Synergism between nuclear receptors bound to specific hormone response elements of the hepatic control region-1 and the proximal apolipoprotein C-II promoter mediate apolipoprotein C-II gene regulation by bile acids and retinoids

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We have shown previously that the hepatic control region 1 (HCR-1) enhances the activity of the human apolipoprotein C-II (apoC-II) promoter in HepG2 cells via two hormone response elements (HREs) present in the apoC-II promoter. In the present paper, we report that the HCR-1 selectively mediates the transactivation of the apoC-II promoter by chenodeoxycholic acid (CDCA) and 9-*cis*-retinoic acid. CDCA, which is a natural ligand of farnesoid X receptor α (FXRα), increases the steady-state apoC-II mRNA levels in HepG2 cells. This increase in transcription requires the binding of retinoid X receptor α (RXRα)–FXRα heterodimers to a novel inverted repeat with one nucleotide spacing (IR-1) present in the HCR-1. This element also binds hepatocyte nuclear factor 4 and apoA-I regulatory protein-1. Transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα heterodimers in the presence of CDCA was abolished by mutations either in the IR-1 HRE of the HCR-1 or in the thyroid HRE of the proximal apoC-II promoter, which binds RXRα–thyroid hormone receptor β (T3Rβ) heterodimers. The same mutations also abolished transactivation of the HCR-1/apoC-II promoter cluster by RXRα–T3Rβ heterodimers in the presence of tri-iodothyronine. The findings establish synergism between nuclear receptors bound to specific HREs of the proximal apoC-II promoter and the HCR-1, and suggest that this synergism mediates the induction of the HCR-1/apoC-II promoter cluster by bile acids and retinoids.

Key words: apolipoprotein C-II (apoC-II), bile acids, gene regulation, hepatic control region 1 (HCR-1), hypertriglyceridaemia.

INTRODUCTION

Apolipoprotein C-II (apoC-II) is an activator of lipoprotein lipase, the enzyme that hydrolyses triacylglycerols that are present in chylomicrons and very-low-density lipoproteins (VLDLs). Patients with inherited apoC-II deficiency are unable to clear triacylglycerol-rich lipoprotein particles from their plasma and develop severe hypertriglyceridaemia [1–7].

The major site of apoC-II mRNA and protein synthesis in humans is the liver and a minor site is the intestine [8–10]. The human *apoC-II* gene has been mapped on the long arm of chromosome 19, in a 45 kb gene cluster that also contains the genes encoding apolipoprotein E (*apoE*), apolipoprotein C-I (*apoC-I*) and apolipoprotein C-IV (*apoC-IV*) [11–13]. Functional studies using transgenic mice have provided evidence for the existence of common regulatory regions within this gene cluster that control the tissue-specific expression of the four genes of the cluster [14–16]. Two such regions that are required for the hepatic expression of the four genes were identified and designated hepatic control region 1 and 2 (HCR-1 and HCR-2 respectively) [17]. *HCR-1* has been mapped within a 320 bp region approx. 15 kb 3′ of the *apoE* gene, in the intergenic region between the *apoC-I* gene and the *apoC-I* pseudogene (*apoCI′*). *HCR-2* has been mapped 27 kb 3′ to the *apoE* gene, in the intergenic region between the *apoC-I′* pseudogene and the *apoC-IV* gene. *HCR-2* is 85 % homologous with *HCR-1* and is believed to have arisen from the duplication of *HCR-1* [17]. Deletion of either *HCR-1* or *HCR-2* did not affect the hepatic expression of the genes of the cluster in transgenic mice; however, deletion of both regions abolished the hepatic expression of the genes of the cluster [16]. In previous studies, we have shown that the 0.55 kb intergenic region between the *apoC-II* and *apoC-IV* genes is a strong hepatocyte-specific promoter, and its activity is enhanced by HCR-1 [18]. An important role in *apoC-II* gene regulation and transcriptional enhancement by HCR-1 is played by two hormone response elements (HREs), which map within the footprinted regions CIIB (− 102/− 81) and CIIC (− 156/− 116) and have different specificities for nuclear receptors [18,19]. The HRE in element CIIC is a direct repeat with four nucleotide spacing and serves as a thyroid HRE (TRE) that binds retinoid X receptor α (RXRα)–thyroid hormone receptor β (T3Rβ) heterodimers, as well as the orphan nuclear receptors apoA-I regulatory protein 1 (ARP-1) and vErb-A-related protein 2 (EAR-2). Element CIIB is recognized exclusively by hepatocyte nuclear factor 4 (HNF-4) [18,19]. RXRα–T3Rβ heterodimers transactivate in the presence of tri-iodothyronine (T3), as well as HNF-4, whereas ARP-1 and EAR-2

Abbreviations used: apoC-I (etc.), apolipoprotein C-I (etc.); apoC-I′, apoC-I pseudogene; ARP-1, apoA-I regulatory protein 1; ARP-PO, acidic ribosomal phosphoprotein; CA, cholic acid; CAT, chloramphenicol acetyltransferase; CDCA, chenodeoxycholic acid; DMEM, Dulbecco's modified Eagle's medium; EAR-2, vErb-A-related protein 2; EMSA, electrophoretic mobility shift assay; FBS, foetal bovine serum; FXRα, farnesoid X receptor α; HCR-1, hepatic control region 1; HNF-4, hepatocyte nuclear factor 4; HRE, hormone response element; HSV-tk, herpes simplex virus thymidine kinase; I-BABP, intestinal bile-acid-binding protein; IR-1: inverted repeat with one nucleotide spacing; luc, luciferase promoter; RA, 9-*cis*-retinoic acid; RT, reverse transcriptase; RXRα, retinoid X receptor α; T3, tri-iodothyronine; T3Rβ, thyroid hormone receptor β; VLDL, very-low-density lipoprotein; WT, wild-type.

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repress the apoC-II promoter activity in transient transfection assays [18,19]. These findings indicate that hormone nuclear receptors are essential for apoC-II gene expression in hepatocytes and, as a consequence, control triacylglycerol homoeostasis.

In the present study, we demonstrate that HCR-1 selectively mediates the activation of the apoC-II promoter by chenodeoxycholic acid (CDCA) and 9-*cis*-retinoic acid (RA), which are the natural ligands of farnesoid X receptor α (FXRα) and RXRα respectively, but does not activate the apoE promoter or the heterologous herpes simplex virus thymidine kinase (HSV-tk) promoter. Bile acids are the major products of cholesterol catabolism and are essential for the solubilization of and transport of dietary lipids [20]. A number of studies have shown that the primary bile acid CDCA, the secondary bile acids deoxycholic acid and lithocolic acid, as well as the conjugated bile acids glycochenodeoxycholic acid and taurochenodeoxycholic acid, are the natural ligands of FXRα [21,22]. FXRα is specifically expressed in tissues actively involved in bile-acid metabolism, such as liver, intestine and kidney, and activates gene transcription by forming heterodimers with RXRα [23]. Among the genes regulated by RXRα–FXRα heterodimers in response to bile acids are those encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis, and intestinal bile-acid-binding protein (I-BABP), a protein that is actively involved in the enterohepatic circulation of bile acids [24,25].

In the present study, we established that the transactivation of the apoC-II promoter by CDCA is mediated by the synergism between RXRα–FXRα heterodimers, which bind to a novel inverted repeat with one nucleotide spacing (IR-1) HRE present in HCR-1, and other nuclear receptors, which bind to the TRE of the proximal apoC-II promoter. Inactivation of either the IR-1 HRE of the HCR-1 or the proximal TRE by mutations abolished the transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα in the presence of CDCA. The same mutations also abolished the transactivation of the HCR-1/apoC-II promoter cluster by RXRα–T3Rβ in the presence of T3. These findings suggest that HCR-1, in addition to its role as a strong hepatic transcriptional enhancer of genes of the *apoE*/*apoC-I*/*apoC-IV*/*apoC-II* cluster, also mediates the expression of the *apoC-II* gene in response to bile acids and retinoids.

EXPERIMENTAL

Materials

Restriction enzymes and modifying enzymes (T4 DNA ligase, the Klenow fragment of DNA polymerase I, calf intestinal alkaline phosphatase) were purchased from Minotech (Heraklion, Crete, Greece), New England Biolabs or Life Technologies. Vent DNA polymerase was purchased from New England Biolabs. Poly(dI-dC) · (dI-dC), acetyl-CoA and dNTPs were purchased from Pharmacia. [α-^{32}P]dATP and [α-^{32}P]dCTP were purchased from Amersham Biosciences or DuPont–New England Nuclear. All reagents for cell culture [Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), trypsin-EDTA and PBS] were purchased from Life Technologies. *o*-Nitrophenyl β-D-galactopyranoside, PMSF and the anti-FLAG M2 antibody were purchased from Sigma. The anti-HNF-4 (C-19), anti-RXRα (D-20) and anti-T3Rβ (J51) antibodies were purchased from Santa Cruz Biotechnology. Oligonucleotides used in this study were synthesized at the Microchemistry Facility of the Institute of Molecular Biology and Biotechnology (University of Crete, Heraklion, Crete, Greece). The luciferase assay kit was purchased from Promega. CDCA, cholic acid (CA) and T3 were purchased from Sigma-Aldrich. RA was a gift from Dr Hinrich Gronemeyer [Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) Illkirch, France].

Plasmid constructions

Plasmids (−388/+18) apoC-II chloramphenicol acetyltransferase (CAT), (11/324) HCR-1/(−388/+18) apoC-II CAT, (11/324) HCR-1/(−388/+18 Bmut) apoC-II CAT, (11/324) HCR-1/(−388/+18 Cmut) apoC-II CAT and (11/324) HCR-1/(−388/+18 B/Cmut) apoC-II CAT have been described previously in [18]. Plasmids (−545/+18) apoC-II luciferase promoter (luc) and (11/324) HCR-1/(−545/+18) apoC-II luc were constructed as follows: the (−545/+18) apoC-II CAT and (11/324) HCR-1/(−545/+18) apoC-II CAT plasmids [18] were digested with *Xba*I and *Xho*I and the resulting fragments were blunt-ended with Klenow DNA polymerase I and inserted into the *Sma*I site of the vector pGL3-basic (Promega). Plasmid (11/324) HCR-1/(−85/+1) tk CAT was constructed by digesting the plasmid (11/324) HCR-1/(−545/+18) apoC-II CAT with *Xba*I and *Kpn*I and ligating the excised fragment containing the (11/324) HCR-1 sequence into the vector (−85/+1) tk CAT at the corresponding sites. Plasmid (−500/+77) apoE luc was constructed as follows: the −500/+77 region of the apoE promoter was PCR-amplified from human genomic DNA using the primers apoE (−500) sense and apoE (+77) antisense (Table 1) bearing *Kpn*I and *Hin*dIII sites respectively. The amplification conditions included an initial denaturation step at 94 °C for 5 min followed by 35 repeated cycles of denaturation (94 °C for 1.5 min), annealing (65 °C for 1.5 min) and extension (72 °C for 2 min). The PCR product containing the apoE promoter was gel-purified, digested with *Kpn*I and *Hin*dIII and inserted into the corresponding sites of vector pGL3-Basic. Plasmid (11/324) HCR-1/(−500/+77) apoE luc was constructed by subcloning the HCR-1 fragment into the *Sal*I/*Bam*HI sites of vector (−500/+77) apoE luc, downstream of the luciferase gene. Plasmid (211/231) HCR-1/(−388/+18) apoC-II CAT and (211/231 mut) HCR-1/(−388/+18) apoC-II CAT were constructed by inserting the wild-type (WT) or mutant (211/231) HCR-1 double-stranded oligonucleotides (Table 2) bearing *Xba*I and *Kpn*I at the 5′ and 3′ ends respectively into the corresponding sites of plasmid (−388/+18) apoC-II CAT. Plasmid (211/231) HCR-1/(−388/+18 B/Cmut) apoC-II CAT was constructed by inserting the WT (211/231) HCR-1 double-stranded oligonucleotide into the *Xba*I/*Kpn*I sites of plasmid (−388/+18 B/Cmut) apoC-II CAT. Reporter plasmids bearing deletions of HCR-1 fused with the (−545/+18) apoC-II promoter were constructed as follows: first, HCR-1 fragments of different lengths were amplified with specific 5′ and 3′ primers (Table 1). The amplification conditions included an initial denaturation step at 94 °C for 5 min, followed by 25 repeated cycles of denaturation (94 °C for 1 min), annealing (61 °C for 30 s) and extension (72 °C for 2 min), and a final extension at 72 °C for 10 min. The resulting PCR fragments were gel-purified, digested with *Xba*I and *Kpn*I and inserted into the (−545/+18) apoC-II CAT plasmid at the corresponding sites. The expression vector pCMX-FXRα was constructed by cloning the FXRα cDNA amplified from HepG2 RNA by reverse transcriptase (RT)-PCR into the vector pCMX. Expression vectors for HNF-4, ARP-1, RXRα and T3Rβ have been described previously in [26,27].

Cell cultures, transient transfections, CAT and luciferase assays

Human hepatoma HepG2 cells, monkey kidney COS-7 cells and human embryonic kidney HEK-293 cells were cultured

Table 1 Oligonucleotide primers for PCR

Oligonucleotide name	Sequence	Purpose
HCR-I/II sense	5′-GGG **TCT AGA** GGC ACA CAG GAG TTT CTG GGC TCA-3′	Used as 5′ primer for the amplification of the 11/260, 11/206, 11/180 and 11/108 HCR-1 regions, Sense orientation. *Xba*I site is shown in bold.
HCR-I/260 antisense	5′-GG**G GTA CC**T CGA GTG GAT GTT GGA GGT GGC AT-3′	Used as 3′ primer for the amplification of the 11/260 and 118/260, HCR-1 regions. Antisense orientation. *Kpn*I site is shown in bold.
HCR-I/206 antisense	5′-GG**G GTA CC**A GCT CCA AGG TCA GCA GGC AGG-3′	Used as 3′ primer for the amplification of the 11/206 HCR-1 region. Antisense orientation. *Kpn*I site is shown in bold.
HCR-I/185 antisense	5′-GG**G GTA CC**G GAG GGC TGT GTG TTT GCT GTT TGC-3′	Used as 3′ primer for the amplification of the 11/185 HCR-1 region. Antisense orientation. *Kpn*I site is shown in bold.
HCR-I/118 antisense	5′-GG**G GTA CC**G ATA TCG TTT GTT CTG TGT GGA CTT CAG AGG CAG CA-3′	Used as 3′ primer for the amplification of the 11/118 HCR-1 region. Antisense orientation. *Kpn*I site is shown in bold.
HCR-I/118 sense	5′-GGG **TCT AGA** GCC TCT GAA GTC CAC ACT GAA CAA ACT TCA GC-3′	Used as 3′ primer for the amplification of the 118/260 HCR-1 region. Antisense orientation. *Xba*I site is shown in bold.
ApoE (− 500) sense	5′-TTT **GGT ACC** GCT GGT CTC AAA CTC CTG ACC TTA AGT GAT TCG-3′	Used as 5′ primer for the amplification of the − 500/ + 77 apoE promoter region. Sense orientation. *Kpn*I site is shown in bold.
ApoE (+ 77) antisense	5′-TTT **AAG CTT** AAC TCG TGG AGT CCT GCT ATG TAC ATG CC-3′	Used as 3′ primer for the amplification of the − 500/ + 77 apoE promoter region. Sense orientation. *Hind*III site is shown in bold.

Table 2 Oligonucleotide probes in gel mobility shift and competition assays

Oligonucleotide name	Sequence	Location
HCR-1 (211/231) sense	5′-CTA GAT ATC AGA GGT CAG AGA CCT CTC TG-3′	Corresponds to the 211/231 region of HCR-1. Sense strand.
HCR-1 (231/211) antisense	5′-GAT CCA GAG AGG TCT CTG ACC TCT GAT AT-3′	Corresponds to the 211/231 region of HCR-1. Antisense strand.
HCR-1 (211/231) mut sense	5′-CTA GAT ATC AGA **AT**T C**T**G A**CG** CCT CTC TG-3′	Corresponds to the 211/231 region of HCR-1 and contains mutations shown in bold. Sense strand.
HCR-1 (231/211) mut antisense	5′-GAT CCA GAG AGG **CG**T C**A**G A**AT** TCT GAT AT-3′	Corresponds to the 211/231 region of HCR-1 and contains mutations shown in bold. Antisense strand.
I-BABP (− 163/− 145) sense	5′-CTA GCC AGG GTG AAT AAC CTC GG-3′	Corresponds to the − 163/− 145 region of the human I-BABP promoter. Sense strand.
I-BABP (− 145/− 163) antisense	5′-CTA GCC GAG GTT ATT CAC CCT GG-3′	Corresponds to the − 145/− 163 region of the human I-BABP promoter. Antisense strand.
WAF(− 123/− 88) sense	5′-GGG AGG GCG GTC CCG GGC GGC GCG GTG GGC CGA GCG-3′	Corresponds to the − 123/− 88 region of the human p21/WAF1 promoter. Sense strand.
WAF(− 88/− 123) antisense	5′-GGG CGC TCG GCC CAC CGC GCC GCC CGG GAC CGC CCT-3′	Corresponds to the − 88/− 123 region of the human p21/WAF1 promoter. Antisense strand.

in DMEM (Glutamax) supplemented with 10% FBS, and penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere. For experiments involving treatment of the cell culture with CDCA, CA, RA or T3, cells were grown in DMEM containing 5% of charcoal-stripped serum. Transient transfections were performed using the $Ca_3(PO_4)_2$ co-precipitation method [28]. At 40 h following transfection, cells were harvested and lysed in 1× lysis buffer, included in the luciferase assay kit, followed by a freeze–thaw cycle and centrifugation at 9500 ***g*** for 5 min at room temperature (25 °C). CAT and β-galactosidase assays were performed as described previously in [29,30]. Luciferase assays were performed using the Promega luciferase assay kit following the manufacturer's instructions.

Preparation of whole-cell extracts from transfected COS-7 cells

COS-7 cells were maintained as stocks in DMEM supplemented with 10% foetal calf serum. Cells at 50–60% confluence in 150 mm diameter dishes were transfected with 42 μg of pMT2-HNF4, pMT2-ARP1, pMT2-RXRα, pMT2-T3Rβ and pCMX-FXRα plasmids. At 40 h after transfection, cells were collected in 40 mM Tris/HCl, pH 7.4, 1 mM EDTA and 0.15 M NaCl, and were pelleted by low-speed centrifugation (2400 ***g*** for 5 min at room temperature). Cells were resuspended in 400 μl of a buffer containing 20 mM Tris/HCl, pH 7.4, 0.4 M KCl, 2 mM dithiothreitol and 10% (v/v) glycerol, and were broken by freeze–thaw three times. Cell debris was removed by centrifugation at 9500 ***g*** for 5 min at 4 °C in a microfuge, and the supernatant (whole-cell extracts) was divided into 100 μl aliquots and stored at − 70 °C.

Western blotting

For Western blotting analysis, extracts from HEK-293 cells were resolved by SDS/PAGE (10.5%). Electrophoresis was performed in 500 ml of 1 × TGS (1l of 10 × TGS contains 30.3 g Tris, pH 8.3, 144.2 g glycine and 10 g SDS), using the Bio-Rad Protean electroblotting apparatus. Proteins on the membrane were visualized by Ponceau S staining. Nitrocellulose membranes were washed with TBST [TBS (0.5 M Tris/HCl, pH 8, and 8.8 mg/ml NaCl) containing 0.05% (v/v) Tween 20] for 10 min at room temperature. Non-specific sites were blocked by soaking the membrane in TBS blocking buffer (TBB) [1 × TBS with 5% (w/v) non-fat dry milk powder and 0.05% (v/v) Tween 20] for 2 h at 4 °C. Western blotting was performed with a 1 : 5000 dilution of the anti-RXRα polyclonal and the anti-T3Rβ monoclonal antibodies in TBB overnight at 4 °C. The membranes were washed three times with TBST for 10 min at

room temperature. As a secondary antibody, we used anti-mouse or anti-rabbit horseradish peroxidase-conjugated at a 1 : 10000 dilution in TBST for 1 h at room temperature. After three washes of 15 min with TBST at room temperature, bands were visualized by enhanced chemiluminescence detection.

Electrophoretic mobility shift, competition and supershift assays

Electrophoretic mobility shift assays (EMSAs) were performed as described previously in [31]. Sense and antisense oligonucleotides were annealed to generate the double-stranded probe and labelled with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dCTP and [α-^{32}P]dATP. The sequence of the oligonucleotides used as probes in the EMSAs is shown in Table 2. For competition and supershift assays, a 100-fold or 200-fold molar excess of non-labelled probe or 1 μl of anti-HNF-4 or anti-RXRα polyclonal antibodies were included in the reaction for 15 min before the addition of the labelled probe.

RT-PCR analysis

HepG2 cells were treated with 10^{-4} M CDCA for various lengths of time, total RNAs were prepared and treated with 10 units of DNaseI (Life Technologies) for 30 min at room temperature. The RNA was extracted with phenol/chloroform and precipitated with ethanol. cDNA synthesis was performed with SuperScript RT (Life Technologies) using oligo(dT) as primer. The cDNAs were used directly for PCR amplification (20 cycles) in the presence of 2.5 μCi of [α-^{32}P]dCTP with primers corresponding to exons 3 and 4 of the human *apoC-II* gene. The sequences of the primers were: sense, 5′-GAGATGCCTAGCCCGACCTTCCTCAC-3′, antisense, 5′-GCTCAGTCTGAACCTGGGGGATCAGG-3′. For normalization, the human acidic ribosomal phosphoprotein (ARP-PO) was amplified as described in [32]. PCR products were analysed by electrophoresis in 5 % native polyacrylamide gels followed by autoradiography and quantification by phosphorimage analysis.

RESULTS

Induction of *apoC-II* gene transcription in HepG2 cells by CDCA

In previous studies, we have shown that the activity of the apoC-II promoter can be modulated by orphan nuclear receptors, such as HNF-4, ARP-1 and EAR-2, as well as by ligand-dependent nuclear receptors such as RXRα–T3Rβ heterodimers in response to T3 [18,19]. In the present study, we investigated the mechanism of *apoC-II* gene induction by CDCA, a bile acid that serves as a ligand for FXRα. Consistent with previous findings [33], RT-PCR analysis of mRNAs extracted from HepG2 cells that were treated with 10^{-4} M CDCA for 12–36 h, as well as those from untreated HepG2 cells, showed that CDCA caused a 3.5-fold increase in steady-state apoC-II mRNA levels, whereas the steady-state mRNA levels of ARP-PO remained nearly constant (Figure 1).

To determine whether or not the observed increase in the steady-state apoC-II mRNA level by CDCA is the result of the transcriptional activation of the apoC-II gene, a series of transfections in HepG2 cells were performed using CAT or luciferase constructs containing the apoC-II, apoE and HSV-tk promoters alone, or the same promoters linked to the HCR-1 (Figure 2A). This analysis showed that the activity of the $-545/+18$ apoC-II promoter (Figure 2B) was not affected by treatment of HepG2 cells with 10^{-5} or 10^{-4} M of CDCA or

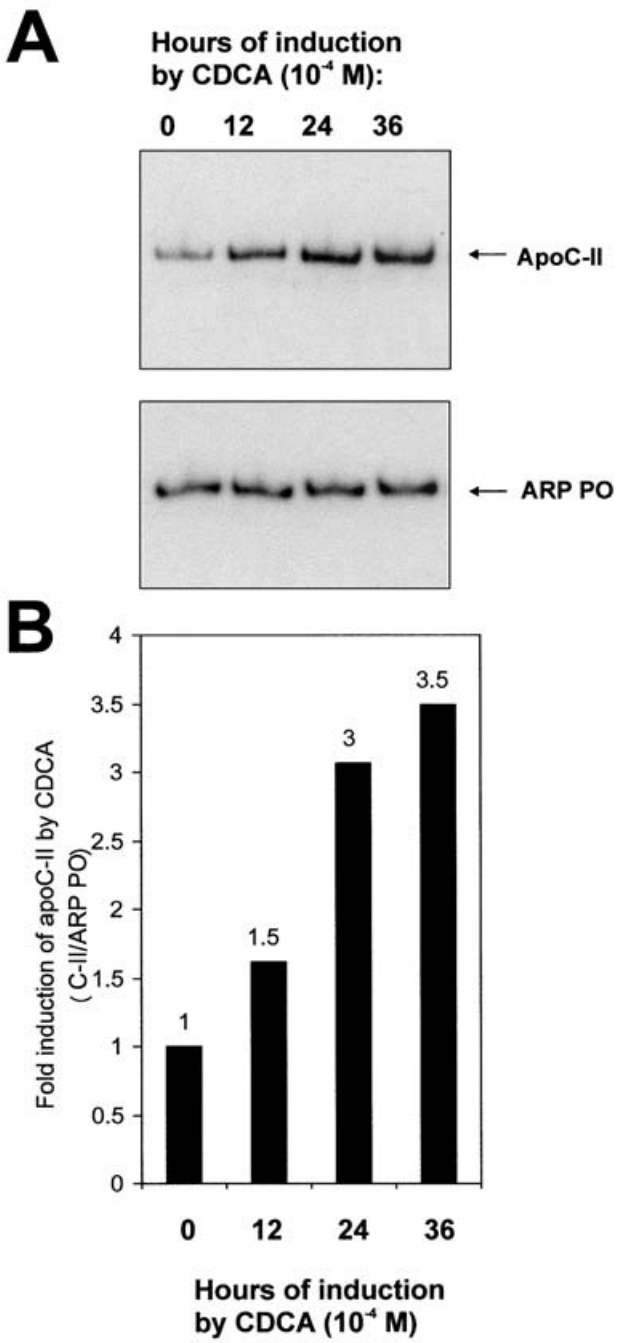

Figure 1 Induction of *apoC-II* gene transcription in HepG2 cells by CDCA

(**A**) RT-PCR analysis of apoC-II and ARP-PO gene expression in HepG2 cells treated with 10^{-4} M CDCA for the indicated time periods. Conditions for RT-PCR and the sequence of the apoC-II and ARP-PO specific primer sets are described in the Experimental section. The arrows show the position of the apoC-II and ARP-PO amplified cDNAs. (**B**) Quantification of the relative apoC-II and ARP-PO mRNA levels determined by RT-PCR using phosphoimage analysis, expressed as the fold induction of *apoC-II* gene expression by CDCA, normalized for ARP-PO expression levels.

CA, suggesting that the proximal $-545/+18$ apoC-II promoter is not sufficient to mediate transcriptional induction in response to bile acids. However, the (11/324) HCR-1/($-545/+18$) apoC-II promoter cluster was transactivated 1.6- and 1.8-fold respectively by 10^{-5} and 10^{-4} M CDCA (Figure 2B). This nearly 2-fold transactivation indicates the effect of the ligand on the endogenous

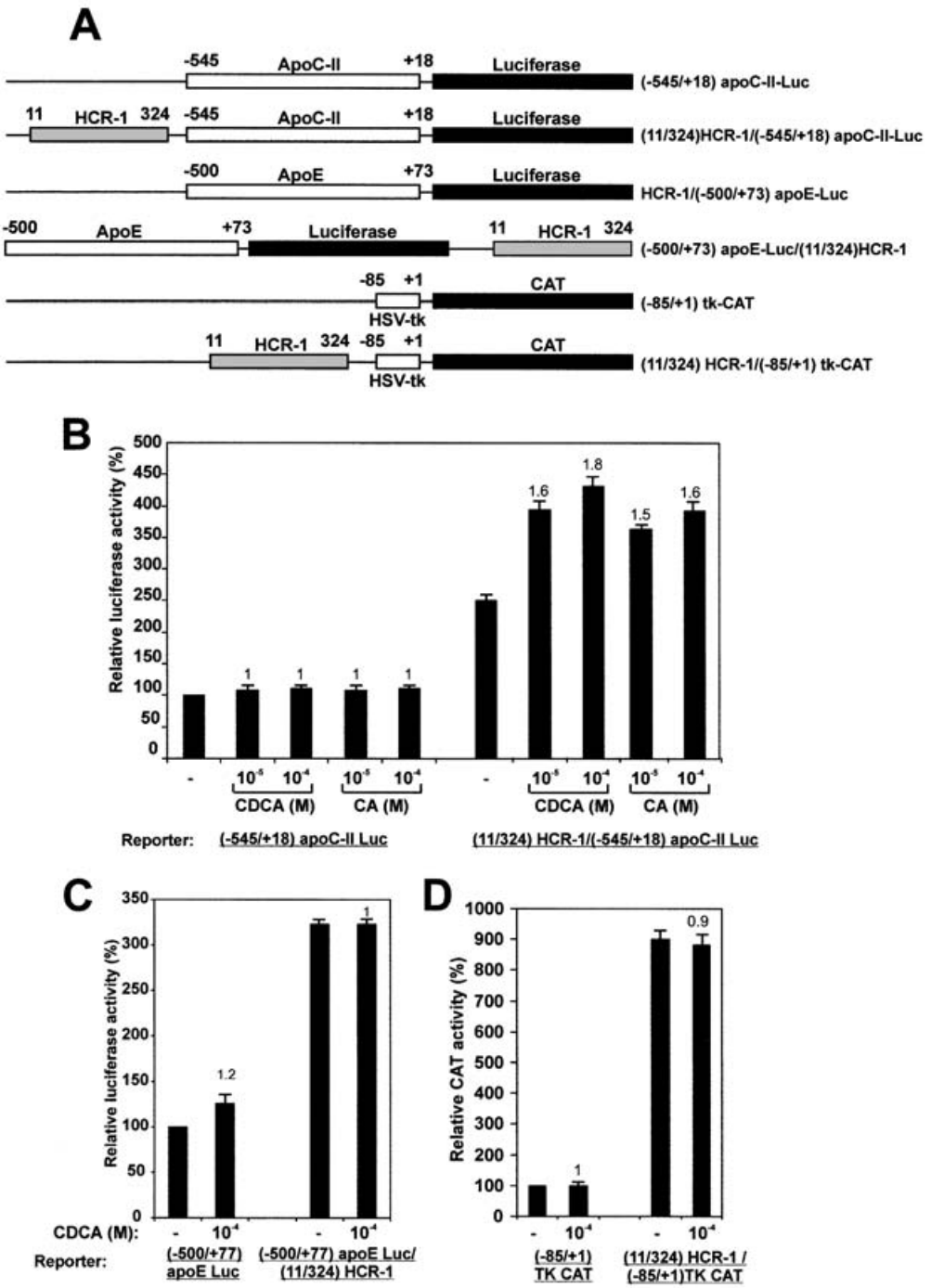

Figure 2 HCR-1 mediates transcriptional activation of the (− 545/+ 18) apoC-II promoter by the bile acids CDCA and CA

(**A**) Schematic representation of the reporter constructs used in the transient transfection experiments of (**B**)–(**D**). Grey rectangles represent the HCR-1 (nucleotides 11–324), open rectangles represent the apoC-II promoter (− 545/+ 18), the apoE promoter (− 500/+ 77) or the thymidine kinase promoter (− 85/+ 1), as indicated. Black rectangles represent the CAT and luciferase reporter genes, as indicated. Numbering of the promoter is relative to the transcription-initiation site of each gene. (**B**) HepG2 cells were transfected with the reporter constructs indicated (1 μg) and treated with 10^{-5} M or 10^{-4} M CDCA or CA for 24 h. In each experiment, the cytomegalovirus (CMV)–β-galactosidase plasmid was included for normalization of the transfection efficiency. Results are means ± S.D. of the relative luciferase activity from two independent experiments performed in duplicate. The fold induction in promoter activity by CDCA or CA is indicated above each bar. (**C**) and (**D**) HepG2 cells were transfected with the reporter constructs indicated (1 μg) and treated with 10^{-4} M CDCA for 24 h. In each experiment, the CMV–β-galactosidase plasmid was included for normalization of the transfection efficiency. Results are means ± S.D. of the relative luciferase (**C**) or CAT (**D**) activity from two independent experiments performed in duplicate. The fold induction in promoter activity by CDCA is shown above each bar.

nuclear receptors present in HepG2 cells. In contrast, no transcriptional enhancement of the activity of the (−500/+77) apoE promoter or the (−85/+1) HSV-tk promoter by CDCA in the absence or the presence of the HCR-1 was observed (Figures 2C and 2D). The results shown in Figure 2 suggest that HCR-1 selectively mediates the transactivation of the apoC-II promoter by the bile acids CDCA and CA.

RXRα–FXRα heterodimers transactivate the HCR-1/apoC-II promoter cluster in response to CDCA and RA

CDCA is the natural ligand for FXRα and induces gene transcription via RXRα–FXRα heterodimers bound to IR-1 HREs present in the promoters of target genes [21,22]. In order to assess the role of RXRα–FXRα heterodimers in the transactivation of the HCR-1/apoC-II promoter cluster by CDCA, we performed transient transfections in COS-7 cells. Treatment of COS-7 cells with 10^{-4} M CDCA or 10^{-6} M RA had a minor effect on the −545/+18 apoC-II promoter activity either in the absence or the presence of RXRα–FXRα heterodimers (Figure 3A). In contrast, the HCR-1/apoC-II promoter cluster was transactivated 18- and 11-fold by overexpression of RXRα–FXRα heterodimers in the presence of CDCA and RA respectively (Figure 3A). Furthermore, in agreement with the results presented in Figure 2, the (−500/+77) apoE promoter or the HCR-1/(−500/+77) apoE promoter cluster was not transactivated by RXRα–FXRα heterodimers in the presence or absence of either CDCA or RA (Figure 3B)

The findings shown in Figure 3 suggested that the transactivation of the HCR-1/apoC-II promoter cluster by CDCA or RA is mediated by RXRα–FXRα heterodimers and that HCR-1 is essential for this transactivation.

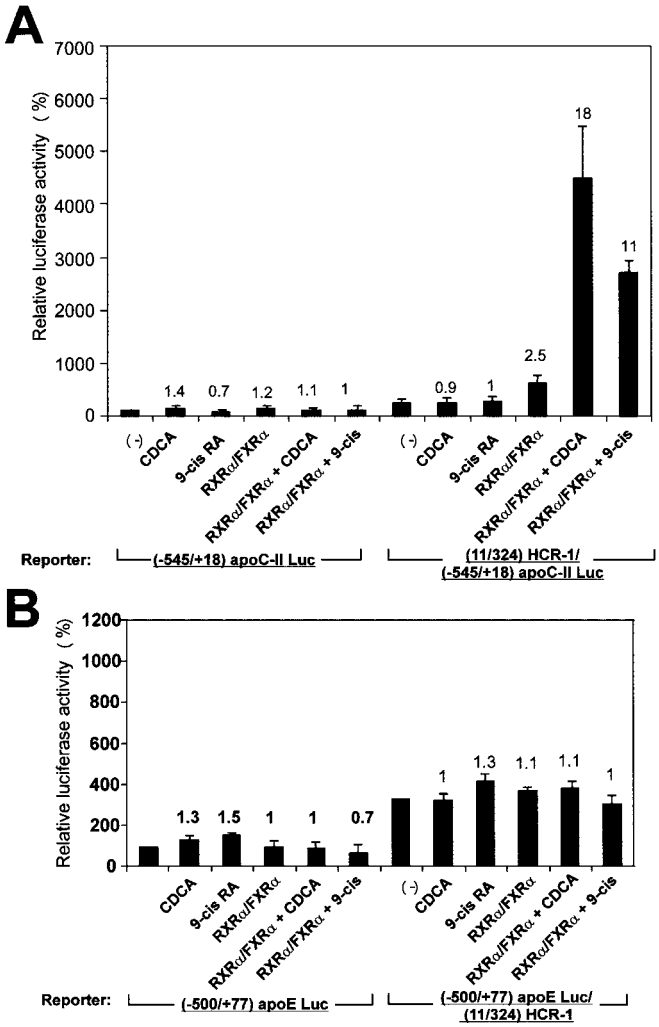

Figure 3 Transcriptional activation of the (−545/+18) apoC-II promoter by RXRα–FXRα heterodimers in response to CDCA and RA in COS-7 cells

(**A**) and (**B**) COS-7 cells were co-transfected with the reporter constructs indicated (1 μg) in the absence or presence of expression vectors expressing RXRα and FXRα (1 μg of each) and treated with 10^{-4} M CDCA or 10^{-6} M RA (9-cis RA or 9-cis) for 24 h. In each experiment, the cytomegalovirus (CMV)–β-galactosidase plasmid (1 μg) was included for normalization of the transfection efficiency. Results are means ± S.D. of the relative luciferase activity from two independent experiments performed in duplicate. The fold induction in promoter activity by the hormone nuclear receptors and ligands is shown above each bar.

Transactivation of the HCR-1/apoC-II promoter cluster by CDCA is mediated by the 206/260 regulatory region of the HCR-1

In order to identify potential RXRα–FXRα-responsive elements in HCR-1, we constructed a series of CAT reporter plasmids containing the −545/+18 apoC-II promoter linked to WT or truncated forms of HCR-1 (Figure 4A). Transient transfections of COS-7 cells in the absence or presence of RXRα–FXRα heterodimers and CDCA showed a 18-, 33-, and 6.5-fold transactivation of the constructs containing the WT (11/324) HCR-1, the (11/260) HCR-1 and the (118/260) HCR-1 regions respectively (the numbers in parentheses indicate the nucleotide sequence of HCR-1 based on the numbering system of [34]). In contrast, constructs containing the HCR-1 deletions 11/206, 11/185 or 11/118 were not transactivated by RXRα–FXRα in the presence of CDCA. No transactivation of the above HCR-1/apoC-II promoter constructs by RXRα–FXRα heterodimers was observed in the absence of CDCA (results not shown).

The findings shown in Figure 4 suggested that the transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα heterodimers in the presence of CDCA requires the 206/260 region of HCR-1. The involvement of regulatory elements and factors bound to the proximal apoC-II promoter was explored with additional experiments that are presented below.

The 214/226 region of HCR-1 is an IR-1 HRE that specifically binds RXRα–FXRα heterodimers and the orphan nuclear receptors HNF-4 and ARP-1

The 206/260 region of HCR-1 contains an inverted repeat, 5′-AGGTCA(G)AGACCT-3′, located between nucleotide positions 214 and 226 (Figure 5A). This inverted repeat is almost identical with the consensus IR-1 HRE, 5′-AGGTCA(N)TGACCT-3′. The receptor-binding specificity of the IR-1 HRE of the HCR-1 was determined by EMSAs using a double-stranded synthetic oligonucleotide corresponding to the 211/231 region of the HCR-1 and extracts of COS-7 cells transfected with plasmids expressing various nuclear receptors. This analysis showed that the (211/231) HCR-1 probe bound RXRα–FXRα heterodimers and the orphan nuclear receptors HNF-4 and ARP-1, but did not bind RXRα, FXRα and T3Rβ homodimers, or RXRα–T3Rβ heterodimers (Figure 5B). Control experiments showed that RXRα–T3Rβ heterodimers bind to the CIIC element of the human apoC-II promoter (Figure 5C). No specific binding was observed using extracts from untransfected COS-7 cells (Figure 5B). In order to characterize further the binding characteristics of RXRα–FXRα

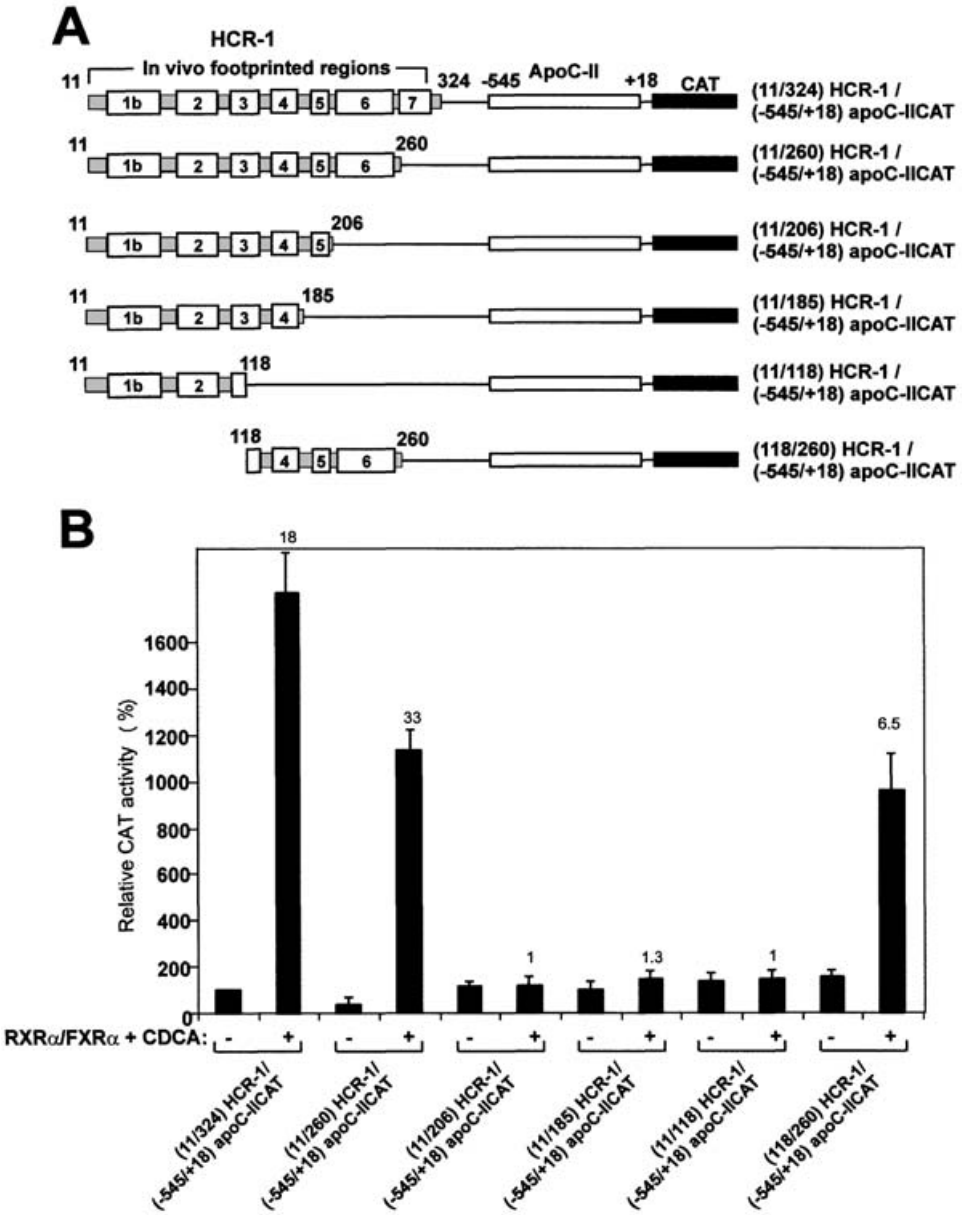

Figure 4 Induction of the apoC-II promoter by CDCA is mediated by the 206/260 regulatory region of HCR-1

(**A**) Schematic representation of the reporter constructs used in the transient transactivation experiments of (**B**). Grey rectangles represent different segments of the HCR-1, open rectangles represent the apoC-II (−545/+18) promoter and black rectangles represent the CAT gene. Open rectangles in HCR-1 numbered 1–7 represent *in vivo* footprinted elements identified previously in HCR-1 [34]. (**B**) COS-7 cells were transfected with the reporter constructs indicated (1 μg) in the absence (−) or presence (+) of expression vectors for RXRα and FXRα (1 μg of each) and treated with 10^{-4} M CDCA for 24 h as indicated. In each experiment, the cytomegalovirus (CMV)–β-galactosidase plasmid (1 μg) was included for normalization of the transfection efficiency. Results are means ± S.D. of the relative CAT activity from two independent experiments performed in duplicate. The fold induction in promoter activity by RXRα–FXRα with CDCA is shown above each bar.

heterodimers on the 211/231 region of HCR-1, a mutated oligonucleotide was synthesized having nucleotide substitutions in the two half repeats of the IR-1 HRE of HCR-1. This oligonucleotide, designated (211/231 mut) HCR-1 (Figure 5A) was used as a probe in EMSAs. As shown in Figure 5(D), RXRα–FXRα heterodimers did not bind to the (211/231 mut) HCR-1 probe. Control experiments showed that a synthetic IR-1 oligonucleotide corresponding to the −163/−145 region of the I-BABP promoter (designated IR-1/I-BABP; Figure 5A), bound to RXRα–FXRα heterodimers.

The specificity of binding of RXRα–FXRα heterodimers to the (211/231) HCR-1 probe was further evaluated by EMSA and supershift assays as well as by competition experiments. As shown in Figure 5(E), binding of RXRα–FXRα heterodimers to the (211/231) HCR-1 probe was competed by 100-fold or 200-fold excess of unlabelled (211/231) HCR-1 oligonucleotide or unlabelled IR-1/I-BABP oligonucleotide. In contrast, no competition of RXRα–FXRα heterodimers was observed using 100-fold or 200-fold excess of the (211/231 mut) HCR-1 oligonucleotide or a non-specific oligonucleotide (WAF-1)

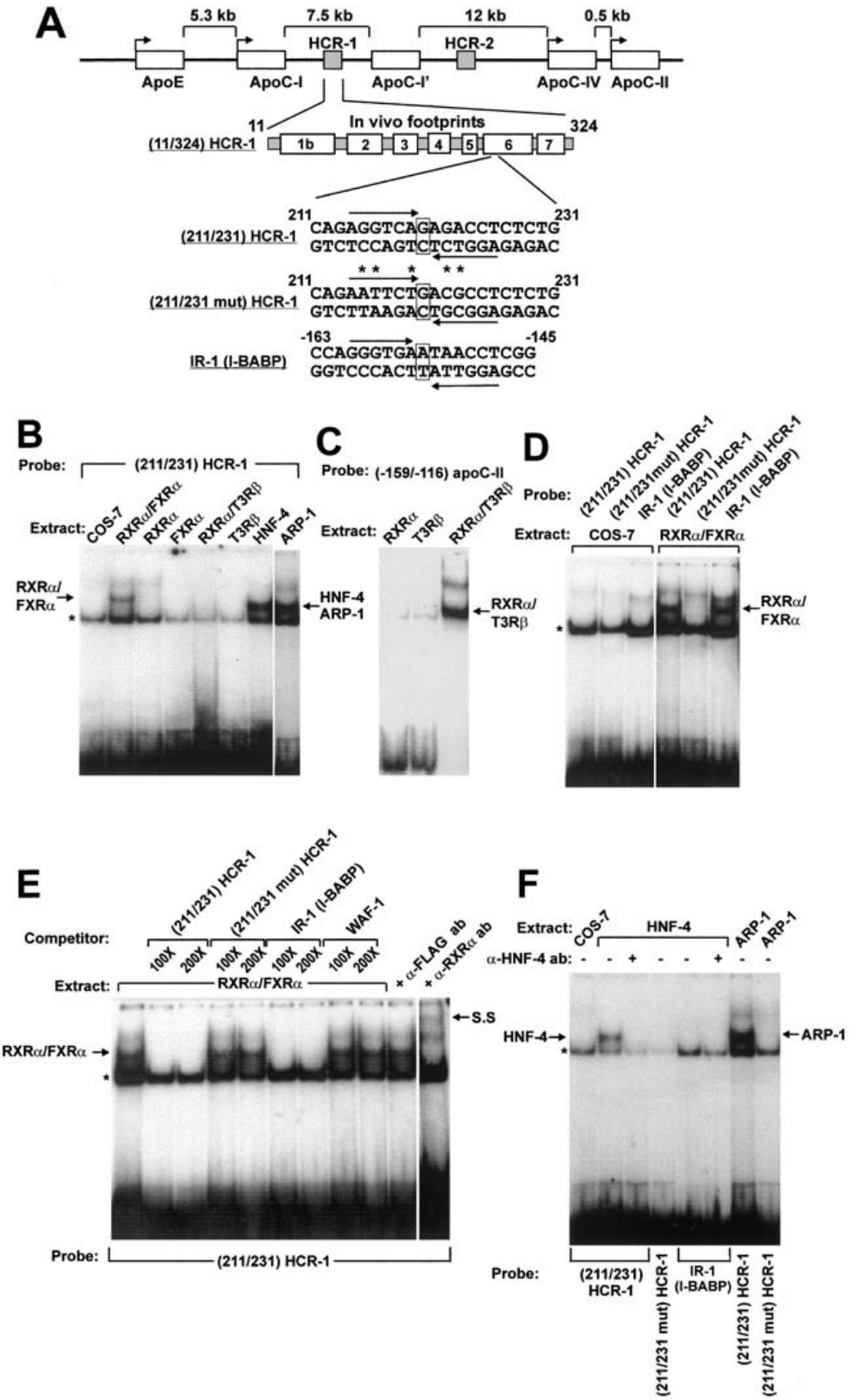

Figure 5 **For legend, see facing page.**

corresponding to the −123/−88 region of the human $p21^{WAF-1}$ promoter which contains two tandem binding sites for the ubiquitous transcription factor Sp1 [35]. Furthermore, the protein–DNA complex formed by RXRα–FXRα and the (211/231) HCR-1 probe disappeared when an anti-RXRα antibody was used, suggesting the presence of RXRα in this complex (Figure 5E). Moreover, a faint band with slower electrophoretic mobility appeared in the presence of the anti-RXRα antibody, which presumably corresponds to a supershifted RXRα–FXRα–DNA complex. In contrast, a non-specific antibody (anti-FLAG M2) had no effect on the formation of the RXRα–FXRα–DNA complex (Figure 5E).

Finally, the specificity of binding of the orphan nuclear receptors HNF-4 and ARP-1 to the (211/231) HCR-1 probe was evaluated. As shown in Figure 5(F), binding of HNF-4 to the (211/231) HCR-1 probe was abolished by an anti-HNF-4 antibody, whereas no binding of HNF-4 to the (211/231 mut) HCR-1 probe or the IR-1/I-BABP probe was observed. ARP-1 also did not bind to the mutant (211/231 mut) HCR-1 probe (Figure 5F). In all reactions, a non-specific protein–DNA complex was observed (indicated with an asterisk in Figures 5B and 5D–5F) that could not be competed by any oligonucleotide tested.

The combined DNA-binding experiments shown in Figures 5(B)–5(F) indicate that the 214/226 region of HCR-1 is an IR-1 HRE that binds specifically to RXRα–FXRα heterodimers and the orphan nuclear receptors HNF-4 and ARP-1.

The 214/226 region of HCR-1 is an RXRα–FXRα response element that mediates transactivation of the HCR-1/apoC-II promoter cluster by CDCA

To characterize further the transcriptional properties of the novel IR-1 HRE present in HCR-1, a synthetic oligonucleotide containing a single copy of this (211/231) HCR-1 HRE, as well as the corresponding mutated oligonucleotide (211/231 mut) HCR-1 that does not bind RXRα–FXRα, HNF-4 or ARP-1 (sequence shown in Figure 5A) were cloned upstream of the (−388/+18) apoC-II promoter CAT construct (Figure 6A). Transient co-transfections in HEK-293 cells showed that the expresssion of RXRα–FXRα heterodimers in the presence of CDCA resulted in a 15.5-fold transactivation of the (211/231) HCR-1/(−388/+18) apoC-II promoter cluster by RXRα–FXRα and CDCA (Figure 6B). In contrast, the (−388/+18) apoC-II promoter or the (211/231 mut) HCR-1/(−388/+18) apoC-II promoter cluster were transactivated 4.4- and 3.2-fold respectively by RXRα–FXRα in the presence of CDCA. No transactivation by RXRα–FXRα heterodimers in the absence of CDCA was observed (results not shown). The transactivation of the (−388/+18) apoC-II promoter by RXRα–FXRα heterodimers in the presence of CDCA and in the absence of HCR-1 was unique to this promoter deletion mutant and was not observed in the (−545/+18) apoC-II promoter (compare Figure 3A with Figure 6B).

These findings confirmed that the IR-1 HRE present in the (214/226) region of HCR-1 is an RXRα–FXRα response element that mediates transactivation of the HCR-1/apoC-II promoter cluster by CDCA.

The effect of orphan nuclear receptors HNF-4 and ARP-1 on the activity of the HCR-1/apoC-II promoter cluster

As shown in Figure 5, the IR-1 HRE present in the (214/226) region of HCR-1 binds, in addition to RXRα–FXRα heterodimers, the orphan nuclear receptors HNF-4 and ARP-1. To assess the effect of ARP-1 and HNF-4 on the HCR-1/apoC-II promoter activity, we performed an additional series of co-transfection experiments in HEK-293 cells. As shown in Figure 6(C), ARP-1 did not alter the observed 4-fold transactivation of the (−388/+18) apoC-II promoter by RXRα–FXRα in the presence of CDCA, but it inhibited strongly the observed 21-fold transactivation of the (211/231) HCR-1/(−388/+18) apoC-II promoter cluster by RXRα–FXRα and CDCA (approx. 75% inhibition). These findings suggest that ARP-1 represses the RXRα–FXRα-mediated transactivation of the (211/231) HCR-1/(−388/+18) apoC-II promoter cluster, possibly due to competition between ARP-1 and RXRα–FXRα heterodimers for the same binding site in the IR-1 HRE of the HCR-1.

Similar experiments showed that HNF-4 transactivated the apoC-II promoter and the (211/231) HCR-1/apoC-II promoter cluster 9- and 24-fold respectively, but had only a small effect on the activity of the (211/231 mut) HCR-1/apoC-II promoter cluster in which the 211/231 element of HCR-1 was mutated (3.5-fold) or on the activity of the (11/324) HCR-1/(−85/+1) tk enhancer/promoter combination in which the apoC-II promoter was replaced by the heterologous HSV-tk promoter, which lacks HNF-4 binding sites (2.5-fold) (Figure 6D).

The findings shown in Figure 6(D) suggest functional co-operation between HNF-4 bound to the IR-1 HRE of the HCR-1 and factors bound to the proximal apoC-II promoter in the transactivation of the HCR-1/apoC-II promoter cluster.

Figure 5 EMSAs established that the 214/226 region of HCR-1 is an IR-1 HRE that specifically binds RXRα–FXRα heterodimers and the orphan nuclear receptors HNF-4 and ARP-1

(**A**) Upper section: schematic representation of the *apoE/apoC-I/apoC-I′/apoC-IV/apoC-II* gene cluster. Open rectangles in the gene cluster represent apolipoprotein genes, distances (in kb) of intergenic regions in the cluster are indicated above each region. The orientation of transcription of each gene is indicated by arrows. Grey squares represent HCR-1 and HCR-2. Middle panel: regulatory elements in HCR-1 identified by *in vivo* footprinting. *In vivo* footprinted areas in (11/324) HCR-1 are shown by open rectangles numbered 1–7. Lower panel: sequence of the novel HRE present in HCR-1. Arrows above or below the DNA sequence of the putative HRE denote the orientation of the HRE half repeats. Asterisks above the HRE sequence denote base substitutions that were introduced in (211/231) HCR-1 region. The sequence of the RXRα–FXRα responsive element present on the I-BABP gene promoter (−163/−145) that was used in this study is also shown. (**B**) EMSAs using extracts from untransfected COS-7 cells or COS-7 cells transfected with expression vectors for the hormone nuclear receptors indicated and the HCR-1 (211/231) oligonucleotide as a probe. The position of the RXRα–FXRα, HNF-4 and ARP-1 protein–DNA complexes are shown by arrows. (**C**) EMSAs using extracts from COS-7 cells transfected with expression vectors for RXRα and T3Rβ as indicated and the −159/−116 apoC-II oligonucleotide as a probe. The arrow shows the position of the RXRα–T3Rβ protein–DNA complex. (**D**) EMSAs using extracts from untransfected COS-7 cells or COS-7 cells transfected with expression vectors for RXRα and FXRα and the oligonucleotide probes indicated that are described in (**A**). The arrow shows the position of the RXRα–FXRα protein–DNA complex. (**E**) EMSAs using extracts from COS-7 cells transfected with expression vectors for RXRα and FXRα and the (211/231) HCR-1 oligonucleotide as a probe in the absence or presence of 100-fold or 200-fold molar excess of the competitor oligonucleotides as indicated. In the supershift experiments, an antibody specific for RXRα (α-RXRα ab) or a non-specific antibody (α-FLAG ab) was included in the reaction mix. S.S. indicates the supershifted DNA–protein complex. The arrows show the position of the shifted and supershifted RXRα–FXRα protein–DNA complexes. (**F**) EMSAs using extracts from untransfected COS-7 cells or COS-7 cells transfected with expression plasmids for HNF-4 or ARP-1 and the synthetic oligonucleotide probes, as indicated. Two of the reactions were performed in the presence of an antibody specific for HNF-4 (α-HNF-4 ab). The arrows show the position of the HNF-4 and ARP-1 protein–DNA complexes. The asterisk in (**B**) and (**D**)–(**F**) shows a non-specific band.

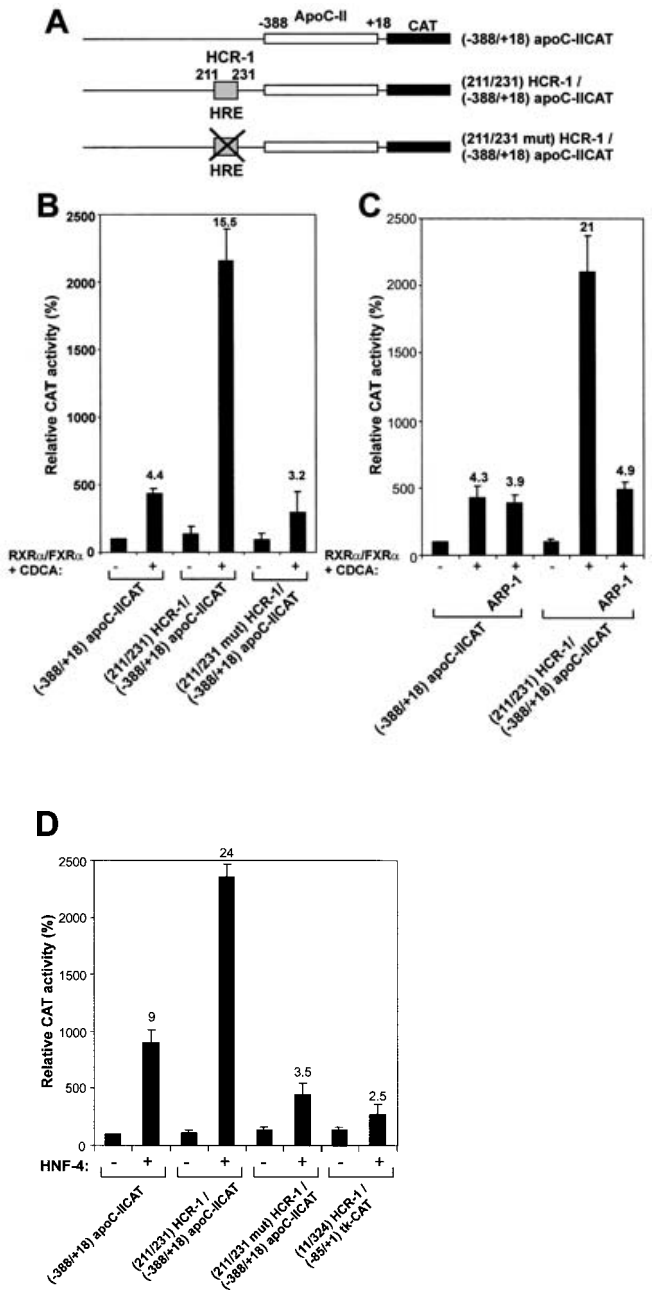

Figure 6 The 214/226 region of HCR-1 is an RXRα–FXRα and HNF-4 responsive element

(**A**) Schematic representation of the reporter constructs used in the transient transfection experiments of (**B**)–(**D**). Grey rectangles represent WT or mutated (211/231) HCR-1 regions, open rectangles represent the apoC-II (−388/+18) promoter and black rectangles represent the CAT gene. (**B**) HEK-293 cells were transfected with the reporter constructs indicated (1 μg) in the absence (−) or presence (+) of expression vectors for RXRα and FXRα (1 μg of each) and treated with 10^{-4} M CDCA for 24 h as indicated. In each experiment, the cytomegalovirus (CMV)–β-galactosidase plasmid (1 μg) was included for normalization of the transfection efficiency. Results are means ± S.D. of the relative luciferase activity from two independent experiments performed in duplicate. The fold transactivation of the promoter by RXRα–FXRα with CDCA is shown above each bar. (**C**) HEK-293 cells were transfected with the reporter constructs indicated (1 μg) in the absence (−) or presence (+) of expression vectors for RXRα–FXRα and ARP-1 (1 μg of each) and treated with 10^{-4} M CDCA for 24 h as indicated. In each experiment, the CMV–β-galactosidase plasmid (1 μg) was included for normalization of the transfection efficiency. Results are means ± S.D. of the relative luciferase activity from two independent experiments performed in duplicate. The fold transactivation of the promoter by RXRα–FXRα with CDCA in the absence or presence of ARP-1 is shown above each bar. (**D**) HEK-293 cells were transfected with the reporter constructs indicated (1 μg) in the absence (−) or presence (+) of an expression vector for HNF-4. In each experiment, the CMV–β-galactosidase plasmid (1 μg) was included for normalization of the transfection efficiency. Results are means ± S.D. of the relative luciferase activity from two independent experiments performed in duplicate. The fold transactivation of the promoter by HNF-4 is shown above each bar.

Synergism between hormone nuclear receptors bound to the HRE of the HCR-1 and the TRE of the proximal apoC-II promoter can account for the transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα heterodimers in the presence of CDCA or by RXRα–T3Rβ heterodimers in the presence of T3

We have shown previously [19] that the proximal apoC-II promoter contains two HREs, designated CIIB and CIIC, with binding specificities for HNF-4 and ARP-1, and RXRα–T3Rβ heterodimers respectively (Figure 7A). In order to evaluate the putative role of these HREs in the mechanism of transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα heterodimers and CDCA, a series of WT or mutant HCR-1/apoC-II promoter constructs (shown schematically in Figure 7A) were utilized in transient co-transfection experiments in HEK-293 cells. In these constructs, the 11/324 or 211/231 HCR-1 regions were placed upstream of the WT (−388/+18) apoC-II promoter or the apoC-II promoter mutated in the HREs of element CIIB (Bmut), element CIIC (Cmut) or both elements (B/Cmut). The activity of these constructs in the absence or presence of RXRα–FXRα and CDCA was evaluated by CAT assays. As shown in Figure 7(B), RXRα–FXRα heterodimers transactivated the (11/324) HCR-1/(−388/+18) apoC-II promoter cluster 6-fold in the presence of CDCA. Mutagenesis of the proximal apoC-II element CIIB had a minor effect on the RXRα–FXRα-mediated transactivation (5-fold transactivation of the mutant apoC-II promoter compared with 6-fold transactivation of the WT apoC-II promoter). Interestingly, mutagenesis of the TRE of the proximal apoC-II promoter on element CIIC, which binds RXRα–T3Rβ heterodimers, abolished the transactivation of the (11/324) HCR-1/(−388/+18) apoC-II promoter cluster by RXRα–FXRα and CDCA.

Finally, mutagenesis of both proximal HREs CIIB and CIIC of the apoC-II promoter abolished the transactivation of the (211/231) HCR-1/(−388/+18) apoC-II promoter cluster by RXRα–FXRα in the presence of CDCA (Figure 7C). Similar co-transfection experiments in HEK-293 cells showed that mutagenesis of either the IR-1 HRE of the HCR-1 or the two HREs of the proximal apoC-II promoter abolished the transactivation of the (211/231) HCR-1/(−388/+18) apoC-II promoter cluster by RXRα–T3Rβ heterodimers in the presence of T3 (Figure 7D). Control immunoblotting experiments showed that HEK-293 cells express endogenous RXRα and T3Rβ (Figure 7E).

The combined results shown in Figure 7 suggest that the transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα heterodimers in the presence of CDCA results from a functional co-operativity between RXRα–FXRα heterodimers bound to the IR-1 HRE present in the 214/226 region of the HCR-1 and heterodimers of RXRα with T3Rβ or possibly other hormone nuclear receptors bound to the TRE of the proximal apoC-II promoter (element CIIC), as illustrated in Figure 8.

DISCUSSION

Background

ApoC-II, at physiological concentrations, is an activator of lipoprotein lipase, the enzyme that catalyses the hydrolysis of the

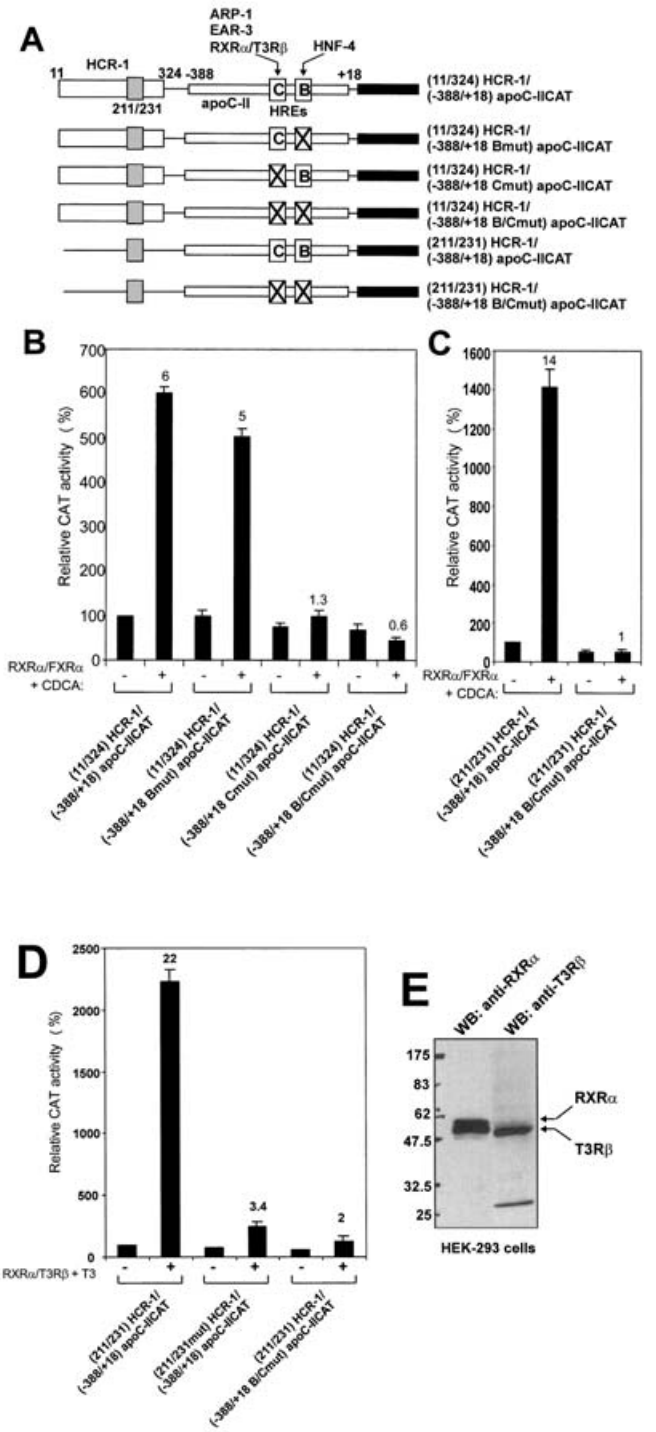

Figure 7 Synergism between hormone nuclear receptors bound to the proximal apoC-II promoter and the HCR-1

(**A**) Schematic representation of the reporter constructs used in the transient transfection experiments of (**B**)–(**D**). Tall open rectangles represent the (11/324) HCR-1 regions, grey squares represent the (211/231) HRE present in HCR-1, short open rectangles represent the apoC-II (−388/+18) promoter, open squares represent the WT or mutated HREs CIIB and CIIC present in the apoC-II promoter and black rectangles represent the CAT gene. The hormone nuclear receptors that bind to the apoC-II HREs are shown above. EAR-3, vErb-A-related protein 3 (**B**) and (**C**) HEK-293 cells were transfected with the reporter constructs indicated (1 μg) in the absence (−) or presence (+) of expression vectors for RXRα and FXRα (1 μg each) and treated with 10^{-4} M of CDCA for 24 h as indicated. In each experiment, the cytomegalovirus (CMV)–β-galactosidase plasmid (1 μg) was included for normalization. Results are means ± S.D. of the relative CAT activity from two independent experiments performed in duplicate. The fold induction in promoter activity by RXRα–FXRα + CDCA is shown above each bar. (**D**) HEK-293 cells were transfected with the reporter constructs indicated (1 μg) in the absence (−) or presence (+) of expression vectors for RXRα and T3Rβ (1 μg of each) and treated with 0.1 μM of T3 for 24 h, as indicated. In each experiment, the CMV–β-galactosidase plasmid (1 μg) was included for normalization of transfection efficiency. Results are means ± S.D. of the relative CAT activity from two independent experiments performed in duplicate. The fold induction in promoter activity by RXRα–T3Rβ with T3 is shown above each bar. (**E**) Analysis of the levels of expression of RXRα and T3Rβ in HEK-293 cells by immunoblotting. Confluent cultures of HEK-293 cells were lysed and protein expression was monitored by SDS/PAGE and Western blotting (WB) using a polyclonal antibody specific for RXRα and a monoclonal antibody specific for T3Rβ as indicated. The positions of RXRα and T3Rβ are indicated by arrows.

triacylglycerols of VLDL and chylomicrons. Structural or regulatory mutations in the apoC-II gene are associated with familial hypertriglyceridaemia in humans [1–7]. Unexpectedly, studies in transgenic mice showed that overexpression of apoC-II also causes severe hypertriglyceridaemia [36]. Thus understanding the factors that control apoC-II gene transcription is important for the management of hypertriglyceridaemia in man.

We have shown previously that hormone nuclear receptors may modulate positively or negatively the activity of the proximal apoC-II promoter [18,19]. Positive regulation is exerted by HNF-4 that binds to an HRE present on the element CIIB located between nucleotides −101 and −85 of the apoC-II promoter. We have shown that the activity of the apoC-II promoter is drastically repressed in a HepG2 cell clone that permanently expresses an anti-HNF-4 ribozyme construct [19]. In addition, the *apoC-II* gene expression is totally abolished in foetal or adult liver cells derived from HNF-4 knockout mice [37,38]. Furthermore, a point mutation at nucleotide position −86 of the apoC-II promoter, which maps within the HNF-4 binding site (element CIIB), has been described in patients with hyperchylomicronaemia [6]. Thus HNF-4 seems to be a key modulator of apoC-II gene transcription.

Positive regulation of apoC-II promoter activity is also exerted by the nuclear receptors RXRα and T3Rβ, which have, as natural ligands, the hormones RA and T3 respectively. The RXRα–T3Rβ heterodimers bind to a TRE present on element CIIC of the apoC-II promoter between nucleotides −165 and −135 and transactivates the apoC-II promoter in the presence of T3, but not in the presence of RA [19].

On the other hand, the apoC-II promoter activity is repressed by the orphan nuclear receptors ARP-1 and EAR-2 that bind to the TRE of element CIIC and compete for the binding of RXRα–T3Rβ heterodimers [18,19]. These early studies suggested that ligand-dependent, as well as orphan nuclear receptors with still unidentified ligands, may have an important role in *apoC-II* gene transcription and could be potential targets for the drug therapy of hyperlipidaemias.

Bile acids as regulators of cholesterol and triacylglycerol homoeostasis

In an effort to identify additional ligands of hormone nuclear receptors that could potentially modulate *apoC-II* gene expression in the liver, we found that treatment of HepG2 cells with the bile acid CDCA increased the steady state apoC-II mRNA levels and HCR-1/apoC-II promoter activity (Figures 1 and 2), in agreement with previous findings [33]. Bile acids are the major end products of cholesterol catabolism and are essential for the solubilization of dietary lipids in the intestine [20]. In addition, bile acids were found to be potent inducers of gene transcription by binding to and activating FXRα, a member of the hormone nuclear receptor superfamily [21,22].

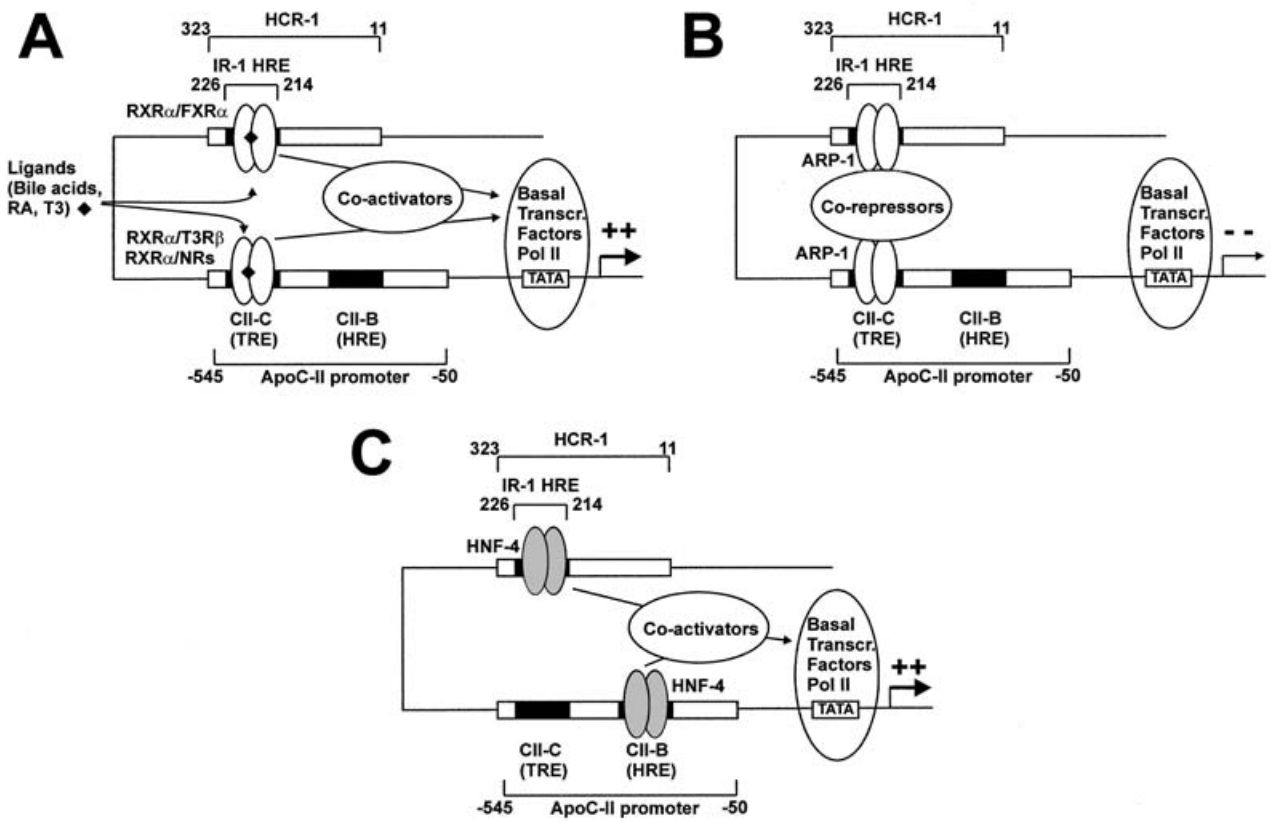

Figure 8 Schematic representation of putative synergistic interactions between nuclear receptors bound to the IR-1 of the (214/226) HCR-1 and the HREs CIIB and CIIC present in the proximal apoC-II promoter

These interactions lead to synergistic transactivation of the HCR-1/apoC-II promoter cluster in response to bile acids, RA and T3 (**A**), HNF-4 (**C**) and to repression of the HCR-1/apoC-II promoter cluster in response to ARP-1, which antagonizes with RXRα–FXRα heterodimers for the same HRE in HCR-1 (**B**). See the text for details. The potential involvement of transcriptional co-activators or co-repressors that may be recruited by the RXRα heterodimers, HNF-4 and ARP-1 bound to the proximal and/or distal sites are also shown in this scheme. Pol II, RNA Polymerase II; NRs, other nuclear receptors that could potentially form heterodimers with RXRα and bind to the proximal regulatory element CIIC of the apoC-II promoter.

Studies in FXRα knockout mice showed that the hepatic and plasma triacylglycerol levels were increased 2–2.5-fold relative to their WT littermates in response to atheromatic diets, suggesting that FXRα may be a modulator of triacylglycerol homoeostasis in mammals [39].

Identification and characterization of a novel IR-1 HRE in the HCR-1 that mediates transactivation of the HCR-1/apoC-II promoter cluster by CDCA, RA and HNF-4

Deletion analysis of the HCR-1, in combination with DNA-binding assays, led to the identification of a putative FXRα responsive element within the 206/260 region of HCR-1 (Figure 4). This region contains the sequence 5′-AGGTCA(G)AGACCT-3′ between nucleotides 214 and 226, which defines an IR-1 HRE (Figure 5A). This HRE differs from the idealized IR-1 HRE, with the sequence 5′-AGGTCA(N)TGACCT-3′, only at the sixth nucleotide of the 3′ half repeat (T instead of an A). IR-1 HREs obtained by a selected and amplified sequence imprinting technique (Selection and Amplification of Binding Sequences; ‘SAAB’) were shown to represent high-affinity binding sites for RXRα–FXRα heterodimers [40]. The novel HRE present in HCR-1 selectively binds RXRα–FXRα heterodimers, but no RXRα, FXRα or T3Rβ homodimers, or RXRα–T3Rβ heterodimers (Figure 5B). Interestingly, this HRE binds the orphan nuclear receptor HNF-4, shown previously to bind preferentially to direct repeats with one nucleotide spacing HREs [41] and ARP-1 (Figure 5B). However, HNF-4 did not bind to the IR-1 HRE present in the promoter of the I-BABP gene (Figure 5E). Thus it appears that binding of HNF-4 to the HRE present in HCR-1 is specific and does not reflect a general preference of HNF-4 for IR-1 elements, but rather reflects a unique property of this type of IR-1.

Functional interactions between RXRα–FXRα heterodimers bound to HCR-1 and hormone nuclear receptors bound to the proximal TRE of the apoC-II promoter

An elegant series of *in vivo* studies have established that HCR-1 is a strong hepatic enhancer of the genes of the *apoE/apoC-I/apoC-IV/apoC-II* gene cluster [14–16]. In HepG2 cells, HCR-1 enhanced the activity of the apoC-II and apoE promoters as well as the activity of the heterologous HSV-tk promoter (Figure 2 and [18]). Furthermore, a double mutation in the HREs CIIB and CIIC of the apoC-II promoter totally abolished the transcriptional enhancement of the apoC-II promoter by HCR-1 [18]. These previous studies had suggested that the hepatic enhancement by HCR-1 is mediated by co-operative interactions between hormone

nuclear receptors bound to elements CIIB and/or CIIC of the apoC-II promoter and unidentified transcription factors bound to HCR-1.

In the present study, a series of experiments suggest that RXRα–FXRα heterodimers bound to HCR-1 could be involved in synergistic interactions with factors bound to the proximal apoC-II promoter elements CIIC and CIIB under conditions of stimulation by bile acids.

One set of experiments showed strong transactivation of the apoC-II promoter by CDCA in cells co-transfected with expression vectors for FXRα and RXRα (Figure 3A) only when the (−545/+18) apoC-II promoter was linked to full-length HCR-1 or to the 211/231 HCR-1 fragment containing the putative FXRα responsive element (Figures 3 and 6). In contrast, the apoE promoter could not be transactivated by RXRα–FXRα and CDCA either in the absence or in the presence of HCR-1 (Figure 3B), suggesting that the proximal promoter context is important for the RXRα–FXRα-mediated transactivation. Significant induction of the HCR-1/apoC-II promoter cluster was also observed by RXRα–FXRα heterodimers in the presence of RA (Figure 3). This is in agreement with a previous study which has shown that RXRα–FXRα is a permissive heterodimer (i.e. is activated by both ligands) [42]. RA could not transactivate the apoC-II promoter in the absence of HCR-1 (Figure 3A and [19]). These findings suggest that RA, similar to CDCA, could also have a role in triacylglycerol homoeostasis.

A second set of experiments showed that the transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα heterodimers in the presence of CDCA was abolished by mutations in the TRE present in the regulatory element CIIC of the proximal apoC-II promoter (Figures 7B and 7C). This finding suggested that endogenous RXRα–T3Rβ heterodimers and possibly other RXRα heterodimers which bind to the TRE of element CIIC are important for the transactivation of the HCR-1/apoC-II promoter cluster by RXRα–FXRα heterodimers in the presence of CDCA. DNA-binding experiments established that RXRα–FXRα heterodimers do not bind to the regulatory elements CIIB and CIIC [33]. In addition, RXRα–FXRα heterodimers in the presence of CDCA could not transactivate synthetic reporter constructs consisting of the regulatory elements CIIC or CIIB of the apoC-II promoter fused with the adenovirus major late ('AdML') minimal promoter (results not shown). These observations favour synergistic interactions between RXRα–FXRα heterodimers bound to HCR-1 and heterodimers of RXRα with T3Rβ or other nuclear receptors bound to the element CIIC of the proximal apoC-II promoter.

Another point to emphasize here is the key role of HNF-4 in the hepatic activity of the apoC-II promoter. HNF-4 binds to both the IR-1 HRE of the HCR-1 (Figure 5) and the proximal HRE of element CIIB of the apoC-II promoter [18,19]. In the present study, we found that the transactivation of the apoC-II promoter by HNF-4 is greatly enhanced in the presence of the 211/231 region of HCR-1 which contains the novel IR-1 HRE (Figure 6D). Thus the novel IR-1 HRE present in HCR-1 could also modulate the overall hepatic activity of the HCR-1/apoC-II promoter cluster. However, the existence of additional binding sites for HNF-4 in the HCR-1 cannot be ruled out.

Based on our findings, a model is proposed that could account for the transcriptional induction of the apoC-II promoter by heterodimers of RXRα with FXRα and T3Rβ or other unidentified partners in the presence of their corresponding ligands (Figure 8). Co-operative interactions between this specific set of hormone nuclear receptors could result from either direct physical contact between the receptors themselves or the co-operative recruitment of common nuclear receptor co-regulators such as cAMP-response-element-binding protein ('CREB')-binding protein ('CBP')/p300, p300/CBP-associated factor ('P/CAF') and/or Src-1 shown previously to interact with ligand-bound RXRα, T3Rβ and FXRα [21,22].

Functional interactions between hormone nuclear receptors bound to proximal and distal sites, similar to those described in the present study, have been described recently for the genes of the apoA-I/apoC-III/apoA-IV cluster in transgenic mice [43]. These *in vivo* studies showed that the HRE of the apoC-III enhancer is required for the intestinal expression of the *apoA-I* and *apoC-III* genes and also enhances the hepatic expression of the two genes [44].

Similar *in vivo* approaches could be applied to establish that the synergistic interactions between RXRα–FXRα heterodimers or HNF-4 that bind to the HCR-1 and RXRα heterodimers bound to the TRE of the apoC-II promoter, demonstrated in the present study, also occur *in vivo*. Selective regulation of the genes of the *apoE/apoC-I/apoC-IV/apoC-II* gene cluster *in vivo* by extracellular ligands will provide new pharmacological targets for the control of hypertriglyceridaemia in humans.

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