Binding Specificity and Modulation of the Human ApoCIII Promoter Activity by Heterodimers of Ligand-Dependent Nuclear Receptors†

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ABSTRACT: Human apolipoprotein CIII (apoCIII) is a major determinant of plasma triglyceride metabolism. The regulatory elements that control both hepatic and intestinal transcription of the human apoCIII gene are localized between nucleotides −792 and −25 of the apoCIII promoter. Elements important for apoCIII promoter activity are three hormone response elements (HREs) and three SP1-binding sites. Orphan members of the nuclear hormone receptor superfamily can bind the HREs and strongly enhance or repress apoCIII promoter activity. In the present study we have investigated the ability of ligand-dependent nuclear hormone receptors to bind and modulate the human apoCIII promoter activity. Experiments using DNA binding and competition assays showed that the proximal element B (−87/−72) binds strongly, in addition to HNF-4, ARP-1, EAR-2, and EAR-3, heterodimers of RXRα with RARα, and less efficiently, homodimers of RARα and heterodimers of RXRα with $T_3R\beta$ or PPARα. Element G (−669/−648), which was shown previously to bind ARP-1 and EAR-3 but not HNF-4, binds strongly heterodimers of RXRα with either RARα or $T_3R\beta$. Finally element I_4 (−732/−712), which was shown to bind HNF-4, also binds strongly ARP-1 and EAR-3, as well as RXRα/RARα heterodimers and less efficiently, RXRα/$T_3R\beta$ heterodimers. Methylation interference experiments have identified the protein−DNA interactions between different nuclear receptors and the respective HREs on the apoCIII promoter. RXRα/RARα heterodimers and HNF-4 homodimers bind to DR-1 motifs on elements B and I_4, respectively. RXRα/$T_3R\beta$ heterodimers and ARP−1 bind to DR-5 and DR-0 motifs respectively on element G. Cotransfection experiments in HepG2 cells showed that RXRα or a combination of RXRα and RARα increased the apoCIII promoter activity approximately 2-fold in the presence of the ligands 9-*cis* or all-*trans* RA. In contrast, a combination of RXRα and $T_3R\beta$ transactivated the apoCIII promoter 1.5-fold in the presence of 9-*cis* RA but it repressed the apoCIII promoter activity in the presence of T_3. Mutations in the HREs of elements B, G, or I_4 or in the SP1-binding site of element H, which abolished the binding of nuclear hormone receptors or SP1 to their cognate site, reduced the promoter strength and exhibited different responses to the ligand-dependent nuclear receptors. The findings suggest that modulation of the apoCIII promoter activity by orphan and ligand-dependent nuclear receptors involves complex interactions among nuclear receptors, SP1 and possibly other factors bound to the enhancer and the proximal promoter region.

Plasma apoCIII[1] is a 79 amino acid protein of known primary structure (*1*, *2*) and gene sequence (*3*−*5*). ApoCIII is a major component of VLDL and a minor component of HDL (*6*). ApoCIII is involved in the modulation of binding of lipoproteins to cell receptors and subsequent catabolism of triglyceride-rich lipoproteins (*7*−*11*), and thus has been implicated in the development of hypertriglyceridemia. This concept was further supported by findings showing that overexpression of the apoCIII gene in transgenic mice is associated with severe hypertriglyceridemia and accumulation in plasma of $apoB_{48}$ containing lipoprotein remnants (*12*, *13*).

ApoCIII gene expression is tissue-specific (*14*−*16*) and developmentally regulated (*17*). The apoCIII promoter contains four proximal (A−D) and six distal (E−J) regulatory elements (*18*). The distal apoCIII regulatory elements F−J (located between nucleotides −792 and −592) act as a general enhancer that increases the strength of the proximal apoCIII as well as the apoA-I and apoA-IV promoters (*18*−*21*). This region may also be essential for the intestinal expression of the apoA-I and apoA-IV genes *in vivo* (*22*). Three HREs, located in the proximal promoter region on element B, as well as in the apoCIII enhancer region on elements G and I_4 (*18*−*21*), play an important role in the

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[1] Abbreviations: apoCIII, apolipoprotein CIII; ARP-1, apolipoprotein AI regulatory protein 1; β-gal, β-galactosidase; CAT, chloramphenicol acetyl transferase; DMS, dimethyl sulfate; EAR-3, v-ErbA-related protein 3; HDL, high-density lipoprotein; HNF-4, hepatocyte nuclear factor 4; HRE, hormone response element; PPARα, peroxisome proliferator-activated receptor α; RARα, all-*trans* retinoic acid receptor α; RSV, Rous sarcoma virus; RXRα, 9-*cis* retinoic acid receptor α; $T_3R\beta$, thyroid hormone receptor β; VLDL, very low-density lipoprotein.

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Table 1: Sequences of Wild-Type and Mutated Oligonucleotides Used in DNA Binding−Competition Experiments, in Methylation Interference Analyses, and in Generating Mutated ApoCIII Promoter Reporter CAT Constructs

oligo	sequence[a]
CIII−B wild type	−96-TCGACACTGGTCAGCAGGTGACCTTTGCCCAGCGCCCTGG-61
CIII−BM5	−96-TCGACACTGGTCAGCAGGTGAC**GACAGA**CCAGCGCCCTGG-61
CIII−G wild type	−669-TCGACCTTGGCTTCTCCACCAACCCC-648
CIII−GM1	−672-CGGCCTT**ACAG**TCTCCACCAACCCC-648
CIII−GM4	−679-CTCTGAGCGGCCTT**ACAGGTCTCGAG**CAACCCCTGCCCT-642
CIII−HM1	−714-GGGAGCCTGGTG**TCTGTCTT**GGCAAAGGC-685
CIII−I_4 wild type	−766-TCGAGAGACCAGCTCCTCCCCCAGGGATGTTATCAGTGGGTCCAG-726
CIII−I_4M	−766-TCGAGAGACCAGCTCCTCCCCCAGGGATGTTATCAGTGGGT**TAC**GA**TAT**CAAAATA-714

[a] The sequence of the sense strand is shown. Alterations of sequences in the mutated oligonucleotides are in bold and underlined.

apoCIII promoter strength. In this study we report that the HREs present in the proximal promoter and enhancer apoCIII regions have different binding specificities for orphan and ligand-dependent nuclear receptors. Heterodimers of RXRα/RARα and RXRα/$T_3R\beta$, in the presence of 9-cis or all-trans RA, transactivate, whereas RXRα/$T_3R\beta$ heterodimers, in the presence of T_3, repressed the apoCIII promoter activity in HepG2 cells. Ligand-dependent transactivation of apoCIII promoter activity by RXRα heterodimers is mainly mediated by the HRE present in element B. These findings suggest that modulation of apoCIII transcription may occur in response to extracellular stimuli that may affect plasma apoCIII and triglyceride levels.

EXPERIMENTAL PROCEDURES

Materials. Reagents were purchased from the following sources: T4 polynucleotide kinase from New England Biolabs; transformation-competent bacterial HB101 cells from Life Technologies, Inc.; [γ-^{32}P] ATP (3000 Ci/mmol), ^{14}C-chloramphenicol (250 Ci/mmol), and Econofluor scintillation fluid from DuPont-New England Nuclear; reagents for automated DNA synthesis from Applied Biosystems, Inc.; IB2 TLC silica gels from J. T. Baker, Inc.; bactotryptone and bacto yeast extracts from Difco Laboratories; plasmid purification kit from Qiagen; *O*-nitrophenyl-β-D-galactopyranoside, dimethyl sulfate (DMS), and TEMED from Sigma; double-stranded poly (dI-dC) and acetyl-CoA from Pharmacia Biotechnology Inc.; and agarose, acrylamide, bis-acrylamide, ammonium persulfate, Tris-base, sodium dodecyl sulfate (SDS), and urea from Biorad Laboratories. Autoradiography film was purchased from Kodak. Chroma spin columns were from Clontech Laboratories Inc. Anti-RARα antibodies were a gift of Dr. Hinrich Gronemeyer (University Louis Pasteur, Strasbourg, France).

Plasmid Constructions. The wild-type (−890/+24) apoCIII−CAT reporter plasmid has been described previously (*18*). The reporter plasmids containing mutations in the regulatory elements B (CIII−BM5), G (CIII−GM1), H (CIII−HM1), and I_4 (CIII−I_4M) shown in Table 1 have been described previously (*18*, *19*, *21*). Constructs containing the full-length cDNAs for HNF-4, ARP-1, EAR-3, RXRα, RARα, PPARα, and $T_3R\beta$ in the expression vector pMT2 (*23*) were described previously (*24*−*26*) and were the generous gift from Dr. J. A. A. Ladias, Harvard Medical School.

Preparation of Rat Liver Nuclear Extracts and Cell Extracts from Transfected COS-1 Cells. Nuclear extracts were prepared from livers of 10 rats (approximately 120 g of liver) as described (*27*, *28*). Extracts from COS-1 cells transfected with the pMT2 vector carrying full-length cDNAs for HNF-4, ARP-1, EAR-3, RXRα, RARα, PPARα, and $T_3R\beta$ were prepared as described (*24*, *25*).

DNA-Binding Gel Electrophoretic Mobility Shift Assay. This analysis was performed using either rat liver nuclear extracts or COS-1 whole cell extracts as described (*18*, *25*, *29*). Specifically, the double-stranded probes CIII−B, CIII−G, or CIII−I_4 (Table 1) were end labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP and were purified using the Chroma spin columns. The extracts were preincubated for 15 min at 4 °C with the nonspecific competitor poly (dI-dC) and specific competitors, where indicated. We used 3 μg of poly (dI-dC), and the specific competitors were used at 100-fold excess over the probe. The radiolabeled probe (200 ng, approximately 30 000 cpm) was added to the preincubation mixture, and the reactions were incubated at 4 °C for 30 min. The binding reactions were electrophoresed through a 4% or 5% polyacrylamide gel, and the DNA−protein complexes were detected by autoradiography.

Methylation Interference. For this assay, each strand of the DNA probes CIII−B, CIII−G, or CIII−I_4 (Table 1) (5 pmol) was labeled at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P] ATP and was annealed to its complementary nonlabeled strand. Double-stranded DNA (10^7 cpm) was partially methylated at G residues using DMS for 5 min at room temperature in the presence of 2 μg of salmon sperm DNA (*25*, *30*). The methylated probes were incubated with whole cell extracts from COS-1 cells expressing the indicated nuclear receptors, and the complexes were analyzed by a preparative gel electrophoretic mobility shift assay. The protein−DNA complexes and the free probe, visualized by autoradiography, were excised from the gel, purified by electroelution, and treated with 1 M piperidine for 30 min at 95 °C. The samples were then dried and dissolved in 98% formamide dye. Equal counts from all samples were analyzed by electrophoresis on a 20% polyacrylamide−urea sequencing gel, and the bands were visualized by autoradiography (*31*).

Cell Transfections and CAT Assays. Monolayers of HepG2 cells were maintained as stocks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Fifty to 60% confluent 30 mm dishes, plated in DMEM supplemented with 5% charcoal-stripped FCS, were transfected using the calcium−phosphate DNA coprecipitation method (*32*). The transfection mixture contained a total of 7.5 μg of plasmid DNA: 3 μg of the apoCIII promoter reporter plasmid, 1 μg of RSV-β-galactosidase (RSV-β-gal) plasmid as internal control, and various concentrations of pMT2 expression plasmids carrying the cDNAs of the different nuclear hormone receptors. In each

transfection, vector DNA was added as necessary to achieve a constant amount of transfected DNA. HepG2 cells were incubated with the transfection mixture for 24 h after which the cells were washed 3 times with PBS and re-fed with DMEM supplemented with 5% charcoal-stripped FCS, in the presence of 10^{-6} M 9-*cis* RA, 10^{-6} M all-*trans* RA, or 10^{-7} M T_3. Control reactions received equal volumes of ethanol. Forty hours post-transfection the cells were washed with PBS and were collected in TEN solution (0.04 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl). Cell extracts were prepared in 0.25 M Tris-HCl, pH 7.8, by three sequential freeze−thaw cycles (*33*). The CAT activity of the cell extracts was determined as described previously (*33*), using ^{14}C-chloramphenicol and acetyl-CoA. The reaction times and extract concentrations were selected to ensure linear conversion of the chloramphenicol to the acetylated forms. The acetylated and nonacetylated chloramphenicol forms were separated on IB2 silica gel plates using chloroform−methanol for development. The radioactive spots detected by autoradiography were excised from the thin-layer plates and counted. The β-galactosidase activity of cell lysates was determined as described (*34*) to normalize for total cell number and variability in transfection efficiency.

RESULTS

Ligand-Dependent Nuclear Receptors Bind to Three Sites on the Human ApoCIII Promoter. The regulatory elements B, G, and I_4 of the human apoCIII promoter contain sequences that have high similarity with the AGG/TTCA half-site motif found in the HRE of a variety of genes (*35*−*37*). Previous studies have shown that the regulatory element B is recognized by the orphan nuclear receptors HNF-4, ARP-1, and EAR-3, element G by ARP-1, and EAR-3 and element I_4 by HNF-4 (*19*, *21*, *25*). In the present study, we investigated the binding specificity of ligand-dependent nuclear receptors to these regulatory elements and have identified the corresponding binding motifs.

DNA-binding gel electrophoresis assays showed that the regulatory element B, in addition to HNF-4, binds strongly RXRα/RARα heterodimers and less efficiently, RARα homodimers and RXRα/PPARα and RXRα/$T_3R\beta$ heterodimers, but does not bind RXRα homodimers. Anti-RARα antibodies supershifted the RARα homodimers and RXRα/RARα heterodimers (Figure 1A). Similar analysis showed that element G, in addition to ARP-1, binds strongly the heterodimers of RXRα with RARα or $T_3R\beta$ but does not bind RXRα/PPARα heterodimers or HNF-4 homodimers (Figure 1B). Finally, the regulatory element I_4 binds strongly the homodimers of orphan nuclear receptors HNF-4, ARP-1, and EAR-3 as well as the heterodimers of RXRα/RARα and less efficiently RXRα/$T_3R\beta$, but it does not bind RXRα homodimers or RXRα/PPARα heterodimers (Figure 1C).

Competition assays showed that binding of RXRα heterodimers on element B is competed by the wild-type probe CIII−B but not by the mutant probe CIII−BM5 which, as it is shown in the bottom of Figure 1A, carries a mutation that affects both half-sites of the HRE binding motif. Similarly, the binding of RXRα/$T_3R\beta$ heterodimers and ARP-1 homodimers is competed by the wild-type probe CIII−G but not by the mutant probe CIII−GM4 which, as shown in Figures 1B and 2C−F, affects the half-sites of both RXRα/$T_3R\beta$ and ARP-1 recognition motifs. Interestingly, mutant probe CIII−GM1, which as shown in Figures 1B and 2C-F, carries a mutation that affects the recognition motif of ARP-1, competes for RXRα/$T_3R\beta$ binding but does not compete for ARP-1 binding, implying that the heterodimers of RXRα/$T_3R\beta$ and the homodimers of ARP-1 may occupy slightly different half-sites on element G (Figure 2B). Finally, the binding of RXRα/$T_3R\beta$ and HNF-4 on element I_4 is competed with by the wild-type probe CIII−I_4 but not by the mutated probe CIII−I_4M which, as shown at the bottom of Figure 1C, carries a mutation that affects both half-sites of the HRE binding motif.

Mode of Binding of RXRα/RARα and RXRα/$T_3R\beta$ Heterodimers and ARP-1 and HNF-4 Homodimers to the Regulatory Elements B, G, and I_4 as Determined by Dimethyl Sulfate Interference Assays. The precise binding motifs of representative nuclear receptors to these sites were delineated by dimethyl sulfate interference analysis. In this analysis we have determined the DNA−protein interactions of RXRα/RARα and RXRα/$T_3R\beta$ heterodimers, as well as ARP-1 and HNF-4 homodimers expressed in COS-1 cells, with elements B, G and I_4, respectively. Dimethyl sulfate interference analysis using element B as probe and a mixture of COS-1 cell extracts expressing RXRα and RARα showed that RXRα/RARα heterodimers form six strong and one weak DNA−protein interactions with nucleotides located within the −82 to −70 region (Figure 2A). These nucleotides define a direct TGGGCA(A)AGGTCA repeat on the noncoding strand with one spacer nucleotide between the repeats (DR-1). Strong interactions make the G residues 3 of repeat A and 2 and 3 of repeat B, of the noncoding strand as well as the G residues 5 of repeat A, 5 of repeat B, and −1 located 3′ of repeat B on the coding strand. Weak interactions makes the G residue 4 of repeat A on the noncoding strand (Figure 2A,B).

Dimethyl sulfate interference analysis using element G as probe and COS-1 extracts expressing RXRα and $T_3R\beta$ showed that RXRα/$T_3R\beta$ heterodimers make eight strong and four weak DNA protein interactions with the nucleotides located in the −672 to −656 region (Figure 2C,D). These interactions define a direct TGGAGA(AGCCA)AGGCCG repeat on the noncoding strand, with five spacer nucleotides between the repeats (DR-5). Strong interactions make the G residues 2, 3, and 5 of repeat A, the G residues −3 and −4 located between the two repeats, and the G residues 2 and 3 of repeat B on the noncoding strand. Weak interactions make the G residues 4 and 5 of repeat B on the coding strand (Figure 2C,D).

To determine whether the orphan nuclear receptors such as ARP-1, which also bind to element G, recognize precisely the same motif as the RXRα/$T_3R\beta$ heterodimers, we used dimethyl sulfate interference analysis with element G as probe and COS-1 extracts expressing ARP-1. As shown in Figure 2E,F, ARP-1 homodimers form six strong and one weak DNA−protein interactions with nucleotides located within the −672 to −660 region. These nucleotides define a direct repeat on the noncoding strand AAGCCAAGGCCG with no spacer nucleotides between the half-repeats (DR-0) (Figure 2F). Strong interactions make the G residues 3 of repeat A and 2, 3, and 6 of repeat B on the noncoding strand, as well as the G residues 5 of repeat A and 4 of repeat B on the coding strand. Weak interactions make the G residues 4 of repeat A on the coding strand and residue −1 located 5′ of repeat A on the noncoding strand (Figure 2E,F).

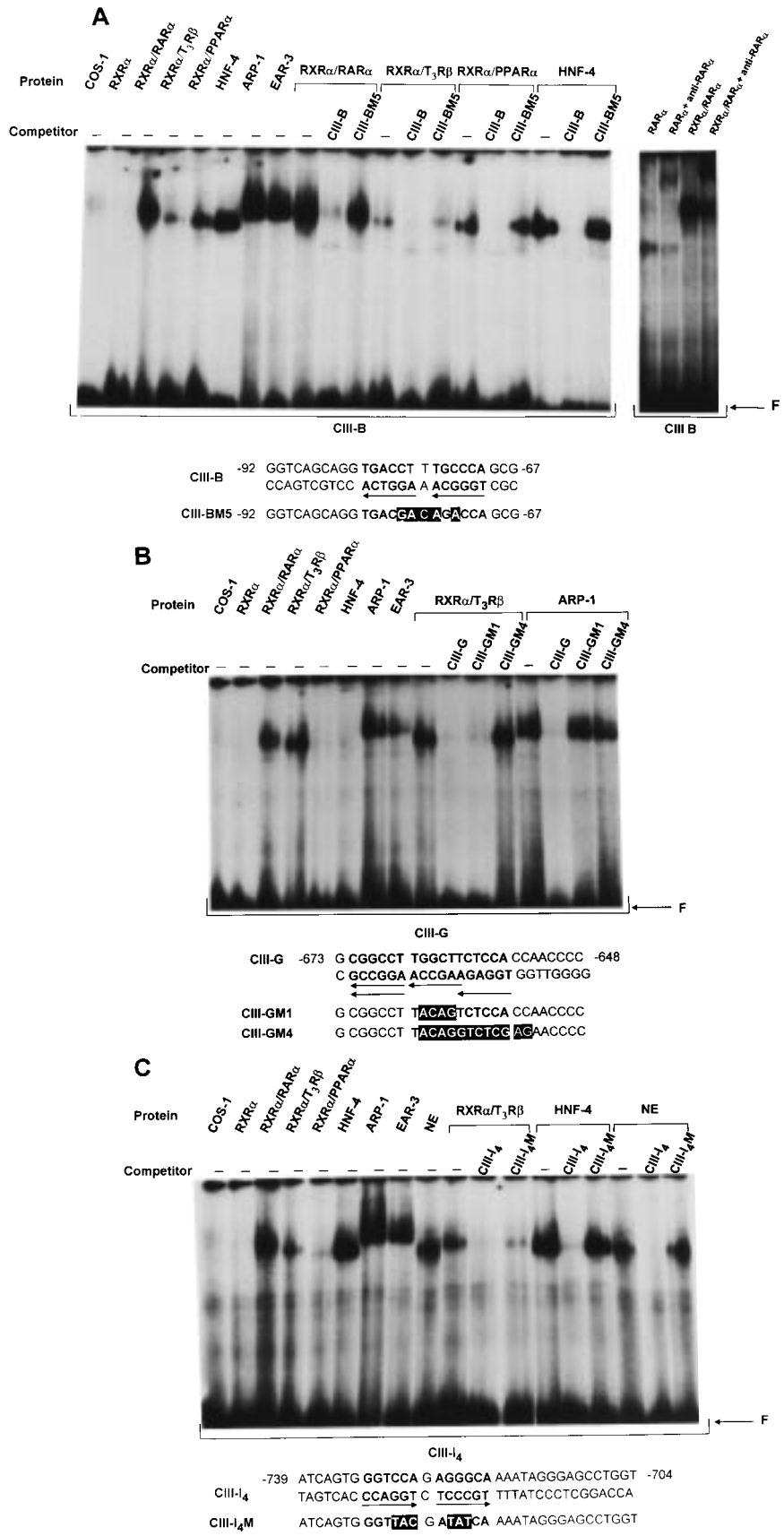

FIGURE 1: DNA-binding gel electrophoresis, supershift, and competition assays of ligand-dependent and orphan nuclear receptors using the wild-type and mutated oligonucleotides corresponding to the regulatory elements CIII−B (A), CIII−G (B), and CIII−I_4 (C) as probes. The probes utilized are indicated in the bottom of each figure along with the mutated oligonucleotides used in the competition assays. Extracts of COS-1 cells transfected with expression vectors for the various ligand-dependent and orphan receptors utilized in the binding assays are indicated at the top of the figure. NE indicates rat liver nuclear extracts. F indicates the free probe. The arrows indicate the position of putative half-sites within the HREs present in these elements.

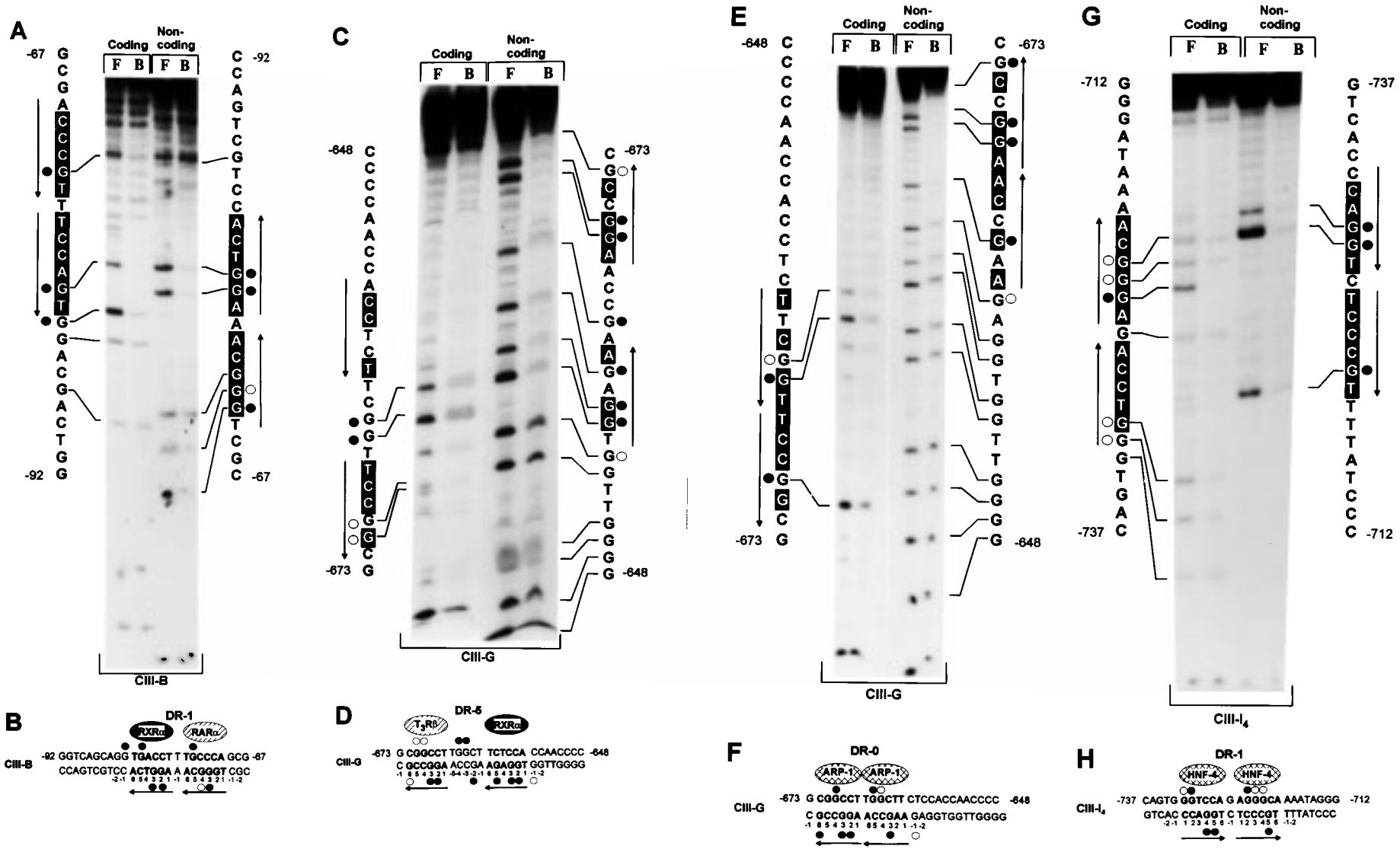

FIGURE 2: DMS modification pattern of the DNA−protein complexes formed with the RXRα/RARα and RXRα/$T_3R\beta$ heterodimers and HNF-4 and ARP-1 homodimers using the elements CIII−B, CIII−G, and CIII−I_4 as robes (Table 1). The RXRα heterodimers and HNF-4 and ARP-1 homodimers were produced by expression of the corresponding cDNAs in COS-1 cells. Panels A and C show the DMS modification pattern of RXRα/RARα and RXRα/$T_3R\beta$ heterodimers with both the coding and noncoding strands of elements CIII−B and CIII−G. Panels B and D are the summaries of the interference patterns deduced from the findings of panels A and C. The DMS modification pattern of ARP-1 with the coding and noncoding strands of element CIII−G is shown in panel E, and the summary of the interference pattern is shown in panel F. Panel G shows the DMS modification pattern of HNF-4 homodimers with the coding and noncoding strands of element CIII−I_4, and the summary of the interference pattern is shown in panel H. F indicates free probe; B indicates probe recovered from the DNA−protein complex after chemical treatment. Strong interactions are illustrated with filled circles, and weak interactions are illustrated with open circles. The nucleotide sequences of the coding and noncoding strands of elements CIII−B, CIII−G, and CIII−I_4 are indicated on each side of Panels A, C, E, and G. The nucleotides that are homologous to the consensus AGG/TTCA motif are highlighted in black. The nucleotides of the repeats defined are numbered 1−6 on the noncoding strand. Nucleotides in the spacer region or nucleotides 5′ or 3′ of repeats A and B are numbered −1 and −2.

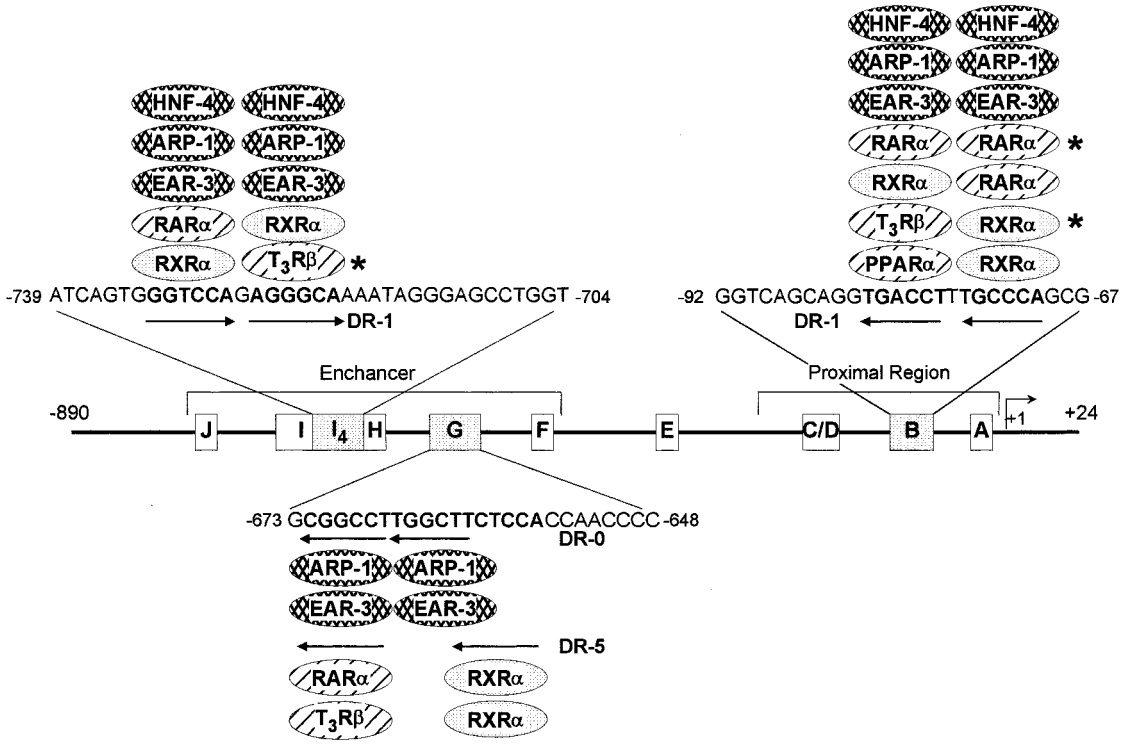

FIGURE 3: Organization of the HREs on the human apoCIII promoter. The panel shows the nucleotide sequence of the wild-type regulatory elements B, G, and I_4. Ovals show the nuclear receptor homo- or heterodimers that bind to each HRE based on the data of Figures 1 and 2. The symbol * indicates heterodimers of hormone nuclear receptors which bind to the HRE with low affinity.

Finally, dimethyl sulfate interference analysis using element I_4 as probe and COS-1 extracts expressing HNF-4 showed that HNF-4 homodimers make five strong and three weak DNA−protein interactions with the nucleotides located within −732 to −720 region (Figure 2G,H). These nucleotides define a direct GGTCCA(G)AGGGCA repeat with one spacer nucleotide between the two repeats (DR-1) on the coding strand. Strong interactions make the G residues at position 2 of both repeats A and B on the coding strand as well as residues 4 and 5 of repeat A and 5 of repeat B on the noncoding strand. Weak interactions make the residues 1 of repeat A and 3 and 4 of repeat B on the coding strand (Figure 2G,H).

In summary, the findings of Figures 1 and 2 demonstrate that elements B and I_4 contain DR-1s that support the binding of HNF-4, ARP-1, and EAR-3 homodimers as well as the binding of heterodimers of RXRα with either RARα or $T_3R\beta$, or heterodimers of RXRα with PPARα and homodimers of RARα, in the case of element B (Figure 3). However, element G consists of three half-sites comprising two different HREs, one DR-0, and one DR-5. Heterodimers of RXRα with RARα or $T_3R\beta$ are recognized by the DR-5 motif, whereas homodimers of ARP-1 are recognized by the DR-0 motif. Thus, the HRE in element G contains information that is interpreted differently by bound ARP-1 homodimers or RXRα/$T_3R\beta$ heterodimers. Previous studies using a dimethyl sulfate and potassium permanganate interference analysis showed that nearly all of the nucleotides of a hexameric repeat, as well as the spacer nucleotides, participated in DNA−protein interactions with homo- or heterodimers of RXR (*30*).

Effect of Ligand-Dependent Nuclear Receptors on the ApoCIII Promoter Strength. We have performed cotransfection titration experiments in HepG2 cells with the wild-type −890/+24 apoCIII−CAT promoter construct and plasmids expressing various combinations of ligand-dependent nuclear receptors in the presence or absence of their corresponding ligands. The experiments with RXRα were performed in the presence of either 9-*cis* RA or all-*trans* RA and increasing amounts of RXRα expression plasmid with concentrations ranging from 100 to 700 ng. This analysis showed that cotransfection with RXRα transactivated the −890/+24 apoCIII promoter approximately 2-fold in the presence of either 9-*cis* RA or all-*trans* RA. In the absence of exogenously added ligand, RXRα had no effect at low concentrations and displayed slight repression at high concentrations (Figure 4A). Optimal transactivation was obtained with 100 ng of plasmid. Since RXRα homodimers do not bind to any of the apoCIII HREs, the observed transactivation may be due to formation of heterodimers of RXRα with RARα or other nuclear receptors present in HepG2 cells.

Cotransfection experiments involving RXRα/RARα heterodimers were performed with increasing amounts of RXRα and RARα expression plasmids with concentrations ranging from 50 to 350 ng for each of the hormone nuclear receptors. This analysis showed that RXRα/RARα heterodimers transactivated 2.5-fold the −890/+24 apoCIII promoter activity in the presence of 9-*cis* or all-*trans* RA (Figure 4B). Optimal ligand-dependent transactivation was observed with 100 ng of each of the RXRα and RARα plasmids. In the absence of ligand there was a trend toward repression at the higher RXRα/RARα concentrations.

Finally, the experiments involving RXRα/$T_3R\beta$ heterodimers were performed with increasing amounts of RXRα and $T_3R\beta$ expression plasmids, with concentrations ranging from 50 to 350 ng for each of the hormone nuclear receptors. These experiments were performed in the absence of ligand

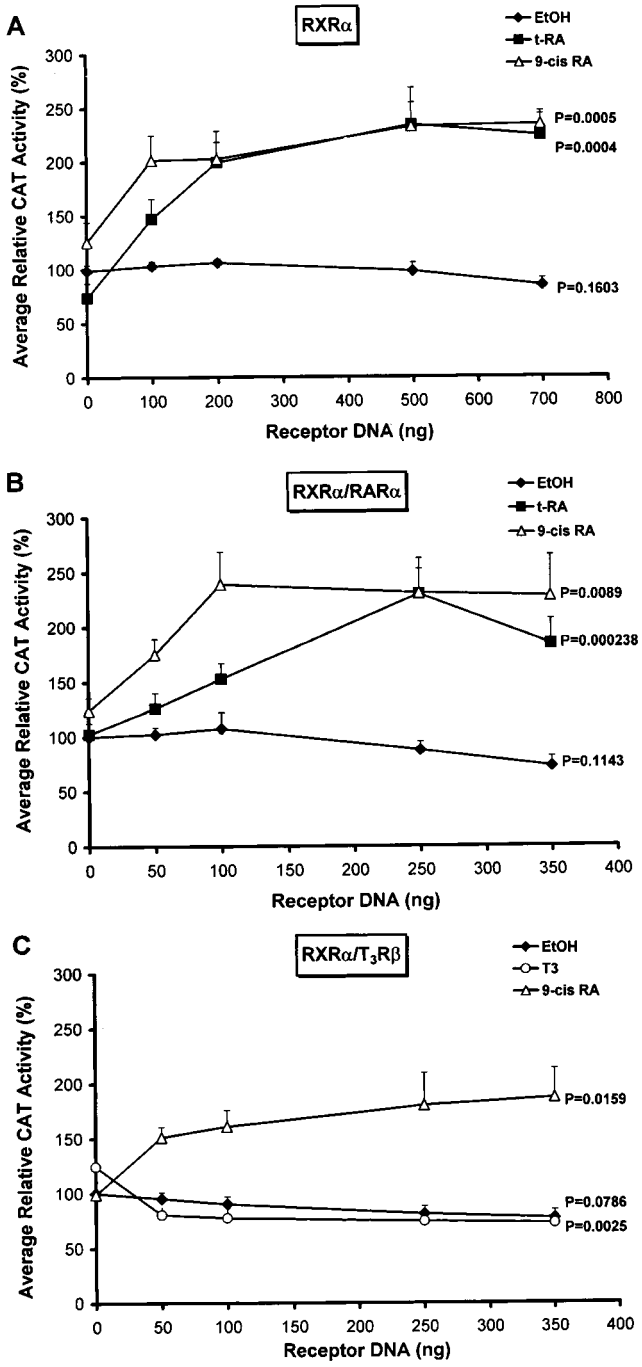

FIGURE 4: Effect of RXRα homo- and heterodimers on the −890/+24 apoCIII promoter activity in HepG2 cells, in the presence or absence of ligands. Panel A shows the effect by RXRα in the presence of 10^{-6} M 9-*cis* RA (triangles) or 10^{-6} M all-*trans* RA (squares) and in the absence of any ligand (diamonds). The experiments were performed with increasing concentrations of RXRα ranging from 100 to 700 ng. Panel B shows the effect by RXRα/RARα heterodimers in the presence of 10^{-6} M 9-*cis* RA (triangles) or 10^{-6} M all-*trans* RA (squares) and in the absence of any ligand (diamonds). Panel C shows the effect by RXRα/T_3Rβ heterodimers in the presence of 10^{-6} M 9-*cis* RA (triangles) or 10^{-7} M T_3 (circles) and in the absence of ligand (diamonds). The experiments in Panels B and C were performed with equal concentrations of each of the expression plasmids ranging from 50 to 350 ng. The total concentration of the expression plasmid for each of the experimental points is the same in panels A, B, and C.

or in the presence of either 9-*cis* RA or T_3 (Figure 4C). This analysis showed that combination of RXRα and T_3Rβ resulted in 1.7-fold transactivation of the apoCIII promoter in the presence of 9-cis RA at concentrations as low as 50 ng and repressed the apoCIII promoter activity in the presence of 10^{-7} M T_3. In the absence of T_3 the apoCIII promoter activity remained unaffected and showed a trend toward repression at higher concentrations of expressing plasmids (Figure 4C).

Previous studies have established the importance of the HREs and SP1-binding sites for apoCIII promoter strength (*19*, *21*, *25*). To assess the contribution of these sites in ligand-dependent transactivation of the apoCIII promoter, we performed cotransfection experiments in HepG2 cells using apoCIII promoter constructs carrying mutations on either element B (CIII−BM5) or I_4 (CIII−I_4M), which abolished the binding of nuclear receptors. In addition, apoCIII reporter construct mutations on elements H (CIII−HM1), which prevented binding of SP1, and G (CIII−GM1), which abolished binding of a yet unidentified factor and ARP-1 homodimers (*19*) but did not affect the binding of RXRα heterodimers (Figure 1B), were used. This analysis showed that transactivation by 9-*cis* RA or all-*trans* RA was abolished in mutant CIII−BM5 either in the absence or in the presence of RXRα heterodimers (Figure 5A,C). Interestingly, a 2-fold transactivation was observed by 9-*cis* or all-*trans* RA in mutant CIII−I_4M both in the absence and in the presence of RXRα heterodimers (Figure 5A−C). These results indicated that the observed ligand-dependent transactivation by RXRα heterodimers in the apoCIII promoter is mainly mediated by element B. In addition, this analysis showed that ligand-dependent transactivation was abolished or significantly reduced in mutants CIII−HM1 and CIII−GM1 (Figure 5D). This indicated that the SP1 factor and factor(s) that recognize element G are also essential for ligand-dependent transactivation of the apoCIII promoter. Figure 6 is a schematic representation that highlights the importance of HREs present in both the proximal and distal sites as well as the SP1 sites present on the apoCIII enhancer for the ligand-dependent transactivation of the apoCIII promoter. It is possible that binding of SP1 to element H and other sites of the enhancer is essential for proper binding and function of the hormone nuclear receptors. Thus, putative interactions of different RXRα heterodimers, SP1, and other factors of the promoter/enhancer cluster may lead to either transcriptional activation as in the case of RXRα/RARα heterodimers (Figure 6) or repression of the apoCIII promoter activity, as in the case of RXRα/T_3Rβ heterodimers.

DISCUSSION

Background on the in vitro and in vivo Regulation of the Human ApoCIII Gene. ApoCIII is a tissue-specific protein that is synthesized predominantly in the liver and to a lesser extent in the intestine (*14*−*16*). Overexpression of this protein in transgenic mice leads to hypertriglyceridemia (*12*, *13*). Thus, understanding the transcriptional regulatory mechanisms which control the expression of the apoCIII gene is important. A series of *in vitro* and *in vivo* studies have pointed out that the distal apoCIII regulatory elements may act as homologous enhancers for apoCIII as well as for the other two genes of the apoA-I, apoCIII, and apoA-IV gene cluster (*19*, *21*, *22*, *38*, *39*). The in vitro experiments showed that deletion of the distal apoCIII promoter region reduced the strength of the proximal promoter to 10−20% of its original value, implying that these elements are required to

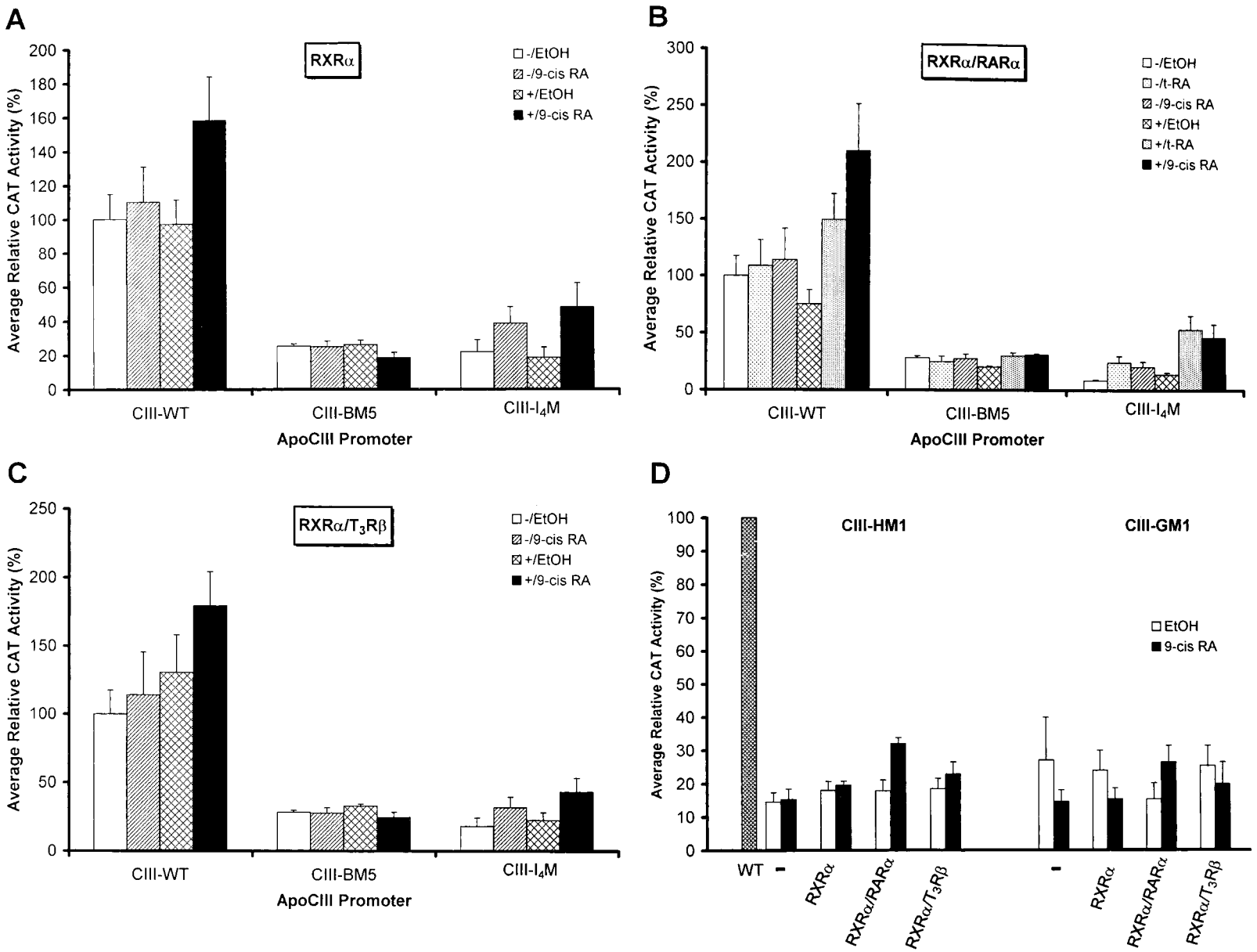

FIGURE 5: Effect of selected mutations in the HREs of elements B and I_4 as well as in elements H and G (Table 1) on the ligand-dependent transactivation of the −890/+24 apoCIII promoter by hormone nuclear receptors. The mutations listed in Table 1 were introduced in the −890/+24 apoCIII promoter. Panels A−C: Effect of RXRα (panel A), RXRα/RARα (panel B), and RXRα/T_3Rβ heterodimers (panel C) on −890/+24 apoCIII promoter activity carrying the mutation CIII−BM5 and CIII−I_4M. Panel D shows the effect of RXR heterodimers on the −890/+24 apoCIII promoter activity carrying the mutations CIII−HM1 and CIII−GM1.

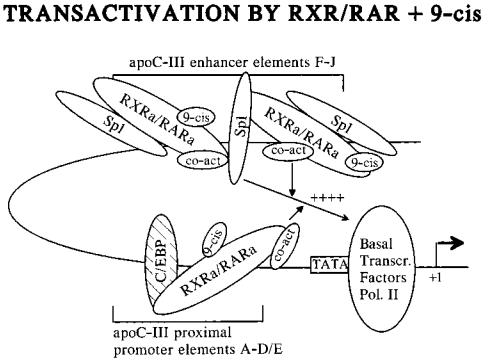

FIGURE 6: Schematic presentation showing the putative interactions of ligand-dependent nuclear receptors and SP1, which may lead to transactivation or repression of the human apoCIII promoter. The diagram assumes participation of transcriptional co-activators and basal transcription factors that may exert positive or negative effects on the function of nuclear hormone receptors. It also assumes protein−protein interactions between nuclear hormone receptors, SP1, and other transcription factors.

enhance the transcription of the apoCIII gene (*18*). Additional studies indicated that the upstream apoCIII regulatory elements F through J increased the strength of the other two promoters of the cluster, apoA-I and apoA-IV, as well as the strength of the heterologous apoB promoter (*21*). A common feature of all these promoters is that they contain one or more proximal HREs which are essential for the function of the enhancer (*20*). The apoCIII enhancer contains multiple binding sites for the transcription factor SP1 as well as two HREs within elements G and I_4 (*19*, *21*). Promoter mutagenesis indicated that the SP1 sites as well as the HREs are important for the overall promoter strength, as well as the functions of the apoCIII enhancer (*19*, *21*).

Orphan nuclear receptors as well as receptors for retinoids and thyroids are members of a nuclear receptor superfamily that controls diverse biological functions including growth, development, and homeostasis (*36*, *40*). They recognize specific hexameric AGG/TTCA motifs with variations in sequence, spacing and orientation, designated HREs (*35*, *37*). This study defined the mode of binding, as well as the specificity of three HREs present in the apoCIII promoter and enhancer region for different types of homo- and heterodimers of ligand-dependent nuclear receptors. Furthermore, it demonstrated transactivation of the apoCIII promoter by RXRα heterodimers in the presence of 9-*cis* RA and repressed the promoter activity in the presence of T_3.

Previous studies have shown that administration of T_3 to hypothyroid animals increased hepatic triglyceride synthesis and secretion (*41*). *In vivo* data indicate that acute administration of T_3 increased the levels of apoCIII gene transcription to 1.6-fold at 2 h post-administration and returned to baseline 24 h later without changes in the steady-state levels of the nuclear or total apoCIII mRNA. The finding suggests that the hormonal effects on apoCIII transcription are counteracted by post-transcriptional events. In contrast, administration of physiological doses of T_3 decreased significantly the apoCIII transcription rate 6 h post-administration of the hormone. Finally, chronic administration of T_3 decreased significantly both the transcriptional rate by 28% and the abundance of apoCIII mRNA by 50−60% (*42*). Consistent with these findings, the rate of apoCIII transcription increased to 1.7-fold of control in hypothyroid rats without changes in the nuclear and total apoCIII mRNA levels (*42*). The effects of retinoids on apoCIII gene expression in vivo have not been studied. Thus the physiological importance of our findings require further investigation.

The HREs on the Regulatory Elements B, G, and I_4 Are the Binding Sites of Orphan and Ligand-Dependent Nuclear Receptors. Mode of Binding and Specificity of Nuclear Receptors for Different HREs. The present study establishes that HREs with DR-1 motifs exist in elements B and I_4, and HREs with DR-5 or DR-0 motifs exist in the regulatory element G of the apoCIII promoter. These motifs are the binding sites for homo- or heterodimers of ligand-dependent nuclear receptors and for homodimers of orphan receptors. We demonstrate that homodimers of RXRα do not recognize any of the three HREs, whereas homodimers of RARα bind weakly to element B. Heterodimers of RXRα/RARα bind strongly to element B and less efficiently to elements G and I_4. In addition, heterodimers of RXRα with $T_3R\beta$ bind mainly to element G and to a lesser extent to elements B and I_4. Finally, heterodimers of RXRα with PPARα bind with moderate affinity only to element B. Binding of RARα homodimers and RXRα/RARα heterodimers to element B was verified with supershift assays using anti-RARα antibodies. Previous studies have shown that the HREs of elements B and I_4 are also recognized by HNF-4 (*21*, *25*) whereas the HRE of element G is recognized only by ARP-1 and EAR-3 (*19*). These orphan receptors were also shown to recognize the regulatory element B (*25*).

An important feature of the HREs is the number of nucleotides separating the two half-repeats (spacer region). It has been proposed that spacing determines the type of homo- or heterodimers of receptors that bind to an HRE (*35*−*37*). It has been suggested that RXRα homodimers bind to direct repeats with a spacing of one nucleotide (DR-1) (*43*, *44*) and RXRα/RARα heterodimers can bind to DR-1, DR-2, or DR-5s (*44*−*47*), whereas RXRα/$T_3R\beta$ heterodimers prefer DR-4s for binding (*36*, *48*, *49*). Nevertheless, exceptions to this rule have been observed (*35*, *50*). To delineate the binding motifs of nuclear receptors to these sites, we utilized dimethyl sulfate interference assays. This analysis showed that the heterodimer RXRα/RARα recognizes a DR-1 TGGGCA(A)AGGTCA on element B that is present between nucleotides −70 and −82 of the antisense strand. Similarly, element I_4 contains a DR-1 GGTCCA(G)-AGGGCA between nucleotides −732 and −720 on the sense strand. Finally, element G contains two different HREs, one DR-0 and one DR-5. The DR-5 TGGAGA(AGCCA)-AGGCCG is located between nucleotides −672 and −654 and recognizes the heterodimers of RXRα. In this motif three of six G residues that participate in strong DNA−protein interactions are located in the spacer region between the two half-repeats. Thus, nucleotides outside the AGG/TTCA motif influence the selectivity of HREs for specific heterodimers of nuclear hormone receptors. Interestingly, the spacer of DR-5 motif serves as a half-site for the binding of ARP-1 homodimers (Figure 3). Therefore, the HRE in element G contains information that dictates differential recognition for binding of ARP-1 homodimers or RXRα/$T_3R\beta$ heterodimers.

This study also showed that the HREs of elements B and I_4 do not bind RXRα homodimers, despite the fact that they

contain a DR-1 type motif. It was shown that binding of homodimeric RXRα is dramatically influenced by the nature of the nucleotide preceding both AGG/TTCA motifs (*51*). Specifically, RXRα homodimers preferentially interact with direct repeats containing either an A or a G immediately upstream of the AGG/TTCA motif, whereas repeats which contain either a T or a C at the same position have greatly reduced binding (*51*). As shown in Figure 2B, repeats A and B in the HRE present on the noncoding strand of element B are preceded by a C and an A nucleotide, respectively. In addition, repeats A and B of the HRE present on the coding strand of element I_4 are both preceded by a G residue, and repeats A and B of the HRE present on the noncoding strand of element G are preceded by G and A residues, respectively. The findings suggest that exceptions to the above rule are possible (Figure 2F,H).

Modulation of the ApoCIII Promoter Activity by Heterodimers of RXRα with RARα or T₃Rβ. To assess the effects of ligand-dependent nuclear receptors on the activity of the −890/+24 apoCIII promoter, we performed cotransfection titration experiments using different combinations of nuclear hormone receptors, in the presence and absence of their ligands. The titration experiments were essential in order to establish the minimum concentration of the expression plasmid required for activation or repression of transcription. Previous studies have shown that RARs bind both 9-*cis* RA and all-*trans* RA with similar high affinity (K_d = 0.2−0.7 nM), whereas RXRs bind only 9-*cis* RA, and with a lower affinity than RARs (K_d = 11.7 nM for RXRα) (*52*, *53*). Thyroid hormone receptors have also been shown to bind their ligand with very high affinity. The reported K_d values of $T_3R\beta$ for T_3 range between 0.1 and 0.01 nM. Most of the studies were performed with nuclear hormone receptors using the ligands at a concentration of 10^{-6} or 10^{-7} M for all-*trans* and 9-*cis* RA and 10^{-7} M for T_3. These concentrations are approximately 10−100 times the K_d value and are well above the saturation levels for the receptors (*52*). The serum concentration of T_3, which is the ligand of $T_3R\beta$, is 1.8 nM. The serum concentration of retinols in the plasma is 1.1−2.4 μM. Thus, the ligand concentration levels we and others have used are well above physiological levels and were designed to ensure saturation of the target HRE with the receptor of choice, in order to assess its effect on gene regulation *in vitro*.

Under these experimental conditions a 2-fold ligand-dependent transactivation of apoCIII promoter was observed by RXRα in the presence of 9-*cis* RA or all-*trans* RA. Since RXRα homodimers do not bind to any of the HREs in this promoter, transactivation probably results from the recruitment by exogenous RXRα of endogenous RAR and/or other heterodimer partner(s). Similar experiments with RXRα/RARα heterodimers resulted in over 2-fold transactivation of the apoCIII promoter by combination of 100 ng each of RXRα and RARα in the presence of either 9-*cis* or all-*trans* RA. Finally, combination of RXRα/$T_3R\beta$ heterodimers resulted in 1.5-fold transactivation of the apoCIII promoter in the presence of 9-*cis* RA and a slight repression of the promoter activity in the presence of T_3, at high concentrations of the heterodimer. In the absence of the ligand there was a trend toward repression at higher nuclear receptor, concentrations. The repression could be the result of displacement of other positive activators from the HREs of the apoCIII promoter by the exogenously added nuclear receptors, which require a specific ligand for their activation. Repression of apoCIII promoter activity was previously reported in the presence of fibrates, probably through the heterodimer of RXRα with PPARα (*54*).

It has been previously proposed that differentially spaced half-site motifs produce distinct transcriptional responses from the retinoic acid receptor. Specifically, RXRα/RARα heterodimers activate transcription in the presence of ligands, when bound to elements consisting of direct repeats separated by five base pairs (DR-5), and exhibit little or no response to activating ligands when bound to elements consisting of direct repeats separated by one base pair (DR-1) (*55*). However, exceptions to this rule have been observed in the promoter of the cellular retinoic acid-binding protein II gene, where activation of transcription is mediated by RAR/RXR heterodimers bound to DR-1 motifs, in the presence of all-*trans* and 9-*cis* RA (*44*, *46*). Our studies also showed that RXRα/$T_3R\beta$ heterodimers either can be activators in the presence of 9-*cis* RA or confer slight repression in the presence of T_3. This repression is consistent with limited *in vivo* observation in hyper- or hypothyroid animals (*41*, *42*).

The thyroid hormone receptor typically represses transcription in the absence of hormone. Hormone binding releases the corepressor and promotes activation (*56*). However, in various promoters containing HREs (*57*−*59*), transcription is activated in the absence but not in the presence of thyroid hormone. It has been proposed that the DNA-binding motifs can act as allosteric effectors that alter the affinity of the receptors for their ligands, as well as coactivators or corepressors (*60*). It is possible that these HREs may inhibit allosterically the interaction of $T_3R\beta$ with the corepressor in the absence of hormone, and may promote the association of the receptor with the corepressor when the hormone is present (*60*).

The present study suggests that, in the presence of RXRα/$T_3R\beta$ heterodimers, allosteric interactions may lead to activation when 9-*cis* RA binds to RXRα, or to slight repression when T_3 binds to $T_3R\beta$. Transcriptional repression was also observed upon the binding of RXRα/$T_3R\beta$ heterodimers to the HREs of the apoA-I promoter which has a DR-1 and a DR-2 HREs (*30*). Such repression in the presence of T_3 may be the result of unfavorable conformation of the $T_3R\beta$ partner brought about by its binding to a DR-1 or DR-2 repeat (*30*). Other studies have shown that ideal binding sites of RXRα/$T_3R\beta$ heterodimers are DR-4s consisting of two AGGTC/AA repeats with four intervening base pairs (*61*). Such elements were shown to confer T_3-dependent transactivation of target promoters such as the growth hormone (*61*) and the human apoC−II promoter (*62*). It is possible that binding of the heterodimers to such sites and allosteric interactions which result from the binding of T_3 affect positively the activation domain of $T_3R\beta$ (*56*).

Mutagenesis analysis established the importance of the HRE present in element B for the ligand-dependent transactivation of the human apoCIII promoter. Although mutations in all three HREs reduced severely the promoter strength in HepG2 cells, only mutations in element B abolished the ligand-dependent transactivation of the promoter. This study also showed that the ability of the nuclear hormone receptors to transactivate the apoCIII promoter was reduced by a mutation in one of the three SP1-binding sites

of the apoCIII promoter. It is possible that, similar to the hormone nuclear receptors, SP1 may also assume a distinct conformation upon binding to the different apoCIII elements F, H, and I. SP1 binding may also affect the conformation of the adjacent hormone nuclear receptors and thus, may specify which of their surfaces are available to contact target factors, including transcription intermediary factors, coactivators, corepressors, and the basal transcription factors (*63*−*65*). Such protein−protein interactions may be favorable or unfavorable, depending on the promoter context, thus resulting in either transcriptional activation, as in the case of RXRα/RARα heterodimers (Figure 4A,B), or repression.

Overall, the present study establishes the binding specificity, as well as the mode of binding of ligand-dependent nuclear receptors to the apoCIII promoter and their effects on the apoCIII promoter activity. Ligand-dependent transactivation is complex and includes putative interactions among nuclear receptors, SP1, and other factors bound to proximal promoter and distal enhancer sites. These *in vitro* findings are consistent with limited *in vivo* data of hyper- or hypothyroid animals and suggest that modulation of apoCIII gene transcription may occur in response to extracellular stimuli, which may in turn affect plasma apoCIII and triglyceride levels.

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