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# Transcriptional Regulation of Human Apolipoprotein Genes ApoB, ApoCIII, and ApoAII by Members of the Steroid Hormone Receptor Superfamily HNF-4, ARP-1, EAR-2, and EAR-3\*

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Apolipoproteins B, CIII, and AII are synthesized primarily in the liver and intestine and play an important role in lipid and cholesterol metabolism. It was previously shown that the cis-acting elements BA1 (-79 to -63), CIIIB (-87 to -63), and AIIJ (-740 to -719) present in the regulatory regions of the human apoB, apoCIII, and apoAII genes, respectively, are recognized by common transcription factors present in hepatic nuclear extracts. This report shows that four members of the steroid receptor superfamily, ARP-1, EAR-2, EAR-3, and HNF-4, bind specifically to the regulatory elements BA1, CIIIB, and AIIJ. Dissociation constant measurements showed that ARP-1, EAR-2, and HNF-4 bind to elements BA1 and CIIIB with similar affinities ( $K_d$  1-3 nM). Cotransfection experiments in HepG2 cells revealed that ARP-1, EAR-2, and EAR-3 repressed the BA1, CIIIB, and AIIJ element-dependent transcription of the reporter gene constructs and the transcription driven by homopolymeric promoters containing either five BA1 or two CIIIB elements. In contrast, HNF-4 activated transcription of reporter genes containing the elements BA1, CIIIB, and AIIJ and reversed the ARP-1-mediated repression of the apoB and apoCIII genes. These results suggested that the opposing transcription effects observed between HNF-4 and ARP-1 may be due to competition for binding to the same regulatory element. Mutations which affected the binding of HNF-4 to elements BA1 and CIIIB affected its ability to activate transcription of the apoB and apoCIII reporter genes, respectively. Transcriptional activation by HNF-4 depended on the presence of elements II (-112 to -94) and III (-86 to -62) of the apoB and H (-705 to -690), I (-766 to -726), and J (-792 to -779) of the apoCIII promoters, indicating that transcriptional activation of apoB and apoCIII genes by HNF-4 requires the synergistic interaction of factors binding to these elements. The finding that HNF-4, ARP-1, EAR-

2 and EAR-3 can regulate the expression of the apoB, apoCIII, and apoAII genes suggest that these nuclear hormone receptors may be an important part of the signal transduction pathways modulating lipid metabolism and cholesterol homeostasis.

Apolipoproteins play a central role in lipid transport and metabolism and are intimately associated with the pathogenesis of atherosclerosis (1, 2). Knowledge of the regulatory mechanisms that control expression of the apolipoprotein genes is important since these proteins represent the main structural components of the low and high density lipoprotein particles and improper concentrations in the plasma may lead to cardiovascular disorders (3, 4). Previous studies have indicated that transcriptional regulation of the human apoB, apoCIII, apoAII, and apoAI genes is accomplished through the interaction of proteins that bind to multiple elements present in the 5' upstream region (5-17). Specifically, the expression of apoB gene in hepatic and intestinal cells is controlled mainly by the interaction of factors that bind to three regulatory elements BCB, BA1, and BA4 present in the -120 to -33 proximal promoter region (7). Furthermore, transcription of the apoB gene may be modulated by an enhancer present in the second intron of the gene (18, 19). Transcription of the apoCIII gene in hepatic and intestinal cells is dependent on the interaction of factors that bind to DNA elements J, I, and H present in the distal promoter region and element B proximal to the transcription start site (9, 10). Transcription of the apoAII gene in hepatic and intestinal cells is controlled by 14 DNA-binding elements (A to N) spaced throughout the 911-base pair promoter region (11, 12). The region -903 to -671 that includes elements N to I is essential for the transcriptional activation of the ApoAII gene in both HepG2 and Caco-2 cells and confers tissue-specific expression when placed in front of heterologous promoters, thus playing the role of a tissue-specific enhancer (11, 12, 20). DNA binding and cross-competition experiments demonstrated that the elements BA1, CIIIB, AIIJ, and A1D of apoB, apoCIII, apoAII, and apoAI promoters bind common nuclear factors (6, 10, 21). Substitution mutations on elements BA1 and CIIIB that abolished the binding of nuclear factors present in hepatic extracts also reduced the transcription of apoB and apoCIII reporter gene constructs to 1.5 and 8%, respectively (6, 10). Therefore, transcription from both promoters is strongly dependent on the specific interactions of nuclear factors with the elements BA1 and CIIIB. In contrast

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to the apoB and apoCIII promoters, deletion of elements A1D and AIIJ from the apoA1 and apoAII promoters reduced transcription of the reporter constructs to 50 and 70% of control, respectively (14, 22, and present study). These results indicated that binding of factors to those elements seem to play a central role in the activation of apoB and apoCIII genes and a less important role in the regulation of apoAII and apoA1 genes. The nuclear factor NF-BA1, that binds to element BA1 on the apoB promoter, was purified from rat liver nuclear extracts and was identified as a polypeptide of 60 kDa (21). *In vitro* transcription with BA1-depleted extracts indicated that purified NF-BA1 retained the ability to activate transcription of the apoB promoter fragment -268 to +8. In addition, DNase I footprinting and gel retardation experiments showed that NF-BA1 protein binds avidly to elements CIIB, A1D, and AIIJ present in the apoCIII, apoA1, and apoAII promoters (21). A heat stable nuclear activity, that binds specifically only to element CIIB, was also purified from rat liver nuclear extracts and designated CIIB1 (23). DNase I footprinting and methylation interference assays performed with the purified CIIB1 factor revealed that this protein recognizes the sequence CAGGTGAC, overlapping but distinct to the NF-BA1 recognition sequence GTGACCTTT. Substitution mutations around the common recognition sequence of GTGAC that dramatically reduced the binding of either the CIIB1 or the NF-BA1 binding activities, revealed that the nuclear activities which bind to the NF-BA1 recognition motif are substantially more important in the activation of the apoCIII promoter (10). CIIB1 had a much lower transactivation potential than the NF-BA1 binding factor, and it was hypothesized that the CIIB1 factor may act as a modulator of the ApoCIII gene transcription by competing for the same binding site.

Recent studies have also shown that the regulatory elements A1D, CIIB, and BA1 of the apoA1, apoCIII, and apoB promoters can bind to transcription factors ARP-1 and HNF-4, members of the steroid-thyroid receptor gene superfamily (22, 24). Cotransfection experiments showed that ARP-1 repressed the transcription of apoA1 gene in HepG2 cells (22). Nuclear factor HNF-4 was shown to interact with the apoCIII promoter region -66 to -87 and to stimulate transcription of a reporter construct containing two tandem apoCIII elements in HeLa cells, indicating that it may be involved in regulating its transcription (24). ARP-1 was shown to have 89 and 98% sequence homology in the DNA-binding domain with the *v-erbA*-related human receptors EAR-2 and EAR-3 (25). Despite their strong sequence homology, ARP-1, EAR-2, and EAR-3 have different chromosomal localization indicating that these factors are products of different genes (22, 25). HNF-4, ARP-1, EAR-2, and EAR-3 belong to the subfamily of orphan receptors for which no ligand has been identified as yet.

In the present study we investigated the binding properties of HNF-4, ARP-1, EAR-2, and EAR-3 on the elements BA1, CIIB, and AIIJ and studied their involvement in regulating the apoB, apoCIII, and apoAII gene transcription.

#### MATERIALS AND METHODS

**Plasmid Construction**—Construct pMARP1 containing the full-length cDNA of ARP-1 in the expression vector pMT2 (26) was described previously (22). Construct pMARP1 was previously referred to as pMA. All other plasmid constructs were made using standard procedures (27). The structures of the resulting constructs were verified by restriction mapping and limited nucleotide sequencing.

The EAR-3 cDNA was derived from a HeLa  $\lambda$ gt11 library (Clontech), using as probe a DNA fragment corresponding to ARP-1 DNA-binding domain. The 5'-untranslated region of EAR-3 was modified

using the polymerase chain reaction (PCR)<sup>1</sup> (28) with the primer 5'-GACGCAGAATTCAAGCTTCCGCGCCCATGGCAATGGTAGTTAGCAGCTGGCGAG-3' which provides a strong translation initiation sequence specified by Kozak (29), a primer corresponding to the SP6 promoter and the EAR-3 cDNA cloned in pGEM-7Zf(+) as a template. The PCR product was cloned in the *EcoRI* site of pMT2 vector, to generate construct pMEAR3.

Plasmid ev2 that contains the human EAR-2 cDNA in the pGEM-3Zf(-) vector, was a kind gift from Dr. Tadashi Yamamoto of the University of Tokyo. The EAR-2 cDNA was excised from the vector and cloned in the *EcoRI* site of pMT2, using *EcoRI* linkers (New England Biolabs), to generate construct pMEAR2.

HNF-4 was cloned from a rat liver  $\lambda$ gt11 library (Clontech) using PCR and primers (Genosys) based on the published sequence (24). The forward primer 5'-GACAGAATTCGCCGCCCATGGACATGGCTGACTACAGTGCT-3' which provides a strong translation initiation sequence specified by Kozak, and the reverse primer 5'-GACAGAATTCAGCTTTCTCTGAGGGTGTGAGCCAGCAGAGCCT-3' were used in a PCR containing  $\sim 2 \times 10^7$  recombinants as described previously (24). The PCR product was digested with *EcoRI* and cloned in the *EcoRI* site of pMT2 vector to generate construct pMHNF4.

The construction of a series of reporter plasmids containing 5' deletion or substitution mutations of the apoB, apoCIII, and apoAII promoters ligated to the chloramphenicol acetyltransferase gene have been previously described (6, 10, 12).

**Cell Transfections and Chloramphenicol Acetyltransferase Assays**—HepG2 cells were maintained as stocks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fifty to 60% confluent 60-mm dishes were transfected using the calcium-phosphate DNA coprecipitation method (30). The transfection mixture contained 6  $\mu$ g of promoter plasmid DNA, 6  $\mu$ g of either pMHNF4, pMARP1, pMEAR2, and pMEAR3 plasmids, and 5  $\mu$ g of  $\beta$ -galactosidase plasmid (31). Cells were harvested 42 h later and lysed by freeze-thawing. Chloramphenicol acetyltransferase assays were performed in a total volume of 150  $\mu$ l, 0.47 M Tris-HCl buffer, pH 7.8, containing 0.5  $\mu$ Ci of [<sup>14</sup>C]chloramphenicol and 0.53 mM acetyl-CoA as described (32). The reaction times and extract concentrations were selected to ensure linear conversions of the chloramphenicol to the acetylated forms. The non-acetylated and acetylated chloramphenicol forms were separated on IB2 silica gel plates using chloroform/methanol 95:5 for development. The radioactive spots, detected by autoradiography, were scraped from the thin layer plates and counted. The  $\beta$ -galactosidase activity of the cell lysates was determined as described (31), and the values were used to normalize variabilities in the efficiency of transfection.

**Preparation of Extracts from COS-1 Transfected Cells**—COS-1 cells were maintained as stocks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fifty to 60% confluent 150-mm dishes were transfected with 42  $\mu$ g of pMHNF4, pMARP1, pMEAR2, and pMEAR3 plasmids. Forty h after transfection cells were collected in 40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl and pelleted by low speed centrifugation. Cells were resuspended in 400  $\mu$ l of a buffer containing 20 mM Tris-HCl, pH 7.4, 0.4 M KCl, 2 mM dithiothreitol, 10% glycerol, and were broken by freeze and thaw three times (33). Cell debris was removed by centrifugation at 4  $^{\circ}$ C for 5 min in a microfuge, and the supernatant (whole cell extracts) was aliquoted and stored at -70  $^{\circ}$ C.

**DNase I Footprinting Assays**—The apoB promoter fragment extending from position -268 to +8 was amplified by the polymerase chain reaction procedure using as 5' and 3' primers the oligonucleotides rev-5-26 and PCR-B8R, respectively (7). The 3' primer was labeled with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase prior to the PCR amplification. The apoCIII promoter fragment -163 to +24 was obtained by digestion of the recombinant pUC-SHCAT plasmid containing this promoter region by *XbaI* and *XhoI* (10). The fragment was labeled with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP either at the *XhoI* or the *XbaI* sites. Labeled fragments were purified by polyacrylamide gel electrophoresis prior to use.

DNase I footprinting (34) was performed in 20  $\mu$ l of reaction volume containing 25 mM HEPES pH 7.6, 40 mM KCl, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 3  $\mu$ g of poly(dI-dC) competitor DNA, and 3-6  $\mu$ l of whole cell extracts obtained from transiently transfected COS-1 cells expressing HNF-4, ARP-1, EAR-2, or EAR-3. After 15 min on ice, 10,000-15,000 cpm of end-labeled frag-

<sup>1</sup> The abbreviations used are: PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ment was added and the incubation continued for 90 min on ice. Two  $\mu$ l of DNase I, freshly diluted to a final concentration of 150  $\mu$ g/ml in 10 mM CaCl<sub>2</sub> was added, and the digestion was allowed to proceed for 5 min on ice. The reaction was stopped by the addition of 4  $\mu$ l of 125 mM Tris-HCl, pH 8.0, 125 mM EDTA and 3% sodium dodecyl sulfate. Forty  $\mu$ g of proteinase K and 5  $\mu$ g of carrier tRNA were added, and the reaction mixture was incubated for 20 min at 65 °C. The DNA was precipitated with 1 volume of 5 M ammonium acetate, pH 8.0, and 2 volumes of Ethanol, resuspended in formamide dye (98% formamide, 0.1%, bromphenol blue, 0.1% xylene cyanol, 5 mM EDTA), electrophoresed on a 6% polyacrylamide, 7 M urea sequencing gel and analyzed by autoradiography.

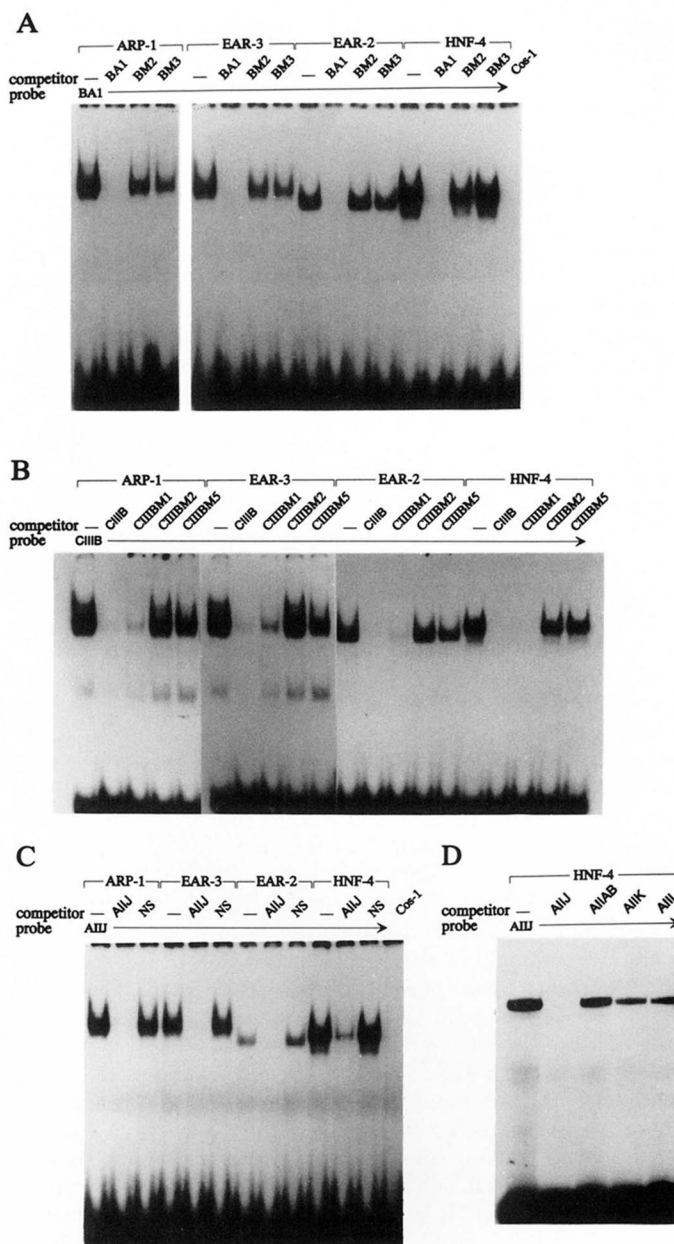
**Gel Electrophoretic Mobility Shift Assays**—Gel electrophoretic mobility shift assays (35, 36) were performed in a 20- $\mu$ l reaction volume containing 25 mM HEPES, pH 7.6, 8% Ficoll 400, 40 mM KCl, 1 mM dithiothreitol, 5 mM MgCl<sub>2</sub>, 1  $\mu$ g of poly(dI-dC), and varying amounts (1–5  $\mu$ l) of whole cell extracts diluted 10-fold. When indicated 20 ng of competitor oligonucleotides were added 15 min prior to the addition of the labeled probe. Following a 15-min incubation on ice, 3 fmol (30,000 cpm) of labeled double-stranded oligonucleotide was added and the incubation continued for 30 min on ice. The reaction mixture was then loaded directly onto a 4% polyacrylamide gel in 1  $\times$  TAE (10  $\times$  TAE = 67 mM Tris, 33 mM sodium acetate, 10 mM EDTA, pH 7.9) and electrophoresed at 10 volts/cm of gel for 2–3 h at 4 °C with recirculation of the buffer (36). Alternatively, gels were run in 0.5  $\times$  TBE (10  $\times$  TBE = 0.89 M Tris, 0.89 M boric acid, 20 mM EDTA) at 4 °C without recirculation. After the run, gels were dried and analyzed by autoradiography.

The dissociation constant values ( $K_d$ ) of ARP-1, EAR-2, and HNF-4 were obtained from binding reactions performed with a constant amount of protein extract and increasing concentrations of radiolabeled BA1 probe (specific radioactivity  $0.5 \times 10^9$  cpm/ $\mu$ g). After gel electrophoresis and autoradiography, the radioactive bands corresponding to the bound and free oligonucleotide were excised, and the radioactivity was measured by scintillation spectroscopy. The  $K_d$  values were calculated by using the equation  $B_p/F_p = -1/K_d \times [B]_p + T_p/K_d$  where  $B_p$ ,  $F_p$ , and  $T_p$  are the bound, free, and total (bound + free) radiolabeled oligonucleotide BA1.

**Methylation Interference**—The coding strands of synthetic oligonucleotides BA1 and CIIIB were labeled at the 5' end with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. Each labeled strand was annealed with the unlabeled complementary strand as described previously (37). End-labeled double-stranded oligonucleotides ( $4 \times 10^6$  cpm) were partially methylated at G residues using dimethyl sulfate (37). The methylated probes were incubated with whole cell extracts from COS-1 cells expressing HNF-4, ARP-1, EAR-2, or EAR-3, and the complexes were analyzed in a preparative mobility shift gel. The gel was exposed for 1 h and both the complexed and free oligonucleotides were excised from the gel, electroeluted and treated with 1 M piperidine at 90 °C for 30 min (37). The samples were dried, dissolved in 98% formamide dye, and electrophoresed on 20% polyacrylamide/urea sequencing gels. Bands were visualized by autoradiography.

## RESULTS

**Comparison of DNA Binding Properties among HNF-4, ARP-1, EAR-2, and EAR-3**—To investigate the possible interactions of the HNF-4, ARP-1, EAR-2, and EAR-3 proteins with the elements BA1, CIIIB, and AIIJ of the apoB, apoCIII, and apoAII genes, respectively, we employed gel mobility shift assays, DNase I protection, and methylation interference studies. The first step was to determine if indeed these proteins had the ability to bind to the BA1, CIIIB, and AIIJ elements. To examine the binding of HNF-4, ARP-1, EAR-2, and EAR-3 to the different elements from the apoB, apoCIII, and apoAII promoters, we used the BA1, CIIIB, and AIIJ probes in a gel mobility shift assay with extracts from COS-1 cells expressing these proteins. All proteins HNF-4, ARP-1, EAR-2, and EAR-3 formed strong specific complexes with elements CIIIB, BA1, and AIIJ (Fig. 1, A–C). Specifically, the factors HNF-4, ARP-1, EAR-2, and EAR-3 bind avidly to the element BA1 of the apoB promoter (Fig. 1A), which was shown previously to bind the NF-BA1 factor. Competition with the homologous oligonucleotide BA1 eliminated the complexes formed, indicating specific binding to the cognate site



**FIG. 1. DNA-binding gel electrophoretic and competition assays (A–D).** The oligonucleotides used as probes or competitors correspond to normal and mutated elements BA1 (–88 to –62) (panel A), CIIIB (–92 to –67) (panel B), and AIIJ (–739 to –716) (panels C and D) of the apoB, apoCIII, and apoAII promoters, respectively. Transcription factors HNF-4, ARP-1, EAR-2, and EAR-3 were produced by transient expression of the corresponding cDNAs in COS-1 cells. Competitor oligonucleotides were used at 200-fold molar excess (20 ng/reaction). *N.S.* indicates oligonucleotide with a nonspecific sequence.

(Fig. 1A). In contrast, very little competition was observed with the mutant oligonucleotides BM2 and BM3, which were shown previously to abolish the binding of the NF-BA1 factor (6). Oligonucleotide CIIIB binds both the NF-BA1 and CIIIB1 nuclear activities. It was shown previously that the mutated oligonucleotide CIIIBM1 affected the binding of only the CIIIB1 factor while it did not affect the binding of the NF-BA1 factor (10). Mutant oligonucleotide CIIIBM2 affected the binding of both the CIIIB1 and NF-BA1 factors, and mutant CIIIBM5 affected the binding of only the NF-BA1 factor (10). To further investigate the relative affinity of HNF-4, ARP-1, EAR-2, and EAR-3 for each of the variant

sequences, we used excess amounts of unlabeled oligonucleotide CIIIBM1, CIIIBM2, and CIIIBM5 as competitors in a gel mobility shift assay with the CIIIB oligonucleotide as the probe (Fig. 1B). Competition with variant oligonucleotide CIIIBM1 competed effectively for the complexes formed between CIIIB and EAR-2, EAR-3, and ARP-1, while it eliminated the CIIIB-HNF-4 complex (Fig. 1B). No competition was observed with variant oligonucleotides CIIIBM2 and CIIIBM5 for any of the complexes formed between CIIIB and HNF-4, ARP-1, EAR-2, and EAR-3 (Fig. 1B). These results indicated that factors HNF-4, ARP-1, EAR-2, and EAR-3 bind with high affinity to the CIIIB and variant CIIIBM1 elements and with much reduced affinity to the variant CIIIBM2 and CIIIBM5 elements. The competition data indicated that the factors HNF-4, ARP-1, EAR-2, and EAR-3 bind to the element CIIIB via the NF-BA1 recognition sequence rather than the CIIIB1 recognition sequence.

Specific binding of HNF-4, ARP-1, EAR-2, and EAR-3 to the AIIJ element of the apoAII promoter which is also capable of binding the NF-BA1 factor (21) was demonstrated in the binding experiment presented in Fig. 1C. The complexes formed between element AIIJ and proteins HNF-4, ARP-1, EAR-2, and EAR-3 were eliminated upon the addition of 20-fold excess of unlabeled AIIJ oligonucleotide competitor but not with a nonspecific oligonucleotide (Fig. 1C).

Three regions along the apoAII promoter have sequence homology with the CIIIB1 recognition site present in the apoCIII promoter. It was recently shown, that partially purified CIIIB1 binds to elements AB, K, and L of the apoAII promoter (38). The motif TGAC is part of a recognition sequence for multiple factors including the members of the steroid receptors. Flanking sequences around this motif will determine the specificity of binding for any one factor. For instance the flanking sequences around the TGAC motif in the L domain of the apoAII promoter create an AP1-binding site that overlaps with the CIIIB1-binding site (11, 38). To test whether domains AB, K, and L of the apoAII gene can also interact with HNF-4, ARP-1, EAR-2, and EAR-3, we performed DNA binding competition experiments. Fig. 1D shows that the binding of HNF-4 to oligonucleotide AIIJ is partially competed by oligonucleotides corresponding to elements K and L but not by the AB element. Similar results were obtained with EAR-2, EAR-3, and ARP-1 (data not shown) indicating that these factors bind with low affinity only to elements K and L.

DNase I footprinting and methylation interference analyses were used to locate the site of interaction of these proteins within the apoB and apoCIII promoters. DNase I footprint analysis of the apoB promoter fragment -268 to +8 with COS-1 extracts expressing EAR-2, ARP-1, and HNF-4 identified a protected area extending between nucleotides -86 to -62 (Fig. 2A). Previous studies showed that purified NF-BA1 protected the region -79 to -63 on the apoB promoter and created four hypersensitive sites that are absent in the footprint obtained by EAR-2, HNF-4, and ARP-1 (21). In addition DNase I footprint analysis of the -214 to +24 apoCIII promoter fragment identified a strong footprint spanning the regions -86 to -63 and -89 to -63 of the coding (Fig. 2B) and non-coding (Fig. 2C) strands, respectively. This footprinting area is similar to the one obtained previously with the purified NF-BA1 factor (21).

Methylation interference of HNF-4, ARP-1, EAR-2, and EAR-3 binding to the oligonucleotide BA1 of the apoB promoter was observed in the region encompassed by the DNase I footprint between nucleotides -80 to -63 (Fig. 3A). There was strong interference of HNF-4 binding by methylation of

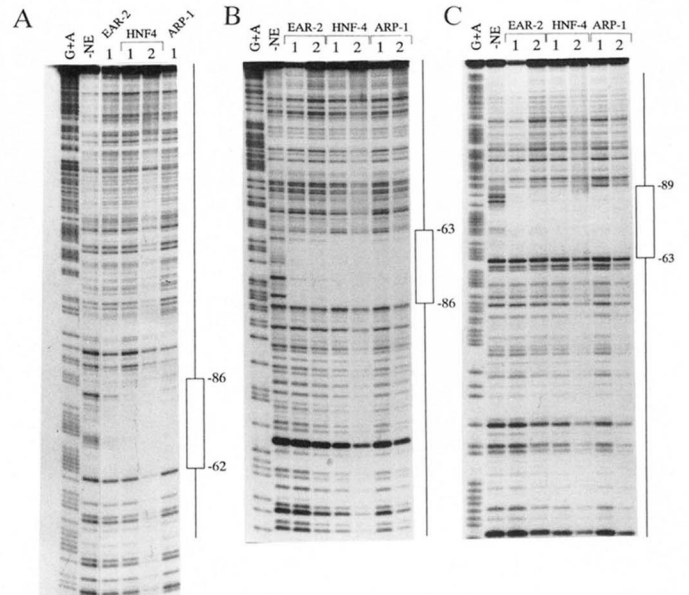


FIG. 2. DNase I footprinting analysis of apoB and apoCIII promoter fragments -268 to +8, and -214 to +24, respectively, performed with extracts from COS-1 cells expressing transcription factors EAR-2, HNF-4, or ARP-1 (A-C). Panel A, the apoB probe was labeled with  $^{32}\text{P}$  in the sense strand. Panels B and C, the apoCIII probe was labeled with  $^{32}\text{P}$  in the sense and antisense strands, respectively. In lanes 1 and 2, footprinting reactions were performed with 3 and 6  $\mu\text{l}$  of extracts. G+A are chemical cleavages of the same DNA fragment used in footprint analysis. -NE shows reactions performed in the absence of extracts. Boxes indicate regions protected from the DNase I digestion. Numbers are with respect to the transcription start site.

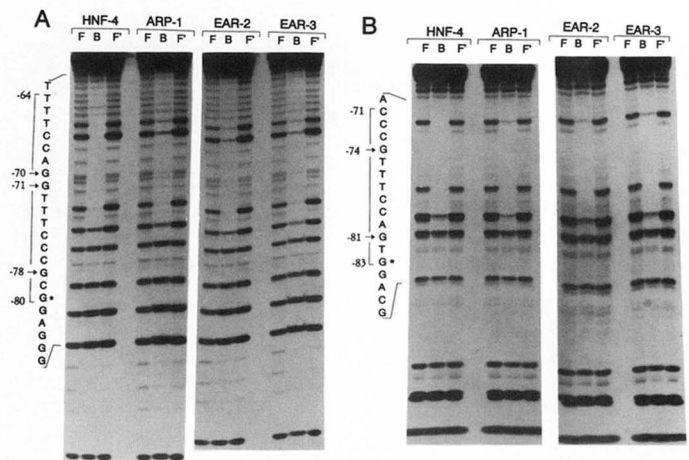


FIG. 3. Methylation interference pattern of the DNA-protein complexes obtained with the element BA1 (-88 to -62) of apoB (panel A) and CIIIB (-92 to -67) of apoCIII (panel B). The analysis was performed following labeling of the coding strand. F, free DNA before binding; F', free DNA recovered after binding; B, bound DNA recovered from the DNA-protein complexes with ARP-1, EAR-2, EAR-3, and HNF-4. The arrows and asterisks indicate the position of guanine residues with strong and weak interference of binding, respectively. The numbers indicate the position of nucleotides with respect to the transcription initiation site.

the G residues at positions -78, -71, and -70 on the coding strand of oligonucleotide BA1 (Fig. 3A). Strong interference of binding by methylation of the same residues was also observed with ARP-1, EAR-2, and EAR-3 although interfer-

ence at residues -71 and -70 was somewhat weaker, as compared to HNF-4. Weak methylation interference was also evident at the G residue at position -80 with all factors (Fig. 3A). Methylation interference of HNF-4, ARP-1, EAR-2, and EAR-3 binding to the CIIIB oligonucleotide was observed in the region encompassed by the DNase I footprint between nucleotides -83 to -71 (Fig. 3B). There was strong interference of HNF-4 binding by methylation of the G residues at positions -81 and -74 of the coding strand of oligonucleotide CIIIB (Fig. 3B). Strong interference of binding with ARP-1, EAR-2, and EAR-3 was also observed at the G residues -81 and -74, although interference at residue -74 was somewhat weaker. Weak methylation interference was also obtained with all factors at the G residue at position -83 (Fig. 3B). Comparison of the DNase I footprint and the methylation interference data revealed that the binding of the steroid receptor proteins is stronger between nucleotides -80 to -63 in the apoB and -83 to -71 in the apoCIII promoters.

The DNA binding affinities of ARP-1, EAR-2, and HNF-4 were calculated by performing saturating binding assays in which a constant amount of factor was titrated with increasing amounts of labeled BA1 oligonucleotide. The binding reactive mixtures were subjected to a gel mobility shift assay to separate the BA1-protein complex from the free BA1 oligonucleotide (Fig. 4, A-C, left panel). Gel segments corresponding to the bound and free BA1 oligonucleotide were excised from the gel, and radioactivity was measured by liquid scintillation counting. The data were plotted as the amount of bound oligonucleotide (BA1-protein complex) versus the total amount of BA1 oligonucleotide present in the incubation reaction (Fig. 4, A-C, middle panel). The dissociation constants ( $K_d$ ) were obtained from linear plots of the BA1-protein complex versus free oligonucleotide according to Scatchard (Fig. 4, A-C, right panel) (39). The dissociation constants obtained are 1.78 nM for ARP-1, 0.98 nM for EAR-2, and 0.705 nM for HNF-4. Similar experiments have been performed using the CIIIB oligonucleotide as a probe. The dissociation constant values obtained ranged from 1.5 to 3 nM (data not shown). This analysis indicated that the affinities of ARP-1, EAR-2, and HNF-4 for their cognate sequences on the apoB and apoCIII promoters are very similar.

The confirmation that HNF-4, ARP-1, EAR-2, and EAR-3 bind with relatively high affinity to the same DNA elements of the apoB, apoCIII, and apoAII genes prompted us to study whether these proteins are capable of regulating their transcription. The slight differences in the binding properties observed between HNF-4 and the ARP-1, EAR-2, or EAR-3 are probably a consequence of the stronger sequence similarities shared by ARP-1, EAR-2, and EAR-3 in the DNA-binding domain as compared to the more divergent sequence present in the DNA-binding domain of HNF-4. These homologies are shown in Fig. 5. The overall sequence similarities shared by ARP-1, EAR-2, and EAR-3 may provide strong evidence relevant to the functions of these proteins. However, since the amino-terminal regions of HNF-4, ARP-1, EAR-2, and EAR-3 have limited similarity, it is possible that each of these proteins may be capable of mediating different regulatory processes. For that reason, the function of HNF-4, ARP-1, EAR-2, and EAR-3 was studied independently by conducting cotransfection experiments in HepG2 cells. The full-length cDNAs for HNF-4, ARP-1, EAR-2, and EAR-3 were cloned into expression vector pMT2 and then cotransfected with reporter plasmids containing various promoters driving the expression of the bacterial chloramphenicol acetyltransferase gene. As a control, the mammalian expression vector

pMT2 lacking the cDNA inserts was cotransfected with the reporter genes.

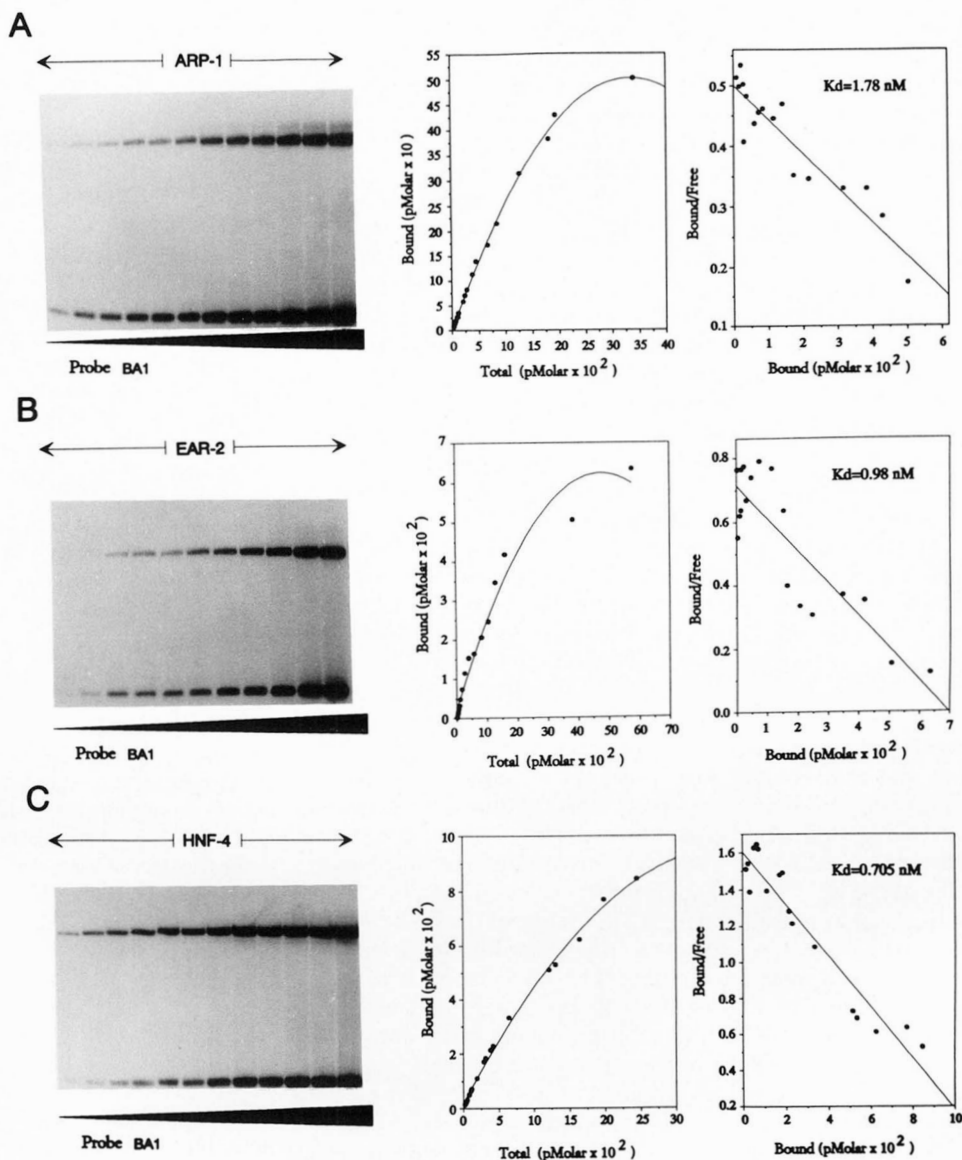
**Repression of ApoB Gene Transcription by ARP-1 and EAR-3**—The regulatory elements in the apoB promoter proximal region and the apoB reporter constructs used in the cotransfection experiments are presented in Fig. 6A. As shown in Fig. 6B, expression of EAR-3 and ARP-1 in HepG2 cells decreased the transcription of the chloramphenicol acetyltransferase gene driven either by the apoB -1800/+24, or the -150/+24 promoter fragments to 1.5% of control. Cotransfection experiments performed with various concentrations of ARP-1 expression plasmid indicated that the repression effect of ARP-1 is dependent on the amount of the cotransfected ARP-1 plasmid and transcriptional repression reached plateau with 4  $\mu$ g of ARP-1 (Fig. 6C). Furthermore, the dramatic decrease in transcription of the apoB gene by the expression of EAR-3 and ARP-1 was very similar with the decrease in transcription observed in mutated promoter constructs BM2 and BM3 lacking a functional BA-1-binding site (7). These experiments indicated that the nuclear factors EAR-3 and ARP-1 act as negative regulators by repressing the BA1-dependent transcription of the apoB gene.

**HNF-4 May Act as Positive Regulator in ApoB Gene Transcription**—Cotransfection experiments in HepG2 cells showed that CAT gene expression driven by the -1800/+124 and -150/+124 apoB promoter fragments is very similar in the presence or absence of HNF-4 (Fig. 6B). In the cotransfection experiments, the amount of HNF-4 expressed in HepG2 cells is high enough to bind and prevent other nuclear factors such as the NF-BA1 to interact with this site. Therefore, the transcription observed in the presence of HNF-4 is predominantly due to the ability of HNF-4 to transactivate the apoB promoter by interacting with site BA1. To further test this hypothesis, we performed cotransfection experiments using a constant amount of ARP-1 and increasing amounts of transfected HNF-4. As seen in Fig. 6C, 1  $\mu$ g of ARP-1 repressed transcription of the apoB promoter fragment -150/+124 in HepG2 cells to 20%. Increasing concentrations of HNF-4 counteracted the repression effect of ARP-1 and transcription levels approached to 80% (Fig. 6D). Since HNF-4 cannot heterodimerize with ARP-1,<sup>2</sup> the increase in transcription is due to the interaction of HNF-4 with element BA1. In addition, HNF-4 increases the transcription of a homopolymeric promoter which contains five BA1 sites by 17-fold (Fig. 6E). The same homopolymeric promoter is repressed in the presence of EAR-2, EAR-3, and ARP-1 (Fig. 6E). These data indicated that HNF-4 may act as a transcriptional activator of the apoB gene by binding to element BA1.

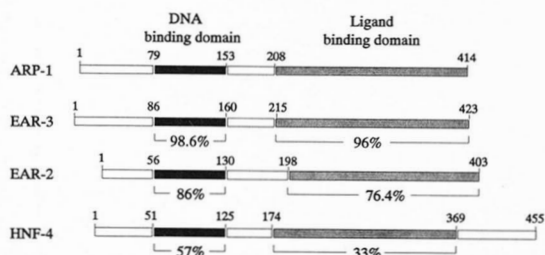
**HNF-4-dependent Transcription of the ApoB Promoter Requires the Synergistic Interaction of Proteins That Bind to Elements II and IV Present in the Proximal Region**—Previous studies have indicated that three elements located between nucleotides -112 to -94 (element II), -86 to -62 (element III) and -72 to -53 (element IV) (Fig. 6A) are essential and may act synergistically to promote transcription driven by the -268/+8 apoB promoter fragment (7). To test whether HNF-4 requires the synergistic interaction of transcription factors that interact with elements II, III, and IV to promote transcription, we have tested the effect of HNF-4 on the transcription of promoter constructs (carrying the BM2, BM6, LM5, and LM6 mutations (Fig. 6A)). As mentioned above, the BM2 mutation which was shown previously to diminish the binding of NF-BA1 factor, dramatically reduces the binding of HNF-4 to site BA1 (Fig. 1A). In addition, the LM5 and LM6 mutations which abolish the binding of hepatic nuclear factors

<sup>2</sup> J. A. A. Ladias, unpublished results.

**FIG. 4. DNA binding affinity measurements of transcription factors HNF-4, ARP-1, and EAR-2 with element BA1, present in the apoB promoter.** The dissociation constant values were obtained from binding reactions containing a constant amount of protein extract and increasing concentrations of radiolabeled BA1 probe (specific radioactivity  $0.5 \times 10^9$  cpm/ $\mu$ g). After gel electrophoresis and autoradiography the radioactive bands corresponding to the bound and free oligonucleotide were excised, and the radioactivity was measured by scintillation spectroscopy. The left part of panels A-C shows the autoradiographic images of 12 binding reactions performed with extracts containing ARP-1, EAR-2, and HNF-4. The middle part of panels A-C are saturation curves where the amount of radioactive probe bound was plotted versus the amount of the total probe present in the reactions. As the amount of the total probe increases the amount of the probe bound reaches a plateau indicating apparent saturation. The right part of panels A-C are Scatchard plots where the amount of bound probe was plotted versus bound/free.



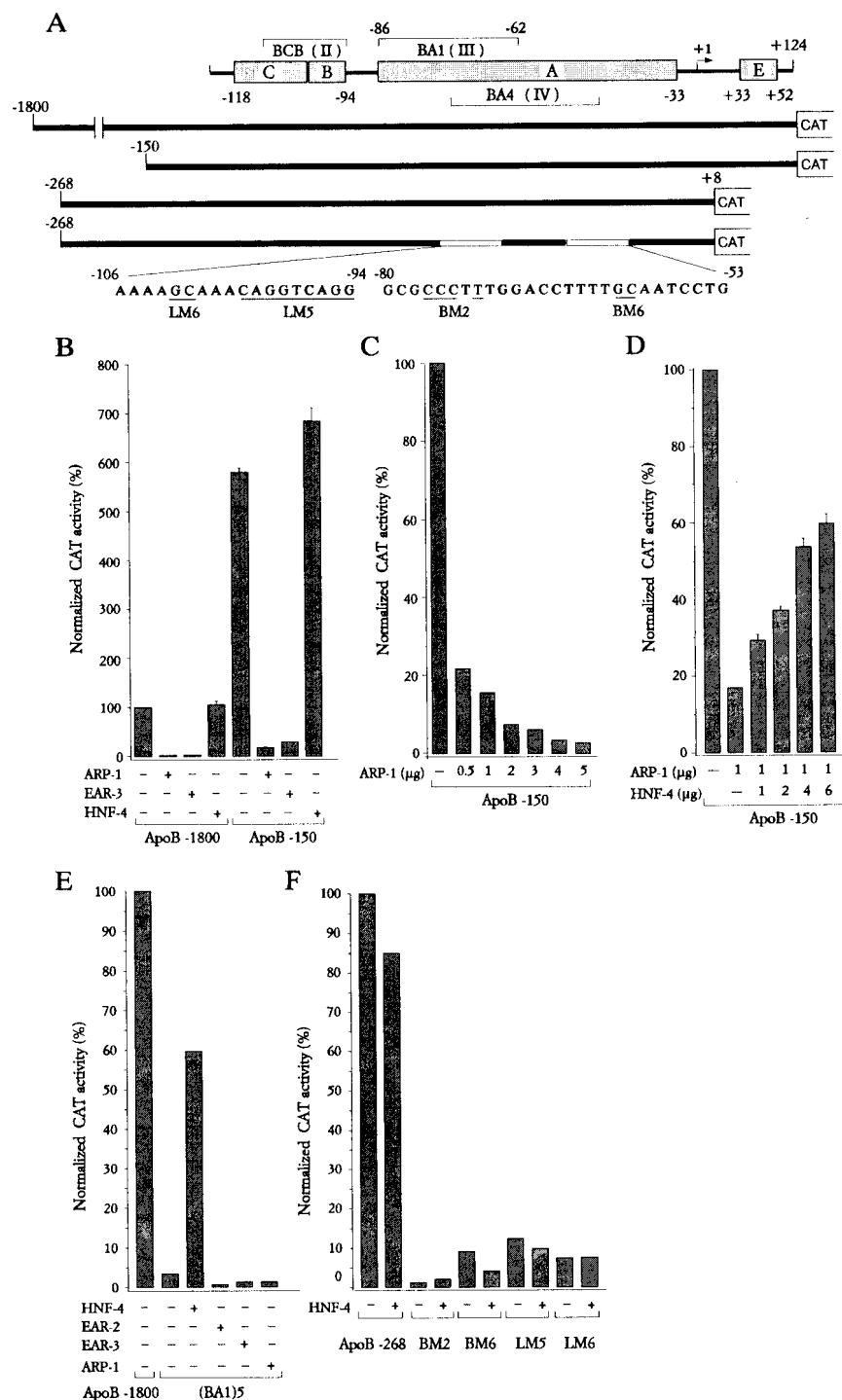
**FIG. 5. Schematic representation of the ARP-1, EAR-2, EAR-3, and HNF-4 proteins and sequence homology comparisons of the different domains to those of ARP-1.**



BCB1-3 that interact with element II reduce transcription to 10% of control (7). Mutation BM6 affects the binding of heat stable activities NF-BA2,3 and C/EBP and reduces transcription to 13% of control (7). Cotransfection experiments with HNF-4 showed that HNF-4 does not affect transcription of the mutant apoB promoters (Fig. 6F). These data indicated that the induction by HNF-4 can be the result of the synergistic interactions with other transcription factors that bind to elements II and IV. As seen in Fig. 6F, the BM2 mutation reduces the apoB transcription in HepG2 cells to 1.5% of control and cotransfection with HNF-4 had no effect on the

transcription driven by the promoter carrying the BM2 mutation. The results obtained with HNF-4, ARP-1, EAR-2, and EAR-3 along with existing evidence on the transcriptional regulation of the apoB gene, are summarized in Fig. 7. Activation of apoB gene transcription requires the synergistic interaction of factors HNF-4 and/or NF-BA1 that bind to element III with the factors BCB1-3, and NF-BA2,3 or C/EBP that bind to elements II and IV, respectively. Mutations that prevent factors BCB1-3, HNF-4, or NF-BA1 and NF-BA2,3 or C/EBP from interacting with their cognate elements lead to transcriptional repression (7). In addition, the apoB promoter is transcriptionally repressed by the binding of factors ARP-1, EAR-2, and EAR-3 to element III. Due to the close proximity of elements II and IV with element III, it is not known whether the binding of ARP-1, EAR-2 and EAR-3 to element III in addition to displacing positive activators such as HNF-4 or NF-BA1 may also affect the binding of neighboring factors to elements B and IV.

**Repression of the ApoCIII Gene Transcription by ARP-1, EAR-2, EAR-3, and Activation by HNF-4**—The effect of HNF-4, ARP-1, EAR-2, and EAR-3 on the transcriptional activity of the apoCIII promoter was monitored by cotransfection experiments in HepG2 cells. The regulatory elements



**FIG. 6. Effect of proteins HNF-4, ARP-1, EAR-2, and EAR-3 transiently transfected in HepG2 cells in the transcription of the apoB gene.**

Transient transfection experiments in HepG2 cells were performed with the apoB promoter constructs and plasmids pMHN4, pMARP1, pMEAR2, and pMEAR3 expressing their corresponding cDNAs. Forty-eight h after transfection the cell lysates were prepared and analyzed for chloramphenicol acetyltransferase activity. The bar graphs are mean values plus standard deviation of at least three independent transfections and show the relative transcription of the promoter fragments in the absence (-) and presence (+) of expression plasmids pMHN4, pMARP1, pMEAR2, and pMEAR3. *Panel A*, wild type and mutant apoB promoter constructs used in the cotransfection experiments. The position of the LM6, LM5, BM2, and BM6 mutations are indicated. LM6 and LM5 mutants are within element II (BCB). Mutation BM2 is within element III (BA1) and mutation BM6 is within the element IV (BA4). *Panel B*, repression of transcription by ARP-1 and EAR-3, and activation by HNF-4. *Panel C*, concentration-dependent repression of transcription by ARP-1. *Panel D*, partial reversal of repression of ARP-1 by HNF-4. *Panel E*, activation of a homopolymeric promoter containing five BA1 sites by HNF-4 and repression by EAR-2, EAR-3, and ARP-1. Normalized chloramphenicol acetyltransferase values are percent of the activity obtained with the -1800 apoB reporter construct. *Panel F*, promoter requirements for the activation of apoB gene by HNF-4 using mutated promoter constructs BM2, BM6, LM5, and LM6.

of the apoCIII promoter and the apoCIII reporter constructs used in the cotransfection experiments are presented in Fig. 8A. As seen in Fig. 8B, EAR-2, EAR-3, and ARP-1 decreased the transcription of the -904/+24 apoCIII promoter fragment to 8% of control. This dramatic decrease in transcription of the apoCIII gene by the expression of EAR-2, EAR-3, and ARP-1 is very similar to the decrease in transcription observed with the mutated apoCIII promoter constructs CIIIBM2 and CIIIBM5 lacking a functional CIIIB element (Fig. 8, A and F). In contrast, HNF-4 expressed in HepG2 cells increased the transcription of the -904 to +24 apoCIII promoter fragment by 8-fold (Fig. 8C), indicating that HNF-4 is a potent activator of apoCIII gene transcription. In addition, a homopolymeric promoter containing two CIIIB

sites was activated 5-fold by HNF-4 and repressed to 10–25% of control by ARP-1, EAR-2, and EAR-3 (Fig. 8D). These results indicated that activation by HNF-4 and repression by ARP-1, EAR-2, and EAR-3 of the apoCIII promoter is mediated by the binding of these factors to site CIIIB. To further test this hypothesis, we performed cotransfection experiments using a constant amount of ARP-1 and increasing amounts of transfected HNF-4 (Fig. 8E). As seen in Fig. 8E, 1 μg of ARP-1 repressed transcription of the -871 apoCIII promoter fragment to 8% of control. Increasing concentrations of HNF-4 counteracted the repression effect of ARP-1, and transcription was induced approximately by 70-fold. As mentioned above, since HNF-4 cannot heterodimerize with ARP-1, tran-



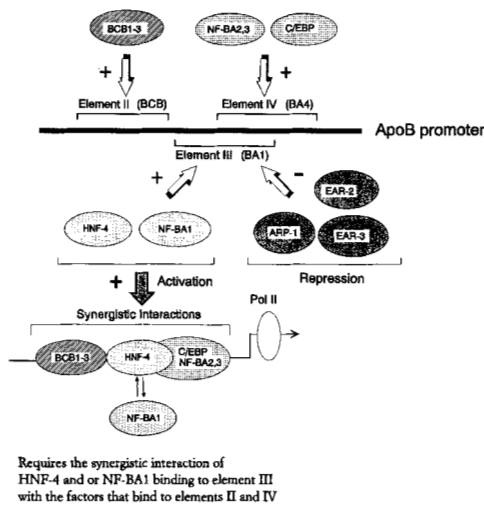


FIG. 7. Schematic representation of promoter elements and factors which participate in the transcriptional regulation of the human apoB gene. + indicates activation. - indicates repression.

scriptional increase is due to the interaction of HNF-4 with element CIIIB.

**Transcriptional Activation of the ApoCIII Promoter by HNF-4 Requires the Presence of Factors Binding to Distal Promoter Elements H, I, and J**—A series of 5' deletion mutants of the apoCIII promoter ligated to chloramphenicol acetyltransferase was used to identify sequences which were required for HNF-4 transactivation (Fig. 8A). Mutants with promoter end points -904 and -871 responded to HNF-4 with an 8–10-fold induction of chloramphenicol acetyltransferase expression (Fig. 8, C and F). Deletion to -755, which eliminates element J and part of element I, reduced the promoter activity to 50% of control (Fig. 8F). However, the truncated promoter could be activated to the same extent as the intact -904 to +24 promoter, indicating that proteins binding to element H may be important for the HNF-4-mediated activation of the apoCIII gene. Further deletions extending from nucleotide -686 to -214 (Fig. 8A) sharply decreased HNF-4-stimulated CAT expression (Fig. 8F). A 2-fold increase observed in the transcription of the truncated -686, -553, and -214 constructs in the presence of HNF-4 may indicate that high concentrations of HNF-4 can drive apoCIII gene transcription independently from elements H, I, and J. HNF-4 responsiveness was also studied by analyzing three promoter constructs with mutations in the CIIIB-binding domain (CIIIBM1, CIIIBM2, and CIIIBM5) (Fig. 8A). Mutation CIIIBM1 which abolished the binding of CIIIB1 factor (10), did not affect the binding of HNF-4 (Fig. 1B). The promoter construct carrying the CIIIBM1 mutation was activated by HNF-4 to the same extent as the normal promoter (Fig. 8F). However, promoter constructs carrying the mutations CIIIBM2 and CIIIBM5 which affected the binding of NF-BA1 and HNF-4 (Fig. 1B) resulted in modest activation of the mutant promoters by HNF-4 (Fig. 8F). This activation of transcription by HNF-4 may reflect the same synergistic interactions of HNF-4 with the factors that bind the distal regulatory elements H, I, and J. These findings suggest that transcriptional activation of the apoCIII gene by HNF-4 requires binding of HNF-4 to element CIIIB and the presence of factors which bind to the regulatory element H and possibly those binding in element I and J (Fig. 9). Negative regulation of the apoCIII gene can result from the binding of ARP-1, EAR-2, and EAR-3 to the CIIIB region or the absence of factors which recognize elements H, I, and J (Fig. 9).

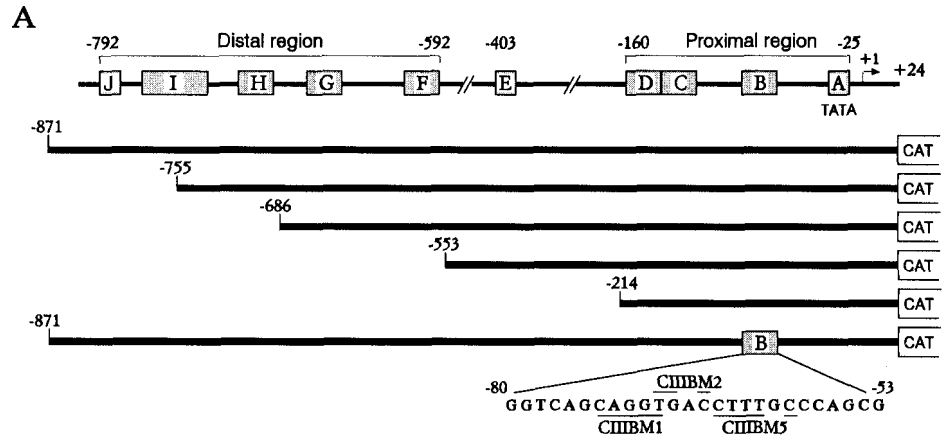
**Regulation of ApoAII Gene Transcription by HNF-4, ARP-1, EAR-2, and EAR-3**—Cotransfection experiments in HepG2 cells were also performed with the -911/+29 and the apoAIIΔJ reporter constructs and expression plasmids carrying the HNF-4, ARP-1, EAR-2, and EAR-3 cDNAs (Fig. 10A). The -911/+29 apoAII promoter chloramphenicol acetyltransferase construct was capable of directing tissue-specific expression in hepatic and intestinal cells. The apoAIIΔJ promoter construct contained the same promoter region with an internal deletion of the element J (-740 to -719) (Fig. 10A) which is the binding site for NF-BA1, HNF-4, ARP-1, EAR-2, and EAR-3 (Fig. 1C) (21). Transfection experiments in HepG2 cells showed that deletion of element J reduced transcription to 70% of control, indicating that transcription of the apoAII gene is not completely dependent on the element J. Cotransfection experiments with ARP-1, EAR-2, and EAR-3 decreased the transcription of the -911 apoAII promoter construct to 30–40% of control (Fig. 10B) whereas HNF-4 activated transcription to 210% of control (Fig. 10B). As seen in Fig. 10B, deletion of the J element reduced transcription of apoAII gene in HepG2 cells to 70% as compared with the wild type promoter. HNF-4, ARP-1, EAR-2, and EAR-3 reduced even more the expression driven from the AIIΔJ promoter. If HNF-4, ARP-1, EAR-2, and EAR-3 could mediate enhancement or repression of the apoAII promoter by binding to element J, then the transcription driven by the AIIΔJ promoter fragment should have remained unaffected by these factors. These results can be interpreted by the ability of HNF-4, ARP-1, EAR-2, and EAR-3 to interact with other elements in the apoAII promoter and such interactions may interfere with the normal transcription driven by this promoter fragment. As shown in Fig. 1D, binding of HNF-4 to element J is partially competed by the elements K and L, indicating that HNF-4 has a weak affinity for the elements K and L. It is possible therefore that the reduction in transcription of apoAIIΔJ promoter by HNF-4 may be due to this interaction.

## DISCUSSION

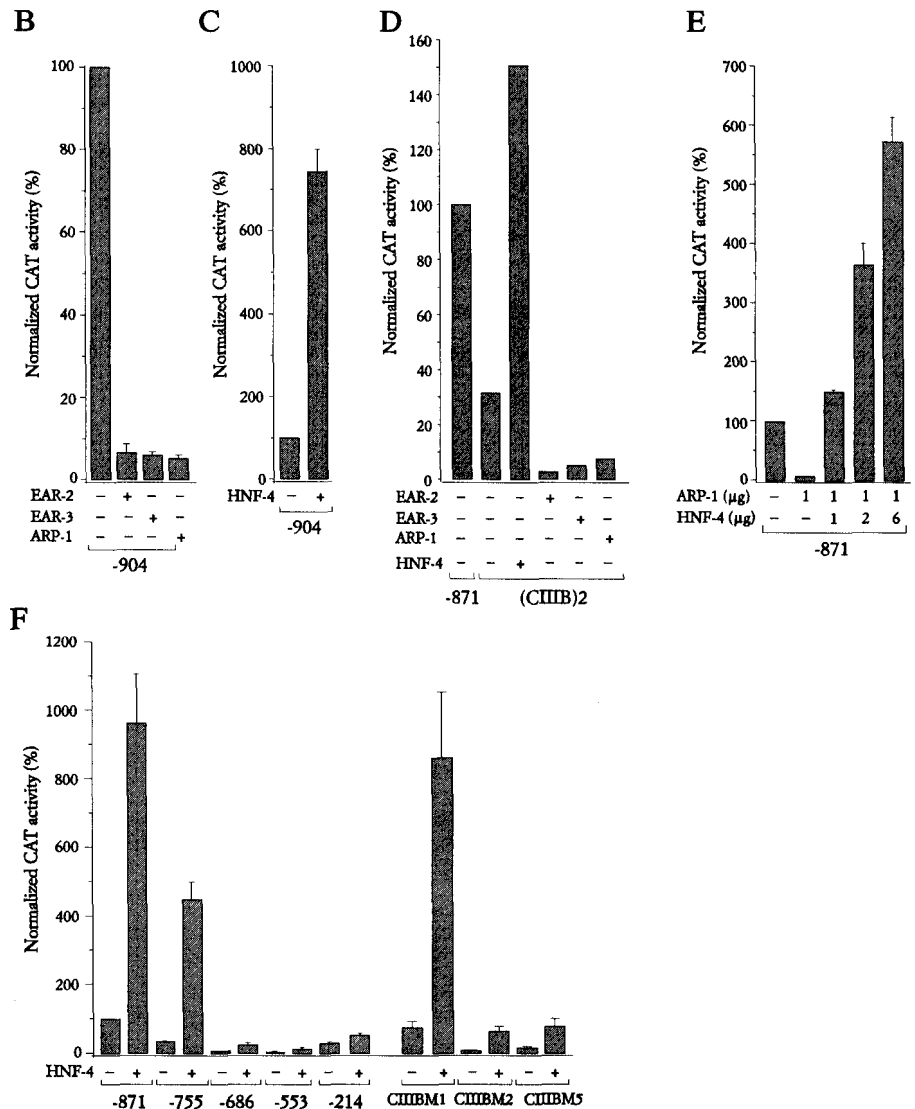
Transcription factors HNF-4, ARP-1, EAR-2, and EAR-3 belong to the superfamily of steroid-thyroid receptor proteins (22, 24, 25, 40). Members of this superfamily regulate gene transcription in response to hormonal and other ligand signals and play a crucial role in developmental and cell differentiation processes (41–43). HNF-4, ARP-1, EAR-2, and EAR-3 belong to the class of the "orphan" receptors for which no ligand has been identified as yet.

In the present study we have shown by DNA binding studies that the proteins HNF-4, ARP-1, EAR-2, and EAR-3 interact effectively with three elements BA1, CIIIB, and AIIJ present in the apoB, apoCIII, and apoAII promoters, respectively. Although elements BA1, CIIIB, and AIIJ have low sequence homology, they bind all four transcription factors with very similar affinities.

It was shown previously that ARP-1 and HNF-4 bind to their recognition sites as homodimers (22, 24). The DNA-binding domains of ARP-1, EAR-3, and EAR-2 are very similar (Fig. 5), and these proteins have identical sequences in the P-box (44–47) of the first zinc finger (EGCKS) (22, 25). In contrast, the DNA-binding domain of HNF-4 has limited similarity and contains a different P-box (DGCKG) than ARP-1, EAR-3, and EAR-2 proteins (24). Since the P-box determines the sequence specificity of DNA binding of the receptor, it is interesting that ARP-1, EAR-2, EAR-3, and HNF-4 all recognize the same regulatory elements in the apoB, apoCIII, apoAII, and apoAI genes. Table I shows align-

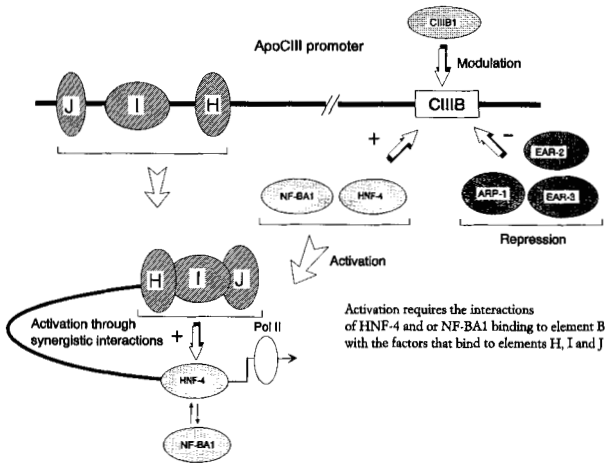


**FIG. 8. Effect of proteins HNF-4, ARP-1, EAR-2, and EAR-3 transiently transfected in HepG2 cells on the transcription of the apoCIII gene.** Transient transfection experiments in HepG2 cells were performed with the apoCIII promoter constructs and plasmids pMHNF4, pMARF1, pMEAR2, and pMEAR3 expressing their corresponding cDNAs. Forty-eight h after transfection cell lysates were prepared and analyzed for chloramphenicol acetyltransferase activity. The bar graphs are mean values plus standard deviation of at least three independent transfections and show the relative transcription of the promoter fragments in the absence (-) and presence (+) of expression plasmids pMHNF4, pMARF1, pMEAR2, and pMEAR3. *Panel A*, apoCIII promoter constructs used in the cotransfection experiments. Mutations CIIBM1, CIIBM2 and CIIBM5 are indicated. *Panel B*, repression of transcription by ARP-1, EAR-2, EAR-3. *Panel C*, activation of transcription by HNF-4. *Panel D*, activation of a homopolymeric promoter containing two CIIB sites by HNF-4 and repression by EAR-2, EAR-3, and ARP-1. *Panel E*, activation of a homopolymeric promoter containing five BA1 sites by HNF-4, and repression by EAR-2, EAR-3, and ARP-1. *Panel F*, reversal of ARP-1 repression by HNF-4. *Panel G*, promoter requirements for activation of the apoCIII gene by HNF-4.

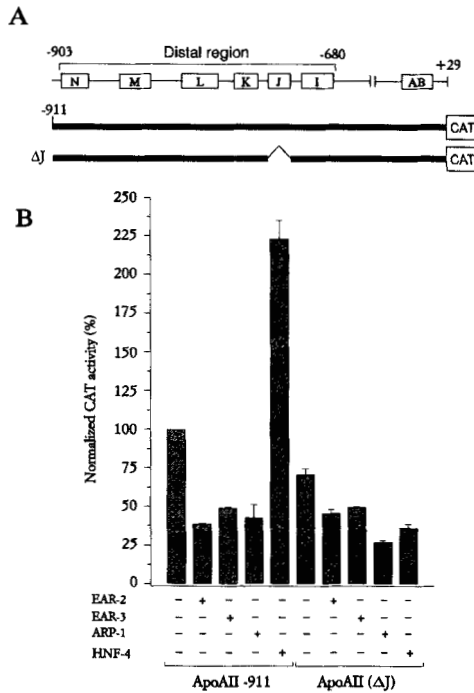


ment and comparisons of oligonucleotide sequences corresponding to regulatory elements of apoCIII, apoB, apoAII, and apoA1 promoters which can bind all four nuclear receptors. In addition, other elements corresponding to the apoA1, transthyretin, and  $\alpha$ 1-antitrypsin genes can discriminate between ARP-1 and HNF-4 (22, 24 and data presented in Table I). Therefore, it is reasonable to speculate that genes having regulatory elements which can interact with both the positive (HNF-4) and negative (ARP-1, EAR-2, and EAR-3) regulatory proteins will be susceptible to the antagonistic action of

these proteins, whereas elements that interact with one of these receptors will be regulated exclusively by that protein. Consistent with previous studies (22, 24) comparison of the elements of Table I which are recognized by the nuclear hormone receptors HNF-4, ARP-1, EAR-2, and EAR-3 generated a consensus binding domain for these factors consisting of two directly repeated motifs 5'-T(C)G(A)ACCC(T)TTG-ACCC(T)T(C)-3'. The present study showed that mutations CIIBM2, CIIBM5, BM2, and BM3 (Table I) within the consensus sequence abolish the binding of all factors. An



**FIG. 9. Schematic representation of promoter elements and factors which participate in the transcriptional regulation of the human apoCIII gene.** + indicates activation. - indicates repression.



**FIG. 10. Effect of proteins ARP-1, EAR-2, EAR-3, and HNF-4 transiently expressed in HepG2 cells on the transcription of the apoAII gene.** Panel A, apoAII promoter elements and constructs used in the cotransfection experiments. ΔJ is a promoter construct in which element J is deleted. Panel B, effect of proteins EAR-2, EAR-3, ARP-1, and HNF-4 transiently transfected in HepG2 cells on the transcription of apoAII gene. The transient transfection experiments in HepG2 cells were performed with the apoAII promoter constructs and plasmids pMHNF4, pMARP1, pMEAR2, and pMEAR3 expressing their corresponding cDNAs. Forty-eight h after transfection the cell lysates were prepared and analyzed for chloramphenicol acetyltransferase activity. The bar graphs are mean values plus standard deviation of at least three independent transfections and show the relative transcription of the promoter fragments in the absence (-) and presence (+) of expression plasmids pMHNF4, pMARP1, pMEAR2, and pMEAR3. The left side shows activation of apoAII gene by HNF-4 and partial repression by ARP-1, EAR-2, and EAR-3. The right side shows that when element J is deleted there is relative repression of transcription of the apoAII gene by ARP-1, EAR-2, EAR-3, and HNF-4.

oligonucleotide sequence JL-12 (Table I) containing two direct repeats of the motif TGACCCCT binds strongly HNF-4, ARP-1, EAR-2, and EAR-3. A spacer of three nucleotides between the repeats in oligonucleotide JLSP-12 (Table I) diminishes the binding of HNF-4 without affecting the binding of ARP-1, EAR-2, and EAR-3. It has been previously suggested that the steroid hormone-responsive elements have arisen from duplication of a progenitor sequence motif 5'-TGACC-3' (48). Mutation of this sequence and introduction of spacing between the two repeated motifs may have led to the generation of numerous regulatory elements that are recognized by the steroid receptors (48). The observed sequence variation among the elements presented in Table I and considerable deviation from a consensus may have arisen due to developmental, cell-specific, or other physiological constraints unique for the particular promoter. It is possible, for example, that the sequence divergence of these elements may have been evolved to accommodate interactions with additional transcription factors, thus creating an individualized spatial and temporal expression pattern for each gene. Furthermore a particular transcription regulatory protein may adopt distinct conformations as a result of binding to different sequences, and this in turn may influence the functional activity of this protein.

The function of HNF-4, ARP-1, EAR-2, and EAR-3 on apoB, apoCIII, and apoAII gene expression was studied by cotransfection experiments in HepG2 cells. It was demonstrated that HNF-4 is a positive activator for the apoB, apoCIII, and apoAII gene transcription. The apoCIII promoter was highly induced by HNF-4. Although transcription of the apoB promoter remained almost unaffected by the overexpression of HNF-4, three lines of evidence support that HNF-4 is involved in the transcriptional stimulation of both the apoB and apoCIII genes and this induction is mediated through elements BA1 and CIIB. The first is the ability of HNF-4 to counteract the repression effect of ARP-1; second HNF-4 cannot stimulate apoB and apoCIII gene transcription from promoter constructs with mutations in elements BA1 and CIIB that eliminate its binding; third, the homopolymeric promoters (BA1)<sub>5</sub> and (CIIB)<sub>2</sub> are induced 15- and 5-fold by HNF-4. In addition, transactivation of the apoB promoter by HNF-4 depends on the synergistic interactions between HNF-4 and other transcription factors which bind to two closely adjacent elements II and IV. This was demonstrated by the inability of HNF-4 to activate transcription in mutated promoter constructs which abolish the binding of factors BCB1-3, NF-BA2,3 and C/EBP in elements II and IV, respectively.

The high inducibility by HNF-4 in apoCIII gene expression is obtained in promoter constructs that have present elements H, I, and J, indicating that HNF-4 requires the synergistic interaction of the factors that bind to elements H, I, and J. This type of functional synergism between HNF-4 and the factors that bind to distal elements H, I, and J may occur by looping out the intervening DNA. It was speculated that such a process involves protein-protein interactions to bring the transcription factors in proximity with the basal initiation complex. Stabilization of this complex would then allow multiple initiation events (49).

Expression of HNF-4 in HepG2 cells increased by 2.5-fold the transcription of apoAII gene promoter and repressed to 50% the transcription of the apoAIIΔJ promoter construct lacking a high affinity binding site for HNF-4. This indicated that HNF-4 has a dual effect on the apoAII gene transcription, a repression and an induction effect. The repression effect observed in the apoAIIΔJ promoter construct has been attrib-

TABLE I

Alignment and comparisons of oligonucleotide sequences corresponding to regulatory elements of apoCIII, apoB, apoAII, apoAI, transthyretin, and  $\alpha$ 1-antitrypsin genes that are recognized by the nuclear hormone receptors HNF-4, ARP-1, EAR-2, and EAR-3.

ELEMENT	GENE	POSITION	REFERENCE	SEQUENCE	HNF-4	ARP-1	EAR-2	EAR-3
CIIB	ApoCIII (H)	-92/-67	10	5'-GGTCAGCAGG TGACCTT TGCCCAG CG-3'	+	+	+	+
BA1	ApoB (H)	-88/-62	6	5'-CCCGGGAGG CGCCCTT TGGACCT TTTG-3'	+	+	+	+
AIIJ	ApoAII (H)	-739/-715	12	5'-TGCCTT CAACCTT TACCCTG GTAGA-3'	+	+	+	+
CIIBM1	ApoCIII (H)	-92/-67	10	5'-GGTCAGCAGG TGACCTT TGCCCAG CG-3'	+	+	+	+
A (A1D)	ApoAI (H)	-214/-192	13, 15	5'-ACT GAACCTT TGACCCG TGCCCT-3'	+	+	+	+
C (A1B)	ApoAI (H)	-136/-114	13, 15	5'-GC TGATCCT TGAACCT TTAAGTT-3'	-/+*	+	ND	+
HNF4	Transthyretin (M)	-151/-130	58	5'-CTACACAAATA TGAACCT TGCC -3'	+	-	ND	ND
LF-A1	$\alpha$ 1-Antitrypsin (H)	-128/-99	59	5'-CCAGCCA GTGGACT TAGCCCG TGTTGCTC-3'	+	-	ND	ND
JL-12	Artificial			5'-C TGACCCCT TGACCCCT G-3'	+	+	+	+
JLSP-12	Artificial			5'-C TGACCCCT CGC TGACCCCT G-3'	-/+*	+	+	+
CONSENSUS								
					TGACCTT		TGACCTT	
					CA	T	TC	TC
CIIBM2	ApoCIII (H)	-92/-67	10	5'-GGTCAGCAGG ATATCTT TGCCCAG CG-3'	-	-	-	-
CIIBM5	ApoCIII (H)	-92/-67	10	5'-GGTCAGCAGG TGACGAC TGACCAG CG-3'	-	-	-	-
BM2	ApoB (H)	-88/-62	6	5'-CCCGGGAGG CGATATC TGGACCT TTTG-3'	-	-	-	-
BM3	ApoB (H)	-88/-62	6	5'-CCCGGGAGG CGCCCTT GATATCT TTTG-3'	-	-	-	-

\*Data not shown; ND, Not determined; H, human; M, mouse

uted to the ability of HNF-4 to interact, although weakly, with elements K and L. Such an interaction may interfere with the binding of other proteins that are needed for stimulating apoAII gene transcription. Alternatively, HNF-4 may inhibit transcription due to protein-protein interactions. Such a repression mechanism that is independent of DNA binding has been described for the glucocorticoid receptor and the transcription factor AP-1/Jun (50-52). Overexpression of c-Jun prevents the glucocorticoid-induced activation of genes (e.g. collagenase gene) carrying a functional glucocorticoid response element and *vice versa*. The element L of the apoAII promoter is very complex and multiple factors such as AP-1, CIIB1, NFY, NF1, and a family of proteins related to C/EBP can interact with highly overlapping boundaries (11, 38). It is possible that HNF-4 may bind to transcription factor AP-1 or another protein capable of interacting with element L. This interaction may prevent the factor(s) from binding element L and promote apoAII transcription.

The induction effect observed by HNF-4 in the apoAII gene transcription may involve synergistic interactions between HNF-4 and other transcription factors that interact with elements similar to the ones described in the apoCIII gene. For instance, element M (5'-CTCTCCCCCTCCCCACC-3') in the apoAII promoter is very homologous with the element H (5'-CTTTGCCCTCCCTCCACC-3') in the apoCIII promoter in reverse orientation and may bind the same or similar factors. As described above, element H has been involved in the transcriptional regulation of the apoCIII gene by HNF-4. It is possible that the induction observed in the apoCIII and apoAII reporter constructs by HNF-4 may involve similar protein-protein interactions.

In contrast to HNF-4, the factors ARP-1, EAR-2, and EAR-3 repressed the transcription of apoB, apoCIII, and apoAII genes. Repression was also observed in constructs (CIIB)<sub>2</sub> and (BA1)<sub>5</sub> containing two and five copies of the elements CIIB and BA1, respectively, suggesting that the repression effect may be mediated primarily through elements BA1 and CIIB.

Several mechanisms for negative control of gene transcription by steroid hormones have been described. In most cases the effect is mediated through a hormone response element binding its cognate receptor. Subsequently, negative regulation may occur by binding of the receptor to the same sequence

recognized by the positive regulators and therefore interfering or competing with the activating factors (53). Negative regulation may also result from different receptor-DNA interactions due to sequence divergence of the response element. In the case of pro-opiomelanocortin gene, glucocorticoid-dependent repression of transcription may be due to binding of the receptor abnormally to an atypical GRE response element thus occupying a positive regulatory element of the promoter (54). In addition, negative regulation may result from protein-protein interactions rather than a direct DNA binding event. For example, a down-regulation mechanism has been observed by progestins only in the presence of their receptor, but in the absence of any detectable progesterone receptor-binding site (55). Such a mechanism has also been described for the human prolactin gene (56) and for the glucocorticoid inhibition of AP1 activity (50-52). We propose that the first mechanism, namely binding of the receptor to the hormone-response element and competition with the activating factors accounts for the results obtained by ARP-1, EAR-2, and EAR-3 transcription factor.

The finding that ARP-1, EAR-2, EAR-3, and HNF-4 are all present in liver (22, 25, 57) raises the interesting possibility that in hepatic cells, containing all the positive and negative regulators, the net effect on the transcription of apoB, apoCIII, and apoAII genes would depend on the concentration and affinity of factors for their recognition sites, as well as protein-protein interactions with other factors binding to proximal or distal regulatory elements.

In addition, the finding that the nuclear hormone receptors HNF-4, ARP-1, EAR-2, and EAR-3 are involved in the regulation of apoB, apoCIII, apoAII, and apoAI genes *in vitro*, may suggest that these receptors could play an important role in lipid and cholesterol metabolism. An interesting possibility is that these receptors may bind intracellular molecules generated during cholesterol and lipid metabolism, thus sensing the metabolic state of the cell and bringing about the appropriate changes in gene expression. Identification of the ligands or other factors that regulate these receptors may reveal novel physiological signal transduction pathways that control apolipoprotein gene expression and contribute to cholesterol homeostasis. Finally, further characterization of the mode of

action of these receptors and the signals mediated by them may be of potential diagnostic and therapeutic value in monitoring common lipid disorders.

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