Molecular Cloning and Functional Analysis of the Promoter of the Human Squalene Synthase Gene*

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We have cloned and characterized the 5'-flanking region of the gene encoding human squalene synthase. We report here the promoter activity of successively 5'-truncated sections of a 1.3 kilobase of this region by fusing it to the coding region of a luciferase reporter gene. DNA segments of 200 base pairs (bp) 5' to the transcription start site, as determined by primer extension analysis, show a strong promoter effect on the expression of the luciferase chimeric gene and a high response to the presence of sterols when transiently transfected into the human hepatoma cell line HepG2 or to the hamster-derived CHO-K1 cells. An approximately 50-fold induction of luciferase activity, in the absence of sterols, was observed in transiently transfected HepG2 cells for fusion constructs containing sections of 200, 459, and 934 bp of the putative human squalene synthase promoter. Loss of promoter activity and response to sterols was localized to a 69-bp section located 131 nucleotides 5' to the transcription start site. Sequence analysis of this region showed that it contained a sterol regulatory element 1 (SRE-1) previously identified in other sterol regulated genes (Smith, J. R., Osborne, T. F., Brown, M. S., Goldstein, J. L., and Gil, G. 1988). J. Biol. Chem. 263, 18480–18487) and two potential NF-1 binding sites. Additional CCAAT box, SRE-1 element, and two Sp1 sites were identified 3' to this section. Sequences within this 69-bp DNA, including the SRE-1 cis-acting element, show strong binding to the purified nuclear transcription factor ADD1 (Tonzonoz, P., Kim, J. B., Graves, R. A., and Spiegelman B. M. 1993) Mol. Cell Biol. 13, 4753–4759) by mobility shift assay and footprinting analyses.

Squalene synthase (farnesyl-diphosphatasefarnesyl-diphosphatasefarnesyltransferase, EC 2.5.1.21) is the first enzyme specific to the cholesterol biosynthetic pathway. The activity of rat hepatic squalene synthase is regulated by dietary cholesterol and by the dietary 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)1 reductase inhibitors, lovastatin, or fluvastatin (1, 2). The activity of human squalene synthase (HSS) and the level of its mRNA are regulated by sterols in the human hepatoma cell line HepG2.

Sterol-mediated regulation has been localized to a 10-base pair (bp) element in the 5'-flanking region of other sterol-regulated genes. This 10-bp sterol regulatory element 1 (SRE-1) mediates increased transcription of the genes encoding HMG-CoA synthase and the low density lipoprotein (LDL) receptor in sterol-depleted cells and its activity is inhibited by sterols (3, 4). Proteins that bind to the SRE-1 of the LDL receptor (SREBPs) were purified by DNA affinity chromatography from nuclear extracts of HeLa cells. A cDNA for SREBP-1 was isolated from adipocyte cDNA library (5). This cDNA, designated ADD1, activated transcription of a reporter gene containing an "E-box" sequence present in the promoter of fatty acid synthase in transfectted NIH 3T3 cells. Cloned SREBP cDNA contain two major classes of proteins, SREBP-1 (5) and SREBP-2 (8). Three different cDNAs for SREBP-1 were isolated, suggesting multiple forms of the mRNA and perhaps different proteins as well. The physiological significance of these subclasses is unclear (6). Different SREBP-1 proteins may have specific physiological roles because mRNAs for the various isoforms are differentially regulated by sterol depletion in HepG2 cells (8).

Proteolytic cleavage of the C-terminal membrane-associated domain of the nascent SREBP-1 (125 kDa) forms its nuclear form (68 kDa). This proteolytic maturation was proposed to be accomplished by a sterol-inhibited protease. The calpain inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) induced the mRNA for HMG-CoA synthase and was proposed to inhibit the degradation of the mature SREBP-1 (9).

In other sterol-regulated genes, the SRE-1 is not involved in sterol-mediated transcriptional regulation. Although the promoter region of farnesyl diphosphate synthase contains multiple forms of the SRE-1 element, these elements are not involved in the sterol-mediated transcriptional regulation (10). Similarly, the promoter of the hamster HMG-CoA reductase contains unique sites for sterol regulation. Red 25, a nuclear hamster liver protein, binds to this regulatory region but did not bind to the sterol regulatory regions of the LDL receptor and HMG-CoA synthase promoters (11).

In this report we characterize the 5' region of the HSS gene. The promoter activity and the sterol-mediated regulation of this DNA were assessed by fusing 5'-flanking DNA to a luciferase reporter gene and transfecting it into HepG2 cells and Chinese hamster ovary (CHO-K1) cells. A 69 bp DNA sequence confers transcriptional competence and sterol regulation. ADD1 binds to this 69 bp sequence in two places, one of which contains an SRE-1 element.
Cloning and Analysis of Promoter of HSS Gene

MATERIALS AND METHODS

Standard recombinant technology procedures were used (12). DNA sequencing was performed by the dideoxy chain-termination method (13) employing Sequenase 7-Diaza-dGTP DNA sequencing kit (U.S. Biochemical Corp.).

Probes for DNase I Footprinting and Electrophoretic Mobility Shift Assay—Double-stranded DNA probes used in DNase I footprinting were generated by restriction enzyme digestions of the 1-kb promoter of the HSS gene cloned into pBluescript. The plasmid was first digested with either BanI (noncoding strand) or NheI (coding strand). Then, the two fragments were end-labeled by incubating with dATP, dGTP, dTTP, [α-32P]dTCTP, and 5 units of Klenow fragment for 30 min at room temperature. After purification by ethanol precipitation, the fragments were digested with PstI (for NheI fragment) or HindIII (for BanI fragment). The 368-bp fragment from the NheI to the PstI digestion sites (nucleotides 246 to 613) and the 319-bp fragment from the HindIII digestion sites (nucleotides 73 to 246, plus 4 bp of the pBluescript vector sequence) were isolated on a 1% agarose gel and purified by Qiaex gel purification column (Qiagen).

Three probes were used in the electrophoretic mobility shift assay. One is a 156-bp DNA fragment generated by digesting the 1-kb promoter of human squalene synthase with NheI and BanI (nucleotides 91 to 246). The other two probes are synthetic oligonucleotides. One of them is a DNA containing a wild-type SRE-1 sequence existing in the HSS promoter (HSS-SRE-1), and the other has the same sequence except that four bases were mutated (nucleotides 183, 184, 186, and 187) and inserted into the SRE-1 site (HSS-SRE-1-mut). The complementary oligonucleotides were reannealed and the probes were 3' and 5' end-labeled by the Klenow fill-in reaction. Described above, the sequences of the two synthetic DNA probes are shown below with boldface letters indicating the mutated bases: HSS-SRE-1, 5'-TCACGGCATCTCCCT-3' and 3'-TCACAAATAGGCGGTACAGAGGAAGG-5'.

Cloning of the 5'-Flanking Region of the HSS Gene—A kH1 human placenta genomic library (Stratagene) was screened by plaque hybridization using the 21-nucleotide, 5'-TCACGGCATCTCCCT-3' as the hybridization probe. One of the four isolated clones was designated pHSS2kbBS, was sequenced. Location of sequencing primers is shown (see Fig. 2). These clones contain approximately 1.5-kb sequence from 5' to the transcription start site (see below), the first exon of the HSS gene including the 0.8-kb untranslated region, and approximately 400 bp of the first intron. A 2-kb restriction fragment of pHSS2kbBS was isolated and subcloned into an EcoRV site of pBluescript to give the correctly oriented insert (pHSs1kb-BS) in this construct for the further preparation of the luciferase expression plasmid in the pXP1 vector (repeatedly, inserted into the Smal site of pBluescript resulted in the reversed orientation of the insert). pHSs1kb-BS was digested with HindIII and BamHI, and the 1-kb DNA fragment was ligated to the same restriction sites in pXP1, a luciferase vector (S. K. Nordeen, University of Colorado, CO), to form pHs1kb-Luc. pHs1kb-BS was digested with PstI to remove the 5' 477-bp of the HS5 insert and ligated to form pHSS326-BS. A HindIII-BamHI fragment was removed from pHSS326-BS and ligated to same site in pXP1 vector to form pHSS326-Luc. pHSS273-BS was prepared from pHSs1kb-BS by an XbaI digestion followed by self-replication. The corresponding pHSS273-Luc plasmid was prepared from the linear plasmids by a HindIII-Sacl 273-bp DNA fragment from pHSS273-BS into an HindIII-Sacl digestion product of pXP1. The next chimeric gene pHSS204-BS was prepared by inserting a 204-bp HindIII-MsdI DNA fragment of pHSS235BS into an HindIII-Sacl-digested pXP1 vector. To prepare the smallest chimeric reporter gene, pHSS164-Luc, we first digested the pHSS1kb-BS DNA with a NheI. The 1-kb fragment was blunt-ended with Klenow and ligated to form pHSS164-BS containing a 1-kb HindIII insert (relative to pHSS1kb-BS) was prepared by adding the 5'nuclease and 3'P-dNTPs to 100-MU activity. After digestion by HindIII, the 1-kb DNA was isolated from a 0.8% agarose gel. The corresponding pHSS164-Luc reporter gene was prepared from the latter by inserting a HindIII-BamHI 1-kb DNA fragment from pHSS1kbRev-BS into an HindIII-BamHI-digested pXP1 vector. All luciferase reporter genes except pHSS1kbRev-Luc had the same 3' ends in a HindIII site of pXP1 and varying 5' ends. All plasmids were verified by sequencing.

Tissue Cultures and Transfection of Cells—HepG2 cells were grown in Dulbecco's modified Eagle's medium 121 (Flow Laboratories) supplemented with 10% fetal bovine serum (FBS), 1 mm pyruvate, 100 units/ml penicillin G, and 100 mg/ml streptomycin in 5% CO2 incubator at 37°C. HepG2 cells were grown on 60-mm tissue culture plates to about 30% confluency. The medium was changed 18 h prior to transfection. On day 0, cells were plated at 2 × 10^5 cells/plate and grown for 24 h. On day 1, cells were transfected with Lipofectamine (Life Technologies, Inc.). For transfection, 5 µg of test DNA (5 µg), pCMV-CMV-jGC (5 µg) (a nonregulated β-galactosidase expression vector) and Lipofectin (10 µl) in 1 ml total volume were added to each plate according to the manufacturer's protocol. The cells were transfected in serum-free media for 6 h and then allowed to recover in 5 ml of media containing FBS. On day 2, the medium was replaced with: (a) same medium as above containing 10% (v/v) FBS; (b) medium with 10% lipoprotein-depleted calf serum (LPDS) substituted for FBS (16); (c) LPDS media supplemented with 5 µg/ml lovastatin; (d) LPDS medium supplemented with 5 µg/ml 25-hydroxycholesterol; (e) LPDS medium supplemented with 15 µg/ml cholesterol; and (f) LPDS medium supplemented with 15 µg/ml cholesterol and 5 µg/ml 25-hydroxycholesterol. Cholesterol and 25-hydroxycholesterol were added in 5 µl of ethanol 5 h after incubation for the assay. The cells were harvested, and extracts were assayed for luciferase and β-galactosidase activities. CHO-K1 cells were transfected and treated the same as the HepG2 cells, except that Ham's F-12 medium (Life Technologies, Inc.) was substituted for the Dulbecco's modified Eagle's medium, and 5% of both FBS and LPDS were used.

Preparation of Cell Extracts and Enzyme Assays—All assays were in triplicate. Following transfection and treatment, the cells were washed twice with phosphate-buffered saline and harvested by scraping the cells into 0.4 ml of phosphate-buffered saline. Cells suspensions were sonicated, and debris was removed by centrifugation at 14,000 × g. Portions of the clear supernatant (20–30 µg of protein) were assayed for β-galactosidase activity using the β-galactosidase activity assay kit (Analytical Luminescence Laboratory). Relative luciferase activity was expressed as the ratio of luciferase activities (in RLU) to β-galactosidase activity (in RLU) in each well.

First Extension Analysis—Transcription start site(s) were determined by primer extension analysis. Poly(A) RNA was prepared from HepG2 cells grown in media-containing LPDS and lovastatin and served as a template for the reaction. The 20-nucleotide primer 5'-GGAACTGTTGCAGGCTTG-3' (called H10), located 164 nucleotides 3' to the AUG translation initiator, was 5'-32P-labeled (using T4 polynucleotide kinase (U. S. Biochemical Corp.) and [γ-32P]ATP) and hybridized to 4 cpm of poly(A) RNA were used for the extension reaction. After hybridization for 45 min at 60°C, the reaction was carried out at 45°C for 60 min with RNA reverse transcriptase (100 units) and 2.5 µm dNTPs in a 40-µl reaction volume. The radioactive extension product was analyzed on 5% sequencing gel using a known M13 single-stranded DNA sequencing product for size determination.

DNase I Footprinting—DNase I footprinting was performed using the Core Footprinting system kit (Promega). Briefly, 1 × 10^7 cpm of single end-labeled probes were incubated with 1 or 2 µg of ADD1 protein in binding buffer containing 25 mM Tris (pH 8.0), 50 mM KCl, 6.25 mM MgCl2, 0.5 mM EDTA, 10% glycerol, the appropriate dNTPs, and 2 µg of Poly(D)-d(C)-Poly(D)-d(C) on ice for 15 min in a total volume of 50 µl. Then, 50 µl of a solution containing 5 mM CaCl2 and 10 mM MgCl2 was added to the mixture. After incubating at room temperature for 1 min, 0.8 units of DNase I was added to each tube, and the incubation was continued at room temperature for an additional 5 minutes. After 5 minutes of incubation, 80 µl of a solution containing 200 µM NaCl, 30 mM EDTA, 1% SDS, and 100 µg/ml yeast RNA. The DNA samples were purified by a phenol/chloroform extraction and ethanol precipitation and resuspended in loading buffer (0.1 mM NaOH/formamide, 0.1% xylene cyanol, and 0.1% bromphenol blue). After denaturation at 75°C, the samples were electrophoresed on a 6% sequencing gel.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay was carried as described previously (17). Binding conditions were as described under "DNase I Footprinting," except that 4 × 10^7 cpm of
end-labeled probe was used in each reaction, and the final reaction volume was 20 μl. In competition assays, an excess amount of unlabeled HSS-SRE-1 DNA was added 5 min prior to addition of the labeled probe. Following binding, the mixture was electrophoresed on a nondenaturing 3% polyacrylamide gel at 24 mA for 3 h in a buffer containing 50 mM Tris, 100 mM glycine, and 2 mM EDTA. Detection of radiolabeled signals was by autoradiography.

RESULTS

Cloning and Analysis of Promoter of HSS Gene

We previously reported the transcriptional regulation of HSS in HepG2 cells (14). To determine the involvement of the 5' flanking region of the gene for HSS in the transcriptional regulation of this enzyme, we first isolated a genomic clone containing this region. The genomic clone (pHSS2kbBS) from a λfixII human placenta genomic library was isolated using HSS cDNA as a probe as described under "Materials and Methods." A partial digestion of the isolated clone with PstI restriction endonuclease followed by Southern analysis led to the isolation of a 2-kb fragment, which was subcloned into a PstI site of pBluescript KS vector and sequenced. This clone (designated pHSS2kbBS) contained at its 3' end the most 5' exon of the HSS gene, which included the 5' untranslated region of the HSS cDNA (14) as well as a portion of the first intron of the gene (data not shown). The 5' end sequence of this clone, down to and including the ATG translation start site, is shown in Fig. 1. This parental clone was used to generate all other constructs and fusion reporter plasmids. A schematic representation of the location of the primers used for the sequencing of this genomic clone in pBluescript is shown in Fig. 2.

Determination of HSS Transcription Initiation Sites

For the preparation of the various luciferase reporter constructs, we first determined the most 5' transcription initiation site. This site was determined by primer extension analysis using poly(A) RNA as a template. A 20-nucleotide primer (designated H10) located 164 nucleotides 3' to the AUG translation initiation site was 5'-32P-end labeled and used for the extension reactions. The two larger primer extension products detected were 262 and 261 nucleotides long. The transcription initiation site was, therefore, calculated to be at either one of the cytidines located 97 or 98 nucleotides 5' to the adenosine of the AUG initiation codon.
For size determination. The two largest 32P-labeled extension products regulated the luciferase reporter fusion genes in these two characteristics of the HSS promoter might display cell type specificity. We chose to examine both hepatic-derived and fibroblast cell lines in order to test the possibility that the transcriptional regulation characteristics of the HSS promoter might display cell type specificity and, therefore, might exhibit a different transcriptional regulation of the luciferase reporter fusion genes in these two cell lines. Thus, we introduced the various chimeric constructs by transfection into both the human hepatoma cell line HepG2 and the hamster-derived CHO-K1 fibroblast cell line. For normalization of the activity in the different transfected cells, we co-transfected the cells with pCMV-β-gal, a non-sterol-regulated β-galactosidase expression vector, and the results were calculated as the ratio of the luciferase to the β-galactosidase activities.

In HepG2 cells, the highest expression of luciferase, relative to the normalizing β-galactosidase activity, was obtained with the pHSS1kb-Luc fusion gene. This expression resulted when the transfected cells were treated with LPDS and lovastatin in the growth media. High luciferase expression was also observed in cells transfected with pHSS532-Luc and pHSS273-Luc. However, further reduction in the size of the synthase promoter resulted in a substantially lower expression of the luciferase activity. Accordingly, pHSS204-Luc and pHSS164-Luc showed relatively low reporter activity (Fig. 5). As expected, reversal of the orientation of the HSS promoter resulted in complete loss of luciferase activity in HepG2 cells (see pHSS1kbRev-Luc in Fig. 5). With slight, although reproducible, differences, a similar pattern was observed for the expression of these constructs in CHO cells (Fig. 5). For further studies of the regulation of the synthase promoter, we chose to use the HepG2 cells.

When transfected HepG2 cells were treated with cholesterol and 25-hydroxycholesterol in LPDS-containing medium (fully suppressed conditions) or with lovastatin in LPDS medium (fully induced conditions), a definite regulation was observed. A 47.6-fold increase in luciferase activity was observed between fully induced and fully suppressed conditions in extracts of pHSS1kb-Luc transfected cells treated for 24 h (see Fig. 4). Thus, the 934-bp 5‘-flanking region of the HSS promoter contained sequence elements that conferred sterol regulation. A similar regulatory response was observed for the pHSS532-Luc and pHSS273-Luc constructs. A 42.9- and a 51.3-fold increase between fully induced and fully suppressed conditions in HepG2 cells was observed for these two constructs, respectively (Fig. 4). Since in fully suppressed conditions the luciferase activity in all of the above three constructs is marginal, it is assumed that it may affect the accuracy of this ratio. Nonetheless, it reflects the pronounced effect sterols have on the regulation of these reporter constructs.

The relative synthase promoter activities in pHSS1kb-Luc, pHSS531-Luc, and pHSS276-Luc constructs under fully induced conditions are very similar. Repeatedly, pHSS1kb-Luc produced a somewhat higher luciferase response than the latter two (see Fig. 6). Since the amounts of the three chimeric DNA constructs as well as the amount of the DNA of the normalizing pCMV-β-gal were kept constant in all transfections, the molar DNA equivalents driving the three luciferase expressions are not equal and are higher in the cells containing the smaller constructs. But even with this consideration, the luciferase expression in the smaller vectors is still considerable. Insertion of the 1-kb 5‘-flanking sequence in a reverse orientation into the pX1 expression vector completely failed to induce luciferase activity. The resulting pHSS1kbRev-Luc construct showed background levels of luminescence either under fully suppressed or fully induced conditions (Fig. 4).

Truncation of the 5‘ HSS flanking sequences at the Mscl site to produce a 204 bp insert almost completely abolished its promoter activity. Only 6.8% of the luciferase activity remained, and a mere 3.4-fold increase in activity was observed for the pHSS204-Luc between fully induced and fully suppressed conditions. Similar results were also obtained for the smaller pHSS164-Luc construct.

**Expression of Luciferase Reporter Genes**

For the construction of the various 5‘ deletion sequences used for the preparation of the different chimeric luciferase reporter plasmids, we took advantage of the conveniently located Smal site 73 nucleotides 3‘ to the transcription start site. This site was located within the 5‘-untranslated region of the HSS cDNA and was used as the 3‘ end of the HSS DNA insert in the various luciferase fusion constructs. The different 5‘ ends of the various sequences were prepared using various endonuclease restriction sites in the promoter of the HSS gene. The schematic representation of the different chimeric genes containing various lengths of the HSS promoter is shown in Fig. 4. Using this methodology, we generated five fusion constructs with HSS promoter inserts, varying from approximately 1 kb (pHSS1kb-Luc) to the smallest fusion construct containing a 164-bp insert (pHSS-164-Luc). All of the five constructs included a 73-nucleotide section of the 5‘-untranslated region of the synthase gene. In addition, we prepared a fusion gene containing the longest HSS insert in a reverse orientation (pHSS1kbRev-Luc) to be used as a negative control.

The expression of the different chimeric HSS-luciferase constructs was tested in transiently transfected cells. We chose to examine both hepatic-derived and fibroblast cell lines in order to test the possibility that the transcriptional regulation characteristics of the HSS promoter might display cell type specificity and, therefore, might exhibit a different transcriptional regulation of the luciferase reporter fusion genes in these two cell lines.
Cholesterol is a much weaker regulator than 25-hydroxycholesterol. Suppression of luciferase activity with 15 μg/ml cholesterol supplementation to the LPDS-containing media resulted in a decrease of activity to 17.9% of fully induced

FIG. 6. Expression of luciferase reporter gene, containing varying lengths of the HSS promoter, in HepG2 cells maintained in different sterol-containing media. HepG2 cells were transfected with one of the chimeric genes pHSS1kb, −532, −273, −204, −164, and −1kbRev-Luc, containing varying lengths promoter fragments of the human squalene synthase fused to a luciferase reporter gene and with a pCMV-βGAL plasmid for the expression of nonregulated β-galactosidase activity. The cells were maintained in LPDS containing media in the presence of 25-hydroxycholesterol and 15 μg/ml cholesterol, according to the protocol described under “Materials and Methods.”

Relative luciferase activities were determined as a ratio of RLU to the activity of β-galactosidase and was normalized to 100 for pHSS1kb-Luc in both cell lines. The relative activity of pHSS1kb-Luc in HepG2 was 1.35 times higher than in CHO-K1 cells.

Cholesterol is a much weaker regulator than 25-hydroxycholesterol. Suppression of luciferase activity with 15 μg/ml cholesterol supplementation to the LPDS-containing media resulted in a decrease of activity to 17.9% of fully induced
Fig. 7. **DNase I footprinting of human squalene synthase promoter.** A, footprinting of coding strand of a DNA fragment corresponding to −91 to −459 of the gene. B, footprinting of noncoding strand of a probe (+73 to −246). Both probes were end-labeled with [α-32P]dCTP as described under "Experimental Procedures." The probe was incubated with or without ADD1 (1 or 2 μg as indicated), followed by DNase I digestion and analysis on a 6% sequencing gel. Lane 1 is the chemical cleavage of the probe at A + G residues, which served as a sequence marker. Lane 2 is the probe digested with DNase I in the absence of ADD1. Lanes 3 and 4 correspond to the DNase I-digested probe in the presence of 1 and 2 μg of ADD1. The sequence position of the probe is shown to the left of the gel. The protected region is indicated on the right side of the gel, and SRE-1 sequence is marked by a bracket.

**Fig. 8. Binding of ADD1 to human squalene synthase promoter.** A probe, corresponding to nucleotides −91 to −246 of the human squalene synthase promoter and containing the SRE-1 sequence element, was generated by the digestion of the HSS promoter with NheI and BanI. This probe was end-labeled with [α-32P]dCTP. The labeled probe was incubated with various amounts of ADD1, as indicated on the top of the gel, under the condition described under "Experimental Procedures." Separation of the free and bound probes was done by electrophoresis on a 4% native polyacrylamide gel. The retarded band, which corresponds to the ADD1-bound probe and the free probe band is indicated by arrows.

Interaction of ADD1 with Promoter Sequences of HSS

In order to identify ADD1-binding regions within the HSS promoter, we performed two assays, footprinting (18, 19) and gel mobility shift assays (17).

DNase I Footprinting—Fig. 7 shows that ADD1 protects DNase I digestion of promoter sequences. Both footprinting of the 368-nucleotide probe of the coding strand, including nucleotides −91 to −559 (A), and the 319-nucleotide probe of the noncoding strand, which includes nucleotides +73 to −246 (B), showed digestion protected sequences. A footprint in the coding probe extending from nucleotide −176 to −196 is visible and includes the SRE-1 element 5′-ATCACGCCAG-3′. A strong footprinting signal, extending from nucleotides −165 to −191 of the noncoding probe and including the SRE-1 element, was also detected. A second footprint was detected 3′ to the SRE-1-containing sequence. This footprint was especially visible for the noncoding strand and included nucleotides −131 to −149 containing the sequence 5′-TCTTAGTGTGAGCCGCC-3′ (see Fig. 78).

Electrophoretic Mobility Shift Assay—The binding of ADD1 to the HSS promoter sequences was also verified by the mobility shift assay. Fig. 8 is an ADD1 dose-response and shows increased mobility shift of a 32P-labeled probe. The 156-bp probe, containing the sequence of nucleotides −91 to −246, which includes the SRE-1 sequence element, was shorter than the probes used for the footprinting. However, from the retardation pattern it was clear that it contained sufficient sequence for the binding of ADD1. To further elucidate the role of the SRE-1 sequence element in the binding of the DNA probe to ADD1, a shorter probe was used in the electrophoretic mobility shift assay. The probe HSS-SRE-1 is a 28-bp DNA centered with the SRE-1 sequence element found in the promoter of HSS. Fig. 9 shows that this probe binds efficiently to ADD1 in conditions, whereas the addition of 5 μg/ml 25-hydroxycholesterol instead resulted in 3.5% remaining activity in cells transfected with pHSS1kb-Luc. A similar, albeit not identical ratio was observed for the cells grown in 10% FBS (see Fig. 6). Similar ratios were also observed in cells transfected with pHSS532-Luc and pHSS273-Luc. It is interesting to note that the shortest promoter in pHSS164-Luc failed completely to respond to the presence of cholesterol, but its relative activity did decrease somewhat in the presence of 25-hydroxycholesterol (Fig. 6).
Fig. 9. Binding of ADD1 to HSS-SRE-1 and HSS-SRE-1-mut. Two 32P-labeled DNA probes, which include either a wild-type SRE-1 sequence found in HSS promoter (HSS-SRE-1) or the same sequence except with four bases mutated at nucleotides 183, 184, 186, and 187 (HSS-SRE-1-mut) were incubated with ADD1 in a competition assay as described under “Materials and Methods.” Lanes 1-3 show the binding of 1 ng of labeled HSS-SRE-1 to increasing concentrations of ADD1. Lane 1, unbound HSS-SRE-1 probe; lanes 2 and 3, HSS-SRE-1 probe incubated with 0.5 and 1.0 μg of ADD1, respectively. Lanes 4-6 show competition assays of 1 ng of labeled HSS-SRE-1 with increasing concentrations of the same unlabeled probe. The molar excess of the unlabeled competing probe for each lane is as follows: lane 4, 100; lane 5, 1,000; lane 6, 10,000. Lanes 7-9 show the binding of 1 ng of labeled HSS-SRE-1-mut probe to increasing concentrations of ADD1. Lane 7, unbound HSS-SRE-1-mut probe; lanes 8 and 9, HSS-SRE-1-mut probe with 0.5 and 1.0 μg of ADD1, respectively. The binding products were analyzed on 4% native polyacrylamide gel.

The mobility shift assay and it is clearly competed for by the same unlabeled probe. Mutation of four bases at positions 183, 184, 186, and 187 in the HSS-SRE-1-mut probe, which were previously shown to be nonpermissive mutations for the binding of the SRE-1 element in the LDL receptor promoter to the SREBP-1 transcription protein (4, 6), totally abolished its binding to ADD1.

DISCUSSION

Squalene synthase was shown to be a highly regulated enzyme in mammals (1, 2, 14, 20). The sterol-mediated regulation of HSS in HepG2 cells was shown to be primarily transcriptional (14). Therefore, to determine whether the 5'-flanking region of the HSS gene contained sterol regulatory elements, we prepared chimeric fusion constructs containing various lengths of the 5'-flanking HSS sequences fused to a luciferase reporter gene. These fusion constructs were introduced to human hepatoma (HepG2) and nonhepatic CHO-K1 cell lines by transfection for transient expression.

Current studies show that the 5'-flanking region of the HSS gene confers a strong promoter activity in the fusion luciferase gene. The 1-kb, 352-bp, and 273-bp 5'-flanking sequences of the HSS gene have comparatively similar promoter function activity. However, deletion of 69 bp at the 5' end of the pHSS273-Luc construct almost completely diminished the promoter activity of the resulting pHSS204-Luc chimeric gene.

The transient expressions of pHSS1kb-Luc, pHSSXP532-Luc, and pHSS273-Luc are found to be highly responsive to the presence of sterols in both CHO (data not shown) and HepG2 cells (see Fig. 6). The sterol-mediated response of the luciferase activity in transiently transfected HepG2 and CHO cells exceeded the reported responses of similar CAT constructs containing the 5'-flanking regions of the genes for farnesyl diphosphate synthase in transiently transfected HepG2 cells (10); that of the LDL receptor and HMG-CoA synthase in transiently transfected CV-1 cells (4); and stably transfected CHO cells with HMG-CoA reductase or HMG-CoA synthase CAT fusion genes (21, 22).

Since there is no significant difference between pHSS532-Luc (containing HSS nucleotides +73 to 459) and pHSS273-Luc (with nucleotides +73 to 200) in their promoter activity and the sterol-mediated response, we can assume that the two distal CCAAT sequences at -210 and at -243 are marginally important, if at all, to the synthase promoter activity and response to sterols. The pHSS204-Luc chimeric gene (nucleotides +73 to -131) retained little of the promoter activity (Fig. 5). Thus, we have to assume that essential cis-acting elements, necessary for the transcription and the sterol-mediated regulation, are present in the 69-bp sequence between nucleotides -131 and -200. There are some recognizable potential cis-acting transcription elements located within this sequence. Starting at nucleotide -187, there is a 7:8 base pair match of the octamer cis-acting element Ser-1, which was shown to confer response to sterols in several genes involved in cholesterol homeostasis (23, 24). This cis-acting element is preceded by AT at its 5' end, which was also shown to be essential, in conjunction with the SRE-1 sequence, for sterol-mediated regulation in the promoter of the LDL receptor (4). At nucleotide -163, the sequence ATGGA is a reverse CCAAT box in the opposite strand. This sequence was shown to be recognized by a family of CTF/NF-1 cellular binding proteins that are known to be involved in transcription initiation (25-28). Finally, the Wood restriction site at nucleotide -128 used for the preparation of pHSS204-Luc disrupted a cis-acting TGGC-CATAT sequence, which is also a binding sequence for a CTF/NF-1 initiation site. Thus, elimination of any one of these three sequences may explain the loss of promoter activity and the response to sterols observed for pHSS204-Luc.

The TGGC-CATAT sequence is located 15 bp 5' to the octamer CACCCAC, an SRE-1 consensus sequence element at nucleotide -108. However, this element lacks the AT sequence at its 5' end, which is essential for the binding and activity of the SREBP-1 transcription factor. The pHSS164-Luc construct was designed to eliminate this SRE-1 element. Since most of the promoter activity was lost by the deletion of sequences 5' to this SRE-1 element, there is no way of assessing, based on the present data, its importance as a regulatory element of the promoter for HSS. An indication that this SRE-1 sequence may not be important for sterol-mediated regulation comes from the observation that this sequence does not show footprinting in the presence of ADD1. Future experiments, involving mutational substitution and single nucleotide mutagenesis should elucidate the involvement and the functional interrelationship of the different regulatory elements.

The 5'-flanking region of the HSS gene also contains two adjacent Sp1 transcription elements with the sequence GGGCCGGG at the core of each. These two elements are present at nucleotides -57 to -40 and are located approximately 50 nucleotides 3' to the consensus SRE-1 sequence. Sp1 sites were shown to be promoter-specific transcription activation sequences of RNA polymerase II (29). These two elements are present in pHSS164-Luc and apparently are not sufficient to confer by themselves any significant promoter activity.

The promoter for HSS does not contain a TATA box 5' to the transcription initiation site. In this respect, the HSS gene is similar to the HMG-CoA reductase gene and different than other highly regulated genes in the cholesterol biosynthetic pathway, such as the genes for HMG-CoA synthase, farnesyl diphosphate synthase, and the LDL receptor (10, 24, 30, 31).
There are multiple forms of the trans-acting sterol regulatory element binding protein (SREBP) factors, which bind to the SRE-1 elements of the promoters of the LDL receptor and the HMG-CoA synthase genes. The two major forms are the SREBP-1 (6) and SREBP-2 (7). However, the existence of different cDNAs for SREBP-1 may indicate the cellular existence of different forms of this protein (6). The processing and translocation of the SREBP-1 from the membrane to the nucleus was shown to be initiated by a proteolytic process. It was also reported that its further degradation by the calpain protease inhibitor ALLN increased the level of the mRNA for HMG-CoA synthase (9). In early experiments, we failed to observe an increase in the level of HSS mRNA when HepG2 cells were exposed to ALLN (data not shown). Having an HSS-luciferase reporter construct enabled the determination of the effect of ALLN in the transcription of this chimeric gene. Again, we failed to observe an increase in luminescence in response to ALLN in HepG2 cells treated with either 10% serum or LPDS. This observation is in agreement with the recent report that the inhibition of SREBP-1 degradation by ALLN treatment of HepG2 cells increased the mRNA levels for the LDL receptor (5, 32), may actually have a different mode of interaction with a promoter element that contains an SRE-1 sequence as shown by the gel mobility shift (Figs. 8 and 9) and footprinting (Fig. 7) assays brings up the interesting possibility that ADD1, which is considered to be the rat homologue for SREBP-1 (5, 32), may actually have a different mode of maturation. To test that, future studies will have to be done in cultured cells with transfected ADD1 precursor.

The footprinting assay indicates that purified ADD1 interacts with two sequences at the HSS promoter. One at nucleotides 165 to 191, which contains the SRE-1 element, and another at nucleotides 149 to 131. This later promoter DNA sequence does not contain an SRE-1 element nor does it contain the E-box motif CANNTG that was used in the digonucleotide screening procedure for the isolation of the ADD1 protein (5). Thus, if this sequence is involved in the binding of ADD1 and the regulation of its promoter activity, the essential DNA motif for its binding will have to be elucidated, in future studies, by mutagenesis methodology. However, the results available clearly indicate the binding of the DNA sequence containing the SRE-1 element to ADD1 (Figs. 8 and 9). As shown, mutation of this sequence in nucleotides that were previously shown to be essential for the binding of the LDL receptor promoter to SREBP-1 (4, 6) also abolished the binding of the HSS SRE-1 sequence to ADD1.