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Hepatocyte Nuclear Factor-4α Regulates the Human Apolipoprotein AV Gene: Identification of a Novel Response Element and Involvement in the Control by Peroxisome Proliferator-Activated Receptor-γ Coactivator-1α, AMP-Activated Protein Kinase, and Mitogen-Activated Protein Kinase Pathway

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The recently discovered apolipoprotein AV (apoAV) gene has been reported to be a key player in modulating plasma triglyceride levels. Here we identify the hepatocyte nuclear factor-4α (HNF-4α) as a novel regulator of human apoAV gene. Inhibition of HNF-4α expression by small interfering RNA resulted in down-regulation of apoAV. Deletion, mutagenesis, and binding assays revealed that HNF-4α directly regulates human apoAV promoter through DR1 [a direct repeat separated by one nucleotide (nt)], and via a novel element for HNF-4α consisting of an inverted repeat separated by 8 nt (IR8). In addition, we show that the coactivator peroxisome proliferator-activated receptor-γ coactivator-1α was capable of stimulating the HNF-4α-dependent transactivation of apoAV promoter. Furthermore, analyses in human hepatic cells demonstrated that AMP-activated protein kinase (AMPK) and the MAPK signaling pathway regulate human apoAV expression and suggested that this regulation may be mediated, at least in part, by changes in HNF-4α. Intriguingly, EMSAs and mice with a liver-specific disruption of the HNF-4α gene revealed a species-distinct regulation of apoAV by HNF-4α, which resembles that of a subset of HNF-4α target genes. Taken together, our data provide new insights into the binding properties and the modulation of HNF-4α and underscore the role of HNF-4α in regulating triglyceride metabolism. (*Molecular Endocrinology* 19: 3107–3125, 2005)

HYPERTRIGLYCERIDEMIA IS AN independent risk factor for coronary heart disease, which remains one of the leading causes of morbidity and mortality in the Western world (1). Recently, the gene of a new member of the apolipoprotein family, named apolipoprotein AV (apoAV), was identified approximately 30 kb downstream from the apoAI/CIII/AIV gene cluster, and it was found to be an important determinant of plasma triglyceride (TG) levels (2–4). In mice, serum TG concentrations were decreased to one third when a human apoAV transgene was overexpressed (2) or adenoviral vectors expressing mouse apoAV were injected (4). Conversely, plasma TG levels were 4-fold elevated in knockout mice lacking apoAV compared with their wild-type littermates (2). Studies in mice that either overexpressed or completely lacked both apolipoproteins suggested that apoAV and apoCIII independently influence plasma TG concentrations but in an opposing manner (5). In humans, single-nucleotide polymorphisms (SNPs) across the locus of apoAV gene were found to be significantly associated with elevated plasma TG and very low-density lipoprotein (VLDL) particles mass levels in several ethnic groups (2, 6, 7). apoAV is a very hydrophobic protein that circulates in plasma at much lower concentrations than other apolipoproteins and appears to reduce plasma TG by inhibiting VLDL-TG production and stimulating lipoprotein lipase (LPL)-mediated VLDL-TG hydrolysis (2, 3, 8).

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Abbreviations: AMPK, AMP-activated protein kinase; AICAR, 5-amino-4-imidazolecarboxamide ribonucleoside; apo, apolipoprotein; apoAV, apolipoprotein AV; BA, bile acid; CoA, coenzyme A; CPT-Iα, carnitine palmitoyltransferase Iα; CYP7A1, cholesterol 7α-hydroxylase; DR, direct repeat; FXR, farnesoid X-activated receptor; G6Pase, glucose-6-phosphatase; HNF-4α, hepatocyte nuclear factor-4α; IR, inverted repeat; LDLr, low-density lipoprotein receptor; LPL, lipoprotein lipase; mitHMGS, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase; MODY1, maturity onset diabetes of the young 1; MTP, microsomal triglyceride transfer protein; nt, nucleotides; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPARα, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; SDS, sodium dodecyl sulfate; SHP, small heterodimer partner; siRNA, small interfering RNA; SNP, single nucleotide polymorphism; TG, triglycerides; VLDL, very low-density lipoprotein.

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Because the amount of apoAV is an important determinant of plasma lipid levels, understanding the factors that control apoAV gene expression is of major importance and may provide new opportunities for therapeutic intervention in atherogenic dyslipidemia. So far, it has been shown that the apoAV gene is regulated by peroxisome proliferator-activated receptor-α (PPARα; NR1C1), farnesoid X-activated receptor (FXR; NR1H4) and sterol regulatory element-binding protein 1c, which are transcription factors implicated in TG metabolism (9–11). In addition, apoAV was identified as a positive acute-phase protein in mouse (12). However, although the apoAV gene expression is restricted to the liver and is essential for lipid homeostasis, its potential regulation by hepatocyte nuclear factor-4α (HNF-4α) has not been reported.

HNF-4α (NR2A1) is a highly conserved member of the nuclear receptor superfamily that was initially identified as a transcriptional factor required for liver-specific gene expression (13), although it is also expressed in kidney, intestine, and pancreas (14). HNF-4α has critical roles in hepatocyte differentiation during liver development and in regulating the transcription of genes involved in glucose and lipid metabolism including the apoAI/CIII/AIV gene cluster, apoAII, apoB, apoCII, microsomal triglyceride transfer protein (MTP), liver-fatty acid binding protein, cholesterol 7α-hydroxylase (CYP7A1), glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), carnitine palmitoyltransferase Iα (CPT-Iα), pregnane X receptor (PXR; NR1I2), and PPARα (15, 16). Furthermore, naturally occurring mutations in a single allele of the HNF-4α gene cause an inherited form of diabetes known as maturity onset diabetes of the young 1 (MODY1). This syndrome is characterized by impaired glucose-induced insulin secretion, underscoring the importance of HNF-4α in pancreas (17). Homodimers of HNF-4α bind to specific DNA sequence elements consisting of a direct repeat of the hexanucleotide core motif PuGGTCA separated by 0, 1, or 2 nt (DR0, DR1, or DR2) to activate transcription in the absence of exogenously added ligands (18–22). Some reports have provided convincing evidence that coenzyme A (CoA) derivatives of certain fatty acids could activate HNF-4α (21, 22). Nevertheless, structural models and cofactor recruitment studies have shown that these derivatives are not traditional ligands and suggested that HNF-4α is constitutively bound to endogenous fatty acids that are essential for the structure of the protein (23, 24). Indeed, recent investigations indicate that coactivator rather than ligand binding locks the active conformation of HNF-4α (25). Thus, the interplay with coactivators is a major concern for the understanding of HNF-4α-dependent regulation of genes.

PPAR-γ coactivator-1α (PGC-1α) is a versatile coactivator for numerous nuclear receptors and is implicated in diverse biological activities including glucose and lipid metabolism. Recently, PGC-1α has been shown to enhance the HNF-4α-mediated transactivation of PEPCK, G6Pase, L-CPT I, *CYP7A1*, and FXR genes (26–30).

Regulation of HNF-4α-dependent transactivation has also been achieved by modulation of HNF-4α gene expression. We have learned from Leff and coworkers (31) that the apoCIII gene can be regulated by the MAPK signaling pathway and that this regulation is mediated by modulation of HNF-4α expression. These findings indicate that hormonal and metabolic signals can control HNF-4α target genes by changes in the amount of the receptor.

Because there are numerous potential phosphorylation sites in HNF-4α, another possibility for the regulation of its activity is posttranslational modification. AMP-activated protein kinase (AMPK) is a central sensor of a cellular signaling system that regulates multiple metabolic enzymes in response to reduced intracellular energy levels. It has been reported that AMPK directly phosphorylates HNF-4α and represses its activity by reducing its ability to form homodimers and bind DNA, and by increasing its degradation rate (32, 33). Consequently, activation of AMPK catalytic activity by 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) down-regulates the expression of most, although not all, HNF-4α target genes (32).

The current studies were undertaken to provide a comprehensive analysis of the potential regulation of human apoAV gene expression by HNF-4α. We presently demonstrate that human apoAV is a *bona fide* HNF-4α target gene. We show that inhibition of HNF-4α expression results in down-regulation of human apoAV and describe that HNF-4α directly activates the human apoAV gene promoter through a DR1 and a novel HNF-4α response element. Furthermore, the coactivator PGC-1α is capable of stimulating this HNF-4α-dependent transactivation of human apoAV promoter. In addition, our data demonstrate that AMPK and the MAPK cascade regulate human apoAV expression and suggest that this regulation may be mediated, at least in part, by changes in HNF-4α function. Furthermore, we presently report that the regulation of apoAV by HNF-4α is somehow species specific, a phenomenon also present in a subset of HNF-4α target genes. Finally, we discuss the implications that the identification of apoAV as an HNF-4α target gene may have in the regulation of TG levels by bile acids (BAs).

RESULTS

HNF-4α Enhances the Activity of the Human ApoAV Gene Promoter

ApoAV is an important determinant of plasma TG levels that appears to be expressed exclusively in the liver (2, 3, 10, 11). Inasmuch as the nuclear receptor HNF-4α is essential for maintenance of hepatic gene expression and is a crucial regulator of genes involved in lipid metabolism (16), including particularly the

apoAI/CIII/AIV gene cluster (34, 35), we evaluated whether this nuclear receptor modulates human apoAV gene expression. To study the direct effect of HNF-4α on transcription, transient transfection studies were performed in Hep3B cells, which express moderate levels of apoAV (10) and HNF-4α (36). Cotransfection of a human HNF-4α expression plasmid resulted in a significant increase of the activity of the firefly luciferase reporter gene driven by the −617/+18 sequence of the human apoAV promoter (Fig. 1A). The effect of HNF-4α overexpression was promoter dependent because it was not observed with the promoter-less pGL3-basic vector. To further confirm the induction by HNF-4α, monkey kidney CV-1 cells, a cellular model frequently used to avoid the background of endogenous HNF-4α (19, 37, 38), were also employed. As shown in Fig. 1B, increasing amounts of the expression plasmid for HNF-4α resulted in a dose-dependent induction of firefly luciferase activity. Activation by exogenously supplied HNF-4α was already appreciable at low doses (Fig. 1B).

Mapping of the Human ApoAV Promoter Regions Conferring Responsiveness to HNF-4α

To localize the promoter region required for HNF-4α activation, a series of progressively larger 5′-deletion human apoAV promoter constructs from nt −2455 to +18 in front of the firefly luciferase reporter gene were transiently transfected into CV-1 cells together with a human HNF-4α expression plasmid. As shown in Fig. 2A, the sequence upstream to position −437 could be removed without preventing strong activation of the reporter gene by HNF-4α. However, deletion of the fragment spanning −437 to −242 reduced HNF-4α responsiveness. Moreover, further deletion up to nt −82 almost abolished the induction of apoAV promoter activity by HNF-4α, indicating that at least two regions mediate the response.

On the other hand, a T→C SNP at nt −600 relative to the transcription start site (−1131 nt upstream from the predicted translation start codon) of the human apoAV gene, called SNP3 in Ref. 2, has been associated with elevated plasma TG levels (2, 6, 7). Analysis of the sequence surrounding this SNP3 revealed no apparent transcription factor binding sites, but assuming a high degree of degeneration one might consider the occurrence of two potential hexamer binding sites separated by a single nt between nt −611 and −599, especially with a C at −600 (GGGGCA A ATCTCA), thereby conforming to the DR1 response element for HNF-4α. Luciferase reporter assays performed in Hep3B (data not shown) and CV-1 cells (Fig. 2A) revealed that change in common allele T to C had no significant effect on the induction by HNF-4α. Consistent with this observation, the deletion of a fragment containing this SNP, nt −617 to −535, did not affect the response to HNF-4α (Fig. 2A).

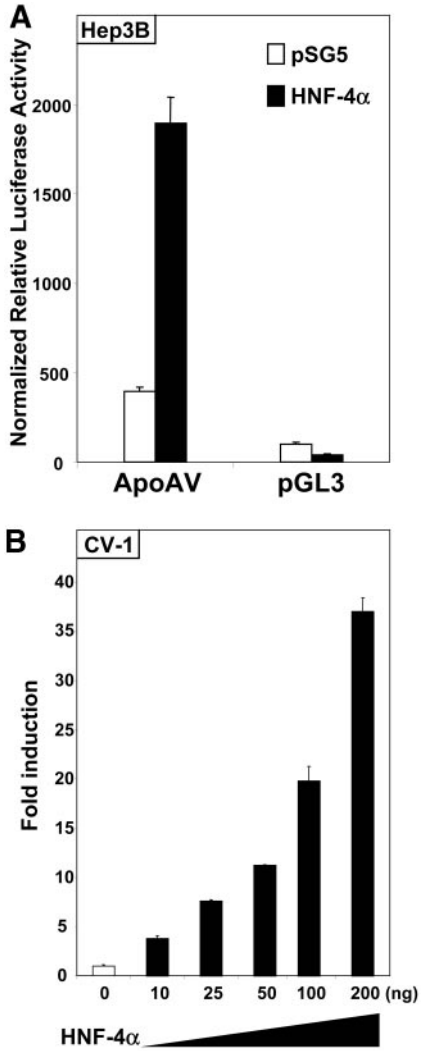

Fig. 1. HNF-4α Induces the Activity of the Human apoAV Gene Promoter

A, Hep3B cells were transiently transfected with a plasmid containing a luciferase reporter gene driven by the 5′ flanking region (−617/+18) of the human apoAV gene or the empty pGL3-basic vector along with a plasmid expressing human HNF-4α (200 ng) or the empty expression vector pSG5 as control. B, The reporter plasmid containing the −617/+18 region of human apoAV cloned in front of the luciferase gene was cotransfected with increasing amounts of an HNF-4α expression plasmid into CV-1 cells. Plasmid dosage was kept constant by addition of empty expression vector. Luciferase activities were measured and expressed as described in *Materials and Methods*.

Identification of a DR1 Site and a Novel Element for Transcriptional Activation by HNF-4α in the Human ApoAV Promoter

Analysis of the sequence in the −437/−242 fragment revealed two hexamer binding sites separated by a single nt between nt −271 and −259 (Fig. 2B), which correspond to a canonical DR1 response element for HNF-4α. In contrast, analysis of the sequence in the

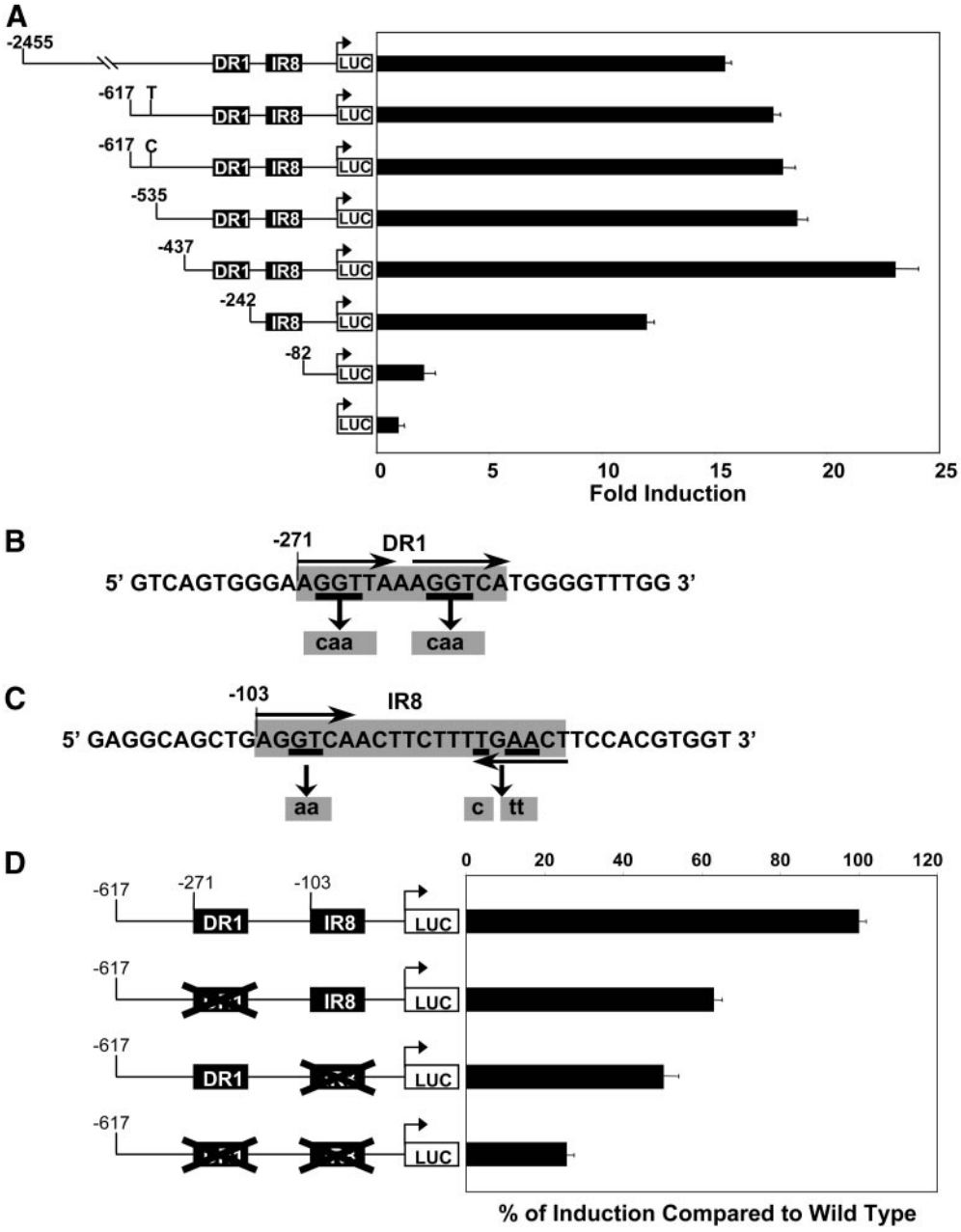

Fig. 2. Identification of the Human ApoAV Promoter Elements Conferring Its Responsiveness to HNF-4α

A, Localization of the human ApoAV promoter regions conferring responsiveness to HNF-4α by progressive deletion analysis. CV-1 cells were cotransfected with reporter plasmids containing the firefly luciferase gene driven by progressively 5′ shortened fragments of the apoAV promoter as indicated together with a plasmid expressing human HNF-4α or the empty vector pSG5 as control. Luciferase activities were measured as described in *Materials and Methods*. Results are expressed as fold induction by HNF-4α over control. The presence of T or C at −600 relative to the transcription start site, corresponding to SNP3 alleles, is indicated. B and C, Human apoAV gene promoter sequences surrounding the DR1 (B) and the IR8 (C) elements. The *gray boxes* denote the DR1 and IR8 sequences. The AGGTCA half-sites are indicated by *horizontal arrows*. The wild-type nucleotides that were modified by site-directed mutagenesis are *underlined*. The corresponding mutated nucleotides are shown *below* the *vertical arrows* and within the *gray squares*. D, Functional effects of mutations in the DR1 and IR8 elements on the response to HNF-4α. CV-1 cells were transfected with a plasmid expressing human HNF-4α or the empty pSG5 vector as control together with reporter constructs containing the wild-type or site-directed mutated 5′ flanking regions (−617/+18) of the human apoAV gene. Luciferase activities were measured as described in *Materials and Methods*. Results are expressed as percentage of induction by HNF-4α obtained with wild type. The *crosses* depict the presence of site-directed mutations in the DR1 and/or IR8 elements.

−242/−82 fragment did not reveal any of the previously described HNF-4α response elements DR0, DR1 or DR2 motifs (19, 20, 39). The only repeat of the hexanucleotide core motif PuGGTCA with a low degree of degeneration present within this region corresponds to an inverted repeat separated by 8 nt (IR8) between nt −103 and −84 (Fig. 2C), which has been previously described to respond to FXR (10). To characterize both DR1 and IR8 sites as HNF-4α response elements, CV-1 cells were cotransfected with a human HNF-4α expression vector and −617/+18 apoAV promoter-luciferase reporter plasmids in which DR1 and/or IR8 elements were mutated (Fig. 2, B and C). The data shown in Fig. 2D indicate that both sites confer HNF-4α response to the apoAV promoter and that they contribute similarly, albeit somewhat more IR8 than DR1, to the HNF-4α-mediated induction. The reporter construct bearing mutations in both DR1 and IR8 sites retained a transactivation by HNF-4α of about 25% compared with wild-type. Similar results were obtained with DR1 and IR8 mutant constructs containing the sequence spanning from −305 to +18 of apoAV promoter (data not shown). The basis for this remaining response is unclear because systematic EMSAs performed with numerous fragments within this region showed no binding to HNF-4α (see below), and it might be the result of an indirect action of HNF-4α.

Binding Analysis of HNF-4α to the Human apoAV Promoter

To investigate whether HNF-4α homodimers directly bind to the human apoAV gene promoter, radiolabeled double-stranded oligonucleotides corresponding to fragments of the DNA sequence spanning from nt −617 to +18 were used as probes in EMSA experiments (Fig. 3A). As expected, the addition of *in vitro*-translated human HNF-4α protein resulted in the appearance of retarded complexes when using a −275/−247 fragment containing the apoAV DR1 site (Fig. 3A, lane 6) and a −109/−80 fragment containing the apoAV IR8 element (lane 12), but not when −399/−374, −169/−147, −144/−117, −52/−31 probes (Fig. 3A, lanes 4, 8, 10, and 14, respectively), or −298/−263, −258/−221, −220/−183, −183/−143, −142/−104, −240/−202, −202/−166, −166/−131, −141/−103 fragments were employed (data not shown). On the other hand, in line with the results from cell transfection assays, no protein-DNA complex band appeared when using a −617/−593 fragment containing the SNP3 with a T (Fig. 3A, lane 2) or a C (data not shown) at −600.

HNF-4α Binds Specifically to the Human ApoAV DR1 and IR8 Elements

We next investigated whether HNF-4α was able to bind to apoAV DR1 and IR8 elements in hepatic nuclear extracts. For this purpose, we performed EMSAs with nuclear extracts obtained from human liver-derived HepG2 cells, which express apoAV (10) and HNF-4α (39), in the presence or absence of a specific HNF-4α antibody. As expected, the slower migrating complexes obtained with apoAV IR8 (Fig. 3B, lane 1) and DR1 (lane 3), or a control probe containing a known HNF-4α-binding element present in the human apoCIII gene promoter (lane 5), were supershifted upon addition of the HNF-4α antibody (lanes 2, 4, and 6, respectively). In contrast, double-stranded oligonucleotides that are equivalent to apoAV IR8 and DR1 probes (mt3 AvIR8 and mt3 AvDR1, respectively) but harbor point mutations in the half-sites (Fig. 2, B and C) could not form the same slower migrating complexes (Fig. 3B, lanes 7 and 9). Furthermore, a shift with faster mobility of mt3 AvDR1 probe (lane 9), which had a similar migration to a faint band appeared with the wild-type AvDR1 probe (lane 3), was not supershifted (lane 10). Additionally, because essentially most of the slower DNA-protein complexes were supershifted by the HNF-4α antibody, these results indicated that endogenous HNF-4α in these complexes was the major factor in nuclear extracts that could interact with apoAV DR1 and IR8 elements.

To unequivocally characterize the apoAV DR1 and IR8 as HNF-4α-binding sites, the specificity of these interactions was demonstrated by competition analysis. The formation of the retarded complex of apoAV DR1 was inhibited by the addition of increasing concentrations of either the unlabeled wild-type probe or a cold probe containing the HNF-4α-binding element of the human apoCIII gene promoter (data not shown). In contrast, cold double-stranded oligonucleotides that are equivalent to the apoAV DR1 probe but harbor point mutations (see Table 1) in the upstream half-site (mt1 AvDR1), in the downstream half-site (mt2 AvDR1) or in both half-sites (mt3 AvDR1 and mt4 AvDR1) could not displace the labeled wild-type element (data not shown). The HNF-4α-IR8 binding was also specific, as demonstrated by competition of an excess of either unlabeled wild-type apoAV IR8 oligonucleotide or a cold probe containing the HNF-4α-binding element of the human apoCIII gene promoter (Fig. 4B, wt AvIR8 and CONT, respectively). Furthermore, mutation of either half-site (Fig. 4A, mt1 AvIR8, mt2 AvIR8, mt3 AvIR8) abolished the ability of the probe to compete (Fig. 4B). Because an HNF-4α binding site with this type of organization has not been reported before, we extended the analysis of this element by introducing a point mutation in the downstream half-site to eliminate the only degeneration present in this hexanucleotide (Fig. 4A, pft AvIR8). As shown in Fig. 4C, the binding of HNF-4α to a radiolabeled probe corresponding to this perfect IR8 of the core motif AGGTCA (lane 6) is even more apparent than with the wild-type apoAV IR8 (lane 5). In addition, mutation of nt in the half-site spacing to leave the two hexanucleotide core-binding sites separated by 8 T (Fig. 4A, pft tttIR8), resulted in a modest reduction, not exclusion, of the binding (Fig. 4C, lane 7). As a control, no binding is observed with a labeled probe bearing point mutations that increase degener-

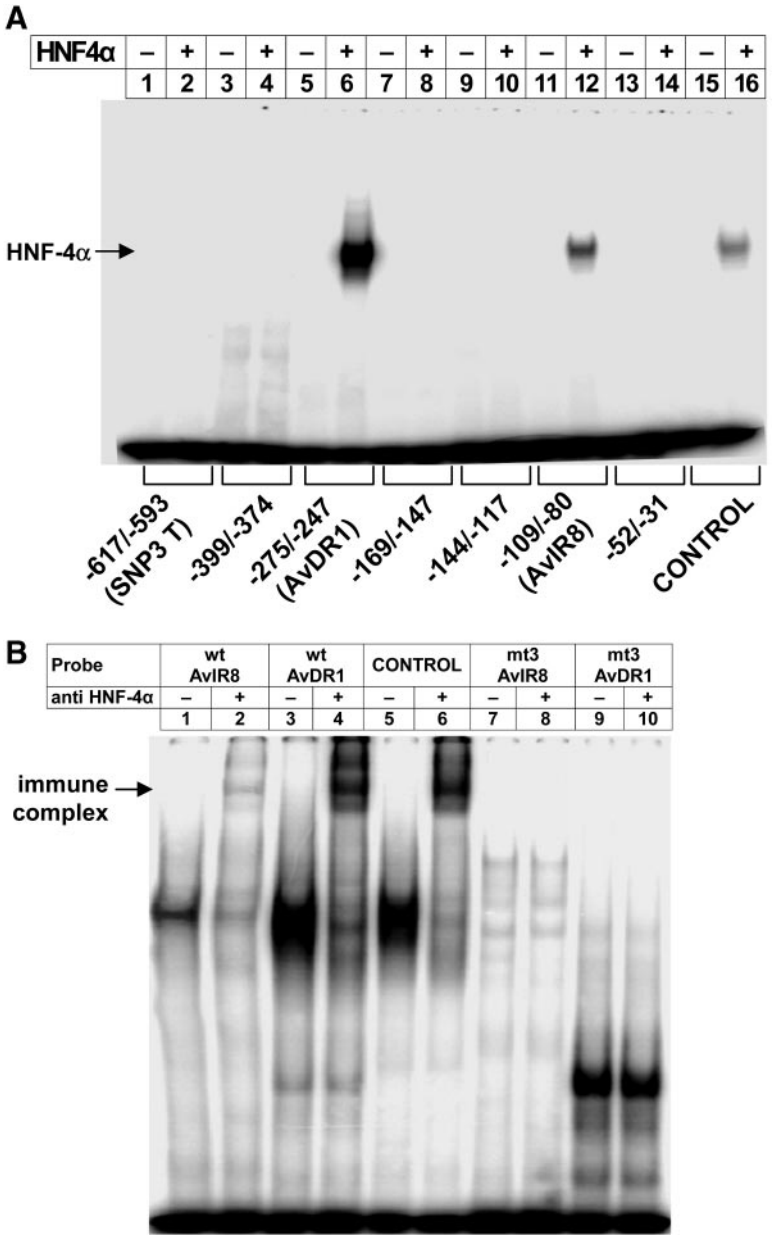

Fig. 3. Binding Analysis of HNF-4α and HepG2 Nuclear Extracts to the Human apoAV Gene Promoter

A, EMSAs were performed using *in vitro*-transcribed/translated HNF-4α (2.5 μl) or unprogrammed reticulocyte lysate (−), when indicated, and labeled double-stranded oligonucleotides corresponding to the indicated fragments of the human apoAV gene promoter as described in *Materials and Methods*. CONTROL, PPARα/HNF-4α site of mitHMGS gene (60). B, Endogenous HNF-4α binds specifically to the apoAV DR1 and IR8 elements. EMSAs were performed using HepG2 nuclear extracts and labeled double-stranded oligonucleotides corresponding to nt −109 to −80 (wt AvIR8) and −275 to −247 (wt AvDR1) of the human apoAV gene promoter, and an antibody against HNF-4α, when indicated, as described in *Materials and Methods*. mt3 AvIR8 and mt3 AvDR1 are modified versions of wt AvIR8 and wt AvDR1, respectively, whose mutations are shown in Fig. 2, B and C. Supershifted immune complexes are indicated by an *arrow*. CONTROL, HNF-4α-binding element of apoCIII gene (39).

ation of the two hexanucleotides (lane 8). Taken together, these results demonstrate that HNF-4α homodimers are able to directly and specifically interact *in vitro* with the apoAV IR8 and not with cryptic binding motifs embedded within this site. Next, we examined whether HNF-4α binds human apoAV IR8 *in vivo* by chromatin immunoprecipitation (ChIP) assay. For technical reasons, this may not be possible on the endogenous apoAV promoter due to the closeness of the DR1 and IR8 sites. Thus, we carried out a DNA immunoprecipitation assay on plasmid p-242/+18hAvLUC, which is an apoAV promoter-luciferase reporter construct that contains IR8 but not DR1. HepG2 cells were transfected, and formaldehyde was

Table 1. Oligonucleotides Used in this Study

Name		Sequence (5′ → 3′)	Use in this Study
JCR 169	hapoAV dirmut1 DR1 F	GCAGGTCAGTGGGAAGGTTAAA**caa**CATGGGGTTTGGGAG	Site-directed mutagenesis
JCR 170	hapoAV dirmut1 DR1 R	CTCCCAAACCCCATG**ttg**TTTAACCTTCCCACTGACCTGC	Site-directed mutagenesis
JCR 188	hapoAV dirmut2 DR1 F	GCAGGTCAGTGGGAA**caa**TAAA**caa**CATGGGGTTTGGGAG	Site-directed mutagenesis
JCR 189	hapoAV dirmut2 DR1 R	CTCCCAAACCCCATG**ttg**TTTA**ttg**TTCCCACTGACCTGC	Site-directed mutagenesis
JCR 167	hapoAV dirmut3 IR8 F	GCTGGGAGGCAGCTGAG**aa**CAACTTCTTT**c**G**tt**CTTCCACGTGG	Site-directed mutagenesis
JCR 168	hapoAV dirmut3 IR8 R	CCACGTGGAAG**aa**C**g**AAAGAAGTTG**tt**CTCAGCTGCCTCCCAGC	Site-directed mutagenesis
JCR 117	hapoAVC −617/−593 F	GATCCTCATGGGGCAAATCTCACTTTCGA	EMSA
JCR 118	hapoAVC −617/−593 R	GATCTCGAAAGTGAGATTTGCCCCATGAG	EMSA
JCR 119	hapoAVT −617/−593 F	GATCCTCATGGGGCAAATCTTACTTTCGA	EMSA
JCR 120	hapoAVT −617/−593 R	GATCTCGAAAGTAAGATTTGCCCCATGAG	EMSA
JCR 123	hapoAV −399/−374 F	GATCCTGGTAGGTGAACACTGTCCATCTTGA	EMSA
JCR 124	hapoAV −399/−374 R	GATCTCAAGATGGACAGTGTTCACCTACCAG	EMSA
JCR 155	hapoAV −169/−147 F	GATCCAGGGCACTCATTAACCCTCTGA	EMSA
JCR 156	hapoAV −169/−147 R	GATCTCAGAGGGTTAATGAGTGCCCTG	EMSA
JCR 153	hapoAV −144/−117 F	GATCCTGCCAGGGAAAGGGCAGGAGGTGAGTGCA	EMSA
JCR 154	hapoAV −144/−117 R	GATCTGCACTCACCTCCTGCCCTTTCCCTGGCAG	EMSA
JCR 151	hapoAV −52/−31 F	GATCCAGAGGCTCAGGGCCCTGGAGA	EMSA
JCR 152	hapoAV −52/−31 R	GATCTCTCCAGGGCCCTGAGCCTCTG	EMSA
JCR 157	mitHMGS −113/−82 F	GATCCTTGTTCTGAGACCTTTGGCCCAGTTTTTCTGA	EMSA
JCR 158	mitHMGS −113/−82 R	GATCTCAGAAAAACTGGGCCAAAGGTCTCAGAACAAG	EMSA
JCR 127	hapoAV −275/−247AvDR1 F	GATCCGGGAAGGTTAAAGGTCATGGGGTTTGGGA	EMSA
JCR 128	hapoAV −275/−247AvDR1 R	GATCTCCCAAACCCCATGACCTTTAACCTTCCCG	EMSA
JCR 165	hapoAV mt1 AvDR1 F	GATCCGGGAAGGTTAAA**caa**CATGGGGTTTGGGA	EMSA
JCR 166	hapoAV mt1 AvDR1 R	GATCTCCCAAACCCCATG**ttg**TTTAACCTTCCCG	EMSA
XP 27	hapoAV mt2 AvDR1 F	GATCCGGGAA**caa**TAAAGGTCATGGGGTTTGGGA	EMSA
XP 28	hapoAV mt2 AvDR1 R	GATCTCCCAAACCCCATGACCTTTA**ttg**TTCCCG	EMSA
XP 25	hapoAV mt3 AvDR1 F	GATCCGGGAA**caa**TAAA**caa**CATGGGGTTTGGGA	EMSA
XP 26	hapoAV mt3 AvDR1 R	GATCTCCCAAACCCCATG**ttg**TTTA**ttg**TTCCCG	EMSA
XP 31	hapoAV mt4 AvDR1 F	GATCCGGGAAG**c**TTAAAG**a**TCATGGGGTTTGGGA	EMSA
XP 32	hapoAV mt4 AvDR1 R	GATCTCCCAAACCCCATGA**t**CTTTAA**g**CTTCCCG	EMSA
XP 69	mapoAV AvDR1 F	GATCCGGGAAGGTTAAAGGTCATGGGGTTTGGGA	EMSA
XP 70	mapoAV AvDR1 R	GATCTCCCAAACCCCATGACCTTTAACCTTCCCG	EMSA
JCR 61	hapoCIII −90/−68C3PRE2 F	GATCCTCAGCAGGTGACCTTTGCCCAGCA	EMSA
JCR 62	hapoCIII −90/−68C3PRE2 R	GATCTGCTGGGCAAAGGTCACCTGCTGAG	EMSA
JCR 139	hapoAV −109/−80AvIR8 F	GATCCAGCTGAGGTCAACTTCTTTTGAACTTCCA	EMSA
JCR 140	hapoAV −109/−80AvIR8 R	GATCTGGAAGTTCAAAAGAAGTTGACCTCAGCTG	EMSA
JCR 159	hapoAV mt1 IR8 F	GATCCAGCTGAG**aa**CAACTTCTTTTGAACTTCCA	EMSA
JCR 160	hapoAV mt1 IR8 R	GATCTGGAAGTTCAAAAGAAGTTG**tt**CTCAGCTG	EMSA
JCR 161	hapoAV mt2 IR8 F	GATCCAGCTGAGGTCAACTTCTTT**c**G**tt**CTTCCA	EMSA
JCR 162	hapoAV mt2 IR8 R	GATCTGGAAG**aa**C**g**AAAGAAGTTGACCTCAGCTG	EMSA
JCR 163	hapoAV mt3 IR8 F	GATCCAGCTGAG**aa**CAACTTCTTT**c**G**tt**CTTCCA	EMSA
JCR 164	hapoAV mt3 IR8 R	GATCTGGAAG**aa**C**g**AAAGAAGTTG**tt**CTCAGCTG	EMSA
JCR 190	pft AvIR8 F	GATCCAGCTGAGGTCAACTTCTTTTGA**c**CTTCCA	EMSA
JCR 191	pft AvIR8 R	GATCTGGAAG**g**TCAAAAGAAGTTGACCTCAGCTG	EMSA
JCR 194	pft tttIR8 F	GATCCAGCTGAGGTCA**tt**TT**t**TTTTGA**c**CTTCCA	EMSA
JCR 195	pft tttIR8 R	GATCTGGAAG**g**TCAAAA**a**AA**aa**TGACCTCAGCTG	EMSA
LU 1	hHNF-4α S	GAGTCCTATAAGCTCCTGCCG	Real-time RT-PCR
LU 2	hHNF-4α AS	CCAGCGGCTTGCTAGATAAC	Real-time RT-PCR
LU 3	hcyclophylin S	CATCTGCACTGCCAAGACTGA	Real-time RT-PCR
LU 4	hcyclophylin AS	CCACAATATTCATGCCTTCTTTCA	Real-time RT-PCR
LU 5	hapoCIII S	CTTCTCAGCTTCATGCAGGGTTA	Real-time RT-PCR
LU 6	hapoCIII AS	ACGCTGCTCAGTGCATCCTT	Real-time RT-PCR
LU 7	hLDLr S	GTTGCTGGCAGAGGAAATGAGAAG	Real-time RT-PCR
LU 8	hLDLr AS	CAAAGGAAGACGAGGAGCACGAT	Real-time RT-PCR
LU 9	hapoAI S	CTCGGCATTTCTGGCAGCAA	Real-time RT-PCR
LU 10	hapoAI AS	AGTGGCCAGGTCCTTCACTCGA	Real-time RT-PCR
XP 3	hapoAV S	AGCTGGTGGGCTGGAATTT	Real-time RT-PCR
XP 4	hapoAV AS	GGCCACCTGCTCCATCAG	Real-time RT-PCR
XP 100	hapoAV −197/−178 F	CACTTCTCTACTGGGGCAGC	ChIP
XP 101	pGL3-bv R	CCATGGTGGCTTTACCAACAGTACCG	ChIP
siQI 1	siControl 1022076 S	UUCUCCGAACGUGUCACGUdTdT	RNA interference
siQI 2	siControl 1022076 AS	ACGUGACACGUUCGGAGAAdTdT	RNA interference
siLU 1	sihHNF-4α S	CCACAUGUACUCCUGCAGAdTdT	RNA interference
siLU 2	sihHNF-4α AS	UCUGCAGGAGUACAUGUGGdTdT	RNA interference

Mutated nucleotides are denoted in *bold* and *lowercase type.*

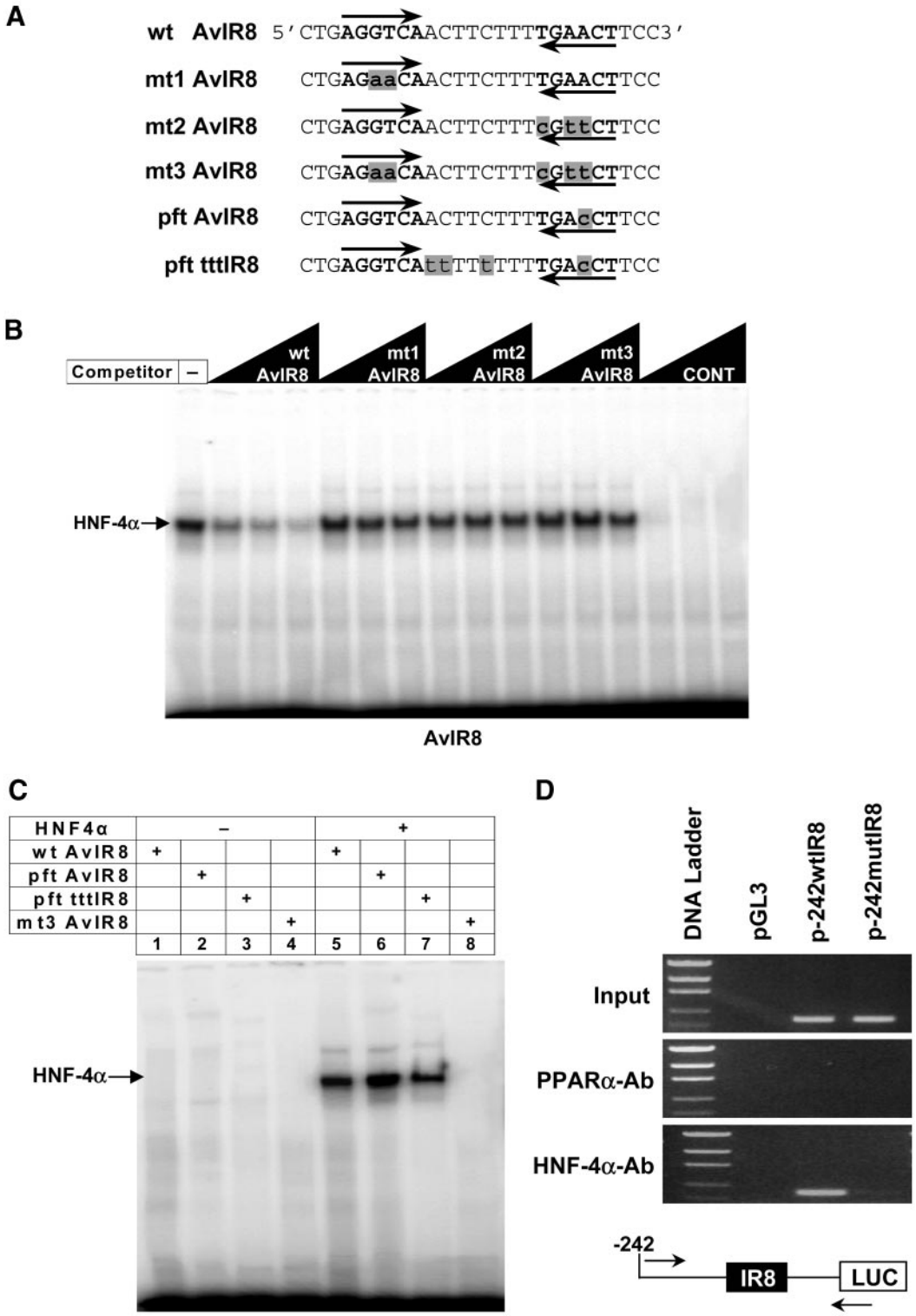

Fig. 4. HNF-4α Binds Specifically to the IR8 Element of apoAV Gene Promoter

A, Human apoAV gene promoter sequence surrounding the IR8 element and mutated versions used in experiments B and C. The hexameric consensus sites are in *boldface type*, and their orientations are indicated by *arrows*. Mutations in half-sites are shown in *lowercase type* and within *gray squares*. B, Competition experiments for binding of *in vitro*-transcribed/translated HNF-4α to the labeled probe corresponding to nt −109 to −80 (AvIR8) of the human apoAV gene promoter were performed by adding 50-, 250-, and 500-fold molar excess of the indicated unlabeled double-stranded oligonucleotides. CONT, HNF-4α-binding element of apoCIII gene (39). C, EMSAs were performed as described in *Materials and Methods* using *in vitro*-transcribed/translated HNF-4α (2.5 μl) or unprogrammed reticulocyte lysate (−), when indicated, and labeled double-stranded oligonucle-

added to cross-link the DNA-protein complexes *in vivo*. After immunoprecipitation, and reversal of cross-linking, the DNA was purified and used as a template for PCR amplification with primers that encompass the region from nt −197 of apoAV promoter to a sequence in the pGL3-basic vector. As shown in Fig. 4D, this region was precipitated by antibodies directed against HNF-4α. In contrast, no signal was obtained when the immunoprecipitation was carried out with anti-PPARα antibodies in the same conditions, which is consistent with reports showing that the DR1 element at −271 is required for PPARα-mediated transactivation of human apoAV promoter (9, 10). Moreover, when ChIP was performed on an equivalent construct harboring mutations within the IR8 site, the signal was dramatically diminished (Fig. 4D, p-242mutIR8/HNF-4α-Ab).

Inhibition of HNF-4α Expression Results in Down-Regulation of Human ApoAV

To further provide evidence for the involvement of HNF-4α in the regulation of human apoAV expression, we used RNA interference technology to knock down endogenous HNF-4α. Transfection of HepG2 with increasing concentrations of small interfering RNA (siRNA) targeting HNF-4α expression resulted in a dose-dependent decrease of apoAV mRNA levels (Fig. 5A). This effect appeared to be specific because the expression of apoCIII, a well-known HNF-4α target gene, was also diminished, whereas cyclophilin, used as internal control, was unaffected by siRNA transfection (Fig. 5A and data not shown). Moreover, immunoblotting experiments revealed that this HNF-4α siRNA-mediated down-regulation of apoAV occurred also at the protein level (Fig. 5, B and C) and confirmed that this effect is specific because the nonsilencing control siRNA did not significantly affect endogenous HNF-4α and apoAV protein expression (Fig. 5B). In addition, actin protein levels, which were measured as a control, were not modified after siRNA transfection, thereby demonstrating the specificity of this effect (Fig. 5B). Taken together, these results establish the essential role of HNF-4α in the regulation of human apoAV expression.

HNF-4α Is Involved in the Stimulation of ApoAV Gene Promoter by the Nuclear Receptor Coactivator PGC-1α

It has been demonstrated that PGC-1α stimulates the promoter activity of multiple hepatic genes involved in gluconeogenesis (26, 27), fatty acid oxidation (28), BA

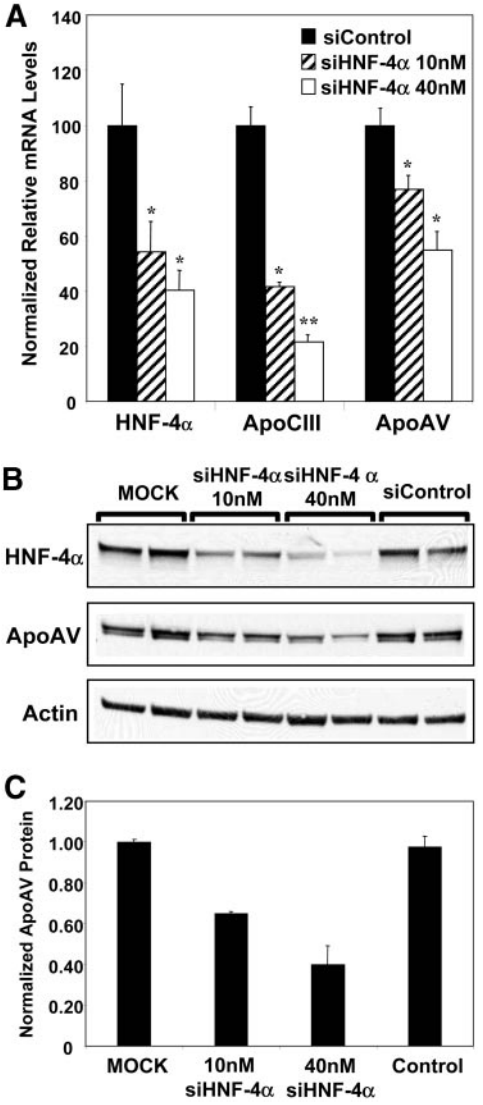

Fig. 5. Inhibition of HNF-4α Expression Results in Down-Regulation of apoAV in HepG2 Cells

A, HepG2 cells were transfected with increasing concentrations of siRNA targeting HNF-4α (siHNF-4α) or 20 nM control siRNA (siControl). Forty-eight hours after transfection, total RNA was extracted for analysis by real time RT-PCR as described in *Materials and Methods*. Specific mRNA levels normalized to cyclophilin are expressed relative to control set as 100 for each gene (mean ± SD). *, $P < 0.05$; **, $P < 0.001$ *vs.* control. B, Western analyses of HNF-4α, apoAV, and actin expression in whole cell lysates from HepG2 48 h after siRNA transfections. Experiments were performed twice with identical results. C, Quantification of apoAV protein levels relative to the actin control by densitometric scanning and ImageQuant.

otides corresponding to the sequences in A. D, ChIP analysis of HNF-4α interaction with apoAV IR8 element *in vivo*. Cross-linked DNA isolated from HepG2 cells transfected with luciferase reporter plasmid p-242/+18hAvLUC (p-242wtIR8), an equivalent construct harboring mutations within the IR8 site (p-242mutIR8), or pGL3-basic vector as a negative control, was immunoprecipitated with the indicated antibodies, and subjected to PCR as described in *Materials and Methods*. PCR products were electrophoresed on 1% agarose gel. The relative location of the PCR primers is depicted as *arrows* in diagram below. LUC, Luciferase. Experiments were performed at least two independent times, and representative data are shown.

synthesis (29), and TG metabolism (30) through coactivation of HNF-4α. To determine whether PGC-1α modulates apoAV promoter activity and to evaluate the potential involvement of HNF-4α, transient transfection assays were performed with the firefly luciferase reporter gene expression vector driven by sequences of the human apoAV promoter. In Hep3B, cotransfection of a human PGC-1α expression plasmid significantly enhanced the −617/+18 apoAV promoter activity (Fig. 6A). Cotransfection of HNF-4α and PGC-1α together resulted in a further activation, although only slightly greater than with HNF-4α alone (Fig. 6A). The effect of PGC-1α overexpression was promoter dependent because it was not observed with the promoter-less pGL3-basic vector. To identify the regulatory elements mediating the effect of PGC-1α, a reporter construct bearing mutations in the DR1 and IR8 elements (Fig. 2, B and C) was tested. Although some remaining response was observed, the mutations markedly diminished the response to PGC-1α and HNF-4α (Fig. 6A). In addition, a truncated construct representing the −82/+18 apoAV promoter was completely unresponsive to HNF-4α and PGC-1α. These results indicate that PGC-1α moderately stimulates human apoAV promoter activity, preferentially via DR1 and IR8 elements. PGC-1α can also coacti-

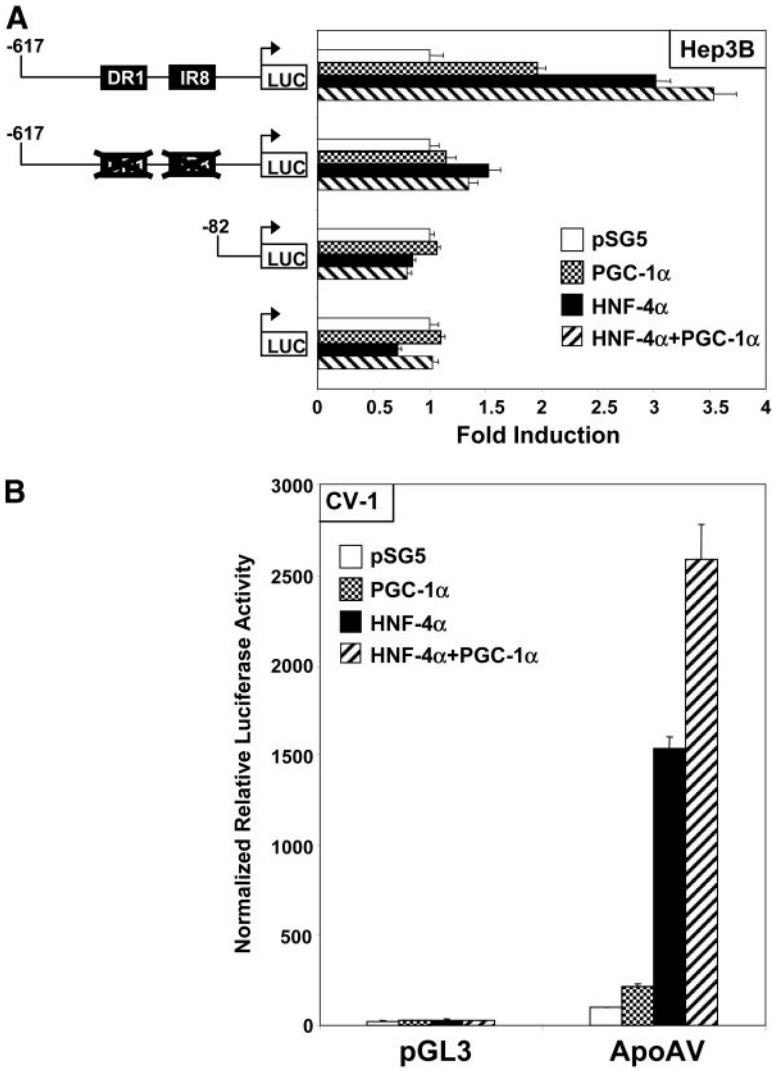

Fig. 6. PGC-1α Stimulates the HNF-4α-Dependent Transactivation of apoAV Gene Promoter

A, Hep3B cells were transfected with plasmids expressing human HNF-4α and/or PGC-1α (100 ng), or the empty vector pSG5 as control, together with reporter constructs containing the wild-type or site-directed mutated 5′-flanking region (−617/+18) or a deletion up to nt −82 of the human apoAV gene. The empty pGL3-basic vector was used as a negative control. The plasmid dosage was kept constant by the addition of empty expression vector. The luciferase activities were measured as described in *Materials and Methods*. Results are expressed as fold induction over control. LUC, luciferase. B, CV-1 cells were transfected as in A. The luciferase activities were measured and expressed as described in *Materials and Methods*. pGL3, the empty pGL3-basic vector used as a negative control. ApoAV, A luciferase reporter construct driven by the 5′-flanking region (−617/+18) of the human apoAV gene.

vate PPARα (40) and FXR (30). Because apoAV DR1 and IR8 have been previously described as PPARα and FXR response elements, respectively (10), it is also possible that PGC-1α is acting on the human apoAV promoter through these transcription factors. To prove that PGC-1α-dependent stimuli on apoAV promoter activity can be achieved by coactivation of HNF-4α, we performed cotransfection studies in the CV-1 line, a cellular model frequently used by others in PGC-1α studies (30, 40) to avoid the background of endogenous HNF-4α, PPARα and FXR. As shown in Fig. 6B, cotransfection of PGC-1α alone resulted in a weak activation of apoAV promoter, an effect also observed in other promoters (Fig. 2 in Ref. 30 and Fig. 1 in Ref. 40). However, cotransfection of PGC-1α and HNF-4α together caused a dramatic activation of this promoter that was markedly higher than with either factor alone. These results reveal a clear synergistic effect between PGC-1α and HNF-4α on the activity of the human apoAV promoter.

The MAPK Pathway Regulates the Expression of Human ApoAV

Leff and colleagues (31) have demonstrated that apoCIII expression can be regulated by signals acting through the MAPK signaling pathway and that this regulation is mediated, at least in part, by changes in the amount of HNF-4α. To determine whether this could be also true for apoAV, HepG2 cells were incubated for 24 h in medium containing PD98059 (41), an inhibitor of the upstream activator of Erk1/2, which are members of the MAPK family, or vehicle. As determined by quantitative real-time RT-PCR, treatment with PD98059 caused a significant increase of apoAV mRNA levels (Fig. 7A). These effects were specific because the expression of apoCIII was also increased by PD98059, in accordance with previous studies (31), whereas cyclophilin, used as internal control, remained unaffected by the treatments (Fig. 7A and data not shown). Furthermore, in contrast to apoAV and apoCIII, low-density lipoprotein receptor (LDLr) mRNA levels were slightly reduced by PD98059 after 24 h of treatment under standard serum conditions, in agreement with published results (42). ApoAI mRNA levels were affected similarly to apoAV and apoCIII (Fig. 7A).

Next, Western blot analysis performed on whole cell lysates from treated HepG2 revealed that the quantity of apoAV and HNF-4α proteins was notably increased by PD98059, whereas the levels of actin were indistinguishable between treatments (Fig. 7B). Taken together, these observations demonstrate that the MAPK/Erk kinase signaling pathway controls human apoAV expression and suggest that this regulation may be mediated, at least in part, by changes in the amount of HNF-4α.

Activation of AMPK Down-Regulates Expression of Human ApoAV

Activation of AMPK diminishes HNF-4α protein levels and inhibits its ability to form homodimers and bind to DNA, and consequently down-regulates the expression of several HNF-4α target genes (32, 33). To investigate whether activation of AMPK catalytic activity down-regulates human apoAV expression, HepG2 cells were incubated for 24 h in the presence of increasing concentrations of AICAR, which is converted in cells to an analog of AMP that activates the kinase (43). As shown in Fig. 7C, AICAR significantly decreased apoAV mRNA levels. As a positive control, apoCIII mRNA levels were measured, and consistent with the literature (32), a clear down-regulation was observed. ApoAI mRNA levels were also decreased. However, no changes were detected in cyclophilin levels, which was used as internal control, and in contrast to apolipoproteins, LDLr mRNA levels were robustly induced by AICAR treatment (Fig. 7C).

Immunoblotting assays with whole cell lysates revealed that increasing concentrations of AICAR decrease HNF-4α protein levels in HepG2 (Fig. 7D), likewise as described in murine hepatocytes (32). The results, depicted in Fig. 7D, show that apoAV protein levels declined with increasing concentration of AICAR, whereas actin levels were not modified and while the phosphorylation of acetyl-CoA carboxylase increased. Given the key role of HNF-4α in apoAV expression, these data clearly indicate that the activation of AMPK catalytic activity down-regulates human apoAV expression and suggest that this negative regulation occurs, at least in part, via HNF-4α.

AMPK and the MAPK Cascade Modulate the Binding of HepG2 Nuclear Extracts to the DR1 and IR8 Elements of Human ApoAV Promoter

We next examined whether the MAPK/Erk kinase pathway and the activation of AMPK could modify the binding of HepG2 nuclear extracts to the HNF-4α response elements of human apoAV. For this purpose, we carried out EMSAs using nuclear extracts prepared from HepG2 treated with AICAR, PD98059, or vehicle and labeled oligonucleotides corresponding to the apoAV DR1 and IR8 elements described in this report (Fig. 7E, AvDR1 and AvIR8, respectively). As expected, we found that AICAR treatment caused a dramatic reduction in DNA binding of the nuclear extracts to the IR8 (Fig. 7E, compare lane 1 with lane 4) and DR1 (Fig. 7E, compare lane 2 with lane 5), whereas PD98059 robustly increased binding to both HNF-4α sites of apoAV (Fig. 7E, compare lanes 7 and 8 with lanes 10 and 11, respectively). These results further support the hypothesis that HNF-4α is mediating the effects of AMPK and the MAPK/Erk kinase pathway on human apoAV expression.

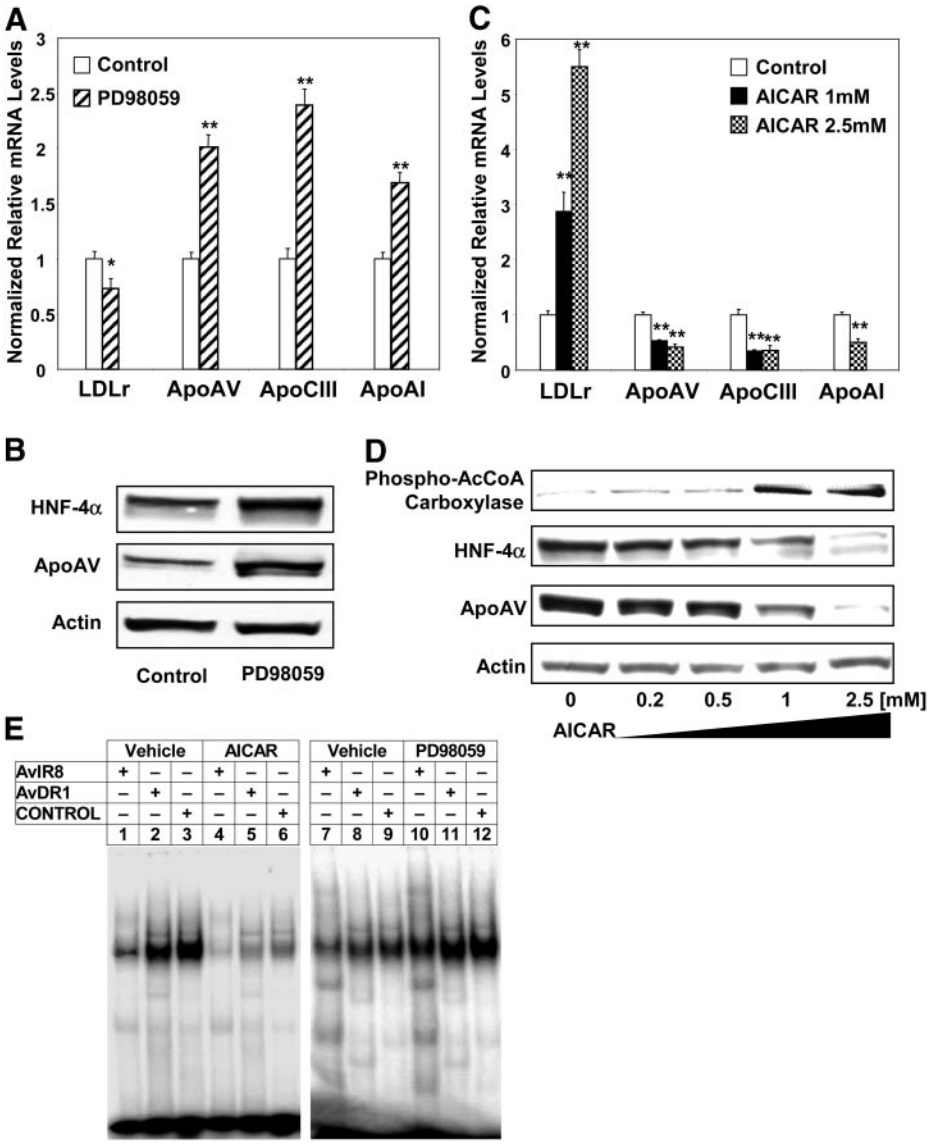

Fig. 7. Regulation of Human apoAV Expression by the MAPK Pathway and AMPK

A, HepG2 cells were treated for 24 h with vehicle (Control) or the Erk1/2 kinase inhibitor PD98059 (20 μM). Total RNA was extracted for analysis by real time RT-PCR as described in *Materials and Methods*. Specific mRNA levels normalized to cyclophilin content are expressed relative to untreated cells set as 1 for each gene (mean ± SD). Significant differences compared with the untreated control are as follows: *, $P < 0.05$; **, $P < 0.001$. B, Western analyses of HNF-4α, apoAV, and actin expression in whole cell lysates from HepG2 treated for 24 h as in A. C, HepG2 cells were treated as in A with increasing concentrations of AMPK activator AICAR. D, Western analyses performed as in B with increasing concentrations of AICAR. The contents of phosphorylated acetyl-CoA carboxylase were also monitored as positive control. E, AMPK and the MAPK pathway modulate the binding of HepG2 nuclear extracts to the DR1 and IR8 elements of human apoAV promoter. HepG2 cells were treated for 24 h with vehicle, AICAR (2.5 mM), or PD98059 (20 μM). Nuclear extracts were prepared and EMSAs were performed using labeled double-stranded oligonucleotides corresponding to nt −109 to −80 (AvIR8) and −275 to −247 (AvDR1) of the human apoAV gene promoter as described in *Materials and Methods*. CONTROL, HNF-4α-binding element of apoCIII gene (39). Experiments were performed twice with identical results.

Species-Distinct Regulation of ApoAV by HNF-4α

Next, we sought to extend the analysis to mouse, an animal model commonly used for apoAV studies (2–5, 8, 11, 12). Sequence comparisons revealed that the human apoAV DR1 element that we describe here as an HNF-4α site is apparently not conserved in the mouse apoAV gene promoter. Indeed, the alignment of the sequences, depicted in Fig. 8A, reveals 3-nt differences that render higher degeneration of the two hexameric PuGGTCA core-binding sites in murine sequences compared with the human DR1. In addition, it has been reported that the occurrence of A as the spacer nucleotide between the hexamers is crucial for

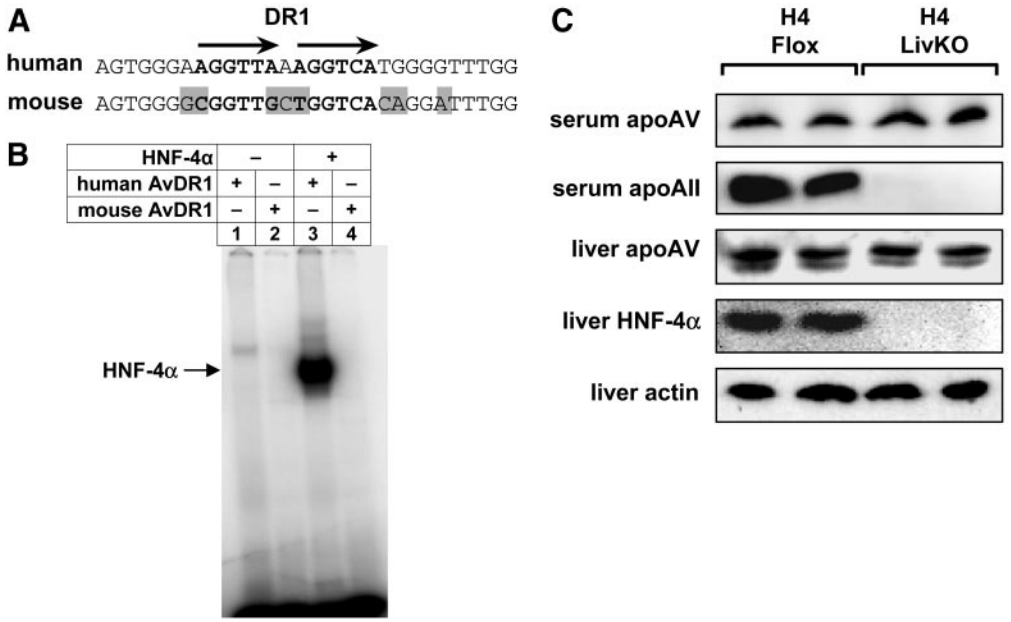

Fig. 8. Species-Distinct Regulation of ApoAV by HNF-4α

A, Alignment of human and mouse apoAV sequences surrounding the DR1 element. The *gray boxes* denote the differences between sequences. The hexameric sites are in *boldface type* and their orientations are indicated by *arrows*. B, EMSAs were performed using *in vitro*-transcribed/translated HNF-4α (2.5 μl) or unprogrammed reticulocyte lysate (−), when indicated, and labeled probes containing the human apoAV DR1 sequence or the mouse counterpart as described in *Materials and Methods*. C, Hepatic and serum apoAV protein levels are not affected by liver-specific disruption of HNF-4α in mice. Liver homogenates and sera of conditionally HNF-4α-null mice (H4 LivKO) and controls (H4 FLOX) were assayed by Western blot for the presence of the indicated proteins as described in *Materials and Methods*. Results from representative experiments are shown.

the binding of HNF-4α (20, 38). To determine whether these nucleotide differences could impair the binding of HNF-4α, EMSA experiments were performed. As shown in Fig. 8B, HNF-4α binds to a human, but not to a mouse, probe containing the DR1 sequence. These results prompted us to explore the possibility of a species-distinct regulation of the apoAV gene by HNF-4α.

To obtain direct evidence for different regulation of murine apoAV by HNF-4α, we measured the levels of apoAV in H4LivKO mice, which are conditionally HNF-4α-null mice lacking hepatic HNF-4α expression (16). Strikingly, immunoblotting assays showed that the absence of HNF-4α in the liver of standard chow-fed adult mice had no effect on liver or serum protein concentrations of apoAV, further confirming the species-distinct regulation of apoAV by HNF-4α (Fig. 8C). As a control, we verified that serum of H4LivKO mice was virtually devoid of apoAII, a canonical HNF-4α target gene (16).

DISCUSSION

Overexpression of apoAV results in a dramatic reduction of plasma TG, whereas the lack of apoAV transcripts causes a marked hypertriglyceridemia (2–5, 8). Therefore, the potential regulation of apoAV expression by hormonal and metabolic signals may play a significant role in controlling plasma TG levels. The present study reveals that a liver-enriched nuclear receptor involved in the regulation of lipid-related genes, namely HNF-4α, controls the human apoAV gene. We show that depletion of the endogenous HNF-4α by siRNA-mediated RNA interference causes down-regulation of apoAV mRNA levels. Our results demonstrate unequivocally that HNF-4α directly regulates the apoAV gene promoter via two elements, a DR1 and an IR8, a hitherto unknown element for HNF-4α. We show for the first time that HNF-4α binds to an IR8 both *in vitro* and *in vivo*, and by using deletion and site-directed mutagenesis we demonstrate that this IR8 is required for full transactivation by HNF-4α. Thus, we conclude that this novel IR8 element is a *bona fide* HNF-4α response element.

Numerous studies have reported that a T→C polymorphism in the 5′-region of the human apoAV gene, called SNP3, is associated with elevated plasma TG levels (2, 6, 7). Assuming a high degree of degeneration, one could consider the occurrence of a DR1 element spanning nt −611 to −599, particularly if a C is present (GGGGCA A ATCTCA). We had previously reported that the basal activity or the PPARα or FXR-mediated induction of the apoAV promoter was not affected by this change of T to C (10). Similarly, our gel shift analyses (Fig. 3A and data not shown) indicate

that HNF-4α is not binding to this fragment. Moreover, luciferase reporter assays revealed that deletion of the sequence surrounding this SNP or the change of the common allele T to C has no significant effect on the induction by HNF-4α of the apoAV promoter activity (Fig. 2A), further arguing against the functional character of this polymorphism.

Two reports on MODY1 showed that, in addition to abnormal pancreatic β-cell function, mutations in a single allele of the HNF-4α gene, which result in truncation of the protein, were associated with mild reductions in serum levels of TG, apoAII, and apoCIII (44, 45). Despite the key role of HNF-4α in hepatic expression, no liver defect was identified in individuals with these HNF-4α mutations. Notably, the serum levels of some HNF-4α-regulated genes, including transferrin, α-1 antitrypsin, and apoAI were not decreased in subjects with HNF-4α mutations (44, 45). The reasons for these findings remain unknown, but compensatory mechanisms related to the diabetic condition have been suggested (45). Our RNA interference data show that apoAV expression is less sensitive than apoCIII to a reduction of HNF-4α levels (Fig. 5A), thereby suggesting that a dramatic reduction of apoAV levels in MODY1 patients is unlikely.

In the present study, we also explored which effects may produce the modulation of HNF-4α activity on the expression of human apoAV. A previous report has shown that inhibition of the Erk1/2 signaling in HepG2 augmented HNF-4α levels, which in turn resulted in an increase of apoCIII expression (31). Here we show that this also may be valid for apoAV (Fig. 7). Furthermore, it has been reported that AMPK represses the activity and increases the degradation of HNF-4α by direct phosphorylation, which consequently results in down-regulation of the expression of many HNF-4α target genes (32, 33). As expected, the activation of AMPK catalytic activity by AICAR led to the down-regulation of apoAV and apoCIII (Fig. 7). Interestingly, in contrast to rodent hepatocytes (32), we observed that AICAR decreases apoAI expression in human HepG2. Moreover, these effects on HNF-4α function were confirmed in gel shift analysis showing that the binding of HepG2 nuclear extracts to the DR1 and IR8 elements of human apoAV (mainly due to HNF-4α, as shown in Fig. 3B) was diminished by activation of AMPK and increased by inhibition of the Erk1/2 signaling (Fig. 7E). Notwithstanding the above, we do not exclude the possibility that besides HNF-4α there might be other yet undiscovered factors contributing to the effects of MAPK and AMPK. At present, the physiological role of the MAPK/Erk kinase cascade in the regulation of apoAV expression remains unknown. We have observed that IL-1β negatively regulates apoAV expression in human hepatocytes and HepG2 (data not shown). Because IL-1β activates Erk1/2 signaling (46) and reduces HNF-4α protein levels in HepG2 (Ref. 47 and data not shown), it is tempting to speculate that the response of human apoAV to this inflammatory cytokine may be mediated, at least in part, by changes in the HNF-4α-dependent regulation. However, further work is required to confirm or rebut this hypothesis. AMPK acts as a fuel sensor that is activated by states of low energy charge (*i.e.* reduced ATP/AMP ratios). When activated, AMPK inhibits ATP-consuming anabolic pathways and stimulates ATP-producing catabolic signals. In the liver, AMPK activation results in reduced lipid and glucose synthesis by repressing gene expression of fatty acid synthase, PEPCK, and G6Pase (48). Because all the effects of AMPK activation that have been described caused reduction of gene expression, it has been proposed that one of the primary functions of AMPK might be to reduce the general rate of transcription, a process that utilizes large amounts of energy (48). The inhibition of HNF-4α activity would indeed reduce the transcription of a relatively large set of genes, which is consistent with this general role of the kinase in reducing the rate of energy-utilizing processes. However, the apoAV down-regulation by AMPK appears counterproductive because it would result in a reduction in the VLDL-TG hydrolysis. Further studies are needed to elucidate the precise physiological significance of the repression of human apoAV by activated AMPK.

Strikingly, we observed that liver and plasma concentrations of apoAV in adult mice were unaffected by liver-specific disruption of the HNF-4α gene (Fig. 8C), whereas expression of other HNF-4α target genes including *CYP7A1*, MTP, apoAII, apoB, and apoCIII genes was found markedly decreased (Ref. 16 and Fig. 8C). We also show that nucleotide differences between human and mouse in the most upstream HNF-4α element of apoAV promoter abolish binding by HNF-4α (Fig. 8B), which could be a sign of a species-distinct regulation of the apoAV gene by HNF-4α. However, the sequence of the IR8 in the human apoAV promoter is perfectly conserved in murine counterpart (data not shown), which is difficult at present to reconcile with the former results in HNF-4α-null livers. One possibility might be that in these modified mice another activator substitutes for HNF-4α in apoAV IR8 site. In addition, given that the expression of some mouse genes containing a functional HNF-4α site in their proximal promoters is down-regulated by disruption of HNF-4α in fetal hepatocytes but not in the adult liver (15, 16, 49, 50), it is tempting to speculate that the apoAV IR8 element might be used by HNF-4α in the fetal liver but not the mouse adult liver. In fact, an HNF-4α binding site was characterized in the mouse PXR gene and found to be required for activation of the PXR promoter in fetal hepatocytes (49). However, the role of this element in the adult liver is uncertain because the expression of PXR is decreased by deletion of the HNF-4α gene in fetal hepatocytes but not in the fully differentiated liver (15, 16, 49). By analogy to apoAV, the case of apoAI is particularly intriguing because the expression of the human gene is clearly regulated by HNF-4α and the promoter contains two HNF-4α functional elements (34–37). In contrast, 3-nt differences abolish activation by HNF-4α through the

most upstream element of the rat and mouse apoAI gene promoters (51). Still, the activity of rodent apoAI is increased by HNF-4α via a proximal element (50, 51) at nt −132/−120. This finding is in direct contradiction with the demonstration that mRNA levels for apoAI were unaffected by disruption of the HNF-4α in the adult mice liver (16), but it is consistent with the abolishment of apoAI expression in fetal HNF-4α-null livers (15). Thus, it will be of considerable interest for future liver development studies to determine whether there is also an HNF-4α-dependent regulation of mouse apoAV expression specific to the fetal stage, and to clarify the physiological significance of the IR8 element in apoAV gene.

It is well established that, in the liver, PGC-1α plays a key role in the nutritional regulation of gluconeogenesis, fatty acid oxidation, ketogenesis, and BA synthesis (26–30). Spiegelman and co-workers (26) have reported that PGC-1α coactivates HNF-4α by direct interaction in a ligand-independent manner. These authors have shown that HNF-4α is absolutely required for PGC-1α-mediated induction of G6Pase and PEPCK, whereas PGC-1α can still activate, albeit at much lesser extent, the expression of the CPT-Iα in the absence of HNF-4α (27). In fact, PGC-1α can stimulate CPT-Iα through both HNF-4α and thyroid hormone receptor-β (28, 52). In reporter assays, transfection of either PGC-1α or HNF-4α activated G6Pase promoter very poorly, whereas cotransfection of both PGC-1α and HNF-4α caused a dramatic activation (27). In similar assays, a synergism between PGC-1α and HNF-4 was clear in the promoters of CPT-Iα (28), CYP7A1 (29) and FXR genes (30), respectively, but to a much lesser extent than in G6Pase promoter. Our studies show that there is also a clear synergism between PGC-1α and HNF-4α in the activation of apoAV promoter, even stronger than those reported for other genes (*i.e.* FXR), but less dramatic than in G6Pase promoter. Whether other transcription factor besides HNF-4α could participate in recruiting PGC-1α to the apoAV promoter is under investigation. Additionally, because the hepatic expression of HNF-4α itself is induced after fasting and PGC-1α abundance is increased in fasting and diabetes (26, 30), it will be of great interest to examine the levels of human apoAV expression in these states.

The identification of apoAV as an HNF-4α target gene has serious implications in the regulation of TG levels by BAs. Several investigators have recently reported that in the liver BAs can regulate the transcription of genes involved in lipoprotein and TG metabolism via a pathway involving FXR and the small heterodimer partner (SHP) (53–56). BAs down-regulate HNF-4α target genes by reducing the expression of HNF-4α itself (53). In addition, BAs reduce MTP and apoB mRNA levels because of increased expression of the FXR target gene SHP, which in turn suppresses HNF-4α activity (53). Notably, BAs suppress hepatic expression of apoCIII, an inhibitor of LPL-mediated lipolysis, via an active repression by FXR and displacement of HNF-4α from the DNA site (54). On the other hand, BAs induce the expression of the HNF-4α target apoCII, an obligate cofactor for LPL, through two IR1 FXR response elements (55). Thus, one may speculate that BAs would repress HNF-4α target genes if no positive BA response elements were present. We have previously reported that the apoAV IR8 element is a positive FXR response element (10). However, we observed that apoAV mRNA levels were not modified by FXR agonists in HepG2, thus suggesting that the regulation of apoAV expression by BAs was not straightforward (10). It is possible that the role of IR8 as a positive FXR element is to counteract a negative BA-triggered signal through HNF-4α, thereby avoiding the down-regulation of apoAV, an activator of LPL-mediated hydrolysis of VLDL-TG.

Finally, while performing this study, a genome-scale location analysis using ChIP assays and DNA microarrays containing 700-bp promoter regions of 13,000 human genes identified the apoAV gene promoter bound by HNF-4α in human hepatocytes (57), further confirming our results.

In conclusion, here we demonstrate that human apoAV is *de facto* an HNF-4α target gene. Our data provide new insights into the binding properties of HNF-4α, recognize HNF-4α as a highly regulated factor that is involved in multiple hormonal and nutritional signaling pathways, and underscore the physiological role of HNF-4α in TG metabolism.

MATERIALS AND METHODS

Plasmids

Constructs p-2455/+18hAvLUC, p-617/+18hAvLUC, p-535/+18hAvLUC, p-437/+18hAvLUC, p-242/+18hAvLUC and p-82/+18hAvLUC containing the corresponding sequences of the 5′ flanking region of human apoAV gene cloned in front of the promoter-less firefly (*Photinus pyralis*) luciferase gene have been previously described (10). Site-directed mutagenesis of the construct p-617/+18hAvLUC was performed using the QuikChange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the recommendations of the manufacturer. For mutation of the DR1 element, the oligonucleotides JCR169 and JCR170 were used to generate a preliminary construct, and then a second site-directed mutagenesis was accomplished with the primers JCR188 and JCR189 (see Table 1). The IR8 site was mutated using the oligonucleotides JCR167 and JCR168. Plasmids expressing human cDNAs for HNF-4α and PGC-1α, respectively, were provided by J. M. Maglich (GlaxoSmithKline, Research Triangle Park, NC). The backbone of those plasmids was the mammalian expression vector pSG5 with a modified polylinker. Plasmid DNA was prepared using the QIAGEN (Valencia, CA) endotoxin-free maxipreparation method, and quantitated spectrophotometrically. The integrities of all plasmids were verified by DNA sequencing.

Cell Transfection and Reporter Assays

Human hepatoblastoma Hep3B and monkey kidney CV-1 cell lines were cultured in Eagle's basal medium supplemented with nonessential amino acids, 2 mM L-glutamine, 100 U/ml

penicillin, 100 μg/ml streptomycin sulfate, and 10% (vol/vol) fetal calf serum (medium A). On d 0, cells were seeded on 24-well plates at a density of 3×10^5 or 5×10^4 cells/well for Hep3B or CV-1, respectively. On d 1, cells were transfected with FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Typically, each well of a 24-well plate received 100 ng of firefly luciferase reporter plasmid and, when indicated, 0–200 ng of plasmids expressing human HNF-4α or PGC-1α. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pSG5. A total of 100 ng/well of a sea pansy (*Renilla reniformis*) luciferase plasmid pRL-TK (Promega, Madison, WI) was included in all transfections as an internal control for transfection efficiency. On d 3, cell lysates were prepared by shaking the cells in 200 μl of 1× Promega Lysis Buffer for 15 min at room temperature. Firefly and *Renilla* luciferase activities were measured using a Dual-Luciferase Reporter Assay System (Promega) and a Lumistar luminometer (BMG Lab Technologies, Offenburg, Germany). Firefly luciferase activity values were divided by *Renilla* luciferase activity values to obtain normalized luciferase activities. To facilitate comparisons within a given experiment, activity data were presented either as relative luciferase activities or as fold induction over the normalized activity of the reporter plasmid in the absence of nuclear receptor cotransfection. All transfection experiments were performed at least three times, and with each experimental point done in triplicate. The data are expressed as the means ± SD. Statistic significance analyses were done with Student's *t* test.

RNA Interference

Twenty-one-nucleotide RNA oligonucleotides targeting the human HNF-4α mRNA (5′-AACCACAUGUACUCCUGCAGA-3′) were chemically synthesized and supplied in the 2′-deprotected, annealed, and desalted form by Dharmacon (Lafayette, CO). Nonsilencing control siRNA was provided by QIAGEN (see Table 1). The sequence of the specific siRNA duplex was designed according to the manufacturer's recommendations and subjected to a basic local assignment and search tool search against the human genome sequence to ensure that non-HNF-4α genes were not targeted. On d 0, human hepatoblastoma HepG2 cells were seeded on 24-well plates at a density of 3×10^5 cells/well in medium A. On d 1, cells were transfected with siRNAs by using TansIT-TKO reagent (Mirus, Madison, WI) following the manufacturer's instructions. Twenty-four hours after transfection, cells were refed with fresh medium A. On d 3, the cells were washed twice with PBS and harvested for isolation of RNA or Western analysis.

Treatment of HepG2

On d 0, human hepatoblastoma HepG2 cells were plated on 24-well plates at 3.5×10^5 in medium A. On d 1, the cells were refed with the corresponding fresh media and vehicle (Me_2SO), 20 μM PD98059 (Sigma, St. Louis, MO) or AICAR (Toronto Research Chemicals, Toronto, Ontario, Canada; A611700) as indicated. On d 2, the cells were washed twice with PBS and harvested for isolation of RNA or Western analysis.

Real-Time PCR Quantification of mRNAs

Total RNA was prepared from primary HepG2 cells, and real-time RT-PCRs were performed as previously described with minor modifications (10). The reactions contained, in a final volume of 20 μl, 4 μl of diluted (1:10) cDNA, a 300 nM concentration of the forward and reverse primers, and 2× SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Real-time PCRs were carried out in 384-well plates by using the ABI PRISM 7900 sequence detection system (Applied Biosystems). Levels of HNF-4α, apoCIII, apoAV, apoAI, and LDLr were normalized to cyclophilin to compensate for variations in input RNA amounts (cyclophilin levels were unaffected by the treatments). All points were performed in triplicate during two independent experiments, and the RT-PCRs were carried out in duplicate for each sample. The relative amounts of mRNAs were calculated using the comparative threshold method.

Nuclear Extracts

On d 0, HepG2 cells were seeded on 100-mm diameter dishes at a density of 1.5×10^7 cells/dish in medium A. On d 1, the cells were treated with vehicle (Me_2SO), 20 μM PD98059, or 2.5 mM AICAR in fresh medium A. On d 2, the cells were washed twice with PBS and harvested in 1.5 ml of ice-cold PBS. The extraction of DNA-binding proteins was performed essentially as described (58). Briefly, cells were pelleted at 4 C and resuspended in 400 μl of hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 10 μl/ml Protease Inhibitor Cocktail from Sigma]. The cells were allowed to swell on ice for 10 min, and then vortexed for 10 sec and centrifuged at 4 C for 1 min. The pellet was resuspended in 100 μl of extraction buffer [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 10 μl/ml Protease Inhibitor Cocktail from Sigma] and incubated on ice for 20 min. Cellular debris was removed by centrifugation for 2 min at 4 C and 2.5 μl of the supernatant fraction (containing 4 μg/μl nuclear proteins) were used for EMSAs.

In Vitro Transcription/Translation and EMSAs

Human HNF-4α protein was synthesized *in vitro* from the expression plasmid in rabbit reticulocyte lysate by using TNT Quick Coupled transcription/translation system (Promega) according to the instructions of the manufacturer. To obtain an unprogrammed lysate as a negative control for EMSA, a reaction was performed with the empty vector pSG5. Double-stranded oligonucleotides were radiolabeled by fill in with the Klenow fragment of DNA polymerase I and used as probes. For competition experiments, increasing fold molar excesses of unlabeled probes were included during a 15-min preincubation on ice. Protein-DNA binding assays were performed as described (10). Supershift reactions were carried out by including 2 μl of HNF-4α antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; SC-6556X). AvDR1 is a double-stranded oligonucleotide corresponding to nt −275 to −247 of human apoAV promoter. The probe AvIR8 contains the sequence spanning nt −109 to −80 of the human apoAV promoter. The mutations present in the modified versions of AvDR1 (mt1 AvDR1, mt2 AvDR1, mt3 AvDR1 and mt4 AvDR1) and AvIR8 (mt1 AvIR8, mt2 AvIR8, mt3 AvIR8, pft AvIR8 and pft tttIR8) are shown in Table 1 and Fig. 4A. The control probe used in EMSA with HNF-4α and several fragments of apoAV promoter contains the PPARα/HNF-4α site of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mitHMGS) gene promoter (59, 60). The probe used as a control for assays with nuclear extracts and competition experiments corresponds to the nt −90 to −68 of human apoCIII gene promoter containing a known HNF-4α-binding element (39). Samples were electrophoresed at 4 C on a 4.5% polyacrylamide gel in 0.5× TBE buffer [45 mM Tris, 45 mM boric acid, 1 mM EDTA (pH 8.0)]. Gels were dried and analyzed using a PhosphorImager STORM 860 and ImageQuant software (Amersham Biosciences, Buckinghamshire, UK).

ChIP Assay

DNA immunoprecipitation assay was performed according to the ChIP protocols of Upstate Biotechnology (Lake Placid, NY) with minor modifications. Briefly, on d 0 HepG2 cells were seeded on 10-cm culture dishes at a density of 8.5×10^6 cells/dish in medium A. On d 1, cells were transfected with 7 μg of wild-type luciferase reporter plasmid p-242/+18hAvLUC (described in Ref. 10), an equivalent construct harboring mutations within IR8 site (see site-directed mutagenesis described in *Plasmids*), or pGL3-basic vector. Sixteen hours after transfection, the cells were washed twice with PBS and protein-DNA complexes were fixed by 1% formaldehyde for 10 min at 37 C in PBS. Cells were washed twice with PBS with protease inhibitors (Protease Inhibitor Cocktail from Sigma) and then collected in ice-cold PBS with protease inhibitors. Cell pellets were obtained by centrifugation at 1000 × *g* in PBS for 4 min, resuspended in 1 ml of hypotonic buffer [50 mM Tris-HCl (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40, 10 μl/ml Protease Inhibitor Cocktail from Sigma], and incubated on ice for 10 min. Nuclei were isolated by centrifugation at 2000 × *g* for 10 min, then resuspended in 1 ml of sodium dodecyl sulfate (SDS)-containing sonication buffer [0.01% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)] with protease inhibitors and incubated on ice for 10 min. Lysates were sonicated for four cycles of 10 sec each. Debris was removed from the samples by centrifugation at 14,000 × *g* for 10 min. Supernatants were collected and diluted 5-fold in ChIP dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl] with protease inhibitors, divided into aliquots of 1 ml and precleared with salmon sperm DNA preabsorbed protein A-Agarose slurry (Upstate Biotechnology) at 4 C for 1 h with rotation. An aliquot was removed as a control (input) and stored at 4 C until decross-linking. Immunoprecipitations were performed overnight with rotation at 4 C with either 4 μg of anti-HNF-4α (Santa Cruz; SC-6556) or 4 μg of anti-PPARα (Abcam, Cambridge, UK; ab-2779) antibodies for each aliquot. Immunoprecipitated complexes were collected with the addition of protein A-Agarose slurry followed by sequential washes for 5 min each time in low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], high-salt wash buffer (same as the low-salt buffer but with 500 mM NaCl), LiCl buffer [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], and Tris-EDTA buffer. Precipitates were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 30 min with rotation. The cross-linking of protein-DNA complexes in precipitates and inputs was reversed by incubation with 5 M NaCl at 65 C for 4 h. DNA-associated proteins were digested with proteinase K (QIAGEN) for 1 h at 45 C. The DNA was extracted with a QIAquick PCR purification kit (QIAGEN) and one tenth of purified sample was subjected to PCR amplification with a forward primer corresponding to a sequence upstream of IR8 in human apoAV promoter and a reverse primer corresponding to a sequence in the pGL3-basic vector (Table 1). PCR products (one fifth for input) were resolved by 1% agarose-ethidium bromide gel electrophoresis and visualized by UV.

Western Blot Analysis

Whole cell lysates were prepared by shaking the cells from in 100 μl/well lysis buffer (PBS, 1% Triton, 50 mM NaF, 5 mM sodium pyrophosphate, 10 μl/ml Protease Inhibitor Cocktail from Sigma) for 30 min at 4 C. The lysates were centrifuged at $10^3 \times g$ at 4 C for 5 min. Equal amounts of protein (40 μg) from supernatant were run on 10% polyacrylamide 3[*N*-morpholino]propanesulfonic acid NuPAGE Nobex gels (Invitrogen Life Technologies, Carlsbad, CA) and transferred onto nitrocellulose membranes in NuPAGE transfer buffer (Invitrogen Life Technologies). Membranes were preincubated for 1 h at room temperature in blocking buffer, 5% nonfat dry milk in TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (vol/vol) Tween 20]. Subsequently, the blots were incubated overnight at 4 C with rabbit antihuman apoAV (8), rabbit antiactin (Sigma; A5060), or goat anti-HNF-4α (Santa Cruz; SC-6556) antibodies in blocking buffer. For phosphorylated acetyl-CoA carboxylase, lysates were run on 7.5% polyacrylamide gels, and blot was incubated with rabbit antiphosphoacetyl-CoA carboxylase (Ser79) antibody (Upstate Biotechnology). After washing five times in TBST for 5 min, blots were incubated with donkey horseradish-peroxidase-conjugated antirabbit IgG antibody (Amersham Biosciences) or mouse horseradish-peroxidase-conjugated antigoat IgG antibody (Pierce Biotechnology, Rockford, IL) for 1 h at room temperature. Finally, blots were washed as above and immunoreactive bands were detected by ECL (Amersham Biosciences). ApoAV contents were quantitated and normalized to actin using a densitometer and ImageQuant software (Amersham Biosciences). Specimens of conditionally HNF-4α-null mice (16) and controls (n = 5/group) were generously provided by Dr. Y. Inoue (National Institutes of Health, Bethesda, MD). Livers were homogenized in PBS containing a protease inhibitor cocktail (Roche), and equal amounts of proteins were pooled. Two hundred micrograms of protein from liver homogenates and 2–5 μl of pooled sera were separated on denaturing polyacrylamide gels. After transfer to polyvinylidene difluoride membrane (Millipore, Billerica, MA), proteins were visualized as described above by using anti-HNF-4α (SC-6556), antirat apoAV (3) and antimouse apoAII (Biodesign, Saco, ME) antibodies.

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REFERENCES

1. Cullen, P 2000 Evidence that triglycerides are an independent coronary heart disease risk factor. Am J Cardiol 86:943–949
2. Pennacchio LA, Olivier M, Hubacek JA, Cohen JC, Cox DR, Fruchart JC, Krauss RM, Rubin EM 2001 An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science 294: 169–173
3. van der Vliet HN, Sammels MG, Leegwater AC, Levels JH, Reitsma PH, Boers W, Chamuleau RA 2001 Apolipoprotein A-V: a novel apolipoprotein associated with an early phase of liver regeneration. J Biol Chem 276: 44512–44520
4. van der Vliet HN, Schaap FG, Levels JH, Ottenhoff R, Looije N, Wesseling JG, Groen AK, Chamuleau RA 2002 Adenoviral overexpression of apolipoprotein A-V reduces serum levels of triglycerides and cholesterol in mice. Biochem Biophys Res Commun 295:1156–1159

5. Baroukh N, Bauge E, Akiyama J, Chang J, Afzal V, Fruchart JC, Rubin EM, Fruchart-Najib J, Pennacchio LA 2004 Analysis of apolipoprotein A5, C3, and plasma triglyceride concentrations in genetically engineered mice. Arterioscler Thromb Vasc Biol 24:1297–1302
6. Pennacchio LA, Olivier M, Hubacek JA, Krauss RM, Rubin EM, Cohen JC 2002 Two independent apolipoprotein A5 haplotypes influence human plasma triglyceride levels. Hum Mol Genet 11:3031–3038
7. Lai CQ, Tai ES, Tan CE, Cutter J, Chew SK, Zhu YP, Adiconis X, Ordovas JM 2003 The APOA5 locus is a strong determinant of plasma triglyceride concentrations across ethnic groups in Singapore. J Lipid Res 44: 2365–2373
8. Schaap FG, Rensen PC, Voshol PJ, Vrins C, van der Vliet HN, Chamuleau RA, Havekes LM, Groen AK, van Dijk KW 2004 ApoAV reduces plasma triglycerides by inhibiting very low density lipoprotein-triglyceride VLDL-TG production and stimulating lipoprotein lipase-mediated VLDL-TG hydrolysis. J Biol Chem 279:27941–27947
9. Vu-Dac N, Gervois P, Jakel H, Nowak M, Bauge E, Dehondt H, Staels B, Pennacchio LA, Rubin EM, Fruchart-Najib J, Fruchart JC 2003 Apolipoprotein A5, a crucial determinant of plasma triglyceride levels, is highly responsive to peroxisome proliferator-activated receptor α activators. J Biol Chem 278:17982–17985
10. Prieur X, Coste H, Rodríguez JC 2003 The human apolipoprotein AV gene is regulated by peroxisome proliferator-activated receptor-α and contains a novel farnesoid X-activated receptor response element. J Biol Chem 278:25468–25480
11. Jakel H, Nowak M, Moitrot E, Dehondt H, Hum DW, Pennacchio LA, Fruchart-Najib J, Fruchart JC 2004 The liver X receptor ligand T0901317 down-regulates APOA5 gene expression through activation of SREBP-1c. J Biol Chem 279:45462–45469
12. Khovidhunkit W, Duchateau PN, Medzihradszky KF, Moser AH, Naya-Vigne J, Shigenaga JK, Kane JP, Grunfeld C, Feingold KR 2004 Apolipoproteins A-IV and A-V are acute-phase proteins in mouse HDL. Atherosclerosis 176:37–44
13. Sladek FM, Zhong WM, Lai E, Darnell Jr JE 1990 Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev 4:2353–2365
14. Drewes T, Senkel S, Holewa B, Ryffel GU 1996 Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. Mol Cell Biol 16:925–931
15. Li J, Ning G, Duncan SA 2000 Mammalian hepatocyte differentiation requires the transcription factor HNF-4α. Genes Dev 14:464–474
16. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ 2001 Hepatocyte nuclear factor 4α (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol Cell Biol 21:1393–1403
17. Yamagata K, Furuta H, Oda N, Kaisaki PJ, Menzel S, Cox NJ, Fajans SS, Signorini S, Stoffel M, Bell G I 1996 Mutations in the hepatocyte nuclear factor-4α gene in maturity-onset diabetes of the young (MODY1) Nature 384:458–460
18. Jiang G, Nepomuceno L, Hopkins K, Sladek FM 1995 Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. Mol Cell Biol 15:5131–5143
19. Carter ME, Gulick T, Moore DD, Kelly DP 1994 A pleiotropic element in the medium-chain acyl coenzyme A dehydrogenase gene promoter mediates transcriptional regulation by multiple nuclear receptor transcription factors and defines novel receptor-DNA binding motifs. Mol Cell Biol 14:4360–4372
20. Fraser JD, Martinez V, Straney R, Briggs MR 1998 DNA binding and transcription activation specificity of hepatocyte nuclear factor 4. Nucleic Acids Res 26:2702–2707
21. Hertz R, Magenheim J, Berman I, Bar-Tana J 1998 DNA binding and transcription activation specificity of hepatocyte nuclear factor 4. Nature 392:512–516
22. Petrescu AD, Hertz R, Bar-Tana J, Schroeder F, Kier AB 2002 Ligand specificity and conformational dependence of the hepatic nuclear factor-4α (HNF-4α). J Biol Chem 277:23988–23999
23. Bogan AA, Dallas-Yang Q, Ruse Jr MD, Maeda Y, Jiang G, Nepomuceno L, Scanlan TS, Cohen FE, Sladek FM 2000 Analysis of protein dimerization and ligand binding of orphan receptor HNF4α. J Mol Biol 302:831–851
24. Ruse Jr MD, Privalsky ML, Sladek FM 2002 Competitive cofactor recruitment by orphan receptor hepatocyte nuclear factor 4α1: modulation by the F domain. Mol Cell Biol 22:1626–1638
25. Duda K, Chi YI, Shoelson SE 2004 Structural basis for HNF-4α activation by ligand and coactivator binding. J Biol Chem 279:23311–23316
26. Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, Spiegelman B M 2001 Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. Nature 413:131–138
27. Rhee J, Inoue Y, Yoon JC, Puigserver P, Fan M, Gonzalez FJ, Spiegelman BM 2003 Regulation of hepatic fasting response by PPARγ coactivator-1α (PGC-1): requirement for hepatocyte nuclear factor 4α in gluconeogenesis. Proc Natl Acad Sci USA 100:4012–4017
28. Louet JF, Hayhurst G, Gonzalez FJ, Girard J, Decaux JF 2002 The coactivator PGC-1 is involved in the regulation of the liver carnitine palmitoyltransferase I gene expression by cAMP in combination with HNF4 α and cAMP-response element-binding protein (CREB). J Biol Chem 277:37991–8000
29. Shin DJ, Campos JA, Gil G, Osborne TF 2003 PGC-1α activates CYP7A1 and bile acid biosynthesis. J Biol Chem 278:50047–50052
30. Zhang Y, Castellani LW, Sinal CJ, Gonzalez FJ, Edwards PA 2004 Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) regulates triglyceride metabolism by activation of the nuclear receptor FXR. Genes Dev 18:157–169
31. Reddy S, Yang W, Taylor DG, Shen X, Oxender D, Kust G, Leff T 1999 Mitogen-activated protein kinase regulates transcription of the ApoCIII gene. Involvement of the orphan nuclear receptor HNF4. J Biol Chem 274: 33050–33056
32. Leclerc I, Lenzner C, Gourdon L, Vaulont S, Kahn A, Viollet B 2001 Hepatocyte nuclear factor-4α involved in type 1 maturity-onset diabetes of the young is a novel target of AMP-activated protein kinase. Diabetes 50: 1515–1521
33. Hong YH, Varanasi US, Yang W, Leff T 2003 AMP-activated protein kinase regulates HNF4α transcriptional activity by inhibiting dimer formation and decreasing protein stability. J Biol Chem 278:27495–27501
34. Zannis VI, Kan HY, Kritis A, Zanni EE, Kardassis D 2001 Transcriptional regulatory mechanisms of the human apolipoprotein genes in vitro and in vivo. Curr Opin Lipidol 12:181–207
35. Naiki T, Nagaki M, Shidoji Y, Kojima H, Imose M, Kato T, Ohishi N, Yagi K, Moriwaki H 2002 Analysis of gene expression profile induced by hepatocyte nuclear factor 4α in hepatoma cells using an oligonucleotide microarray. J Biol Chem 277:14011–14019
36. Fraser JD, Keller D, Martinez V, Santiso-Mere D, Straney R, Briggs MR 1997 Utilization of recombinant adenovirus and dominant negative mutants to characterize hepatocyte nuclear factor 4-regulated apolipoprotein AI and CIII expression. J Biol Chem 272:13892–13898

37. Harnish DC, Malik S, Kilbourne E, Costa R, Karathanasis SK 1996 Control of apolipoprotein AI gene expression through synergistic interactions between hepatocyte nuclear factors 3 and 4. J Biol Chem 271:13621–13628
38. Nakshatri H, Bhat-Nakshatri P 1998 Multiple parameters determine the specificity of transcriptional response by nuclear receptors HNF-4, ARP-1, PPAR, RAR and RXR through common response elements. Nucleic Acids Res 26:2491–2499
39. Mietus-Snyder M, Sladek FM, Ginsburg GS, Kuo CF, Ladias JA, Darnell Jr JE, Karathanasis SK 1992 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. Mol Cell Biol 12:1708–1718
40. Vega RB, Huss JM, Kelly DP 2000 The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor α in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Mol Cell Biol 20:1868–1876
41. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR 1995 A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 92: 7686–7689
42. Kumar A, Chambers TC, Cloud-Heflin BA, Mehta KD 1997 Phorbol ester-induced low density lipoprotein receptor gene expression in HepG2 cells involves protein kinase C-mediated p42/44 MAP kinase activation. J Lipid Res 38:2240–2248
43. Corton JM, Gillespie JG, Hawley SA, Hardie DG 1995 5-Aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells. Eur J Biochem 229:558–565
44. Lehto M, Bitzen PO, Isomaa B, Wipemo C, Wessman Y, Forsblom C, Tuomi T, Taskinen MR, Groop L 1999 Mutation in the HNF-4α gene affects insulin secretion and triglyceride metabolism. Diabetes 48:423–425
45. Shih DQ, Dansky HM, Fleisher M, Assmann G, Fajans SS, Stoffel M 2000 Genotype/phenotype relationships in HNF-4α/MODY1: haploinsufficiency is associated with reduced apolipoprotein (AII), apolipoprotein (CIII), lipoprotein(a), and triglyceride levels. Diabetes 49:832–837
46. Kumar A, Middleton A, Chambers TC, Mehta KD 1998 Differential roles of extracellular signal-regulated kinase-1/2 and p38(MAPK) in interleukin-1β- and tumor necrosis factor-α-induced low density lipoprotein receptor expression in HepG2 cells. J Biol Chem 273:15742–15748
47. Wang B, Cai SR, Gao C, Sladek FM, Ponder KP 2001 Lipopolysaccharide results in a marked decrease in hepatocyte nuclear factor 4α in rat liver. Hepatology 34: 979–989
48. Leff T 2003 AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. Biochem Soc Trans 31:224–227
49. Kamiya A, Inoue Y, Gonzalez FJ 2003 Role of the hepatocyte nuclear factor 4α in control of the pregnane X receptor during fetal liver development. Hepatology 37: 1375–1384
50. Chan J, Nakabayashi H, Wong NC 1993 HNF-4 increases activity of the rat Apo A1 gene. Nucleic Acids Res 21:1205–1211
51. Murao K, Bassyouni H, Taylor AH, Wanke IE, Wong NC 1997 Hepatocyte nuclear factor 4 inhibits the activity of site A from the rat apolipoprotein AI gene. Biochemistry 36:301–306
52. Zhang Y, Ma K, Song S, Elam MB, Cook GA, Park EA 2004 Peroxisomal proliferator-activated receptor-γ coactivator-1 α (PGC-1α) enhances the thyroid hormone induction of carnitine palmitoyltransferase I (CPT-Iα). J Biol Chem 279:53963–53971
53. Hirokane H, Nakahara M, Tachibana S, Shimizu M, Sato R 2004 Bile acid reduces the secretion of very low density lipoprotein by repressing microsomal triglyceride transfer protein gene expression mediated by hepatocyte nuclear factor-4. J Biol Chem 279:45685–45692
54. Claudel T, Inoue Y, Barbier O, Duran-Sandoval D, Kosykh V, Fruchart J, Fruchart JC, Gonzalez FJ, Staels B 2003 Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. Gastroenterology 125: 544–555
55. Kast HR, Nguyen CM, Sinal CJ, Jones SA, Laffitte BA, Reue K, Gonzalez FJ, Willson TM, Edwards PA 2001 Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression. Mol Endocrinol 15:1720–1728
56. Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J 2004 Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. J Clin Invest 113:1408–1418
57. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI, Young RA 2004 Control of pancreas and liver gene expression by HNF transcription factors. Science 303:1378–1381
58. Andrews NC, Faller DV 1991 A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. Nucleic Acids Res 19:2499
59. Rodríguez JC, Gil-Gómez G, Hegardt FG, Haro D 1994 Peroxisome proliferator-activated receptor mediates induction of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by fatty acids. J Biol Chem 269: 18767–18772
60. Rodríguez JC, Ortiz JA, Hegardt FG, Haro D 1998 The hepatocyte nuclear factor 4 (HNF-4) represses the mitochondrial HMG-CoA synthase gene. Biochem Biophys Res Commun 242:692–696

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