vitro* data from HepG2 cells, which show that TR4 can induce apoE expression.

**Influence of Hepatocyte Nuclear Factor 4 (HNF-4) on TR4-induced ApoE Expression**—Many nuclear receptors are able to bind to DR1-HRE sites, although the relative binding affinity could be influenced by the nucleotide sequences of core motifs as well as the spacer nucleotide within the particular DR1-HRE (34, 35). One of these nuclear receptors is HNF-4. We were interested in determining the relative influence of TR4 and HNF-4 on apoE expression. As shown in Fig. 5A, TR4 highly induced transcriptional activity of the reporter gene (pGL-TK-(DR1)<sub>3</sub>-Luc), whereas HNF-4 showed only a marginal effect on this reporter gene. To explore the mechanism of the differential induction effects of TR4 and HNF-4 on apoE expression, we performed gel shift assays. As shown in Fig. 5B, *in*



**FIG. 4. Expression of the apoE gene is reduced in TR4KO mice.** A, RT-PCR analysis of wild-type (+/+) and homozygous TR4KO (-/-) mice using a 5' primer specific to a region of exon 3 present in the TR4 gene in wild-type mice, as well as to a region in the targeting construct present in TR4KO mice, and a 3' primer specific to a region of exon 5 deleted in the TR4 targeting construct. The PCR product (323 bp for wild-type) is amplified from wild-type but not from TR4KO cDNAs. A hypoxanthine phosphoribosyltransferase (HPRT) fragment (249 bp) was amplified as an internal control. B, Northern blot analysis of apoE mRNA from wild-type and TR4KO mouse liver samples. Total RNAs (25  $\mu$ g each) from three sets of wild-type (lanes 1, 3, and 5) and TR4KO (lanes 2, 4, and 6) mouse liver samples were subjected to Northern blot analysis using the indicated  $^{32}$ P-labeled probes followed by quantification with a PhosphorImager. After correction for loading differences using the ratio of apoE to 18 S rRNA, the amount of each sample was expressed relative to the sample in lane 1 (set as 1.0). C, serum apoE levels of wild-type and TR4KO mice. Proteins in the serum (1.0  $\mu$ l) from three sets of wild-type (lanes 1, 3, and 5) and TR4KO mice (lanes 2, 4, and 6) were separated by 10% SDS-PAGE and immunoblotted using an anti-apoE antibody followed by quantification with a VersaDoc imaging system. The apoE level of each sample was expressed as the ratio relative to the sample in lane 1 (set as 100%). Results are means  $\pm$  S.D. of samples from three different animals per group (wild-type or TR4KO).

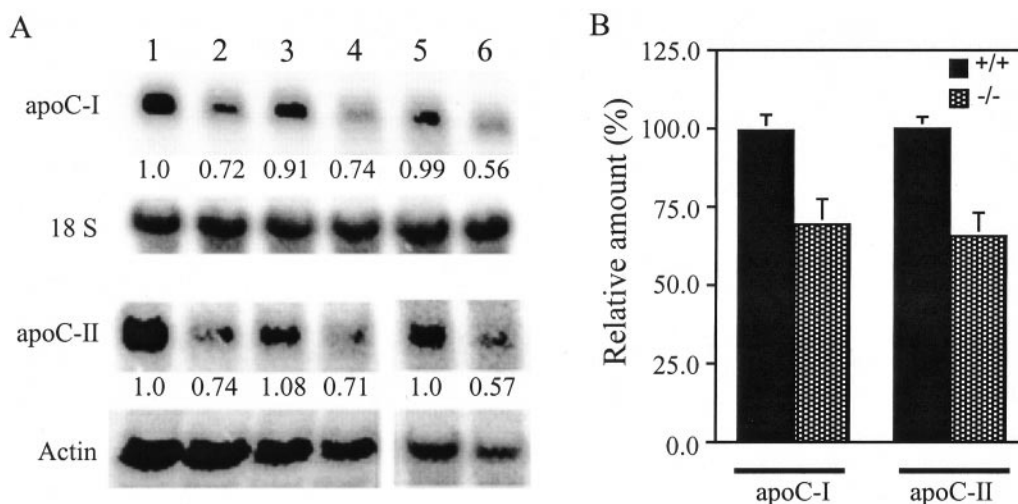
*in vitro* translated TR4 could strongly bind to  $^{32}$ P-TR4RE-DR1-apoE (lane 2, open arrowhead). However, we can only see a very weak binding complex consisting of HNF-4 and TR4RE-DR1-apoE when we replaced *in vitro* translated TR4 with *in vitro* translated HNF-4 (lane 3, arrow). Previous reports have demonstrated that changing the spacer nucleotide from A to G reduces the affinity of HNF-4 for DR1-HRE (34, 35). This suggests that the nucleotide sequence of TR4RE-DR1-apoE may contribute to weak binding affinity for HNF-4. The bind-



**FIG. 5. The influence of HNF-4 on TR4-mediated transcriptional activation through the TR4RE-DR1-apoE element.** A, HepG2 or COS-1 cells were transfected with 300 ng of pGL-TK-(DR1)<sub>3</sub>-Luc, in the presence of 100 ng of pCMX-TR4 or pcDNA1-HNF-4, as indicated. Relative luciferase activity (-fold) was expressed based on the induction -fold relative to transfection of empty vector (pCMX) (set as 1-fold). B, a gel shift assay was performed with *in vitro* translated TR4 or HNF-4 as indicated.  $^{32}$ P-labeled TR4RE-DR1-apoE was used as a probe. Supershift of the specific retarded complex was induced by an anti-TR4 antibody. Retarded complexes of TR4 and HNF-4 and the supershift induced by the anti-TR4 antibody are indicated as open arrowhead, arrow, and closed arrowhead, respectively. n.s., nonspecific binding.

ing affinity of TR4 for TR4RE-DR1-apoE was decreased when *in vitro* translated HNF-4 was added together with *in vitro* translated TR4 and  $^{32}$ P-TR4RE-DR1-apoE (lane 4). This result suggests that HNF-4 may affect TR4 binding affinity for TR4RE-DR1-apoE, although HNF-4 itself shows very weak binding affinity for TR4RE-DR1-apoE. The specific complex consisting of TR4-TR4RE-DR1-apoE was supershifted by addition of an anti-TR4 antibody in the presence of *in vitro* translated HNF-4 (lane 5, closed arrowhead). Together both reporter and gel shift assays suggest that TR4 may strongly bind to this special DR1 site in the HCR-1 region to induce apoE expression.

**ApoC-I and ApoC-II mRNA Expression Is Also Reduced in TR4KO Mice**—In previous studies with transgenic mice, apoC-I and C-II genes, located in the same gene cluster with the apoE gene, were reported to be regulated by tissue-specific enhancer regions, HCRs (8–10). Furthermore Kast *et al.* (11) reported that the FXR/RXR $\alpha$  heterodimer was able to modulate apoC-II gene expression via an IR1 element in the HCR, a region that partially overlaps with the TR4RE-DR1-apoE element in an arrangement of three consensus hexameric AG-GTCA motifs. Since our data showed that TR4 could induce apoE gene expression via binding to the DR1 DNA element in the HCR-1 region, we were interested to see whether TR4 could also regulate the apoC-I and apoC-II genes via this HCR region. Using three sets of liver tissues from wild-type (Fig. 6A, lanes 1, 3, and 5) and TR4KO mice (Fig. 6A, lanes 2, 4, and 6), Northern blot analyses clearly showed that loss of the TR4 gene results in the reduction of apoC-I and apoC-II mRNA levels to 70 and 65% of those in wild-type liver tissues, respectively (Fig. 6, A and B). We next carried out transient transfections of the pGL-apoE/HCR-1-Luc reporter to compare the effects of TR4 and the FXR/RXR $\alpha$  heterodimer on apoE gene expression. As shown in Fig. 7A, TR4 and the FXR/RXR $\alpha$  heterodimer induced transcriptional activity of the reporter gene in HepG2 cells by  $2.32 \pm 0.36$ -fold (lane 2) and  $2.04 \pm 0.38$ -fold (lane 3), respec-



**FIG. 6. Reduction of apoC-I and apoC-II mRNA expression in TR4KO mice.** A, Northern blot analyses of apoC-I and apoC-II mRNAs from wild-type and TR4KO mouse liver samples. Total RNAs (25  $\mu$ g each) from three sets of wild-type (lanes 1, 3, and 5) and TR4KO mouse liver samples (lanes 2, 4, and 6) were subjected to Northern blot analysis using the indicated  $^{32}$ P-labeled probes followed by quantification with a Phosphor-Imager. After correction for loading differences using the ratio of apoC-I or apoC-II to either 18 S rRNA (for apoC-I) or  $\beta$ -actin (for apoC-II), the amount of each sample was expressed relative to the sample in lane 1 (for apoC-I) or the samples in lanes 1 and 5 (for apoC-II) (set as 1.0). B, relative apoC-I and apoC-II mRNA levels were determined relative to the sample in lane 1 (for apoC-I) or the samples in lanes 1 and 5 (for apoC-II) (set as 100%). Results are means  $\pm$  S.D. of samples from three different animals per group (wild type or TR4KO).

tively. However, when the FXR/RXR $\alpha$  heterodimer was co-transfected with TR4, the induced transcriptional activity of the reporter gene was reduced to the basal level (lane 4).

Several nuclear receptor signaling pathways may be involved in this suppressive effect between TR4 and the FXR/RXR $\alpha$  heterodimer relative to transcriptional activity. To determine whether competition between TR4 and RXR $\alpha$  for FXR binding results in these antagonistic effects, we used mammalian two-hybrid assays to test whether TR4 could form heterodimeric complexes with FXR. The expression vector VP16-TR4, consisting of the full-length human TR4 fused to transcriptional activator VP16, was co-transfected with the pG5-Luc reporter plasmid and either GAL4-FXR or GAL4-RXR $\alpha$  into COS-1 cells. As shown in Fig. 7B, co-transfection of VP16-TR4, VP16-RXR $\alpha$ , GAL4-FXR, or GAL4-RXR $\alpha$  together with either GAL4 or VP16 empty vector showed a low background level of transcriptional activity. Consistent with a previous report (28), significant induction was observed when VP16-RXR $\alpha$  was co-transfected with GAL4-FXR (Fig. 7B, lane 6). However, co-transfection of VP16-TR4 with either GAL4-FXR or GAL4-RXR $\alpha$  showed near background levels, suggesting that TR4 does not form a heterodimer with either FXR or RXR $\alpha$  (Fig. 7B, lanes 7 and 8). This result suggests that TR4 and the FXR/RXR $\alpha$  heterodimer may compete with each other for binding to partially overlapping response elements. To confirm this hypothesis, a gel shift assay was performed using  $^{32}$ P-DR1/IR1. As shown in Fig. 7C, both *in vitro* translated TR4 and the FXR/RXR $\alpha$  heterodimer could form complexes, but TR4 showed a higher affinity for the  $^{32}$ P-DR1/IR1 (lane 2, open arrowhead versus lane 5, arrow). Differences in DR1/IR1 binding affinity between TR4 and the FXR/RXR $\alpha$  heterodimer may be due to different expression levels of coupled *in vitro* transcribed and translated TR4, FXR, and RXR $\alpha$ . To rule out this possibility, we examined the protein levels of *in vitro* translated RXR $\alpha$ , FXR, and TR4 with [ $^{35}$ S]Met by SDS-PAGE. As shown in Fig. 7D, TR4 protein level was similar to or lower than the levels of FXR and RXR $\alpha$ , suggesting that TR4 had much higher affinity for the DR1/IR1 element than did the FXR/RXR $\alpha$  heterodimer. When *in vitro* translated TR4 was added together with *in vitro* translated FXR and RXR $\alpha$ , the TR4 binding band was obviously reduced (Fig. 7C, lane 2 versus lane 6), and the

FXR/RXR $\alpha$ -DR1/IR1 complex disappeared (Fig. 7C, lane 5 versus lane 6). To further confirm whether endogenous TR4 can interact with this DR1/IR1 element, we performed a gel shift assay using HepG2 cell nuclear extracts. As shown in Fig. 7E, TR4 binding disappeared when the nuclear extracts were incubated with excessive amounts of cold wild-type DR1 probe but was still intact in the presence of mutant DR1 probe (Fig. 7E, lane 2 versus lane 3). This binding complex showed a supershift with the addition of anti-TR4 antibody (lane 4). This result clearly shows that endogenous TR4 interacts with TR4RE-DR1-apoE even in the presence of an overlapping IR1, the FXR/RXR $\alpha$  response element.

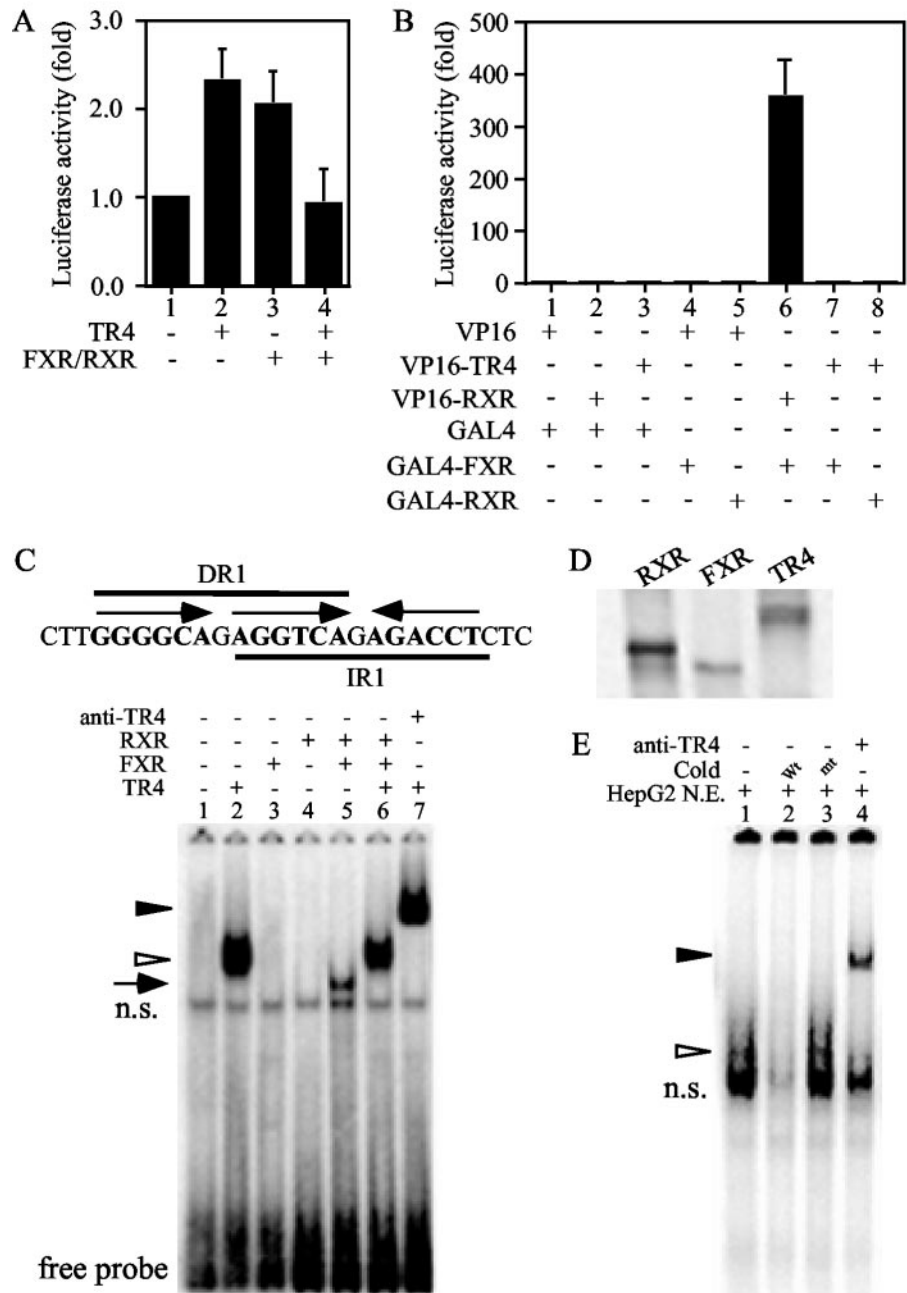
#### DISCUSSION

Hepatic apoE expression has previously been reported to be regulated by its liver-specific enhancer region, HCR (8–10). However, the molecular mechanism mediating HCR regulation of apoE gene expression remains unclear. Based on the presence of a potential DR1 response element (TR4RE-DR1-apoE) in the HCR-1, we demonstrated that TR4 induces apoE expression in HepG2 hepatoma cells via the TR4RE-DR1-apoE element in HCR-1. Data from TR4KO mice further confirmed the importance of TR4 in the regulation of apoE expression as well as in the expression of other apolipoproteins, apoC-I and C-II, in the same gene cluster.

Early studies suggested that DR1-HRE sequences could bind various nuclear receptors, including TR4, TR2, retinoic acid receptors, RXRs, peroxisome proliferator-activated receptors, and HNF-4 (14, 29–33). However, the affinity and specificity of these nuclear receptors for DR1-HRE may depend on the sequences of core motifs and spacer nucleotides of such elements (34, 35). HNF-4, a liver enriched factor, has been shown to play important roles in lipid metabolism and transport via induction of several apolipoprotein genes, including apoE (27, 36–40). While HCR-1 is HNF-4-responsive in gel shift assays, the detailed binding site(s) for HNF-4 in the HCR-1 remain unclear. Here we demonstrate that HNF-4 has only a marginal effect on the modulation of apoE expression via TR4RE-DR1-apoE in the HCR-1, which may explain why we can see a strong TR4 effect on apoE expression in HepG2 cells and in the TR4KO mouse model, yet there is no obvious change in apoE expression in HNF-4KO mice as

**FIG. 7. The effect of the FXR/RXR $\alpha$  heterodimer on TR4-mediated transcriptional activation.**

**A**, a reporter gene construct (300 ng of pGL-apoE/HCR-1-Luc) was co-transfected with constant amounts (150 ng) of TR4 (pCMV-TR4) or FXR and RXR $\alpha$  expression vectors (pSG5-FXR and pCMV-RXR $\alpha$ ) into HepG2 cells as indicated. **B**, mammalian two-hybrid interaction of FXR with RXR $\alpha$ , but not with TR4, in COS-1 cells. COS-1 cells were transiently transfected with 500 ng of pG5-Luc and 200 ng of different fusion plasmids as indicated. The two-hybrid interaction was expressed as -fold induction relative to that of the GAL4/VP16-transfected sample (lane 1, set as 1.0-fold). Data presented in **A** and **B** represent the mean  $\pm$  S.D. of at least three individual assays. **C**, a gel shift assay was performed with *in vitro* translated TR4, FXR, or RXR $\alpha$  as indicated. The  $^{32}$ P-labeled DR1/IR1 element was used as a probe. Retarded complexes containing TR4 and the FXR/RXR $\alpha$  heterodimer as well as the supershift induced by an anti-TR4 antibody are indicated by the open arrowhead, arrow, and closed arrowhead, respectively. Free probes are shown as indicated. *n.s.*, nonspecific binding. **D**, analysis of *in vitro* translated products. After expression with [ $^{35}$ S]Met in a coupled transcription and translation system (50- $\mu$ l reaction), 1.5  $\mu$ l of *in vitro* translated RXR $\alpha$  and FXR or 3  $\mu$ l of *in vitro* translated TR4 were subjected to 10% SDS-PAGE for analysis of relative expression between samples. **E**, a gel shift assay was performed using nuclear extracts of HepG2 cells with the  $^{32}$ P-labeled DR1/IR1 element. Twenty molar excesses of unlabeled DR1 oligonucleotides (*Wt*) or mutated DR1 oligonucleotides (*mt*) were added as competitor DNA (lanes 2 and 3). For the supershift assay, an anti-TR4 antibody was added as indicated. The retarded complex and the supershifted band are indicated by open and closed arrowheads, respectively. *n.s.*, nonspecific binding.



described in recent studies (41). The reason for the lack of apoE response to HNF-4 may be due to the particular sequences of the potential DR1-HRE site in the HCR-1 region. Although it is considered a DR1 consensus site, the specific sequence of the TR4RE-DR1 in the HCR-1, including the spacer nucleotide G, may be unfavorable to HNF-4 binding. Previous reports have demonstrated that changing the spacer nucleotide from A to G reduces the affinity of HNF-4 for DR1-HRE (34, 35).

Early studies have shown that TR4 might be able to modulate gene expression via binding to AGGTCA DRs with variable length spacer sequences, from one to five (DR1-DR5) nucleotides, with its highest affinity for DR1 elements (13-17). Here we show that loss of TR4 results in reduction of the expression of the apoE, apoC-I, and apoC-II genes, which are members of the same gene cluster. We do not know whether TR4 regulates apoE, apoC-I, and apoC-II solely through TR4RE-DR1-apoE in the HCR-1. Recently the FXR/RXR $\alpha$  heterodimer was shown to regulate apoC-II expression via an IR1 element that partially

overlaps the TR4-DR1-apoE element in HCR-1 (11). This complex arrangement suggests the involvement of various nuclear receptors in transcriptional regulation via the HCR. In support of this idea, previous studies have demonstrated that a variety of nuclear receptors regulate many genes through binding either the same, or partially overlapping, response elements (19-22). At present, the effects of the FXR/RXR $\alpha$  heterodimer on transcription of the apoE gene is unclear even though transcription of the apoE and apoC-II genes is under the control of the tissue-specific enhancer region HCR in the liver. In reporter gene assays in HepG2 cells, we were able to see TR4- and FXR/RXR $\alpha$  heterodimer-mediated transcriptional induction of the apoE gene and that these two receptors show antagonistic effects. Many nuclear receptors can form heterodimers with other receptors, such as RXR, and cross-talk between nuclear receptor signaling pathways has occurred via heterodimerization between nuclear receptors (23, 28, 42-45). In this study, TR4 shows no interaction with either FXR or

RXR $\alpha$ , suggesting that antagonistic effects may be through competition between TR4 and the FXR/RXR $\alpha$  heterodimer for binding to partially overlapping response elements (DR1/IR1). Recently we have shown that TR4 can bind to a single AG-GTCA core motif, preceded by an AT-rich sequence, as a monomer (46). Thus, TR4 may not only bind to TR4RE-DR1-apoE as a homodimer but may also occupy a single AGGTCA motif of the IR1 FXR response element as a monomer, thereby moving the FXR/RXR $\alpha$  heterodimer away from this DR1/IR1 element.

However, our results and previous studies show that TR4 and the FXR/RXR $\alpha$  heterodimer can regulate expression of apoC-II, another apolipoprotein gene in the apoE/apoC-I/apoC-IV/apoC-II gene cluster (11). This is a puzzling observation even though it has been reported in many studies (19–22). One possibility is that the availability of RXR $\alpha$  may determine the transition from FXR control to TR4 control of hepatic expression of the apoE gene. RXR $\alpha$  is required for heterodimerization with many liver abundant nuclear receptors, including FXR (28, 42–45). Sequestration of RXR $\alpha$  away from FXR by other nuclear receptors would result in reduction in the numbers of available FXR/RXR $\alpha$  heterodimers for transcriptional induction of the apoE gene.

An alternative explanation is that under physiological conditions various metabolic cues may signal TR4 and the FXR/RXR $\alpha$  heterodimer to regulate these genes dynamically, leading to multiple transcriptional responses. Unfortunately the endogenous ligand for TR4 has not yet been determined, so identification of such a ligand may provide further insight regarding the mechanisms of TR4-mediated gene regulation.

Although further study is necessary to determine the mechanism that controls TR4 regulation of apoE, apoC-I, and apoC-II expression, it seems that multiple nuclear receptors, such as TR4 and the FXR/RXR $\alpha$  heterodimer, regulate the same gene via partially overlapping response elements (DR1 and IR1). This may explain why we are unable to see the disappearance of expression of these genes in either TR4KO or FXR knockout mice (11).

In summary, our data demonstrate that TR4 modulates hepatic apoE expression and the expression of other apolipoproteins, such as apoC-I and apoC-II, via the tissue-specific enhancer HCR-1. The present study suggests that TR4 is involved in regulation of lipoprotein metabolism, and the finding of the natural ligand for TR4 will help in understanding the role of TR4 in various complex, hormone nuclear receptor-related processes in the liver.

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