Cooperative Interaction between Hepatocyte Nuclear Factor 4 α and GATA Transcription Factors Regulates ATP-Binding Cassette Sterol Transporters ABCG5 and ABCG8[∇]

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Cholesterol homeostasis is maintained by coordinate regulation of cholesterol synthesis and its conversion to bile acids in the liver. The excretion of cholesterol from liver and intestine is regulated by ATP-binding cassette half-transporters ABCG5 and ABCG8. The genes for these two proteins are closely linked and divergently transcribed from a common intergenic promoter region. Here, we identified a binding site for hepatocyte nuclear factor 4 α (HNF4 α) in the ABCG5/ABCG8 intergenic promoter, through which HNF4 α strongly activated the expression of a reporter gene in both directions. The HNF4 α -responsive element is flanked by two conserved GATA boxes that were also required for stimulation by HNF4 α . GATA4 and GATA6 bind to the GATA boxes, coexpression of GATA4 and HNF4 α leads to a striking synergistic activation of both the ABCG5 and the ABCG8 promoters, and binding sites for HNF4 α and GATA were essential for maximal synergism. We also show that HNF4 α , GATA4, and GATA6 colocalize in the nuclei of HepG2 cells and that a physical interaction between HNF4 α and GATA4 is critical for the synergistic response. This is the first demonstration that HNF4 α acts synergistically with GATA factors to activate gene expression in a bidirectional fashion.

Cholesterol homeostasis is maintained by a series of regulatory pathways that control the synthesis of endogenous cholesterol, the absorption of dietary sterol, and the elimination of cholesterol and its catabolic end products, bile acids. Transcriptional control of many genes vital to these processes can be attributed to two classes of transcription factors: sterol regulatory element-binding proteins (SREBPs), especially SREBP-2, which control the production of key enzymes in cholesterol biosynthesis (11, 36, 38, 39), and the nuclear hormone receptor family, including liver X receptor (LXR), farnesoid X receptor, small heterodimer partner, liver receptor homolog1 (LRH-1), and hepatocyte nuclear factor 4 α (HNF4 α), which control the expression of genes involved in cholesterol efflux, catabolism, and elimination (3, 27).

HNF4 α is the most abundant nuclear orphan receptor expressed in the liver, and it is involved in early liver development (22). HNF4 α is also expressed in kidney, intestine, and

pancreas and is required for expression of many tissue-specific traits in all of these organs. Transcriptional activation by HNF4 α is mediated by its binding as a homodimer to a DNA sequence composed of two direct repeats (DRs) of the hexanucleotide motif AGGTCA separated by 1 base, referred to as an HNF4 α response element of the DR-1 type. Like other nuclear receptors, HNF4 α exhibits a modular structure with six distinct domains (A to F). The N-terminal A/B domain is highly variable among nuclear receptors and contains a ligand-independent activation function 1 (AF-1) domain. The highly conserved C domain encodes the DNA binding domain of nuclear receptors and confers sequence-specific DNA recognition. By linking the highly structured C and E domains, the hinge D region may allow for flexibility in the conformation of the DNA binding and ligand binding domains. The ligand-dependent nuclear receptors also contain a ligand binding domain in the E region. This domain is also involved in several functions in addition to ligand binding, including dimerization and ligand-dependent transcriptional activation, also referred to as AF-2. The F domain may play a role in discriminating between coactivator and corepressor recruitment to the E domain (35). HNF4 α can activate gene transcription in the absence of exogenous ligand (18, 42, 43); therefore, unlike those of classic nuclear receptors, the transcriptional activity of HNF4 α is largely dependent on the selective interaction of tissue-specific or independently regulated coregulators with its

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AF-2 domain to stimulate target genes in a tissue- and metabolically regulated gene-specific manner (7). Disruption of the HNF4 α gene results in defects in early liver development (22); however, gene inactivation, specifically in adult liver, resulted in the accumulation of hepatic lipids, markedly reduced serum levels of cholesterol and triglycerides, and increased serum bile acids (10). Expression levels of CYP7A1, Na⁺-taurocholate cotransport peptide, organic anion transporter 1, apolipoprotein B100, and scavenger receptor B-1 were all reduced in these mice (10). These results indicate that HNF4 α is a key regulator of bile acid and lipoprotein metabolism and plays a central role in lipid homeostasis (44). HNF4 α is also involved in diabetes, as a mutation of the HNF4 α gene causes maturity onset diabetes of the young type 1 (51). HNF4 α also regulates the expression of the HNF1 α gene, which is also linked to development of maturity onset diabetes of the young type 3 (16). The central role of HNF4 α is further highlighted by the large number of putative HNF4 α target genes, as reported in analysis combining chromatin immunoprecipitation (ChIP) from hepatocytes and pancreatic islets with a promoter microarray (31).

ABCG5 and ABCG8 are ATP-binding cassette half-transporters (2, 20, 24, 41) and regulate the excretion of sterols from the liver and intestine. Mutations in either of these transporters leads to β -sitosterolemia, an autosomal recessive disease characterized by premature coronary atherosclerosis and elevated levels of phytosterols in plasma (9, 21, 25). These defects were attributed to enhanced intestinal absorption and decreased biliary excretion of sterols (40). Mice lacking ABCG5 and ABCG8 proteins have markedly reduced capacities to secrete sterols into bile (53), whereas overexpression of ABCG5 and ABCG8 in the liver dramatically increases biliary cholesterol secretion and decreases dietary cholesterol absorption (54). The human ABCG5 and ABCG8 genes are oriented in a head-to-head configuration, and both genes are transcribed in opposite directions from independent transcription start sites that are separated by only 374 bp of intergenic sequence, which acts as a bidirectional promoter (2, 20, 24). The genes are predominantly and coordinately expressed in liver and small intestine (2, 20, 49). Both proteins reside on the apical plasma membrane of polarized hepatocyte WifB cells and also localize to intestinal microvilli in the gut lumen (34). The coexpression and the coordinated regulation are required for either protein to accumulate to normal levels and be properly transported to the cell surface. The intergenic promoter harbors a number of potential transcription factor binding sites, including two GATA boxes and an LRH-1 element (6); however, the promoter sequences and transcription factors that are responsible for the robust and tissue-restricted expression of the ABCG5 and ABCG8 gene products in hepatocytes and enterocytes are not understood. Their expression is regulated by the oxysterol-sensing LXR nuclear receptor, but an LXR α -responsive region has not been identified (34).

The GATA family of transcription factors also play a crucial role in controlling hepatogenesis. Defined by two evolutionarily conserved DNA zinc finger motifs that bind to the consensus DNA sequence (A/T)GATA(A/G), the GATA family is separated into two subfamilies based on their patterns of expression as well as amino acid conservation. GATA-1, -2, and -3 are expressed primarily in hematopoietic cells (32), whereas

GATA-4, -5, and -6 are expressed in a diverse array of tissues, including liver, small intestine, heart, lungs, and gonads (1, 15, 19, 29, 46). Although the wide-ranging expression patterns of GATA-4, -5, and -6 argue against these proteins being master regulators of tissue- or cell type-specific gene expression, there is increasing evidence that this subfamily might be critical in regulating cell-specific gene expression through unique interactions with other semirestricted transcription factors and coregulators (23, 50).

In this report, we used microarray analysis combined with RNA interference and adenoviral overexpression approaches to identify additional HNF4 α target genes. Because ABCG8 mRNA was induced or repressed by overexpression or inhibition of HNF4 α , respectively, we tentatively identified ABCG8 as a new HNF4 α target gene. An evaluation of the intergenic promoter sequence separating ABCG5 and ABCG8 indicated that it contains conserved sequences that we show bind both HNF4 α and GATA factors in vitro and in vivo. Additionally, the coexpression of the HNF4 α and GATA proteins synergistically activated the expression of a reporter gene through the same HNF4 α and GATA sites, independent of the orientation of the bidirectional intergenic control region. Because the ABCG5 and ABCG8 proteins form a functional complex and are unstable when expressed alone, the utilization of a common control region that functions in a bidirectional fashion provides an unusual but simple mechanism for ensuring their coordinate expression.

MATERIALS AND METHODS

Antibodies. Immunoglobulin G H1415 (IgG-H1415) and IgG-K9218 are mouse monoclonal antibodies directed against amino acids (aa) 394 to 461 of human HNF4 α 2 and the A/B domain of human HNF4 α 2 (aa 3 to 49), respectively. Monoclonal antibodies IgG-H2429 and IgG-H2402 against human GATA4 and IgG-H2617 against human GATA-6 were produced by immunizing separate mice with peptides representing aa 332 to 442 of GATA4 and aa 152 to 382 of GATA6, respectively. The specificities of each antibody have been reported previously by our laboratories (14, 47). Horseradish peroxidase (HRP)-conjugated IgG-H2429 and IgG-H2617 were prepared by a peroxidase labeling kit (Dojindo, Japan) according to the manufacturer's instructions.

Cell culture. The human hepatoblastoma HepG2 cells and human embryonic kidney 293 cells (obtained from the Cell Resource Center for Biomedical Research at Tohoku University) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum and penicillin (100 U/ml)-streptomycin (100 μ g/ml) (Invitrogen, Carlsbad, CA). Chinese hamster ovary (CHO) cells were maintained in Ham F-12 medium (Sigma) containing 5% fetal bovine serum and penicillin (100 U/ml)-streptomycin (100 μ g/ml). All cells were cultured at 37°C in 5% CO₂.

RNA interference. The duplexes of each small interfering RNA (siRNA), targeting HNF4 α mRNA (target sequences of 5'-GGCAGUGCGUGGUGGA CAAdTdT-3' and 5'-UUGUCCACACGACACUGCCdGdG-3') and a negative control (nonsilencing siRNA) (5'-UUCUCCGAACGUGUCACGUdTdT-3' and 5'-ACGUGACACGUUCGGAGAAdTdT-3') were purchased from QIAGEN (Valencia, CA). For transfections, HepG2 cells were cultured overnight at a density of 1.0×10^6 cells/well in six-well tissue culture plates and siRNA transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 48 h, the cells were washed with phosphate-buffered saline (PBS) and processed for total RNA isolation and whole-cell extracts. RNA was extracted using ISOGEN (Wako, Japan). HNF4 α expression was confirmed by immunoblot analysis.

Adenoviral construction and infection. The human HNF4 α 2 construct (described previously by our laboratories [14, 47]) was introduced to the Adeno-X genome for generation of recombinant Adeno-X virus according to the Adeno-X expression system manual (Clontech, Palo Alto, CA). The same strategy was used to generate recombinant LacZ adenovirus (Clontech) expressing the bacterial β -galactosidase gene (Clontech), which served as a negative control. For adenoviral infection, HepG2 cells were cultured overnight at a density of $1.0 \times$

10⁶ cells/well in six-well tissue culture plates, followed by addition of recombinant adenoviruses at multiplicities of infection of 10 to 40. After 48 h of incubation, the cells were processed for total RNA isolation and whole-cell extracts.

Affymetrix oligonucleotide microarray and QRT-PCR. The methods for microarray and quantitative real-time PCR (QRT-PCR) have been described previously (48, 52). We used Affymetrix Genechip Human Genome U133 Plus 2.0 arrays containing more than 54,000 probe sets. Real-time PCRs were performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA). All primer sequences used in this article are available upon request. Total RNA from HepG2 cells and mouse liver was extracted with ISOGEN (Wako, Japan). The generation of adult-liver-specific, *Hnf4α*-null mice by a Cre-loxP-mediated deletion (in which the Cre gene is under the control of the albumin promoter) to remove exons 4 and 5 of the *Hnf4α* gene was previously described (10). Total RNA was extracted from the livers of 45-day-old HNF4α^{flox/flox}; albumin-Cre^{+/+} (HNF4α^{LivKO}) and HNF4α^{flox/flox}; albumin-Cre^{-/-} (FLOX) mice. The mice were housed under a standard 12-h-light/12-h-dark cycle with ad libitum water and chow.

Transient transfection and enzymatic assay. Cells were cultured at a density of 1.0 × 10⁵ cells/well in 24-well tissue culture plates overnight, and transfections were performed using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. The reporter construct (0.2 μg), protein expression plasmids, and a pCMVβ-galactosidase plasmid (0.05 μg) as an internal control were transfected in each well. The pcDNA3 vector was added to normalize the amounts of DNA transfected in each assay. After 24 h, the cells were washed with PBS and assayed for luciferase activity using a luciferase assay system (Promega, Madison, WI), and luminescence was determined using a Lumat Flash and Glow LB955 luminometer (Berthold Systems, Inc., Pittsburgh, PA). Luciferase activities (relative light units) were normalized to β-galactosidase activity (optical density units) by dividing the number of relative light units by the value for β-galactosidase activity expressed from a cotransfected pCMVβ plasmid as described previously (13, 37). All the experiments were performed at least three times, and the most representative results are shown.

Construction of human ABCG5- and ABCG8-luciferase reporter plasmids. Based on the available human ABCG5 sequence (33), the intergenic sequences from position -1 to -374 relative to the translation start site were amplified by PCR, using human genomic DNA from HepG2 cells as a template, and cloned in both orientations into the luciferase reporter vector, pGL3 basic (Promega, Madison, WI). The resulting constructs were designated pABCG5-luc and pABCG8-luc.

Mutations were introduced into the ABCG5 luciferase constructs (position -374 fragments) by PCR-based site-directed mutagenesis, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Oligonucleotides were designed to mutate each element alone or in combination with mutated bases (underlined) in the context of pABCG5-luc as follows: for the site 1 DR-1 motif (bp -176 to -164), from cgcTGGCTAAAGGTACatc to cgcTCCGCGGACGTACatc; for the site 2 DR-1 motif (bp -254 to -242), from ctcCTGCCCCTGGGCCCCgtc to ctcCTGAATTC CGCCGctc; for the site 3 DR-1 motif (bp -267 to -255), from gggACACCT CGGCCTCctg to gggACCGATATCCCTCctg; for the site 4 DR-1 motif (bp -372 to -360), from tggGGCCACAGGTCTgtg to tggGCATATGACGTCTgtg; for GATA-boxB (bp -160 to -155), from atcAGATAAgtg to atcGCTAG Ctg; for GATA-boxA (bp -190 to -185), from tcaAGATAAgga to tcaAAG CTTgga. The same strategy was used to generate ABCG8-luciferase constructs in the context of pABCG8-luc, and each element was mutated as follows: for the site 1 DR-1 motif (bp -164 to -176), from gatGTACCTTTAGCCAgcg to gatGTACGATATCCGAgcg; for the site 2 DR-1 motif (bp -242 to -254), from gacGGGCCCCAGGCGAGgag to gacGCATATGGCGCAGgag; for the site 3 DR-1 motif (bp -255 to -267), from cagGAGGCCGAGGTGTcc to cagGA ACTAGTCGTGTcc; for the site 4 DR-1 motif (bp -360 to -372), from cacAGACCTGTGGGCCcga to cacAGACGAATTCGCCcga; for GATA-boxB (bp -155 to -160), from ccaTTATCTgtg to ccaGCTAGCgtg; for GATA-boxA (bp -185 to -190), from tccTTATCTgtg to tccAAGCTTgtg. The capital letters represent putative DR-1 motifs or GATA binding sites.

Expression plasmids. Cytomegalovirus (CMV) promoter-driven expression vectors carrying the human GATA4 and GATA6 genes were generated by PCR and inserted into pcDNA3. The CMV promoter-driven expression plasmid for human HNF4α2 (pcDNA3-HNF4α) was described previously (47). To create the C-terminal deletion expression vectors encoding aa 1 to 361 and 1 to 368 of HNF4α [pCMV-HNF4α(1-361) and pCMV-HNF4α(1-368), respectively] or aa 1 to 303 and 1 to 332 of GATA4 [pCMV-GATA4(1-303) and pCMV-GATA4(1-332), respectively], stop codons were introduced by site-directed mutagenesis into the open reading frame of pcDNA3-HNF4α2 or pCMV-GATA4, respectively. To create internal deletion mutant expression plasmids pCMV-

HNF4α(Δ359-368), pCMV-GATA4(Δ202-303), pCMV-GATA4(Δ304-332), and pCMV-GATA4(Δ202-332), DNA sequences encoding aa 359 to 368 of HNF4α or DNA sequences encoding aa 202 to 303, 304 to 332, or 202 to 332 of GATA4 were replaced with the NheI site (which encodes Ala-Ser) by site-directed mutagenesis. To create the N-terminal deletion expression plasmid pCMV-GATA4(202-442), DNA sequences corresponding to aa 202 to 442 of GATA4 were amplified by PCR and ligated into pcDNA3. Each mutant was sequenced to confirm the mutation, and at least two independent clones of each mutant plasmid were independently transfected to confirm the results.

Mammalian two-hybrid and glutathione S-transferase (GST) pull-down assays. The full-length GAL4-HNF4α2 and GAL4-HNF4α8 fusion constructs and the various deletion mutants were generated from the pcDNA3-based constructs described above by PCR using appropriate primers and cloned in frame into pBIND. The full-length and deletion mutant VP16-GATA4 fusion constructs were constructed using the same strategy. The reporter plasmid pGAL4-luc contains five GAL4 DNA binding sites plus the adenovirus E1B TATA box fused upstream of the firefly luciferase gene (luc).

For GST pull-down assays, GST fusion vectors containing aa 135 to 465, aa 135 to 361, and aa 135 to 465 with an internal deletion aa 359 to 368 of HNF4α2 were created in the bacterial expression vector pET41 (Novagen, Madison, WI), expressed in BL21-CodonPlus(DE3)-RIL bacteria (Stratagene, La Jolla, CA), and purified using a MagneGST protein purification system (Promega). The same molar levels of purified GST or GST-HNF4α fusion proteins (10 and 25 μg, respectively) were incubated with 10 μl of ³⁵S-labeled GATA4 or GATA6 (TNT Quick coupled transcription and translation kit; Promega) for 2 h at 4°C and washed three times before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out.

ChIP assay. The association of HNF4α with the human ABCG5 and ABCG8 intergenic promoter was determined by ChIP assays as we previously published (12). The HNF4α antibody used for immunoprecipitation in ChIP was IgG-H1415 (14, 47). Mouse IgG was used as a negative control for immunoprecipitation. The primers used to amplify the intergenic promoter were 5'-TGGCTA AAGGTACATCAGATA-3' and 5'-GGCCAACAGGCAGCAAAGCTG-3' (from position -1 to -176) at a final concentration of 150 nM.

Immunoblot analysis. Protein samples were run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (ProBlott) (Applied Biosystems). Membranes were blocked with 5% (wt/vol) nonfat milk in PBS containing 0.1% Tween for 1 h, incubated with the antibodies indicated in the figure legends for 1 h, and detected by chemiluminescence using SuperSignal West Dura extended duration substrate (Pierce, Rockford, IL) according to the manufacturer's instructions. All the reactions were performed at room temperature.

EMSA. Nuclear extracts were prepared as described previously (36). The HNF4α and GATA4 proteins were in vitro translated with a TNT Quick coupled transcription/translation system (Promega). Double-stranded oligonucleotide probes for the electrophoretic mobility shift assay (EMSA) were prepared by heating equal molar amounts of complementary oligonucleotides to 95°C and cooling them to room temperature. The resulting double-stranded fragments were labeled by filling in the overhang incorporated in the synthetic oligonucleotides with [α-³²P]dCTP (3,000 Ci/mmol) (GE Healthcare) with the Klenow fragment of DNA polymerase I. Labeled fragments were purified through Probe-Quant G-50 microspin columns (GE Healthcare). Binding reactions were initiated with the addition of 25 μg of nuclear extracts to 100,000 cpm of labeled oligonucleotide probe dissolved in 10 μl of the buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 0.5 μg of poly(dI-dC). Samples were incubated for 30 min at room temperature. For the supershift assay, antibody supershift was carried out by adding the antibody (2 μg) to the binding reaction mixture and incubating at room temperature for an additional 30 min. The images were analyzed using Typhoon9400 (GE Healthcare). The oligonucleotides used for EMSA and competition assays are listed in Table 1.

Statistical analysis. All values are expressed as the means ± standard errors (SE). All data were analyzed by the unpaired Student *t* test for significant differences between the mean values for each group.

RESULTS

HNF4α regulates the expression of both ABCG5 and ABCG8 in HepG2 cells and mouse liver. To identify novel target genes that are regulated by HNF4α, the expression of ≈54,600 transcripts represented on the Affymetrix Genechip

TABLE 1. Double-stranded oligonucleotides used for gel shift analyses^a

Probe	Sequences
Wild type	5'-GGACGCGCTGGCTAAAGGTAC ATCAGATAATGGTC-3' 3'-TGCGCGACCGATTTCATGTA GTCTATTACCAG-5'
HNF4 α mutant	5'-GGACGCGCTTCAAGCTTCAAC ATCAGATAATGGTC-3' 3'-TGCGCGAAGTTTCGAAGTTGTA GTCTATTACCAG-5'
GATA mutant	5'-GGACGCGCTGGCTAAAGGTAC ATCAGTAAATGGTC-3' 3'-TGCGCGACCGATTTTCCATGT AGTCATTACCAG-5'
CYP8B1	5'-GGCCACAGGGCAAGGTCCAG GTGCTCAGAC-3' 3'-GGTGTCCCGTTCCAGGTCCAC GAGTCTG-5'
CYP8B1 mutant	5'-GGCCACATTTACCTTGACAGG TGCTCAGAC-3' 3'-GGTGTAAATGGAAGTGTCCAC GAGTCTG-5'

^a Underlined base pairs represent the specific mutations in each oligonucleotide.

Human Genome U133 Plus 2.0 microarrays was quantified in samples from HepG2 cells that overexpressed HNF4 α by adenoviral transduction or from those that expressed reduced levels of HNF4 α by the application of siRNA. Overexpression and depletion of cellular HNF4 α protein were confirmed by immunoblotting (Fig. 1A and B, bottom panels). To choose genes that are likely to be direct targets of HNF4 α , we screened mRNAs that were increased by more than 1.52 ($2^{0.6}$)-fold in HNF4 α -overexpressing HepG2 cells and also decreased by less than 0.66 ($2^{-0.6}$)-fold in HNF4 α -siRNA-transfected HepG2 cells. Table 2 lists 39 probe sets that met both of these stringent criteria. This list includes three genes previously identified as direct HNF4 α targets on the basis of short-term transcription reporter assays with cultured cells and/or DNA binding assays (Table 2, footnote c) and three genes reported as HNF4 α candidate genes from ChIP/promoter microarray studies from human hepatocytes (Table 2, footnote b) (31).

ABCG8 represents a novel gene that met these stringent criteria as a putative HNF4 α target gene. To further evaluate *ABCG8* as a candidate HNF4 α target gene, we performed QRT-PCR with primers from the *ABCG8* coding sequence. Because *ABCG8* represents only half of a functional ATP-binding cassette transporter and the other half is encoded by the *ABCG5* gene, which is closely linked and coordinately expressed with *ABCG8* (6), we also examined the mRNA levels of *ABCG5*. The results in Fig. 1A and B show that the expression levels of mRNA for both half-transporters were reduced when HNF4 α protein was knocked down by siRNA and increased when HNF4 α protein was increased through adenovirus expression. To provide further evidence that HNF4 α actually activates *ABCG5* and *ABCG8* transcripts in vivo, we examined their expression in a mouse model where hepatic HNF4 α is knocked out through a liver-specific gene

inactivation event (designated H4LivKO) (10). Figure 1C shows that the expression levels of *ABCG5* and *ABCG8* mRNAs were reduced by 50 and 60% in the H4LivKO liver compared with those in the control liver (HNF4 $\alpha^{\text{flox/flox}}$; albumin-Cre⁻) (designated H4Flox), respectively. These data provide strong support for a role for HNF4 α as a physiologically important regulator of *ABCG5* and *ABCG8* gene transcription in vivo.

FIG. 1. HNF4 α regulates ABCG5 and ABCG8 gene expressions. (A) siRNA-mediated knockdown of HNF4 α . HepG2 cells were transfected with either HNF4 α -specific siRNA (si-HNF4 α) or control siRNA (si-cont). Two days after transfection, the cells were harvested for isolation of total RNA and whole-cell extracts. (B) Dose-dependent activation of ABCG5 and ABCG8 gene expressions by HNF4 α . HepG2 cells were transduced with either HNF4 α gene or control LacZ adenoviruses at the indicated multiplicities of infection (MOI) as described in Materials and Methods. Two days after infection, the cells were harvested for isolation of total RNA and whole-cell extracts. (A and B) Gene expression was analyzed by QRT-PCR using primer sets specific for human ABCG5 and ABCG8 and cyclophilin as an internal control (top). QRT-PCR was performed in triplicate. The values are normalized to cyclophilin mRNA levels and expressed as means $\pm$ SE. *, $P < 0.01$; **, $P < 0.05$. Data represent averages for three independent experiments. Aliquots (10 μ g) of protein were subjected to SDS-PAGE and immunoblot analysis using either anti-HNF4 α (IgG-K9218) or β -actin (AC-15; sigma) antibody (bottom). (C) Reduction of ABCG5 and ABCG8 mRNA levels in the livers of HNF4 α LivKO mice. Total RNA from the livers of control (HNF4 α FLOX) mice or mice lacking liver HNF4 α (H4LivKO) (10) (45 days old, four or five mice per group) was subjected to QRT-PCR quantification for ABCG5 and ABCG8, with HNF4 α as a control. The values are normalized to cyclophilin mRNA levels. The relative mRNA levels are expressed as the means $\pm$ SE. *, $P < 0.01$; **, $P < 0.05$.

TABLE 2. Genes regulated by HNF4 α in HepG2 cells^a

Description	GenBank accession no.	Avg fold change for:	
		HNF4 α adenovirus	HNF4 α siRNA
ATP-binding cassette, subfamily G, member 8 ^b	NM_022437	2.60	-0.77
Hypothetical protein LOC149703	AW299927	2.33	-1.30
Retinol binding protein 2, cellular ^c	NM_004164	2.10	-1.17
Tumor necrosis factor (ligand) superfamily, member 10	NM_003810	1.70	-0.97
Hypothetical protein LOC123876	AV696234	1.57	-1.37
<i>Homo sapiens</i> cDNA clone IMAGE:2544952.3'	AW051599	1.53	-1.70
Solute carrier organic anion transporter family, member 2B1	NM_007256	1.50	-1.00
Solute carrier organic anion transporter family, member 2B1	AB026256	1.47	-1.13
Phospholipase A2, group XIIB	AF349540	1.37	-0.97
<i>N</i> -Myristoyltransferase 2	AL134489	1.33	-0.93
Cytochrome P450, family 4, subfamily F, polypeptide 3	NM_000896	1.33	-1.20
Deiodinase, iodothyronine, type I	NM_000792	1.33	-0.97
cDNA FLJ38824 fis, clone LIVER2008945	AK096143	1.30	-1.07
Interleukin 28 receptor, alpha	AW340139	1.30	-1.30
Interleukin 22 receptor, alpha 1 ^b	NM_021258	1.30	-1.03
Aldehyde dehydrogenase 6 family, member A1	AW612403	1.30	-0.63
Phospholipase A2, group XIIB	BF939574	1.27	-1.07
Hypothetical protein LOC163782	AA456955	1.23	-0.93
Zinc finger protein 22 (KOX 15)	AA744771	1.17	-1.47
Mucin 20	AB037780	1.10	-1.10
Solute carrier family 13, member 5	AI797218	1.10	-1.23
Fatty acid binding protein 1, liver ^c	NM_001443	1.07	-1.03
Meprin A, alpha ^c	NM_005588	1.03	-1.20
Tumor necrosis factor (ligand) superfamily, member 10	U57059	1.00	-1.03
Betaine-homocysteine methyltransferase ^b	NM_001713	1.00	-0.83
3-Hydroxybutyrate dehydrogenase	BC005844	0.97	-0.83
Sulfotransferase family, cytosolic, 1C, member 1	AF026303	0.97	-1.23
Sulfotransferase family, cytosolic, 1C, member 1	AF186255	0.97	-1.10
Paired related homeobox 1	M95929	0.97	-0.90
Aldo-keto reductase family 1, member D1	NM_005989	0.93	-1.20
Solute carrier family 6, member 4	NM_001045	0.90	-1.03
Lectin, galactoside binding, soluble, 2	NM_006498	0.87	-0.73
Abhydrolase domain containing 2	AI557319	0.80	-0.73
Sulfotransferase family, cytosolic, 1C, member 1	NM_001056	0.80	-0.97
Hypothetical protein FLJ10847	NM_018242	0.77	-0.80
Cytochrome P450, family 19, subfamily A, polypeptide 1	NM_000103	0.73	-1.30
Sterol <i>O</i> -acyltransferase 2	AF059203	0.70	-1.27
Chromosome 7 open reading frame 32	AI884867	0.67	-0.90
unc-93 homolog A	AL021331	0.67	-1.57

^a RNAs from HepG2 cells treated with HNF4 α adenovirus or siRNA oligonucleotide were hybridized onto Affymetrix oligonucleotide arrays and quantified. Average change (2nd-fold) represents the regulation level in RNA prepared from control adenovirus or siRNA oligonucleotide-treated HepG2 cells. Overall, 18,595 probe sets (34%) were expressed in HNF4 α -transduced HepG2 cells (identified as "present" by Affymetrix software). The HNF4 α -transduced HepG2 cells expressed 18,595 probe sets and the HNF4 α -siRNA-transfected HepG2 cells 20,278 probe sets. Using the Mann-Whitney ranking feature of the Affymetrix software as criteria for statistical significance, we identified 985 probe sets as increased in the HNF4 α -transduced HepG2 cells and 724 as decreased in the siHNF4 α -treated HepG2 cells.

^b Genes reported as HNF4 α candidate genes from ChIP/promoter microarray studies from human hepatocytes.

^c Genes previously identified as direct HNF4 α targets on the basis of short-term transcription reporter assays with cultured cells and/or DNA binding assays.

ABCG5 and ABCG8 are the direct transcriptional targets of HNF4 α . We next sought to determine whether ABCG5 and ABCG8 expression levels are directly regulated by the binding of HNF4 α to the ABCG5 and ABCG8 intergenic control region. HNF4 α preferentially binds DNA as a homodimer to the HNF4 α -responsive element (HNF4 α -RE), which is composed of two nuclear receptor consensus half-sites of AG(G/T)TCA organized as a DR and separated by a single nucleotide (DR-1). We scanned the sequences of the intergenic control regions from the human, rat, mouse, and bovine genomes for the presence of putative DR-1 motifs and noticed a highly conserved DR-1-like element (namely, the site 1 DR-1 motif) flanked by two conserved GATA boxes. This region is located 176 nucleotides upstream of the translation start site for ABCG5 in all four species examined (Fig. 2A). In addition,

three other potential HNF4 α binding sites were revealed by manually scanning the sequence. These are located at positions -254, -267, and -372 (DR-1 sites 2, 3, and 4, respectively [Fig. 2B]).

To investigate whether HNF4 α binds and transcriptionally activates the ABCG5 and ABCG8 promoters, we generated luciferase reporter constructs of the 374-bp ABCG5/ABCG8 intergenic promoter by inserting this DNA into the promoter-less luciferase reporter gene pGL3-basic in both directions. Promoter-reporter constructs were transiently transfected into HepG2 cells along with increasing amounts of HNF4 α expression plasmid (Fig. 2C). As predicted, the expression levels of luciferase from both reporters were dramatically increased in proportion to the amounts of cotransfected HNF4 α expression plasmid (maxima of 17- and 5-fold, respectively). In a recipro-

FIG. 2. Both ABCG5 and ABCG8 are the direct transcriptional targets for HNF4α. (A) Schematic representation of the ABCG5 and ABCG8 intergenic promoter, illustrating four DR-1 motifs and two GATA boxes (A and B, top). The DR-1 motif at position -176 and two flanking GATA sites are conserved in human, bovine, rat, and mouse promoters. Conserved nucleotides are indicated by asterisks (bottom). (B) Alignment of four DR-1 motifs in the ABCG5 and ABCG8 promoter. The sequences of DR-1 motifs at positions -372, -267, -254, and -176 and the consensus nucleotide(s) found in HNF4α binding sequences are aligned. Since DR-1 motifs at positions -267 and -254 are in the inverted orientation, the bottom strand of the DNA is presented. In the consensus site, the consensus nucleotide(s) found in HNF4α binding sequences is represented with a capital letter; the lowercase letters point out divergences from the consensus that are represented as described in reference 55. (C and D) HNF4α activates the ABCG5 and ABCG8 promoters. The indicated amounts of HNF4α expression plasmids (C) or HNF4α-siRNA (D) were transfected into HepG2 cells together with pABCG5-luc and pABCG8-luc and incubated for 24 h, after which the cells were harvested and assayed for luciferase and β-galactosidase activity as described in Materials and Methods. The numbers above the open and closed circles refer to the induction relative to conditions in the absence of the HNF4α expression plasmid (C) or si-HNF4α (D). (E and F) Identification of an HNF4α-RE in the ABCG5 and ABCG8 promoter. Mutations were generated in the DR-1 motif at positions -372, -267, -254, and -176 in the context of pABCG5-luc (E) and in the context of pABCG8-luc (F), and mutants were assayed for transcriptional activity. Transfection and luciferase assays were performed as described above. The numbers above the bars refer to the increases induced by HNF4α. O.D., optical density.

cal manner, when HNF4α-specific siRNA was cotransfected, the expression levels from both promoter constructs were reduced by 40 to 60% (Fig. 2D). The promoter luciferase reporter activation potencies induced by HNF4α looked unequal, with ABCG5 being more robustly stimulated (~4-fold) than ABCG8. The reason for this is unclear, as the expressions of both mRNAs were equally affected in the HNF4α^{-/-}

mouse. The difference may be due to a higher basal promoter activity for ABCG8 in the luciferase reporter system, as the absolute levels of activity induced by transfection of HNF4α were similar for both constructs. It could also be due to subtle differences related to the opposite orientation of the same DNA fragment in the reporter construct. These results document that the intergenic control region is a bona fide bidirec-

FIG. 3. Specific binding of HNF4α to the ABCG5 and ABCG8 promoter. (A) EMSAs. Nuclear extracts isolated from HepG2 cells (25 μg) were incubated with a ³²P-labeled wild-type (wt) oligonucleotide probe carrying HNF4α-RE in the absence (lane 2) or presence of a 10- to 100-fold excess of unlabeled wild-type (lanes 3 to 5) or mutant (mt) (lanes 6 to 8) oligonucleotide. For the supershift assay, anti-HNF4α antibody K9218 (lane 10) or H1415 (lane 11) was added into the binding reaction mixture. Each anti-HNF4α antibody (2 μg) was added into the reaction 30 min prior to the addition of the probe. The HNF4α-DNA complex and its supershifted complex (S.S) are indicated on the left. (B) In vitro-synthesized HNF4α protein was incubated with a double-stranded, radiolabeled oligonucleotide probe containing the sequence of the high-affinity HNF4α binding site of the CYP8B1 promoter (55), followed by EMSA. Competition experiments were performed in the presence of a 50- to 200-fold molar excess of the unlabeled oligonucleotides for either the wild-type (lanes 2 to 4) or mutant (lanes 5 to 7) binding site 1 or the HNF4α binding site of the CYP8B1 promoter (lanes 8 to 10) as listed in Table 1. (C) The HNF4α-RE of the intergenic promoter of ABCG5 and ABCG8 binds to HNF4α in the context of an intact chromatin structure as demonstrated by ChIP. HepG2 cells were transfected with HNF4α-specific siRNA or control siRNA and harvested for ChIP as described in Materials and Methods. Recovery of the ABCG5 and ABCG8 promoter fragment following ChIP using HNF4α antibody was quantified by QRT-PCR (top). The presence of HNF4α in cell lysate before immunoprecipitation (Pre IP) and in samples immunoprecipitated with HNF4α antibody (Post IP), detected by immunoblot analysis with HNF4α antibody, is shown (bottom). All data represent the recovery of each DNA fragment relative to the total input DNA level. Each bar and symbol represent means ± SE for triplicate experiments. *, *P* < 0.01 compared to the control level. The data represent averages for at least three independent experiments.

tional promoter and that HNF4α is capable of activating expression in both directions, suggesting that both ABCG5 and ABCG8 are HNF4α target genes.

To evaluate which putative HNF4α site(s) in ABCG5 pro-

moter confers the HNF4α responsiveness in the 5'-to-3' orientation corresponding to the ABCG5 promoter (pABCG5-luc), mutations were introduced individually into each of the four putative HNF4α binding sites of the human ABCG5 pro-

motor and the corresponding mutant reporter genes were transfected into HepG2 cells. Mutation of the site 1 DR-1 motif dramatically reduced responsiveness to HNF4 α , while the other mutants responded similarly to the wild-type promoter (Fig. 2E). Conversely, when we evaluated the construct containing the intergenic control region in the 5'-to-3' orientation corresponding to the ABCG8 promoter (pABCG8-luc), the same site 1 DR-1 motif was also the only site that was crucial for reporter expression (Fig. 2F). Together, these data indicate that the site 1 DR-1 motif at position -176 is key to the HNF4 α transactivation of both the ABCG5 and the ABCG8 genes. Other DR-1 sites may also contribute to the basal transcriptional activity since mutations of other sites altered the basal transcriptional activity.

To determine whether HNF4 α is capable of binding to the site 1 DR-1 motif, EMSA was performed using ³²P-labeled oligonucleotide probes covering the site 1 DR-1 motif (double stranded 35-mer, from position -184 to -150) and nuclear extracts from HepG2 cells. The nuclear extracts contained proteins that bound to the site 1 HNF4 α motif (Fig. 3A, lane 2). The intensity of this band was diminished by the addition of a 50- to 100-fold molar excess of unlabeled probes (Fig. 3A, lanes 4 and 5), but no competition was seen with a mutated derivative of the site 1 HNF4 α motif (Fig. 3A, lanes 6, 7, and 8). To further examine whether this specific binding was due to HNF4 α , we included antibodies against HNF4 α (IgG-K9218) (Fig. 3A, lane 10) and IgG-H1415 (Fig. 3A, lane 11) or control IgG (Fig. 3A, lane 9) in the binding reaction. Incubation with anti-HNF4 α antibodies resulted in a clear supershifted band (Fig. 3A, S.S.), and the slowly migrating nonsupershifted band was barely visible (Fig. 3A, lanes 10 and 11). The control IgG had no discernible effect on the migration or intensity of any of the shifted bands (Fig. 3A, lane 9), indicating that the protein bound to the site 1 DR-1 motif was indeed HNF4 α . We further evaluated the ability of the site 1 HNF4 α motif to compete for binding to a consensus HNF4 α -RE (Fig. 3B). An ABCG5 and ABCG8 probe carrying the site 1 DR-1 motif was able to compete for binding to a radiolabeled band corresponding to the CYP8B1 HNF4 α -specific binding site (Fig. 3B, lanes 2 to 4) (55), indicating that the site 1 DR-1 motif is able to displace binding over the well-characterized HNF4 α -RE of the CYP8B1 promoter, albeit with a lower efficiency than the unlabeled self probe. Taken together, these results indicate that the ABCG5/ABCG8 intergenic control region has a single HNF4 α binding site that is critical for activation of gene expression in both directions.

Finally, we assessed binding of HNF4 α to the ABCG5/ABCG8 promoter in HepG2 cells by a quantitative ChIP assay. Fragmented chromatin from formaldehyde cross-linked HepG2 cells was subjected to immunoprecipitation with HNF4 α antibody or with IgG as a control, and the presence of the ABCG5/ABCG8 promoter in the immunoprecipitates was then analyzed by QRT-PCR. To validate the data, we also performed ChIP with HepG2 cells, where HNF4 α was knocked down by RNA interference. In control siRNA-transfected HepG2 cells, endogenous HNF4 α bound to the promoter of the ABCG5/ABCG8 gene (Fig. 3C, top, compare lanes 1 and 2), while in HNF4 α siRNA-transfected HepG2 cells, HNF4 α antibody barely immunoprecipitated the ABCG5/ABCG8 promoter (Fig. 3C, top, compare lanes 2, 3,

and 4). The immunoblot in the bottom panel of Fig. 3C shows that HNF4 α protein levels were decreased in the HNF4 α -specific siRNA-transfected cells (Fig. 3C, Pre IP) and that HNF4 α was efficiently captured by the immunoprecipitation procedure with anti-HNF4 α antibody (Fig. 3C, Post IP) in proportion to the overall levels (Fig. 3C, bottom; compare the gels labeled "Pre IP" for direct immunoblot with those labeled "Post IP"). Consistent with the results of EMSA showing that HNF4 α binds to the site 1 DR-1 motif *in vitro*, the results for the ChIP analysis revealed that HNF4 α associates with the site 1 DR-1 motif-containing region of the ABCG5 and ABCG8 promoter *in vivo*. Therefore, hereafter we refer to the site 1 DR-1 motif as an HNF4 α -RE in the ABCG5 and ABCG8 intergenic promoter region.

GATA binding sites are required for HNF4 α activation of ABCG5 and ABCG8 transcription. There is a consensus GATA box on each side of the site 1 HNF4 α -RE at position -176 in the ABCG5/ABCG8 promoter (the boxes are referred to as GATA-boxA and GATA-boxB) (Fig. 2A). The sequences of both GATA boxes and site 1 HNF4 α elements are completely conserved among the four species noted above. Both the HNF4 α and the GATA factors (GATA4 to 6) play crucial roles in endoderm development and organogenesis as well as the maintenance of differentiated organ function. A complex network of regulation involving GATA factors and HNF4 α is required for organogenesis (4, 17, 28, 30, 45); however, there are no reports that address a direct connection between these two important activator proteins. To examine the influence of GATA factors on the transcriptional activation of the ABCG5 and ABCG8 promoters by HNF4 α , we began by analyzing mutant reporter genes in which either or both of the GATA boxes were mutated in pABCG5-luc for promoter activity and HNF4 α responsiveness (Fig. 4A). The results indicate that GATA-boxB, which is immediately adjacent to the HNF4 α -RE, is indispensable for HNF4 α -mediated promoter activation of the intergenic control region in both orientations. Although the changes were not very profound, mutation of GATA-boxA in isolation caused an increase or decrease in promoter activity for ABCG5 or ABCG8, respectively, suggesting that GATA-boxA may also play a role in the activation of both promoters (Fig. 4A). Since immunohistochemical and gene expression microarray analyses indicated that both GATA4 and GATA6 were expressed in HepG2 cells (data not shown), we evaluated whether GATA4 or GATA6 might function with HNF4 α to activate ABCG5/ABCG8. EMSA demonstrated that *in vitro*-translated GATA4 bound to GATA-boxB (Fig. 4B), and quantitative ChIP assays demonstrated that endogenous GATA4 and GATA6 bind to the chromatin associated with the ABCG5/ABCG8 intergenic control region in HepG2 cells (Fig. 4C). These data suggested that transcriptional regulation of ABCG5 and ABCG8 might involve cooperative interaction between HNF4 α and GATA factors.

Synergistic activation of transcription by HNF4 α and GATA4. We tested the possibility of synergistic activation by using cotransfection, two-hybrid, *in vitro* binding, and coimmunoprecipitation assays. First, we examined the abilities of GATA factors to activate the ABCG5 and ABCG8 genes together with HNF4 α . Figure 4D shows the results for cotransfection assays with HepG2 cells, where we evaluated the roles of GATA4 and HNF4 α in transcriptional activation of the

FIG. 4. Transactivation of the human ABCG5/ABCG8 promoter by HNF4 α requires GATA factors. (A) The GATA box(es) is required for HNF4 α -dependent activation of the ABCG5/ABCG8 promoter. Mutations were generated into each GATA box located at position -190 (ABCG5 orientation, mutA) or -151 (mutB) or both (mutA+B) in the context of the pABCG5-luc and pABCG8-luc reporter constructs and assayed for transcriptional induction by GATA4 (0.15 μ g) as described in the legend to Fig. 2. (B) Mutation of GATA-boxB abolished GATA binding. Competition EMSA was performed as described in Materials and Methods. The 32 P-labeled wild-type (wt) probe specifically binds to GATA4 (lane 2). GATA4 binding is specifically competed by unlabeled wild-type probe (lanes 3 to 5) but not a mutant GATA-boxB oligonucleotide probe (lanes 6 to 8). (C) GATA boxes of the intergenic promoter of ABCG5 and ABCG8 bind GATA4 and GATA6 in the context of an intact chromatin structure as demonstrated by ChIP. Formaldehyde cross-linked, fragmented, chromatin-associated DNA from 1×10^7 HepG2 cells was immunoprecipitated with GATA4, GATA6, or control IgG. The recovery of the ABCG5 and ABCG8 promoter fragment following ChIP using the indicated antibody was quantified by QRT-PCR as described in the legend to Fig. 3C (top). The presence of GATA4 or GATA6 in the samples immunoprecipitated with GATA4 or GATA6 antibody (Post IP) detected by immunoblot analysis with GATA4 and GATA6 antibody (HRP-conjugated IgG-H2429 for GATA4 and HRP-conjugated IgG-H2617 for GATA6) is shown (bottom). All data represent the recovery of each DNA fragment relative to the total input DNA level. Each bar and symbol represent means $\pm$ SE for triplicate experiments. *, $P < 0.01$ compared to the control level. The data represent averages for at least three independent experiments. (D) Synergistic activation of the ABCG5 and ABCG8 gene promoter by HNF4 α and GATA factors, an intact GATA box(es), and the HNF4 α -RE are required for HNF4 α /GATA4 synergy. HepG2 cells were cotransfected with ABCG5 and ABCG8 promoter luciferase reporter plasmids containing the wild type, the indicated mutant GATA-box, or HNF4 α -RE (Mut site 1) in combination with the HNF4 α (0.15 μ g) and GATA4 (0.15 μ g) expression plasmid as indicated.

ABCG5 and ABCG8 gene promoters. When the ABCG5-luc promoter reporter construct was cotransfected with the GATA4 expression plasmid into HepG2 cells, luciferase activity was minimally affected and transfection of the HNF4 α expression construct alone resulted in significant stimulation (15-fold). When both expression constructs were cotransfected, there was a dramatic stimulation of promoter activity (120-fold). In this experiment, mutation of GATA-boxB markedly reduced GATA/HNF4 α synergy, and simultaneous mutation of GATA-boxA and GATA-boxB (G5 orientation) (Fig. 4D, MutA+B) as well as the HNF4 α -RE mutant (Fig. 4D, Mut site1) completely abolished activity. In contrast, mutation of only the GATA-boxA mutant did not alter GATA/HNF4 α synergy. Similar results were obtained for the ABCG8-luc construct, except that mutation of GATA-boxA resulted in a significant decrease in HNF4 α /GATA synergy. Taken together, these results indicate that GATA-boxB, which is adjacent to HNF4 α -RE, is necessary for ABCG5 promoter activity, while GATA-boxA also contributes to GATA/HNF4 α synergy in the ABCG8 promoter. GATA6 also synergistically activated ABCG5 and ABCG8 promoter activity in a similar manner (data not shown).

The AF-2 domain of HNF4 α is required for HNF4 α /GATA4 synergy. To better understand the mechanisms involved in HNF4 α /GATA4 synergy, we carried out structure function analyses of HNF4 α and GATA4. A series of HNF4 α mutants were coexpressed, and ABCG5 promoter activities were tested for their abilities to respond to GATA4. As shown in Fig. 5A, a deletion of 10 aa removing the conserved AF-2 motif [HNF4 α (1-361)] essentially abolished synergy with GATA4, while an analogous construct with an intact AF-2 motif [HNF4 α (1-368)] showed strong synergy (Fig. 5A). The internal deletion of this core of the AF-2 motif [HNF4 α (Δ 359-368)] also abrogated synergy with GATA4 on the ABCG5 promoter. These data demonstrate that the AF-2 domain of HNF4 α is required for synergy with GATA4.

Next, to identify GATA sequences required for HNF4 α /GATA4 synergy, a series of GATA4 mutants were coexpressed and ABCG5 promoter activities were tested for their abilities to respond to HNF4 α . The GATA proteins contain two transcriptional activation domains flanking the two-zinc-finger DNA binding domain (Fig. 5B). Removal of either the first 201 aa or the C-terminal activation domain (aa 333 to 442), which decreases GATA transcriptional activity, reduced but did not abrogate synergy; deletion of the two-zinc-finger domains (aa 202 to 303) or the adjacent basic domain (aa 304 to 332) profoundly decreased GATA transcriptional activity and abolished HNF4 α -mediated transcription over the ABCG5 promoter.

GATA4 interacts with the AF-2 domain of HNF4 α in mammalian cells. The mechanism involved in the synergistic activation mentioned above may involve a direct interaction between the HNF4 α and GATA4 proteins. To test this hypothesis, we employed a two-hybrid approach with mammalian cells using a Gal4-HNF4 α chimera and a fusion of the VP16 transactivation domain to GATA4 (Fig. 5C and D). As indicated in Fig. 5C, coexpression of a GAL4-HNF4 α 2 chimera with the VP16-GATA4 fusion stimulated GAL4 luciferase reporter gene expression beyond that observed with Gal4-HNF4 α alone. Coexpression of HNF4 α 8, an isoform that lacks

the N-terminal AF-1 domain but shares the same C terminus, also increased reporter gene expression, suggesting that the C-terminal domains of both HNF4 α isoforms associate with GATA4. Removal of the first 116 aa (the A-C domain) and deletion of the C-terminal F domain (aa 369 to 465) did not abrogate reporter gene expression; however, a deletion of 10 aa, removing the conserved AF-2 motif in the E domain, essentially abolished stimulation of reporter gene expression with VP16-GATA4. Overall, these data demonstrate that HNF4 α interacts with GATA4 through its AF-2 motif and, consistent with the findings that AF-2 interacts with GATA4, both HNF4 α 2 and HNF4 α 8 exhibit synergy with GATA4 in activation of both the ABCG5 and the ABCG8 promoters (data not shown).

Next, we examined which domains of GATA4 were required for HNF4 α interaction. As shown in Fig. 5D, removal of aa 202 to 303 or aa 202 to 332 but not removal of aa 304 to 332 of GATA4 resulted in the loss of stimulation of GAL4 reporter gene expression, indicating that the domain containing the two zinc fingers is required for the association with HNF4 α .

Using a GST pull-down assay, we examined whether HNF4 α binds directly to GATA4 or GATA6. HNF4 α 2 deletion mutants were expressed in *Escherichia coli* as fusion proteins with GST, and ³⁵S-labeled GATA4 or GATA6 was synthesized in vitro. The data in Fig. 5E show that deletion of the AF-2 domain of HNF4 α 2 abolishes the interaction with GATA4. The same results were obtained with ³⁵S-labeled GATA6 (data not shown). Using similar methods, the HNF4 α binding domain of GATA4 was examined. As shown in Fig. 5F, deletion of aa 202 to 303 of GATA4 but not removal of aa 304 to 332 completely abolished the binding to HNF4 α .

Overall, these results are in agreement with those derived from our mammalian two-hybrid experiments (Fig. 5C) and support our hypothesis that the AF-2 domain of HNF4 α interacts physically with the zinc finger motif of GATA4 to synergistically activate ABCG5/ABCG8 gene expression. Consistent with the current finding that HNF4 α and GATA4 or GATA6 physically and functionally interact, endogenous HNF4 α localized exclusively to the nucleus, presumably the nucleoplasm, and colocalized with GATA4 and GATA6 as determined by double immunofluorescence staining (data not shown).

DISCUSSION

The recent demonstration that mutations in the ABCG5 and ABCG8 genes result in human genetic disorders of cholesterol homeostasis has made it very important to investigate the mechanisms for how these key proteins are expressed and regulated during normal metabolic fluctuations and in response to pathological states. Simultaneous up-regulation of ABCG5 and ABCG8 promotes biliary sterol excretion and prohibits intestinal absorption of luminal cholesterol, thereby leading to the net cholesterol loss from the body (34, 53, 54). These actions point to the possible therapeutic potential of targeting ABCG5/ABCG8 for the treatment of atherosclerosis and other disorders of hypercholesterolemia. The ABCG5 and ABCG8 genes are tightly linked and divergently transcribed. The data presented here clearly demonstrate that the human ABCG5 and ABCG8 intergenic promoter region contains con-

FIG. 5. Structure function analyses of HNF4α and GATA4. (A and B) Mapping of domains required for HNF4α and GATA4 synergy. The ABCG5-luc reporter constructs were cotransfected into HepG2 cells with the wild type or the indicated mutant of either HNF4α (A) or GATA4 (0.15 μg each) (B) and assayed for luciferase activities as described in the legend to Fig. 2. The various HNF4α and GATA4 expression constructs are diagrammed at the left of each graph. Numbers at the bottom indicate the amino acids deleted or retained in each expression construct. Letters in the diagram (A to F) indicate conventional nuclear receptor domains. Nf and Cf, amino- and carboxy-terminal zinc fingers, respectively; TAD, transcriptional activation domain. (C and D) Mapping of the in vivo interaction domains of HNF4α and GATA4 evaluated by the mammalian two-hybrid assay. CHO cells were transfected with the reporter vector pGAL4-luc (0.15 μg) and the hybrid constructs or empty plasmids (0.015 μg each) indicated. Data are representative of four experiments performed in duplicate. All error bars indicate deviation SE. (E and F) Mapping of the in vitro interaction domains of HNF4α and GATA4. GST pull-down assays were performed with ³⁵S-labeled GATA4 in the presence of GST or GST fusions containing the indicated HNF4α deletion mutants immobilized to glutathione beads (E) or various ³⁵S-labeled GATA4 mutants with GST-fused HNF4α(135-465) fusion proteins coupled to glutathione beads (F). After being washed, specifically bound proteins were eluted, separated by SDS-PAGE, and detected by autoradiography. The input samples contain 1% of the material added to each GST assay. O.D., optical density.

served DNA elements that bind HNF4α and GATA factors. We also demonstrate that these two proteins synergistically activate expression from the intergenic control region in a bidirectional fashion, strongly suggesting that coordinate regulation of ABCG5 and ABCG8 occurs through the binding of

HNF4α and GATA factors to the same *cis*-acting elements. This is an unusual mechanism for ensuring coordinate regulation of the two proteins. Because HNF4α is a nuclear receptor that regulates a number of genes involved in removal of sterols and bile acids from liver and intestine (3, 5, 27), ABCG5 and

ABCG8 can be added to the growing list of key lipid metabolism genes that are regulated by HNF4 α .

We also showed that HNF4 α , GATA4, and GATA6 were colocalized in the same compartment of nuclei in HepG2 cells (data not shown) and that a physical interaction between GATA factors and HNF4 α is likely important for synergistic activation of ABCG5 and ABCG8 (Fig. 5). In the adult rat small intestine, GATA4 and GATA6 were localized in the majority of enterocytes and crypt epithelial cells. In the liver, GATA6 was localized in hepatocytes and intrahepatic bile duct epithelial cells (data not shown). HNF4 α was also localized to enterocytes and crypt epithelial cells and hepatocytes and intrahepatic bile duct epithelial cells (47). This localization pattern is consistent with GATA4, GATA6, and HNF4 α being responsible for the robust and tissue-restricted expression of the ABCG5 and ABCG8 gene products in hepatocytes and enterocytes.

Both HNF4 α and the monomeric LRH-1 receptor are liver-enriched nuclear receptors and share overlapping roles for bile acid biosynthesis by regulating the expressions of CYP7A1 and CYP8B1, which are key gene expressions for bile acid biosynthesis (8, 26, 55). It was reported previously that LRH-1 binds to the ABCG5/ABCG8 intergenic promoter to maintain the basal transcriptional activity (6). Our results, using the liver-specific HNF4 α knockout mice and targeted over- and under-expression of HNF4 α in cultured cells, show that the contribution of HNF4 α to the normal promoter activities of ABCG5 and ABCG8 is approximately 40 to 60% (Fig. 1A and C and 2D).

It is conceivable that both LRH-1 and HNF4 α contribute to maintaining the basal transcriptional activities of these genes; however, our experiments indicate that HNF4 α plays a more significant role because HNF4 α strongly induced ABCG5 and ABCG8 promoter activities and functioned synergistically with GATA factors in this process. In contrast, LRH-1 activated the ABCG5 and ABCG8 promoter at only very modest levels of less than twofold and coexpression of GATA did not result in synergistic activation in HepG2 cells (data not shown).

Studies with LXR knockout animals and synthetic LXR agonists suggest that transcription of ABCG5 and ABCG8 genes is under LXR control (34). However, an LXR-responsive element has not been identified in the vicinity of the ABCG5/ABCG8 intergenic control region. Based on our studies, it will be important to determine whether an LXR α -dependent pathway is linked to the HNF4 α /GATA-dependent pathway uncovered here.

The current study revealed that the ABCG5 and ABCG8 intergenic promoter contains the core regulatory elements through which HNF4 α and GATA robustly stimulate transcription in both directions. Because the ABCG5 and ABCG8 proteins form a complex that exports cholesterol from both enterocytes and hepatocytes and limits net cholesterol absorption, therapeutic strategies for changing their expression by modulating HNF4 α /GATA synergy may represent an attractive target for preventing and treating atherosclerosis and hypercholesterolemia.

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