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Antagonism between Apolipoprotein AI Regulatory Protein 1, Ear3/COUP-TF, and Hepatocyte Nuclear Factor 4 Modulates Apolipoprotein CIII Gene Expression in Liver and Intestinal Cells

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Apolipoprotein CIII (apoCIII), a lipid-binding protein involved in the transport of triglycerides and cholesterol in the plasma, is synthesized primarily in the liver and the intestine. A *cis*-acting regulatory element, C3P, located at −90 to −66 upstream from the apoCIII gene transcriptional start site (+1), is necessary for maximal expression of the apoCIII gene in human hepatoma (HepG2) and intestinal carcinoma (Caco2) cells. This report shows that three members of the steroid receptor superfamily of transcription factors, hepatocyte nuclear factor 4 (HNF-4), apolipoprotein AI regulatory protein 1 (ARP-1), and Ear3/COUP-TF, act at the C3P site. HNF-4 activates apoCIII gene expression in HepG2 and Caco2 cells, while ARP-1 and Ear3/COUP-TF repress its expression in the same cells. HNF-4 activation is abolished by increasing amounts of ARP-1 or Ear3/COUP-TF, and repression by ARP-1 or Ear3/COUP-TF is alleviated by increasing amounts of HNF-4. HNF-4 and ARP-1 bind with similar affinities to the C3P site, suggesting that their opposing transcriptional effects may be mediated by direct competition for DNA binding. HNF-4 and ARP-1 mRNAs are present within the same cells in the liver and intestine, and protein extracts from hepatic tissue, HepG2, and Caco2 cells contain significantly more HNF-4 than ARP-1 or Ear3/COUP-TF binding activities. These findings suggest that the transcription of the apoCIII gene in vivo is dependent, at least in part, upon the intracellular balance of these positive and negative regulatory factors.

Apolipoprotein CIII (apoCIII) is a major protein constituent of the triglyceride-rich lipoproteins, very low density lipoproteins, and chylomicrons, and it appears to play an important role in their metabolism by inhibiting the hydrolysis of triglycerides by lipoprotein lipase (4, 12, 56) and inhibiting the removal of chylomicrons and triglyceride-rich lipoproteins by hepatocytes (44, 53, 59). ApoCIII plasma levels are often elevated in hypertriglyceridemic individuals (5, 45). Furthermore, overexpression of apoCIII in transgenic mice results in profound hypertriglyceridemia (21). However, the mechanism whereby apoCIII influences triglyceride metabolism has not been clearly defined.

The apoCIII gene is a member of a dispersed apolipoprotein gene family (31) and is tandemly linked to the apoAI and apoAIV genes in the human (22), rat (19), and chicken (23) genomes. ApoCIII is expressed predominantly in liver and intestine in mammalian and avian species (19, 30, 34), and its expression appears to be developmentally regulated (19).

The human apoCIII promoter contains several distinct regions which together modulate apoCIII gene transcription (38, 46). Two strong positive regulatory elements, from −859 to −686 and from −110 to −68 upstream from the transcription start site (+1), have been defined by deletion experiments, and both are necessary for high levels of apoCIII gene expression in cells of hepatic and intestinal origin (38, 46). A sequence motif within the proximal positive element, designated C3P (29), spanning from −86 to −74, has been shown to bind transactivating proteins present in liver nuclear extracts (24, 29, 39). Comparison of the 5′ flanking sequences in the apoCIII genes of human (38), rat (19), and chicken (34) origin reveals that the TATA box and the C3P site, as well as several upstream elements corresponding to regions of protection in DNase I experiments (38), show significant sequence conservation (34).

The importance of the C3P site for apoCIII gene expression has led to a search for relevant binding proteins. Recent work has suggested that four members of the steroid hormone receptor superfamily of transcription factors (for reviews, see references 3, 9, and 18) may play a role in apolipoprotein gene regulation. Three of the four have been shown to bind the C3P oligonucleotide (oligo C3P). Hepatocyte nuclear factor 4 (HNF-4), which is found in liver, kidney, and intestine but not many other tissues, binds to the C3P site and activates transcription in reporter constructs containing this site in HeLa cells (55). A second protein, apoAI regulatory protein 1 (ARP-1) (27), and a third closely related protein, Ear3/COUP-TF (36, 57), also bind to C3P as well as to a similar site, site A, in the apoAI promoter (27). Both ARP-1 and Ear3/COUP-TF, which are present in most tissues, decrease apoAI gene transcription in HepG2 cells (27, 34). The fourth protein, a recently described retinoic acid-responsive factor, RXRα (32, 47), binds to site A and

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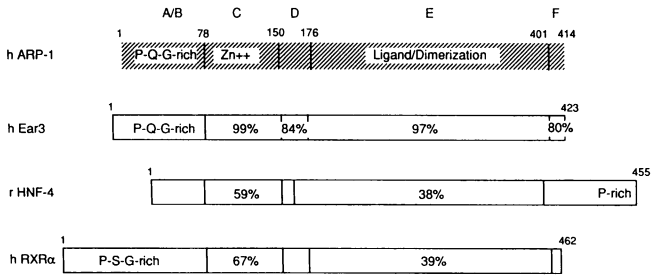

FIG. 1. Comparison of the amino acid sequences of HNF-4, ARP-1, Ear3/COUP-TF, and RXRα. Percentages indicate amino acid identity between ARP-1 and the other three factors; Zn++ signifies the zinc finger/DNA binding domain. P-Q-G-rich in hARP-1 denotes 27% proline (15 of 56 amino acids), 20% glutamic acid (13 of 64), and 28% glycine (14 of 51), with stretches of glycine and glutamic acid. P-Q-G-rich in hEar3 denotes 15% proline (11 of 73), 16% glutamic acid (12 of 73), and 27% glycine (20 of 73), with stretches of glutamic acid and glycine which do not align with those in hARP-1. Most prolines in that domain are conserved between hARP-1 and hEar3, the overall similarity being 54%. P-rich in HNF-4 denotes 26% proline (10 of 38). P-S-G-rich in hRXRα denotes 20% proline (19 of 95), 20% serine (19 of 95), and 13% glycine (12 of 95).

activates transcription from basal promoters (47) but does not appear to interact with the C3P site (34). A comparison of the general structural and functional organizations of HNF-4, ARP-1, Ear3/COUP-TF, and RXR-α transcription factors is shown in Fig. 1.

This report shows that HNF-4, ARP-1, and Ear3/COUP-TF compete for binding to the C3P site within the apoCIII promoter and that while HNF-4 stimulates transcription, both ARP-1 and Ear3/COUP-TF function as transcriptional repressors. Maximal apoCIII gene expression requires the presence of both the C3P site and an upstream element localized to −770 to −722. In situ hybridization revealed that HNF-4, ARP-1, and Ear3/COUP-TF are coexpressed within the same cells in liver and intestine, and gel retardation assays in the presence of HNF-4 and Ear3/COUP-TF antibodies showed that there is significantly more HNF-4 than ARP-1 or Ear3/COUP-TF in liver and intestine, suggesting that the transcriptional activity of the apoCIII gene is at least in part dependent upon the intracellular balance of HNF-4, ARP-1, and Ear3/COUP-TF.

MATERIALS AND METHODS

Plasmid constructions. All plasmids were constructed by using standard procedures as described elsewhere (50). The constructs were verified by restriction mapping and nucleotide sequencing. The −850/+22 apoCIII wild-type promoter fragment was prepared by the polymerase chain reaction (PCR) on a 2.2-kb *Bam*HI-*Eco*RI fragment which contains a portion of the apoCIII gene and its 5′ flanking sequences isolated from a previously described genomic clone, λApoAI-8 (22). The PCR oligonucleotide primers (−850 to −827 and +3 to +22) were designed with nested *Hin*dIII sites, and the resulting PCR fragment was inserted into the *Hin*dIII site of the previously described pUC9CAT vector (51). The −800-, −770-, −722-, and −686CIIIwtCAT upstream deletion mutants were prepared by PCR on the −850CIIIwtCAT template, using the same 3′ oligonucleotide primer designed for the −850 construct, with upstream oligonucleotide primers designed to progressively delete previously described footprint regions in the apoCIII gene promoter (38). These PCR primers (−800 to −780, −770 to −750, −722 to −702, and −686 to −666) were designed with flanking *Xba*I, *Bgl*II, and *Hin*dIII restriction sites, and the fragments were cloned into the *Hin*dIII site of the same pUC9CAT vector. A *Pst*I site located at −200 was used to construct the −200CIIIwtCAT upstream deletion mutant. The −850/+22 apoCIII xh C3P site mutant, designated −850CIIIxhCAT, was constructed as originally described by Leff et al. (29) by altering a *Bst*EII site within the C3P sequence motif so as to eliminate protein binding. Synthetic oligonucleotides spanning both the wild-type and the xh mutant C3P site were generated (see below), annealed, and then cloned in single or multiple copies into the *Bam*HI restriction site of the −41AICAT construct (51), which confers only a low level of basal transcription activity.

To overexpress HNF-4, ARP-1, and Ear3/COUP-TF for footprinting and transient transfection assays, *Eco*RI cDNA inserts for these three proteins were subcloned into the pMT2 eukaryotic expression vector (25). The cDNA clone for Ear3 was of hamster origin, but it shows sequence identity with the human Ear3 (36) and with the independently isolated COUP-TF (57) and is referred to as Ear3/COUP-TF. The resulting constructs were designated pMT2-HNF-4, pMT2-ARP-1, and pMT2-Ear3/COUP-TF, respectively. A 550-bp *Eco*RI fragment from the 3′ untranslated region of the human growth hormone was similarly subcloned into the pMT2 vector, and the resulting control construct was designated pMT2-UT.

Synthetic oligonucleotides. Complementary oligonucleotides spanning from −90 to −59 in the apoCIII wild-type gene (oligo C3P) and from −93 to −59 in the xh mutant (oligo xh) (29) were synthesized with the tetranucleotide 5′-GATC-3′ at their 5′ ends on a Biosearch model 8600 DNA synthesizer, deblocked at 55°C, and purified through a preparative polyacrylamide gel as described elsewhere (50). Sequence coordinates for the oligonucleotides used in this study concur with the revised apoCIII promoter sequence recently published (38).

Gel retardation assays. The ARP-1, Ear3/COUP-TF, and HNF-4 cDNA inserts were subcloned into the *Eco*RI site of pGEM4 (Promega), linearized with *Xba*I, and transcribed in vitro by SP6 RNA polymerase. The resulting RNA was translated in a rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine. For gel retardation analysis, proteins were incubated with ^{32}P-labeled oligo C3P (10 fmol) for 30 min on ice in the presence of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.25 μg of poly(dI-dC) and was electrophoresed in low-ionic-strength polyacrylamide gels as described previously (11). Two filters were placed against the dried gel. The first was used to block the ^{35}S radiation.

For determination of the dissociation constants (K_d) for HNF-4 and ARP-1, gel retardations were performed with increasing amounts of oligo C3P probe. After electrophoresis, the gels were fixed, dried, and exposed to X-ray film. Areas corresponding to complex and free probe were cut from the dried gel and counted by liquid scintillation. To correct for quenching, a control lane was included in each gel to measure background and to correct the specific activity of the probe. Data were analyzed by the method of Scatchard (52).

For the in vitro protein competition experiment, ARP-1 and HNF-4 cDNAs in Bluescript were transcribed with T3 RNA polymerase, and the resulting mRNAs were translated in vitro with rabbit reticulocyte lysate (Promega) in the presence of [^{3}H]leucine. The relative amounts of translated

protein in each reaction were determined by densitometric quantitation of an autoradiogram of a sodium dodecyl sulfate-polyacrylamide gel which had been soaked in En^{3}Hance (NEN Dupont). Gel shift reaction mixtures (15 μl) contained 2 μg of poly(dI-dC) and 25 ng of nonspecific oligonucleotide, and the reactions were performed as previously described (55). Since 1 μl of the HNF-4 translation reaction mixture contained approximately the same molar amount of translated protein as did 1.7 μl of the ARP-1 translation reaction mixture, a bovine serum albumin supplement in the reticulocyte lysate buffer (100 mg/ml in 10 mM Tris (pH 7.4)–113 mM potassium acetate–0.7 mM $MgCl_2$) was used to ensure that each shift reaction contained the same amount of protein.

Crude nuclear extracts from rat liver (55) and Caco2 (58) cells were prepared as previously described. Extracts from HepG2 cells were prepared essentially as were the liver extracts except that instead of being passed over sucrose gradients, the cells were swelled in a hypotonic solution and Dounce homogenized. After the osmolarity was restored, the nuclei were pelleted by centrifugation at 2,000 rpm for 6 min and extracted with 0.4 M KCl as described elsewhere (55). Gel retardation experiments with these extracts were performed with each 15-μl reaction mixture containing 1 to 2 μg of extract, 0.5 ng of probe, 2 μg of poly(dI-dC), and 25 ng of a nonspecific oligonucleotide (−175) (55).

Methylation interference. Methylation interference was performed by using in vitro-translated proteins, prepared as described above, without [^{35}S]methionine labeling. Both the coding and noncoding strands of oligo C3P were labeled at the 5′ end with [γ-^{32}P]ATP, annealed with a 0.1-fold molar excess of unlabeled complementary oligonucleotide, partially methylated with dimethyl sulfate as previously described (49), and used for preparative gel retardation. Protein-bound and free probe were recovered by electroelution onto a NA-45 DEAE membrane (Schleicher & Schuell), incubated in 10 mM sodium phosphate–1 mM EDTA (pH 8.0) at 90°C for 15 min, and then cleaved with 0.1 M NaOH at 90°C for 30 min. The samples were precipitated with ethanol and analyzed on a 15% polyacrylamide–8 M urea gel.

DNase I protection assays. DNase I protection assays were carried out by using as probe a DNA fragment spanning the −200 to +22 region of the apoCIII gene. The fragment was labeled at the 5′ end of the noncoding strand by digesting the −850/+22 pUC9CAT construct with *Hin*dIII, dephosphorylating with bacterial alkaline phosphatase (Bethesda Research Laboratories), and labeling with [γ-^{32}P]ATP and T4 polynucleotide kinase. The proximal 222-base fragment was released by digestion with *Pst*I and was gel purified.

Expression vectors pMT2-HNF-4 and pMT2-ARP-1 (see "Plasmid constructions") were transfected into COS-1 cells, and whole cell extracts were prepared by three cycles of freeze-thawing in buffer G as previously described (58). The resulting COS-1 protein extracts were quantified by the Bradford assay. COS-1 protein extract (10 to 20 μg) was combined with the −200/+22 probe under the DNase I experimental conditions previously described (58).

Cell culture and transfection assays. Human hepatoma (HepG2) and human colon carcinoma (Caco2) cells were maintained in Dulbecco modified Eagle medium (GIBCO), supplemented with 10% fetal calf serum (Sigma) and penicillin in a 5% CO_2 atmosphere. Cells were seeded at 2.0×10^6 cells per 100-mm dish. Plasmid DNA from the various constructs was transfected into cultured cells by the calcium phosphate coprecipitation method (17). To correct for variations in DNA uptake by the cells, 3 μg of plasmid pRSV-βgal (8) was cotransfected with each test construct. At 16 to 18 h after transfection, cell monolayers were treated with 15% glycerol; 48 h later, cells were harvested. Cell protein extracts were made by three freeze-thaw cycles. β-Galactosidase activity and chloramphenicol acetyltransferase (CAT) enzyme activity were assayed by methods previously described (8, 16). For each transfection experiment, CAT enzymatic activity was normalized to β-galactosidase activity.

Tissue in situ hybridization. The tissue in situ hybridization protocol was adapted from Cox et al. (6). Hepatic, intestinal and renal tissues were prefixed in 4% paraformaldehyde and then subjected to cryoprotection in 30% sucrose and cryostat sectioning. Serial sections 5 to 10 μm thick were collected onto poly-L-lysine-coated slides. After pretreatment and acetylation, the samples were incubated in 15 μl of hybridization solution containing 10^5 cpm of ^{35}S-labeled riboprobe, prepared by using HNF-4 or ARP-1 cloned into Bluescript as previously described (26). The HNF-4 probe contained the nucleotides encoding amino acids 187 to 352, which span the dimerization/ligand binding domain. The ARP-1 probe contained a 2.2-kb genomic fragment from the 3′ untranslated region which did not cross-react with Ear3/COUP-TF. The sections were placed on slides, covered, sealed, and stored in a humidified chamber for hybridization at 55°C for 4 to 16 h. Washing with 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C was followed by treatment with 5 μg of RNase A per ml in 2× SSC at 37°C. The slices were dehydrated, coated with Kodak NTB-2 emulsion (diluted 1:1 with H_2O), and exposed for 1 day to 3 weeks. After development, the slides were counterstained with either hematoxylin-eosin or Giemsa stain.

RESULTS

HNF-4, ARP-1, and Ear3/COUP-TF bind to the apoCIII C3P site. Protein-DNA interactions at the C3P site were evaluated by a gel retardation assay using a probe containing the C3P sequence (oligo C3P) and in vitro-translated HNF-4, ARP-1, and Ear3/COUP-TF proteins. As shown in Fig. 2A, each protein formed retardation complexes with the oligo C3P probe (lanes 1, 5, and 9). Formation of these complexes was competed for by oligo C3P (lanes 2, 6, and 10) but not by a mutated version of C3P (oligo xh; lanes 3, 7, and 11) or by an oligonucleotide with an unrelated sequence (NS; lanes 4, 8, and 12).

The purine contact points for these protein-DNA interactions were determined by methylation interference analysis. An identical pattern of purine contacts for ARP-1 and Ear3/COUP-TF was observed (Fig. 2B). The contact points for HNF-4 were identical to those of ARP-1 and Ear3/COUP-TF in the 5′ half of the C3P site (−82/−76), but they included two additional residues in the 3′ half of the C3P site (−75/−69). Binding of HNF-4 and ARP-1 to the C3P site was also evaluated by DNase I protection assays, using proteins expressed in COS-1 cells and the −200 to +22 region of the apoCIII gene promoter as probe. An identical pattern of protection was observed for both ARP-1 and HNF-4 between bases −89 and −64, encompassing the C3P site (Fig. 2C, lanes 3 and 5). This protection was competed for by the addition of unlabeled oligo C3P (lanes 4 and 6). A version of the apoCIII gene promoter which contains the xh mutation (29) at the C3P site and which does not support transcription was only marginally protected by ARP-1 and HNF-4 (lanes 8 and 9). Thus, HNF-4 and ARP-1 bind specifically to the C3P

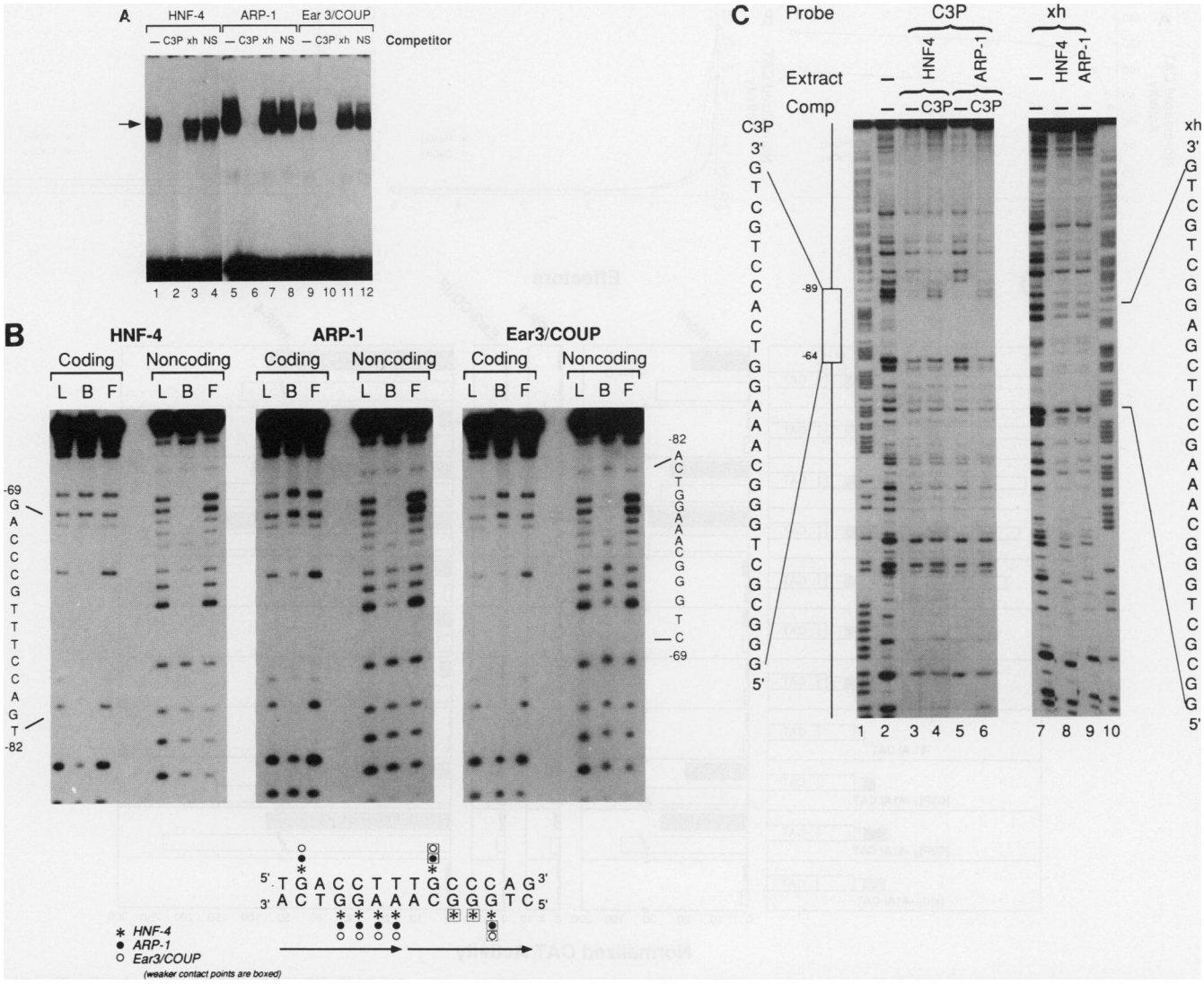

FIG. 2. Analysis of HNF-4, ARP-1, and Ear3/COUP-TF binding to the apoCIII promoter. (A) Gel retardation analysis of in vitro-translated HNF-4, ARP-1, and Ear3/COUP-TF proteins. ^{32}P-labeled oligo C3P was incubated with the proteins shown and with 50-fold molar excesses of competitor oligonucleotides: −, none; C3P, oligo C3P; xh, oligo xh; NS, nonspecific competitor (−178 to −148 from the human apoAI gene promoter [58]). Reticulocyte lysate lacking a template gave no complex (data not shown). (B) Methylation interference analysis of in vitro-translated HNF-4, ARP-1, and EAR-3/COUP-TF proteins with both strands of 5′-^{32}P-labeled oligo C3P probe. The DNA sequences of the coding and noncoding strands are given at the left and right, respectively. Lanes: L, ladder; B, protein-bound probe; F, free probe. The purine contact points are summarized at the bottom. (C) DNase I footprinting of the human apoCIII promoter. Whole cell extracts produced from COS cells transfected with HNF-4 and ARP-1 expression vectors were incubated with ^{32}P-labeled probe from the apoCIII promoter and then treated with DNase I. Probe were wild-type −200 to +22 (C3P; lanes 1 to 6) and xh mutation −203 to +22 (xh; lanes 7 to 10). Lanes 1 and 10 are Maxam-Gilbert G+A sequencing ladders; lanes 2 and 7 lack extract. The footprinted region (−89 to −64) and corresponding sequences are given at the left (C3P) and right (xh). Lanes 4 and 6 are reactions in the presence of C3P oligonucleotide competitor.

site of the apoCIII gene, and they protect a region identical to that protected by crude liver nuclear extracts (29, 38).

ApoCIII gene transcription is activated by HNF-4 but repressed by ARP-1 and Ear3/COUP-TF. The functional consequences of HNF-4, ARP-1, and Ear3/COUP-TF on apoCIII transcription were analyzed by transient transfection assays in cell lines of hepatic (HepG2) and intestinal (Caco2) origin. Increasing amounts of the pMT2-HNF-4, pMT2-ARP-1, or pMT2-Ear3/COUP-TF expression vector were cotransfected into HepG2 cells with a constant amount of a reporter construct containing nucleotides −850 to +22 of the apoCIII gene promoter placed in front of the CAT gene (−850CIIIwtCAT). As shown in Fig. 3A to C, 5 μg of pMT2-HNF-4 induced a 10-fold stimulation of CAT activity (Fig. 3A), while 2 μg of either ARP-1 (Fig. 3B) or Ear3/COUP-TF (Fig. 3C) completely repressed the activity from the apoCIII promoter. Similar results were also obtained using Caco2 cells (Fig. 3A to C).

To show that the effects of HNF-4, ARP-1, and Ear3/COUP-TF are mediated by the C3P site, several reporter constructs were made and assayed by transient transfection in HepG2 and Caco2 cells either alone or in the presence of

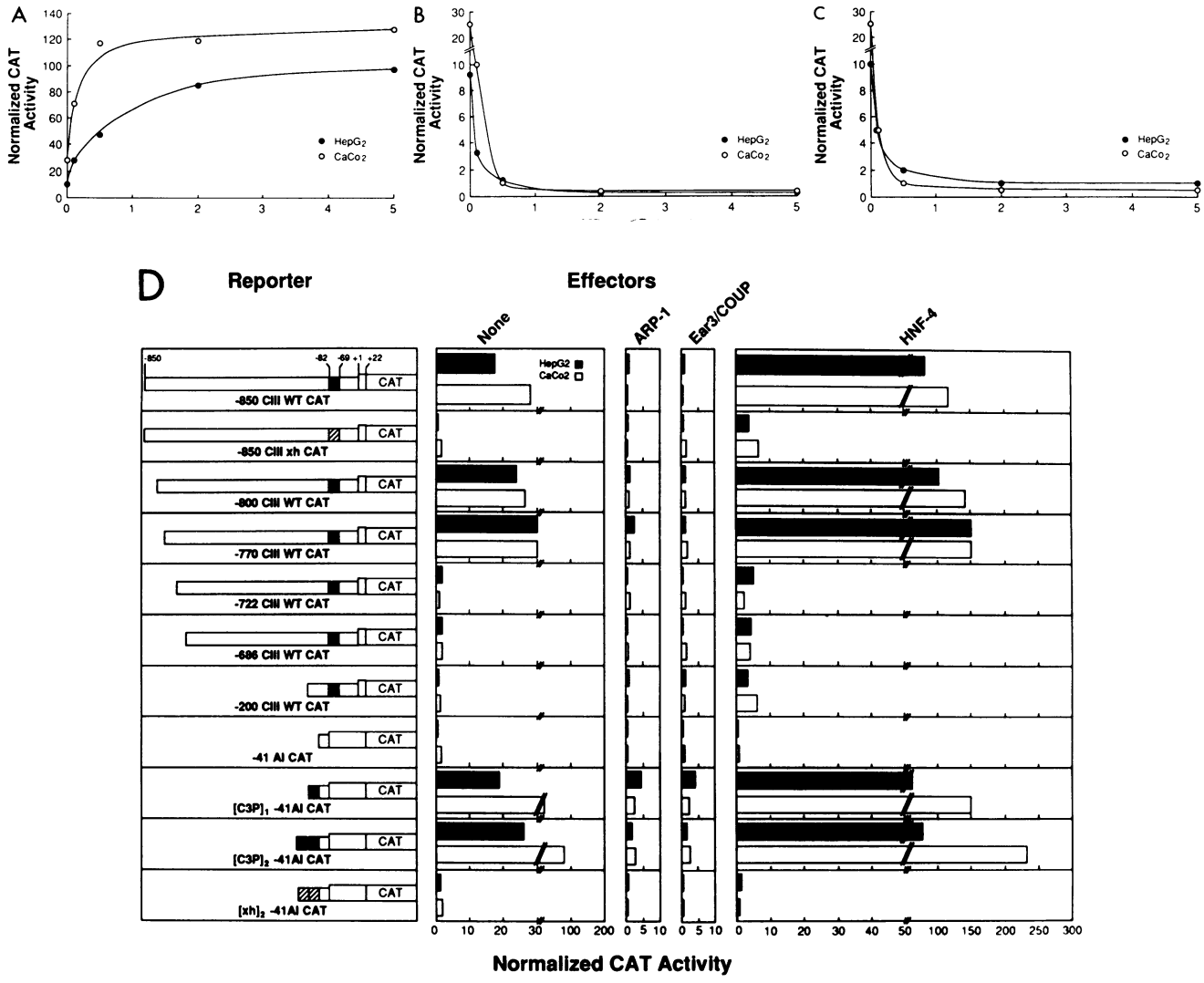

FIG. 3. Activation of the apoCIII promoter by HNF-4 and repression by ARP-1 and Ear3/COUP-TF in HepG2 and Caco2 cells. Transient cotransfection assays with the CAT reporter and transcription factor expression constructs indicated were performed at least three times in HepG2 cells and one to three times in Caco2 cells, and the results were averaged. CAT activity was normalized to the activity of a β-galactosidase internal standard. (A to C) The reporter −850CIIIwtCAT (10 μg) was transfected with increasing amounts of pMT2-HNF-4 (A), pMT2-ARP-1 (B), and pMT2-Ear3/COUP-TF (C). (D) A variety of reporter constructs (10 μg) containing different amounts of the apoCIII promoter, as diagrammed at the left, were transfected into HepG2 and Caco2 with the minimum amount of a given expression vector needed to give a maximal effect, based on the results in panels A to C (2 μg of each except 0.5 μg of HNF-4 in Caco2 cells).

one of the three expression vectors, pMT2-HNF-4, pMT2-ARP-1, or pMT2-Ear3/COUP-TF (Fig. 3D). When a construct containing the xh mutation (construct −850CIIIxh-CAT) was used, the basal expression was dramatically reduced in both cell types and the effects of HNF-4, ARP-1, or Ear3/COUP-TF were almost completely eliminated. Deletion of nucleotide −850 to −770 did not affect transcriptional activity, while deletion of −770 to −722 abolished basal activity and resulted in greatly diminished transactivation by HNF-4. Further deletions to −686 or to −200 did not restore transcriptional activity. These results are in agreement with previous findings that both C3P and upstream regulatory sequences are necessary for maximal apoCIII gene expression (29, 46) and show that the C3P site, in the context of the apoCIII promoter, is necessary but not sufficient for transactivation by HNF-4 and repression by ARP-1 or Ear3/COUP-TF.

When a single copy of oligo C3P was inserted in the sense orientation in front of a basal promoter containing the TATA box of the apoAI gene ([C3P]$_1$-41AICAT), expression was raised above basal levels in both HepG2 and Caco2 cells, presumably because of endogenous HNF-4 present in both cell types (see Fig. 7). Cotransfection with pMT2-ARP-1 or pMT2-Ear3/COUP-TF repressed the activity of this construct in both cell types, while pMT2-HNF-4 augmented expression even further. Virtually identical results were obtained for a construct containing a double C3P site ([C3P]$_2$-41AICAT), while a construct with two copies of the mutated site ([xh]$_2$-41AICAT) showed no transcriptional activity under any condition. The orientation of the C3P site did not affect the activity (data not shown). These results showed that a single C3P site placed in front of a minimal promoter can activate transcription. This is in contrast to the inactivity of the −200-, −686-, and −722CIIIwtCAT con-

structs and raises the possibility that transcription factors bound to the C3P site contact the basal transcriptional machinery in different ways, depending on the promoter context in which the C3P site is located (see Discussion).

ARP-1 and Ear3/COUP-TF oppose HNF-4 transactivation of apoCIII gene expression. Since the transcriptional activities of HNF-4, ARP-1, and Ear3/COUP-TF depend on the C3P site, and since each of these proteins binds to this site, it seemed possible that these proteins, when present in the same cell, might compete with each other for binding. Therefore, transient cotransfection assays were performed with use of the −850CIIIwtCAT reporter construct and various combinations of HNF-4, ARP-1, and Ear3/COUP-TF expression vectors. The results show that repression of the −850CIIIwtCAT construct's expression in HepG2 cells by pMT2-ARP-1 or pMT2-Ear3/COUP-TF was completely overcome with increasing amounts of pMT2-HNF-4 (Fig. 4A and B). Conversely, transactivation by pMT2-HNF-4 was repressed by increasing amounts of pMT2-ARP-1 or pMT2-Ear3/COUP-TF. Virtually identical results were obtained when the cotransfections were done in Caco2 cells (Fig. 4C) and when the $[C3P]_2$-41AICAT construct was used as the reporter (data not shown). These results indicate that ARP-1 and Ear3/COUP-TF antagonize transactivation of the apoCIII gene by HNF-4.

HNF-4 and ARP-1 bind with similar affinities to the C3P site. Since HNF-4 and ARP-1 compete for transcriptional control of the apoCIII promoter, the relative affinities of each protein for the C3P site were examined. This was done first by Scatchard analysis using whole cell extracts from COS-1 cells transfected with either pMT2-HNF-4 or pMT2-ARP-1. The results show that HNF-4 and ARP-1 bind to the C3P site with similar affinities: the K_d values were 14.1 nM for HNF-4 and 13.4 nM for ARP-1 (Fig. 5A). Similar results were obtained with use of in vitro-synthesized HNF-4 and ARP-1 (data not shown).

Competition for binding to the C3P site was then analyzed directly by mixing in vitro-synthesized HNF-4 and ARP-1 protein in various ratios and analyzing their binding to oligo C3P. The different electrophoretic mobilities of HNF-4 and ARP-1 DNA complexes facilitated their discrimination and identification. The results (Fig. 5B) indicate that the HNF-4 complex is converted to an ARP-1 complex by increasing amounts of ARP-1 and, conversely, that the ARP-1 complex is converted to an HNF-4 complex by increasing amounts of HNF-4. Equivalent binding of the two proteins to the probe occurs at a molar ratio of approximately 1 HNF-4/2 ARP-1 (Fig. 5B, lanes 5 and 9). These results are consistent with the Scatchard analysis (Fig. 5A) and, together with the observation that no complexes with mobilities other than those corresponding to either protein alone were evident at any HNF-4/ARP-1 ratio, strongly suggest that these proteins bind to the C3P site in a mutually exclusive manner.

HNF-4 and ARP-1 are coexpressed in liver and intestinal cells. Although the transient transfection assays and the in vitro binding studies indicate that HNF-4 and ARP-1 have opposing effects on apoCIII gene transcription, coexistence of these proteins in the same cell type must be shown in order for these observations to have physiological relevance. Therefore, in situ hybridizations were performed to localize each mRNA in the tissues which express the apoCIII gene, liver and intestine, and in a control tissue, kidney, which contains very little apoCIII mRNA. The results (Fig. 6) show that HNF-4 and ARP-1 mRNAs are present uniformly throughout the hepatocyte population (Fig. 6A and B). Both mRNAs are also present in the epithelial cells lining the villi

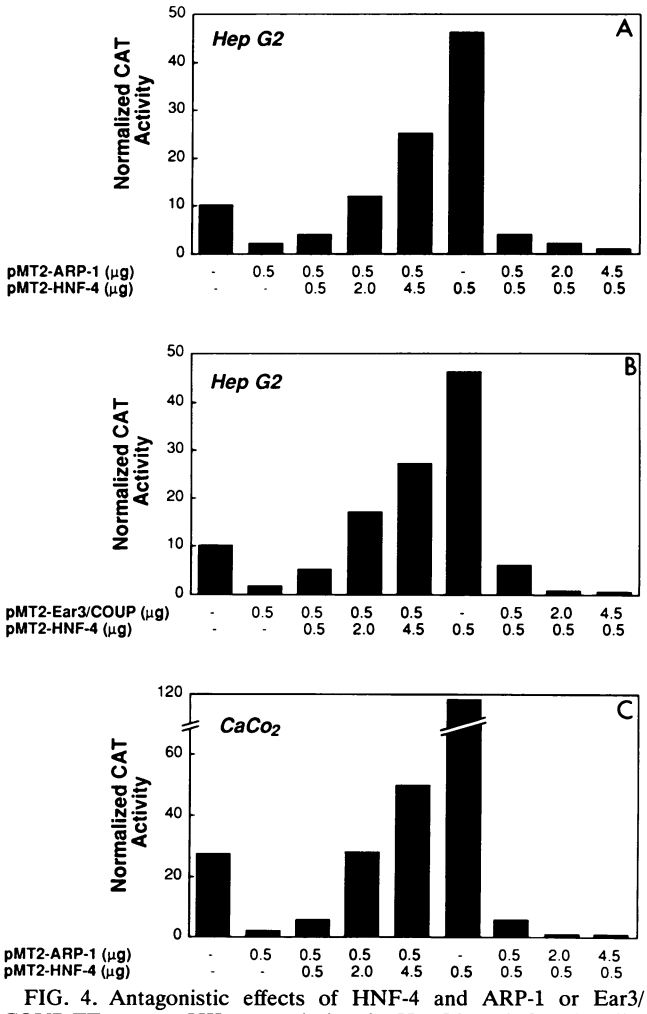

FIG. 4. Antagonistic effects of HNF-4 and ARP-1 or Ear3/COUP-TF on apoCIII transcription in HepG2 and Caco2 cells. Normalized CAT activity (averaged over three independent experiments) is shown for the −850CIIIwtCAT reporter alone (leftmost column) and in the presence of various combinations of expression vectors containing each of the three transcription factors as indicated. For each combination, one factor is held constant while the other is increased from zero, and vice versa. The results of combining Ear3/COUP-TF and HNF-4 in Caco2 cells were virtually identical to the results shown for ARP-1 and HNF-4 (data not shown).

and in the crypts of the intestine (Fig. 6C and D). In situ hybridization in the kidney, on the other hand, gave a very different result. HNF-4 mRNA was confined to the proximal tubules in the cortex (Fig. 6E), while ARP-1 was present in the cortex as well as the medulla (Fig. 6F).

To evaluate the relative amounts of active protein in expressing tissues, nuclear extracts were made from liver and from HepG2 and Caco2 cells and were used in gel retardation assays with the C3P probe. Antisera to HNF-4 and a polyclonal antibody raised against COUP-TF which recognizes both Ear3/COUP-TF and ARP-1, but not HNF-4 (data not shown), were added to the shift reactions to distinguish the factors. The results (Fig. 7) indicate that in liver, HepG2, and Caco2 cells, there is considerably more HNF-4 binding activity than there is binding of ARP-1, Ear3/COUP-TF, or other proteins which might be recognized by the COUP-TF antisera (compare lanes 7, 12, and 17

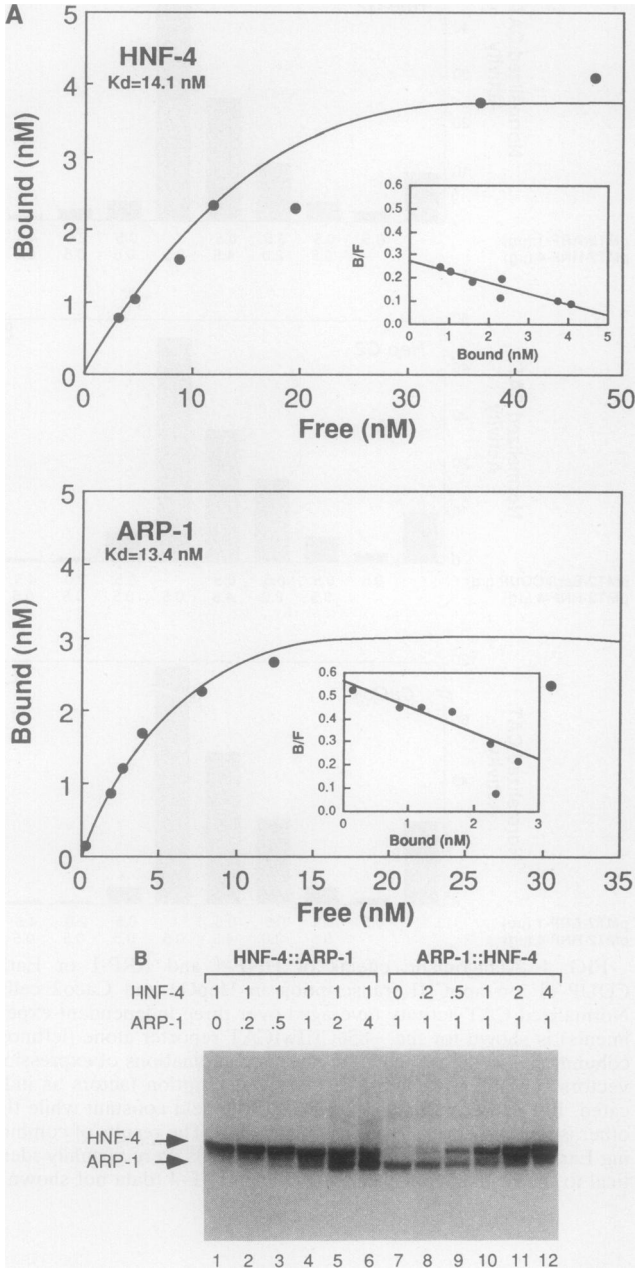

FIG. 5. Comparison of HNF-4 and ARP-1 affinity for binding to C3P. (A) Saturation curves showing binding of HNF-4 and ARP-1 to the apoCIII C3P site as a function of DNA concentration. Gel retardation assays were performed with increasing amounts of radiolabeled site C3P probe with fixed amounts of whole cell extracts from COS-1 cells transfected with pMT2-HNF-4 or pMT2-ARP-1. Binding was quantitated as described in the text. Inserts show Scatchard analyses of the same data. The slope of the best-fit line is equal to $-1/K_d$, K_d representing the dissociation constant of each protein on the C3P oligo. A representative experiment is shown; the experiments were carried out three times with similar results. The K_d values reported represent averages of the three experiments. (B) Protein competition. In vitro-translated HNF-4 and ARP-1 proteins were mixed to yield the molar ratios shown. The mixtures were then used in the gel retardation assay with the C3P probe as described in the text. Arrows indicate positions of the HNF-4 and ARP-1 shifts.

[with HNF-4 antiserum] with lanes 8, 13, and 18 [with COUP-TF antiserum]). It is also noteworthy that HNF-4, ARP-1, and COUP-TF appear to constitute the major species binding to the C3P site in these tissues and cell types. Only a small amount of unaltered shift activity is left when both antisera are used (lanes 9, 14, and 19). Finally, the Caco2 extracts appeared to have significantly (at least two-fold) more HNF-4 DNA binding activity than did the HepG2 extracts, which could explain the higher basal activity of the apoCIII promoter in Caco2 cells (Fig. 3).

DISCUSSION

ApoCIII, the most abundant of the C apolipoproteins and a major protein constituent of very low density lipoproteins and chylomicrons, seems to play an important role not only in triglyceride metabolism and cholesterol homeostasis but also in the initiation and progression of atherosclerosis via its putative role in generating hypertriglyceridemia (2). In this report, HNF-4, ARP-1, and Ear3/COUP-TF, three members of the steroid receptor class of transcription factors, have been shown to bind to the apoCIII promoter at the C3P site, a site which is essential for gene expression in HepG2 and Caco2 cells. HNF-4 was a powerful transcriptional activator, while ARP-1 strongly repressed apoCIII gene expression under the experimental conditions used in our study. Ear3/COUP-TF also functioned as a strong repressor, although it was initially described as a transcriptional activator (57). The observation that HNF-4 and ARP-1 mRNAs are present within the same cells in liver and intestine together with the observation that protein extracts from hepatic tissue, HepG2, and Caco2 cells contain more HNF-4 than ARP-1 or Ear3/COUP-TF binding activities suggests that the transcription of the apoCIII gene is at least in part dependent upon the intracellular balance of these positive and negative regulatory factors.

HNF-4, ARP-1, and Ear3/COUP-TF regulate apoCIII expression by competition for binding to the C3P site. The observation that HNF-4 and ARP-1 bind to C3P with similar affinities and in a mutually exclusive manner suggests that their mechanism of action involves direct competition at the level of DNA binding. Preliminary experiments with an Ear3/COUP-TF mutant in which the DNA binding domain is replaced by the progesterone receptor DNA binding domain (43) demonstrate that in the absence of binding to the C3P site, Ear3/COUP-TF cannot repress apoCIII gene expression and cannot antagonize transactivation by HNF-4 (34). Furthermore, in cells lacking HNF-4 (CV-1), the ARP-1 expression vector has no effect on a reporter construct containing an ARP-1/HNF-4 binding site proximal to the thymidine kinase basal promoter (34), suggesting that ARP-1 is not actively involved in inhibition of basal transcription. It therefore appears that the ability of ARP-1 to repress HNF-4-dependent transactivation is due to a displacement of HNF-4 by ARP-1 from their common binding site. Competition for DNA binding has been described for other members of the steroid receptor superfamily which, because of their highly related zinc finger DNA binding domains, can interact with overlapping or identical DNA elements. For example, estrogen and thyroid hormone receptors exert opposite regulatory effects via their competitive binding to the estrogen response element (13). Similarly, the glucocorticoid receptor has been shown to function as a transcriptional inhibitor of at least three independent genes via competition with positive factors for overlapping binding sites (1, 42, 48), and various retinoic acid receptors compete

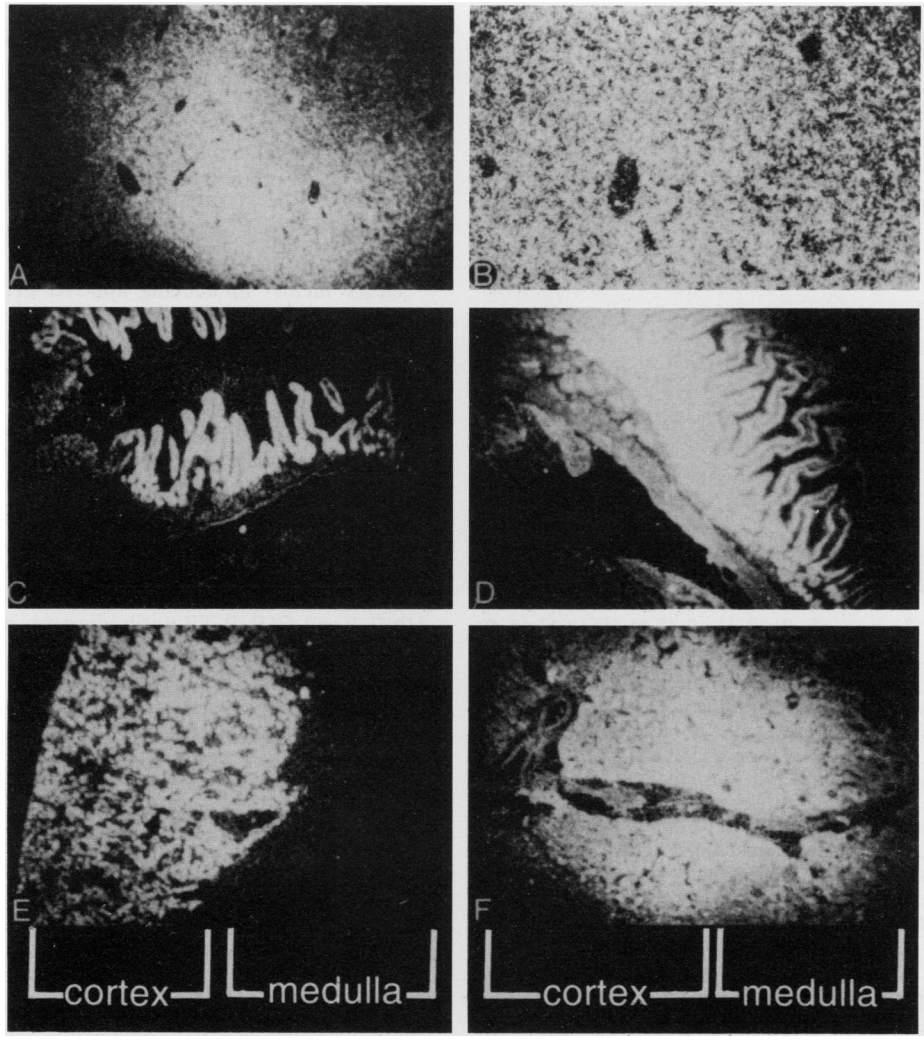

FIG. 6. In situ hybridization of HNF-4 and ARP-1 antisense riboprobe to mouse embryo sections. The dark-field micrographs in panels A, C, and E have been hybridized to HNF-4, and those in panels B, D, and F have been hybridized to ARP-1. (A and B) Sections from liver, showing homogeneous distribution of the signal across liver acini. (C and D) Sections from intestine, taken from the proximal duodenum. Signal is localized to the epithelial lining of the intestinal villi. (E and F) Sections from kidney. HNF-4 is present only in the cortex, while ARP-1 is distributed diffusely across both the cortex and medulla. Magnifications: A and C to F, ×40; B, ×100. (Probes in the sense orientation gave no specific signals.)

for binding to their common response element (7, 20, 33). These observations, together with our findings, suggest that antagonistic interactions between members of the steroid receptor superfamily of transcription factors may be a general mechanism to modulate the expression of relevant target genes.

A potential model for apoCIII gene regulation is that in most cell types, ARP-1 and Ear3/COUP-TF occupy the C3P site but do not significantly affect the basal transcriptional machinery. However, in hepatocytes and enterocytes, in which HNF-4 is present in relatively abundant amounts, ARP-1 and Ear3/COUP-TF are replaced by HNF-4 and apoCIII transcription is increased by some as yet undetermined mechanism. Although this antagonism between ARP-1, Ear3/COUP-TF, and HNF-4 may play a fundamental role in apoCIII gene regulation in cells of hepatic and intestinal origin, it should be noted that in contrast to the C3P site, not all sites that bind HNF-4 can also bind ARP-1 and Ear3/COUP-TF (27, 34, 54, 55). This may constitute an important example of combinatorial mechanisms for gene regulation. Thus, sites like C3P, such as sites in the apoAI and apoB gene promoters where all three of these transcriptional regulators bind (data not shown), may be susceptible to this antagonism, whereas sites which bind HNF-4 but not ARP-1 or Ear3/COUP-TF may not be susceptible. Finally, it seems reasonable that HNF-4 may activate apoCIII gene expression through the proline-rich region at its carboxy-terminal end, since similar sites are important for other transcriptional activators (Fig. 1) (35). However, ARP-1 and Ear3/COUP-TF also have motifs associated with activation, rich in proline, glutamine, and glycine, albeit at their N termini. Therefore, it remains possible that any or all of these proteins function differently in different promoter and cellular contexts.

The −770 to −722 region of the apoCIII gene works in synergy with the C3P site. The necessity of both upstream and downstream regulatory elements for maximal apoCIII gene expression suggests additional levels of interaction

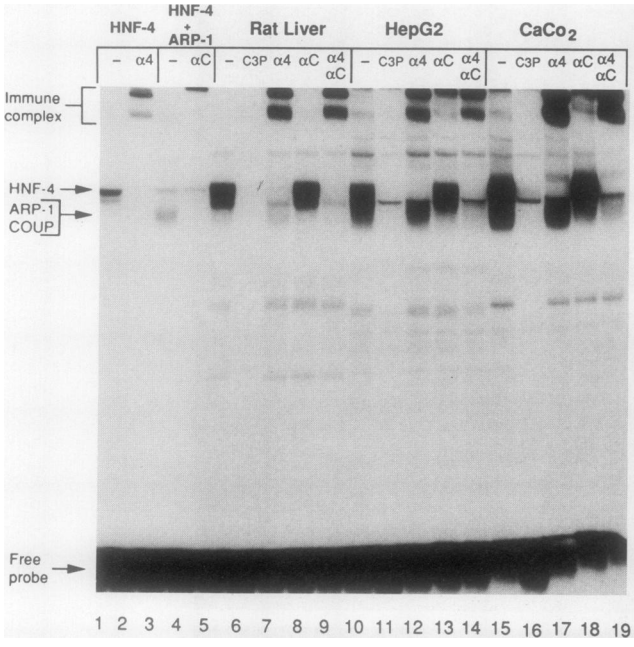

FIG. 7. Comparison of HNF-4, ARP-1, and Ear3/COUP-TF binding activities in liver, HepG2, and Caco2 cells by gel retardation analysis. Crude nuclear extracts from rat liver (1 μg), HepG2 (2 μg), or Caco2 (2 μg) cells were incubated with ^{32}P-labeled oligo C3P (0.5 ng). Additives were as follows: −, none; C3P, 25 ng of unlabeled oligo C3P; α4, antisera to a synthetic C-terminal peptide of HNF-4 (55); αC, polyclonal antisera raised against COUP-TF (57); α4αC, both antisera. The antisera (1 μl of a 1:10 dilution in 3% bovine serum albumin in a saline solution) was added halfway through the reaction at room temperature. HNF-4 and ARP-1 are in vitro-translated proteins. Lanes 3 and 4 contain HNF-4 and ARP-1 proteins mixed together (in unequal amounts).

between binding proteins on the apoCIII promoter. First, there appears to be a restraining effect in the native promoter which prevents the −722CIIIwtCAT, −686CIIIwtCAT, and −200CIIIwtCAT constructs from responding to transactivation by HNF-4 (Fig. 3D). This contrasts with the finding that a single downstream regulatory element (C3P site) in the context of a minimal promoter (the apoAI gene TATA box) is fully responsive to transactivation by HNF-4. While this discrepancy could be explained by different distances between the C3P sites and the TATA boxes in the two reporter constructs, it could also be explained if a negative regulatory region that has been mapped to −200 to −110 (46) is somehow involved in restraining the activity of HNF-4. Second, this transcriptional restraint is overcome once an upstream element (between −770 and −722) is present (−770CIIIwtCAT, −800CIIIwtCAT, and −850CIIIwtCAT constructs), raising the possibility that a protein(s) bound to this upstream site cooperates with HNF-4 bound to C3P to activate apoCIII transcription. While the −766 to −726 region has been shown to be protected from DNase I digestion by crude nuclear extracts from liver (38), the factor(s) acting at this region may not be limited to hepatocytes. Indeed, preliminary gel shift experiments indicate that nuclear extracts from various nonhepatic cells contain a factor other than HNF-4 which binds to this upstream element (34). Furthermore, the −850CIIIwtCAT construct is transactivated by HNF-4 in nonhepatic cells (HeLa and CV-1), and the upstream element is required for this transactivation (34).

The mapping of an upstream positive regulatory element to sequences between −770 and −722 is interesting in light of recent findings which suggest that this element is an integral part of a common enhancer for all three closely linked apolipoprotein genes, apoAI, apoCIII, and apoAIV (28, 37). The dependence of distinct but physically linked genes on a common enhancer is analogous to the shared enhancer element for the α-fetoprotein and albumin genes (15) and the locus control region in the β-globin gene cluster (41).

Other factors influencing apoCIII gene expression via the C3P site. While it seems likely that interplay between HNF-4, ARP-1, and Ear3/COUP-TF at the C3P site plays a major role in apoCIII gene expression, other factors will undoubtably also influence physiologic levels. For example, other transactivating proteins which bind to the C3P site have been described: AF-1 (29) and CIIIB1 and CIIIB2 (24, 38, 39). The relationship, if any, between HNF-4, ARP-1, and Ear3/COUP-TF and these other C3P site binding factors remains to be determined. However, since the electrophoretic mobility of most of the retardation complexes formed with C3P probe and nuclear extracts is altered when both HNF-4 and COUP-TF antibodies are present, it is possible that at least some of the additional C3P site binding factors are related to HNF-4, ARP-1, or Ear3/COUP-TF.

Additionally, it is possible that HNF-4, ARP-1, and Ear3/COUP-TF, like other members of the steroid receptor class of transcription factors, participate in protein-protein interactions leading to heterodimerization (10, 14). Although the current data indicate that ARP-1 and HNF-4 do not heterodimerize with each other, other unpublished observations indicate that ARP-1 and Ear3/COUP-TF do. This is not surprising, considering the sequence similarity between these two proteins (Fig. 1). The possibility that any of these three factors will heterodimerize with other family members remains to be investigated.

Furthermore, although HNF-4, ARP-1, and Ear3/COUP-TF are members of a class of transcription factors that includes ligand-dependent factors, they are all three considered orphan receptors because as yet, no relevant ligands have been identified (reviewed in reference 9). It has been proposed that orphan receptors may respond to ligands indigenous to their cells of function via an intracrine system linking extracellular physiologic events to gene expression (reviewed in reference 40). Finally, other signals may indirectly affect the action of these transcription factors. For example, Ear3/COUP-TF has been implicated in mediating the effects of dopamine via a phosphorylation event triggered by dopamine stimulation of cell surface receptors (43).

Conclusion. In this study, we show that three recently cloned transcription factors, HNF-4, ARP-1, and Ear3/COUP-TF, compete for binding to the apoCIII gene promoter C3P site. While HNF-4 cooperates with an upstream enhancer element of the apoCIII promoter to increase apoCIII gene expression, ARP-1 and Ear3/COUP-TF antagonize this transactivation. Since all three proteins are members of the steroid receptor superfamily of transcription factors, they may respond to as yet unidentified ligands. Identification of such ligands or other signaling events that alter the activities of these transcription factors may provide important insights into the mechanisms linking physiologic signals to triglyceride metabolism and cholesterol homeostasis.

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