Sterol regulation of human fatty acid synthase promoter I requires nuclear factor-Y- and Sp-1-binding sites

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To understand cholesterol-mediated regulation of human fatty acid synthase promoter I, we tested various 5′-deletion constructs of promoter I-luciferase reporter gene constructs in HepG2 cells. The reporter gene constructs that contained only the Sp-1-binding site (nucleotides −82 to −74) and the two tandem sterol regulatory elements (SREs; nucleotides −63 to −46) did not respond to cholesterol. Only the reporter gene constructs containing a nuclear factor-Y (NF-Y) sequence, the CCAAT sequence (nucleotides −90 to −86), an Sp-1 sequence, and the two tandem SREs responded to cholesterol. The NF-Y-binding site, therefore, is essential for cholesterol response. Mutating the SREs or the NF-Y site and inserting 4 bp between the Sp-1- and NF-Y-binding sites both resulted in a minimal cholesterol response of the reporter genes. Electrophoretic mobility-shift assays using anti-SRE-binding protein (SREBP) and anti-NF-Ya antibodies confirmed that these SREs and the NF-Y site bind the respective factors. We also identified a second Sp-1 site located between nucleotides −40 and −30 that can substitute for the mutated Sp-1 site located between nucleotides −82 and −74. The reporter gene expression of the wild-type promoter and the Sp-1 site (nucleotides −82 to −74) mutant promoter was similar when SREBP1a [the N-terminal domain of SREBP (amino acids 1–520)] was constitutively overexpressed, suggesting that Sp-1 recruits SREBP to the SREs. Under the same conditions, an NF-Y site mutation resulted in significant loss of reporter gene expression, suggesting that NF-Y is required to activate the cholesterol response.

Humans, like other animals, derive long-chain fatty acids either from food or by *de novo* synthesis from acetyl-CoA. *De novo* synthesis requires the participation of two multifunctional enzymes, fatty acid synthase (FAS; EC 2.3.1.85) and acetyl-CoA carboxylase (EC 6.4.1.2) (1). The regulation of animal FAS by diet and hormones is well documented (2). A fat-free carbohydrate-rich diet induces the synthesis of FAS and, consequently, an increase in the metabolic levels of long-chain fatty acids (1, 2). The levels of FAS are controlled by the rate of transcription and by the stability of its mRNA (2, 3). The regulation of animal FAS mRNA levels by the thyroid hormone and by insulin has been demonstrated (2, 3).

Recent studies on cholesterol-mediated regulation of rat acetyl-CoA carboxylase (4, 5), rat FAS promoter I (6, 7), and stearoyl-CoA desaturase 1 (8, 9) suggest that lipogenesis and sterol synthesis and uptake are all coordinately regulated by cholesterol. Sterol-mediated regulation is facilitated by sterol regulatory element (SRE)-binding proteins (SREBPs) (10–14). SREBPs were originally characterized as transcription factors that regulate the genes involved in cholesterol uptake from plasma, such as the low-density lipoprotein receptor (15, 16), and the genes involved in the biosynthetic pathway of cholesterol and lipids, such as 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase, HMG-CoA reductase (17, 18), farnesyl diphosphate (FPP) synthase (19, 20), squalene synthase (21, 22), and glycerol-3-phosphate acyltransferase (23).

SREBPs are a novel family of membrane-bound transcription factors that contain the basic helix—loop–helix leucine zipper (24). When sterol levels are low, the membrane-bound precursor form of SREBP undergoes proteolytic cleavage in a two-step process that releases the N-terminal SREBP domain, which then enters the nucleus and binds to the SREs of the target genes (24). SREBPs bind to sequences that contain a direct repeat of 5′-pyCApy-3′, rather than E-boxes, in the promoters of sterol-regulated genes (7, 24). SREBPs are weak activators of transcription and function efficiently only when activated by coactivating transcription factors such as nuclear factor Y (NF-Y) or Sp-1. Consequently, Sp-1- or NF-Y-binding sites are found in close proximity to an SRE. Sp-1 is considered to be the coactivating factor for the low-density lipoprotein receptor (15, 16), for acetyl-CoA carboxylase (4, 5), and for the FAS promoters (6, 7). The promoters in HMG-CoA synthase (19), FPP synthase (20), and glycerol-3-phosphate acyltransferase (23) require NF-Y as a coregulator.

Sp-1 and NF-Y are ubiquitous transcription factors. Sp-1 binds to the consensus sequence CGGGGCCCCG (25). NF-Y, also called CP1, is a heterotrimeric metalloprotein composed of three subunits—NF-Ya, NF-Yb, and NF-Yc (26)—and binds CCAAT boxes in various promoters such as albumin (27), globins (28), β-actin (29), α-collagen (30), and interleukin-4 (31). NF-Y subunit sequences are highly conserved among eukaryotes, and the yeast homolog heme-activated proteins can form heterotrimeric complexes with the corresponding mammalian subunits (32).

We have shown previously that human FAS has two promoters, I and II, and that promoter I is the major promoter (33). Human FAS is regulated by triiodothyronine through the two thyroid hormone response elements that are present in promoter I (34). Recent studies on the cholesterol regulation of the rat FAS promoter have shown that the promoter-proximal SREBP-binding sites (nucleotides −73 to −43) and the Sp-1-binding site (nucleotides −91 to −81) are involved in this regulation (6, 7). These sequences are also present in human FAS promoter I. In addition, there is an NF-Y-binding site in human FAS promoter I; this site, which is located 4 bp upstream of the Sp-1-binding site, is identical to the cAMP response element in the rat FAS promoter (35, 36). In this study, we show that NF-Y is an essential coactivator of the cholesterol response of human FAS promoter I. We also demonstrate that human FAS promoter I

Abbreviations: FAS, fatty acid synthase; SRE, sterol regulatory element; SREBP, SRE-binding protein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; FPP, farnesyl diphosphate; NF-Y, nuclear factor Y; TK, thymidine kinase; CMV, cytomegalovirus; SREBP1a, N-terminal domain of SREBP (amino acids 1−520); RLU, relative luciferase units; EMSA, electrophoretic mobility-shift assay.

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Table 1. Oligonucleotides used to construct plasmids

Oligonucleotide	Sequence
Human FAS promoter I-luciferase reporter gene constructs	
Wild type	GGGCGCAGCCCCGACGCTC**ATTGG**CCTGG**GCGGCGC**AGCC
NF-Ymut	GGGCGCAGCCCCGACGCTC**GGTAC**CCTGGGCGGCGCAGCC
NF-Yinst	GGGGCGCAGCCCCGACGCTC**ATTGG**CCTAGGTGGGCGGCGCAGCC
Sp1mut	GGGGCGCAGCCCCGACGCTCATTGGCCTGAATTCTTCAGCCCAAGCTGTC
SREmut	TCCCCGGGCGGTACCGAATCATGGGCTGACAGC
Luciferase reporter constructs in minimal TK promoter plasmid	
−92/−40wt	TCATTGGCCTGGGCGGCGCAGCCAAGCTGTCAGCCCATGTGGCGTGGCCGCCC
−67/−40wt	GCTGTCAGCCCATGTGGCGTGGCCGCCC
Sp1inst	GGGTACCCCGCCCGGGGATGGCCGCGGCTCGAGA
SRE-1/NF-Ymut	CATCACCCCACCGGCTCATTGGCC

contains two Sp-1-binding sites, and that these sites function redundantly in cholesterol response. We further show that Sp-1 alone cannot activate the sterol response in human FAS promoter I. Herein, we discuss the implication of these results.

Materials and Methods

Materials. Cell culture media and LipofectAmine were purchased from Life Technologies (Grand Island, NY). Lipodeficient fetal bovine serum was purchased from PerImmune (Rockville, MD). The anti-SREBP1a [the N-terminal domain of SREBP (amino acids 1–520)] and the anti-Sp-1 antibodies were purchased from Santa Cruz Biotechnology, and the anti-NF-Ya antibodies were purchased from PharMingen. The sources of all the other chemicals, radioactive materials, and DNA-modifying enzymes were as described (33, 34). The oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). The oligonucleotides used to construct the human FAS promoter I-luciferase reporter gene plasmids and the thymidine kinase (TK)-luciferase reporter gene plasmids are listed in Table 1.

Construction of Reporter Gene Plasmids. The human FAS promoter I-luciferase plasmids LC9–2, LC9–3, LC9–4, LC9–5, and LC11 and the TK-luciferase plasmids TK-272/-67 and TK-272/-40 were constructed as described (33, 34). LC9–2/del was generated by deleting the *Sma*I fragment (nucleotides −110 to −40) from LC9–2. LC9–2/Sp1mut, LC9–2/NF-Ymut, LC9–2/SREmut, and LC9–2/inst were generated by cloning PCR products obtained by using appropriate synthetic oligonucleotides (Table 1) into plasmid LC9–2/del to substitute for the wild-type sequences. Plasmids TK-92/-40, TK-67/-40, TK-110/-40/Sp1mut, TK-92/-40/NF-Ymut, and TK-272/-40/Sp1mut were generated by cloning either the corresponding double-stranded synthetic oligonucleotides or PCR fragments into minimal TK-luciferase. Plasmid TK-92/-30/Sp1inst, which contained an additional 7 bp, was generated by cloning double-stranded synthetic oligonucleotides into TK-92/-40/Sp1mut by using the *Kpn*I and *Xho*I sites. TK-92/-30/Sp1inst, which contained an additional 3 bp, was generated by blunt ending the *Kpn*I site with S1 nuclease and religating. TK-272/-30/Sp1mut was generated by cloning the S1 nuclease-treated *Sac*II fragment of LC9–2/Sp1mut in minimal TK plasmid at the *Sma*I site. The SREBP expression plasmid pCMX-SREBP1a was generated by blunt ending the *Nco*I-*Acc*I fragment that encodes amino acids 1 to 520 of pSREBP1a (American Type Culture Collection, Rockville, MD) and cloning it into the pCMX-promoter vector (Ming-Jer Tsai, Department of Molecular and Cellular Biology, Baylor College of Medicine) at the *Eco*RV site. pCMX is a cytomegalovirus (CMV)-promoter plasmid that contains a T7 promoter sequence to facilitate *in vitro* transcription and translation.

Cell Culture and Transfection. HepG2 cells were grown in DMEM supplemented with 10% fetal bovine serum containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. The cells were seeded in six-well culture plates (2×10^5 cells/well) and grown overnight in the same medium. The cells were then transfected with 5 μl of LipofectAmine complexes containing 2 μg of the luciferase reporter gene constructs and 0.2 μg of pCMV-β-galactosidase. After 5 h, the medium was withdrawn and replaced with DMEM supplemented with 10% lipoprotein-deficient fetal bovine serum (inducing medium) or with DMEM containing 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol (repressing medium). After 20 h, the cells were harvested, and the β-galactosidase and luciferase activities were measured as described (33, 34). To test the effect of SREBP1a on reporter gene expression, pCMX-SREBP1a (10 to 1,000 ng) was used in the transfection assays together with the reporter gene and pCMV-β-galactosidase plasmids; the pCMX-promoter plasmid, which lacks SREBP1a, was used in the same amounts as pCMX-SREBP1a as a control. After transfection, the cells were incubated for 20 h in 10% fetal bovine serum. To normalize the transfection efficiencies in all these experiments, the luciferase activity was divided by the β-galactosidase activity, and the reporter gene expression was reported as relative luciferase units (RLU).

Electrophoretic Mobility-Shift Assay (EMSA). The N-terminal domain of SREBP (amino acids 1−520) was synthesized by using the pCMX-SREBP1a plasmid as a template and the Promega reticulocyte *in vitro* transcription and translation kit as described (34). The nuclear extracts of HepG2 cells were prepared according to the procedure of Attardi and Tjian (37). Either the *in vitro*-synthesized SREBP1a or the nuclear extracts were incubated with ^{32}P-labeled DNA templates, and the DNA–protein complexes that formed were analyzed on a 5% polyacrylamide gel as described (34). The supershift analyses were performed by using either anti-SREBP or anti-NF-Ya antibodies.

Results

Deletional Analysis of Human FAS Promoter I. The sequence of human FAS promoter I contains three SREs. The SRE-1-type element, which is located between nucleotides −136 and −127 (33), is identical to those present in the low-density lipoprotein receptor promoter (15, 16) and in the rat FAS promoter (6, 7). Another two tandem SREs, located between nucleotides −63 and −46 (Fig. 1), are identical to the two tandem SREs that were previously characterized in the rat FAS promoter (6, 7).

To understand the role of these SREs in sterol regulation of human FAS promoter I, we performed 5′-deletion analysis and tested a series of human FAS–luciferase reporter gene constructs (Fig. 1). The reporter gene constructs LC9–2, LC9–3, LC9–4, and LC11 were activated by about 7- to 12-fold in the absence of

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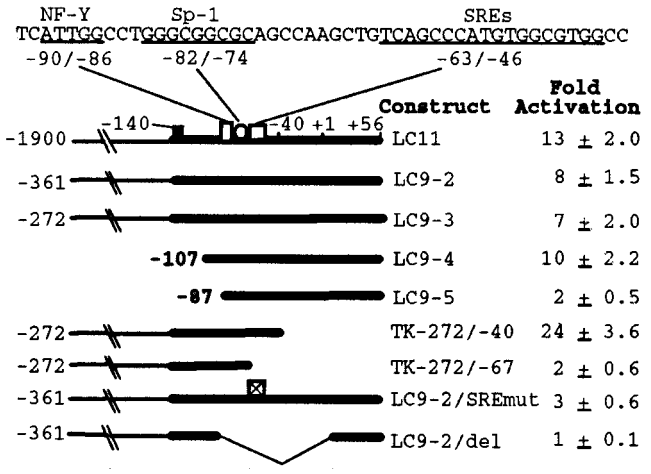

Fig. 1. Identification of the sequences required for the cholesterol response of human FAS promoter I. Details regarding construction of the plasmids, the transfection conditions, the culture media, and the calculation of RLUs are described in *Materials and Methods*. HepG2 cells were used in these experiments. The fold activation was derived by dividing the RLU values obtained with transfected cells grown in inducing medium with the RLU values obtained with transfected cells grown in repressing medium. LC9–2/SREmut contains a mutated SRE but otherwise is identical to LC9–2.

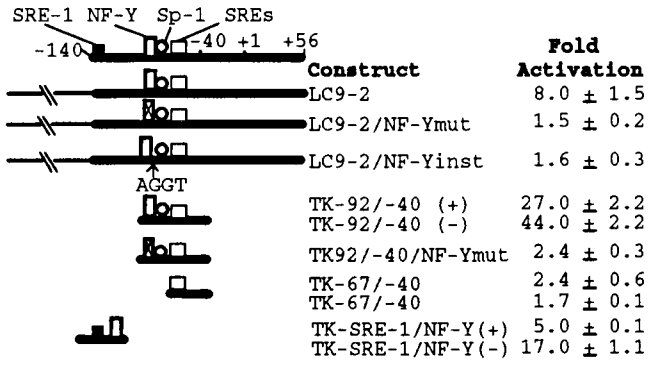

Fig. 2. Essentiality of the NF-Y-binding sequence for the cholesterol response of human FAS promoter I. The experimental conditions were as described in the legend to Fig. 1 and in *Materials and Methods*. LC9–2/NF-Ymut contains GGATC instead of the inverted NF-Y sequence ATTGG. LC9–2/NF-Yinst contains an additional 4 bp that were inserted between the NF-Y- and Sp-1-binding sites. In some TK reporter gene constructs, a plus (+) denotes that the FAS promoter insert is in the same orientation as in human FAS promoter I, and a minus (−) denotes that the FAS promoter insert is in the opposite orientation with respect to human FAS promoter I.

cholesterol (Fig. 1). LC9–4 (−107/+56) lacks the SRE-1 sequence but still responds to cholesterol. Hence, the SRE-1 located between nucleotides −136 and −127 is not essential for the cholesterol response of human FAS promoter I. Its lack of function was attributed to the absence of coactivating factor-binding sequences such as Sp-1 or NF-Y (6, 7, 19, 20, 23). In the rat FAS promoter, the sterol response is mediated through the promoter proximal SRE sequence and an Sp-1-binding site just upstream of it (6, 7). However, LC9–5, which contains the two SREs (nucleotides −63 to −46) and an Sp-1-binding site (nucleotides −82 to −74) did not exhibit a significant sterol response (Fig. 1). Comparing the sterol response of LC9–4 with that of LC9–5 revealed that the sequence between nucleotides −107 and −87 is required for the SREs to function. The promoter activity of LC9–5 in the lipid-depleted serum was about 7- to 10-fold less than that of LC9–4. We had previously established the importance of the sequence between nucleotides −107 and −87 in human FAS promoter activity (33). In the present study, we found within this 20-bp sequence an inverted NF-Y-binding consensus sequence, CCAAT, which is located between nucleotides −90 and −86. The role of this consensus sequence in the sterol response of human FAS promoter I is discussed below.

To confirm that the SREs located between nucleotides −63 and −46 in human FAS promoter I are responsible for cholesterol-mediated regulation, we tested the reporter gene constructs TK-272/-67, which lacks these SREs, and TK-272/-40, which contains them (Fig. 1). In the absence of cholesterol, TK-272/-40 exhibited a 24-fold activation of reporter gene expression, whereas TK-272/-67 showed only a minimal response (Fig. 1). These results suggested that the SRE sequences located between nucleotides −63 and −46 are essential for the cholesterol response. Furthermore, TK-272/-67, which contains SRE-1 (nucleotides −136 to −127) responded minimally to cholesterol, thereby confirming that SRE-1 is nonfunctional.

To further confirm the role of these SREs in the cholesterol response of human FAS promoter I, we mutated the sequence of one of the SREs in LC9–2 from TCAGCCCAT-GTGGCGTGGC to TCAGCCCAT-GATTCGGTAC and generated the construct LC9–2/SREmut. This mutant construct exhibited only a 3-fold cholesterol response, as opposed to the 8-fold response observed with LC9–2 (Fig. 1). Although we mutated only one of the two SREs, these results are consistent with those obtained with the mutated rat FAS SREs (7). The data shown in Fig. 1 confirm that the sequences located between nucleotides −63 and −46 are the SREs of human FAS promoter I. On the basis of the EMSA performed with *in vitro*-synthesized SREBP1a protein, anti-SREBP1a antibodies, and a ^{32}P-labeled 52-bp fragment of human FAS promoter I (nucleotides −92 to −40), we confirmed that these SREs bind SREBP (data not shown).

Essentiality of the NF-Y-Binding Site for the Cholesterol Response of Human FAS Promoter I. The 5′-deletion analysis of human FAS promoter I identified a 20-bp sequence between nucleotides −107 and −87 that is critical for cholesterol response (Fig. 1, LC9–4 and LC9–5). This region contains the sequence ATTGG (nucleotides −90 to −86), which is an inverted CCAAT box and a putative NF-Y-binding site. To determine that this inverted CCAAT sequence is involved in the cholesterol response, we mutated ATTGG to GGTAC in LC9–2 and generated LC9–2/NF-Ymut as described in *Materials and Methods*. The cholesterol response of LC9–2/NF-Ymut was negligible when compared with that of LC9–2, suggesting that the ATTGG sequence is essential for cholesterol response (Fig. 2). To confirm this observation, we also generated luciferase reporter constructs TK-92/-40 and TK-92/-40/NF-Ymut, which respectively contained wild-type and mutated NF-Y-binding sequences. As shown in Fig. 2, the NF-Y mutation reduced the cholesterol response to basal levels (Fig. 2, TK-92/-40/NF-Ymut). The NF-Y-binding sequence CCAAT, therefore, is critical for cholesterol response in human FAS promoter I.

To further demonstrate the role of this NF-Y-binding sequence in cholesterol response, we altered the spacing between the NF-Y-binding site and the SRE by inserting four additional bps between NF-Y and Sp-1 to make the construct LC9–2/NF-Yinst (Fig. 2). This insertion did not alter the spacing between the Sp-1-binding site and the SRE, which was shown to be critical in the cholesterol response of rat FAS (6). As shown in Fig. 2 (LC9–2/NF-Yinst), however, changing the spacing between NF-Y and SRE abolished the cholesterol response. On the basis of these results, we concluded that not only is the CCAAT sequence essential, but the spacing between this sequence and

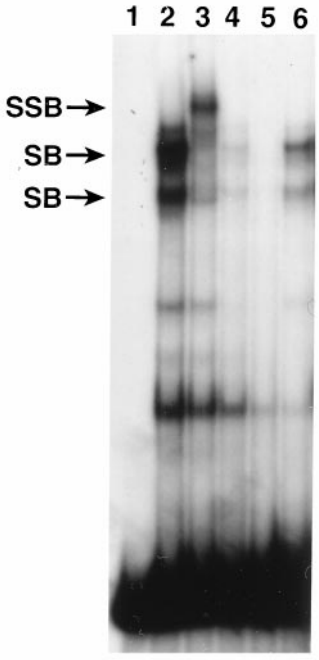

Fig. 3. Confirmation that NF-Y binds to the CCAAT sequence. Nuclear extracts of HepG2 cells were prepared according to the procedure of Attardi and Tjian (34, 36). The ^{32}P-labeled DNA probe (nucleotides −92 to −40) was generated by using the Klenow fragment of DNA polymerase I. All other experimental conditions were as described (34) and as in *Materials and Methods*. Lane 1, DNA probe only; lane 2, DNA probe incubated with cell extract; lane 3, same as in lane 2, except that the DNA–protein complexes were incubated with anti-NF-Ya antibodies; lane 4, as in lane 3, but the binding reaction contained a 50-fold excess of the unlabeled DNA probe; lane 5, as in lane 4, except that the unlabeled DNA probe contained an additional 4 bp that were inserted between the NF-Y- and Sp-1-binding sites; lane 6, as in lane 4, except that the NF-Y-binding site in the unlabeled DNA probe was mutated.

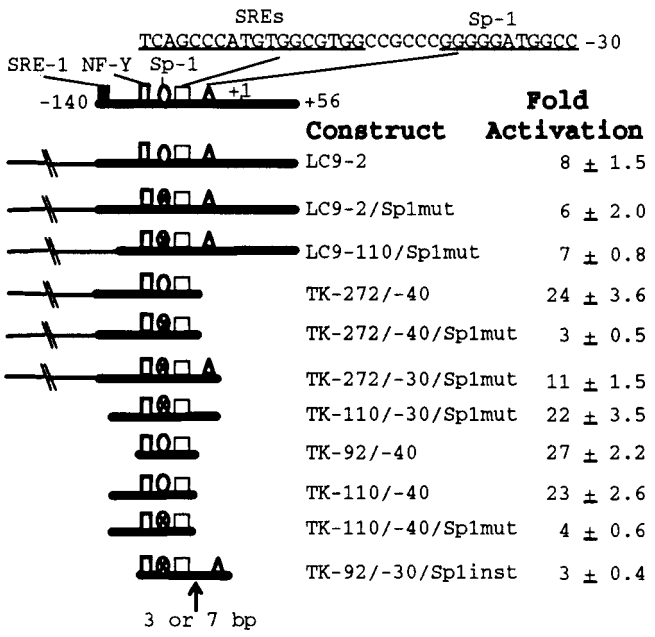

Fig. 4. Identification of two Sp-1-binding sites in human FAS promoter I. The experimental conditions were as described in the legends to Figs. 1 and 3 and in *Materials and Methods*. The Sp1mut constructs contained the mutated sequence GAATTCTTC instead of the wild-type sequence GGGCGGCGC. The Sp1inst constructs contained an additional 3 or 7 bp that had been inserted between the SREs and the downstream Sp-1-binding site.

the SREs is critical for the cholesterol response of human FAS promoter I.

To confirm whether the ATTGG sequence between nucleotides −90 and −86 is the NF-Y-binding site, we used nuclear extracts prepared from HepG2 cells to perform EMSA. As shown in Fig. 3 (lane 2), proteins present in the nuclear extract formed complexes with the DNA probe (nucleotides −92 to −40) and retarded its mobility. That these DNA–protein complexes contain NF-Y was confirmed by incubating them with anti-NF-Ya antibodies and then performing EMSA (supershifted band in Fig. 3, lane 3). The unlabeled DNA probe and the DNA fragment derived from the insertion-mutant LC9–2/NF-Yinst competed with the NF-Y−DNA complex formation (Fig. 3, lanes 4 and 5). However, the DNA containing the NF-Y mutation did not compete with the DNA probe in complex formation (Fig. 3, lane 6). These results suggested that NF-Y binds to the CCAAT sequence in human FAS promoter I.

Role of the Sp-1-Binding Site in the Cholesterol Response of Human FAS Promoter I. We have shown that LC9–5, which contains an Sp-1-binding site and the SREs and lacks the NF-Y-binding site (Fig. 1), had no appreciable cholesterol response. Apparently, Sp-1 alone is insufficient for the activation of SREBP in the cholesterol response of human FAS promoter I. To determine the role of Sp-1 in the cholesterol response of human FAS promoter I, we mutated the Sp-1 site (nucleotides −82 to −74) in LC9–2 and generated LC9–2/Sp1mut. Surprisingly, LC9–2 and LC9–2/Sp1mut had nearly the same cholesterol response (Fig. 4), suggesting that the Sp-1-binding site is not critical for the cholesterol response of human FAS promoter I. To confirm this observation, we used a heterologous promoter and compared the cholesterol responses of TK-92/-40 and the Sp-1 mutant construct TK-110/-40/Sp1mut. Interestingly, TK-110/-40 and TK-92/-40 respectively showed a 23- and 27-fold cholesterol response, whereas TK-110/-40/Sp1mut showed only a 4-fold response (Fig. 4), which is at the level of basal activity of the reporter gene construct TK-67/-40 that contained SRE only (Fig. 2). Sp-1, therefore, is required when the human FAS sterol regulatory region is expressed by using a heterologous promoter.

These seemingly contradictory observations about the role of Sp-1 in cholesterol response may be explained by the lack of nucleotide sequences either upstream of −110 or downstream of −40 in TK-110/-40/Sp1mut. These sequences may be essential for cholesterol response when the Sp-1 site located between nucleotide −82 and −74 is mutated. The sequences upstream of nucleotide −110 were found to be not involved in rescuing the Sp-1 mutation, because LC9–110/Sp1mut, a 5′-deletion construct of LC9–2/Sp1mut, also exhibited a normal cholesterol response (Fig. 4). In addition, TK-272/-40/Sp1mut, which contains the sequences upstream of nucleotide −110, exhibited only a 3-fold cholesterol response (Fig. 4).

To determine whether the sequences downstream of nucleotide −40 in human FAS promoter I are involved in maintaining cholesterol response when the Sp-1 site is mutated, we generated TK reporter gene constructs that contained the downstream sequences of the promoter up to nucleotide −30 on the 3′ side. As shown in Fig. 4, the TK-272/-30/Sp1mut construct exhibited an 11-fold cholesterol response, which is significantly higher than that of the TK-272/-40/Sp1mut construct. Furthermore, TK-110/-30/Sp1mut exhibited a 22-fold cholesterol response, which was comparable to that of TK-110/-40. These results indicated that a sequence between nucleotides −39 and −30 rescues the Sp-1 mutation (nucleotides −82/−74). Within this sequence, we have identified a putative Sp-1-binding site (Fig. 4) and will refer to it as the downstream Sp-1 site. To further demonstrate the role

Table 2. Roles of the NF-Y- and Sp-1-binding sites in reporter gene expression in the presence of various amounts of SREBP

Constructs	Activation by pCMX-SREBP, fold		
	10 ng	100 ng	1 μg
TK-92/−40	21 ± 3.0	42 ± 2.3	91 ± 8.0
TK-92/−40/Sp1mut	9 ± 2.0	42 ± 4.5	ND
TK-92/−40/NF-Ymut	2 ± 0.3	14 ± 1.5	19 ± 1.9

HepG2 cells were transfected with reporter gene plasmid DNA along with 10 ng, 100 ng, or 1 μg of pCMX-SREBP1a plasmid DNA. As a control, transfections were performed with pCMX vector devoid of the SREBP1a insert, instead of pCMX-SREBP1a. The transfected cells were grown in culture medium containing 10% fetal calf serum for 20 h and were then harvested. The RLUs were determined as described in *Materials and Methods*. Fold activation is the ratio of RLUs obtained with pCMX-SREBP1a-transfected cells and those of the pCMX vector. ND, not determined.

of this Sp-1-binding site, we generated another two reporter gene constructs by inserting 3 or 7 bp between the SREs and this downstream Sp-1 site. Both of the TK-92/-30/Sp1inst mutants exhibited a minimal (3-fold) cholesterol response (Fig. 4), suggesting that the spacing between this downstream Sp-1 site and the SREs is also critical. Even though the sequence that contains the SREs and the NF-Y- and Sp-1-binding sites (nucleotides −92 to −40) is sufficient for cholesterol-mediated regulation of human FAS promoter I, the downstream Sp-1 site (nucleotides −39 to −30) is required when the Sp-1 site located between nucleotides −82 and −74 is mutated. It is noteworthy that this downstream putative Sp-1 site is also present in the rat FAS promoter sequence and likely plays a role similar to that of human FAS promoter I.

Roles of NF-Y and Sp-1 in the Sterol Response When SREBP1a Is Expressed Constitutively. It is generally accepted that because of the weak interaction of SREBP with SRE, coactivating factors such as NF-Y or Sp-1 are needed to recruit and stabilize this interaction and activate the promoters (24). As described above, human FAS promoter I has the binding sites for NF-Y and Sp-1 and needs both sequences to respond to sterol. To delineate the roles of Sp-1 and NF-Y, we expressed SREBP1a in HepG2 cells by using increasing amounts of the CMX-SREBP1a plasmid in transient transfection assays. When the mature form of SREBP was constitutively expressed in cultured cells by using an SREBP1a expression plasmid, these promoters were activated, even when cholesterol was present in the medium (20). As expected from previous studies (20), the reporter gene expression of TK-92/-40 increased in serum-containing medium as the amount of the CMX-SREBP1a plasmid used during transfection was increased (Table 2). However, when 10 ng of the CMX-SREBP1a plasmid was used in the transfection assays, TK-92/-40/Sp1mut exhibited significant activity, whereas TK-92/-40/NF-Ymut exhibited only background activity. When 100 ng of the SREBP1a plasmid was used in the transfection assays, the reporter gene activity of TK-92/-40/Sp-1mut was very similar to that of the wild-type plasmid (TK-92/-40). Although the reporter gene activity of TK-92/-40/NF-Ymut also increased, the level of expression was only about one-fourth that of the wild-type plasmid. Although we do not know the physiological concentrations of mature SREBP in HepG2 cells under derepressive conditions, the results presented in Table 2 show that whereas NF-Y is essential, Sp-1 is not needed for target gene activation when the mature form of SREBP1a is expressed at "low" levels.

Discussion

Human and rat FAS promoter sequences are highly conserved, having an unprecedented identity of ≈90% throughout about 170 bp (33). In the rat FAS promoter, SREs and the Sp-1 site are the elements needed for cholesterol response (6, 7). A detailed analysis of the sequences responsible for the cholesterol response of human FAS promoter I was prompted by the observation that the 5′-deletion construct LC9–5, which contains the Sp-1 site and SREs, showed a negligible sterol response (Fig. 1), suggesting that the SREs and the Sp-1 site are not sufficient to promote the sterol response of human FAS promoter I. The analyses described above showed that the NF-Y-binding sequence located between nucleotides −90 and −86 is necessary for the cholesterol response of human FAS promoter I (Fig. 2).

The role of the Sp-1 site located between nucleotides −82 and −74 in the cholesterol-mediated regulation of human FAS promoter I was initially confusing. However, by using various reporter gene constructs, we discovered that the human FAS reporter gene that contained a mutated Sp-1-binding site exhibited a significant cholesterol response (Fig. 4) because of the presence of a downstream Sp-1-like sequence, GGGGATGGC (nucleotides −39 to −30). The heterologous (TK) reporter gene constructs that contained human FAS promoter I sequences, which had been truncated at nucleotide −40 on the 3′ side, responded minimally to cholesterol when the Sp-1-binding site (nucleotides −82 to −74) was mutated because of lack of this downstream Sp-1-like sequence (Fig. 4).

By comparing the sequences of human FAS promoter I and the rat FAS promoter, we found that the downstream Sp-1 sequence (nucleotides −39 to −30) is also present in the rat FAS promoter. Previous studies by other investigators on the cholesterol response of the rat FAS promoter were performed with reporter gene constructs lacking this downstream Sp-1-binding site (6, 7). Consequently, mutating the upstream Sp-1 sequence in the rat FAS promoter abolished the cholesterol response (6, 7). These results are similar to our observations with TK-92/-40/Sp-1mut, which contained a mutation of Sp-1 between nucleotides −82 and −74, and demonstrate that the spacing between the NF-Y site and the SREs is critical for the cholesterol response of human FAS promoter I by inserting an extra 4 bp between the NF-Y and Sp-1 sites (Fig. 2, LC9–2/inst). Because LC9–2/inst contains two Sp-1-binding sites, either of which is sufficient to elicit the cholesterol response, the spacing between the NF-Y- and Sp-1-binding sites does not seem to be critical for this response. The loss of cholesterol response observed when additional bases were introduced into the rat FAS promoter between the Sp-1 site and the SREs (7) could also be because of a change in the distance between the NF-Y site and the SREs.

The distance between the NF-Y- and SREBP-binding sites varies between 10 and 22 bp among different promoters (17, 20–22). Surprisingly, we were able to activate the nonfunctional SRE-1 (nucleotides −140 to −133) in human FAS promoter I when an NF-Y-binding site was inserted 5 bp from this SRE in either orientation (Fig. 2). Insertional mutations that change the distance between the NF-Y- and SREBP-binding sites in human FAS promoter I (Fig. 2) and in the FPP synthase promoter (23) disrupted the sterol response of both promoters. It is difficult, therefore, to predict the optimal distance between the SRE and the NF-Y sequence.

On the basis of sequence conservation, the human FAS promoter is unique in that it has Sp-1- and NF-Y-binding sequences. The results shown in Table 2 indicate that when the mature form of SREBP is expressed at excessive levels, the binding of Sp-1 to the promoter is not essential for its activation, suggesting that Sp-1 recruits SREBP to SRE. However, NF-Y seems to enhance the sterol response by activating SREBP, because the NF-Y site mutant failed to show maximal promoter activation, even when SREBP was overexpressed (Table 2). Hence, NF-Y is also a general transcription activator of human FAS promoter I.

As discussed above, the cholesterol response of several promoters involves SRE and either NF-Y or Sp-1. Because SREBP is a weak transcription activator (38), either NF-Y or Sp-1 must be playing a dual role of recruiting SREBP as well as activating it in these promoters. In the FPP synthase promoter, NF-Y enhances the binding of SREBP to SRE (19, 20). In addition, the interaction of NF-Y and SREBP in the absence of DNA has been demonstrated *in vitro* (39). Recent studies, however, indicate that the sterol response of the HMG-CoA synthase promoter, which requires NF-Y and SREBP, also requires an additional coactivator, CREB, the cAMP response element-binding protein (40). CREB-binding protein (CBP) and p300, a CBP-related protein, both bind the activation domain of SREBP *in vitro* (41). Transfecting HeLa cells with plasmids that express CBP enhanced the ability of SREBP to activate transcription of reporter genes (41). Apparently, NF-Y and Sp-1 interact with other factors in eliciting sterol response (42, 43). The NF-Y-binding site in the rat FAS promoter is also involved in the cAMP-mediated response of the rat FAS promoter (35, 36). The cooperative binding of NF-Y and Sp-1 in rat FAS insulin response has been shown (44). By using the yeast two-hybrid system, the interaction of NF-Ya with Sp-1 has also been demonstrated (45). In addition, NF-Ya has been shown to interact with Sp-1 *in vitro* in the absence of DNA (45). Hence, it is possible that an interaction between NF-Y and Sp-1 might also play a role in the sterol response of human FAS promoter I. Though most of the promoters that respond to cholesterol require either NF-Y or Sp-1 (4–7, 15, 16, 19, 20, and 23), human FAS promoter I is unique in that it requires both NF-Y and Sp-1 for cholesterol response. Furthermore, there are two Sp-1 sites that support cholesterol response in human FAS promoter I. The Sp-1 site located between NF-Y and the SREs has been very well characterized in the rat FAS promoter (6, 7) and is identical to that of human FAS promoter I. It remains to be determined, however, whether the putative Sp-1 site downstream of the SREs binds Sp-1 or other coactivators of the sterol response in human FAS promoter I.

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