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**Genes: Structure and Regulation:  
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Kim, Sang-Kyu Park, Ji-Ho Seo, Jae Bum  
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Hueng-Sik Choi

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

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Han-Jong Kim‡§, Joon-Young Kim‡, Ju-Youn Kim¶, Sang-Kyu Park¶, Ji-Ho Seo||, Jae Bum Kim\*\*, In-Kyu Lee||, Kyung-Sup Kim¶‡‡, and Hueng-Sik Choi‡§§

From the ‡Hormone Research Center, School of Biological Sciences and Technology, Chonnam National University, Kwangju 500-757, Republic of Korea, ¶Department of Biochemistry and Molecular Biology, Institute of Genetic Science, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea, ||Department of Internal Medicine, School of Medicine, Keimyung University, Taegu 700-712, Republic of Korea, and \*\*School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

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, and chromatin immunoprecipitation 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 *SHP* 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)<sup>1</sup> 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, HNF4 $\alpha$ , liver receptor homologue 1, estrogen-related receptor- $\gamma$ , 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), steroidogenic factor-1 (17), HNF4 $\alpha$  (18), liver receptor homologue 1 (14), and estrogen-related receptor- $\gamma$  (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 7 $\alpha$ -hydroxylase (*CYP7A1*) gene expression, which is a rate-limiting enzyme in bile acid biosynthesis. LXR $\alpha$ , 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, LXR $\alpha$  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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§§ To whom correspondence should be addressed. Tel.: 82-62-530-0503; Fax: 82-62-530-0500; E-mail: hsc@chonnam.ac.kr.

<sup>1</sup> The abbreviations used are: SHP, small heterodimer partner; SREBP, sterol regulatory element-binding protein; SRE, sterol regulatory element; HNF4 $\alpha$ , hepatocyte nuclear factor-4 $\alpha$ ; 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 *SHP* 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, squalene 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 the genes involved in fatty acid synthesis (36) and SREBP-2 activates genes involved in cholesterol synthesis (37). In addition, the reports that the *SREBP-1c* promoter is a direct target for regulation by LXR/retinoid X receptor heterodimers provided a straightforward explanation for the ability of LXR ligands to induce hepatic lipogenesis (38–40). Efficient transcriptional activation by SREBPs is dependent on additional transcription factors such as nuclear factor Y (NF-Y) and Sp1 (41, 42). Most SREBP target genes require NF-Y binding to adjacent CCAAT motifs, which are usually located within 21 bp of the SRE (43). *In vitro* studies initially demonstrated that the binding of NF-Y to DNA enhanced the binding affinity of SREBP to adjacent SREs (31), presumably as a result of direct interaction of the two proteins (34). Study of the LDL receptor promoter demonstrated that Sp1 also interacts with SREBP to form a more stable DNA-protein complex (42, 44).

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, adenovirus-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 expression plasmids pCSA10, pCMVhSREBP-1c 436, pCS2, and p5xSRE-tk/Luc 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(SRE1)x2, hSHP(SRE1)x3, and hSHP(SRE1)x4/Luc were constructed by inserting 2, 3, and 4 copies, respectively, of SRE1 fragments into the XhoI-digested pGL2-promoter plasmid (Promega). The primers used to construct SRE1 multi-copy reporters are as follows: 5'-TCGAGACTGAGGTGATATCAG-3' and 5'-TCGACTGATATCAC CTCAGT-C-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 XhoI-digested pGL2-promoter plasmid. The sequences of the oligonucleotides were as follows: SRE1-CCAAT, 5'-CCGGGACTGAGGTGATATCACAGCTGCCAATGCCC-3'; SRE1, 5'-CCGGGACTGAGGTGATATCAC-3'; and CCAAT, 5'-CCGGGCCCAATGCCC-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 XhoI-digested pcDNA3/HA vector.

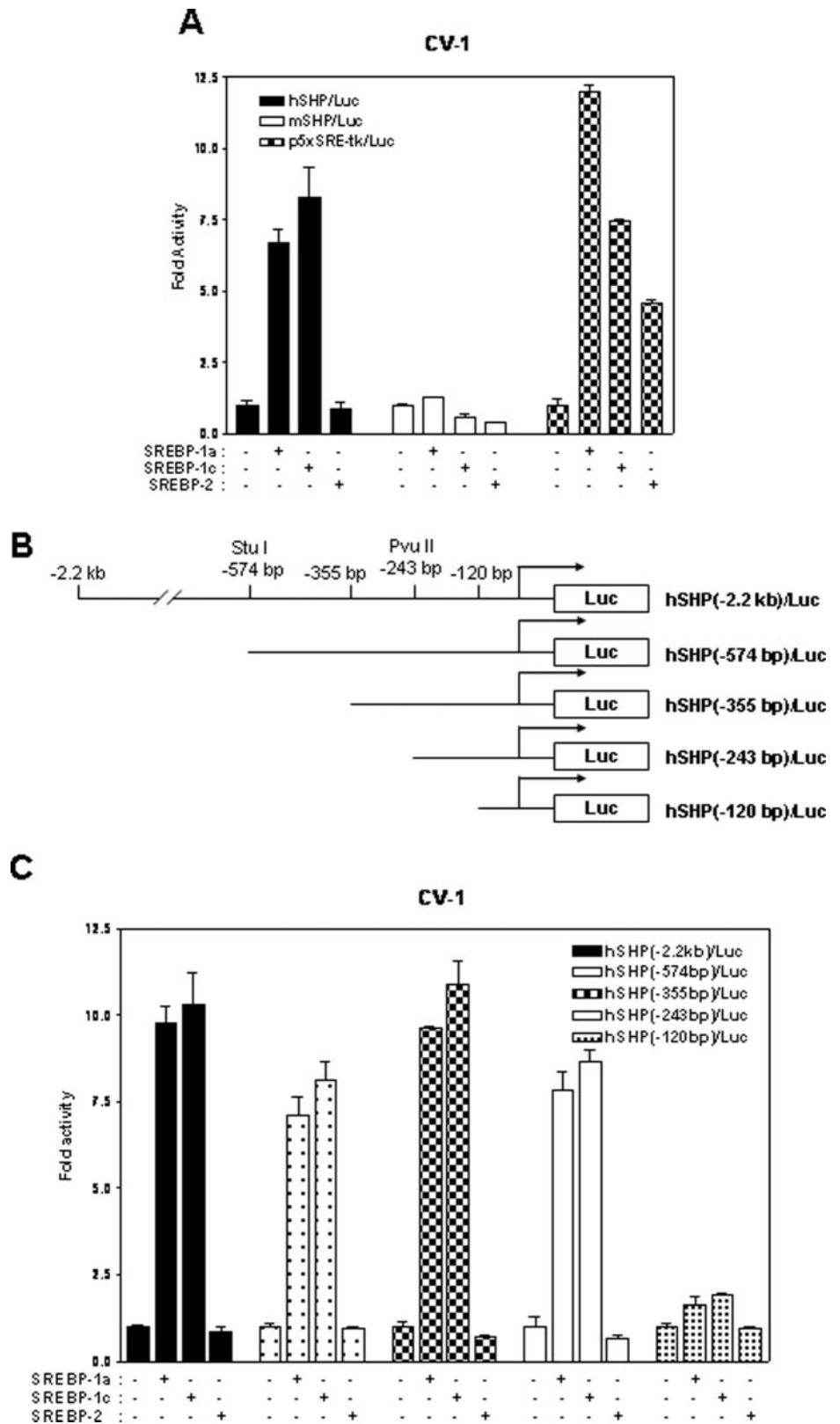
**Preparation of Recombinant Adenovirus**—The recombinant adenovirus was prepared as described previously (46). In brief, the cDNA encoding SREBP-1c/ADD1–403 was cloned into pAd-YC2 shuttle vector, which is under the control of the cytomegalovirus promoter and contains bovine growth hormone polyadenylation signal sequence. For homologous recombination, pAd-YC2 shuttle vector (5  $\mu$ g) and a rescue vector, pJM17 (5  $\mu$ g), 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 293 cells at 37 °C for 1 h. An equal volume mixture of 2 $\times$  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<sub>2</sub> (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 (Invitrogen), 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  $\times$  10<sup>4</sup> 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 0.2  $\mu$ g of various reporters together with 0.2  $\mu$ g of the SREBP expression vectors pCSA10, pCMVhSREBP-1c 436, and pCS2. Total DNA used in each transfection was adjusted to 1  $\mu$ g/well by adding appropriate amount of pcDNA3 empty vectors, and 0.2  $\mu$ g of cytomegalovirus- $\beta$ -galactosidase plasmids were cotransfected as an internal control. Cells were harvested 40–48 h after the transfection for luciferase and  $\beta$ -galactosidase assays. The luciferase activity was normalized by  $\beta$ -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 <sup>32</sup>P. The sequences of the primers for PCR were 5'-CCGCTCGAGCGGGACCAATGGGG-3' and 5'-CCCCGGCAGGAATG-3'. Recombinant SREBP-1 proteins (1, 2, and 4  $\mu$ g), purified as described in Ref. 35, were incubated with 3  $\times$  10<sup>5</sup> cpm of labeled probe for 20 min on ice under the condition of 10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 7% (v/v) glycerol, and 2  $\mu$ g of poly(dI-dC), and then 50  $\mu$ l of diluted DNase I solution (0.002–0.001 units/ $\mu$ l) was added to the DNA-protein binding reactions.



**FIG. 2. SREBP-1 transactivates human but not mouse SHP promoter.** *A*, transcriptional activation of the *hSHP* promoter by SREBP-1 but not by SREBP-2. CV-1 cells were transiently transfected with 200 ng of  $-2.2$ -kb human (black bars), mouse *SHP* promoter (white bars) fused to a luciferase reporter or p5xSRE-tk/Luc (gray bars), together with 100 ng of SREBP expression plasmids pCSA10 (SREBP-1a), pCMVh-SREBP-1c 436 (SREBP-1c), pCS2 (SREBP2), or empty vector. *B*, schematic diagram of the *hSHP* promoter deletion constructs cloned in pGL2-basic vector. The restriction enzyme and primer sites used to make the deletions are indicated at the top. *C*, mapping the sequences required for activation of the *hSHP* promoter by SREBP-1. CV-1 cells were transfected with 200 ng of each reporter plasmid and 100 ng of SREBP expression plasmid as indicated. Approximately 40 h after transfection, the cells were harvested and luciferase activity was measured and normalized against  $\beta$ -galactosidase activity. One representative experiment is shown. All values represent the mean of duplicate samples, and similar results were obtained in at least three independent experiments. The representative results were expressed as -fold activation ( $n$ -fold) over the value obtained with vector alone with the error bars as indicated.



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-1a proteins expressed in *E. coli* (Fig. 3). SREBP-1a 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 (ATCACCCAC) 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-1a 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 to only 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.

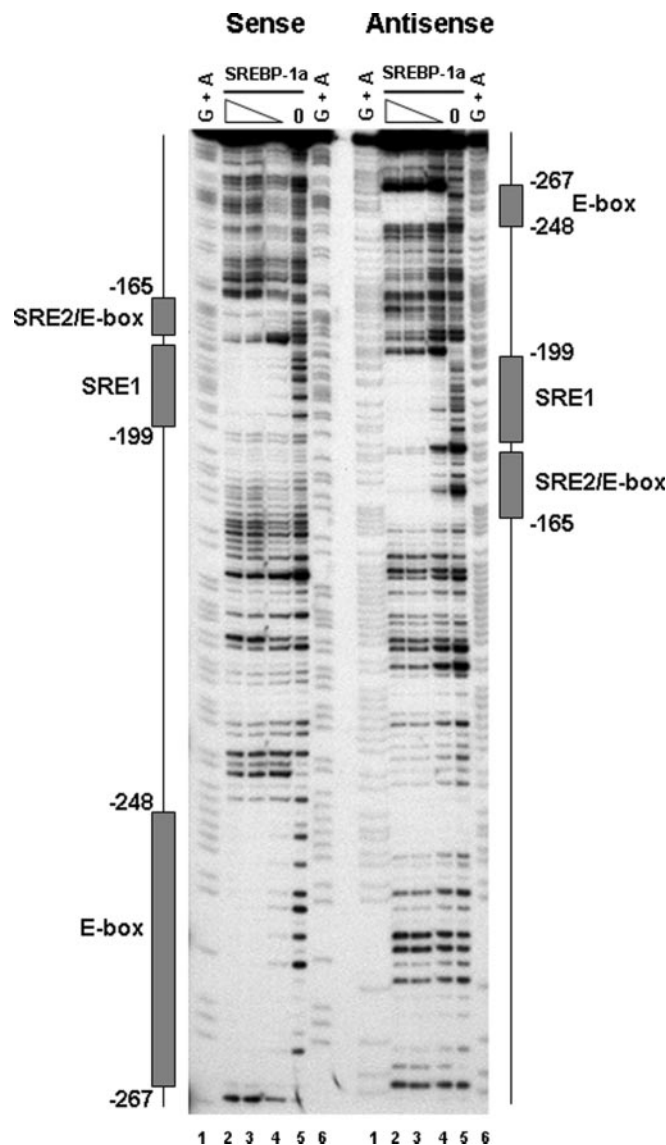
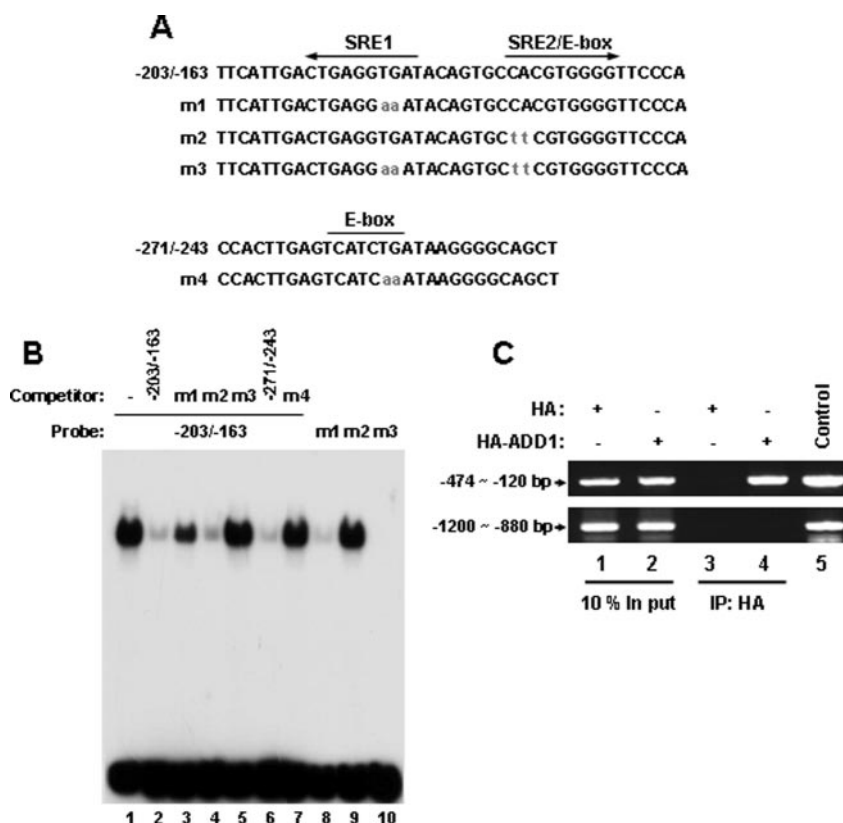


FIG. 3. Determination of the SREBP-1 binding sites in the *hSHP* promoter by DNase I footprinting. The 235-bp DNA fragments of the *hSHP* promoter (nucleotides -355 to -120) were  $^{32}\text{P}$ -labeled at the end of the sense or antisense strands and then PAGE-purified. The radiolabeled probes and the purified human recombinant SREBP-1a expressed in *Escherichia coli* were used for the reaction. The preparation of recombinant protein and DNase I footprinting assay was performed as described under "Experimental Procedures." DNase I-treated reaction mixtures were subjected to electrophoresis on denaturing 6% polyacrylamide gel. The same radiolabeled probes were also subjected to chemical cleavage sequencing reactions (G + A). The reaction products in the lanes were: lanes 1 and 6, G + A reaction; lane 5, reactions without protein; lanes 2, 3, and 4, reactions with 4, 2, and 1  $\mu\text{g}$  of SREBP-1a, respectively. The protected regions (boxes) and their relative positions in the *hSHP* promoter are indicated as SRE1, SRE2, and 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 responsible 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-1c/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-1c/ADD1, but not for HA epitope only, indicating that HA-SREBP-1c/ADD1 formed a specific complex

#### FIG. 4. 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 <sup>32</sup>P-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 ~ -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 ~ -880 bp fragment is used as a negative control, and -2.2 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.

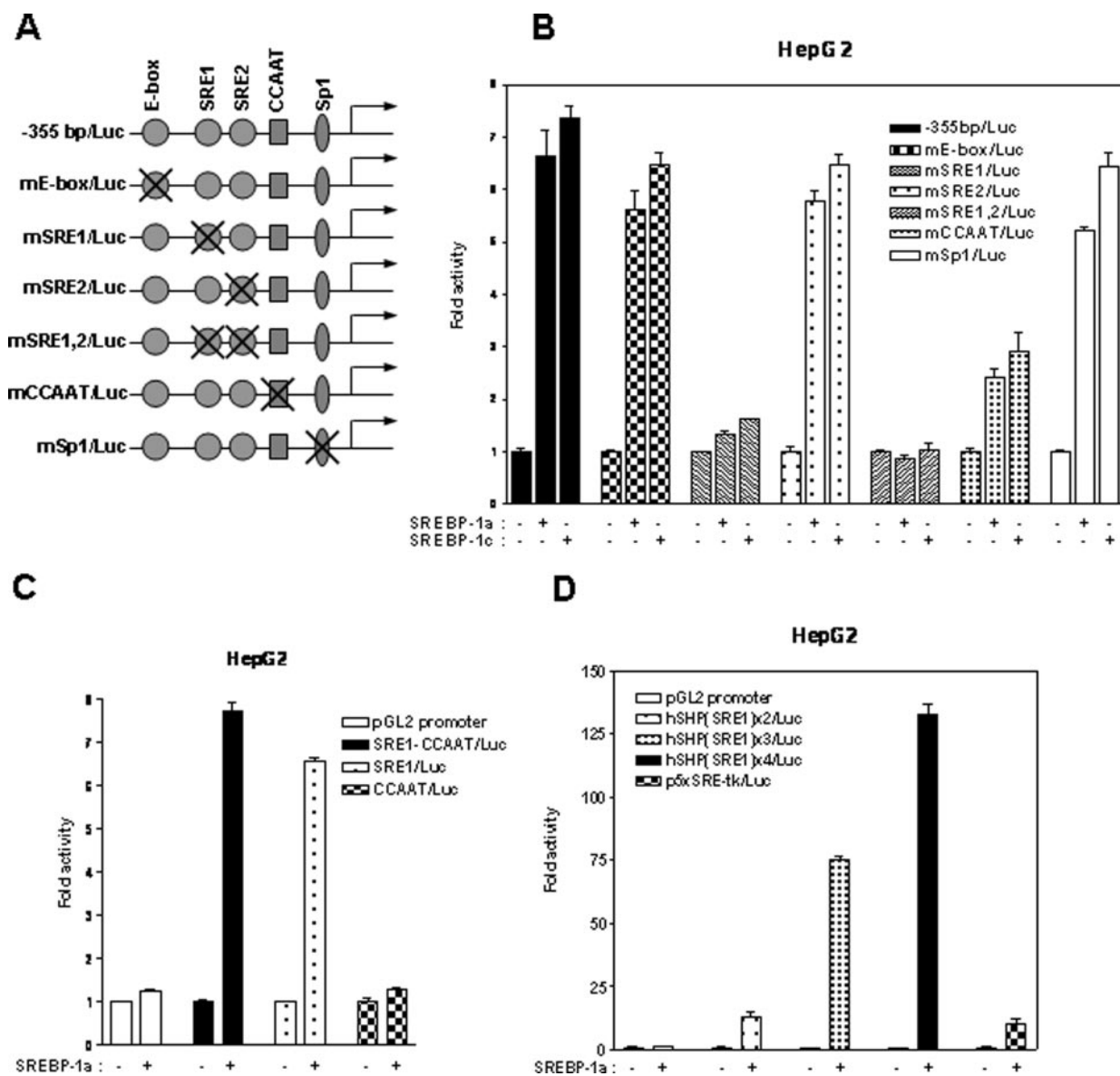
*The SRE1 (-186 to -195 bp) Plays a Critical Role in the Transactivation of the hSHP Promoter by SREBP-1*—To further evaluate the functional significance of the SREBP-1 binding sites (SRE1, SRE2, and E-box) in the *hSHP* promoter that we identified, site-directed mutagenesis was introduced to the -355-bp *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 SREBPs 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

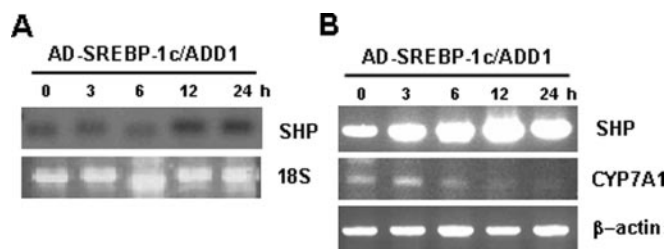
SREBP-1-dependent activation of the *hSHP* promoter. However, mutation of Sp1 binding site had little effect on the SREBP-1-mediated activation of the *hSHP* promoter.

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-1 is directly 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.



**FIG. 5. The SRE1 (–186 to –195 bp) plays a critical role in the transactivation of the *hSHP* promoter by SREBP-1.** A, schematic diagrams of wild-type and mutant *hSHP* promoter constructs from +28 bp to –355 bp as indicated. The putative CCAAT box and Sp1 binding site are indicated by squares and ovals, respectively. B and C, SRE1 is essential for the activation of the *hSHP* promoter by SREBP-1. HepG2 cells were cotransfected with 200 ng of wild type or mutant *hSHP* promoter constructs (B) and SRE1-CCAAT/Luc, SRE1/Luc, and CCAAT/Luc (C) together with 100 ng of SREBP-1 expression plasmids pCSA10, pCMVhSREBP-1c 436, or empty vectors, as indicated. D, SRE1 is sufficient to confer the SREBP-1 responsiveness. HepG2 cells were transfected with 100 ng of multiple copies of SRE1/Luc (two, three, or four copies), p5xSRE-tk or pGL2 promoter together with 100 ng of SREBP-1 expression plasmids pCSA10 or empty vector. Two days after transfection, cells were harvested for luciferase and  $\beta$ -galactosidase assays. The representative results were expressed as -fold activation (*n*-fold) over the value obtained with vector alone with the error bars as indicated.



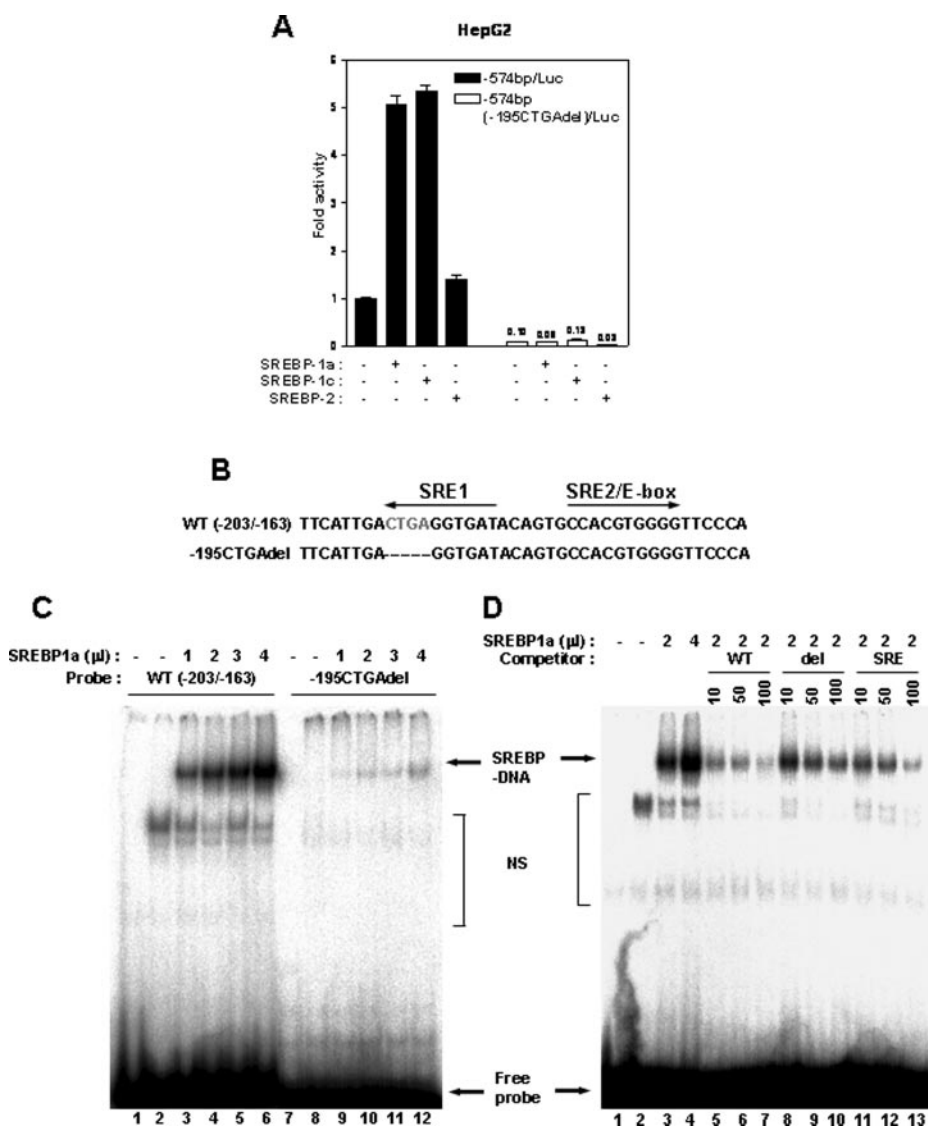
**FIG. 6. Adenovirus-mediated SREBP-1c/ADD1 overexpression stimulated the *hSHP* mRNA expression in the HepG2 cells.** HepG2 cells were infected with adenoviral vector expressing SREBP-1c/ADD1 (100 plaque-forming units/cells). Total RNA was isolated from cells at the indicated time after infection and analyzed by Northern blotting (A) and RT-PCR (B). A, an RNA blot containing 20  $\mu$ g of total RNA was hybridized with  $^{32}$ P-labeled probes for SHP. B, the mRNA levels of SHP, CYP7A1, and  $\beta$ -actin were analyzed by RT-PCR.

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 induced SHP mRNA expression, consistent with Northern blot analysis (Fig. 6B). In addition, 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



**FIG. 7. Naturally occurring mutation in SRE1 drastically reduced the *hSHP* promoter activity and DNA binding affinity by SREBP-1.** **A**, naturally occurring mutation (–195CTGAdel) in the *hSHP* promoter abolished the SREBP-1 dependent activation of the *hSHP* promoter. HepG2 cells were transfected with 200 ng of wild type (–574bp/Luc) or mutant type (–195CTGAdel/Luc) *hSHP* promoter reporter together with 100 ng of expression plasmids encoding SREBP-1a, SREBP-1c, SREBP-2, or empty vector. Approximately 40 h after transfection, the cells were harvested, and luciferase activity was measured and normalized against  $\beta$ -galactosidase activity. **B**, the oligonucleotide sequences of wild-type (nucleotides –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.  $^{32}$ P-labeled wild type (–203 to –163, lanes 1–6) and –195CTGAdel (lanes 7–12) probes were combined with 1 to 4  $\mu$ l 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  $\mu$ l 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  $^{32}$ P-labeled wild type SRE1 and *in vitro* translated SREBP-1a (2  $\mu$ l), as competitors. DNA-protein complexes were analyzed on 4% polyacrylamide gel and analyzed by autoradiography.



mRNA level, which results in the feedback repression of *CYP7A1* 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 transfected a reporter construct (–195CTGAdel/Luc) that harbors 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/–163, containing SRE1 and SRE2) and –195CTGAdel probes (Fig. 7B). SREBP-1a proteins were prepared by *in vitro* translation and incubated with the labeled probes. SREBP-1a binds to WT(–203/–163) probes, as shown by retarded complex compared with unprogrammed lysates. However, SREBP-1a did not show significant binding to the –195CTGAdel probes (Fig. 7C). This result suggests that no significant SREBP-1 binding to SRE2 occurs, supported by the result from Fig. 4B. These results indicate that deletion of 4 bp in SRE1 may result in the reduced SREBP-1 binding to SRE1 in the *hSHP* promoter. To confirm the reduced binding affinity of SREBP-1 to the mutated SRE1, we employed competition assay using unlabeled oligonucleotides of WT(–203/–163), –195CTGAdel, and LDLR-SRE as competitors. As shown in Fig. 7D, unlabeled oligonucleotides of WT (–203/–163) and LDLR-SRE (designated SRE) considerably perturbed SREBP-1a binding to WT(–203/–163) probes. However, increasing amounts of –195CTGAdel (designated del) oligonucleotides had a modest effect on the SREBP-1 binding to WT (–203/–163) probes. Taken together, these observations suggest that the mutation in the *hSHP* promoter, naturally occurring in SRE1, may result in the reduction of the *hSHP* gene transcriptional activation by SREBP-1.

## DISCUSSION

Previous reports have identified several nuclear receptors, such as steroidogenic factor-1, liver receptor homologue-1, HNF4 $\alpha$ , farnesoid X receptor, estrogen-related receptor- $\gamma$ , and LXR $\alpha$ , 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  $\alpha$  (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 carboxylase 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 translocation. 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 CYP7A gene pro-

motor activity in HepG2 cells (52). The inhibitory effect of insulin on the human CYP7A 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 CYP7A 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 CYP7A 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 CYP7A 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 HNF4 $\alpha$  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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