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SREBP-2 and NF-Y are involved in the transcriptional regulation of squalene epoxidase

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Abstract

The expression of squalene epoxidase (SE) is highly regulated transcriptionally by cholesterol. To elucidate these molecular mechanisms, we isolated the human and rat genomic clones. The entire human SE gene was about 24 kb long and organized into 11 exons with 10 introns. Unidirectional deletion analysis of the human 5′-flanking region indicated that the sequence between −264 and −230 bp conferred cholesterol sensitivity on a reporter gene. This region contained a potential copy of consensus sterol regulatory element (SRE) sequence (CCACGCAAC) previously identified in the promoter of cholesterogenic and its related genes. The transcriptional activation observed under overexpression of sterol regulatory element binding protein-2 (SREBP-2) supported the functional role of the SRE sequence. Another deletion analysis showed that the sequence −207 to −192 bp was also active and it contained nuclear factor Y (NF-Y) binding site. Both sites might play critical roles in sterol mediated regulation of SE gene. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Squalene epoxidase; Cholesterol; Transcriptional regulation; SREBP-2; SRE; NF-Y

Squalene epoxidase (SE) [squalene NADPH: oxygen oxidoreductase; 2,3-epoxidizing. EC 1.14.99.7.] catalyzes the conversion of squalene to 2,3-oxidosqualene, the first oxygenase reaction of cholesterol biosynthesis. Cholesterol is well known to be involved in pathogenesis of atherosclerosis. Since the post-mevalonate pathway of cholesterol biosynthesis has a branched pathway supplying prenyl moiety to such as p21 ras [1] and small GTP-binding proteins [2], the post-squalene pathway including squalene synthase, SE, and lanosterol synthase has evoked considerable interest as therapeutic targets for lowering low density lipoprotein (LDL) cholesterol, a risk factor in coronary heart disease.

SE appears to be an important rate-limiting enzyme in this pathway as it has an extremely low specific activity compared to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase or squalene synthase in HepG2 cells [3,4]. Recent DNA micro-array expression analysis also revealed that the SE and HMG-CoA reductase were the unique enzymes involved in cholesterol biosynthesis whose expression was simultaneously suppressed in human fibroblasts within 4 h after serum addition [5]. Previously, we have reported the isolation of rat and mouse SE cDNAs [6,7], the chromosomal mapping of the human SE gene (SQLE) [8] and the transcriptional regulation of SE in comparison with those of HMG-CoA reductase and LDL receptor in human cell lines [9]. Immunoblots and Northern blot analyses showed that sterol produced endogenously can highly regulate SE expression at transcriptional level.

Brown and Goldstein [10] proved that sterol regulatory element-binding protein (SREBP) upregulates both HMG-CoA synthase and LDL receptor gene via 10-bp sequence termed sterol regulatory element (SRE). Squalene synthase, farnesyl diphosphate synthase, fatty acid synthase, and acetyl coenzyme A carboxylase genes are also known to be regulated by SREBP [11–14]. SREBPs are transcription factors derived from precursor proteins in endoplasmic reticulum by two sequential proteolytic cleavages. As a mature form of transcription

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factor, the liberated NH_2-segment activates genes for controlling cholesterol synthesis and uptake after entering into the nucleus [15]. In addition to SREBPs, two transcription factors on cholesterol related genes are noticed. Transcriptional regulation of farnesyl diphosphate synthase and LDL receptor are reported to be dependent in part on a ubiquitous transcription factor, nucleal factor Y (NF-Y) and Sp-1, respectively [16,17].

To determine the functional promoter region and the regulatory mechanism of SE gene, we performed the molecular cloning of human and rat SE genomic DNAs. In this report genomic organization of human SE and the character of its promoter on transcriptional regulation are described.

Materials and methods

Materials. [α-^{32}P]dCTP (3000 Ci/mmol) was purchased from DuPont NEN. Fetal bovine serum (FBS) was from Bioserum (Canterbury, Australia). Lipoprotein-deficient fetal bovine serum (LPDS) ($d > 1.215$ g/ml) was prepared by ultracentrifugation as described by Goldstein et al. [18].

Cell culture medium was purchased from Nissui Pharmaceutical (Tokyo, Japan). SREBP-2 expression vector (pCMVSREBP-2), which encodes amino acids 1–481 of SREBP-2, was kindly provided from Dr. Osborne, T.F. of University of California, Irvine.

Isolation of human and rat squalene epoxidase genomic clones. Human and rat genomic phage library (Clonetech) constructed in the EMBL-3 SP6/T7 vector were screened by hybridization with a human SE cDNA probe (DDBJ/EMBL/GenBank Accession No. D78130) and rat SE cDNA probe (DDBJ/EMBL/GenBank Accession No. D37920) labeled with [α-^{32}P]dCTP.

The solution containing λ phage clones (5×10^4/each 10 cm plate) was mixed with plating *E. Coli* (K802). After the infected bacteria were incubated for 20 min at 37 °C, molten top agarose was added and poured onto agar plates. Then the plates containing the infected bacteria were incubated at 37 °C for 6 h and phage plaques were transferred to cellulose nitrate membrane (Tokyo Roshi, Japan). The membranes were immersed in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 1 min, then in neutralizing solution (1.5 M NaCl, 0.5 M Tris–HCl [pH 7.4]) for 5 min. The membranes were rinsed in 2× SSC, and baked at 80 °C for 2 h. Probes were made with [α-^{32}P]dCTP by random primer method using a Mega Prime Kit (Amersham). Hybridization was carried out at 65 °C in the hybridization solution (5 mM EDTA, 1 × Denhalt, 33 μg/ml salmon sperm DNA, and 2×10^7 cpm SE cDNA probes) over night. The membranes were washed with 3× SSC solution three times at 65 °C for 20 min. After washing, the membranes were exposed to X-ray film (Fuji RX).

Top agarose near positive clone was cut off and suspended in the SM solution. We repeated this procedure three times, and isolated positive clones. Positive clones were collected by plate lysate method and phage DNA was obtained using Lambda Kit (QIAGEN). The human SE genomic clones were digested with the restriction enzymes, and electrophoresed in a 1% agarose gel. Then the digested clones were transferred to a nytran membrane (Schleicher and Schuell, Dassel, Germany) with 3.0 M NaCl, 0.3 M sodium citrate (20× SSC) as the transfer buffer. The membrane was baked at 80 °C for 2 h and Southern blot hybridization analysis was performed with many parts of human and rat SE cDNA probes. Digested fragments were subcloned into pUC 19. DNA was sequenced by the dideoxynucleotide chain termination method (373A Sequencer, Applied-Bio-System).

Construction of human SE promotor-luciferase fusion gene. For unidirectional deletion analysis, we create human SE promoter fragments 741/+49, −298/+49, −264/+49, −230/+49, and −93/+49, which were ligated upstream of the firefly luciferase gene in the promoterless pGVB vector (Tokyo-Ink, Tokyo) in the sense orientation (pGVHSE −741, pGVHSE −298, pGVHSE −264, pGVHSE −230, and pGVHSE −93, respectively).

Deletion fragments −264/−139, −264/−192, and −264/−207 were ligated to silent β Interferon (β-IFN) promoter (−55/+19) and inserted

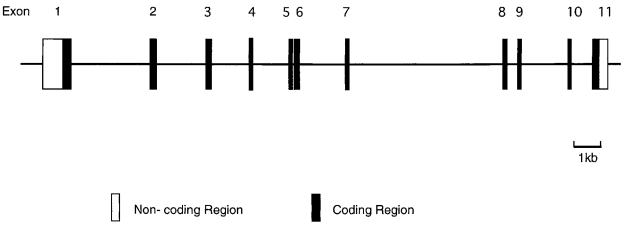

Fig. 1. Organization of the human SE gene. The map shows the positions of exons and introns. The exons are represented by open (5′ and 3′ untranslated regions) and closed (translated regions) boxes; introns and flanking sequences are shown as thin lines.

Table 1
Exon–intron organization of human SE gene

Exon no.	Exon size (bp)	Sequence of exon–intron junction		Intron size (kb)	Amino acid interrupted
		5′ Splice down	3′splice down		
1	1169	AGGAGG	gtatgt------aaacag CGCAGA	3.5	Arg 97/Arg 98
2	253	TTGGAG	gtaggt------ttgcag ATACAG	2.1	Asp 182
3	181	GCCCAA	gttaga------ccccag TGCAAA	1.7	Asn 242
4	97	ATCAAG	gtgaga------ttatag GAACTC	1.5	Lys 274/Glu 275
5	114	ATGAAG	gtactg------ttttag AATGCA	0.087	Lys 312/Asn 313
6	172	TACCTG	gtaaga------ttgcag ATCACC	2.2	Asp 370
7	96	AACGAG	gtatta------tcatag GTGTTC	6.4	Gly 402
8	143	TTCGAG	gtaaga------ttttag GCCAAA	0.4	Glu 449/Ala 450
9	97	CAGATG	gtgggt------ttttag ATTCCC	2.1	Asp 482
10	88	TTCTGT	gtaagt------ttacag ATTGTC	0.9	Val 511
11	529				

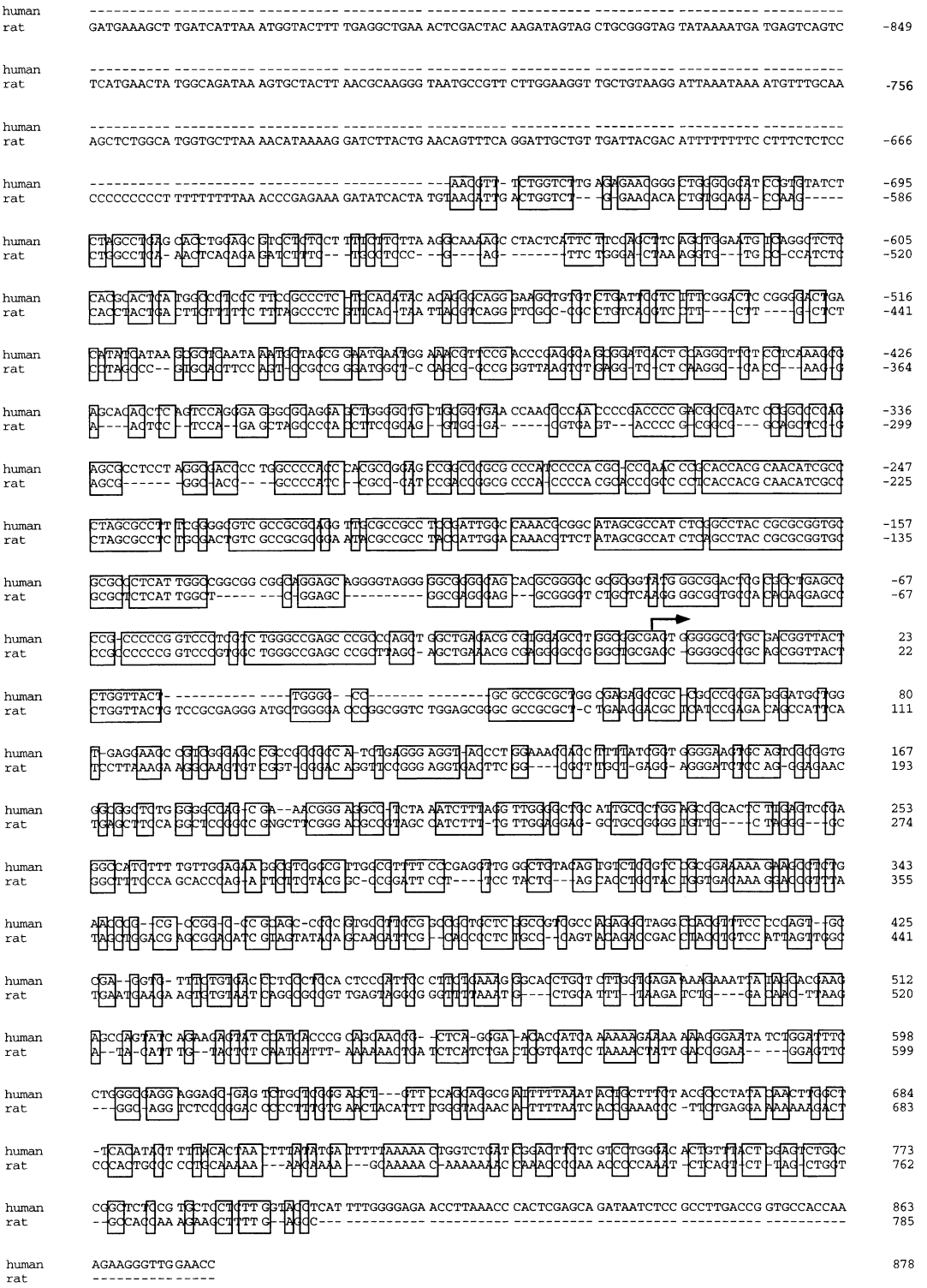

Fig. 2. Nucleotide sequence of the human and rat SE promoters. The humans and rat 5′ flanking sequences of initiation codon are aligned. Conserved nucleotides between humans and rat are boxed. The number at the right represents the positions relative to the initiation site of transcription (+1), and arrow shows the start of numbering.

to pGVB vector which designated as pGVHSE −264/−139, pGVHSE −264/−192, and pGVHSE −264/−207, respectively.

Cell culture, cell transfection, induction, and luciferase assay. HeLa cells were grown in Dulbecco's modified essential medium (DMEM) containing 10% FBS (FBS medium). Cells (5.0×10^5) were seeded in 35 mm dishes containing 2 ml of FBS medium. After HeLa cells reached confluent monolayer in 24 h, transfection was performed by calcium phosphate method with 10 μg of plasmid, also using 10% dimethylsulfoxide (DMSO). The β-galactosidase reporter plasmid (pCMV β 5 μg) was cotransfected with the luciferase fusion constructs

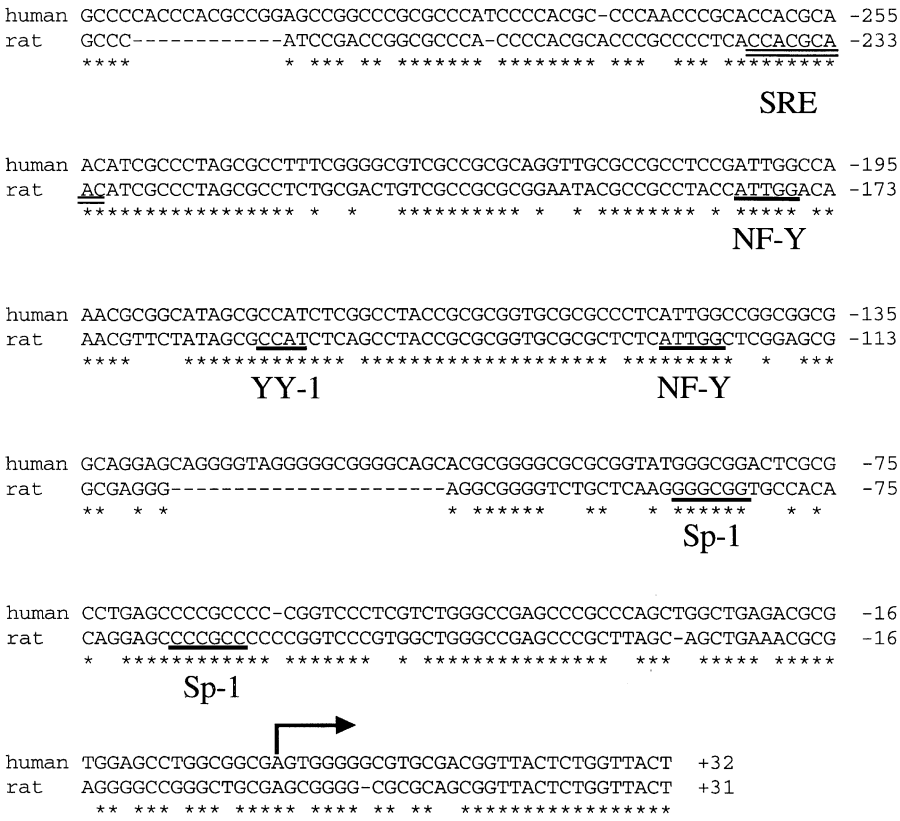

Fig. 3. Conserved region of human and rat SE promoters. Well-conserved regions of human and rat sequences are shown from Fig. 2. The number at the right represents the positions relative to the initiation site of transcription (+1). Conserved bases between humans and rat are identified by star (*) under each nucleotide. The putative SRE is doubleunderlined. The putative NF-Y, YY-1, and Sp-1 binding sites are underlined, respectively.

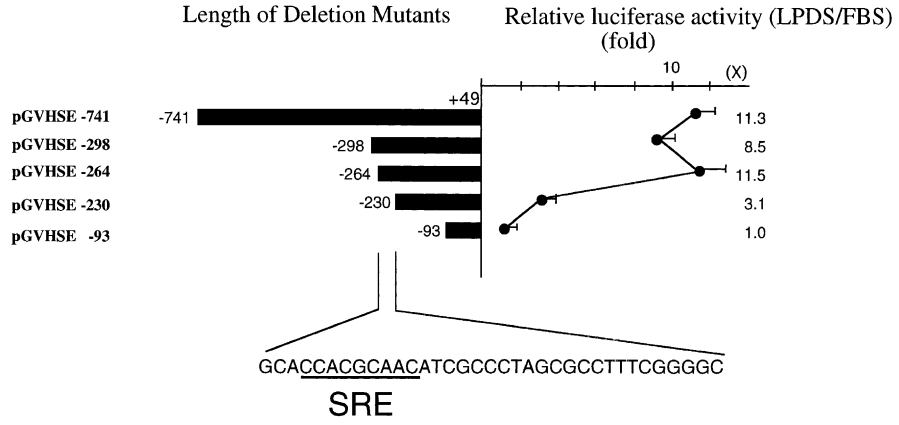

Fig. 4. Luciferase assay of human SE promoter deletion mutants [1]. Schematic length of various human SE promoter and luciferase fusion constructs are shown. The numbers to the left of each construct represent the positions relative to the initiation site of transcription (+1). Luciferase assays were performed and relative activity (Cultured in LPDS medium versus FBS medium) for the constructs is shown in a line graph. The nucleotides below diagrams indicate the sequence between −264 to −230 bp of human SE promoter. Data are presented as means ± SD of three independent experiments.

for monitoring transfection efficiency. The cells were washed with phosphate-buffered saline twice, and the medium was replaced with 3 ml of FBS medium or DMEM medium containing 10% LPDS (LPDS medium) and cultured for 48 h. After that, cells were harvested and luciferase activity was determined with a luminophotometer (Lumat LB 9501, Berthold, Germany) after normalization on the base of β-galactosidase activity with Galacto-Light chemiluminescent reporter assay (Tropix, Bedford, MA, USA).

SREBP-2 over expression analysis. SREBP-2 expression vector (pCMVSREBP-2, 2 μg) was cotransfected to HeLa cells with pGVHSE −264 and pGVHSE −230. Cells were cultured with FBS medium and luciferase activity was measured as previously described.

Results and discussion

Exon–intron organization and nucleotide sequence of the human SE gene promoter

A human genomic phage library was screened with the human SE cDNA probes. Analysis of 2×10^6 clones by plaque hybridization yielded 15 positive clones. Positive clones were digested with the restriction enzymes and digested fragments were subcloned into pUC 19 and sequenced as described in Materials and methods.

The nucleotide sequences of all the exons, part of the introns, and flanking regions were determined. The exon–intron boundaries were deduced by comparison with the human SE cDNA sequence. The entire human SE gene is contained in about 24 kb of genomic DNA, and the human SE gene consists of 11 exons and 10 introns (Fig. 1). The exon–intron boundaries sequence is shown in Table 1. Each displayed canonical GT/AG donor/acceptor for RNA splicing. Exon I and II contains a putative membrane binding domain and an FAD binding domain, respectively. The identical undecapeptide in common with mammalian and yeast SE is located in exon VIII [6].

We also determined 5′-flanking sequences of human and rat SE genes (Fig. 2). The transcription initiation site of exon 1 was comfirmed by primer extension analysis (data not shown). The A was defined as +1 in the numbering of the nucleotide sequence. The well conserved region is noticed at the location around −300 to +30 bp (Fig. 2). There are candidate binding sequences of the known transcription factors identical to Sp-1 (−87 to −82 and −66 to −61), NF-Y (−202 to −198 and −148 to −144), and Ying Yang 1 (YY-1) (−180 to −177). So this conserved region might be important for the regulation of this gene (Fig. 3).

Functional characterization of sterol responsive region in the 5′ flanking sequence of the human SE gene

To determine the regulatory element by sterol of human SE promoter, we prepared the five different chimeric luciferase plasmids which contain unidirectional deletion sequence of human SE 5′ flanking region. Fusion constructs are transfected into HeLa cells. The higher induction was observed with pGVHSE −741, pGVHSE −298, and pGVHSE −264 when HeLa cells were incubated in LPDS medium, comparing with FBS medium, after being normalized by β-galactosidase activity. However, further reduction of SE promoter size resulted in no significant difference in luciferase activities between cells incubated in LPDS medium or FBS medium (Fig. 4). These results suggest that the transcription of SE gene is regulated by sterols through the promoter sequence located in −264 to −230 bp. The promoter consisting of 35 bp is active and sterol sensitive.

We examined whether the sterol mediated regulation of human SE promoter relates to SREBP. When SREBP-2 expression vector (pCMVSREBP-2) was cotransfected with pGVHSE −264, luciferase activity was stimulated 15.9-fold even in FBS medium. However, induction was not observed when pCMVSREBP-2 was cotransfected with pGVHSE −230 (Fig. 5). These results strongly suggest that human SE gene was transcriptionally regulated through SREBP-2 by intracellular levels in vivo and the promoter sequence located in −264 to −230 bp was necessary for SREBP mediated transcriptional regulation. Between −264 to 230 bp, 5′-CAC CACGCAACATCGCCCTAGCGCCT-3′ is identical in both humans and rat. This sequence must contain the major functional sterol regulation site. And the most likely SRE is present in −261 to −253 bp (5′-CCACGC AAC-3′) which is direct repeats of half site of SRE. Very recent transgenic study revealed that SREBP activates almost all steps of cholesterol synthetic pathway including SE gene [19]. This evidence matches our results.

On another deletion analysis, human SE promoter constructs (pGVHSE −264/−139, pGVHSE −264/−192, and pGVHSE −264/−207) were also transfected into HeLa cells. The high expression was observed with pGVHSE −264/−139 and pGVHSE −264/−192 when HeLa cells were treated with LPDS, but with pGVHSE −264/−207, luciferase activity was decreased significantly

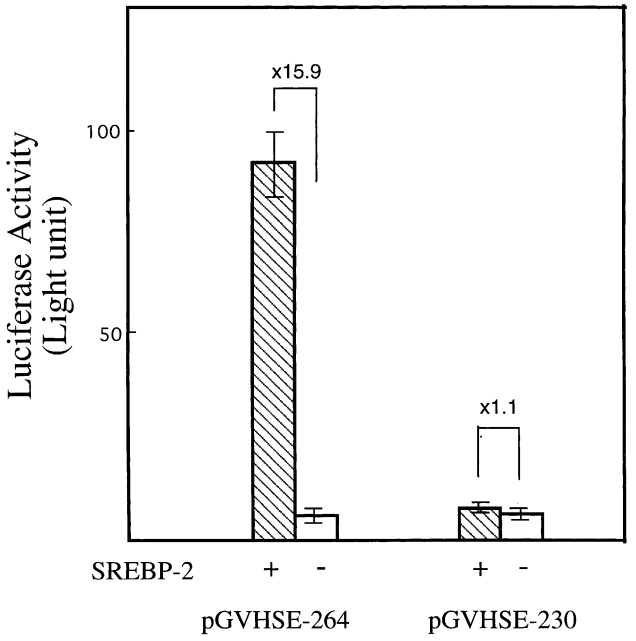

Fig. 5. Stimulation of human SE promoter luciferase by overexpression of SREBP-2. The luciferase-reporter plasmids were co-transfected with 2 μg of pCMVSREBP-2 plasmid expressing transcriptionally active SREBP-2. HeLa cells were transfected and incubated at 37 °C for 47 h in DMEM supplmented with 10% FBS. The fold induction is relative luciferase activity in the presence (+) versus absense (−) of SREBP-2 expression. Data are presented as means ± SD of three independent experiments.

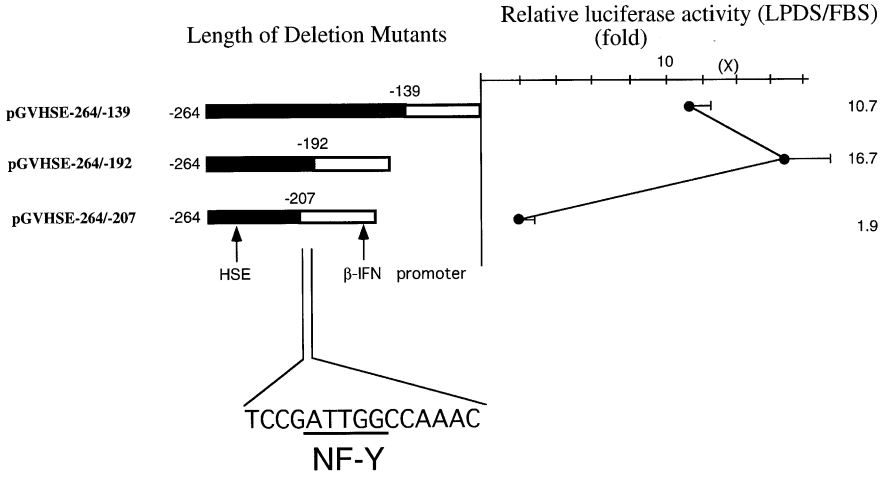

Fig. 6. Luciferase assay of human SE promoter deletion mutants [2]. Schematic diagrams of various human SE promoters, β-IFN promoter, and luciferase fusion constructs are shown. The numbers to the left and middle of each construct represent the positions relative to the initiation site of transcription (+1). Luciferase assays were performed and relative activity cultured in LPDS versus FBS for each construct is shown in a line graph. The nucleotides below diagrams indicate the sequence between −206 to −192 bp of human SE promoter. Data are presented as means ± SD of three independent experiments.

(Fig. 6). The human SE promoter between −206 and −192 bp characterized as 5′-TCCGATTGGCCAAAC-3′ (15 bp) contains a distal NF-Y binding sequence (5′-ATTGG-3′). NF-Y is a ubiquitous transcription factor and it is reported that SREBP and NF-Y interact cooperatively in HMG-CoA syntase gene expression [20]. Our result indicated that distal NF-Y binding site is necessary for sterol regulation of SE gene. There is another NF-Y binding site (proximal NF-Y site; −148 to −144) in SE promoter, but so far, it is not known if it works or not.

Sp-1 is also ubiquitous transcription factor and is considered to be the coactivating factor for LDL receptor gene [17,21]. Recent study indicated Sp-1 and NF-Y are involved in the regulation of fatty acid synthase and 7-dehydrocholesterol reductase gene expression [22,23]. Two Sp-1 binding sites are found in SE promoter (Fig. 3). Sp-1 may participate in transcriptional regulation of SE gene.

Recent study showed that YY-1 inhibits the transcription of specific SREBP-dependent genes [24,25]. Since a YY-1 binding site is also observed in SE promoter (Fig. 3), it may regulate the expression of SE negatively.

Leber et al. [26] reported that two 6-bp direct repeats (AGCTCG) are unique to the ERG1 (Squalene Epoxidase of *Saccharomyces cerevisiae*) and this region is important for ergosterol-regulated expression of *ERG1*. But we could not find this sequence in human and rat promoter of SE gene. The mechanisms of transcriptional regulation may be different between mammal and yeast.

SE catalyzes 2,3(*S*)-oxidosqualene to 2,3(*S*): 22(*S*), 23-squalenedioxide [27], which is a precursor of 24(*S*), 25-epoxycholesterol. 24(*S*), 25-epoxycholesterol is one of ligands of nuclear receptor LXRα which is involved in the homeostasis of cholesterol and steroids [28–30] and also serve as a positive signal for HMG-CoA reductase degradation [31]. To know the transcriptional mechanisms of SE gene more precisely is important, since the generation of oxysterol as a ligand of LXRα and a signal for HMG-CoA reductase degradation may be regulated through the transcription of SE gene.

In the present study, we presented SRE and NF-Y sites in the promoter of human and rat SE gene, which are required for sterol regulation of this gene. These results were demonstrated by the use of constructs containing the promoter of human SE gene and transiently transfected HeLa cells. We are going to continue to study the precise molecular mechanisms of transcriptional regulation of SE.

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