Control of ACAT2 liver expression by HNF1

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Abstract ACAT catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acids. There are two known genes encoding the two ACAT enzymes, ACAT1 and ACAT2 (also known as Soat1 and Soat2). In adult humans, ACAT1 is present in most tissues, whereas ACAT2 is localized to enterocytes and hepatocytes. In this report, we elucidate the mechanisms that control the liver-specific expression of the human ACAT2 gene. We identified hepatic nuclear factor 1 (HNF1) as an important liver-specific *trans*-acting element for the human ACAT2 gene using the human hepatocellular carcinoma cell lines HuH7 and HepG2. Targeted deletion of the HNF1 binding site in the DNA sequence abolished not only the basal promoter function in HepG2 and HuH7 cells but also the induction of the ACAT2 promoter by HNF1. Electrophoretic mobility shift assay and chromatin immunoprecipitation assay demonstrated that the transcription factors HNF1α and HNF1β interact with this region in the human ACAT2 gene in vitro and in vivo. These data indicate that *a*) the identified HNF1 binding site serves as a positive regulator sequence, *b*) the binding site is functionally active both in vivo and in vitro, and *c*) the transcription factors HNF1α and HNF1β, which bind to this site, play an important part in the regulation of the human ACAT2 promoter.—Pramfalk, C., M. A. Davis, M. Eriksson, L. L. Rudel, and P. Parini. **Control of ACAT2 liver expression by HNF1.** ***J. Lipid Res.*** **2005.** 46: **1868–1876.**

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ACAT is an integral membrane protein of the rough endoplasmic reticulum that catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acids (1). The solubility characteristics of cholesteryl esters make them the major choice for the storage of cholesterol, mostly as cytoplasmic lipid droplets inside the cells. Thus, ACAT is a key enzyme in the control of intracellular cholesterol storage and in determining the level of free cholesterol (2). There are two known genes that encode the two ACAT enzymes, ACAT1 and ACAT2, also known by international convention as steroyl *O*-acyltransferase (i.e., Soat1 and Soat2, respectively) (3). ACAT1 has been shown to be present in most tissues, whereas the location of ACAT2 has been relatively unclear. According to previous studies (4–6), ACAT2 is found mainly in the apical region of the intestinal villi and in human fetal liver, whereas ACAT1 is the major ACAT isoenzyme in adult human liver. However, we recently showed that ACAT2 is the major ACAT isoenzyme in adult human liver and that it is located in hepatocytes, whereas ACAT1 is located in Kupffer cells (7). This is consistent with similar findings in mice and nonhuman primates (8, 9) and is also in agreement with the proposed role for ACAT2 in the assembly and secretion of cholesteryl esters in lipoproteins.

Based on this new knowledge, ACAT2 may be a viable target for the treatment and prevention of diseases associated with cholesterol accumulation (e.g., atherosclerosis). Thus, it is of great importance to characterize the mechanisms involved in ACAT2 transcriptional regulation. In the present study, we identify an important liver-specific *cis*-acting element in the promoter region of ACAT2 that acts as a putative binding site for the hepatic nuclear factor 1 (HNF1). This element serves as a positive regulator of gene expression and is functionally active. Finally, by chromatin immunoprecipitation assay, we show that the transcription factors HNF1α and HNF1β bind to the identified promoter region of ACAT2 in vivo in human liver.

EXPERIMENTAL PROCEDURES

Materials

HepG2, HEK293, and HuH7 cells were purchased from the American Type Culture Collection (Manassas, VA). HNF1α ex-

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Abbreviations: Cdx-2, caudal-related homeodomain protein-2; C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation assay; EMSA, electrophoretic mobility shift assay; HNF1, hepatic nuclear factor 1; MODY, maturity-onset diabetes of the young; TESS, transcription element search software.

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pression vector was a generous gift from Prof. Pal R. Njølstad and Dr. Lise Bjørkhaug Gundersen (Haukeland University Hospital, Norway). HNF1β expression vector was a generous gift from Prof. G. U. Ryffel at the Institute for Cell Biology (Essen, Germany). A human liver sample was obtained from a donor under a protocol approved by the ethics committee at Huddinge University Hospital Karolinska. All cell culture reagents were purchased from GIBCO (Paisley, Scotland). All chemicals used for the electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation assay (ChIP) buffers were purchased from Sigma (St. Louis, MO). Protein G-Sepharose 4 Fast Flow was purchased from Pharmacia Biotech (Uppsala, Sweden).

Cell culture

HepG2 and HEK293 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. HuH7 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in 75 cm^2 cell culture flasks and passaged when they reached ~90% confluence.

Construction of ACAT2 promoter constructs

The 5′ untranslated genomic sequence (~4 kb) of the ACAT2 gene was amplified from human genomic DNA (forward primer, 5′-CTGCCCTCAGCCTATCTTGGT-3′; reverse primer, 5′-TGCGGTCTCCAGCGGGCAG-3′). To facilitate directional cloning into pGL3 vector and to isolate the promoter region, the product was digested with *Kpn*I and *Pst*I, because these restriction enzymes generated an ~1.4 kb fragment from −1,305 to +86. 5′ nested deletions of the human ACAT2 promoter construct were obtained by PCR using the ~1.4 kb construct as template. The forward primers for the four truncations were as follows: −269, 5′-GACAAGCTTCTGAGAGG-3′; −782, 5′-CTAATATATGAGCTCATCC-3′; −1,044, 5′-TTAGTCCTTCCTGTGACAGC-3′; and −1,196, 5′-GAGTGTTGGTGTTGGCTGG-3′. All constructs were sequenced to confirm homology with the human ACAT2 promoter region.

Identification of possible transcription factor binding sites and mutagenesis

To search for putative transcription factor binding sites as potential regulators in the human ACAT2 promoter region, the sequence was analyzed using the transcription element search software (TESS) database (www.cbil.upenn.edu) at the University of Pennsylvania (Philadelphia, PA). Plasmids carrying specific point mutations for these *cis*-elements were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. A pair of complementary primers was designed using Stratagene's World Wide Web-based primer design software program (http://labtools.stratagene.com). Mutant constructs of the p−1044 and the full-length promoter (p−1305) constructs were generated by deletion of the six bases indicated with lowercase letters. For the CCAAT/enhancer binding protein (C/EBP), the forward sequence was 5′-CTGCCCACCAGGAgttgggAGTGGGAAGGGGA-3′ and the reverse sequence was 5′-TCCCCTTCCCACTcccaacTCCTGGTGGGCAG-3′. For HNF1, the forward sequence was 5′-GGAGGGGAAGGATTAATAgttaatCCCAGCAGGAACCC-3′ and the reverse sequence was 5′-GGGTTCCTGCTGGGattaacTATTAATCCTTCCCCTCC-3′. These primers were used to amplify the entire plasmid DNA by PCR using Pfu Turbo DNA polymerase (Stratagene). Plasmids were sequenced (Cybergene, Stockholm, Sweden) to confirm that correct deletions were obtained.

EMSA and supershift assay

Nuclear extracts were prepared from HuH7 cells and cultured in 75 cm^2 cell culture flasks, as described by Azzout-Marniche et al. (10). The forward sequence for the HNF1 binding site was 5′-AAGGATTAATAGTTAATCCCAGCAGGAACCC-3′. The underlined bases were deleted in the forward sequence for the mutated HNF1 binding site. Unlabeled double-stranded probes were generated by mixing 1 μg of forward and 1 μg of reverse oligonucleotide, 5 μl of 1 M NaCl, and double-distilled water up to 50 μl and annealed at 95°C for 10 min. Labeled probes were generated by mixing 5 μl of unlabeled probe, 2 μl of 10× PNK buffer (Promega, Madison, WI), 1 μl of 0.1 M DTT, 1.5 μl of T4 polynucleotide kinase (Promega), 2 μl of [γ-^{32}P]ATP, and double-distilled water up to 50 μl and incubated at 37°C for 1 h, before passing through a Sephadex G-50 column (Amersham, Uppsala, Sweden). The labeled probes (40,000 cpm) were combined with 10 or 20 μg of nuclear extract along with 2 μg of poly(dI-dC) (Amersham, Piscataway, NJ), 7.5 μl of DNA binding buffer [80 mM Hepes-NaOH, pH 7.6, 0.2 M NaCl, 40 mM DTT, 20% (v/v) glycerol, 2 mM EDTA, and 1.2 mg/ml bovine serum albumin], 1 μl of 0.1 M DTT, 4 μl of buffer C [10 mM Hepes-KOH, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, and protease inhibitors], and double-distilled water up to 30 μl. Binding reaction mixtures were incubated for 20 min at room temperature and resolved on a nondenatured (5%, w/v) acrylamide gel in 1× Tris borate EDTA at 4°C for 3 h. For competition assays, 100-fold molar excess of unlabeled probe was added to the binding reaction mixture. For supershift assays, 2 μg of HNF1α antibody (catalog number Sc-6547X; Santa Cruz Biotechnology, Santa Cruz, CA) or 2 μg of HNF1β antibody (catalog number Sc-22840X; Santa Cruz Biotechnology) was added to the binding reaction mixture. After electrophoresis, gels were dried and exposed overnight to X-ray film at −80°C.

ChIP

ChIP was performed as described (11), with modifications. Approximately 200 mg of human liver was diced on ice, suspended in PBS, and fixed at room temperature in 1% (v/v) formaldehyde to cross-link proteins to DNA. Reactions were stopped by the addition of glycine (0.125 M) and pelleted two times by centrifugation, first at 3,000 rpm for 10 min and then at 7,500 rpm for 5 min. The final pellet was then suspended in ChIP buffer [50 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, pH 8.0, 1% Triton X, and 0.14 M NaCl supplemented with a protease inhibitor mixture from Roche (Mannheim, Germany)] and incubated for 10 min at 4°C. Genomic DNA was sheared by sonication on ice using a Branson sonifier 250. Supernatant, collected by centrifugation for 10 min at 13,000 rpm, was preimmunocleared by the addition of protein G-Sepharose slurry for 2 h at 4°C. After centrifugation for 2 min at 5,000 rpm, the supernatant was divided into aliquots and immunoprecipitated overnight at 4°C in a solution containing 5 μg/μl sonicated salmon sperm DNA, 10 mg/ml BSA, and the specific antibodies for HNF1α or HNF1β or an IgG antibody (catalog number Sc-2027; Santa Cruz Biotechnology) used as a baseline control. Immunocomplexes were precipitated by the addition of protein G-Sepharose slurry and centrifugation for 2 min at 5,000 rpm. Pellets were washed twice in TSE I (1% Triton X, 2 mM EDTA, 20 mM Tris, pH 8.0, and 0.15 M NaCl), once in LiCl buffer (20 mM Tris, pH 8.0, 1 mM EDTA, 0.25 M LiCl, and 1% NP40), and twice in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). The precipitated immunocomplexes were dissociated by incubation in TE buffer with 1% SDS at room temperature for 30 min, and the cross-linking was reversed by incubation for 6 h at 66°C. DNA fragments were purified using Wizard SV Gel and the PCR Clean-Up System kit (Promega). For real-time PCR, the ABI PRISM 7700 sequence detector and qPCR Mastermix Plus for SYBRGreen (Med Probe, Oslo, Norway) were used. The forward sequence for the human ACAT2 promoter was 5′-TTAATCCCAGCAGGAACCCAG-3′ and the re-

verse sequence was 5′-AGTAACAGAAGGGTATGTGCTTTGAG-3′. Primers used to standardize for DNA loading in the PCR were designed using human exon 9 of ACAT2, with the forward sequence 5′-GCTATAGCCTTGGGCCACC-3′ and the reverse sequence 5′-TCACAAGAATTCGACAGCCAGAT-3′. Cycle numbers for the human ACAT2 promoter minus the respective cycle numbers for human exon 9 of ACAT2 were used to calculate the amount of specific HNF1 *cis*-element sequence in the immunoprecipitated samples, according to the delta-cycle threshold (delta-C_T) calculation. Delta-C_T values were thereafter linearized and expressed as fold induction from baseline control (IgG). After 40 cycles, PCR products were separated on a 2% agarose gel to monitor that a single amplicon of the correct correspondent size was present.

Transfection and reporter activity assays

HepG2, HuH7, and HEK293 cells were plated out on six-well tissue culture plates so that they reached ~70% confluence after 24 h. Transfections of HepG2 cells were performed with 2 μg of promoter construct and 2 μg of pSV-β-galactosidase control vector (Promega) or, in the case of cotransfection assays, 2 μg of promoter construct, 2 μg of pSV-β-galactosidase control vector, and 0.1 μg of expression vector using Fugene-6 reagent (Roche, Indianapolis, IN) at a ratio of 6:1 (Fugene-6/DNA). Transfections of HuH7 cells were performed like those for HepG2 cells, except that Lipofectin reagent (Invitrogen, Carlsbad, CA) at a ratio of 3:1 (Lipofectin/DNA) was used. Transfections of HEK293 cells were performed with 1 μg of promoter construct and 1 μg of pSV-β-galactosidase control vector or, in the case of cotransfection assays, 1 μg of promoter construct, 1 μg of pSV-β-galactosidase control vector, and 0.1 μg of expression vector using Lipofectamine 2000 reagent (Invitrogen) at a ratio of 0.25:1 (Lipofectamine 2000/DNA). The pSV-β-galactosidase control vector was used to correct for variation in transfection efficiency. Forty-eight hours after transfection, cellular lysates were prepared in reporter lysis buffer (Promega). β-Galactosidase and luciferase activities were determined using a β-galactosidase assay kit and a luciferase assay kit, respectively, according to the manufacturer's instructions (Promega). All transfection data are expressed as luciferase activity corrected by β-galactosidase activity. Experiments were performed in five replicates and were repeated at least twice. Data represent means ± SEM.

RESULTS

Cloning and identification of *cis*-acting elements

To be able to identify regions that are important for human ACAT2 gene activity in liver, the ~1.4 kb promoter region was used as template to create four deletion constructs, termed p−1196 (−1,196 to +86), p−1044 (−1,044 to +86), p−782 (−782 to +86), and p−269 (−269 to +86). We then transiently transfected them into HepG2, HuH7, and HEK293 cells using pGL3 basic vector as a baseline control. As shown in **Fig. 1**, the p−1044 construct conferred maximum luciferase activity in both HepG2 and HuH7 cells, and the activity declined appreciably when comparing the p−1044 with the p−782 construct, suggesting the presence of potential positive regulatory elements in this region. Moreover, the promoter activity increased >4-fold with the p−1044 construct compared with the full-length (~1.4 kb) promoter construct (p−1305) in both HepG2 and HuH7 cells, suggesting the presence of potential repressor elements. Further deletions from nucleotide positions −782 to −269 did not have a significant effect on the ACAT2 promoter. Although the HepG2 cells displayed higher basal activity than HuH7 cells, both cell lines followed similar patterns of activity.

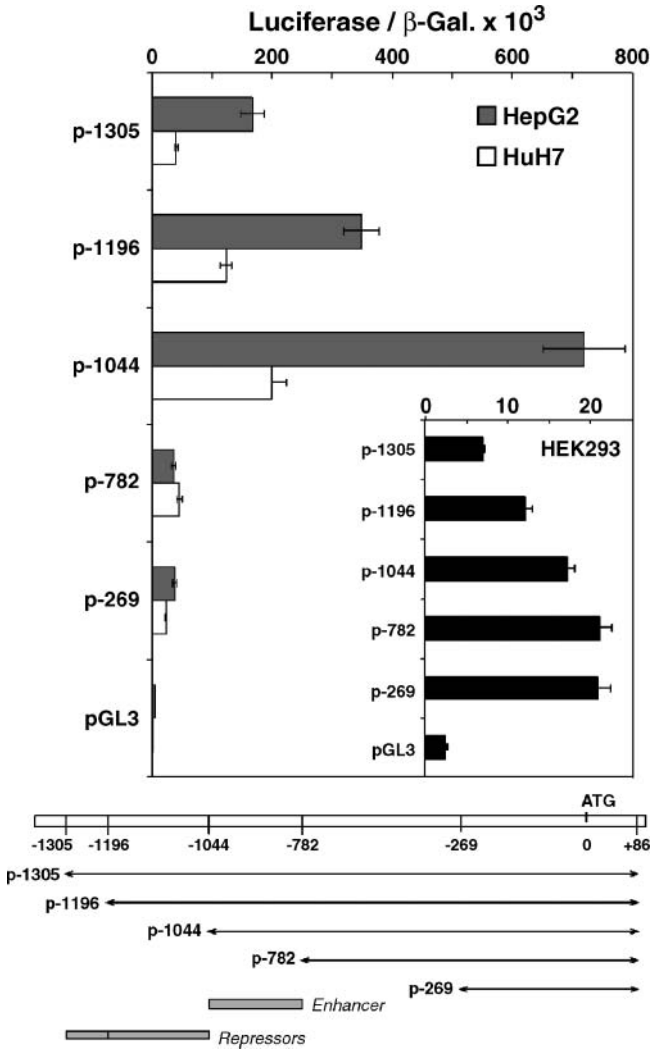

Fig. 1. Effect of ACAT2 promoter length on hepatocyte-specific expression. Nearly 1.4 kb of the 5′ flanking sequence upstream of the start codon ATG of the human ACAT2 gene was cloned into a pGL3 basic vector (−1,305 to +86), and four progressive 5′ nested deletions, termed p−1196 (−1,196 to +86), p−1044 (−1,044 to +86), p−782 (−782 to +86), and p−269 (−269 to +86), were transfected into HepG2, HuH7, and HEK293 cells as described in Experimental Procedures. pSV-β-galactosidase (β-Gal) control vector served as an internal control of transfection efficiency. The empty (pGL3) reporter vector served as a baseline control. Luciferase and β-galactosidase activities were measured in cell lysates 48 h after transfection. Luciferase activity was corrected by β-galactosidase activity. All transfections were performed in five replicates, and data represent means ± SEM.

To investigate the hepatocyte and promoter specificity of these findings, we transfected the human kidney cell line HEK293, which does not express ACAT2 (6), with the same ACAT2 promoter constructs described above. HEK293 cells displayed >20-fold lower luciferase activity than HepG2

cells, with a completely different pattern (Fig. 1). The highest activity was seen in the p−782 and p−269 constructs. This led us to conclude that potential repressor elements could be present in the region between −1,305 and −1,044 bp and that positive regulatory elements are present in the region between −1,044 and −782 bp upstream of the transcription start site and that these are liver-specific. We chose to characterize the positive regulatory elements in detail, without further studies of the potential repressor elements.

We screened the sequence using TESS to search for putative transcription factor binding sites as potential positive regulators in the promoter region between −1,044 and −782 bp upstream of the ATG start codon of the human ACAT2 gene. Several *cis*-elements were located in this core region, two of which displayed a 100% match. The first was a putative binding site for the transcription factors HNF1α and HNF1β, and the second was a putative binding site for the transcription factors C/EBPα and C/EBPβ. Therefore, we performed specific mutation analyses to examine the relative importance of these elements.

Constructs carrying these specific deletions, termed p−HNF1 mutant (deletion of the putative *cis*-element for HNF1α and HNF1β) and p−C/EBP mutant (deletion of the putative *cis*-element for C/EBPα and C/EBPβ), were transiently transfected into HepG2 and HuH7 cells (**Fig. 2A**). For mutation analyses, we used the p−1044 construct because of its high transcription activity. As shown in Fig. 2B, C, deletion of the six bases GTTGGG, which correspond to the putative *cis*-element for C/EBPα and C/EBPβ, did not have a significant effect on the luciferase activity in either HuH7 or HepG2 cells when comparing the p−1044 with the p−C/EBP mutant construct. However, deletion of the bases GTTAAT, which correspond to the putative *cis*-element for HNF1α and HNF1β, decreased the activity by 5- to 6-fold in both HuH7 and HepG2 cells. Furthermore, deletion of the putative binding site for HNF1 decreased the luciferase activity to a level similar to that observed for the p−782 construct. Transfection with a construct carrying deletions for both the putative HNF1 and C/EBP binding sites (p−HNF1 and p−C/EBP mutant constructs) had the same effect on luciferase activity as deletion of the HNF1 *cis*-element alone. We also generated a deletion of the putative *cis*-element for HNF1 in the full-length promoter construct (termed p−HNF1 mutant p−1305) and transiently transfected it into HepG2 cells. Similar to what was observed for the p−1044 construct, deletion of the putative HNF1 binding site decreased the luciferase activity almost 9-fold compared with that in the full-length promoter construct, as shown in Fig. 2D.

Interaction of HNF1α and HNF1β with the human ACAT2 promoter in vitro and in vivo

To investigate whether this HNF1 binding site was functional, we isolated nuclear extracts from HuH7 cells and performed EMSA and supershift assays (**Fig. 3**). Two specific bands, labeled with the HNF1 probe, were detectable (lanes 1 and 2), corresponding to 10 and 20 μg nuclear extracts, respectively. Preincubation with an excess of un-

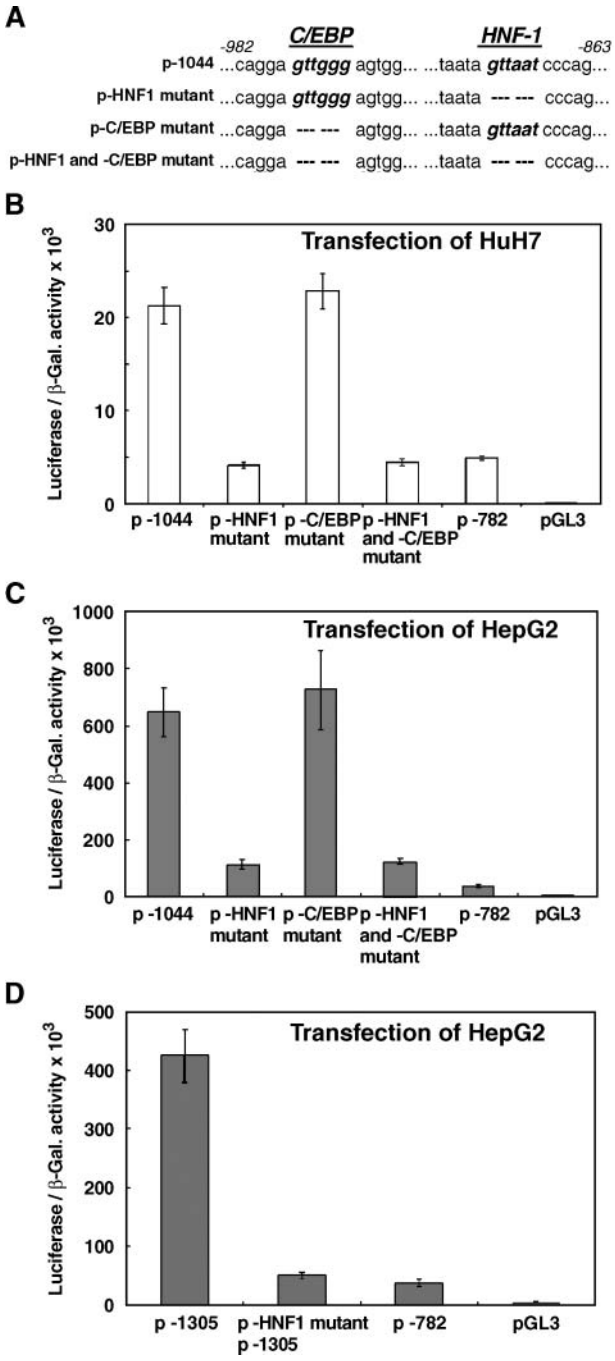

Fig. 2. Effects of deletions of hepatic nuclear factor 1 (HNF1) and CCAAT/enhancer binding protein (C/EBP) elements in the human ACAT2 gene on hepatocyte-specific expression. p−C/EBP mutant, p−HNF1 mutant, and p−HNF1 and −C/EBP mutant constructs (A), carrying deletions for the putative C/EBP, the putative HNF1, and for both putative binding sites in the p−1044 construct, respectively, were transiently transfected into HuH7 cells (B) and HepG2 cells (C). D: p−HNF1 mutant p−1305 construct, carrying a deletion for the putative HNF1 binding site in the full-length promoter construct, was transiently transfected into HepG2. pSV-β-galactosidase (β-Gal) control vector served as an internal control of transfection efficiency. The empty (pGL3) reporter vector served as a baseline control. Luciferase and β-galactosidase activities were measured in cell lysates 48 h after transfection. Luciferase activity was corrected by β-galactosidase activity. All transfections were performed in five replicates, and data represent means ± SEM.

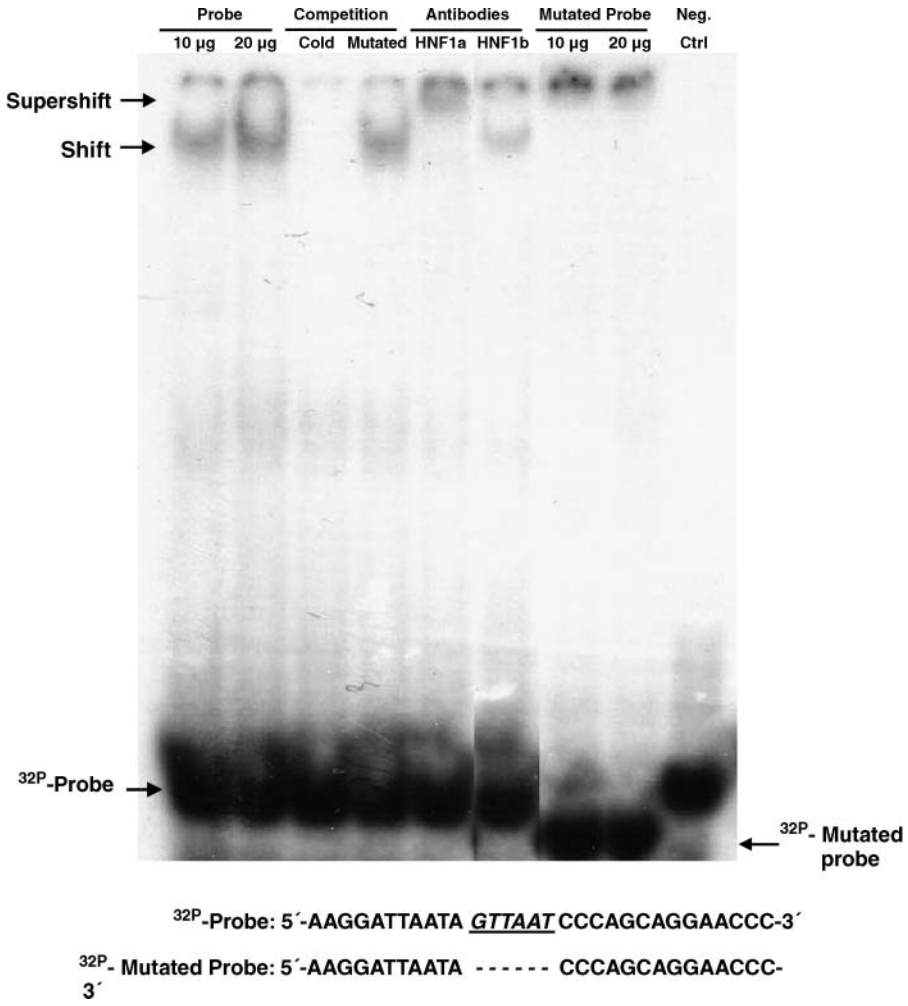

Fig. 3. Functionality of the HNF1 binding site in the human ACAT2 gene. Nuclear extracts, prepared from HuH7 cells, were incubated with ^{32}P-labeled double-stranded probes with or without a mutation for the HNF1 binding site and resolved on a nondenatured (5%, w/v) acrylamide gel. For competition assays, a 100-fold molar excess of unlabeled probe was added to the binding reaction mixture, and for the supershift assays, 2 μg of HNF1α or HNF1β antibodies was added. Lanes 1 and 2, binding reaction between 10 and 20 μg nuclear extracts, respectively, and ^{32}P-labeled HNF1 probe. Lane 3, competition between labeled and unlabeled HNF1 probe. Lane 4, competition between labeled HNF1 probe and unlabeled mutated HNF1 probe. Lane 5, supershift reaction between labeled HNF1 probe and 2 μg of HNF1α antibody. Lane 6, supershift reaction between labeled HNF1 probe and 2 μg of HNF1β antibody. Lanes 7 and 8, binding reaction between 10 and 20 μg nuclear extracts, respectively, and labeled mutated HNF1 probe. Lane 9, 10 μg of BSA and labeled HNF1 probe, serving as a negative control (Neg. Ctrl).

labeled HNF1 probe (lane 3) competed out the binding, demonstrating the specificity of the interaction. When an excess of unlabeled mutated HNF1 probe missing the HNF1 *cis*-element was used as a competitor, a clear band was still present (lane 4). Supershift assays, with the labeled HNF1 probe and HNF1α antibody, shifted the specific band even farther up (lane 5). The supershift was not observed when the HNF1β antibody was used (lane 6). Furthermore, preincubation of 10 and 20 μg nuclear extracts with labeled mutated HNF1 probe did not result in the appearance of any specific band (lanes 7 and 8). Lane 9 represents labeled HNF1 probe incubated with BSA, which served as a negative control.

The ability of the transcription factors HNF1α and HNF1β to bind to the HNF1 element in the human ACAT2 promoter in vitro does not necessarily confirm that these are bound to the HNF1 element in vivo. Access to DNA binding sites may be influenced by chromatin structure, occupancy of the binding sites by other DNA binding proteins, and intranuclear availability of the transcription factors (11). To determine whether HNF1α and/or HNF1β interact with the human ACAT2 promoter in vivo, we performed ChIP using human liver (**Fig. 4**). After cross-linking, DNA fragments were immunoprecipitated with specific antibodies and amplified by PCR. An IgG antibody was used as a baseline control and to compare the levels of specific DNA fragments. Before the immunoprecipitations, a small aliquot of chromatin was saved and used as an input control. Immunoprecipitation with the HNF1α antibody led to an almost 15-fold enrichment of

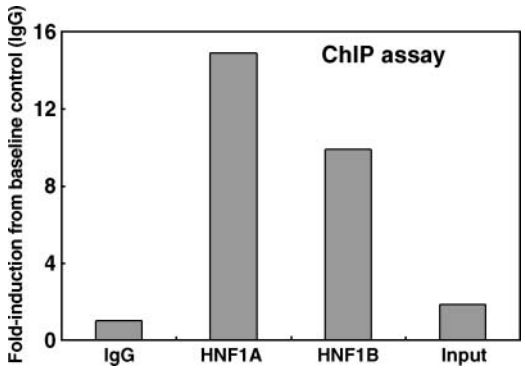

Fig. 4. In vivo association of HNF1α and HNF1β with the human ACAT2 promoter in liver. Soluble chromatin was prepared from 200 mg of human liver and immunoprecipitated with specific antibodies against HNF1α, HNF1β, or IgG and amplified using real-time RT-PCR, conducted in triplicate. The IgG antibody was used as a baseline control and to compare the relative fold enrichment of the ACAT2 promoter by the specific DNA fragments. Before immunoprecipitations, a small aliquot of chromatin was saved and used as an input control. ChIP, chromatin immunoprecipitation.

the ACAT2 promoter, whereas immunoprecipitation with the HNF1β antibody led to a 10-fold enrichment of the promoter. These data strongly suggest that both HNF1α and HNF1β are associated with the human ACAT2 promoter in vivo, despite the fact that we could only show a direct binding of HNF1α to this *cis*-element in vitro.

Effects of HNF1α and HNF1β overexpression on the human ACAT2 promoter

We next determined whether the transcription factors HNF1α and/or HNF1β could regulate expression through the identified binding site in the human ACAT2 gene. We transiently transfected HuH7 and HepG2 cells with the p−1044 or p−HNF1 mutant construct with or without expression vectors for HNF1α and HNF1β. In HuH7 cells, transfection with the p−1044 construct along with HNF1α caused a 3- to 4-fold induction of luciferase activity (**Fig. 5A**). A >2-fold induction was observed when HNF1β expression vector was used. Furthermore, no synergistic effects were observed when both HNF1α and HNF1β expression vectors were cotransfected in HuH7 cells. In HepG2 cells, a 33% increase in luciferase activity was observed when the p−1044 construct and the HNF1α expression vector were cotransfected (data not shown). HNF1β overexpression failed to increase p−1044 promoter activity in HepG2 cells (data not shown). We also transfected HEK293 cells with the p−1044 construct and the HNF1α or HNF1β expression vector. Both expression vectors caused a 3-fold induction of luciferase activity (data not shown).

To identify whether HNF1α and HNF1β could regulate the human ACAT2 promoter through another *cis*-element, we also transfected HuH7 and HepG2 cells with the p−HNF1 mutant construct along with the HNF1α and HNF1β expression vectors. A complete loss of HNF1-dependent stimulation by the expression vectors was seen in

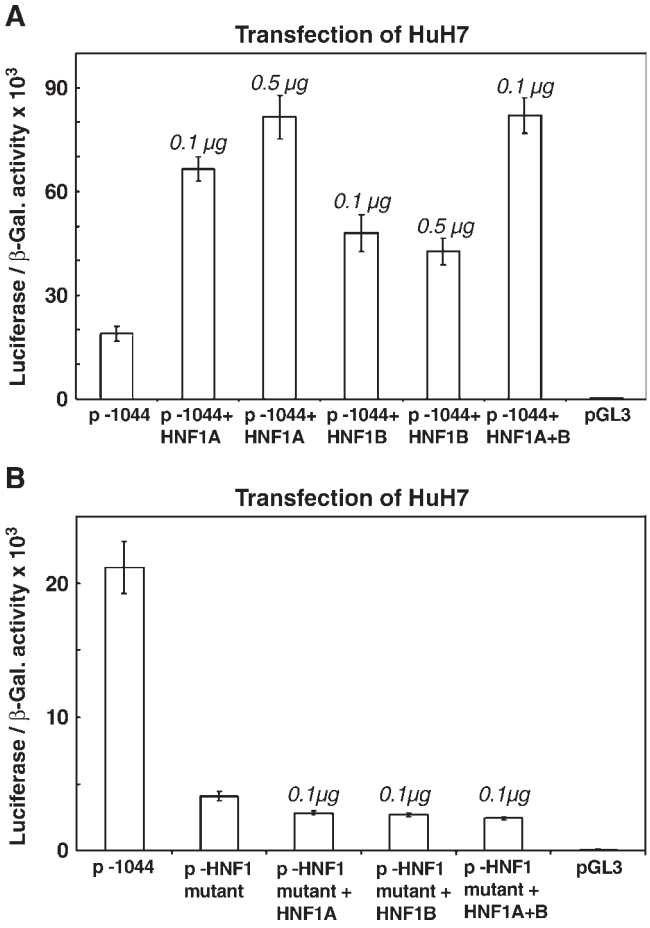

Fig. 5. Importance of the HNF1 binding site for hepatocyte-specific expression. The p−1044 construct (A) or the p−HNF1 mutant construct (B) carrying a deletion for the putative HNF1 binding site, and 0.1 or 0.5 μg of HNF1α and/or HNF1β expression vector, was cotransfected into HuH7 cells. pSV-β-galactosidase (β-Gal) control vector served as an internal control of transfection efficiency. The empty (pGL3) reporter vector served as a baseline control. Luciferase and β-galactosidase activities were measured in cell lysates 48 h after transfection. Luciferase activity was corrected by β-galactosidase activity. All transfections were performed in five replicates, and data represent means ± SEM.

HuH7 cells when the p−HNF1 mutant construct was used, as shown in Fig. 5B, and in HepG2 cells (data not shown). This indicates that deletion of this HNF1 *cis*-element in the 5′ region of the human ACAT2 gene prevents activation by HNF1α and HNF1β in both hepatocellular cell lines.

We also examined whether the ability of HNF1α and HNF1β to activate the promoter pertained to the ∼1.4 kb ACAT2 promoter construct. Therefore, we transfected HepG2, HuH7, and HEK293 cells with the full-length ACAT2 promoter construct (p−1305) along with the HNF1α or HNF1β expression vector. The luciferase activities increased using the HNF1α expression vector and decreased using the HNF1β expression vector for both HepG2 and HuH7 cells, as shown in **Fig. 6A**, **B**. However, the luciferase activity increase was more pronounced in HEK293 cells using the expression vectors for HNF1α and HNF1β, as shown in Fig. 6C.

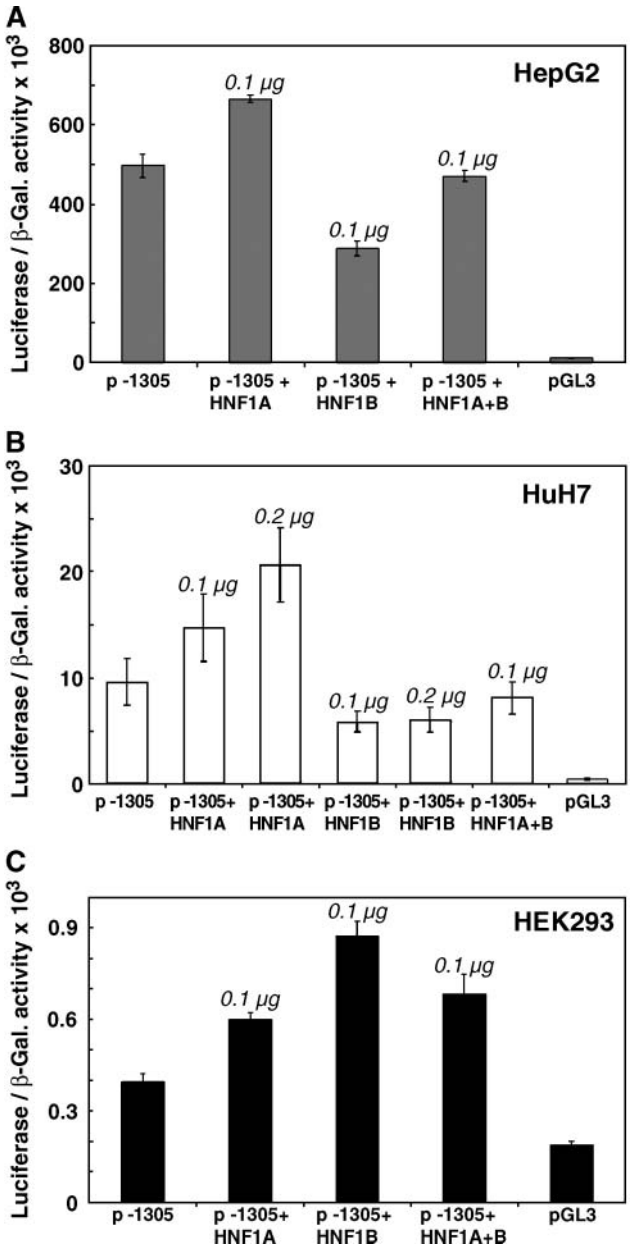

Fig. 6. Influence of HNF1α and HNF1β overexpression on human ACAT2 promoter activity in different cells. The human ACAT2 full-length promoter construct (p−1305), along with 0.1 or 0.2 μg of HNF1α and/or HNF1β expression vector, was transiently cotransfected into HepG2 cells (A), into HuH7 cells (B), or into HEK293 cells (C). pSV-β-galactosidase (β-Gal) control vector served as an internal control of transfection efficiency. The empty (pGL3) reporter vector served as a baseline control. Luciferase and β-galactosidase activities were measured in cell lysates 48 h after transfection. Luciferase activity was corrected by β-galactosidase activity. All transfections were performed in five replicates, and data represent means ± SEM.

DISCUSSION

In this study, the promoter of the human ACAT2 gene was cloned and deletion constructs were created, mutated, and used to transfect different cell lines to find important elements in the DNA sequence that could explain the liver-specific expression of this gene. This revealed an important *cis*-acting element located at position −871 to −866 bp upstream of the transcription start site of human ACAT2, to which HNF1α and HNF1β bind in human liver.

HNF1, also called HNF1α, is a dimeric protein composed of an N-terminal dimerization domain, a DNA binding domain, and a C-terminal domain that is essential for the transactivation of target promoters. HNF1α and vHNF1, also called HNF1β, share strong homologies in both the dimerization domain and the DNA binding domain and thus are able to form heterodimers and bind to the same DNA sequences (12). HNF1α and HNF1β are expressed in various organs, including the liver, kidney, stomach, and pancreas (13–15). The expression of HNF1α and HNF1β overlaps; however, in adult liver, HNF1β is weakly expressed but HNF1α is much more abundant (13–15).

The HNF1 element identified at positions −871 to −866 bp is important for the hepatic-specific expression of the human ACAT2 gene and can bind to and be regulated by the transcription factors HNF1α and HNF1β, as we now have shown in vitro and in vivo. An interesting recent paper by Odom et al. (16) listed genes occupied by the transcription factors HNF1α, HNF4α, and HNF6 in human liver and in pancreatic islets. However, in the list of genes bound by HNF1α in hepatocytes, ACAT2 (or Soat2) is absent. The reason for this absence is uncertain, but it may be explained by the different technical approaches used. In their genome-scale location analysis, Odom et al. (16) targeted the region spanning 700 bp upstream and 200 bp downstream of transcription start sites of putative genes, possibly leading to failure to recognize a control of ACAT2 gene expression by HNF1. Furthermore, the fact that until recently (7) ACAT1 was thought to be the primary cholesterol-esterifying enzyme expressed in human liver also could have played a role.

We were also able to demonstrate the regulation of the ACAT2 promoter region by HNF1β overexpression. However, we failed to show any direct binding of HNF1β in the hepatocyte-derived HuH7 cells using EMSA. This might be attributable to the fact that EMSA experiments commonly reveal the most abundant and/or highest affinity interacting protein (17), which in this case might be HNF1α. However, the binding of HNF1β to the HNF1 *cis*-element in the human ACAT2 gene in liver was shown using ChIP, indicating the involvement of HNF1β in the control of ACAT2 gene expression.

Transfection of HepG2 cells with the HNF1α or HNF1β expression vector did not result in an induction of the activity of the p−1044 construct, as mentioned above. This may be explained by the previously reported presence of intermediate to high endogenous levels of HNF1 in these cells (18, 19). On the other hand, when transfecting HEK293 cells, which do not basally express HNF1α and HNF1β (20) or ACAT2 (6), strong induction of the activity of the ACAT2 promoter construct was observed after overexpression of HNF1α and HNF1β. This again suggests a very strong dependence of ACAT2 gene expression upon HNF1. Nevertheless, other hepatocyte-specific fac-

Fig. 7. Alignment between human and mouse 5′ flanking regions of the ACAT2 gene. Shaded letters show base homology. aataaa, poly-A sequence of the IGFBP-6 gene; *Cdx-2*, caudal-related homeodomain protein-2; framed *HNF1*, sequence for the HNF1 responsible for the hepatocyte-specific expression of the ACAT2 gene in humans; *(HNF1)*, *cis*-element for HNF1 present only in the mouse sequence. The longer underlined sequence shows part of the exon sequence.

tors interacting with HNF1 must also be involved in the regulation of human ACAT2 expression, because the ACAT2 gene is not expressed in organs (i.e., the kidney) with high levels of HNF1α and HNF1β expression (15).

The expression of ACAT2 in humans is confined to enterocytes and hepatocytes, and previous studies by Song et al. (6) showed that caudal-related homeodomain protein-2 (Cdx-2) elements in the 5′ region are responsible for the enterocyte-specific expression of the human ACAT2 gene. Interestingly, one of these Cdx-2 elements is located immediately upstream to the HNF1 binding site (**Fig. 7**). Thus, the cell type-specific expression of the human ACAT2 gene seems to be regulated by a relatively short region of the ACAT2 promoter. In support of this concept, alignment of the untranslated 5′ sequences of the human and mouse genes shows high overall homology (55%) and complete identity of the region containing the HNF1 and Cdx-2 elements (Fig. 7). In the mouse, but not in the human, promoter region of the ACAT2 gene, the untranslated 5′ sequence contains another conserved putative HNF1 binding site, located closer to the transcription start site (Fig. 7). The presence of this second HNF1 *cis*-element may partly explain the higher expression level of ACAT2 observed in livers of mice compared with humans.

In a rodent model of hyperlipidemia, deletion of ACAT2 consistently led to less extensive atherosclerosis (21, 22), and primate species comparison showed that cynomolgus monkeys, which are more susceptible to atherogenic diets, had higher hepatic ACAT2 expression and activity than hyporesponsive African green monkeys (23). Thus, ACAT2 seems to be linked to an increased risk of atherosclerosis. To date, little is known about the regulation of ACAT2 expression in human liver, partly because its expression was only recently identified in this organ (7).

Mutation of the TCF1 gene, which encodes HNF1α, causes maturity-onset diabetes of the young (MODY)-3, a disease characterized by decreased insulin secretion in juveniles (24). Although a detailed study of lipoprotein metabolism has not been performed in this condition, in particular regarding the hepatic production of cholesteryl ester, it is of interest that VLDL secretion may also be reduced in MODY3 patients (25). In mice, deletion of *Tcf1* leads to disturbed bile acid transport and to an increased synthesis of bile acids and cholesterol (26). Buoyant HDLs

accumulate in plasma as a result of disturbed hepatic lipase and LCAT expression (26). Nevertheless, the impact of *Tcf1* deletion on ACAT2 expression and activity has not been evaluated, despite the lower VLDL cholesterol content that seems to be present in *Tcf1* knockout mice (26). Moreover, in vitro studies have identified HNF1α as an essential regulator of bile acid and plasma cholesterol metabolism (26, 27). Thus, it should be of interest to investigate whether factors that regulate the expression of HNF1α may in turn affect the expression of the human ACAT2 gene.

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