Transcriptional regulation of the human hepatic lipase (*LIPC*) gene promoter

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Abstract **Hepatic lipase (HL) plays a key role in the metabolism of plasma lipoproteins, and its level of activity requires tight regulation, given the association of both low and high levels with atherosclerosis and coronary artery disease. However, little is known about the factors responsible for HL expression. Here, we report that the human hepatic lipase gene (*LIPC*) promoter is regulated by hepatocyte nuclear factor 4α (HNF4α), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), apolipoprotein A-I regulatory protein-1 (ARP-1), and hepatocyte nuclear factor 1α (HNF1α). Reporter analysis showed that HNF4α directly regulates the *LIPC* promoter via two newly identified direct repeat elements, DR1 and DR4. PGC-1α is capable of stimulating the HNF4α-dependent transactivation of the *LIPC* promoter. ARP-1 displaces HNF4α from the DR1 site and blocks its ability to activate the *LIPC* promoter. Induction by HNF1α requires the HNF1 binding site and upon cotransfection with HNF4α leads to an additive effect. In addition, the in vivo relevance of HNF4α in *LIPC* expression is shown by the ability of the HNF4α antagonist Medica 16 to repress endogenous *LIPC* mRNA expression. Furthermore, disruption of *Hnf4α* in mice prevents the expression of HL mRNA in liver. The overall effect these transcription factors have on HL expression will ultimately depend on the interplay between these various factors and their relative intracellular concentrations.**—Rufibach, L. E., S. A. Duncan, M. Battle, and S. S. Deeb. **Transcriptional regulation of the human hepatic lipase (*LIPC*) gene promoter. *J. Lipid Res.* 2006.** 47: **1463–1477.**

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Hepatic lipase (HL) is a 477 amino acid glycoprotein that plays an important role in lipoprotein metabolism. The majority of HL is synthesized and secreted by the liver, where it has been shown to act as both a lipase and a ligand. As a lipase, it catalyzes the hydrolysis of triglycerides and phospholipids of intermediate density lipoprotein remnants, large buoyant LDLs, and HDLs to form smaller, denser lipoprotein particles (1, 2). Studies in humans have shown an association between high HL activity and increased plasma concentrations of small, dense LDL and HDL particles, one of the major risk factors for coronary artery disease (3–5). As a ligand, HL contributes to the process of reverse cholesterol transport by participating with surface proteoglycans and the low density lipoprotein receptor like-protein in promoting hepatic uptake of lipoproteins, including remnant LDL and HDL particles (6–8), thus mediating the hepatic uptake of HDL-cholesteryl esters (9). The observed association of low HL activity with coronary artery disease might be attributable to decreased HL-enhanced remnant uptake by the liver (10). Together, these data show that HL is an important enzyme in lipid metabolism that must be highly regulated, because both low and high levels of HL activity appear associated with dyslipidemia.

The expression level of hepatic lipase activity varies widely in normal individuals (5- to 8-fold) and is influenced by genetic variation, obesity, gender, intracellular cholesterol, and lipid-lowering therapy (11). However, the signal transduction pathways and transcription factors that mediate HL regulation by these factors have not been elucidated. To date, only a few transcription factor binding sites have been identified in the hepatic lipase gene (*LIPC*) proximal promoter (**Fig. 1A**). The positive regulators include the upstream stimulatory factor (USF; −527 to −502) (12) and the hepatocyte nuclear factor 1α (HNF1α; −65 to −53) (13). The negative regulators include the estrogen receptor α (−1,557 to −1,175) (14), the activator protein-1 (−564 to −558) (15), and the farnesoid X receptor (FXR). The exact binding site for FXR has not

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Abbreviations: ARP-1, apolipoprotein A-I regulatory protein-1; ChIP, chromatin immunoprecipitation; COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; *CYP7A1*, cholesterol 7α-hydroxylase; DR, direct repeat; EMSA, electrophoretic mobility shift assay; FXR, farnesoid X receptor; HNF1α, hepatocyte nuclear factor 1α; HNF4α, hepatocyte nuclear factor 4α; *LIPC*, hepatic lipase gene; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; RARα, retinoic acid receptor α; RXRα, retinoid X receptor α; USF, upstream stimulatory factor; WCE, whole cell extract; WT, wild-type.

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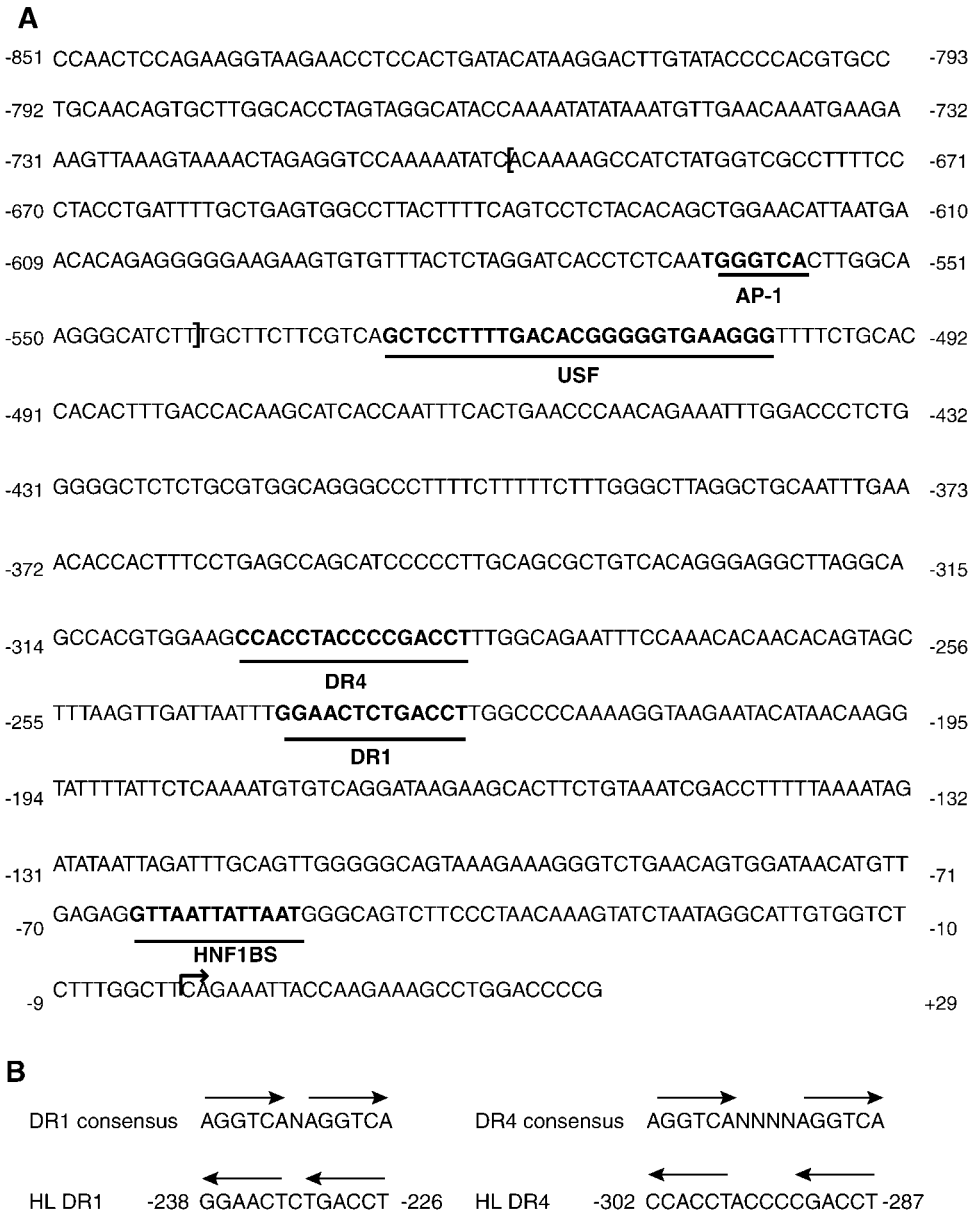

Fig. 1. Nucleotide sequence of the hepatic lipase gene (*LIPC*) proximal promoter region between nucleotides −851 and +29 and comparison of direct repeat 1 (DR1) or DR4 binding sites. A: *LIPC* proximal promoter, indicating the identified transcription factor binding sites (boldface and underlined or between brackets for farnesoid X receptor). The arrow designates the transcription start site. HNF1BS indicates the hepatocyte nuclear factor 1 (HNF1) binding site. AP-1, activator protein-1; USF, upstream stimulatory factor. B: Sequence comparison of the consensus DR1 and DR4 DNA elements and the *LIPC* wild-type (WT) DR1 (HL DR1) and DR4 (HL DR4) sites. Arrows indicate the location and direction of the DRs.

been elucidated, but it is located between −698 and −541 (16). USF, estrogen receptor α, and FXR have been confirmed functionally by transient transfection assays, whereas HNF1α and activator protein-1 are only predicted based on sequence, binding assays, and/or placement within DNase I footprints from hepatic nuclear extracts.

Upon further sequence examination of the proximal *LIPC* promoter, we identified two additional potential transcription factor binding sites between nucleotides −302 and −226 consisting of tandem inverted direct repeats (DRs) that conform to the consensus DNA response element half site 5′-RG(G/T)TCA-3′ (Fig. 1B). The functional importance of these DRs is suggested by the region's 78% sequence conservation between rat and human (17). The first element, located between −226 and −238, is a DR1 (DRs separated by 1 bp; Fig. 1B) and lies within DNase I footprints previously identified with hepatic nuclear extracts (13, 15). The second site is a DR4 (DRs separated by 4 bp; Fig. 1B) located between −287 and −302. DR1 and DR4 DNA response elements are known to bind various members of the nuclear receptor family as either homodimers or heterodimers. Possible DR1 and DR4 binding

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factors include retinoid X receptor α (RXRα), apolipoprotein A-I regulatory protein-1 (ARP-1), also know as the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), hepatocyte nuclear factor 4α (HNF4α), retinoic acid receptor α (RARα), peroxisome proliferator-activated receptor, liver X receptor, thyroid hormone receptor, and the constitutive androstane receptor (18–21).

Among the transcription factors mentioned above, RXRα, RARα, HNF1α, HNF4α, and ARP-1 are of particular interest because they have been shown to act together to regulate the expression of a number of genes involved in lipid metabolism, including the apolipoprotein gene promoters *APOA1/C3/A4* gene cluster, *APOA2*, and *APOB* (22–26). On a DR1 element, RXRα homodimers act as positive regulators in the presence of ligand (9-*cis*-retinoic acid), whereas RXRα-RARα heterodimers repress RXRα homodimer activation, in a ligand-dependent manner, by competition for binding to the DR1 element (27). On DR4 elements, RXRα binds as a heterodimer with various partners and acts as a transcriptional regulator (18). HNF4α is a liver-enriched transcription factor essential for hepatocyte differentiation and liver function (28, 29). Its disruption leads to embryonic lethality (30). Importantly, HNF4α expression has been shown to correlate with HL expression in several hepatic cells lines (17). HNF4α is a constitutively activated transcription factor whose ligand has been identified as tightly bound endogenous fatty acids (31–33). Recent analysis has suggested that binding of coactivators rather than ligand binding locks HNF4α into an active conformation (34).

Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a coactivator for numerous nuclear receptors that regulate a wide range of biological processes, including glucose and lipid metabolism (reviewed in 35). PGC-1α has been shown to stimulate HNF4α-mediated transactivation of the promoters for phosphoenolpyruvate carboxykinase (*PEPCK*), glucose-6-phosphatase (*G6Pase*), liver carnitine palmitoyltransferase I (*L-CPTI*), cholesterol 7α-hydroxylase (*CYP7A1*), *FXR*, and *APOA5* (36–41). ARP-1 is widely expressed in multiple tissues during embryonic development, and like *Hnf4α*, disruption of the *Arp-1* gene is embryonic lethal (42). HNF4α activates transcription by binding as a homodimer to DR1 sequences (43, 44), whereas ARP-1 can bind to either DR1 or DR4 and activate (45, 46) or repress (44) transcription by various direct and indirect actions (47). ARP-1 has also been shown to specifically influence transcriptional activation by HNF4α either negatively or positively, depending on the promoter context. ARP-1 interferes with the activation by HNF4α on the apolipoprotein gene promoters *APOA1* (48), *APOA2* (43), *APOB* (43), and *APOC3* (43, 49) by competing with HNF4α for occupancy of the DR1 binding site. Conversely, ARP-1 acts synergistically with HNF4α on the promoters of *CYP7A1* (20), *APOC2* (50), and *HNF1* (51) by protein-protein interactions through the ligand binding domain of HNF4α.

HNF1α is a homeodomain-containing transcription factor important for diverse metabolic functions in pancreatic islets, liver, intestine, and kidney (52, 53). It is enriched in the liver, where it transcriptionally modulates numerous liver-specific genes (54). That HNF1α plays a role in HL expression is suggested by the reduction of HL expression in HNF1α-deficient mice (53) and the association of mutations in *HNF1α* with variations in plasma lipoprotein metabolism (55). However, whether HNF1α plays a direct or indirect role in HL expression has not been elucidated. Lockwood and Frayling (56) used expression data from HNF1α-deficient mice to write software that searched for potential HNF1α binding sites 2 kb upstream of 28 genes downregulated in HNF1α-deficient mice compared with wild-type (WT) mice. This analysis identified *LIPC* as one of eight genes likely to be directly regulated by HNF1α. Similar to ARP-1, HNF1α can both negatively (57) and positively (58, 59) affect transcriptional regulation by HNF4α.

In this study, we investigated the potential regulation of the *LIPC* proximal promoter by these factors. We demonstrate that the *LIPC* proximal promoter is regulated by HNF4α, PGC-1α, HNF1α, and ARP-1 via the DR1, DR4, and HNF1 binding sites but is not regulated by RARα and RXRα. In addition, we demonstrate the importance of HNF4α for endogenous hepatic lipase expression in vivo.

EXPERIMENTAL PROCEDURES

Plasmids and cloning

Plasmid constructs containing the human *LIPC* proximal promoter segment from −851 to +29 were prepared by PCR amplification using total human genomic DNA as a template and the following primers: forward, 5′tcatgagctcCCAACTCCAGAAGGTAAGAACC3′; reverse, 5′tagtaagcttCGGGGTCCAGGCTTTCTTGG3′. The lowercase letters indicate non-*LIPC* sequences, and the underlined sequences are *Sst*I and *Hind*III cleavage sites, respectively. The restriction sites were used for directional cloning of the *LIPC* proximal promoter initially into the pXP1 vector. The pGL4-851 (WT) construct was made by excising the −851 to +29 *LIPC* proximal promoter region from pXP1 with *Xho*I and *Hind*III and cloning it into the pGL4 luciferase vector from Promega. Dr. Vassilis I. Zannis (Boston University Medical Center) kindly provided the expression vectors pMT2-ARP-1 and pMT2-HNF4α and the control empty vector pMT2, and Dr. Anastasia Kralli (Scripps Institute) provided the pcDNA3/HA-hPGC-1α expression vector (60). The pcDNA3.1-HNF1α expression plasmid was constructed by cloning the HNF1α cDNA, prepared by PCR from cDNA made from HuH7 mRNA, into the pcDNA3.1/V5-His TOPO TA expression vector (Invitrogen). The expressed HNF1α protein does not contain the V5 epitope or the His tag, because the natural stop codon for HNF1α is present in the cDNA clone. The sequences of all constructed plasmids were confirmed by sequencing in both orientations.

Site-directed mutagenesis of the *LIPC* WT promoter construct

All mutants were generated by site-directed mutagenesis using the Quikchange site-directed mutagenesis kit (Stratagene) and the following primers (boldface letters indicate transcription factor binding regions, and lowercase letters show mutated bases): DR1 mutant, 5′GCTTTAAGTTGATTAATTT**Gttcga**C**TttCga**TGGCCCCAAAAGG3′ and 5′CCTTTTGGGGCCA**tcGaaAGtcgaaC**AAATTAATCAACTTAAAGC3′; DR4 mutant,

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5′GCAGCCACGTGGAAG**CagtCgACCCCtAtgg**TTGGCAGAATTTCC3′ and 5′CGTCGGTGCACCTTC**GtcaGcTGGGGaTacc**AACCGTCTTAAAGG3′; HNF1 mutant, 5′GTGGATAACATGTTGAGAG**GTTAAgccggAAT**GGGCAGTCTTCCCTAACAAAGT3′ and 5′ACTTTGTTAGGGAAGACTGCCC**ATTccggcTTAAC**CTCTCAACATGTTATCCAC3′. Site-directed mutagenesis was performed according to the manufacturer's protocol. The sequences of all mutant promoter constructs were confirmed by sequencing in both orientations.

Cell culture and transient transfection assays

The human hepatoma HuH7 and African green monkey kidney COS7 cell lines were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc.).

For transient transfection assays, HuH7 cells were seeded at 1×10^5 cells/well in 24-well plates 24 h before transfection. Transfections in HuH7 cells were carried out using the Lipofectamine 2000 (Invitrogen) reagent in DMEM without fetal bovine serum at a 1:2.5 μg DNA/μl Lipofectamine ratio. A total of 600–850 ng of plasmid DNA was used per well, consisting of the appropriate promoter construct plasmid, 10 ng of phRG-TK renilla reporter vector (Promega), used to correct for differences in transfection efficiencies, and the appropriate combination of expression plasmids (see figure legends for the exact amounts used). The total amount of DNA was kept constant by supplementing with the pMT2 empty vector. The DMEM-transfection mixture was replaced 4–6 h after transfection with complete medium (listed above).

Luciferase activity was determined 24 h after transfection using the Dual-Luciferase kit (Promega). Briefly, the cells were lysed directly in the 24-well plate using 120 μl of 1× passive lysis buffer provided with the Dual-Luciferase kit. One hundred microliters of LARII reagent (firefly luciferase substrate) was added to 50 μl of lysis supernatant, and firefly luciferase activity was measured with a Lumat LB 9507 luminometer for 10 s. Then, 100 μl of Stop-n-Glo reagent (renilla luciferase substrate) was added to the reaction, and renilla luciferase activity was measured for an additional 10 s. Firefly luciferase activities were normalized by renilla activities to correct for differences in transfection efficiencies. All transfection experiments were repeated at least two times in triplicate. Statistical significance was analyzed by Student's *t*-test. Firefly luciferase activities normalized by renilla activities are presented as fold induction relative to the normalized firefly luciferase activity in cells transfected with the pMT2 empty vector only, which was taken as 1.0.

Preparation of whole cell and nuclear extracts

Whole cell extracts (WCEs) were prepared using transiently transfected COS7 cells in 10 cm plates using 10 μg of expression vector in the presence of 30 μl of Lipofectamine 2000 (Invitrogen). After 24 h, cells were washed and collected at 200 *g* in 40 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.15 M NaCl (43). Cells were lysed in 20 mM Tris-HCl, pH 7.4, 0.4 mM KCl, 2 mM DTT, and 20% (w/v) glycerol by three rounds of freezing and thawing in liquid nitrogen, essentially as described previously (61). Cell debris was removed by centrifugation at 4°C (10,000 *g*) for 15 min. Phenylmethylsulfonyl fluoride was added to the supernatant (WCE) to a final concentration of 1 mM, and aliquots were frozen at −70°C. Nuclear extracts from HuH7 cells were prepared using the CelLytic NuCLEAR extraction kit (Sigma-Aldrich, Inc.) according to the manufacturer's protocol. Protein concentrations of whole cell and nuclear extracts were determined using the 96-well format of the Coomassie Protein Assay kit (Pierce) and quantified using the SpectraCount plate reader (Packard).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed using the Gel Shift Assay system (Promega) at room temperature according to the manufacturer's protocol. Double-stranded synthetic probes (see **Fig. 2** for sequences of individual probes) were prepared by annealing equal amounts of complementary synthetic oligonucleotides with single-stranded 5′ overhangs (cgcg), as described previously (20). The resulting double-stranded fragments were labeled with (^{32}P)dCTP using the Klenow fragment of DNA polymerase I. MicroSpin G-25 spin columns (Amersham Pharmacia Biotech) were used to remove the unincorporated label. Unlabeled double-stranded oligonucleotides were used as cold competitors (see Fig. 2 for sequences of individual competitors). Specific antisera were added to the DNA binding reaction for "supershifting" of DNA transcription factor complexes. Antibodies against HNF4α (sc-6557X), RARα (sc-551X), and RXRα (sc-774X) were obtained from Santa Cruz Biotechnology, Inc., and anti-Coup-TF, which recognizes both ARP-1/Coup-TFII and Coup-TFI, was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine). One microliter of anti-HNF4α, anti-RARα, and anti-RXRα antibodies and 1 μl of diluted anti-CoupTF (1:10 dilution in 1 mg/ml BSA) was used in supershift assays. Competition and supershift assays were carried out by preincubating cold competitors and antisera with protein extracts for 20 min before adding the binding probe. After addition of the binding probe, incubation was continued for an additional 20 min. Binding reactions were electrophoresed on 4% polyacrylamide gels, dried, and autoradiographed using the Molecular Dynamics Storm 820 PhosphorImager.

The dissociation constant (K_d) values of HNF4α and ARP-1 were calculated from binding reactions performed with a constant amount of WCE from COS7 cells overexpressing either HNF4α or ARP-1 (0.5 or 1 μg, respectively) and increasing concentrations of radiolabeled *LIPC* DR1 probe (see Fig. 2 for probe sequence), as described previously (43). Binding reactions were incubated at room temperature for 30 min. After gel electrophoresis and PhosphorImager scanning, the bands corresponding to the bound and free oligonucleotides were quantified using ImageQuant 5.2 software (Molecular Dynamics).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ ChIP kit from Upstate. The HNF4α antibody (H171X, sc-8987X) and HNF1α antibodies (C19X, sc-6547X or H140, sc10791) were obtained from Santa Cruz Biotechnology, Inc. Briefly, HuH7 cells were seeded in 100 mm plates with 10 ml of medium and grown to 7×10^6 cells/plate. The HuH7 cells on each plate were cross-linked with 1% formaldehyde for 10 min at room temperature. Glycine was added to a final concentration of 0.125 M for 5 min at room temperature to quench the reaction. Each 100 mM plate was washed two times with 5 ml of 1× PBS plus protease inhibitors. Cells were then scraped from each plate into a microfuge tube and spun at 700 *g* at 4°C for 4 min. The cell pellet was resuspended in 350 μl of SDS lysis buffer containing protease inhibitors per 7×10^6 starting cells. Cell lysates were divided into two tubes and sonicated at 30% amplitude for 10 pulses (10 s pulse followed by a 30 s pause) using a Branson model 102C sonicator. The sonicated cell lysate was spun at 12,000 *g* at 4°C for 10 min, and the supernatant was separated into 100 μl aliquots of $\sim 2 \times 10^6$ cell equivalents each.

One 100 μl aliquot (2×10^6 cell equivalents) was used per ChIP assay. Each 100 μl aliquot was diluted with 900 μl of ChIP dilution buffer plus protease inhibitors. To preclear the chromatin, 60 μl of protein A or G agarose (plus sonicated salmon sperm DNA) was added to each sample and incubated for 1 h at

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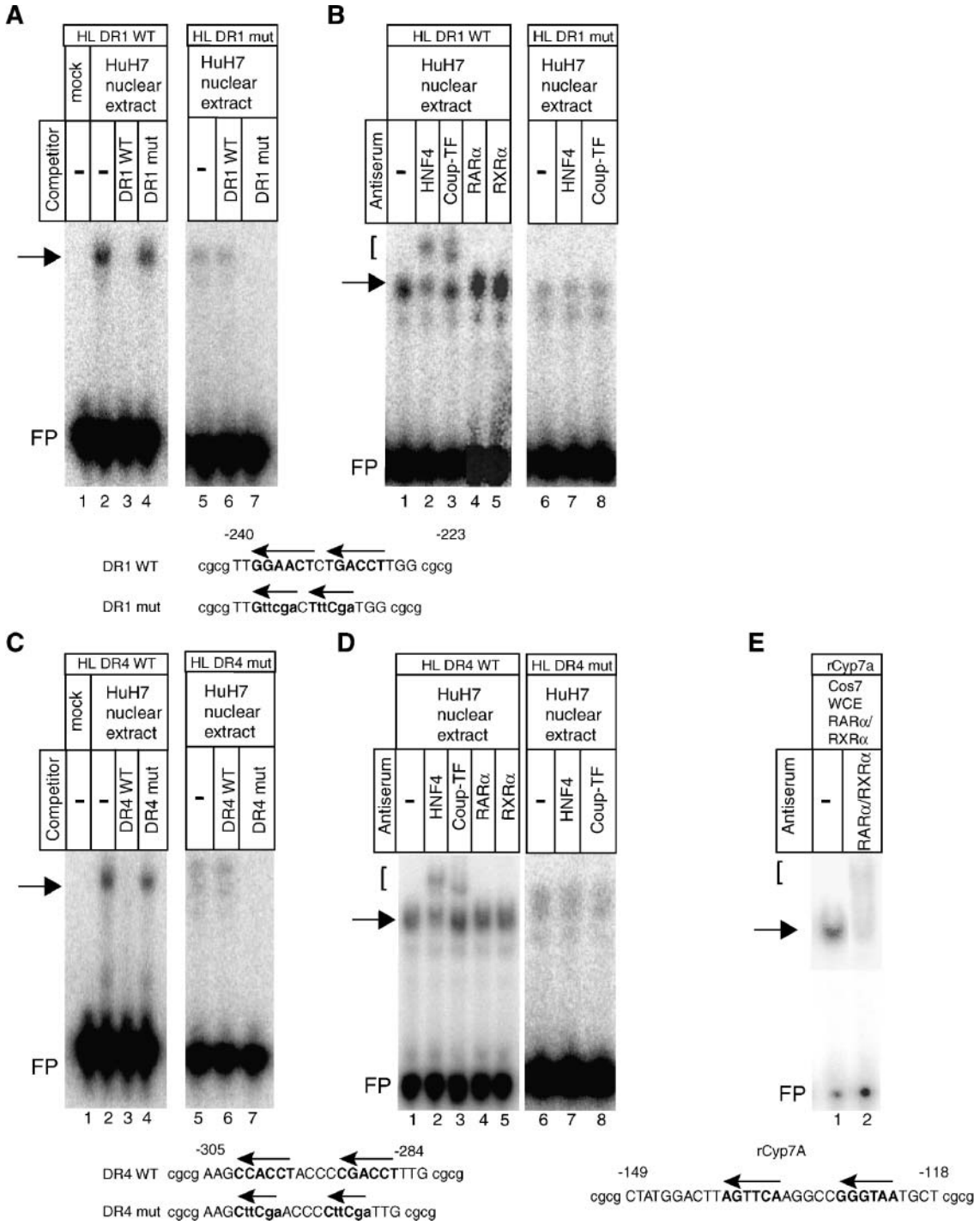

Fig. 2. Binding of nuclear extract proteins from HuH7 cells to the *LIPC* DR1 or DR4 WT and mutant sequences, and supershifting electrophoretic mobility shift assays (EMSAs) to identify factors present in the resulting DNA/protein complex. A, C: Autoradiographs of EMSA gels using no nuclear extract (mock) or 1 μg of nuclear extract proteins from HuH7 cells bound to a double-stranded WT or mutant HL DR1 (A) or HL DR4 (C) probe. Nuclear extracts were competed with and without a 250-fold molar excess of cold WT competitor (A or C, lanes 3 and 6) or mutant (mut) competitor (A or C, lanes 4 and 7), as specified above the lanes. B, D: Supershifting EMSA assays to identify factors present in the DNA/protein complex from the nuclear extracts of HuH7 cells. Nuclear proteins were bound to WT or mutant HL probes with and without antibodies against HNF4α, chicken ovalbumin upstream promoter transcription factor (COUP-TF) [apolipoprotein A-I regulatory protein-1 (ARP-1)], retinoic acid receptor α (RARα), and retinoid X receptor α (RXRα), as indicated above the lanes. E: Supershifting EMSA assay to show that antibodies against RXRα and RARα can detect RXRα/RARα binding to the known DR5-responsive element of the rat cholesterol 7α-hydroxylase promoter (rCyp7a). Arrows indicate the DNA/protein bands shifted by the protein extracts, brackets denote bands supershifted by the indicated antibodies, and FP designates the free probe. Under the autoradiographs are the sequences of the probes and cold competitors. Boldface letters and arrows indicate the two inverted half-sites. Uppercase letters represent WT sequences, and lowercase letters represent mutated bases.

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4°C with rotation. The beads were pelleted by centrifugation at 4,000 *g* for 1 min. The precleared chromatin was immunoprecipitated with no antibody, 10 μg of the appropriate antibody, or corresponding IgG and incubated with rotation at 4°C overnight. The protein/antigen/DNA complexes were collected by adsorption to 60 μl of protein A or G agarose (plus sonicated salmon sperm DNA) and incubated for 1 h at 4°C with rotation. The agarose beads were pelleted by brief centrifugation at 4,000 *g* for 1 min, and the supernatant fraction was removed. The beads were washed once with 1 ml of low-salt immune complex wash buffer, once with 1 ml of high-salt immune complex wash buffer, once with 1 ml of LiCl immune complex wash buffer, and twice with 1 ml of Tris-EDTA (TE) buffer. After every wash, the beads were pelleted by brief centrifugation at 4,000 *g* for 1 min and the supernatant fraction was removed. The protein/DNA complexes were eluted in 200 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$ in water) and reverse cross-linked at 65°C overnight by the addition of 8 μl of 5 M NaCl, 4 μl of 0.5 M EDTA, 8 μl of 1 M Tris-HCl, and 1 μl of proteinase K. RNA was eliminated by the addition of 1 μl of RNase A and incubation at 37°C for 30 min. The DNA was purified using the PCR purification kit from Qiagen and eluted in 50 μl of elution buffer (EB; Qiagen).

Immunoprecipitated DNA and input DNA (from reverse cross-linked and purified 2×10^6 cell equivalents) were analyzed by PCR using primers from sequences for the human *LIPC* proximal promoter DR1 region (DR1forward, 5′TTGGCAGAATTTCCAAACACAACAC3′; DR1reverse, 5′CCACTGTTCAGACCCTTTCTTTACTGC3′), the *LIPC* HNF1 binding region (HNF1 forward, 5′GCAGTTGGGGGCAGTAAAGAAAGG3′; HNF1 reverse, 5′GCTTTGTCCAAGGGCACTTGATTG3′), a region from HL exon 6 (HLexon6 forward, 5′CCATCACCCAGACCATAAAATGCT3′; HLexon6 reverse, 5′GACGTGGTAGCCCAGCGTGT3′), and primers surrounding the DR1 region of the human MTP promoter as a positive control for HNF4α binding (MTP forward, 5′CTGGTTTGGTTTAGCTCTC3′; MTP reverse, 5′GACCCTCTTCAGAACCTG3′) (58) or surrounding the human HNF4α proximal promoter as a positive control for HNF1α binding (HNF4α forward, 5′GGTGAGTCAAGGGTCAAATGAGTGC3′; HNF4α reverse, 5′CCTAGCCTCTGTGAAGGGGTGGAG3′) (62). Results were verified by real-time PCR using the primers listed above and SYBR Green PCR Master Mix (Applied Biosystems) on a 7500 Real-Time PCR System from Applied Biosystems. Values were assigned using a standard curve and normalized by human GAPDH values (GAPDH forward, 5′TACTAGCGGTTTTACGGGCG3′; GAPDH reverse, 5′TCGAACAGGAGGAGCAGAGAGCGA3′; Upstate).

Medica 16 treatment, RNA isolation, reverse transcription, and real-time PCR

HuH7 cells were seeded in six-well plates and, after reaching 60–70% confluence, were treated with 100, 250, or 400 μM MEDICA 16 (M16; Cayman Chemical) or the vehicle DMSO for 48 h. Total RNA was extracted 48 h after treatment using the TRIzol reagent (Invitrogen). Five hundred nanograms of each RNA preparation was reverse-transcribed to cDNA using the iScript cDNA synthesis kit from Bio-Rad. The cDNA from each preparation was diluted 1:50, and 2 μl of each dilution was used for real time PCR. Real-time PCR was performed on the 7500 Real-Time PCR System from Applied Biosystems using the Universal PCR Master Mix (Applied Biosystems) and primer/probe mixtures from Applied Biosystems for hGAPDH, hLIPC (hepatic lipase), or hAPOC3. The housekeeping gene GAPDH was used as an internal control for sample normalization. The mRNA values were calculated using a standard curve method and are presented as mRNA levels relative to the mock level, which was set at 1.0.

Detection of HL mRNA by RT-PCR in livers of mice with liver-specific targeted disruption of HNF4α

RNA from livers of the recently generated (29) mouse embryos whose livers lacked HNF4α (*Hnf4*$^{loxP/loxP}$ *Alfp.cre*) and control mouse embryos (*Hnf4*$^{loxP/+}$ *Alfp.cre*) were tested for the expression of HL. RT-PCR was carried out as described previously (28) using mouse HL primers (forward, 5′GCTGTCGTCTCAGACCTCAGC3′; reverse, 5′GAGCAGGATCAACTCGCCGATC3′) and primers for mouse Hprt, Hnf4α, aldolase B, and albumin (described in 28).

RESULTS

Binding of nuclear receptors to the DR4 and DR1 elements of the proximal *LIPC* gene promoter

Sequence evaluation of the proximal *LIPC* gene promoter revealed the presence of two inverted DR elements (Fig. 1A) that conform to the consensus DNA response element half-site 5′-RG(G/T)TCA-3′ (Fig. 1B). The first element was a DR1 located between −226 and −238. The second site was a DR4 located between −287 and −302. DR elements are known to bind a number of nuclear receptors, including HNF4α, ARP-1, RARα, and RXRα. Binding of these nuclear receptors to the HL DR1 and DR4 elements was evaluated by EMSA using nuclear extracts from the human hepatocarcinoma cell line, HuH7, which expresses all of these nuclear receptors as well as HL (Fig. 2). For DR1, significant amounts of a single DNA/protein complex were observed with nuclear extracts from HuH7 cells with the WT probe (Fig. 2A, lane 2) but not with the mutant DR1 probe (Fig. 2A, lane 5). The nuclear extract binding was specific to DR1, being effectively outcompeted by an excess of nonlabeled DR1 WT probe (Fig. 2A, lane 3) but not by an excess of nonlabeled DR1 mutant probe (Fig. 2A, lane 4). The DR1 DNA/protein complex from HuH7 nuclear extracts on the WT DR1 probe, but not the mutant DR1 probe, was shown by supershifting with specific antiserum to contain the nuclear receptors HNF4α and Coup-TF (ARP-1) (Fig. 2B, compare lanes 2, 3 with lanes 7, 8) but neither RARα nor RXRα (Fig. 2B, lanes 4, 5). Given that the anti-COUP-TF antibody used in this study does not discriminate between ARP-1/Coup-TFII and Coup-TFI, one or both COUP-TFs could be components of the DNA/protein complex. Similarly, nuclear extracts from HuH7 cells formed a single major complex with the WT probe (Fig. 2C, lane 2) but not the mutant DR4 probe (Fig. 2C, lane 5). Binding specificity for the DR4 element was shown by competition for binding by an excess of nonlabeled DR4 WT probe (Fig. 2C, lane 3) but not by an excess of the DR4 mutant probe (Fig. 2C, lane 4). The DR4 DNA/protein complex on the WT DR4, but not the mutant DR4 probe, was supershifted with antiserum specific for HNF4α and Coup-TF (ARP-1) (Fig. 2D, compare lanes 2, 3 with lanes 7, 8) but not by anti-RARα or anti-RXRα (Fig. 2D, lanes 4, 5). Figure 2E shows the binding and supershifting of RXRα and RARα to the *rCyp7a1* DR5 site, a known RXRα/RARα responsive element (19).

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Direct binding of HNF4α and ARP-1 to the DR1 and DR4 probes was next investigated using WCEs from the nonhepatic cell line COS7 overexpressing these recombinant transcription factors. COS7 cells were used because they express no HNF4α and only a small amount of ARP-1 (61, 63). EMSA and supershift assays with specific antisera confirmed the findings from HuH7 cells (**Fig. 3**). WCEs from COS7 cells overexpressing either HNF4α or ARP-1 bound to the DR1 (Fig. 3A, lanes 2–4 and 8–10) and DR4 (Fig. 3B, lanes 2–4 and 8–10) probes, and binding was effectively out-competed by an excess of nonlabeled WT probe (Fig. 3A, B, lanes 5, 11) but not by an excess of nonlabeled mutant probe (Fig. 3A, B, lanes 6, 12). In addition, the DNA/protein bands were supershifted by HNF4α (Fig. 3A, B, lane 3) or Coup-TF (ARP-1) (Fig. 3A, B, lane 9).

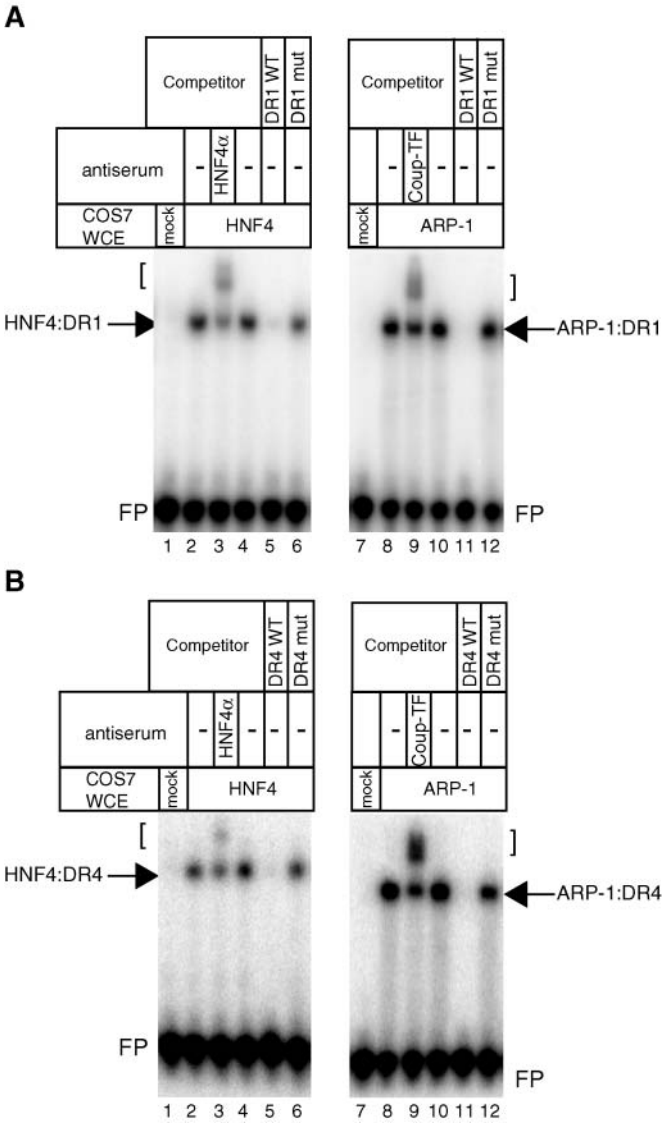

Fig. 3. Direct binding of HNF4α and ARP-1 to the DR1 and DR4 elements. Binding of whole cell extracts (WCEs) from COS7 cells transfected with empty vector (mock), HNF4α, or ARP-1 expression vectors to radiolabeled double-stranded HL DR1 (A) or HL DR4 (B) probe. EMSA assays were performed using 1 μg of WCEs expressing HNF4α or 0.2 μg of WCEs expressing ARP-1 with or without antibodies against HNF4α and COUP-TF (ARP-1), as specified above the lanes. Binding competitions were carried out as indicated in the presence of 250-fold excess of unlabeled double-stranded WT or mutant (mut) competitors. Arrows indicate the DNA/protein bands shifted by the WCEs, brackets denote the bands supershifted by the indicated antibodies, and FP designates the free probe. See Fig. 2 for sequences of probes and competitors.

Transcriptional regulation of the *LIPC* promoter by HNF4α, HNF1α, and ARP-1

Based on the EMSA data described above and the previously identified binding of HNF1α to the *LIPC* promoter (13), we investigated, by transient transfection assays, the ability of HNF4α, ARP-1, and HNF1α to modulate the expression of the *LIPC* promoter in HuH7 cells. HuH7 cells were cotransfected with the pGL4-851 (WT) luciferase reporter construct and vectors expressing HNF4α, HNF1α, or ARP-1 (**Fig. 4A**). Transfection of either HNF4α or HNF1α resulted in a 2.5-fold increase of *LIPC* promoter activity, whereas transfection with ARP-1 showed a slight, but statistically significant, repression of ~15%. Because both HNF1α and ARP-1 have been shown to affect transcriptional regulation by HNF4α, we also tested the effect of cotransfecting HNF4α, HNF1α, and ARP-1 in various combinations (Fig. 4A). Coexpression of HNF4α and HNF1α had an additive effect, whereas ARP-1 repressed induction of the *LIPC* promoter by HNF4α but not induction by HNF1α. These results provide functional support for the importance of these transcription factors in modulating *LIPC* expression.

Because both HNF4α and ARP-1 are known to bind to the DR1 site, the loss of *LIPC* induction by HNF4α upon cotransfection with ARP-1 could be the result of competition for DR1 binding. To test this hypothesis, transient transfection assays were performed with the pGL4-851 promoter construct and various ratio combinations of HNF4α and ARP-1 expression vectors (Fig. 4B). The results showed that increasing amounts of ARP-1 led to greater repression of pGL4-851 promoter construct expression. Conversely, ARP-1 repression of *LIPC* promoter induction by HNF4α was completely overcome by increasing amounts of transfected HNF4α expression vector. Interestingly, cotransfection of equal amounts of ARP-1 and HNF4α expression vectors did not increase the ARP-1 repression. Only when HNF4α was in excess compared with ARP-1 was the repression overcome.

Because HNF4α and ARP-1 appear to compete for transcriptional control of the *LIPC* promoter via the DR1 element, the relative binding affinities of each protein to the DR1 site were examined. Saturation binding assays were performed by incubating increasing concentrations of radiolabeled DR1 probe with a constant amount of WCEs from COS7 cells that were transiently transfected with HNF4α or ARP-1 expression vectors (Fig. 4C). The K_d values were determined from the Scatchard plots depicted in Fig. 4C in the panels at right. The results indicate that ARP-1 binds to the DR1 element with a much higher affinity (K_d = 1.5 nM) than HNF4α (K_d = 13.8 nM). This could explain way HNF4α needs to be in excess to outcompete ARP-1 for binding to DR1. These results support

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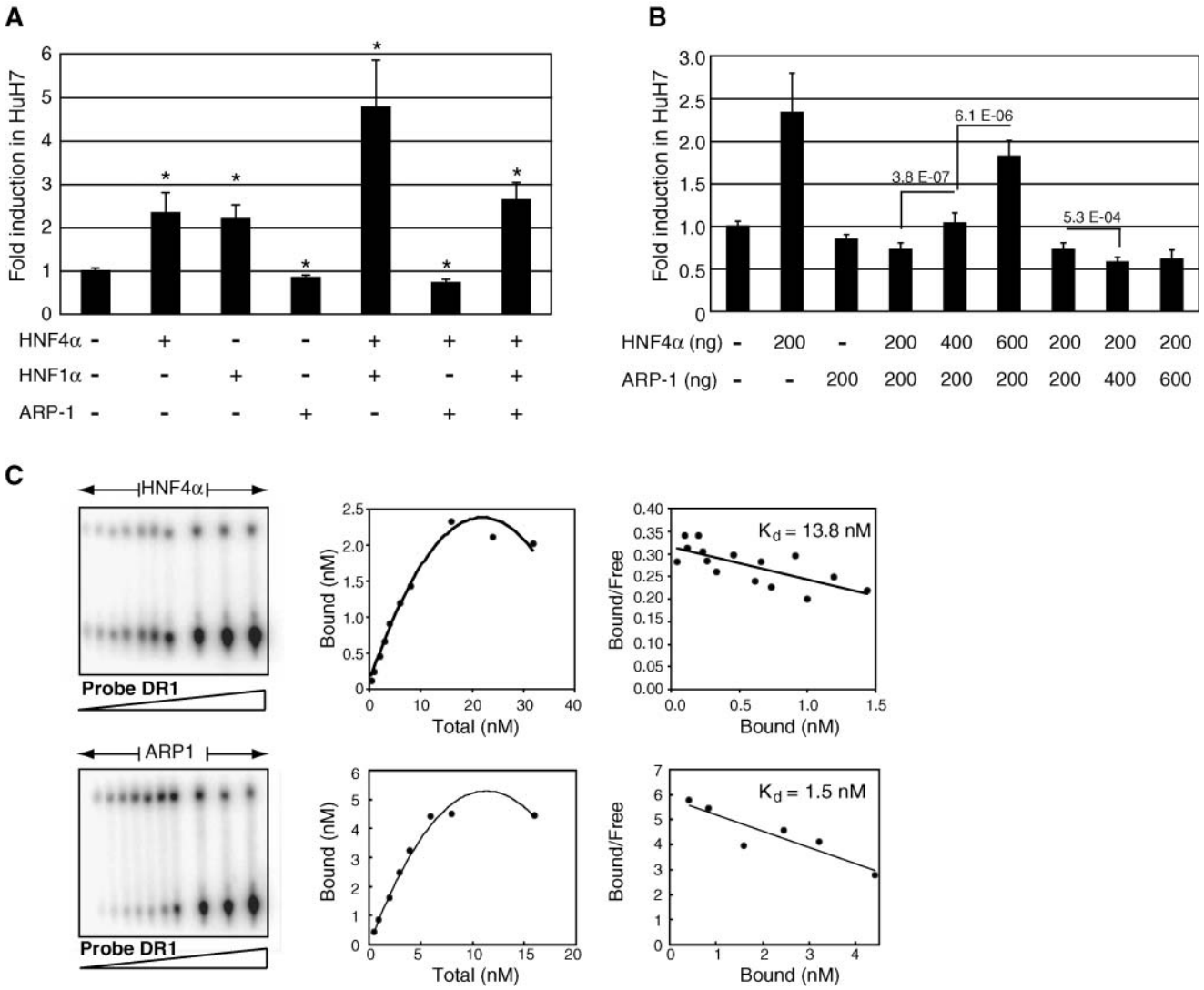

Fig. 4. Effect of overexpressing HNF4α, HNF1α, and ARP-1 on the activity of the *LIPC* proximal promoter in HuH7 cells, and binding affinities of HNF4α and ARP-1 to the *LIPC* DR1. A: Cotransfections were carried out in HuH7 cells using 200 ng of the WT *LIPC* promoter construct (pGL4-851) with 200 ng of each expression vector listed. Asterisks denote statistically significant differences ($P < 0.001$), as determined by Student's *t*-test, compared with empty vector-transfected cells (first bar). Results are presented as fold induction relative to the activity of cells cotransfected with the pMT2 empty vector only, taken as 1.0 (first bar). Error bars represent standard deviations of at least two experiments done in triplicate. B: Cotransfections of HuH7 cells were done using 50 ng of the pGL4-851 *LIPC* promoter construct and the indicated amounts of HNF4α or ARP-1 expression vectors. Results are presented as described for A. Values on the graph indicate the *P* values associated with the differences between the indicated bars, as determined by Student's *t*-test. Error bars represent standard deviations of at least two experiments done in triplicate. C: Autoradiographs of 10 binding reactions for HNF4α (top) and ARP-1 (bottom) are presented at left. The corresponding saturation curves presenting plots of the amount of radioactive bound probe versus total probe present in the reactions are presented in the middle. Scatchard plots of the amount of bound probe versus the fraction of bound/free probe are presented at right and were used to calculate the dissociation constants (K_d).

the notion that ARP-1 antagonizes HNF4α transactivation of the *LIPC* promoter via competition for the DR1 element.

Identification of the transcription factor binding sites responsible for HNF4α and HNF1α induction of the *LIPC* promoter

To determine whether the transactivation by HNF4α and HNF1α occurs through the identified DR1, DR4, and HNF1 binding elements (Fig. 1), transient cotransfections were carried out in HuH7 cells using promoter constructs with mutations in the DR4 (pGL4-851DR4mut), DR1 (pGL4-851DR1mut), and HNF1 (pGL4-851HNF1BSmut) binding sites as well as combinations of these mutants (pGL4-851-DR4/DR1mut, pGL4-851-DR4/DR1/HNF1BSmut) (**Fig. 5**). Analysis of the basal level of activity of these promoter constructs without the addition of any exogenously expressed vectors showed a significant reduction upon mutation of the DR4 and HNF1 binding site but not upon mutation of the DR1 site (Fig. 5A). The combination of all three mutations nearly eliminated all basal activity of the promoter. These data suggest a role for the DR4 and HNF1 binding sites in the endogenous expression of the *LIPC* proximal promoter in HuH7 cells. The lack of effect on basal activity of the *LIPC* promoter by the DR1

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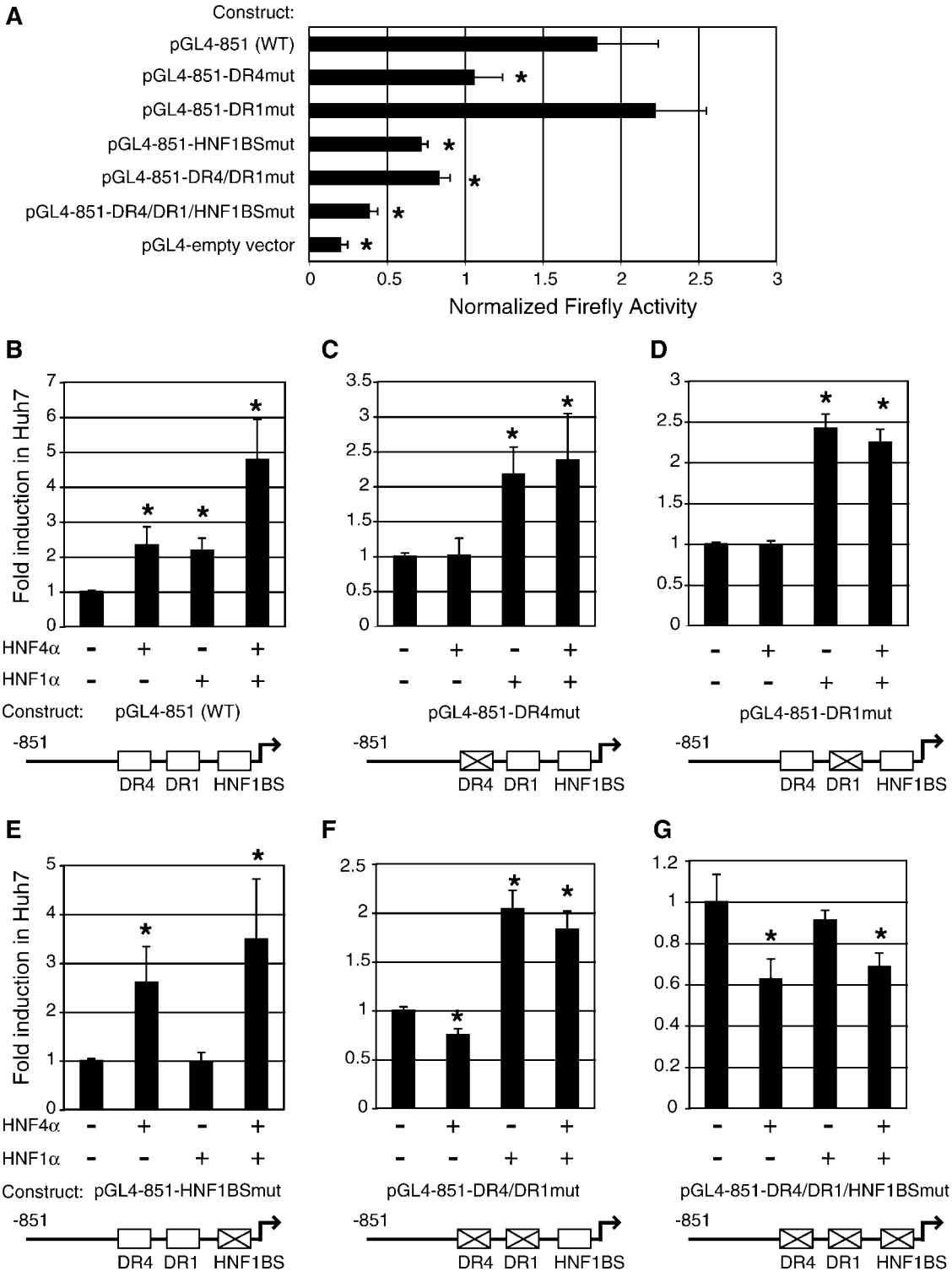

Fig. 5. Effect of mutations in the DR4, DR1, and HNF1 binding sites on *LIPC* promoter expression in HuH7 cells. A: HuH7 cells were transfected with 200 ng of the indicated promoter construct and pMT2 empty vector to a total of 600 ng. Luciferase values were determined 24 h after transfection. Values for each promoter construct are graphed as normalized firefly activity, which represents the firefly luciferase values normalized by the control renilla values (see Experimental Procedures for explanation). Asterisks reflect statistical differences ($P < 0.001$) compared with the pGL4-851 construct. Error bars represent standard deviations of at least two to three triplicate experiments. B–G: HuH7 cells were cotransfected with 200 ng of the indicated promoter construct and 200 ng each of the indicated expression vectors. Results are presented as fold induction relative to the activity of cells cotransfected with the pMT2 empty vector only, taken as 1.0 (first bar in each graph). Error bars represent standard deviations of at least two to three triplicate experiments. Asterisks reflect statistical differences ($P < 0.001$) compared with pMT2 empty vector-transfected cells. Below panels B–G are diagrams of the individual promoter constructs with the mutated elements indicated by X.

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mutant suggests that DR1 is not required for the basal level of expression.

Cotransfection of these mutant constructs with HNF4α revealed that both DR4 (Fig. 5C) and DR1 (Fig. 5D) are essential for the observed 2.5-fold induction of the *LIPC* proximal promoter by HNF4α (Fig. 5B), because mutation of either site resulted in a complete loss of induction. Neither mutation had an effect on *LIPC* induction by HNF1α (Fig. 5C, D, F). Interestingly, the DR4/DR1 double mutant showed a slight repression upon addition of HNF4α (Fig. 5F, G). Mutation of the HL HNF1 binding site completely abolished the induction produced by overexpression of HNF1α but had no effect on induction by HNF4α (Fig. 5E). These data corroborate the EMSA findings and show that binding of these proteins to the DR1, DR4, and HNF1 binding sites is functionally relevant.

Stimulation of HNF4α-mediated transactivation by PGC-1α

It has been shown that PGC-1α stimulates the induction of numerous promoters through coactivation of HNF4α. To elucidate whether PGC-1α can stimulate the HNF4α-mediated transactivation of the *LIPC* promoter, transient cotransfections were carried out in HuH7 cells using PGC-1α and HNF4α. PGC-1α alone was unable to stimulate expression of the pGL4-851 WT *LIPC* promoter construct. However, cotransfection of PGC-1α with HNF4α resulted in a significantly greater activation than transfection of HNF4α alone (**Fig. 6**). The induction by PGC-1α and HNF4α was promoter-dependent, because no activation was observed with the promoterless pGL4 vector. To identify the regulatory elements mediating the effect of PGC-1α, reporter constructs containing mutations in the HL DR1, DR4, and HNF1 elements were tested (Fig. 6). Although some remaining response was observed, mutations in both the DR1 and DR4 elements, but not in the HNF1 site, markedly diminished the response to PGC-1α and HNF4α. These results show that PGC-1α moderately stimulates the HNF4α-mediated induction of the *LIPC* proximal promoter preferentially via the DR1 and DR4 elements.

Determination of whether endogenously expressed HNF4α and HNF1α interact with the *LIPC* proximal promoter in vivo

In vivo interaction of endogenously expressed HNF4α and HNF1α with the *LIPC* proximal promoter was evaluated by ChIP analysis of anti-HNF4α or anti-HNF1α antiserum in HuH7 cells. Endogenous HNF4α was not observed to be associated with the *LIPC* DR1 region or any other region of the *LIPC* proximal promoter, as determined by the lack of enrichment by the HNF4α antibody over rabbit IgG (data not shown). ChIP analysis for binding of endogenously expressed HNF1α to the *LIPC* proximal promoter was inconclusive, because both HNF1α antibodies tested failed with the *LIPC* promoter region as well as with the positive control (the HNF4α proximal promoter) for HNF1α antibody binding (data not shown). Because ChIP assays have a narrow window of detection and may not work on all DNA elements, these results do not necessarily exclude endogenous HNF4α and HNF1α binding to the HL proximal promoter.

Suppression of *LIPC* expression by HNF4α antagonists

Fatty acyl-CoAs longer than C12 specifically bind to the ligand binding domain of HNF4α and result in activation or suppression of its transcriptional activity based on the degree of saturation of the fatty acyl-CoAs (64). Fibrates and Medica analogs (fatty acyl-CoAs C18–C22) are HNF4α antagonists that suppress the expression of HNF4α-responsive genes. We treated HuH7 cells with M16 to test whether endogenously expressed HNF4α is important for hepatic lipase expression in vivo (**Fig. 7**). Endogenous hepatic lipase mRNA expression in HuH7 cells was inhibited in a dose-dependent manner upon treatment with 250–400 μM M16. Expression of the *APOC3* gene, which has been shown to be an HNF4α-responsive gene (64, 65), was also repressed by M16. Surprisingly, treatment with 100 μM M16 resulted in a slight, but statistically significant, increase in hepatic lipase mRNA expression. This was not seen with *APOC3*, and further analysis will be required to determine the cause of this increase. The repression observed by M16 supports the notion that HNF4α is involved in *LIPC* regulation in vivo.

HNF4α is essential for in vivo hepatic *LIPC* expression in mice

We next examined the role of HNF4α in the regulation of *LIPC* expression in an animal model with a liver-specific targeted disruption of the mouse *Hnf4a* gene. Disruption of *Hnf4a* in the mouse was observed to cause lethality during early embryogenesis (30). Recently, however, this

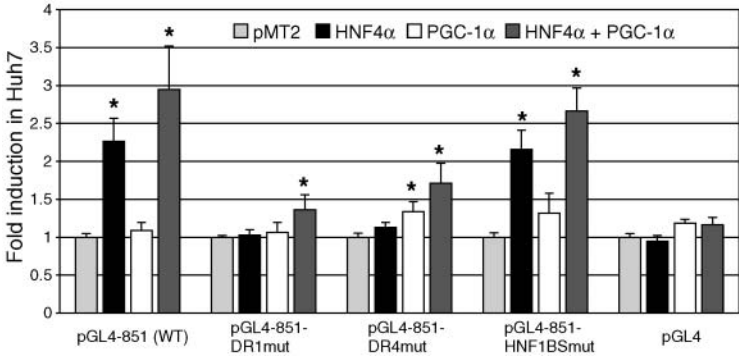

Fig. 6. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) stimulates HNF4α-mediated transactivation of the *LIPC* gene promoter. HuH7 cells were transfected with 200 ng of the indicated promoter construct and 200 ng each of the indicated expression vectors. Results are presented as fold induction relative to the activity of cells cotransfected with pMT2 empty vector only (light gray bars), taken as 1.0. Error bars represent standard deviations of two triplicate experiments. Asterisks reflect statistical differences ($P < 0.001$) compared with pMT2 empty vector-transfected cells.

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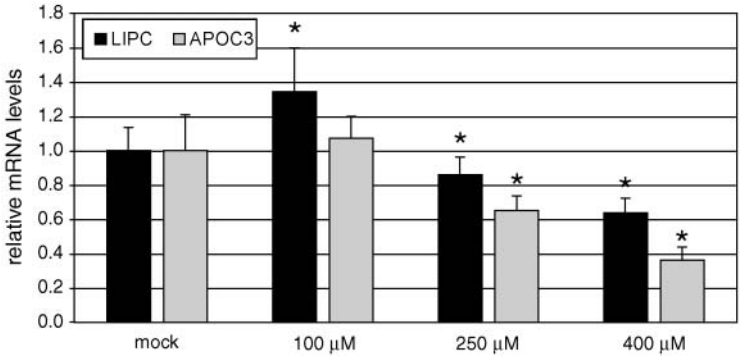

Fig. 7. Suppression of hepatic lipase expression by the HNF4α antagonist Medica 16 (M16). HuH7 cells were incubated for 48 h in the absence (mock) or presence of 100, 250, or 400 μM M16. *LIPC* or apolipoprotein gene promoter *APOC3* mRNA levels were determined by real-time PCR as described in Experimental Procedures and normalized to hGAPDH levels. Values represent averages from two independent experiments graphed as relative mRNA values compared with mock, taken as 1. Asterisks reflect statistical differences ($P < 0.001$) compared with mock values.

early embryonic arrest was circumvented (66) by the generation of a liver-specific targeted disruption of *Hnf4a* using the Cre-lox system (29, 67). This allowed for the generation of mouse embryos with livers lacking HNF4α that are viable at 18.5 days after coitus. To determine whether HNF4α is essential for hepatic *Lipc* expression in mice, we compared the steady-state level of HL mRNA by RT-PCR in livers from mouse embryos both containing and lacking HNF4α. HL mRNA was undetectable in total cellular RNA from livers of *Hnf4a*-deficient mice. Expression of *Hprt* was included as a control for the amount of mRNA used in the RT-PCR, and expression of the albumin and aldolase B genes was used as examples of HNF4α-independent and -dependent genes, respectively (28) (**Fig. 8**). Like the M16 data, these results support the importance of HNF4α for hepatic lipase expression in vivo but do not indicate whether the effect is direct or indirect.

DISCUSSION

Hepatic lipase plays a key role in the modulation of plasma lipoprotein particle size and density. High levels of HL are associated with an atherogenic lipoprotein profile composed of high levels of small, dense LDL and HDL particles. Both genetic and physiologic factors influence the level of HL. However, very few *cis*-regulatory elements that regulate *LIPC* expression have been identified. Sequence analysis of the human *LIPC* proximal promoter revealed the presence of two phylogenetically conserved inverted DRs (DR1 and DR4) with the motif 5′-RG(G/T)TCA-3′ as well as a putative HNF1 binding site. These sites bind proteins such as RXRα, RARα, HNF4α, HNF1α, and ARP-1, which are known to regulate genes important in lipid metabolism. In this report, we present evidence that HNF4α, PGC-1α, ARP-1, and HNF1α may play roles in regulating *LIPC* gene expression.

Transient cotransfection experiments showed that HNF4α induced the *LIPC* proximal promoter by 2.5-fold (Fig. 4A). Site-directed mutagenesis of either the DR1 or DR4 element abrogated this induction (Fig. 5). Because HNF4α has been shown to exclusively form homodimers that bind DRs separated by zero, one, or two nucleotides (DR0, DR1, or DR2) (31, 68–70), the requirement of both the DR4 and DR1 elements for activation by HNF4α suggested potential interaction between HNF4α bound to the DR1 element and another transcription factor bound to DR4 (Fig. 5C, D). There is precedent for this type of interaction in regulating gene expression by HNF4α. Numerous studies have shown that transactivation by HNF4α depends on synergistic interactions between HNF4α and additional factor(s) bound to other regulatory elements. For example, transactivation of the *APOC3* gene is mediated by direct physical interaction between HNF4α bound to the I4 element of the *APOC3* enhancer with the specificity protein 1 bound to multiple sites (71, 72). In addition, cooperative binding of USF and HNF4α has been shown to drive the transcription of *APOA2* (73) and *APOC3* (74). Interestingly, USF has been shown to activate the *LIPC* proximal promoter (12), but its ability to transcriptionally synergize with HNF4α has not been tested. Moreover, activation of the *APOB* promoter by HNF4α requires the interaction of HNF4α bound to element III with C/EBPα bound to the adjacent element IV (43). Although specificity protein 1, USF, and C/EBPα are good

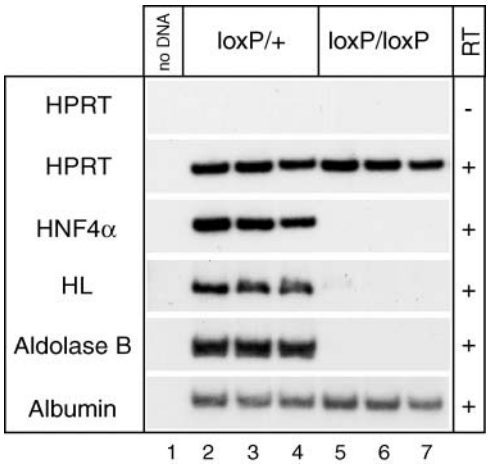

Fig. 8. HNF4α is essential for HL mRNA expression in mouse liver. Steady-state levels of mRNA in normal embryonic day 18.5 days post coitus (*HNF4*$^{loxP/+}$*;AlfpCre*) livers (lanes 2–4) and *Hnf4a*-deficient (*HNF4*$^{loxP/loxP}$*;AlfpCre*) livers (lanes 5–7) were determined by RT-PCR. All samples contained comparable amounts of mRNA, as indicated by *Hprt* amplification in the presence of reverse transcriptase (RT). No amplification was observed in the absence of mRNA (lane 1). In mouse embryos whose livers lack HNF4α (lanes 5–7), the mRNA levels of HL and the positive control, aldolase B, in liver were undetectable. The same mRNA level of the negative control, albumin, was present in both normal and *Hnf4a*-deficient livers.

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examples of factors that show transcriptional synergism with HNF4α, none of them are known to bind DR4 elements.

Because DR4 elements are known to bind homodimers and heterodimers, the factor binding to DR4 could represent more than one protein. We tested HNF4α, ARP-1, RXRα, and RARα for their ability to bind and induce the *LIPC* promoter via the DR4 site. EMSA results showed binding of HNF4α and ARP-1 to the DR4 site (Figs. 2, 3). However, despite these results, we do not believe that HNF4α or ARP-1 represents the factor hypothesized to bind to DR4. To begin with, HNF4α has not been shown to bind DR4 elements (68, 70). However, it is possible that this is a novel finding and that the HNF4α-mediated activation of the HL promoter requires HNF4α binding to both the DR1 and DR4 elements. Numerous promoters contain two HNF4α binding sites [e.g., *APOC3* (70), ornithine transcarbamylase (44), and microsomal triglyceride transfer protein (58)]. However, the HNF4α binding sites are generally both DR1 elements, and loss of activation by HNF4α requires mutation of both sites, not just one, as seen with the HL promoter. Additionally, despite the fact that homodimers of ARP-1 have been shown to bind DR4 elements (19, 20) and actively induce or repress transcription (reviewed in 47), overexpression of ARP-1 alone showed little effect on *LIPC* promoter expression (Fig. 4A). Because the factor binding to HL DR4 appears to play a positive role in HL expression, the lack of activation upon overexpression of ARP-1 suggests that it is not the factor binding to the HL DR4 element. DR4 elements are also known to bind RXRα homodimers and heterodimers with various partners. However, supershifting studies using anti-RXRα antiserum showed no RXRα in the DNA/protein complex bound to the DR4 element (Fig. 2D, lane 5). It is possible that RXRα may not be expressed at high enough levels in HuH7 cells to see binding from nuclear extracts. However, the lack of HL promoter activation by RXRα in transient transfection assays (data not shown) suggests that it is not involved in the regulation of the HL promoter. Therefore, our analysis has ruled out the proteins most commonly known to bind DR4 sequences as being the DR4-bound factor. Sequence analysis of the DR4 region failed to reveal any additional transcription factor consensus sites. We are in the process of trying to determine the identity of the unknown factor(s) binding to DR4 (protein X in **Fig. 9**).

PGC-1α has been shown to coactivate HNF4α by direct interaction in a ligand-independent manner (36). Synergism between PGC-1α and HNF4α has been clearly shown for the promoters of *PEPCK*, *G6Pase*, *CPT-Iα*, *CYP7A1*, *FXR*, and *APOA5* (36–41). Our studies show a slight synergism between PGC-1α and HNF4α in the activation of the *LIPC* promoter similar to that reported for other genes (i.e., *CYP7A1*). The slight response could be attributable to high background caused by endogenously expressed PGC-1α.

HNF4α also appears essential for in vivo hepatic *LIPC* expression. HL mRNA expression was shown to be completely abolished in livers of *Hnf4a*-deficient mice (Fig. 8). Moreover, treatment of HuH7 cells with the HNF4α antagonist M16 led to the reduction of endogenous HL mRNA expression as a result of the inhibition of transactivation by HNF4α (Fig. 7). These data show the importance of HNF4α to the in vivo expression of HL but do not indicate whether the role of HNF4α is direct, indirect, or both. To further elucidate the in vivo role of HNF4α, we tested the ability of endogenously expressed HNF4α to bind the DR1 element by ChIP. ChIP analysis failed to show binding of endogenous HNF4α to the DR1 region in HuH7 cells. This lack of endogenous HNF4α binding to the *LIPC* DR1 element is supported by the lack of effect on basal *LIPC* promoter activity upon mutation of the DR1 site (Fig. 5A). However, it is possible that, as a result of the DR1 binding competition between HNF4α and ARP-1, the amount of endogenous HNF4α binding to the HL DR1 is below the detection limit of the assays performed. On the other hand, if endogenously expressed HNF4α does not regulate the *LIPC* proximal promoter in vivo, it still could play a direct role in *LIPC* expression by binding to sequences outside the −851 to +29 *LIPC* proximal promoter. However, we failed to detect any further increase in *LIPC* promoter activation by HNF4α over that found with the proximal promoter in transient transfection assays using *LIPC* promoter constructs containing sequence up to −4.7 kb upstream of the transcription start site (data not shown). HNF4α could also have an indirect effect whereby HNF4α could activate the transcription of another protein that is important for direct regulation of the *LIPC* proximal promoter. This other protein could be the factor binding to DR4 or HNF1α, which is positively regulated by HNF4α (75, 76).

ARP-1 appears to block binding and activation by HNF4α through competition for DR1 binding. Blocked binding through direct competition with HNF4α for the DR1 site is a commonly observed mechanism of repression for ARP-1 (47). Data from various experiments support the competition model. EMSA studies showed that both ARP-1 and HNF4α bind the DR1 site (Figs. 2, 3), and determination of the relative DR1 binding affinities indicated that ARP-1 binds the DR1 element with much higher affinity than HNF4α (Fig. 4C). The ability of ARP-1 to out-compete HNF4α for binding to DR1 explains how cotransfection of equal amounts of ARP1 and HNF4α results in the loss of *LIPC* promoter induction by HNF4α (Fig. 4A). However, when HNF4α is expressed in excess compared with ARP-1, induction by HNF4α is restored (Fig. 4B). The higher binding affinity of ARP-1 could explain why higher concentrations of HNF4α are needed to out-compete ARP-1 for binding to DR1.

We have also determined that HNF1α is important for *LIPC* expression. Transfection studies showed a significant loss of basal activity of the *LIPC* promoter construct upon mutation of the HNF1 binding site (Fig. 5A) and a 2.5-fold induction upon overexpression of HNF1α (Fig. 4A). Site-directed mutagenesis of the HNF1 binding site abolished this induction (Fig. 5E). Coexpression of both HNF1α and HNF4α normally leads to a synergistic effect (58, 59) or repression of HNF4α-induced transcription (57) attributable to physical interaction between these two proteins. However, we have demonstrated that cotransfection of

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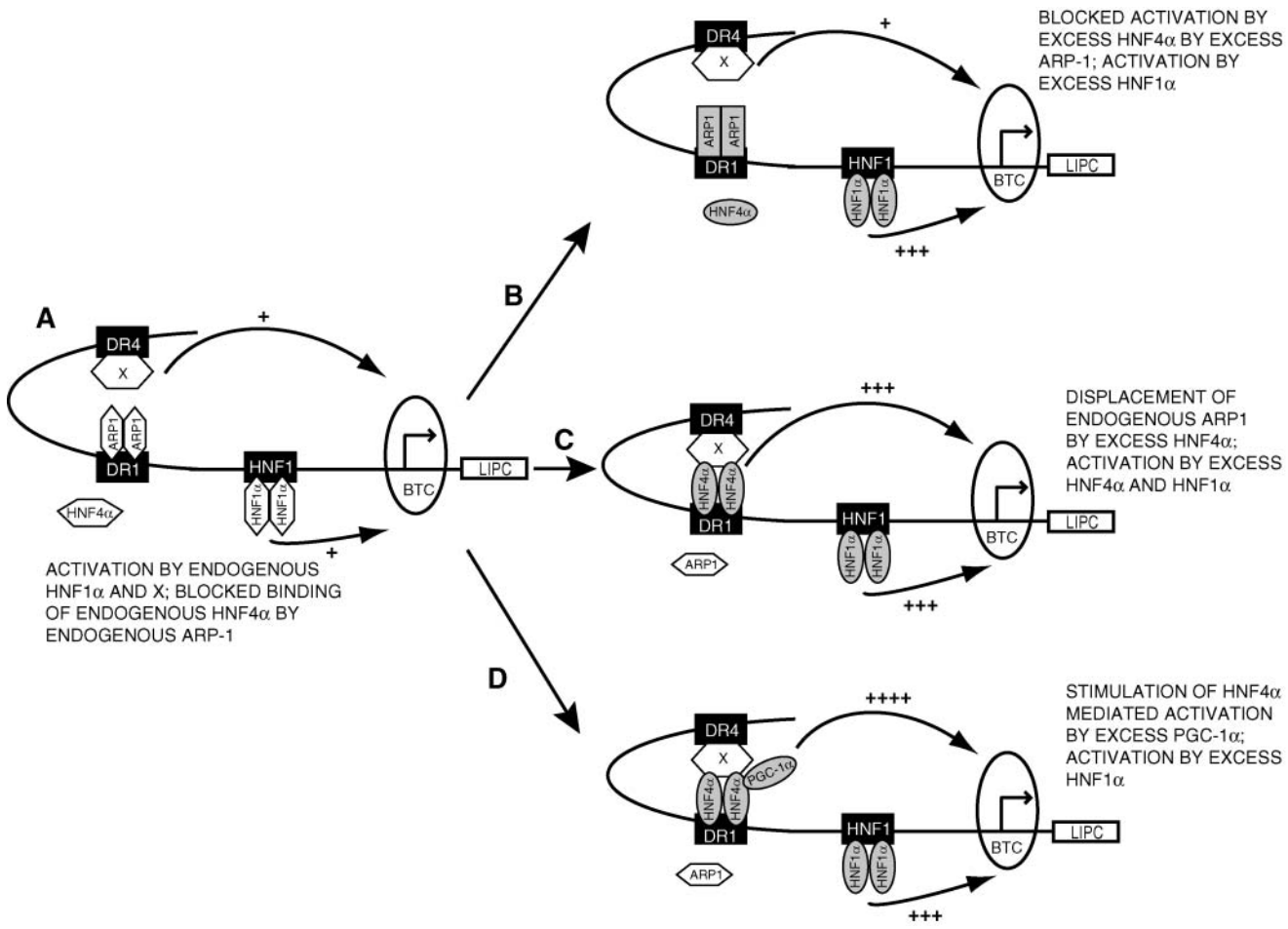

Fig. 9. Schematic model showing the proposed role of unknown protein X, HNF1α, HNF4α, PGC-1α, and ARP-1 in the transactivation of the human *LIPC* promoter. A: Endogenously expressed proteins. B: Overexpression of HNF4α, HNF1α, and ARP-1. C: Overexpression of HNF4α and HNF1α. D: Overexpression of HNF4α, HNF1α, and PGC-1α. White hexagons represent endogenously expressed proteins; gray ovals and rectangles represent exogenously expressed proteins; DR1, DR4, and HNF1 denote the indicated binding sites in the *LIPC* promoter; the number of + signs indicates the strength of promoter activation; and BTC indicates the basal transcription complex.

both HNF1α and HNF4α produces an additive effect on the *LIPC* proximal promoter (Fig. 5B). This could indicate that on the *LIPC* promoter these two proteins do not interact physically but instead interact with the basal transcription machinery individually. Despite the fact that we have been unable to confirm the binding of endogenous HNF1α to the *LIPC* proximal promoter by ChIP analysis, we feel that the loss of basal activity and induction by HNF1α upon mutation of the *LIPC* HNF1 binding site and the fact that only members of the HNF1 family (HNF1α and HNF1β) have been shown to bind the HNF1 consensus sequence (54) strongly support the argument that HNF1α is important to the in vivo expression of HL. In addition, microarray analysis of HNF1α-deficient versus WT mice showed a dramatic reduction (80%) in mouse *LIPC* mRNA expression, further supporting the importance of HNF1α for the regulation of *LIPC* expression in vivo (53).

In conclusion, we have provided in vitro and in vivo evidence for the importance of HNF4α in the activation of the *LIPC* proximal promoter via the DR1 and DR4 elements. The coactivator PGC-1α appears to moderately stimulate this HNF4α-mediated transactivation. We have also shown that HNF1α independently activates the *LIPC* proximal promoter using the HNF1 binding site and that ARP-1 acts as an antagonist to induction by HNF4α through competition for DR1 binding. Based on these results, we propose the model shown in Fig. 9.

The level of hepatic lipase in each individual will ultimately be determined by the interplay between these various transcription factors and modulated by signals that affect the levels of each factor. HNF4α, HNF1α, and ARP-1 are part of a complex regulatory network responsible for defining the hepatic phenotype. Cross-regulation and autoregulation between these and other factors are important mechanisms for the establishment of strictly controlled interdependent regulatory pathways that lead to balanced high-level expression of the main factors needed in hepatocytes (51, 57, 62, 75–77). Therefore, signals that affect the expression of one or all of these transcription factors will in turn influence the expression of the others and ultimately determine the level of expression of the many proteins involved in lipid metabolism that they regulate. The better we understand how HNF4α, HNF1α, and

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ARP-1 are regulated and interact to modulate hepatic lipase expression, the better we will be able to help in the management of lipoprotein profiles that predispose to coronary artery disease and atherosclerosis.

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