Differential Regulation of Human and Mouse Orphan Nuclear Receptor Small Heterodimer Partner Promoter by Sterol Regulatory Element Binding Protein-1*

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Small heterodimer partner (SHP; NR0B2) is an unusual orphan nuclear receptor that lacks a conventional DNA-binding domain and acts as a modulator of transcriptional activities of a number of nuclear receptors. Herein, we report that the human SHP promoter (hSHP) is activated by sterol regulatory element-binding protein-1 (SREBP-1), which regulates the expression of various genes involved in cholesterol and fatty acid synthesis. Overexpression of SREBP-1 activated the human but not mouse SHP promoter, although SREBP-2 had little effect on the SHP promoter in CV-1 cells. Serial deletion reporter assays revealed that SREBP-1-responsive region is located within the sequences from −243 to −120 bp in the hSHP promoter. DNase I footprinting, gel shift assays demonstrated that SREBP-1 binds directly to the hSHP promoter. Site-directed mutagenesis made it clear that the hSHP promoter activation by SREBP-1 is mostly mediated by the SRE1 (−186 to −195 bp) in the hSHP promoter, which is not conserved in the mouse SHP promoter. Moreover, adenovirus-mediated overexpression of SREBP-1c/ADD-1 induced hSHP mRNA expression and repressed CYP7A1 expression in HepG2 cells. Finally, we found that a four-nucleotide deletion (−195CTGAdel) in the hSHP promoter, which is reported to be associated with altered body weight and insulin secretion in human, coincides with the SRE1. This mutation strongly decreased both basal and SREBP-1-dependent activities of the hSHP promoter, because of the reduced binding of SREBP-1 to the mutated SRE1. Overall, our results demonstrate a differential regulation of human and mouse SHP promoters by SREBP-1. We propose a possible role of SREBP-1 in the species differential regulation of cholesterol and bile acid homeostasis via a novel mechanism of up-regulation of the hSHP gene expression.

Small heterodimer partner (SHP; NR0B2) is a member of the large nuclear receptor family of transcriptional factors that lacks a conventional DNA binding domain (1). Various studies have reported SHP to be a repressor of transcriptional activities of a number of nuclear receptors, including glucocorticoid receptor, estrogen receptor, thyroid hormone receptor, retinoic acid receptor, retinoid X receptor, constitutive androstane receptor, pregnane X receptor, HNF4α, liver receptor homologue 1, estrogen-related receptor-γ, and liver X receptor (LXR) (2–10). The very broad range of receptors sensitive to inhibition by SHP suggests a central role for SHP in modulation of nuclear receptor signaling pathways. Although the mechanisms underlying this repressor function remain unclear, recent results demonstrate that SHP can compete with coactivators for binding to the AF-2 surface (7, 11). In addition, a direct transcriptional repression domain contributes significantly to the inhibitory function of SHP. SHP is expressed in a wide variety of tissues, including heart, brain, liver, spleen, adrenal gland, small intestine, and pancreas (2, 12). The human SHP gene is located on chromosome 1p36.1 and consists of two exons separated by an intron (13). SHP gene transcription is regulated by several members of the nuclear receptor superfamily, including the bile acid receptor farnesoid X receptor (14–16), steroidalogenic factor-1 (17), HNF4α (18), liver receptor homologue 1 (14), and estrogen-related receptor-γ (9). However, other families of transcription factors that can regulate the SHP gene expression have not yet been fully characterized.

Previous reports have suggested that SHP plays a pivotal role in the regulation of cholesterol homeostasis via bile acid-activated regulatory cascade in the liver (14–16). The farnesoid X receptor-mediated SHP gene induction has been shown to inhibit the activity of orphan nuclear receptor liver receptor homologue 1, a positive regulator of cholesterol 7α-hydroxylase (CYP7A1) gene expression, which is a rate-limiting enzyme in bile acid biosynthesis. LXRα, which is activated by metabolites of cholesterol, has been shown to activate the mouse and rat but not human CYP7A1 gene promoters by direct binding to conserved LXR response element, was lacking in human CYP7A1 promoter (19–21). More recently, LXRα has been shown to induce human but not rat SHP gene expression via direct binding to the LXR response element in human SHP.

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1 The abbreviations used are: SHP, small heterodimer partner; SREBP, sterol regulatory element-binding protein; SRE, sterol regulatory element; HNF4α, hepatocyte nuclear factor-4α; LXR, liver X receptor; LDL, low density lipoprotein; NF-Y, nuclear factor Y, Sp1, stimulatory protein 1; HA, hemagglutinin; RT, reverse transcriptase; WT, wild type.
promoter (22), which in turn represses CYP7A1 gene expression. This report gives another example of the interspecies difference in the regulation of CYP7A1 gene expression. However, loss-of-function studies of SHP demonstrated that redundant pathways independent of the SHP-mediated pathway are implicated in the negative feedback regulation of bile acid production (23, 24). In addition, it was reported previously that mutations in the coding region of SHP gene are associated with mild hyperinsulinemia, moderate obesity, and decreased insulin sensitivity in human (12). However, a recent report has shown that genetic variations in the hSHP gene are unlikely to be predisposed to diabetes, obesity, or increased birth weight (25). In addition, it is reported that homozygous mutation (−195CTGAdel) in the SHP gene promoter may be associated with lower birth weight and insulin secretion in human (26).

Sterol regulatory element-binding proteins (SREBPs) are a family of membrane-bound transcription factors that regulate cholesterol and fatty acid homeostasis (27, 28). In mammals, three SREBP isoforms have been identified that are designated SREBP-1a, SREBP-1c/ADD1, and SREBP-2. SREBP-1a and SREBP-1c/ADD1 are derived from the same gene by virtue of alternative splicing of the first exon (27). SREBP-1a has a longer transcription-activating domain and is a more potent transcriptional activator than SREBP-1c in cultured cells and liver (29). Nascent SREBPs reside in the endoplasmic reticulum and the nuclear envelope as precursor forms (27). The transcriptionally active amino-terminal segments (mature forms) are released from the precursor SREBPs by a sequential two-step cleavage process. Once cleaved, the mature forms translocate into the nucleus, where they bind to sterol regulatory elements (SREs) in the promoters of target genes. SREBPs regulate the transcription of many genes involved in cholesterol and fatty acid synthesis, such as low density lipoprotein (LDL) receptor, farnesyl-pyrophosphate synthase, squaleric synthase, hydroxymethylglutaryl-CoA reductase, hydroxymethylglutaryl-CoA synthase, fatty acid synthase, acetyl-CoA carboxylase and ATP citrate-lyase (30–35). Previous reports suggested that SREBP-1 and SREBP-2 have different effects on their target genes. SREBP-1 preferentially activates SREBP-1c has a longer transcription-activating domain and is a more potent transcription-activating domain than SREBP-1c in cultured cells and liver (35). SREBP-1a has a shorter transcription-activating domain and is a more potent transcription-activating domain than SREBP-1c in cultured cells and liver (35). SREBP-1a has a longer transcription-activating domain than SREBP-1c in cultured cells and liver (35). SREBP-1a has a shorter transcription-activating domain than SREBP-1c in cultured cells and liver (35). Three SREBP isoforms have been identified that are designated SREBP-1a, SREBP-1c/ADD1, and SREBP-2. SREBP-1a and SREBP-1c/ADD1 are derived from the same gene by virtue of alternative splicing of the first exon (27). SREBP-1a has a longer transcription-activating domain and is a more potent transcriptional activator than SREBP-1c in cultured cells and liver (29). Nascent SREBPs reside in the endoplasmic reticulum and the nuclear envelope as precursor forms (27). The transcriptionally active amino-terminal segments (mature forms) are released from the precursor SREBPs by a sequential two-step cleavage process. Once cleaved, the mature forms translocate into the nucleus, where they bind to sterol regulatory elements (SREs) in the promoters of target genes. SREBPs regulate the transcription of many genes involved in cholesterol and fatty acid synthesis, such as low density lipoprotein (LDL) receptor, farnesyl-pyrophosphate synthase, squalelic synthase, hydroxymethylglutaryl-CoA reductase, hydroxymethylglutaryl-CoA synthase, fatty acid synthase, acetyl-CoA carboxylase and ATP citrate-lyase (30–35). Previous reports suggested that SREBP-1 and SREBP-2 have different effects on their target genes. SREBP-1 preferentially activates transcription of the CYP7A1 gene via direct stimulation of the hSHP promoter. In this study, we demonstrate that SREBP-1, but not SREBP-2, stimulates the hSHP promoter through its binding to SRE1, which is located in the region from −186 to −195 bp in the hSHP promoter but is not conserved in the mouse SHP promoter. We also found that a naturally occurring mutation (−195CTGAdel) in the hSHP promoter, corresponding to SRE1 mutation, results in the complete loss of activation by SREBP-1 because of the reduced binding of SREBP-1 to the altered SRE1. In addition, adenosine-mediated overexpression of SREBP-1c/ADD1 repressed CYP7A1 expression by induction of SHP expression in HepG2 cells. Our results suggest that SREBP-1 can be involved in the species differential regulation of CYP7A1 gene expression via direct stimulation of the hSHP promoter.

**EXPERIMENTAL PROCEDURES**

**Plasmids and DNA Construction—** SREBP-1 expression plasmids pCSA10, pCMVhSREBP-1c 436, p8BS2, and p5xSRE-hLuc were described previously (35), and the cDNA encoding SREBP-1c/ADD1–403 for adenovirus recombinant was described previously (45). The wild-type and mutant reporter plasmids of the hSHP promoter were cloned into the pGL2-basic plasmid (Promega) using restriction enzymes and specific primers. Wild-type and serial deletion constructs were made by fusing various length of 5′-flanking sequences of the hSHP promoter to the luciferase gene at +30 (~2.2 kb/Luc, −574 bp/Luc, −355 bp/Luc, −243 bp/Luc, and −120 bp/Luc). Mutant reporters mE-box/Luc, mSRE1/Luc, mSRE2/Luc, mSRE1/2/Luc, mCCAAT/Luc, and mSp1/Luc were constructed by introducing site-directed mutagenesis into the hSHP promoter −355 bp/Luc using the primers. 195CTGAdel/Luc was made from the −574 bp/Luc by using primers. The mutated sequences are shown in Figs. 4A and 7A. SRE1 multi-copy reporters, hSHP/SRE1x2, hSHP/SRE1x3, and hSHP/SRE1x4/Luc were constructed by inserting 2, 3, and 4 copies, respectively, of SRE1 fragments into the Xhol-digested pGL2-promoter plasmid (Promega). The primers used to construct SRE1 multi-copy reporters were as follows: 5′-TCG- GCCGGACTGATATCACAGCTGCCCAATGCCC-3′; SRE1-CCAAT/Luc, SRE1/Luc, and CCAAT/Luc reporters were constructed by inserting a single copy of double-stranded oligonucleotides into the XmaI- and Xhol-digested pGL2-promoter plasmid. The sequences of the oligonucleotides were as follows: SRE1-CCAAT, 5′-CCGGGACTGATATCACAGCTGCCCAATGCCC-3′; SRE1, 5′-CCGGGACTGATATCACAGCTGCCCAATGCCC-3′; and CCAAT, 5′-CCGGGCAATGCCCC-3’. pcDNA3/HA-ADD1 was constructed by inserting a PCR fragment encoding the N terminus (403 amino acids) of SREBP-1c/ADD1 into EcoRI- and Xhol-digested pcDNA3/HA vector.

**Preparation of Recombinant Adenovirus—** The recombinant adenoviruses were prepared as described previously (46). In brief, the cDNA encoding SREBP-1c/ADD1–403 was cloned into pAd-YC2 shuttle vector, which is under the control of the cytomegalovirus promoter and contains bovine growth hormone polyadenylation signal sequence. For homologous recombination, pAd-YC2 shuttle vector (5 μg) and a rescue vector, pJM17 (5 μg), were cotransfected into human embryonic kidney 293 cells. To purify pure plaques, cell culture supernatant was serially diluted in serum-free media and incubated with 37 °C and 1 h. An equal volume mixture of 2× medium and 1% agarose was overlaid on 293 cells. After 7 days, plaques that were well isolated were further purified and propagated in 293 cells and screened by PCR using upstream primers derived from the cytomegalovirus promoter and downstream primers from the bovine growth hormone polyadenylation sequence. Then, the recombinants were amplified in 293 cells and were purified and isolated using CsCl (Sigma). The preparations were collected and desalted, and titers were determined by the measurement of plaque counts.

**Cell Culture and Transient Transfection Assay—** HepG2 and CV-1 cells were maintained with Dulbecco’s modified Eagle’s medium (In-vitrogen), supplemented with 10% fetal bovine serum (Cambrex Bio Science, Walkersville, Inc., Walkersville, MD) and antibiotics (Invitrogen). Cells were split onto 24-well plates at densities of 2–8 × 10^4 cells/well the day before transfection. Transient transfection assays were performed using the SuperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. Cells were transfected with various reporters together with 0.2 μg of the SREBP expression vectors pCSA10, pCMVhSREBP-1c 436, and p8BS2. Total DNA used in each transfection was adjusted to 1 μg/well by adding appropriate amount of pcDNA3 empty vectors, and 0.2 μg of cytomegalovirus β-galactosidase plasmids cotransfected as an internal control. Cells were harvested 40–48 h after the transfection for luciferase and β-galactosidase assays. The luciferase activity was normalized by β-galactosidase activity.

**DNaSe I Footprinting Assay—** DNA fragment covering the region from −355 to −120 bp was labeled in one strand by PCR with a primer set, one of which was labeled with 32P. The sequences of the primers for PCR were 5′-CCCCTGACTGATATCACAGCTGCCCAATGCCC-3′ and 5′-CCGGGACTGATATCACAGCTGCCCAATGCCC-3′.

DNaSe I footprinting assay was performed using the SuperFect transfection reagent (Qiagen) according to the manufacturer’s instructions. Cells were transfected with various reporters together with 0.2 μg of the SREBP expression vectors pCSA10, pCMVhSREBP-1c 436, and p8BS2. Total DNA used in each transfection was adjusted to 1 μg/well by adding appropriate amount of pcDNA3 empty vectors, and 0.2 μg of cytomegalovirus β-galactosidase plasmids cotransfected as an internal control. Cells were harvested 40–48 h after the transfection for luciferase and β-galactosidase assays. The luciferase activity was normalized by β-galactosidase activity.
After 2 min of digestion at room temperature, the reaction was stopped by adding 100 μl of stop buffer containing 1% (v/v) SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0, and 0.1 g/μl glycogen. The DNA was extracted with phenol/chloroform and recovered by ethanol precipitation. The pellets were dissolved in sequencing gel loading buffer and then resolved on denaturing 6% polyacrylamide/6 M urea gel. The footprints were compared with G + A ladder produced by the chemical cleavage sequencing reaction of the same probe to determine the corresponding nucleotide sequences.

**Gel Mobility Shift Assay**—The probes used in gel shift assay cover the region from −203 to −163 bp of the hSHP promoter. Mutated sequences are shown in Figs. 4A and 7A as indicated. Double stranded oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The labeled probes were incubated with 1 μg of purified recombinant SREBP-1a (Fig. 4B) or 1–4 μl of in vitro translated SREBP-1a proteins (Fig. 7, C and D) in 20 μl of each reaction containing 10 mM HEPES, pH 7.6, 75 mM KCl, 1 mM EDTA, 10 mM dithiothreitol, 10% (v/v) glycerol, 1 μg of poly (dI-dC), and 0.5% bovine serum albumin.

In vitro-translated SREBP-1a proteins were prepared by using a coupled transcription and translation system (TNT-coupled reticulocyte lysate system; Promega) according to the manufacturer’s instructions. For unlabeled competition assays, unlabeled oligonucleotides were added to the reactions at ~100-fold molar excess (Fig. 4B) or 10, 50, and 100-fold molar excess (Fig. 7D). After a 20-min incubation at room temperature, the samples were resolved on a 4% polyacrylamide gel in 1X TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) at 250 V for 1 h at room temperature. After electrophoresis, the polyacrylamide gel was dried and exposed to Fuji HR-G30 X-ray film for 3 h at −70 °C with intensifying screen.

**Chromatin Immunoprecipitation Assay**—The chromatin immunoprecipitation assay was performed as described previously (47). In brief, HepG2 cells were transfected with 10 μg of pcDNA3/H-A SREBP-1c/ADD1 and pcDNA3/H-A empty vectors. At 30 h after transfection, the cells were fixed with 1% formaldehyde and harvested. For immunoprecipitation, anti-HA antibodies (Roche Molecular Biochemicals) and protein A-Sepharose beads CL-4B (Amersham Biosciences) were used. The final DNA extractions were amplified by PCR using pairs of primers. The primers used for PCR as follows: −120 to −474 bp, 5'-CCCTTGACGAGAATG-3' and 5'-AGGTTAGGCAAAACAAGC-3'; and −880 to −1200 bp, 5'-CAGCTACTTCTAGGAGCTA-3' and 5'-CATAAAGTGAATCTCTAG-3'. The PCR products were a 354-bp fragment (nucleotides −120 to −474 bp of the hSHP promoter) containing SREBP-1 response region (−243 and −120 bp) and a 320-bp fragment (nucleotides from −880 to −1200, used as a negative control).

**Northern Blot and Reverse Transcriptase PCR Analyses**—HepG2 cells were maintained with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum for 24 h and infected with 1 ml of adenovirus containing Dulbecco’s modified Eagle’s medium at a titer of 10 plaque-forming units/cell for 12 h at 37 °C. Then, culture medium was adjusted to 2 ml with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. After viral infection for 0, 3, 6, 12, and 24 h, infected cells were harvested for RNA isolation using the TRIzol reagent (Invitrogen). Northern blot analysis was carried out as described previously (13). The mRNAs of hSHP and hCYP7A1 were analyzed by RT-PCR as described previously (22). In brief, first-strand cDNA was synthesized from 1 μg of total RNA using an anchored oligo(dT) primer and reverse transcriptase. The resulting first-strand cDNA was then amplified to measure mRNA levels of SHP, CYP7A1, and β-actin by 25, 30, and 25 cycles of PCR, respectively, using specific primers. The mRNA levels of β-actin served as an internal control for the RT-PCR analysis. The primers used for PCR were as follows: SHP forward primer, 5’-CAAGAAGATTCTGCTTGGAGG-3’, SHP reverse primer, 5’-GGATGGCAACATCTTCACCTG-3’; CYP7A1 forward primer, 5’-CTAAGGGGTTTCTAATTGGC-3’; CYP7A1 reverse primer, 5’-ACTGTTCCAAAGGTGGCACT-3’; β-actin forward primer, 5’-GTCACTACATTCTGCTTGCTG-3’; and β-actin reverse primer, 5’-CCTGTTTGTCTGCTGCTG-3’. The sizes of the SHP, CYP7A1, and β-actin PCR products were 390, 350, and 350 bp, respectively.

**RESULTS**

**Identification of SREBP-1 as Transcriptional Activators of the Human SHP Promoter**—In an effort to identify the transcription factors that regulate the SHP promoter, we found putative binding sites for SREBP2s (two SREs), NF-Y (CCAT box), and Sp1 (GC box) in the human SHP (hSHP) promoter region spanning from upstream −572 bp to downstream +28 bp of the putative transcription start site (Fig. 1A). We aligned the sequences containing putative SREs in the human and mouse SHP (mSHP) promoters and noticed that mouse putative SRE1 differs from human and consensus SRE sequences, whereas both human and mouse SRE2/E-box are identical in the core E-box (Fig. 1B). To determine whether SREBP2s regul-
late SHP promoter, we performed transient transfection assay using the reporter gene containing −2.2 kb of the human and mouse SHP promoters. The p5xSRE-tk/Luc reporter, which contains five copies of SRE elements from the LDL receptor gene promoter at the upstream of herpes simplex thymidine kinase promoter, was used as a positive control. Overexpression of both pCSA10 and pCMVhSREBP1c 436 encoding the mature form of human SREBP-1a and -1c strongly activated the hSHP promoter in CV-1 cells. It is interesting that neither SREBP-1a nor SREBP-1c activated the mSHP promoter, implying that the difference in SRE1 sequence between human and mouse SHP promoter might result in the differential regulation of human and mouse SHP promoter by SREBP-1. It was noteworthy that SREBP-2 had no significant effect on either human or mouse SHP promoter activity, whereas SREBP-2 activated the p5xSRE-tk/Luc activity, as reported
previously (Fig. 2A). This result indicates that the *hSHP* gene is a specific target of SREBP-1 but not of SREBP-2, supported by the previous reports that SREBP-1 and SREBP-2 have different effects on their target genes (36, 37). Taken together, our results suggest that human and mouse *SHP* promoter can be differentially and specifically regulated by SREBP-1. To map the sequences required for SREBP-1-mediated activation, we made a series of 5’ deletions of the *hSHP* promoter as indicated in Fig. 2B and performed a transient transfection assay. As shown in Fig. 2C, SREBP-1 responsiveness was continuously retained with a deletion up to −243 bp but was lost with the −120-bp construct. These results indicate that the sequences required for SREBP-1 response lie in the region between −243 and −120 bp within the *hSHP* promoter.

**Determination of SREBP-1 Binding Regions by DNase I Footprinting and Gel Shift Assays**—To localize the direct SREBP-1 binding sites in the region from −355 to −120 bp of the *hSHP* promoter containing the SREBP-1 responsive region (−243 to −120 bp), DNase I footprinting assay was performed using purified recombinant human SREBP-1α proteins expressed in *E. coli* (Fig. 3). SREBP-1α protected three regions, from −160 to −179 (SRE2; designated as SRE2/E-box), from −186 to −195 (SRE1) and from −248 to −267 (designated as E-box). The inverse sequence (ATCACCTCAG) of SRE1 has 80% homology to SRE (ATCACCCCAC) identified in the LDL receptor promoter (30). The −165 to −179 bp (SRE2/E-box) and −248 to −267 bp (E-box) regions correspond to E-box motifs (CANNTG) that are binding sites for basic helix-loop-helix proteins. However, the result shown in Fig. 2C suggested that the upstream E-box (−248 to −267 bp) region might not be necessary for SREBP-1-mediated transactivation of the *hSHP* promoter, although SREBP-1 binds to E-box (−248 to −267 bp) in DNase I footprinting assay.

To confirm the specific binding of SREBP-1 to these three footprinted regions, gel shift assays were performed. We designed wild-type (−203/−163) probes covering the protected regions (SRE1 and SRE2/E-box) (Fig. 4A). The oligonucleotides of mutant SRE1 (m1), mutant SRE2/E-box (m2), double mutant SRE1/SRE2 (m3), wild-type (−271/−243) containing upstream E-box, and the E-box mutant (m4) were prepared for unlabeled competition assay. As shown in Fig. 4B, purified SREBP-1α proteins produced one major retarded band with wild-type (−203/−163) probe (lane 1), although the probe contains two potential SREs, suggesting that SREBP-1 may bind only to one of the potential SREs. For competition assay, unlabeled oligonucleotides of −203/−163, m1, m2, m3, −271/−243, or m4 were added to the reactions as competitors. When 100-fold molar excess of unlabeled oligonucleotides of −203/−163 and −271/−243 (lanes 2 and 6) were added, retarded bands almost disappeared. The unlabeled oligonucleotides of m3 and m4 had no significant effects on the SREBP-1 binding to wild-type (−203/−163) probes. However, SREBP-1-DNA complex formation was slightly inhibited by the excess of unlabeled mutant SRE1 (m1, lane 3) oligonucleotides, whereas the retarded band almost disappeared by addition of mutant SRE2/E-box (m2, lane 4) oligonucleotides. Thus, to exclude the fold excess used as competitors and to directly confirm whether SREBP-1 binds to individual SRE1 or SRE2 within the −203/−163 sequences, the labeled oligonucleotides of m1, m2, or m3 were used as probes in gel shift assay. The m2 probe, containing intact SRE1, avidly formed the complex with SREBP-1 comparable with that of wild probe (lane 9), whereas SRE1 mutation (m1) drastically inhibited the complex formation (lane 8) and the double mutation of SRE1 and SRE2 completely blocked the binding to SREBP-1 (lane 10). These results indicated that SREBP-1 binds predominantly to SRE1 and weakly to SRE2/E-box.

To verify that SREBP-1 binds to the *hSHP* promoter in *vivo*, we performed chromatin immunoprecipitation assay using two sets of PCR primers specific for −120 to −474-bp region containing SREBP-1 responsive region and the primers encompassing the −880 to −1200 bp of the *hSHP* promoter as a negative control. Expression vectors encoding HA epitope only or HA-SREBP-1α/ADD1 were transfected into HepG2 cells, and cell lysates were immunoprecipitated with the anti-HA antibody. As shown in Fig. 4C, the 354 bp of PCR product (−120 to −474 bp) was observed in cells transfected with expression vectors for HA-SREBP-1α/ADD1, but not for HA epitope only, indicating that HA-SREBP-1α/ADD1 formed a specific complex.
Regulation of Human SHP Promoter by SREBP-1

SREBP-1 predominantly binds to SRE1 in the hSHP promoter in gel mobility shift assay. A, the sequences of wild-type (nucleotides −203 to −163 and −271 to −243) and mutant (m1, m2, m3, and m4) oligonucleotides used for the gel retardation assays are shown as indicated. Lower case letters indicate the substituted nucleotides in the SRE1, SRE2/E-box, and E-box. B, gel mobility shift and unlabeled competition assays were performed with the 32P-labeled wild-type (lanes 1–7), mutant (lanes 8–10), probes, and recombinant SREBP-1a (1 µg) protein as indicated. Unlabeled oligonucleotides were added as competitors at 100-fold molar excess where indicated. C, chromatin immunoprecipitation assay. HepG2 cells were transfected with expression vectors for HA epitope only or HA/SREBP-1c/ADD1. Soluble chromatin from these cells was prepared and immunoprecipitated with monoclonal antibody against HA (lanes 3 and 4) as described previously (48). The −474 to −120 bp fragment (354 bp) contains SREBP-1 binding site and 10% of the soluble chromatin used in the reaction was used as inputs (lanes 1 and 2). The −1200 to −880 bp fragment is used as a negative control, and −22 kb/Luc plasmids were amplified as a positive control (lane 5) as indicated.

with the hSHP promoter in vivo. No bands were detected for the region spanning −880 to −1200 bp of hSHP promoter used as a negative control. These results demonstrated that SREBP-1 binds to the hSHP promoter in vivo. Taken together, we suggest that SREBP-1 regulates the hSHP promoter by direct binding to SRE1.

The SRE1 (−186 to −195 bp) Plays a Critical Role in the Transactivation of the hSHP Promoter by SREBP-1—To further evaluate the functional significance of the SREBP-1 binding sites (SRE1, SRE2, and E-box) in the hSHP promoter that we identified, site-directed mutagenesis was introduced to the −355-bp hSHP promoter using the primers as indicated (Fig. 5A). Wild-type −355 bp/Luc and the mutant reporter plasmids were transiently transfected with SREBP-1 expression vectors into HepG2 cells. The SRE1 mutation (mSRE1/Luc) and double SRE1 and 2 mutation (mSRE1,2/Luc) completely abolished the SREBP-1-mediated activation of the hSHP promoter (Fig. 5B). However, SRE2 mutation had little effect on the activation of the hSHP promoter by SREBP-1, supporting the results from Fig. 4B showing that SREBP-1 predominantly binds to SRE1 rather than SRE2. In addition, the E-box mutant (mE-box/Luc) reporter was still activated by SREBP-1. This observation was consistent with the result from Fig. 2C, showing that the SREBP-1 responsive region, located between −243 and −120 bp, does not contain the E-box.

The SREBP-1s have been shown to work efficiently depending on the promoters along with ubiquitous coregulatory proteins such as Sp1 (42) or NF-Y (41). To test whether adjacent SRE related elements (CCAAT box and Sp1 binding site) are involved in SREBP-1-mediated activation of the hSHP promoter, mutant reporters of CCAAT box (mCCAAT/Luc) and Sp1 binding site (mSp1/Luc) were constructed by introducing site-directed mutagenesis to −355 bp/Luc. As shown in Fig. 5B, SREBP-1 dependent reporter activity was significantly decreased by mutation of CCAAT box (mCCAAT/Luc), suggesting that the CCAAT box binding proteins can be involved in the SREBP-1-dependent activation of the hSHP promoter. However, mutation of Sp1 binding site had little effect on the SREBP-1-mediated activation of the hSHP promoter.

To further confirm whether the CCAAT box is directly required for the ability of SREBP-1 to transactivate the hSHP promoter, we constructed the reporters, the SRE1/Luc, CCAAT/Luc, and SRE1-CCAAT/Luc, containing one copy of individual SRE1, CCAAT box, and both SRE1 and CCAAT box, respectively, which originated from the hSHP promoter. The reporters were cotransfected with expression vectors for SREBP-1a into HepG2 cells. As shown in Fig. 5C, the SRE1/Luc reporter was significantly activated by SREBP-1a, whereas SREBP-1a had little effect on either CCAAT/Luc or the pGL2 basal promoter. SREBP-1-mediated reporter activity of SRE1-CCAAT/Luc was unexpectedly similar to that of SRE1/Luc, suggesting that CCAAT box may be indirectly involved in the ability of SREBP-1 to transactivate the hSHP promoter in the natural promoter context. To test specifically whether SRE1 was sufficient to confer SREBP-1 responsiveness, we constructed multiple copies of SRE1 reporters containing two, three, or four copies of SRE1 and performed a transient transfection assay using the reporter genes as indicated (Fig. 5D). SREBP-1a activated multiple copies of SRE1 as well as p5xSRE-tk reporter from the LDL receptor gene promoter, whereas SREBP-1a had no effect on the pGL2 basal promoter. These results indicate that SRE1 may be sufficient to mediate the activation of the hSHP promoter by SREBP-1. Taken together, we conclude that SRE1 plays a major role in the activation of the hSHP promoter by SREBP-1.

Induction of SHP mRNA by SREBP-1c/ADD1 in HepG2 Cells—To examine whether SREBP-1c is directed involved in the regulation of the hSHP gene expression, we overexpressed SREBP-1c/ADD1 into HepG2 cells via adenovirus infection. As shown in Fig. 6A, SHP mRNA was induced by overexpression of AD-SREBP-1c/ADD1 at 3 h and peaked at 12 h after infection.
This result suggests that SREBP-1 can stimulate the hSHP gene promoter in the natural promoter context.

Based on the previous report showing that overexpression of SHP represses the hCYP7A1 promoter (22), we examined whether AD-SREBP-1c/ADD1 could repress the transcription of hCYP7A1 gene via induction of the hSHP gene expression. RT-PCR analysis was performed to detect CYP7A1 mRNA, because we could not detect CYP7A1 expression by Northern blotting in HepG2 cells. RT-PCR results showed that AD-SREBP-1c/ADD1 significantly repressed endogenous CYP7A1 mRNA expression in HepG2 cells, indicating that induction of the hSHP gene by SREBP-1 may contribute to the repression of CYP7A1 gene expression. Taken together, these results suggest that SREBP-1 can increase endogenous human SHP.
mRNA level, which results in the feedback repression of CYPT7A1 gene expression.

Effect of Naturally Occurring Mutation in SRE1 on SREBP-1-Dependent Activation of SHP Promoter—It has been reported that mutations in the SHP gene in the Japanese subjects are associated with mild hyperinsulinemia and obesity (12, 25). More recently, it is reported that a genetic variation in the hSHP promoter results in the lower birth weight and insulin secretion. The 4-bp (CTGA) deletion at −195 bp upstream of the transcription start site of human SHP promoter (designated −195CTGAdel) was found in subjects from the UK (26). We were intrigued to find that −195CTGAdel occurs in the region corresponding to SRE1, suggesting that the deletion can affect the SREBP-1-dependent activation. To address this, we made and transfection a reporter construct (−195CTGAdel/Luc) harboring CTGA nucleotide deletion from the hSHP promoter (−574bp/Luc). As shown in Fig. 7A, basal activity of −195CTGAdel/Luc was remarkably diminished about 10-fold less than that of wild-type promoter in HepG2 cells. Furthermore, SREBP-1 responsiveness was almost completely abolished with −195CTGAdel/Luc reporter. This result suggests that the naturally occurring −195CTGAdel mutation corresponding to SRE1 mutation may have significant effect on the SREBP-1 mediated transactivation of the hSHP promoter.

To investigate whether the loss of activation of −195CTGAdel/Luc by SREBP-1 can be at least partly caused by the alteration in SREBP-1 binding potential to DNA, we performed gel shift assay using the wild-type (−203 to −163, containing SRE1 and SRE2) and mutant (−195CTGAdel) probes used for the gel retardation assay. C, naturally occurring mutation in the hSHP promoter markedly diminished the binding ability of SREBP-1 to DNA. 32P-labeled wild type (−203 to −163, lanes 1–6) and −195CTGAdel (lanes 7–12) probes were combined with 1 to 4 μl of SREBP-1a proteins prepared by in vitro translation system as indicated. The total amounts of proteins used in the reactions were adjusted to 4 μl by adding unprogrammed lysates, except for lanes 1 and 7. D, for unlabeled competition assays, 10-, 50-, and 100-fold molar excess of the unlabeled oligonucleotides (WT, −195CTGAdel, and LDLR-SRE) were added to the reaction containing 32P-labeled wild type SRE1 and in vitro translated SREBP-1a (2 μl), as competitors. DNA-protein complexes were analyzed on 4% polyacrylamide gel and analyzed by autoradiography.
**DISCUSSION**

Previous reports have identified several nuclear receptors, such as steroidogenic factor-1, liver receptor homologue-1, HNF4α, farnesoid X receptor, estrogen-related receptor-γ, and LXRα, as potent regulators of SHP gene expression (9, 14–18, 22). We recently reported that basic helix-loop-helix transcription factor E2A and orphan nuclear receptor steroidogenic factor-1 synergistically activate the human, but not mouse SHP gene promoter (48). However, whether other families of transcription factors regulate SHP gene transcription has not been fully characterized. In this report, we have demonstrated that SREBP-1 is a novel regulator of human SHP gene expression. It was interesting that SREBP-1 activated the human, but not the mouse, SHP promoter (Fig. 2A). SRE1 located in −186–−195 bp within the hSHP promoter, which is not conserved in mouse SHP promoter, is identified as SREBP-1-responsive element. In addition, we showed that the hSHP promoter was activated by SREBP-1 but not by SREBP-2. Our finding is supported by the previous reports suggesting that SREBP-1 and SREBP-2 have different effects on their target genes. SREBP-1 preferentially activates genes involved in fatty acid synthesis (36) and SREBP-2 activates genes involved in cholesterol synthesis (37). The mechanism for the differences in sterol regulation between SREBP-1c and −2 is currently unknown; however, the relative low sequence homology between their cholesterol regulatory carboxyl-terminals might be involved (49). In addition, previous reports have suggested that SREBP-1 and -2 have different binding affinities to SREs in ATP citrate-lyase promoter (35). However, we could not observe any difference in the binding affinities of SREBP-1 and -2 to SRE1 within the hSHP promoter (data not shown). It is still possible that amino-terminal activation domains of these factors have different compatibility to the hSHP promoter.

Optimal transcriptional activation by SREBPs depends on additional transcription factors such as Sp1, NF-Y, cAMP response element-binding protein, or CCAAT/enhancer-binding protein α (41, 42, 45, 50). Activation of SREBP target genes require NF-Y binding to adjacent CCAAT motifs that are usually located within 21 bp of the SRE (43). In addition, it has been demonstrated that SREBP and Sp1 cooperatively transactivate the LDL receptor and phosphoenolpyruvate carboxykinase promoters (42, 44, 51). In vitro studies have demonstrated that the binding of NF-Y or Sp1 to DNA is associated with synergistic binding of SREBP to an adjacent SRE (31, 42), presumably as a result of direct protein-protein interaction (34, 44). It is interesting that Sp1 does not seem to be involved in the SREBP-1-mediated activation of the hSHP promoter (Fig. 5B). We showed that mutation in putative CCAAT box significantly reduced the activity of the hSHP promoter by SREBP-1. However, we could observe no significant effect of the dominant-negative form of NF-YA on the SREBP-1 dependent activation of the hSHP promoter (data not shown), suggesting that other CCAAT box binding proteins can be implicated in the SREBP-1 activation. We also showed that the presence of CCAAT box had little effect on SREBP-1-dependent transactivation of SRE1 (Fig. 5C), suggesting that CCAAT box may be indirectly involved in SREBP-1 dependent transactivation of the hSHP promoter in a natural promoter context. However, the CCAAT box binding proteins associated with the regulation of the hSHP promoter by SREBP-1 remain to be determined.

It has been reported that a number of factors, including cholesterol, insulin, glucose, and polyunsaturated fatty acids, are involved in SREBP-1 gene expression or its nucleus translation. Thus, human SHP promoter is likely to be regulated by those factors. For example, insulin, an activator of SREBP-1 expression, has been shown to repress the CYP7A1 gene promoter activity in HepG2 cells (52). The inhibitory effect of insulin on the human CYP7A1 gene expression was much stronger than the rat gene. However, little has been studied on the apparent molecular mechanism of species differences in the regulation of CYP7A1 gene by insulin. We found that adenovirus-mediated overexpression of SREBP-1 repressed the hCYP7A1 gene expression, probably via induction of hSHP gene in HepG2 cells (Fig. 6B). Therefore, we assume that SREBP-1 induced by insulin can activate the hSHP promoter, a negative regulator of CYP7A1 gene expression and that up-regulation of hSHP gene by SREBP-1 may provide a molecular mechanism by which insulin more strongly suppresses the human CYP7A1 gene expression than rat gene. In addition, LXR agonists have been reported to differentially regulate the CYP7A1 gene expression in human and rat/mouse (53). We demonstrated that hSHP mRNA is directly activated by overexpression of SREBP-1c (Fig. 6A), which is a well known target gene of LXR. Thus, we propose an alternative molecular pathway in which LXR agonists repress the human CYP7A1 gene expression via sequential induction of SREBP-1 and SHP gene. It was previously reported that mutations in SHP gene are associated with mild hyperinsulinemia and obesity as a result of impaired inhibition of HNF4α activity, which is known as a positive regulator of insulin secretion (12). More recently, it was also reported that SHP gene mutation might not be predisposed to diabetes, obesity, or increased birth weight (25). In addition, although mutations in SHP are unlikely to be a common cause of severe obesity, homozygous mutation (−195CTGAdel) in the SHP gene promoter may influence birth weight, body mass index, and insulin secretion in human (26). The −195CTGAdel mutation in the SHP gene promoter noticeably coincides with the SRE1, which we here characterized as an SREBP-1-responsive element in the hSHP promoter. It is interesting that this mutation caused a significant reduction of both basal and SREBP-1-dependent activity of the hSHP promoter as a result of reduced binding of SREBP-1 to the mutated SRE1. Therefore, we suggest that the reduction of SREBP-1 dependent SHP gene expression by SRE1 mutation within the hSHP promoter may result in lower body weight and insulin secretion in human. It is necessary to study further the physiological significance between the promoter mutation and the loss of hSHP gene transactivation by SREBP-1. Furthermore, the physiological role of SHP induced by SREBP-1 in fatty acid synthesis or cholesterol synthesis remains to be elucidated and the occurrence of interspecies differences in those pathways that might be mediated by SHP remains to be studied.

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