HNF1α and SREBP2 are important regulators of NPC1L1 in human liver

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Abstract **Niemann-Pick C1-like 1 (NPC1L1), a key regulator of intestinal cholesterol absorption, is highly expressed in human liver. Here, we aimed to gain more insight into mechanisms participating in its hepatic regulation in humans. Correlation analysis in livers from Chinese patients with and without gallstone disease revealed strong positive correlations between NPC1L1 and sterol regulatory element binding protein 2 (SREBP2) ($r = 0.74$, $P < 0.05$) and between NPC1L1 and hepatic nuclear factor α (HNF4α) ($r = 0.53$, $P < 0.05$) mRNA expression. HNF4α is an upstream regulator of HNF1α; thus, we also tested whether HNF1α participates in the regulation of NPC1L1. We showed a dose-dependent regulation by SREBP2 on the NPC1L1 promoter activity and mRNA expression in HuH7 cells. Chromatin immunoprecipitation assay confirmed the binding of SREBP2 to the promoter in vivo. Surprisingly, HNF4α slightly decreased the NPC1L1 promoter activity but had no effect on its gene expression. By contrast, HNF1α increased the promoter activity and the gene expression, and an important HNF1 binding site was identified within the human NPC1L1 promoter. ChIP assays confirmed that HNF1α can bind to the NPC1L1 promoter in vivo.**—Pramfalk, C., Z-Y. Jiang, Q. Cai, H. Hu, S-D. Zhang, T-Q. Han, M. Eriksson, and P. Parini. **HNF1α and SREBP2 are important regulators of NPC1L1 in human liver.** ***J. Lipid Res.*** **2010.** 51: **1354–1362.**

Supplementary key words Niemann-Pick C1-like 1 • sterol regulatory element binding protein 2 • hepatic nuclear factor • cotransfection • chromatin immunoprecipitation • correlation analysis

Niemann-Pick C1-like 1 (NPC1L1) is a key regulator of intestinal cholesterol absorption and is supposed to be the target of the cholesterol-lowering drug ezetimibe (1, 2). Mice deficient in NPC1L1 have ~70% reduction in cholesterol absorption (1) and resistance to diet-induced hypercholesterolemia (3). NPC1L1 is widely expressed in many human tissues, with the highest expression in small intestine and in the liver (3, 4). In mice and rats, *npc1l1* is predominantly expressed in the small intestine, whereas all others tissues showed expression levels <10% of the intestinal expression (1, 5). The exact function of NPC1L1 in the human liver is currently unknown. It was recently reported that NPC1L1 facilitates the uptake of free cholesterol from the culture medium in human (6) and rat (7) hepatoma cells. Previous reports also showed that NPC1L1 localizes to the canalicular membrane in hepatocytes (6, 8). Transgenic mice overexpressing human NPC1L1 in the liver had dramatically decreased biliary cholesterol concentration, which was returned to normal with ezetimibe treatment (8). This suggests that hepatic NPC1L1 could be another target of ezetimibe in humans.

Several genes involved in cholesterol synthesis and uptake are regulated by sterol regulatory element binding protein 2 (SREBP2). Activation of SREBP2 is dependent on the cholesterol status of the cell (9). When cellular cholesterol levels are low, SREBP2 is proteolytically cleaved to release the N-terminal portion to generate the mature form that can enter the nucleus and bind to sterol regulatory elements (SREs) or E-boxes in the promoter of various genes and affect gene expression (9, 10).

Hepatic nuclear factors (HNFs) 1 and 4 are expressed in various organs, including the liver, intestine, and pan-

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Abbreviations: ChIP, chromatin immunoprecipitation; GS, gallstone disease; GSF, gallstone-free; HNF, hepatic nuclear factor; NPC1, Niemann-Pick C1; NPC1L1, Niemann-Pick C1-like 1; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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creas (11). Deficiency of HNF1α in mice (12) results in defect bile acid transport, increased bile acid and liver cholesterol synthesis, and impaired HDL metabolism. HNF4α knockout mice die before birth (13), and conditional liver-specific disruption of HNF4α (14) results in hepatomegaly, lipid deposition in the liver, reduced serum cholesterol and triglyceride levels, and elevated serum bile acid concentrations. Thus, both HNF1α and HNF4α play important roles in lipid homeostasis.

Since the physiological significance in human liver remains to be clarified, the aim of this study was to gain more insight into the mechanisms that participate in the transcriptional regulation of hepatic NPC1L1.

EXPERIMENTAL PROCEDURES

Materials

2×SYBRGreen Mastermix was purchased from MedProbe (Oslo, Norway). HuH7 and HEK293 cells were purchased from American Type Culture Collection (Manassas, VA). SREBP2, HNF1α, and IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The human NPC1L1 promoter (an ∼1,700 bp fragment, ranging from −1570 to +137 bp, cloned into pGL3 promoter vector) was a generous gift from Dr. Charlotte Murphy and Dr. Mats Gåfvels (Karolinska Institutet, Sweden). The SREBP2 expression vector, which is not the full length but encodes the transcriptionally active form of the protein, was from American Type Culture Collection (pCMV-SREBP2-468 No.63452). The HNF4α expression vector (15) was a generous gift from Dr. Theodore C. Simon (Washington University School of Medicine, St. Louis, MO). The HNF1α expression vector (16) was a generous gift from Professor Pal R. Njølstad and Dr. Lise Bjørkhaug Gundersen (Haukeland University Hospital, Norway). Lipoprotein deficient serum (LPDS) and LDL were isolated using FBS or plasma from a healthy blood donor, respectively, by density gradient ultracentrifugation (17).

Methods

Subjects. Liver biopsies were from subjects who have previously been investigated in a study by Jiang et al. (18). In brief, 22 normolipidemic and nonobese Chinese patients (11 females and 11 males) with cholesterol gallstone disease (GS) and 12 Chinese gallstone-free patients (GSF; nine females and three males) were included. None of the patients were subjected to any lipid-lowering treatment. Informed consent to participate in the study and to collection of a liver biopsy was obtained. Patients were fasted overnight before a biopsy of ∼0.5–1.0 g was taken from the right lobe of the liver, snap-frozen in liquid nitrogen, and stored at –70°C. The study protocol was approved by the ethics committees at Ruijin Hospital, Shanghai Jiaotong University School of Medicine, and at Karolinska University Hospital in Huddinge.

RNA preparation and real-time RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. One microgram of RNA was transcribed into cDNA using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The cDNA was diluted 1:10 in DEPC-H_2O. Real-time RT-PCR was performed in triplicate with 5 μl cDNA, 12.5 μl SYBRGreen Mastermix, and forward and reverse primers. Primer sequences are available upon request. Arbitrary units were calculated by linearization of the C_T values.

Cell experiments, mutagenesis, and transfections. HuH7 and HEK293 cells were cultured as described (19). For all cell experiments, HuH7 or HEK293 cells were plated out on 6-well tissue culture plates so that they reached ∼70% confluence after 24 h (i.e., ∼700,000 cells/well). The human NPC1L1 promoter (−1570 to +137) was screened using the transcription factor TESS database (www.cbil.upenn.edu at University of Pennsylvania, Philadelphia, PA) to search for putative HNF1 binding sites, and specific point mutations were generated as described (primer sequences are available upon request) (19).

Transfections of HuH7 cells were performed using 2 μg promoter construct (or mutated constructs) and 2 μg pSV-β-galactosidase control vector (Promega, Madison, WI) with or without increasing concentrations of SREBP2, HNF1α, and HNF4α expression vectors or with 0.5 μg of each expression vector using Lipofectin reagent (Invitrogen, Carlsbad, CA) at a ratio of 3:1 (Lipofectin:DNA). pGL3 empty vector (Promega) was used to adjust for differences in amount of DNA added to the cells. Transfections of HEK293 cells were performed like those for HuH7 cells, except that Lipofectamine 2000 reagent (Invitrogen) at a ratio of 0.25:1 (Lipofectamine:DNA) was used. The pSV-β-galactosidase control vector was used to correct for variation in transfection efficiency. Forty-eight hours after transfection, cell lysates were prepared in reporter lysis buffer (Promega). β-Galactosidase and luciferase activities were determined using β-galactosidase or luciferase assay kits, respectively, according to the manufacturer's instructions (Promega). All transfection data are expressed as luciferase activity corrected by β-galactosidase activity.

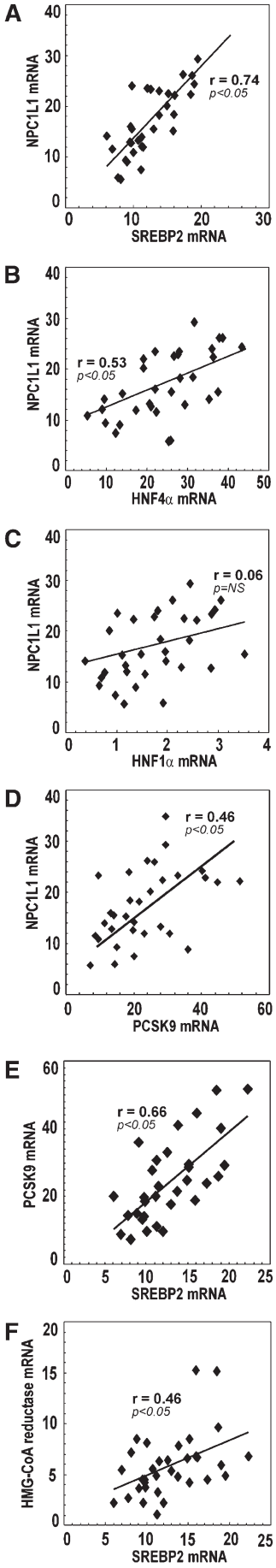
For gene expression analysis, HuH7 cells were transfected with 0, 0.05, 0.1, 0.5, 1, and 2 μg SREBP2, HNF1α, or HNF4α expression vector using Lipofectin reagent (Invitrogen). Forty-eight hours after transfection, total RNAs were prepared using TRIzol reagent according to the manufacturer's protocol.

To study the effect of cholesterol on NPC1L1 gene expression, HuH7 cells were incubated for 12 h with 0, 0.1, 0.5, 1, and 2 mM LDL cholesterol or with 10% LPDS prior to RNA extraction.

All cell experiments were performed in quadruplicates, and data represent means ± SEM.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assays were performed using ∼200 mg liver from a healthy donor as described (19). Specific antibodies for HNF1α (sc-6547×; Santa Cruz Biotechnology), SREBP2 (sc-8151×; Santa Cruz Biotechnology), and an IgG antibody (sc-2027; Santa Cruz Biotechnology), as a (baseline) control, were used (4 μg). Primers used for detection of the in vivo binding of SREBP2 to the two SREBP2 binding sites (SRE1 and SRE2) in the human NPC1L1 promoter were as follows: forward sequence (SRE1) 5′-GAAGGGGAGGAGGCTGCCTT-3′, and the reverse sequence 5′-TCAGGAACAGCCAAGGGCTG-3′; and forward sequence (SRE2) 5′-CTAGGGGTGACCGGTGGGAC-3′, and the reverse sequence 5′-CTTCTCCCTCTTGTCCCTTGCC-3′. Primers used for detection of the in vivo binding of HNF1α to the human NPC1L1 promoter were as follows: forward sequence 5′-GGAGCAGGGCTGCTGCTCAA-3′, and the reverse sequence 5′-CTTCGATGACAACCCTGGCC-3′. Also, in order to correct for different DNA loading (input versus immunoprecipitated samples), primers were designed using the human exon 7 of NPC1L1, with the forward sequence 5′-CCACGAGAGGTCCACATTGG-3′ and the reverse sequence 5′-GAAGAAGCAGATGGCCTCAGA-3′, and used as internal control in the PCR.

Electrophoretic mobility shift assay and supershift assay. Nuclear extracts were prepared from HuH7 cells as described by Azzout-Marniche et al. (20). Forward sequences for primers



used to generate double-stranded probes were as follows: (−769 bp) 5′-CTCAAGGCTCCCAGCCATGCTCTGTCC-3′; (−665 bp) 5′-AGCAAAGTGAGCAAAGGAGAGTCTTCCA-3′; (−598 bp) 5′-CTGTTCTTTCCCTTTGCTTCCTGGAG-3′; (−360 bp) 5′-AGGCCGGGTCCCAGCCAGTGCCTGGTC-3′; (−158 bp) 5′-CAGTGGGAGTGGTGGATCATTAACCAGCAGGGCTC-3′; (−119 bp) 5′-TCATTGGTCCCTAACCCAGTCAGGCC-3′. The underlined bases in the forward sequences were deleted for the mutated HNF1 binding sites. Unlabeled double-stranded probes were generated by mixing 1 μg forward and 1 μg reverse oligonucleotide, 5 μl 1 M NaCl, and ddH_2O up to 50 μl, and annealed at 95°C for 10 min. Labeled probes were generated by mixing 5 μl unlabeled probe, 2 μl 10× PNK buffer (Promega), 1 μl 0.1 M DTT, 1.5 μl T4 polynucleotide kinase (Promega), 2 μl [γ-^{32}P ATP], and ddH_2O 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 μg nuclear extract along with 2 μg poly (dI-dC) (Amersham, Piscataway, NJ), 7.5 μl 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 BSA), 1 μl 0.1 M DTT, 4 μl 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 ddH_2O up to 30 μl. Binding reaction mixtures were incubated for 20 min at room temperature and resolved on a nondenatured (4% w/v) acrylamide gel in 1× TBE at 4°C for 3 h. For supershift assays, 2 μg HNF1α (sc-6547×; Santa Cruz Biotechnology) or 2 μg SREBP2 (sc-8151×; Santa Cruz Biotechnology) antibodies were added to the binding reaction mixtures. After electrophoresis, gels were dried and exposed over night to X-ray film at −80°C.

Statistical analysis. Data are expressed as means ± SEM for all cell experiments. Correlation analyses in patients were calculated by least square regression analysis. A P value of <0.05 was regarded as statistically significant. For cell experiments, multiway ANOVA, followed by posthoc comparisons according to Dunnett test, was used with the exception of treatment of the cells with LPDS in which the Student's t-test was used (Statistica software; Stat Soft, Tulsa, OK).

RESULTS

NPC1L1 correlates with SREBP2 and HNF4α in human livers

Gene expression analyses were performed in liver samples from 22 Chinese patients with cholesterol GS and 12 Chinese GSF patients. As we recently reported (18), no significant differences were observed in NPC1L1, SREBP2, and HNF1α mRNA expression between GS and GSF patients, though GS patients had 43% ($P < 0.05$) higher HNF4α mRNA expression compared with GSF patients.

A strong positive correlation between NPC1L1 and SREBP2 mRNA expression was present. As shown in **Fig. 1A**, when all patients were considered the regression coef-

Fig. 1. Correlation of mRNA expression in human liver from Chinese patients with and without cholesterol GS (n = 34): NPC1L1 and SREBP2 (A); HNF4α (B); HNF1α (C); PCSK9 (D), and between PCSK9 and SREBP2 (E) and HMG-CoA reductase and SREBP2 (F). Gene expressions were related to cyclophilin A mRNA. The correlation values were determined by least square regression analysis.

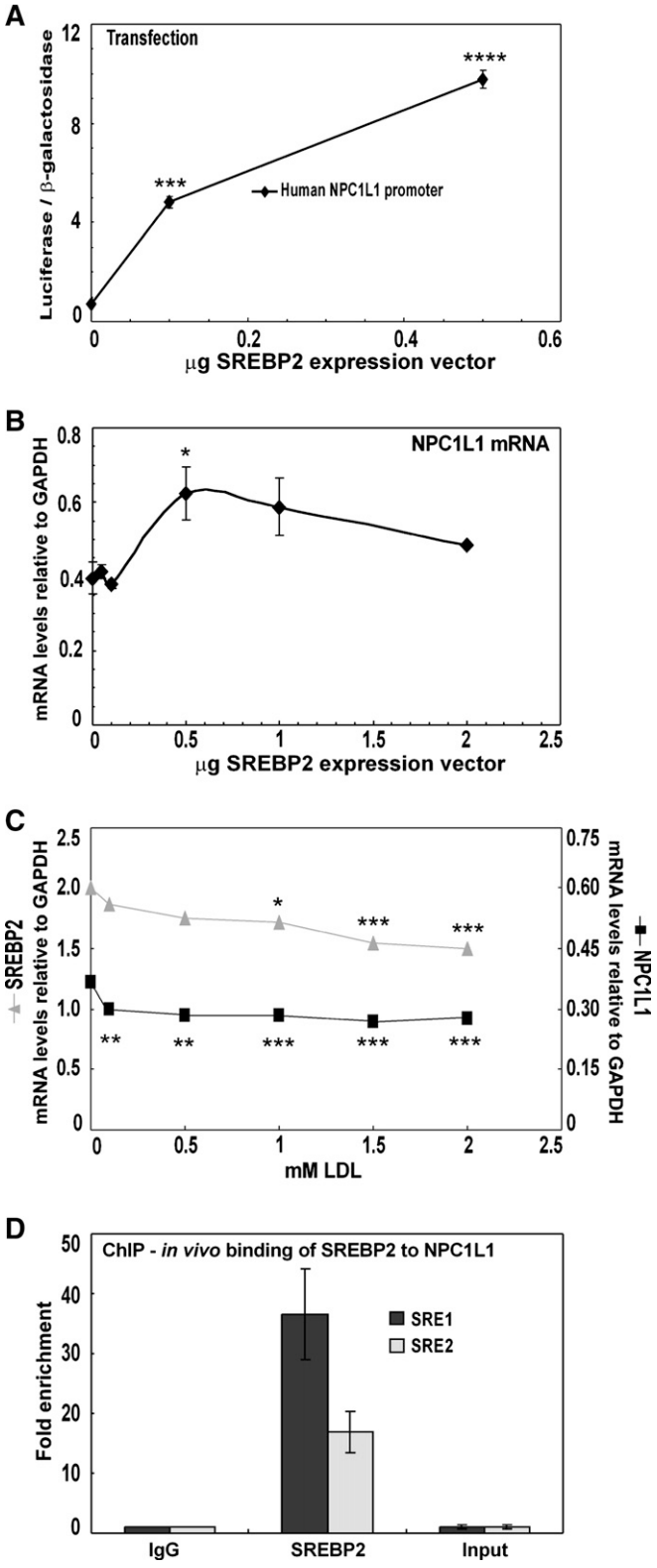


Fig. 2. SREBP2 binds to and regulates the human NPC1L1 promoter. A: The human NPC1L1 promoter (−1,570 to +137) was used for cotransfection analysis along with 0.1 or 0.5 μg SREBP2 expression vector in HuH7 cells. HuH7 cells were transfected with increasing amounts of SREBP2 expression vector (B) or treated with increasing concentrations of LDL cholesterol (C). Gene expressions were normalized to GAPDH mRNA. Experiments in A–C were performed in quadruplicates, and data are expressed as means ± SEM. Differences between groups were tested by one-way ANOVA followed by posthoc comparisons according to the Dunnett test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. D: Soluble chromatins (~60 μg DNA, ~500 bp in length) were prepared from human liver and immunoprecipitated with 4 μg antibodies against SREBP2 or IgG and amplified by real-time RT-PCR using primers for detection of SRE1 (−91/−81 bp) and SRE2 (−748/735 bp) in the NPC1L1 promoter. The IgG antibody was used as a baseline control and used to compare the relative fold enrichment of the NPC1L1 promoter by the specific DNA fragments. Before immunoprecipitation, a small aliquot of chromatin was saved and used as an input control. Experiments were performed in triplicate, and data are expressed as means ± SEM.

ficient was 0.74 ($P < 0.05$). When the patients were divided by the presence of gallstone disease, the correlation was still present ($r = 0.71$ in GS, and $r = 0.89$ in GSF, $P < 0.05$).

Also, a positive correlation between NPC1L1 and HNF4α mRNA expression was observed. When all the patients were considered, the regression coefficient was 0.53 ($P < 0.05$) (Fig. 1B), and when the patients were divided by the presence of gallstone disease, the correlation was still present ($r = 0.44$ in GS, and $r = 0.79$ in GSF, $P < 0.05$) also for this transcription factor.

No significant correlation between NPC1L1 and HNF1α mRNA expression was observed ($r = 0.06$ in all patients; $r = -0.07$ in GS, and $r = 0.47$ in GSF; Fig. 1C).

SREBP2 is known to regulate HMG-CoA reductase and PCSK9 (9, 21, 22). Thus, this gene was used as a control of our model; also, we tested the correlation between NPC1L1 and PCSK9 (Fig. 1D). In agreement with previous studies, we showed strong positive correlations between NPC1L1 and PCSK9 ($r = 0.46$, $P < 0.05$) (Fig. 1D), PCSK9 and SREBP2 ($r = 0.66$, $P < 0.05$) (Fig. 1E), and between HMG-CoA reductase and SREBP2 ($r = 0.46$, $P < 0.05$) (Fig. 1F) mRNA expression when all patients were considered.

SREBP2 interacts with the human NPC1L1 promoter in vitro and in vivo

The above observations prompted us to investigate whether SREBP2, HNF4α, and HNF1α may participate in the hepatic regulation of NPC1L1 in humans. Cotransfection experiments in human hepatoma cells (HuH7) using the human NPC1L1 promoter (−1,570 to +137 bp) and the SREBP2 expression vector were performed. A strong dose-dependent regulation by SREBP2 on the human NPC1L1 promoter activity (**Fig. 2A**) was observed. Also, the NPC1L1 mRNA expression increased ~60% ($P < 0.05$) using 0.5 μg SREBP2 expression vector (Fig. 2B). Activation of SREBP2 is dependent on the free cholesterol levels inside the cell. To study the effect on the endogenous NPC1L1 gene expression under more physiological conditions, HuH7 cells were depleted or loaded with cholesterol. Loading of the cells with LDL cholesterol decreased both the NPC1L1 and the SREBP2 mRNA expression (Fig. 2C). A strong positive correlation ($r = 0.94$, $P < 0.05$) between NPC1L1 and SREBP2 mRNA expression was also observed (data not shown). Cholesterol depletion, on the other hand, resulted in an insignificant trend toward increased NPC1L1 mRNA levels (data not shown). Recently, Alrefai et al. (23) identified two SREs, SRE1 and SRE2, in

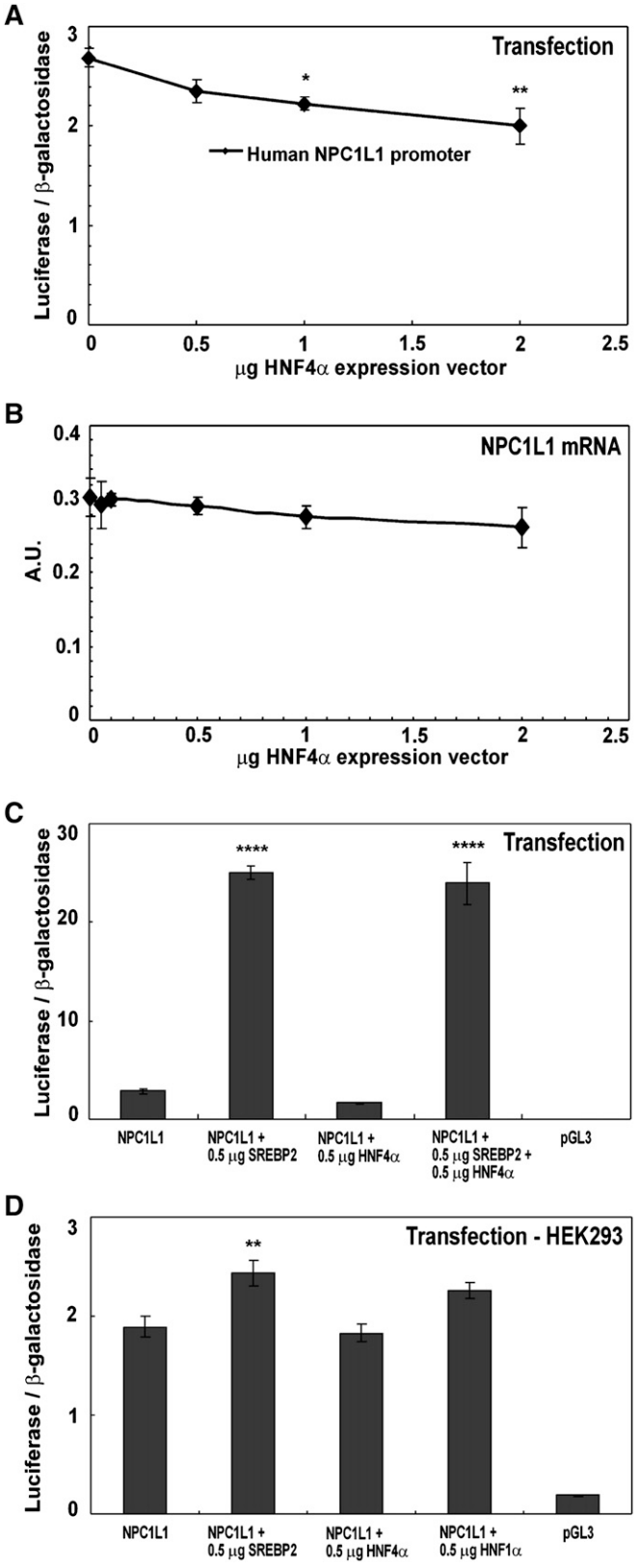


Fig. 3. Effects of HNF4α on the human NPC1L1 promoter. A: The human NPC1L1 promoter (−1,570 to +137) was used for cotransfection analysis along with 0.5, 1, or 2 μg HNF4α expression vector in HuH7 cells. B: HuH7 cells were transfected with increasing amount of HNF4α expression vector. The NPC1L1 gene expression was normalized to GAPDH mRNA. HuH7 (C) or HEK293 (D) cells were cotransfected with NPC1L1 promoter along with 0.5 μg expression vectors. Experiments in A–D were performed in quadruplicates, and data are expressed as means ± SEM. Differences between groups were tested by one-way ANOVA followed by posthoc comparisons according to the Dunnett test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$.

the human NPC1L1 promoter. To determine whether SREBP2 can bind to these SREs in the NPC1L1 promoter in vivo, we performed ChIP assay using human liver. Immunoprecipitation with a specific antibody against SREBP2 and primers designed to target the two previously identified SREs were used in the PCR analysis. This led to >36-fold enrichment of SRE1 (−91/−81 bp) and ∼17-fold enrichment of SRE2 (−748/−738 bp) sequences present in the human NPC1L1 promoter (Fig. 2D). Collectively, these results show that SREBP2 can regulate and bind to the NPC1L1 promoter in human liver.

Effects of HNF4α overexpression on NPC1L1 promoter activity and gene expression

Due to the positive correlation between NPC1L1 and HNF4α in the patients, we also performed cotransfections in HuH7 cells using the human NPC1L1 promoter along with the HNF4α expression vector. Surprisingly, HNF4α decreased the human NPC1L1 promoter activity in a dose-dependent fashion (**Fig. 3A**), whereas HNF4α overexpression had no effect on its mRNA expression (Fig. 3B). Iwayanagi, Takada, and Suzuki (24) showed that the transcription of NPC1L1 was stimulated by HNF4α together with SREBP2, but not by HNF4α alone. Cotransfection of HuH7 cells with both SREBP2 and HNF4α expression vectors was performed to test a possible synergism in the activation of the NPC1L1 promoter. However, no further activation of the promoter activity occurred (Fig. 3C).

HNF1α interacts with the human NPC1L1 promoter in vitro and in vivo

HNF4α is an essential positive regulator of HNF1α (25). HNF4α and HNF1α can bind directly to one another, and both contain a binding site in their promoter region for the other (26), suggesting a reciprocal regulation. Thus, we wanted to test whether HNF1α might participate in the regulation of NPC1L1. We performed cotransfections in HuH7 cells using the human NPC1L1 promoter along with the HNF1α expression vector. These experiments revealed a dose-dependent regulation by HNF1α on the NPC1L1 promoter activity (**Fig. 4A**) and increased mRNA expression (Fig. 4B). Next, we screened the sequence using TESS to search for putative HNF1 binding sites in the human NPC1L1 promoter. Six HNF1 *cis*-elements were found located −119/−114, −158/−144, −360/−354, −598/−593, −665/−660, and −769/−763 bp upstream of the ATG start codon (**Fig. 5**). We performed mutagenesis on these HNF1 *cis*-elements and used the mutant constructs in transfection experiments with or without the HNF1α expression vector. These experiments showed that mutation of one HNF1 binding site (−158/−144) almost completely abolished the regulatory effect of HNF1α on the NPC1L1 promoter activity (Fig. 4C). However, muta-

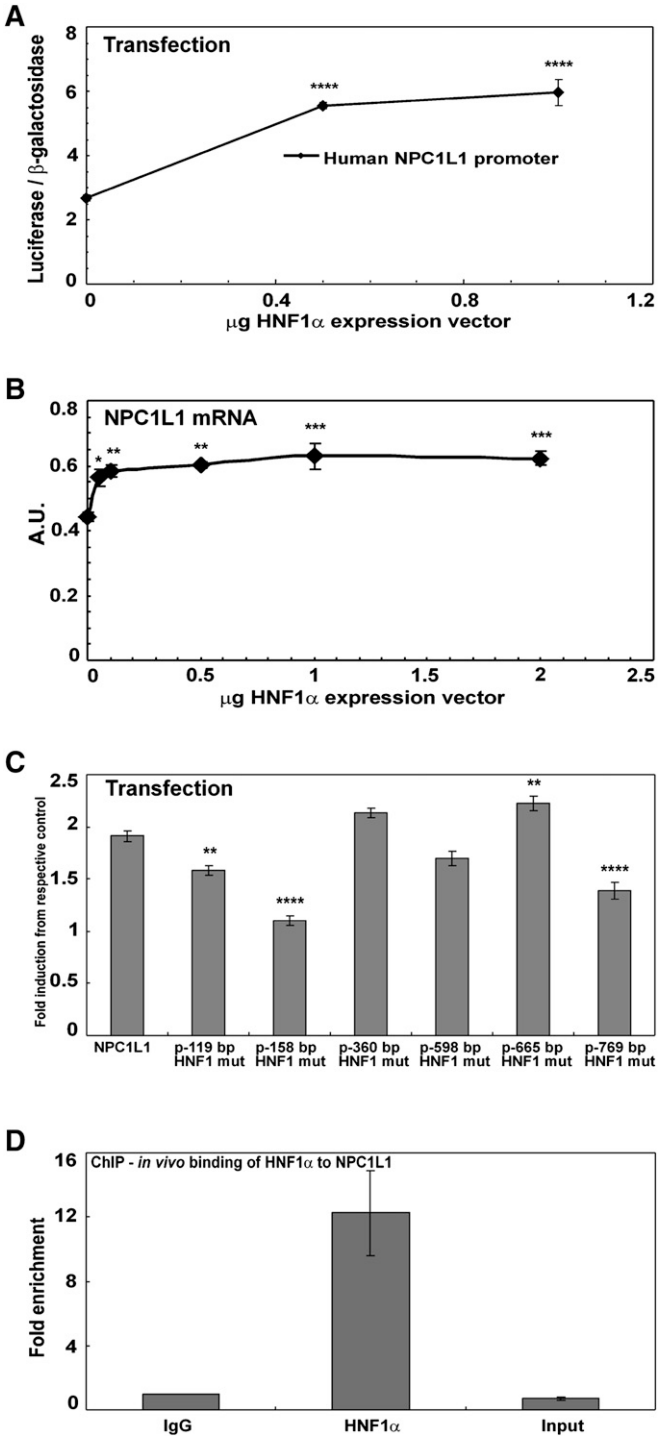


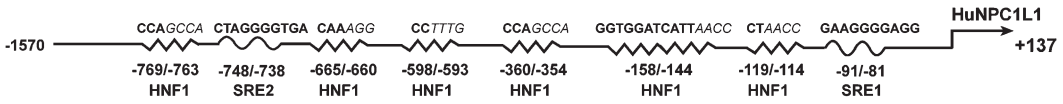
Fig. 4. HNF1α binds to and regulates the human NPC1L1 promoter. A: The human NPC1L1 promoter (−1,570 to +137) was used for cotransfection analysis along with 0.5 or 1 μg HNF1α expression vector in HuH7 cells. B: HuH7 cells were transfected with increasing amounts of HNF1α expression vector. The NPC1L1 gene expression was normalized to GAPDH mRNA. C: Transfection of HuH7 cells with mutant constructs, carrying deletions in the −119 bp (−119/−114), −158 bp (−158/−144), 360 bp (−360/−354), −598 bp (−598/−593), −665 bp (−665/−660), and −769 bp (−769/−763) HNF1 site in the NPC1L1 promoter, with or without HNF1α expression vector. Experiments in A–C were performed in quadruplicates, and data are expressed as means ± SEM. Differences between groups were tested by one-way ANOVA followed by posthoc comparisons according to the Dunnett test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. D: Soluble chromatins (~60 μg DNA, ~500 bp in length) were prepared from human liver and immunoprecipitated with 4 μg antibodies against HNF1α or IgG and amplified by real-time RT-PCR using primers to target the region spanning over the six HNF1 binding sites (−769 to −119 bp) in the NPC1L1 promoter. The IgG antibody was used as a baseline control and used to compare the relative fold enrichment of the NPC1L1 promoter by the specific DNA fragments. Before immunoprecipitations, a small aliquot of chromatin was saved and used as an input control. Experiments were performed in triplicate, and data are expressed as means ± SEM.

tion of two of the other HNF1 binding sites (−119/−114 and −769/−763) also decreased the induction by HNF1α on the NPC1L1 promoter activity, although to a lesser extent. Furthermore, mutation of the −665/−660 bp HNF1 site increased the induction by HNF1α, suggesting that this site may work as a repressor element. Also, to determine whether HNF1α can bind to the human NPC1L1 promoter in vivo, we performed ChIP analysis using human liver (Fig. 4D). Immunoprecipitation with a specific antibody against HNF1α and primers designed to target the region spanning over the six HNF1 sites (−769 to −119 bp) were used in the PCR analysis. This led to >12-fold enrichment of the human NPC1L1 promoter. Finally, to be able to distinguish which of the six putative HNF1 binding sites is responsible for the transactivation by HNF1α, we isolated nuclear extracts from HuH7 cells and performed electrophoretic mobility shift assay and supershift assays for each site. These experiments revealed a direct binding of HNF1α to the −158/−144 bp HNF1 binding site in the NPC1L1 promoter (**Fig. 6**) but no binding of HNF1α to the other five HNF1 sites (data not shown). Specific bands, labeled with the HNF1 probe, were detectable (lanes 1 and 5). Supershift assay, with the labeled HNF1 probe and HNF1α antibody, shifted the specific bands even further (lanes 2 and 6). Furthermore, preincubation of 10 μg nuclear extract with labeled mutated HNF1 probe did not result in appearance of any specific band (lanes 3), and no supershift occurred with either the labeled mutated HNF1 probe and HNF1α antibody (lane 4) or with the labeled HNF1 probe and SREBP2 antibody (lane 7). Collectively, these results show that HNF1α can regulate and bind to the NPC1L1 promoter in human liver and that these effects are mediated by the −158/−144 bp HNF1 binding site.

DISCUSSION

In this study, we investigated whether SREBP2, HNF4α, and HNF1α might be important regulators of the NPC1L1 promoter in human liver in vitro and in vivo.

SREBP2 regulates genes involved in cholesterol biosynthesis (e.g., HMG-CoA reductase) and uptake (e.g., LDL-receptor), and it is activated upon low cellular cholesterol levels (9). Sané et al. (4) reported that SREBP2 mRNA expression was increased in Caco-2 cells deprived of NPC1L1. Alrefai et al. (23) reported that NPC1L1 mRNA expres-



NPC1L1 promoter HNF1A and SREBP2 binding sites

Fig. 5. Putative HNF1 and SRE sites in the human NPC1L1 promoter. Six putative HNF1 binding sites, located at −769/−763, −665/−660, −598/−593, −360/−354, −158/−144, and −119/−114 bp upstream of the ATG transcription start site in the human NPC1L1 promoter. Also, two SREs (SRE1 at −91/−81 and SRE2 at −748/738) were identified.

sion decreased by cholesterol loading and increased in response to cholesterol depletion in Caco-2 cells, and two SREs in the NPC1L1 promoter region were identified. These studies suggest that low levels of cholesterol activate the transcription of SREBP2, leading to an upregulation of the LDL-receptor and HMG-CoA reductase, which in turn increases NPC1L1 expression with a subsequent increase in cholesterol absorption. Furthermore, in miniature pigs treated with a combination of ezetimibe plus simvastatin, Telford et al. (27) reported increased NPC1L1 expression in liver and intestine, which was positively correlated with the SREBP2, HMG-CoA reductase, and LDL receptor expressions. In line with previous reports, we showed *a*) strong positive correlation between NPC1L1 and SREBP2 mRNA expression in liver from Chinese patients with and without gallstone, as well as divided by the presence of gallstone disease; *b*) a strong dose-dependent regulation of SREBP2 on the NPC1L1 promoter activity in HuH7 cells and that this finding is liver specific (>13-fold in HuH7 vs. <30% in HEK293 cells using 0.5 μg SREBP2 expression vector; Fig. 3D); *c*) increased NPC1L1 mRNA expression following transfection with SREBP2 expression vector; *d*) decreased NPC1L1 and SREBP2 mRNA expression following cholesterol loading of the cells with LDL; and, finally, *e*) binding of SREBP2 to the human NPC1L1 promoter in human liver. The in vivo binding of SREBP2 to NPC1L1 may thus participate in a coordinated regulation wherein hepatocytes may acquire more cholesterol. Thus, SREBP2 seems to be an important regulator of both the intestinal and the hepatic NPC1L1 expression in humans.

Misawa et al. (28) reported a direct interaction between SREBP2 and HNF4α to enhance the sterol isomerase gene expression in Caco-2 and HepG2 cells. Iwayanagi, Takada, and Suzuki (24) showed that HNF4α small interfering RNA reduced the mRNA expression and abolished the cholesterol-dependent regulation of NPCL1 in HepG2 cells; also, the transcription of NPC1L1 was stimulated by HNF4α together with SREBP2, but not by HNF4α alone. In our study, cotransfection with the human NPC1L1 promoter along with HNF4α expression vector slightly decreased the promoter activity but had no effect on the NPC1L1 gene expression. Also, no synergistic activation of NPC1L1 promoter activity was seen in HuH7 cells after cotransfection with SREBP2 and HNF4α. The discrepancies might be due to different amounts of expression vector; we used up to 2 μg, whereas Iwayanagi, Takada, and Suzuki (24) used 0.5 μg of HNF4α expression vector, which had no effect on the promoter activity in our experiments. In the cotransfection experiments, we used 0.5 μg of each expression vector, whereas Iwayanagi, Takada, and Suzuki used 0.1 μg SREBP2 and 0.5 μg HNF4α expression

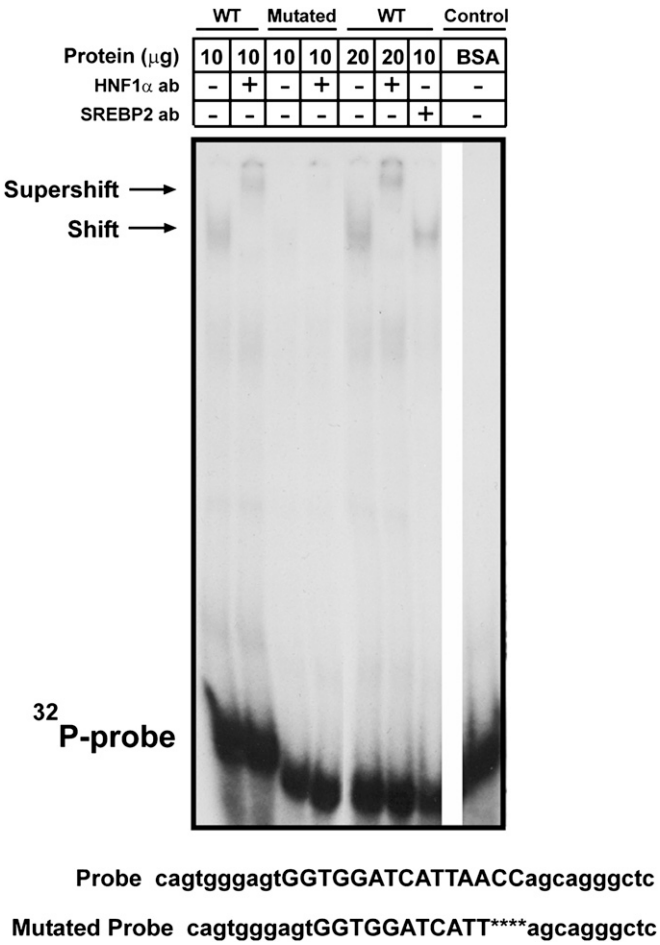


Fig. 6. Functionality of the HNF1 binding sites in the human NPC1L1 gene. Nuclear extracts, prepared from HuH7 cells, were incubated with ^{32}P-labeled double-stranded probes, with or without mutation of the HNF1 binding site and resolved on nondenatured (4% w/v) acrylamide gel. Lanes 1 and 5, binding reactions between 10 or 20 μg nuclear extract and ^{32}P-labeled HNF1 probe, respectively. Lanes 2 and 6, supershift reactions between labeled HNF1 probe and 2 μg HNF1α antibody. Lane 3, binding reaction between 10 μg nuclear extract and labeled mutated HNF1 probe. Lane 4, supershift reaction between labeled mutated HNF1 probe and 2 μg HNF1α antibody. Lane 7, supershift reaction between labeled HNF1 probe and 2 μg SREBP2 antibody. Lane 8, 10 μg BSA and labeled HNF1 probe, serving as a negative control.

vector. Also, different plasmids were used to create the HNF4α expression vectors. Furthermore, we used the human hepatoma cell line HuH7, whereas Iwayanagi, Takada, and Suzuki (24) used HepG2 cells.

The strong correlation between NPC1L1 and HNF4α mRNA expression in our Chinese patients suggests that HNF4α may function by *trans*-activating NPC1L1 via binding to other transcription factors, including SREBP2. HNF4α is an upstream regulator of HNF1α, and both contain binding sites for each other in their promoter region (26, 29), suggesting a reciprocal regulation. HNF1α was recently shown to be an important regulator of ACAT2 (19), which is another important gene involved in cholesterol homeostasis. By coimmunoprecipitation of nuclear extracts from human liver with an antibody raised against HNF1α and detection by Western blot with an HNF4α antibody, we were able to show that a protein-protein interaction between HNF4α and HNF1α occurs in human liver (30). Thus, it is possible that HNF4α also exerts its action on NPC1L1 by *trans*-activating NPC1L1 via binding to HNF1α or by increasing its expression. Interestingly, Odom et al. (29) reported that HNF4α can bind to the NPC1 gene but not to NPC1L1 in primary human hepatocytes. The NPC1L1 protein shares 42% identity and 51% similarity with NPC1 (31); despite this, mice deficient in NPC1 did not show altered functional activity of NPC1L1 implying their independent roles and regulations (32).

Odom et al. (29) also reported that HNF1α can bind to the NPC1L1 gene in primary human hepatocytes. This is in line with our results in HuH7 cells, showing a dose-dependent regulation of HNF1α on the NPC1L1 promoter activity and increased mRNA expression following HNF1α overexpression. The liver specificity of this finding was tested by cotransfection of HEK293 cells with the human NPC1L1 promoter and 0.5 μg HNF1α expression vector (Fig. 3D); no induction by HNF1α on the promoter activity was identified in these cells. Also, we identified an HNF1 binding site located −158/−144 bp upstream of transcription start site and showed that mutation of this site almost completely abolished the induction by HNF1α. Electrophoretic mobility shift assay and supershift assays showed a direct binding of HNF1α to this site but not to the other five *cis*-elements. To show the in vivo binding of HNF1α to the NPC1L1 promoter in human liver, we performed ChIP analysis. The six putative HNF1 binding sites (located at −119, −158, −360, −598, −665, and −769 bp upstream of the ATG start codon) are closely located in the NPC1L1 promoter region, and the sonication procedure in the ChIP analysis generally generates DNA fragments of ∼500–800 bp. Thus, primers designed to span over the six HNF1 binding sites were used in the PCR analysis and revealed that HNF1α can bind to the human NPC1L1 promoter in vivo. Collectively, these data suggest that HNF1α may be an important regulator of the hepatic expression of NPC1L1 in humans.

In summary, in this study, we showed that SREBP2 and HNF1α are important transcription factors for the hepatic NPC1L1 promoter activity that can bind to and regulate its expression in humans.

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