THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 272, No. 15, Issue of April 11, pp. 10295–10302, 1997
Printed in U.S.A.

Multiple Sequence Elements are Involved in the Transcriptional Regulation of the Human Squalene Synthase Gene*

(Received for publication, August 19, 1996, and in revised form, February 7, 1997)

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The expression of human squalene synthase (HSS) gene is transcriptionally regulated in HepG-2 cells, up to 10-fold, by variations in cellular cholesterol homeostasis. An earlier deletion analysis of the 5′-flanking region of the HSS gene demonstrated that most of the HSS promoter activity is detected within a 69-base pair sequence located between nucleotides −131 and −200. ADD1/SREBP-1c, a rat homologue of sterol regulatory element-binding protein (SREBP)-1c binds to sterol regulatory element (SRE)-1-like sequence (HSS-SRE-1) present in this region (Guan, G., Jiang, G., Koch, R. L. and Shechter, I. (1995) *J. Biol. Chem.* 270, 21958–21965). In our present study, we demonstrate that mutation of this HSS-SRE-1 element significantly reduced, but did not abolish, the response of HSS promoter to change in sterol concentration. Mutation scanning indicates that two additional DNA promoter sequences are involved in sterol-mediated regulation. The first sequence contains an inverted SRE-3 element (Inv-SRE-3) and the second contains an inverted Y-box (Inv-Y-box) sequence. A single mutation in any of these sequences reduced, but did not completely remove, the response to sterols. Combination mutation studies showed that the HSS promoter activity was abolished only when all three elements were mutated simultaneously. Co-expression of SRE-1- or SRE-2-binding proteins (SREBP-1 or SREBP-2) with HSS promoter-luciferase reporter resulted in a dramatic increase of HSS promoter activity. Gel mobility shift studies indicate differential binding of the SREBPs to regulatory sequences in the HSS promoter. These results indicate that the transcription of the HSS gene is regulated by multiple regulatory elements in the promoter.

Squalene synthase (farnesyl diphosphate-farnesyl diphosphate farnesyltransferase, EC 2.5.1.21) catalyzes the reductive head-to-head condensation of two molecules of farnesyl diphosphate (FPP)[1] to form squalene, the first specific intermediate in the cholesterol biosynthesis pathway. The expression of squalene synthase, as that of several other key enzymes in the pathway, such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, HMG-CoA synthase, and FPP synthase, is highly regulated by the cholesterol homeostasis in cells (1–4). It has previously been demonstrated that the expression of squalene synthase is regulated transcriptionally (5), but the mechanism for this regulation is unknown.

Low density lipoprotein receptor (LDLR) and HMG-CoA synthase are regulated by interaction between recently described transcriptional factors, sterol regulatory element-binding-proteins (SREBPs). These interact with sequences called sterol regulatory elements (SRE-1 and SRE-2) that exist in the promoters of the two genes (6–8). Two functionally related SREBPs, SREBP-1 and SREBP-2, have been purified from human cells and hamster cells, and the mechanism by which they regulate the expression of LDLR and HMG-CoA synthase has been studied extensively (9–11). Human SREBP-1 and SREBP-2 are 47% identical. At the NH_2-terminal region of each protein, there is a basic-helix-loop-helix leucine zipper (bHLH-Zip) structure that serves as a transcriptionally active domain. Next to the bHLH-Zip domain there are two membrane attachment domains. Nascent SREBP-1 and SREBP-2 are localized in the ER by these domains, and they are inactive in stimulating transcription. At lower concentrations of sterol in cells, an ER-associated, sterol-sensitive protease is activated and proteolytically activates the SREBPs by a cleavage at a site between the leucine zipper and the membrane attachment domains to release the bHLH-Zip domain (12). The active bHLH-Zip segment of SREBP-1 was shown to localize at the nucleus. This form of SREBP binds to SRE-1 in promoters of LDLR and HMG-CoA synthase, which results in the transcriptional activation of the two genes (13, 14). The sterol-regulated release of the active form of SREBP-2 from cell membranes was shown to require two sequential proteolytic cleavages of the transmembrane segment of the nascent protein (12). Human SREBP-1 and SREBP-2 were chromosomally mapped to 17p11.2 and 22q13, respectively (15). It was recently demonstrated that the expression of fatty acid synthase (16–19), an essential enzyme in fatty acid biosynthesis, and acetyl-coenzyme A carboxylase (20) are also transcriptionally activated by the binding of SREBP-1 to the sterol regulatory elements in their promoters. This observation provides evidence that directly links the metabolism of fatty acids and cholesterol and the importance of the SREBP family of transcription factors in the regulation of lipid metabolism. More recently, another sterol regulatory element, SRE-3, was defined in FPP synthase promoter (21). This SRE-3, with a limited identity to SRE-1, also interacts with SREBP-1 and thereby initiates the transcription of the gene. The same sequence exists in the promoter of HSS as well (22).

* This work was supported by Grants HL48540 and HL50628 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: FPP, farnesyl diphosphate; SREBP, sterol regulatory element-binding protein; HSS, human squalene synthase; SRE, sterol regulatory element; Inv-SRE-3, inverted SRE-3; Inv-Y-box, inverted Y-box; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; bHLH-Zip, basic-helix-loop-helix leucine zipper; ADD1, adipocyte determination- and differentiation-dependent factor 1; CMV, cytomegalovirus; pCMV-βGAL, pCMV-β-galactosidase; bp, base pair(s); kb, kilobase pair(s); ER, endoplasmic reticulum.

The difference in cellular function of two different SREBPs is presently unknown, since they share similar structure. The two proteins bind *in vitro* to SRE-1 with the same specificity. In cultured cells they stimulate SRE-1-containing promoters in an additive fashion (10). Both undergo proteolytic activation processing and presume to stimulate the expression of the sterol-regulated enzymes by the same mechanism.

Similar sterol-related transcription regulation may exist in rodents as well. The rat homologue of SREBP-1c has been independently cloned as adipocyte determination- and differentiation-dependent factor 1 (ADD1) (18). ADD1/SREBP-1c is expressed predominantly in white adipose tissue, brown adipose tissue, and liver, and its expression is induced at a very early stage of adipogenesis (18, 19). Unlike other bHLH-Zip transcription factors, ADD1/SREBP-1c has a unique dual DNA binding specificity to the E-box (CANNTG) and SRE-1 elements (23). This dual DNA binding specificity of ADD1/SREBP-1c homodimer is conferred by the presence of atypical tyrosine residue in the basic domain instead of an arginine residue, which is present in all other bHLH proteins (23). Recently, it has been demonstrated that ADD1/SREBP-1c has an important role in adipocyte differentiation as well as fatty acid metabolism. Retroviral expression of ADD1/SREBP-1c stimulates adipocyte differentiation and induces the expression of adipocyte-specific genes under strong differentiation conditions (19). Furthermore, in several cell lines, ADD1/SREBP-1c can induce expression of fatty acid synthase and lipoprotein lipase, two key enzymes involved in fatty acid metabolism (19). However, its detailed involvement in the regulation of cholesterol homeostasis is presently unclear.

In an earlier report, we demonstrated the importance of a 69-bp sequence in the promoter region of human squalene synthase (HSS) gene in its sterol-mediated transcriptional regulation. Gel retardation and DNase I footprinting verified that ADD1/SREBP-1c binds to the modified SRE-1 (HSS-SRE-1) element present within the 69-bp region. An 8-bp sequence identical to 8 out of the 10 bp of the SRE-1 element (SRE-1(8/10)) at nucleotide −101 did not show protection in the footprinting assay (22). These findings suggest that the transcription of HSS may be regulated similarly to the LDLR and HMG-CoA synthase, namely by the interaction between SRE-1 and SREBPs.

The present study focuses on identifying sequences of the *cis*-acting DNA elements in the HSS promoter involved in the sterol-mediated regulation of the HSS gene, and their regulatory relationship. We also describe the differential interaction between these elements and the *trans*-acting SREBPs.

EXPERIMENTAL PROCEDURES

Materials and General Methods—Standard molecular biology methods were used (24). DNA sequencing was performed using the Sequenase 7-diaza-dGTP DNA sequencing kit (Amersham Corp.). Restriction enzymes and modification enzymes were purchased from New England Biolabs. [α-^{32}P]dCTP was obtained from DuPont NEN. Lipofectin reagent was purchased from Life Technologies, Inc. Anti-NF-YA (a antibody against the A-subunit of human NF-Y protein) was purchased from Biodesign.

pHSS1kb-Luc, the fully functional HSS promoter-luciferase reporter gene, was constructed as described previously (22). ADD1–403 is an expression vector containing a cDNA fragment of the active transcriptional factor ADD1/SREBP-1c (amino acids 1–403) controlled by an SV40 promoter (pSVSPORT1). ADD1-DN, an inactive protein, is the same construct as ADD1–403 with an alanine point mutation replacing tyrosine in the bHLH motif of ADD1/SREBP-1c at residue 320 (19). pCMV-CSA10 contains the coding sequence from human SREBP-1a from amino acids 1–490 inserted into the pCMV5 expression vector as described previously (25). pCMV-CS2 is a similar expression plasmid that produces amino acids 1–481 of the human SREBP-2 protein. RSABB (SREBP-1 amino acids 234–490) and C2BB (SREBP-2 amino acids 236–401) are bacterial fusion proteins for SREBP-1 and SREBP-2, respectively, and are designed to express the indicated protein fragments as fusion proteins in the pRSET vector (invitrogen). The recombinant proteins were purified from *Escherichia coli* by nickel chelation chromatography.

Cell Culture and Transient Transfection—Human hepatoma HepG-2 cells were maintained in 35-mm plates in a minimum essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 1 mM glutamine, 1 mM pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2. Transient transfection of HepG-2 cells was conducted using the Lipofectin reagent transfection procedure according to the manufacturer's manual and previously described (22). Each transfection included 5 μg of HSS-promoter-luciferase reporter DNA and 4 μg of DNA of pCMV-β-galactosidase (pCMV-βGAL) expression vector for calculation of transfection efficiency. When indicated, various amounts of one of the following DNAs was also included in the transfection mixture: pCMV-CSA10, pCMV-CS2, ADD1–403, or ADD1-DN. On the day following transfection, the cells were treated for 24 h with either sterol(+) (1 μg/ml 25-OH cholesterol plus 10 μg/ml cholesterol in 10% lipid-depleted serum-containing medium) or sterol(−) (5 μg/ml lovastatin substituting the sterols in the same medium). The procedures for cell harvesting and the assays for luciferase activity and β-galactosidase activity have been described previously (22). Relative luciferase activity is expressed as the ratio of luciferase activity in relative light units to β-galactosidase activity (in A_{574}).

Oligonucleotide-directed Mutagenesis—The construct used for the generation of the replacement mutations is a pBluescript phagemid containing the 1-kb 5′-flanking region of HSS gene (pHSS1kb-BS, +73 to −934) (22). First, single-strand DNA was prepared from pHSS1kb-BS following the procedure recommended in the Sculptor *in vitro* mutagenesis kit manual (Amersham Corp.). Briefly, TG1 host cells were transformed with pHSS1kb-BS to obtain fresh transformants. On the following day, one colony was selected and grown in 2 × YT medium for 3 h. VCS-M13 helper phage (Stratagene) was then added to the culture and the incubation continued for an additional 4 h. The cells were then lysed and the single-strand DNA was purified by polyethylene glycol 8000 precipitation, phenol extraction, and finally by ethanol precipitation. Oligonucleotide-directed mutagenesis was performed as described previously (24). To overcome the high GC content in the mutation site, annealing and extension of mutation primers were performed at a relatively higher temperature. Therefore, the following procedure was employed. Single-strand DNA and mutation primer containing a unique restriction enzyme site (see Table I) were denatured at 94 °C for 5 min and slowly cooled to 25 °C to allow annealing. The annealed primer was then extended in a buffer containing 2 mM DTT, 6 mM $MgCl_2$, 0.2 mM each of dNTP, and 5 units of Vent™ DNA polymerase in a total volume of 100 μl. The reaction was kept at 0 °C for 5 min, incubated at room temperature for 10 min, 55 °C for 30 min, and finally, 70 °C for 2 h. Then, 400 units of T4 DNA ligase was added and the reaction incubated at 37 °C for 1 h to allow for extension and ligation. The resulting DNA was used to transform XL-1Blue competent cells. The mutated colonies were selected by colony hybridization and restriction enzyme digestion using the unique site introduced into mutation primers (Table I) and verified by DNA sequencing. The mutated plasmids were used for the subsequent subcloning of the mutated 1-kb HSS insert into *Hin*dIII and *Bam*HI sites of a pXP1 luciferase reporter gene vector as described previously for the native promoter (22). The resulting mutations in pXP1 constructs were confirmed again by DNA sequencing, and the plasmid DNAs were prepared for transient transfections. This procedure was utilized for the introduction of a single transversal replacement mutation in the pHSS1kb-Luc promoter. The various single replacement mutations that were introduced in the region −48 to −190 relative to the transcription starting site are shown in Fig. 1.

The primers described in Table I were used in the procedure described above to generate single replacement mutations. To prepare replacement mutations in multiple regions, oligonucleotides containing several of the mutated sequences were employed. Thus, the doubly mutated pHSSm12-Luc is a combination mutation of both the HSS-SRE-1 and the Inv-SRE-3 sequences (containing mutations present in pHSSm10-Luc and pHSSm1-Luc); the doubly mutated pHSSm13-Luc is a combination mutation of both the HSS-SRE-1 and the Inv-Y-box sequences (containing mutations present in pHSSm1-Luc and pHSSm6-Luc); the doubly mutated pHSSm23-Luc is a combination mutation of both the Inv-Y-box and the Inv-SRE-3 sequences (containing mutations present in pHSSm6-Luc and pHSSm10-Luc); and finally, the triply mutated pHSSm123-Luc is the combination mutation of all three sequence elements (containing mutations present in pHSSm6-Luc and pHSSm12-Luc).

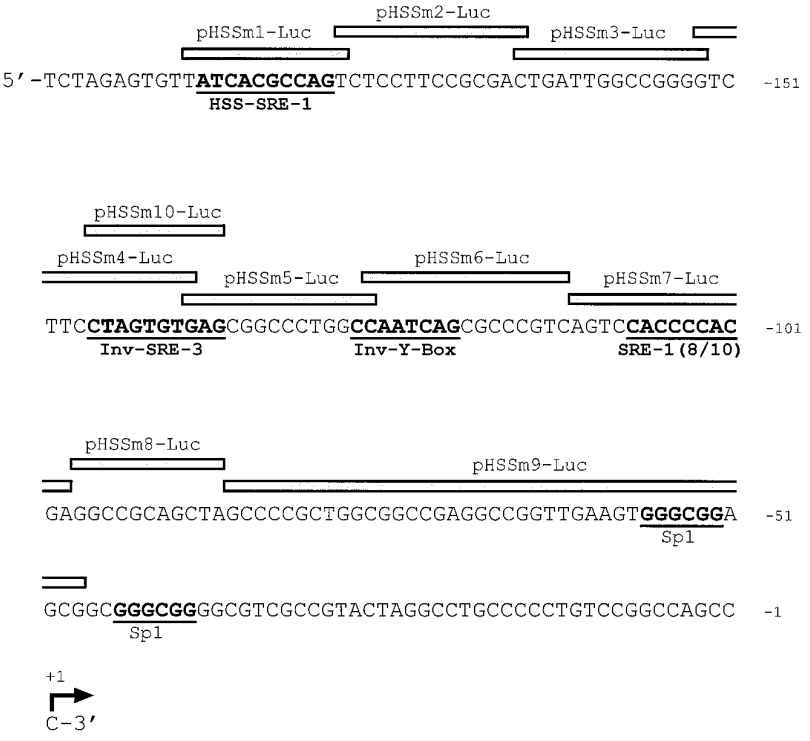

FIG. 1. **Mutation scanning scheme for regulatory elements in HSS promoter.** The sequence shows the 5′-flanking region of the HSS gene from the transcriptional initiation site up to the *Xba*I site at nucleotide −200 of the promoter. Transversal replacement mutations were introduced into the HSS-Luciferase chimeric construct, pHSS1kb-Luc, which contains the fully functional promoter. The oligonucleotide-directed mutagenesis method used for the preparation of the replacement mutations is described under "Experimental Procedures." The position of each mutated DNA section is represented by the *gray bar* above the sequence with the designated name of the construct containing the mutation. The names and sequences of potential regulatory elements are *underlined*. Exact replacement sequence is given in Table I.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from HepG-2 cells treated with either 5 μg/ml 25-OH cholesterol or 5 μg/ml lovastatin based on a protocol described originally by Dignam *et al.* (26). The probes used in the shift assays are synthetic oligonucleotides. The sequences of these oligonucleotides are shown below. Binding condition and labeling of probes have been described previously (22). In each assay, 0.2 ng of DNA from a ^{32}P-end-labeled probe (8×10^4 cpm) was incubated on ice for 30 min with one of the following proteins: 1.5 μg of RSABB, 0.1 μg of C2BB, or 4 μg of nuclear extract. In the competition assay, excess amounts (as indicated for different experiments) of non-labeled competing DNAs were added to the reaction just before the addition of the probe. Mobility immunosupershift assays were carried out under similar conditions except the nuclear extracts were incubated with anti-NF-YA (2 μg) on ice for 1 h prior to addition of the probe. After the binding reaction, the mixture was electrophoresed on a 4% polyacrylamide gel and the binding signal was detected by autoradiography. The following DNA probes were used in the above assays (sequence name indicated by underlining, mutations indicated in bold).

LDL-SRE-1: 5′-TAGAGTGTTATCACCCCACTCTCCTT-3′
3′-TCACAATAGTGGGGTGAGAGGAAGG-5′

HSS-SRE-1: 5′-TAGAGTGTTATCACGCCAGTCTCCTT-3′
3′-TCACAATAGTGCGGTCAGAGGAAGG-5′

HSS-SRE-1-mut: 5′-TAGAGTGTTATC**TAGGA**AGTCTCCTT-3′
3′-TCACAATAG**ATCCT**TCAGAGGAAGG-5′

FPP-SRE-3: 5′-GGGTCTTCCTCGTGTGAGCGGCCCT-3′
3′-CAGAAGGAGCACACTCGCCGGGAC-5′

Inv-SRE-3: 5′-GGGGTCTTCCTAGTGTGAGCGGCCCTG-3′
3′-CCAGAAGGATCACACTCGCCGGGACCG-5′

Inv-Y-box: 5′-CGGCCCTGGCCAATCAGCGCCCGTCAGTCCAC-3′
3′-GGGACCGGTTAGTCGCGGGCAGTCAGGTGGG-5′

SRE-1(8/10): 5′-GCCCGTCAGTCCACCCCACGAGGCCG-3′
3′-GGGCAGTCAGGTGGGGTGCTCCGGCGTC-5′

SEQUENCES 1–7

RESULTS

We have previously reported that the transcriptional regulation of the HSS gene is localized within a 69-bp sequence in HSS promoter. In addition, we have shown that ADD1/SREBP-1c binds to an HSS-SRE-1 element existing in this region (22). To verify the regulatory effect of this interaction between ADD1/SREBP-1c and HSS-SRE-1 in cultured cells, the effect of ADD1/SREBP-1c on HSS promoter activity was tested by expressing different ADD1/SREBP-1c constructs together with pHSS1kb-Luc in HepG-2 cells grown in the presence of sterol. A 3.6-fold increase in HSS promoter activity was observed in the presence of a transcriptionally active portion of ADD1/SREBP-1c protein (ADD1–403). However, its effect was much weaker than that observed in cells grown in the absence of sterols (Fig. 2). In addition, when a point mutation is introduced into the bHLH motif of ADD1/SREBP-1c (ADD1-DN), the protein loses most of its activation effect. These results indicate that the HSS-SRE-1 element in the HSS promoter may be functional.

To further confirm that HSS-SRE-1 is involved in sterol-dependent regulation of HSS transcription, a mutation replacing the HSS-SRE-1 element was introduced into pHSS1kb-Luc. The resulting effect of the mutant (pHSSm1-Luc) on HSS promoter activity was tested in a transient transfection system using HepG-2 cells both in the absence (sterol-depleted, sterol(−)) or presence (sterol (+)) of sterol. Unexpectedly, mutation of the HSS-SRE-1 sequence element still allowed 70% regulation of HSS promoter activity in response to sterol depletion (Fig. 3*A*). This result suggests that in addition to the HSS-SRE-1 element, other sequences in the promoter may also contribute to the sterol-related regulation of HSS transcription. Therefore, to identify DNA sequence elements that are involved in this regulation, a series of transversion mutations were made in which sequences within −48 to −190 promoter region were successively replaced. The effect of these mutations on the HSS promoter activity in both the presence and absence of sterols was investigated. As expected, in addition to the sequence mutated in pHSSm1-Luc, a DNA region that

TABLE I

Oligonucleotides used for the mutagenesis of HSS promoter

Displayed are oligonucleotides used as primers for the mutations of the HSS promoter. The most 3′ and 5′ nucleotide are shown, and their relative positions in the promoter are numbered. Mutated sequences are bold; the restriction enzyme sites introduced are underlined.

Mutation	Oligonucleotide sequence
pHSSm1	$^{-205}$G . . . TGT**ACGGTATGATCA**CTC . . . G^{-164} (*Bcl*I)
pHSSm2	$^{-196}$G . . . CAG**AGGAGGAGGTACCA**TGA . . . C^{-148} (*Kpn*I)
pHSSm3	$^{-184}$G . . . CGA**GGCTAGTCGACCTT**TCT . . . C^{-137} (*Sal*I)
pHSSm4	$^{-171}$C . . . GGG**TAAGGGTACCACGT**AGC . . . C^{-123} (*Kpn*I)
pHSSm5	$^{-157}$C . . . TGT**CCTGTCGGTACCAG**AAT . . . C^{-109} (*Kpn*I)
pHSSm6	$^{-144}$G . . . GGC**GTCGACCATGAATAG**AGT . . . C^{-95} (*Sal*I)
pHSSm7	$^{-130}$G . . . GTC**CTAGACAAGGTACC**GGC . . . C^{-81} (*Kpn*I)
pHSSm8	$^{-116}$C . . . CGA**TCGGTACCGGT**GCC . . . G^{-69} (*Kpn*I)
pHSSm9	$^{-116}$C . . . CTA**TAAGGCGATTGTCGACTTCAATTGATCCTGCCTGTTTCGT**GCG . . . C^{-18} (*Sal*I)
pHSSm10	$^{-160}$G . . . TTC**AGCTGCATTC**CGG . . . C^{-127}

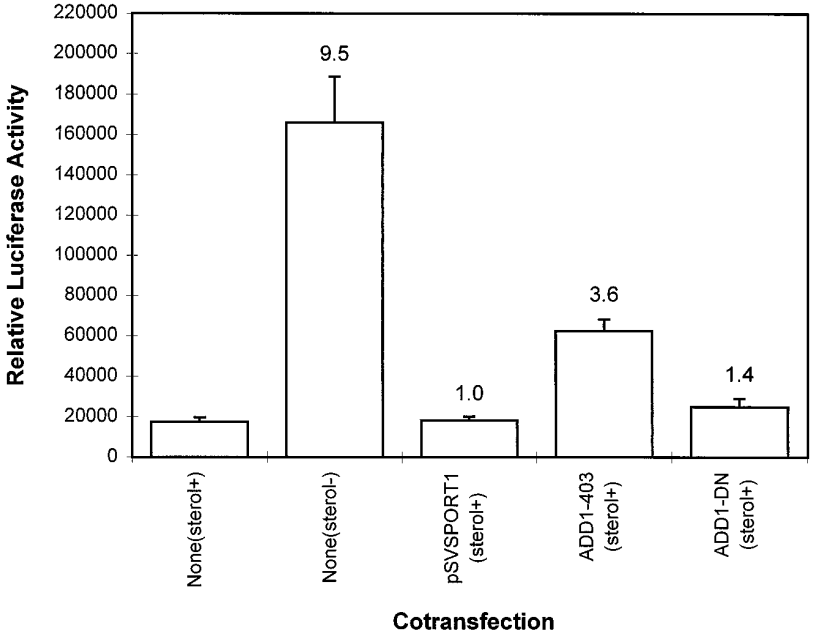

FIG. 2. **Activation of the HSS promoter by ADD1/SREBP-1c.** HepG-2 cells were cotransfected with 5 μg of pHSS1kb-Luc, 4 μg of pCMV-βGAL and 1 μg of one of the three different ADD1/SREBP-1c expression constructs. ADD1–403 contains a cDNA fragment encoding the active part of ADD1/SREBP-1c transcription factor, and ADD1-DN is an ADD1–403 expression vector with a mutation in its bHLH motif. pSVSPORT is the original vector used for the cloning of all ADD1/SREBP-1c fragments. The transfected cells were treated with 1 μg/ml 25-OH cholesterol and 10 μg/ml cholesterol for 24 h. Following this treatment the cells were harvested and cells' extracts were assayed for luciferase and β-galactosidase activities. The relative luciferase activity is expressed as the ratio of luciferase to β-galactosidase activities. Each bar represents the mean ± S.D. of six samples obtained from two separate experiments. The figure depicts the activation by both the functional ADD1/SREBP-1c (*ADD1–403*) and by sterol depletion (*sterol−*). The *numbers* at the top of each column show the -fold activation compared with sterol(+) treatment.

includes the sequences mutated in pHSSm4-Luc, pHSSm5-Luc, and pHSSm6-Luc (nucleotides −113 to −153) also showed significant involvement in the regulation of HSS promoter activity (Fig. 3*A*). The largest decrease in sterol-mediated regulation was observed for pHSSm6-Luc. The relative luciferase activity in cells transfected with pHSSm6-Luc was reduced to about 30% of that observed for pHSS1kb-Luc transfected cells. The mutations in pHSSm2-Luc, pHSSm3-Luc, pHSSm7-Luc, pHSSm8-Luc, and pHSSm9-Luc did not significantly affect the promoter activity or the sterol-mediated response.

Sequence analysis reveals that an SRE-3 element, previously reported in FPP synthase promoter (21), also exists in the HSS promoter (nucleotides −138 to −147) and overlaps the region covered by pHSSm4 and pHSSm5 (Fig. 1). This SRE-3 element has an inverted orientation in comparison to the SRE-3 in FPP synthase promoter. Therefore, it is named Inv-SRE-3. In addition, an A (at position −145) in the HSS element substitutes a C present in the FPP gene. An inverted perfect Y-box sequence CCAATCAG (Inv-Y-box, −121 to −132) is present 3′ to the latter sequence. Y-box element is reported to be involved in major histocompatibility complex class II gene regulation (27). The mutation in both pHSSm5-Luc and pHSSm6-Luc modified this Inv-Y-box. Mobility shift assays indicate that the Inv-Y-box sequence found in the HSS promoter does, in fact, bind the NF-Y transcription factor present in nuclear extracts of HepG-2 cells. When labeled Inv-Y-box probe is used, a clear mobility shift signal is observed (Fig. 4, *lanes 1* and *2*). This signal diminishes in the presence of a competing non-labeled Inv-Y-box DNA probe (*lanes 5–8*) but not with a competing HSS-SRE-1 probe (*lanes 9–12*). The final verification that the interacting transcription factor is indeed NF-Y comes from an immunological assay in which a specific anti-NF-YA interaction with the nuclear extract is shown to supershift the signal to a more retarded position (*lanes 3* and *4*).

The observation that none of these single region replacement mutations resulted in complete loss of HSS promoter activity made us consider the possibility that multiple elements in HSS promoter, and a few transcription proteins factors that bind to these elements, may act in concert for maximal sterol-mediated regulation of HSS transcription. To verify this possibility, five

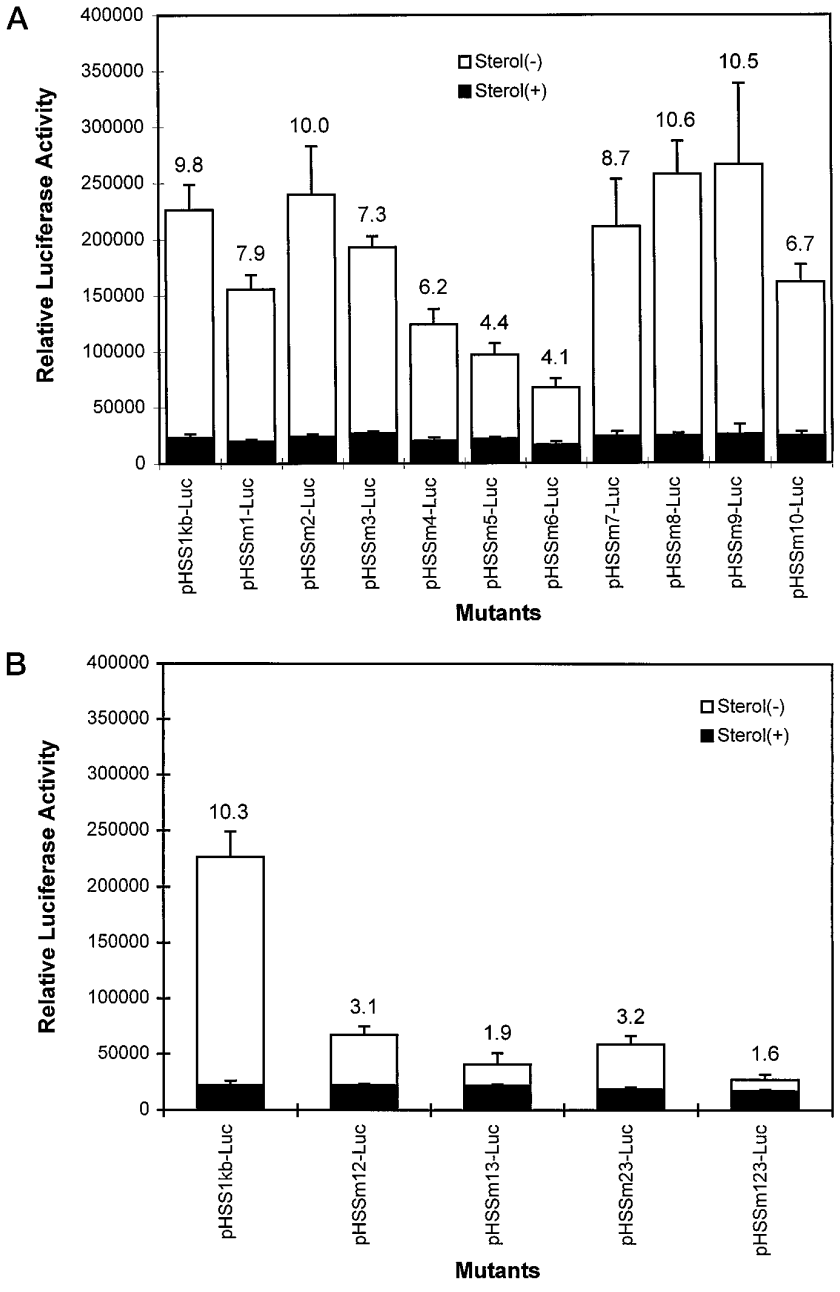

FIG. 3. **The effect of successive replacement mutations within nucleotides −48 to −190 region of the HSS promoter on its activity.** Five μg of DNA from each of the different mutant constructs together with 4 μg of pCMV-βGAL DNA were transfected into HepG-2 cells in 35-mm plates using Lipofectin reagent. After the transfection, the cells were treated with either 1 μg/ml 25-OH cholesterol plus 10 μg/ml cholesterol in a medium containing 10% lipid-depleted serum for 24 h (*Sterol(+)*; *solid bar*) or with 5 μg/ml lovastatin in the same medium for 24 h (*Sterol(−)*; *open bar*). The relative luciferase activity is expressed as the ratio of luciferase activity to the activity of β-galactosidase. pHSS1kb-Luc is a chimeric HSS-luciferase reporter construct that contains the native, fully functional promoter. *Panel A* depicts the relative luciferase activity of the single replacement mutations. The position of the single mutations is shown in Fig. 1. *Panel B* depicts the relative luciferase activities of multiple mutations in the promoter. The locations and sequences of the multiple mutations are described in the text. The results are expressed as the means ± S.D. of 9–12 samples from three to four independent experiments. The numbers at the top of each column show the -fold induction by sterol depletion above the sterol(+) treatment.

more mutants were prepared and their effects on HSS promoter activity were investigated in the transiently transfected HepG-2 cells. First, a mutation that exactly replaced the Inv-SRE-3 element was introduced into pHSS1kb-Luc (pHSSm10-Luc). Transient expression of this construct in cells demonstrated that this replacement mutation indeed decreases the response of the HSS promoter to sterol depletion (Fig. 3*A*). Mutations in multiple regions were then generated. Thus, a mutation of both the HSS-SRE-1 and the Inv-SRE-3 sequences (pHSSm12-Luc), a mutation of both the HSS-SRE-1 and the Inv-Y-box sequences (pHSSm13-Luc), a mutation of both the Inv-Y-box and the Inv-SRE-3 sequences (pHSSm23-Luc), and finally, a mutation of the above three elements (pHSSm123-Luc) were tested.

The effects of these combination mutations on HSS promoter activity are depicted in Fig. 3*B*. pHSSm12-Luc and pHSSm23-Luc reduced luciferase activity in sterol-depleted cells by more than 70%. pHSSm13-Luc reduced activity in these cells by about 90%. Finally, pHSSm123-Luc transfected into the cells failed almost completely to show sterol-mediated transcriptional regulation. These results indicate an additive regulatory effect of the three transcription elements in the HSS promoter.

The transcriptional protein factors that bind to SRE-1 in the LDLR and the HMG-CoA synthase promoters and SRE-3 in the FPP synthase promoter are SREBPs (8, 9, 21). To determine whether the known SREBPs play a similar role in the regulation of HSS gene transcription, we expressed two vector constructs that encode transcriptionally active forms of SREBP-1 and SREBP-2. A dose response in the activation of the pHSS1kb-Luc of the plasmids of three transcription factors encoding ADD1/SREBP-1c, SREBP-1, and SREBP-2 shows maximal activation at 0.5 μg of DNA/plate for SREBP-2 and approximately 1 μg of DNA/plate for the other two. However, the level of activation observed for the rat-derived ADD1/SREBP-1c is only about 40% of that observed for the human-derived SREBPs at 1 μg of DNA/plate (Fig. 5).

We then investigated the binding of the two human-derived SREBP protein factors to the regulatory *cis*-DNA sequence elements present in the HSS promoter. Fig. 6 shows the result of electrophoretic mobility shift assay using synthetic oligonu-

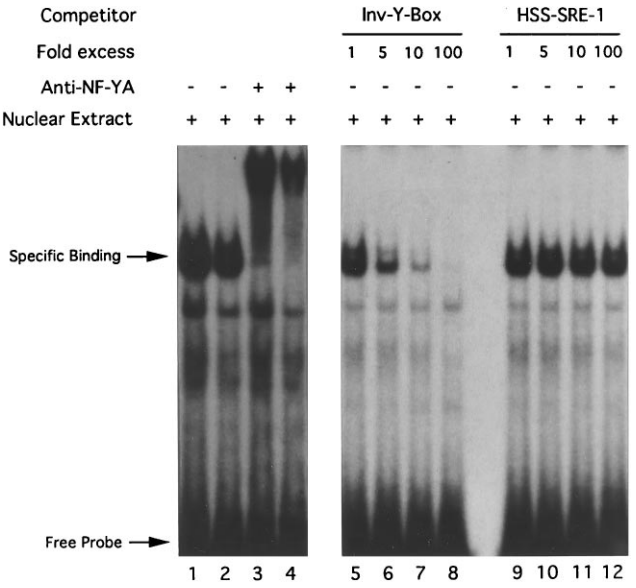

FIG. 4. **Evidence for the presence of NF-Y transcriptional factor in HepG-2 cells' nuclear extracts that bind to the Inv-Y-box sequence of the HSS promoter.** 0.2 ng of ^{32}P-end-labeled oligonucleotide probe (8×10^4 cpm), which contains the Inv-Y-box sequence, was incubated with nuclear extracts from HepG-2 cells grown in the presence of either 5 μg/ml lovastatin or 5 μg/ml 25-OH cholesterol. The mixture was electrophoresed on a 4% polyacrylamide gel. When shown, non-labeled competing oligonucleotides containing either the Inv-Y-box sequence or the HSS-SRE-1 sequence were included in the indicated amounts. For the immuno-supershift assays (*lanes 3* and *4*), an anti-NF-YA antibody was incubated on ice with the nuclear extracts for 1 h prior to the addition of the probe. In *lanes 1* and *3* nuclear extract from cells treated with 25-OH cholesterol was used. In all other experiments nuclear extract from cells treated with lovastatin was used. *Lanes 5–8*, competition assays using various amount of Inv-Y-box competitor; *lanes 9–12*. competition assays using various amount of HSS-SRE-1 competitor. The excess fold of competing DNA is indicated at the *top*.

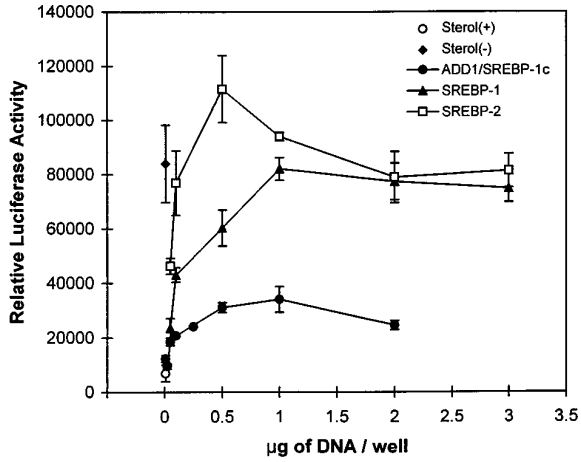

FIG. 5. **Differential activation of the HSS promoter by the transcription factors ADD1/SREBP-1c, SREBP-1, and SREBP-2.** HepG-2 cells, in 35-mm plates, were transfected with 5 μg of pHSS1kb DNA, 4 μg of pCMV-βGAL DNA, and various amounts of one of the following DNAs: ADD1–403, CSA10, or CS2. The cells were treated with 1 μg/ml 25-OH cholesterol and 10 μg/ml cholesterol in 10% lipid-depleted serum-containing medium for 24 h. Luciferase and β-galactosidase activities were measured as described under "Experimental Procedures." The relative luciferase activity is expressed as the ratio of luciferase to the β-galactosidase activities. The relative luciferase activity in non-transfected cells grown in the presence of 5 μg/ml lovastatin (*Sterol(−)*) or in the presence of 1 μg/ml 25-OH cholesterol and 10 μg/ml cholesterol (*Sterol(+)*) are also shown.

cleotides with various sequences of the regulatory elements. SREBP-1 is shown to bind strongly to oligonucleotides containing both the LDL-SRE-1 and the HSS-SRE-1 sequences. It also shows a weak, yet significant, binding to the FPP-SRE-3 sequence of the FPP synthase promoter as well as a weak binding to an oligonucleotide containing the SRE-1(8/10) element. It does not bind to the oligonucleotides containing either the Inv-SRE-3 or the Inv-Y-box sequences, which are present in the HSS promoter.

In comparison, SREBP-2 shows similar binding to SRE-1, HSS-SRE-1, and a much stronger binding signal to SRE-3 and the Inv-SRE-3 sequences. In addition, it also displays a strong binding signal to the oligonucleotide containing the SRE-1(8/10) sequence.

DISCUSSION

Previous studies identified a 69-bp sequence within the HSS promoter region that is important for the transcriptional regulation of the gene. We have previously hypothesized that an SRE-1-like element (HSS-SRE-1) at −180 to −189 is responsible for this regulation (22). In the present report, we demonstrate that this HSS-SRE-1 is not the only element regulating HSS promoter activity and gene transcription. A second sterol response element, Inv-SRE-3, located at nucleotides −138 to −147, originally described in an inverted orientation for FPP synthase promoter, is also shown to contribute to the activation of HSS promoter. Mutation of this Inv-SRE-3 sequence element reduced the activation of the HSS promoter in response to sterol depletion. This reduction in activation is similar to the effect caused by mutation of the HSS-SRE-1 element, suggesting that both elements are involved in the sterol-mediated regulation of HSS transcription. Mutations in both the HSS-SRE-1 and Inv-SRE-3 sequences decreased the sterol depletion response by more than 70%, indicating an additive effect of the two elements. Although these two elements are known to exist and function in other genes involved in cholesterol production, this study of HSS is the first report to show the presence of both transcriptional elements in a gene in this pathway.

The functional relationship of the regulatory sequences HSS-SRE-1 and Inv-SRE-3 in HSS promoter is unknown. The observation that 70% of the regulation remains, when either of these sequences remains unmodified, may indicate that they may also function independently and not necessarily regulate HSS transcription in concert. Our multiple sequences mutation studies demonstrate the importance of the sequence modified in pHSSm6-Luc for HSS promoter activity. We hypothesize that the Inv-Y-box located within this region may function with both the HSS-SRE-1 and Inv-SRE-3 elements and, thereby, enhance their transcriptional regulatory activity. It has been demonstrated that the transcriptional factor NF-Y, which binds to the Y-box sequence, is important in the regulation of FPP synthase and HMG-CoA synthase: two additional genes involved in the production of sterols (28). Evidence also shows that the activation of transcription of the LDLR gene requires a synergistic cooperation of SREBP and the common transcriptional factor Sp1 (25, 29). Therefore, the emerging theme suggests that there is a synergistic interaction between SREBPs and one of the more constitutively expressed transcriptional factors, such as Sp1 or NF-Y, for the regulation. Since no regulatory transcriptional sequence is identified immediately 3′ to the HSS-SRE-1, and since mutation of the Sp1 sequence at nucleotides −52 to −57 does not affect the regulation, the possibility is raised that both the HSS-SRE-1 and the Inv-SRE-3 elements function coordinatively with the Inv-Y-box located 3′ to both. Alternatively, we cannot eliminate the possibility of yet another, hitherto unknown, regulatory element that may exist within the DNA region −113 to −153, be sterol-respon-

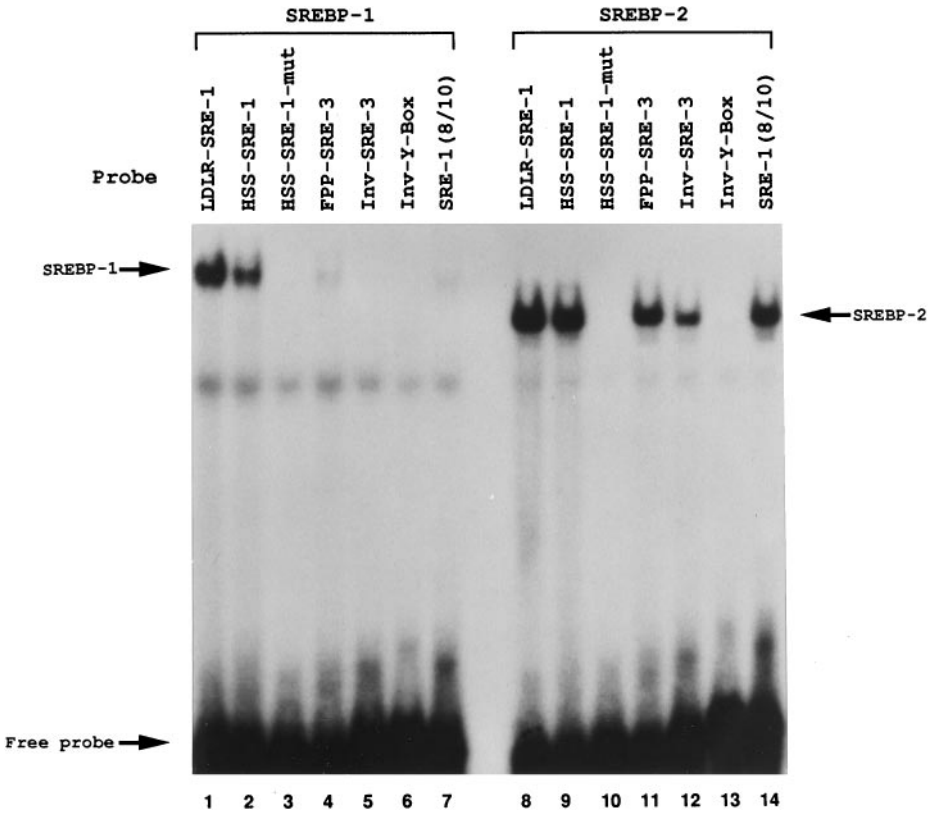

FIG. 6. **Binding of SREBP-1 and SREBP-2 to synthetic oligonucleotides containing sequences of different regulatory elements.** 32P-labeled oligonucleotide probes (0.2 ng of DNA, 8×10^4 cpm), which contain sequences of the LDLR-SRE-1, HSS-SRE-1, HSS-SRE-1-mut, FPP-SRE-3, Inv-SRE-3, Inv-Y-box, and SRE-1(8/10), were incubated with either 1.5 μg of RSABB or 0.1 μg of C2BB. At the end of the reaction, the proteins were electrophoresed on a 4% polyacrylamide gel. The specific sequences in the oligonucleotides used are indicated at the *top* of each lane. *Lanes 1–7*, gel mobility shift of the different regulatory elements by SREBP-1. *Lanes 8–14*, gel mobility shift of the same oligonucleotides using SREBP-2.

sive, and function in the regulation of HSS transcription.

It is of interest that the SRE-3 and the Y-box in the HSS promoter are both in inverted orientation compared with those found in FPP synthase. Their relative position in the promoter is reversed as well. In FPP synthase, the Y-box is 5′ to the SRE-3 sequence and they are separated by a 21-bp spacer. In HSS, the Inv-SRE-3 is 5′ to the Inv-Y-box with a 9-bp separation. In both genes, the two elements are arranged sequentially to allow orientation in the same direction. It is possible that this arrangement is required to enable the interaction between SREBP and NF-Y.

There are two adjacent perfect Sp1 binding sites (GC-box) within the promoter region of HSS. The observations that pHSSm9-Luc did not show repressed activity and that pHSSm123-Luc loses activity completely suggest that the most 5′ Sp1 sequence element may not be important in the regulation of HSS. The maximal activation of pHSS1kb-Luc by the two SREBPs as well as by the rat-derived ADD1/SREBP-1c was obtained at approximately 1 μg plasmid DNA/plate. However, this maximal effect is not the same for all three. A much lower activation was obtained by ADD1/SREBP-1c at this concentration. This activation could not be increased by addition of plasmid DNA. While the actual concentrations of activating factors within the cells is unknown, these results indicate that HSS may be relatively unresponsive to ADD1/SREBP-1c.

The Inv-Y-box sequence is shown to bind to the NF-Y transcription factor, which is present in nuclear extracts of HepG-2 cells (Fig. 4). No apparent difference was observed in the amount of this transcription factor present in cells treated with either lovastatin or 25-OH cholesterol (Fig. 4). Since the NF-Y factor is not tissue-specific, and the Y-box is reported to exist in nonsterol-related genes (27, 30, 31), it raises the possibility that NF-Y may interact with one of the SREBPs for the sterol-mediated regulation of HSS transcription similar to the interaction reported for the FPP gene (28).

The work described here, which provides evidence for differences in interaction with regulatory sequences between SREBP-1 and SREBP-2 (Fig. 6), may indicate distinct functional specificity for the two proteins. This corresponds with the report that the expression of SREBP-1 and SREBP-2 in the hamster liver is not coordinated during variations in sterol concentrations (32). Accordingly, it was proposed that SREBP-1 is responsible for basal transcription of LDLR and HMG-CoA synthase, whereas SREBP-2 is primarily responsible for the sterol-mediated inducible transcription of these genes in whole animal.

We have also observed differences between the two SREBPs in their binding to the identified transcription sequences in the gel-shift mobility assays (Fig. 6). Both the LDLR-SRE-1 and the HSS-SRE-1 sequences are shifted by the two SREBPs. However, while SREBP-2 shifts the oligonucleotide containing the SRE-3 sequence, the binding to SREBP-1 is very weak (for FPP-SRE-3) or completely absent (for Inv-SRE-3). In fact, there are distinct differences between the strength of the signal for FPP-SRE-3 and Inv-SRE-3. Clearly, the FPP-SRE-3 gene sequence generates a stronger signal in the gel mobility shift assays as compared with the HSS gene sequence. However, there is an indication that the study of binding of specific sequences to SREBPs by gel shift mobility assays may have limited significance in the understanding of the activation mechanism of the HSS promoter since the SRE-1(8/10) sequence showed differential mobility shift effects for the two SREBPs. It strongly binds to SREBP-2 and shows a very weak signal with SREBP-1. However, this 8-bp sequence by itself was reported to be non-functional for the LDLR (9) and its replacement mutation in the HSS promoter (pHSSm7-Luc) retained promoter activity and its response to sterols (Fig. 3).

The presence of multiple sterol regulatory elements in the HSS promoter may explain the high sterol-mediated transcriptional regulation of this gene that we have previously reported (5). The detailed interaction between these regulatory elements and the corresponding transcription factors is currently under investigation.

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Multiple Sequence Elements are Involved in the Transcriptional Regulation of the Human Squalene Synthase Gene

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J. Biol. Chem. 1997, 272:10295-10302.
doi: 10.1074/jbc.272.15.10295