THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 40, Issue of October 6, pp. 31078–31085, 2000

Printed in U.S.A.

Promoter Analysis of the Mouse Sterol Regulatory Element-binding Protein-1c Gene*

Received for publication, June 20, 2000, and in revised form, July 25, 2000
Published, JBC Papers in Press, July 28, 2000, DOI 10.1074/jbc.M005353200

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Recent data suggest that sterol regulatory-binding protein (SREBP)-1c plays a key role in the transcriptional regulation of different lipogenic genes mediating lipid synthesis as a key regulator of fuel metabolism. SREBP-1c regulates its downstream genes by changing its own mRNA level, which led us to sequence and analyze the promoter region of the mouse SREBP-1c gene. A cluster of putative binding sites of several transcription factors composed of an NF-Y site, an E-box, a sterol-regulatory element 3, and an Sp1 site were located at −90 base pairs of the SREBP-1c promoter. Luciferase reporter gene assays indicated that this SRE complex is essential to the basal promoter activity and confers responsiveness to activation by nuclear SREBPs. Deletion and mutation analyses suggest that the NF-Y site and SRE3 in the SRE complex are responsible for SREBP activation, although the other sites were also involved in the basal activity. Gel mobility shift assays demonstrate that SREBP-1 binds to the SRE3. Taken together, these findings implicate a positive loop production of SREBP-1c through the SRE complex, possibly leading to the overshoot in induction of SREBP-1c and its downstream genes seen in the livers of refed mice. Furthermore, reporter assays using larger upstream fragments indicated another region that was inducible by addition of sterols. The presence of the SRE complex and a sterol-inducible region in the same promoter suggests a novel regulatory link between cholesterol and fatty acid synthesis.

Sterol regulatory element-binding proteins (SREBPs)[1] are transcription factors that belong to the basic helix-loop-helix leucine zipper family (1, 2). In contrast to other members of this family, SREBPs are synthesized as precursor proteins that remain bound to the endoplasmic reticulum and the nuclear envelope in the presence of sufficient sterol concentrations. Upon sterol deprivation, the precursor protein undergoes a sequential two-step cleavage process to release the NH_2-terminal portion (3). This mature SREBP then enters the nucleus and activates the transcription of genes involved in cholesterol and fatty acid synthesis by binding to sterol regulatory elements (SREs) or to palindromic sequences called E-boxes within their promoter regions (4, 5). Currently, there are three forms of SREBP that have been characterized; SREBP-1a and -1c are derived from a single gene through the use of alternate promoters and SREBP-2 from a different gene. SREBP-1a is the more common isoform and is a stronger activator of transcription with a wider range of target genes than SREBP-1c because of a longer transactivation domain (6). Transgenic mouse studies have shown that SREBP-1c plays a more active role in regulating the transcription of genes involved in fatty acid synthesis than those involved in cholesterol synthesis, whereas SREBP-1a activates both (6, 7). SREBP-2 is known to be actively involved in the transcription of cholesterogenic enzymes (8). It has been shown that all cultured cells analyzed to date exclusively express SREBP-2 and the -1a isoform of SREBP-1, whereas most organs, including the liver, express predominantly SREBP-2 and the 1c isoform of SREBP-1 (9).

Lipogenic enzymes, including fatty acid synthase and acetyl-CoA carboxylase, are a group of genes involved in energy storage through synthesis of fatty acids and triglycerides (10, 11). Excess amounts of carbohydrates taken up by cells are converted to triglycerides through these enzymes in lipogenic organs such as liver and adipose tissue. The lipogenic enzymes are coordinately regulated at the transcriptional level during different metabolic states (10, 11). Recent *in vivo* studies demonstrated that SREBP-1c plays a crucial role in the dietary regulation of most hepatic lipogenic genes. These include studies of the effects of the absence or overexpression of SREBP-1 on hepatic lipogenic gene expression (6, 7, 12), as well as physiological changes of SREBP-1c protein in normal mice after dietary manipulation such as placement on high carbohydrate diets, polyunsaturated fatty acid-enriched diets, and fasting-refeeding regimens (12–17, 27). All these *in vivo* data established distinct roles of SREBP-1c and -2 in hepatic lipo-

* This work was supported by the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research and Health Sciences Research Grants (Research on Human Genome and Gene Therapy) from the Ministry of Health and Welfare. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‖ Recipient of a fellowship under the Postdoctoral Fellowship Program for Foreign Researchers from the Japan Society for the Promotion of Science.

[1] The abbreviations used are: SREBP, sterol regulatory element-binding protein; SRE, sterol regulatory element; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction; CMV, cytomegalovirus; DMEM, Dulbecco's minimally modified medium.

genesis and cholesterogenesis, respectively. The similar coordinated changes in SREBP-1c and lipogenic gene expression at fasting and refeeding were also observed in adipose tissue (18). In the fat tissue, SREBP-1c/ADD1 appears to be involved in adipocyte differentiation and insulin resistance (19, 20). Recent studies suggest that insulin-facilitated glucose uptake mediates lipogenesis through SREBP-1c induction (21–24). In addition, insulin supplement in streptozotocin-induced diabetic animals caused marked induction of SREBP-1c mRNA in the liver (25). DNA microarray analysis of ob/ob mice suggested that leptin might regulate expression of SREBP-1c and its downstream genes in adipose tissue (26). These data suggest that SREBP-1c plays an important role in fuel metabolism involving a multi-organ and complex milieu of metabolites and hormones. Previous reports on the regulation of SREBP-1c have all demonstrated the induction to be at the mRNA level (13–18, 21, 22, 24, 27). Up regulation of hepatic SREBP-1 was observed in rodent livers on a fasting-refeeding regimen, a chronic high carbohydrate diet, and in primary hepatocytes with a high glucose medium. Meanwhile, down regulation of SREBP-1c was observed in livers from fasted rodents, insulin-depleted diabetic rats with streptozotocin treatment, and mice on a diet containing polyunsaturated fatty acids. In contrast to SREBP-2, which mediates sterol regulation completely at the cleavage level through interaction with SCAP and site-1 protease, SREBP-1c seems to control the transcriptional regulation of lipogenic enzymes by self-regulating the nuclear concentration of its mature form, which is highly correlated to its precursor and mRNA levels. These observations prompted us to analyze the promoter of SREBP-1c to understand the regulation of SREBP-1c itself and, thus, that of lipogenic enzymes.

EXPERIMENTAL PROCEDURES

Materials—Standard molecular biology techniques were used. We obtained cholesterol and 25-hydroxycholesterol from Sigma, Redivue [α-^{32}P]dCTP (6000 Ci/mmol) from Amersham Pharmacia Biotech, and restriction enzymes from New England Biolabs. Plasmid DNAs for transfection were prepared with EndoFree Plasmid Maxi kits (Qiagen).

Mouse Genomic SREBP-1c Gene Promoter and Construction of Luciferase Gene Reporter Genes—A *Sac*I fragment of mouse SREBP-1 genomic DNA that contains most of 5′-flanking region of SREBP-1c exon 1 was sequenced from the transcription start site that was tentatively assigned from 5′ rapid amplification of cDNA ends of mouse SREBP-1c (28). A *Bam*HI-*Avr*II fragment (0.55 kb) extending from 5′-untranslated region to the promoter region was subcloned into *Sma*I site of pGL2 basic vector (Promega) (pBP1c550-Luc). The adjacent upstream *Bam*HI fragment (2 kb) was further cloned into pBP1c550-Luc to generate a 2.6-kb fragment construct (pBP1c2600Luc). Other constructs were produced by PCR using this construct as DNA template and subcloning the PCR products into the pGL2 basic vector. The primers used for PCR were as follows: 3′ primer, 5′-TAAGAGCTCGGTACCTCCCCTAGGGC-3′; 5′ primer, pBP1c400-Luc 5′-GGGCCAGGAGTGGGTAAA-3′; pBP1c150-Luc, 5′-GGGAGAAACCCGAGCT-3′; pBP1c-90(NESSp)-Luc, 5′-CTGCTGATTGGCCAT GTG-3′; pBP1c-ESSp-Luc, 5′-CCATGTGCCCTCACCCGA-3′; pBP1c-SSp-Luc, 5′-CGCTCACCCGAGGGGCGGGGCA-3′; pBP1c-Sp-Luc, 5′-CGGGGCACGGAGGCG ATC-3′; pBP1c-mE-Luc, 5′-CTGCTGATTGGCAAAGTGCGCTCACCCGAG-3′; pBP1c-mSRE-Luc, 5′-CTGCTGATTGGCCATGTGCGCTACACCGAGGGGCGGGGCA-3′; pBP1c-mES-Luc, 5′-CTGCTGATTGGCAAAGTGCGCTACACCGAGGGGCGGGGC A-3′; and pBP1c-mSp-Luc, 5′-CTGCTGATTGGCCATGTGCGCTCACCCGAGTTTCGGGGCACGGAGGCG-3′. The primers were tailed with *Sma*I site (5′ primers) or *Kpn*I site (3′ primer). The PCR products were digested with *Sma*I and *Kpn*I and subcloned into the pGL2 basic vector.

Transfections and Luciferase Assays—Human embryonic kidney 293 were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate supplemented with 10% fetal calf serum. On day 0, cells were plated on a 12 well plate at 4.5×10^4 cells/well. On day 2, each luciferase reporter plasmid (0.5 μg), and an SV-β-galactosidase reference plasmid (pSV-β-gal, Promega, 0.5 μg) were transfected into cells using SuperFect Transfection Reagent (Qiagen) according to the manufacturer's protocol. In some experiments, expression plasmid (CMV-SREBP-1a, -1c, or -2 or their respective YR mutant versions) (0.5 μg) or basic plasmid CMV7 as negative control (29) was also co-transfected. 5 h after transfection, the cells were incubated in the condition as indicated for an additional 16 h prior to harvest. For the suppressed condition, DMEM with 10% fetal calf serum with cholesterol (10 μg/ml) and 25-hydroxycholesterol (1 μg/ml) was used to suppress endogenous SREBP activity. For induced condition, DMEM with 10% delipidated calf serum with 10 μM pravastatin was used. The amount of luciferase activity in transfectants was measured and normalized to the amount of β-galactosidase activity as measured by standard kits (Promega).

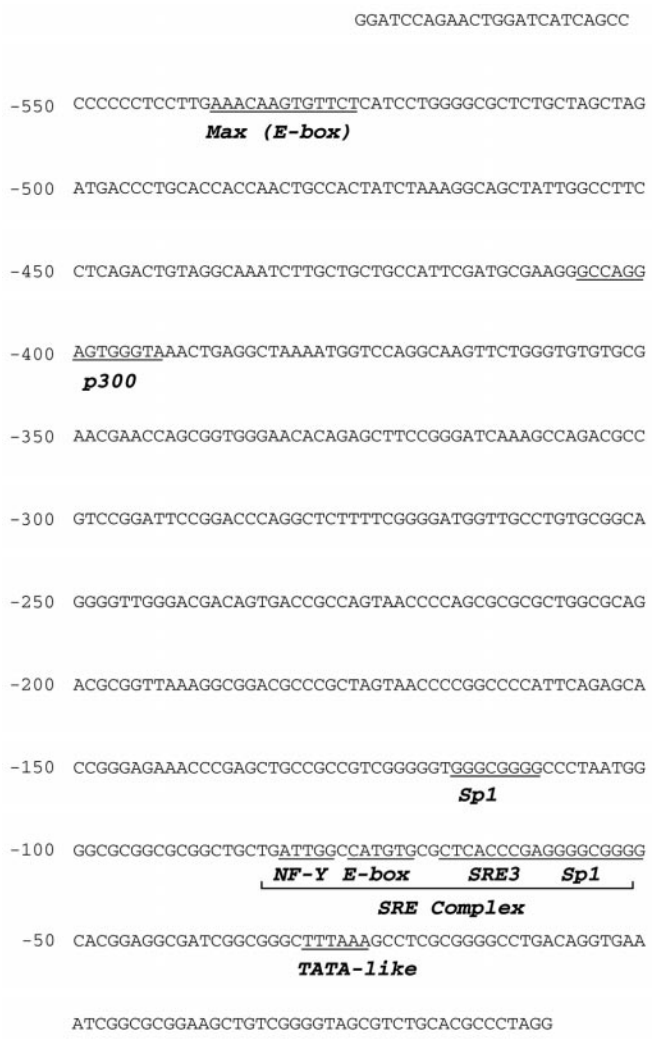

FIG. 1. **DNA sequence of the 5′-flanking region of the mouse SREBP-1c gene.** The sequence of a *Bam*HI-*Avr*II fragment of SREBP-1 gene (28) is shown and numbered in relation to the putative transcription initiation site. This DNA was used for the luciferase reporter gene construct designated pBP1c550-Luc (Fig. 2). The TATA-like box is located at −25 bp. There are two putative Sp1 sites and E-boxes and a p300 site. At −90 bp, there is a region with high probability of being an SREBP target, designated SRE complex that is composed of an NF-Y site (inverted CCAAT), an E-box, an SRE3-like (31) sequence, and an Sp1 site.

Gel Mobility Shift Assay—The DNA probe was prepared by annealing both strands of the SRE complex, TGCTGATTGGCCATGTGCGCTCACCCGAGGGGCGGGG, and was labeled with [α-^{32}P]dCTP by Klenow enzyme, followed by purification on G50 Sephadex columns. The labeled DNA was incubated with a recombinant SREBP-1 protein (100 ng) in a mixture containing 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.5 μg/ml poly(dI-dC), 0.1% Triton X-100, and 1 mg/ml nonfat milk for 30 min on ice. The DNA-protein complexes were resolved on a 4.6% polyacrylamide gel.

RESULTS

Mouse SREBP-1c Promoter Sequence—We sequenced and analyzed 550 base pairs upstream from the transcription ini-

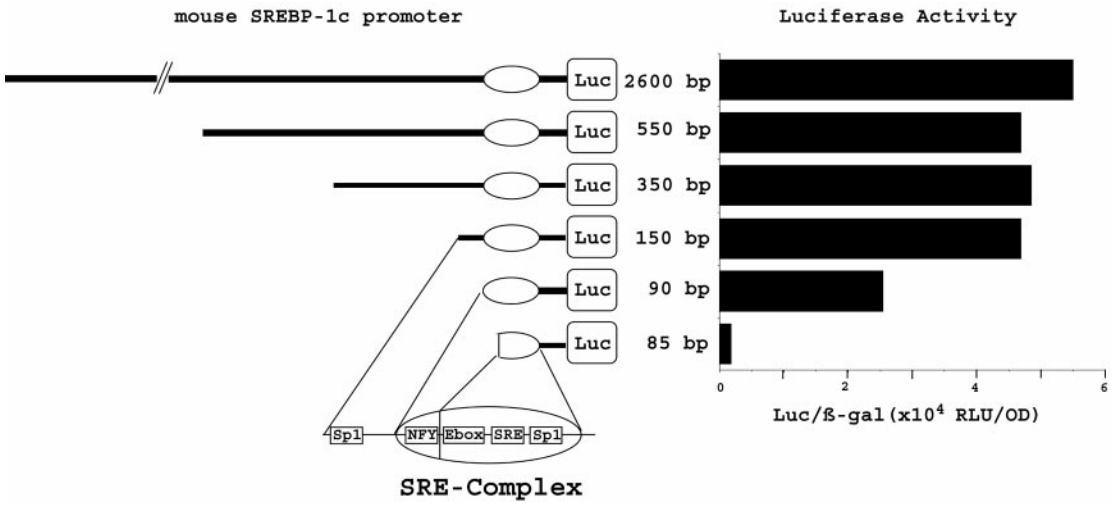

FIG. 2. **Promoter activities of SREBP-1c genomic 5′-flanking fragments with different sizes as measured by luciferase reporter gene assay in 293 cells.** Each promoter genomic fragment of the mouse SREBP-1c gene was prepared as described under "Experimental Procedures" and was subcloned upstream of the firefly luciferase gene of the promoter-less plasmid GL2 basic. The size of the promoter fragment is shown. The relative position of functional units (Sp1 site and the SRE complex) is shown. The 293 cells were transfected with each reporter and a reference plasmid, pSV-β-gal, and thereafter were cultured for 24 h in DMEM with 10% fetal bovine serum. Luciferase activity was measured in and normalized by measuring β-galactosidase activity elicited by a cotransfected pSV-β-gal. The values are the means of three independent experiments.

tiation site of the mouse SREBP-1c gene (Fig. 1). A computer-assisted search (the TFSEARCH program based upon the TRANSFAC data base (30)) of the 5′-flanking region of the mouse SREBP-1c promoter revealed some elements that have potential roles in its transcriptional regulation. It did not pick up any TATA box; however, there was a TATA-like sequence (CTTTAAAG) 25 bp upstream from the putative initiation site. No CAAT box has been found to lie within the expected distance from the start site. Two Sp1-binding sites were found at −102 and −59 bp. Adjacent to the downstream Sp1 site, there was a sequence highly similar (9 of 10 bp) to one of classic sterol regulatory elements, SRE3 (CTCACCCCAG), which was found in the farnesyl diphosphate synthase gene promoter and was extensively analyzed in terms of sterol regulation (31, 32). Interestingly, this SRE3-Sp1 sequence was directly connected to an E-box (CATGTG), which was found as a core sequence of the two SREBP-binding sites of the fatty acid synthase promoter (33). Following the E-box was the NF-Y-binding site (inverted CCAAT box). Thus, this cluster consisting of the NF-Y, E-box, SRE3, and Sp1 sites has a very high probability of being SREBP-binding and activation site and was tentatively designated an SRE complex.

Basal Promoter Activity of the Mouse SREBP-1c Gene—To determine the location of functionally essential sequences required for maximal promoter activity, especially in relation to the SRE complex, we utilized deletional analysis using luciferase gene reporter assays in 293 cells. As shown in Fig. 2, the luciferase gene fused to the 2.6-kb flanking fragment, the longest of the promoter fragments used in this study, gave substantial luciferase activity in 293 cells. The SREBP-1c promoter activities of different length promoters were compared in the cells cultured at subconfluency in DMEM containing high (25 mM) glucose and 10% fetal bovine serum without addition of cholesterol derivatives as a basal condition. Shorter versions containing 0.55, 0.4, and 0.15 kb of the promoter showed essentially the same activity as the 2.6-kb fragment. The 90-bp construct containing the intact SRE complex but not the upstream Sp1 site showed approximately 50% of the activity of the 2.6-kb construct. This suggests that although the first Sp1 site contributes to some considerable promoter activity, the 90-bp promoter region contains nearly maximal activity in this cell culture condition. The deletion of the NF-Y site completely abolished the basal activity, demonstrating that this NF-Y site is essential for basal promoter activity of SREBP-1c.

SREBP Activation of the Mouse SREBP-1c Gene Promoter—The SRE containing promoters analyzed to date in sterol-regulated genes contain NF-Y or Sp1 sites adjacent to SREs, and these cofactors were shown to be indispensable for sterol regulation (32, 34–37). The initial deletion study suggests that the components of the SRE complex are crucial for both basal activity and sterol regulation. Therefore, we focused on the 90-bp construct. We constructed sequential deletion and mutant versions for each component, the NF-Y site, the E-box, SRE-3, and the Sp1 site and measured their promoter activities to evaluate their contributions. First, each luciferase construct was tested in presence of nuclear SREBP-1a that was highly expressed by cotransfection (Fig. 3). Medium was supplemented with cholesterol and 25-hydroxycholesterol to minimize the effects of endogenous SREBPs. As predicted, co-expression of SREBP-1a robustly (20-fold) increased the luciferease activities of the intact 90-bp construct (NESSp-Luc). Deletion of the NF-Y site (ESSp-Luc) caused a marked reduction in the basal activity and a complete disappearance in the SREBP-1a induction, suggesting that NF-Y is indispensable for SREBP-1 activation. Deletion of both the NF-Y-binding site and the E-box (SSp-Luc), leaving just the SRE-3 and the Sp1 site also caused a complete loss of both basal activity and SREBP-1a induction, indicating that the SRE3 and adjacent Sp1 sites, which would appear to be a complete functioning SREBP target site, are not sufficient for SREBP activation. Interestingly, mutation of E-box (mE-Luc) caused a 3-fold decrease in a basal activity but retained a high induction level similar to that of native 90-bp construct after SREBP-1a overexpression, rendering this E-box mutant construct the highest responder (40-fold) to SREBP-1a induction. These data demonstrate that the E-box is involved in some basal activity by a factor(s) other than SREBP. In contrast, when a mutation was introduced into the SRE3 site (mSRE-Luc), the basal activity was minimally affected, but SREBP-1a induction was completely abolished. Additional mutation in the E-box (mES-Luc) did not cause any further change in SREBP-1a induction. Finally, introduction of a mutation in the Sp1 site, which abolished Sp1-binding (mSp1-Luc), caused a substantial decrease in the basal activity but did not affect the fold change by

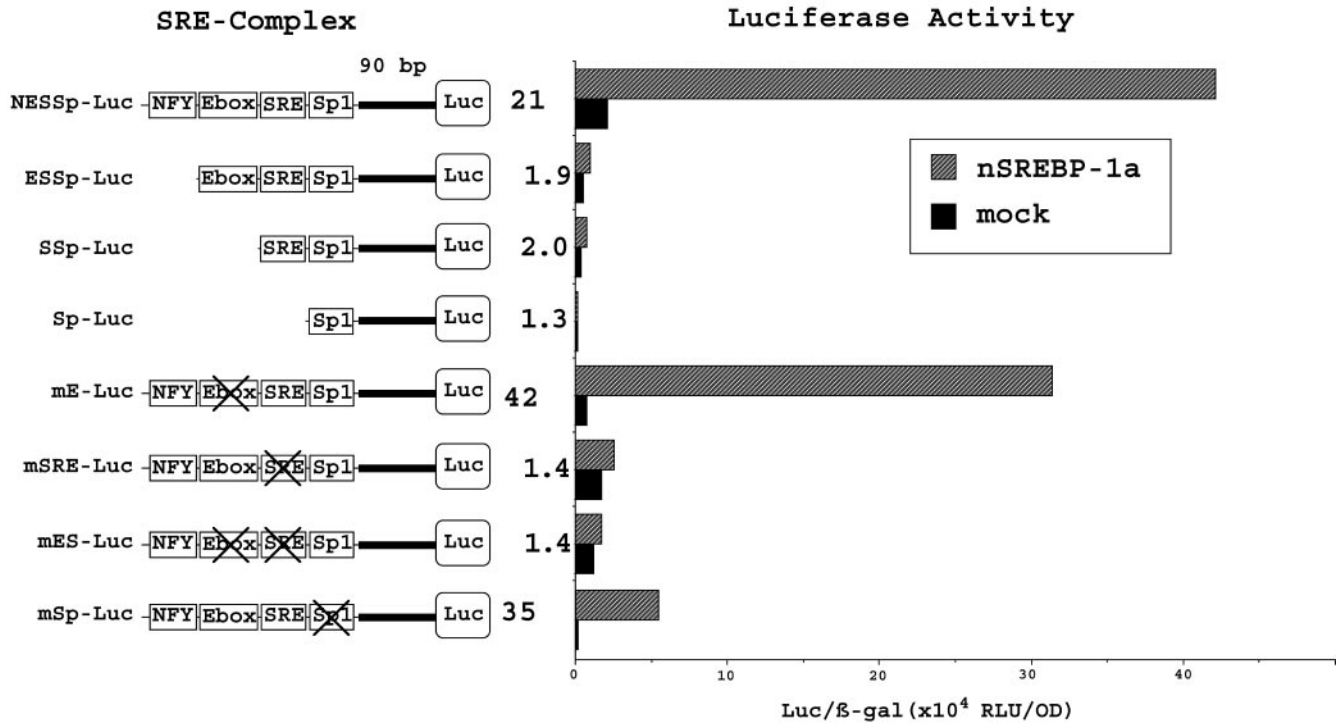

FIG. 3. **Activation of the SREBP-1c promoter by SREBP-1a co-expression in luciferase reporter gene assay.** The luciferase reporter gene pBP-1c90-Luc (NESSp-Luc), which contains the SRE complex, and its various deletion or mutation constructs where each putative binding site was deleted or mutated were constructed as described under "Experimental Procedures." Each reporter gene was cotransfected with a reference plasmid, pSV-β-gal and pCMV-SREBP1a, expression vector of nuclear human SREBP-1a (6), or empty expression vector CMV7 (29) into 293 cells. After transfection, the cells were incubated in DMEM with 10% fetal bovine serum supplemented with 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol (suppressed condition) to suppress endogenous SREBPs for 24 h. The luciferase activity was measured and normalized by β-galactosidase activity. Indicated values are fold changes of the values from SREBP-1a expression experiments relative to controls. The values were means of two independent experiments.

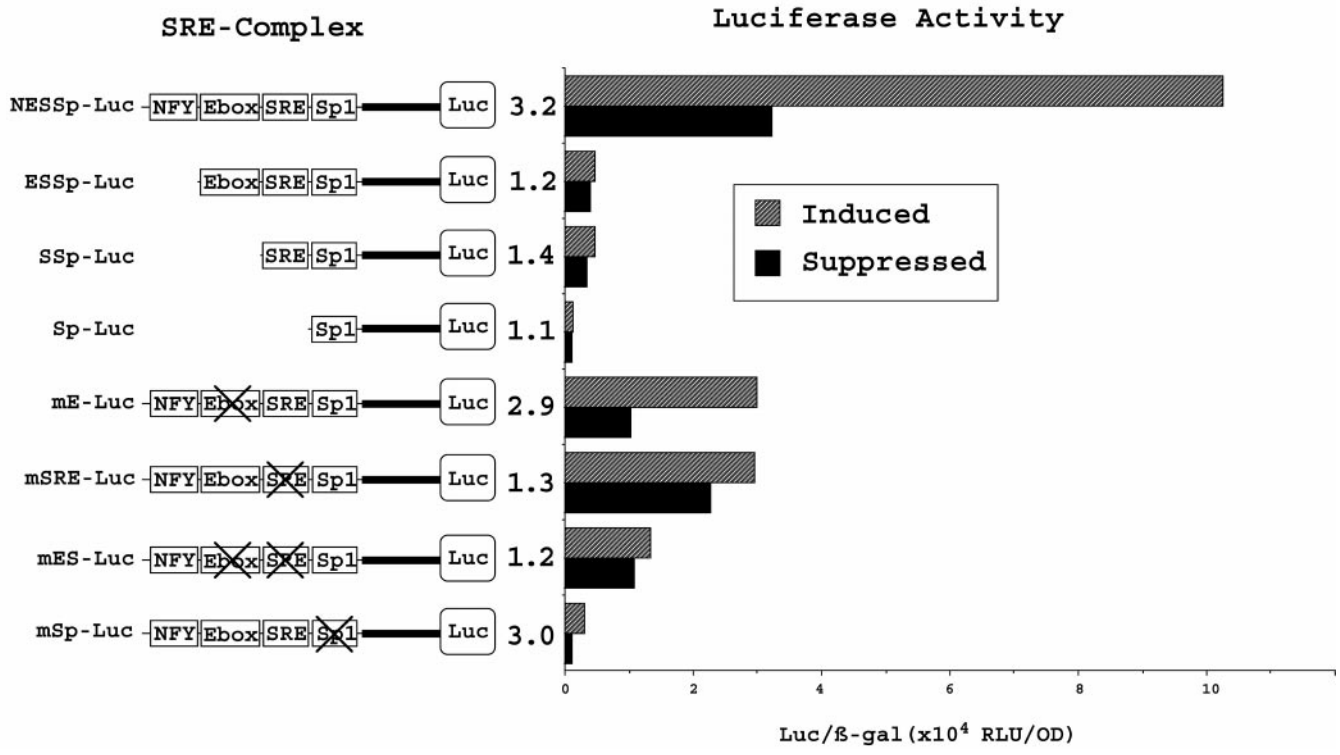

FIG. 4. **Activation of the SREBP-1c promoter through the SRE complex in absence of sterols (an induced condition) in luciferase reporter gene assay.** The same set of experiments was performed as for Fig. 3 except that the cells were incubated in induced or suppressed condition. The induced condition consists of DMEM with 5% delipidated fetal calf serum plus 10 μM pravastatin, and the suppressed condition is DMEM with 10% fetal bovine serum and 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol. Relative fold changes of values from induced condition *versus* suppressed condition are shown. The values are the means of three independent experiments.

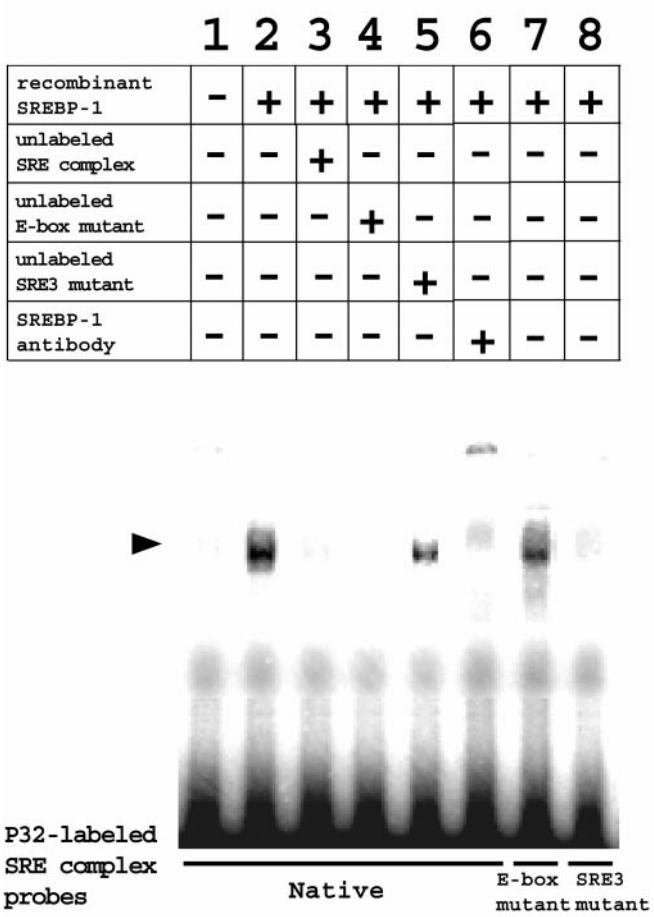

FIG. 5. **Gel shift assay for SREBP-1 binding to SRE complex.** A double-stranded DNA fragment corresponding the SRE complex (Fig. 1) was labeled with [α-^{32}P]dCTP and incubated in the reaction mixture with (*lanes 2–8*) or without (*lane 1*) recombinant nuclear SREBP-1c protein. Specificity of SREBP-1 binding (indicated by the *arrow*) to the SRE complex probe was confirmed by a supershift after the addition of SREBP-1 antibody (*lane 6*). In competition assays (*lanes 3–5*), a 1000-fold molar excess of an unlabeled SRE complex DNA (*lane 3*) or mutated DNA in which E-box (*lane 3*) or SRE3 (*lane 4*) was modified to abolish binding to leucine zipper proteins or SREBP. Mutant probes in which E-box (*lane 7*) or SRE3 (*lane 8*) were also tested for SREBP-1 binding.

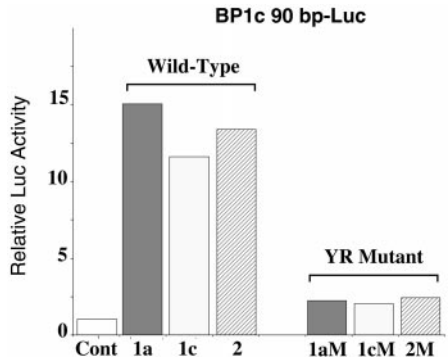

FIG. 6. **Transcriptional activities of nuclear SREBP-1a, -1c, and -2 on the SRE complex in the SREBP-1c promoter in luciferase reporter gene assay in 293 cells.** The luciferase reporter gene pBP-1c90-Luc that contains the SRE complex was cotransfected with each CMV promoter expression vector and a reference plasmid, pSV-β-gal, into 293 cells as described under "Experimental Procedures." The expression vectors used were pCMV-SREBP-1a, pCMV-SREBP-1c, pCMV-SREBP-2, and their corresponding mutant plasmids in which a conserved tyrosine residue in the basic region was replaced by arginine. The luciferase activities were normalized by β-galactosidase activity and the values are expressed as fold change relative to that of control vector (pCMV7).

SREBP-1a activation. Together, these data clearly demonstrate that SREBP induction of the SREBP-1c promoter is completely attributed to the NF-Y site and SRE3 and not to the E-box or the Sp1 site. Meanwhile, both E-box and Sp1 sites contribute to the basal promoter activity independent of SREBP.

Next, the promoter activities of those constructs were compared in transfection studies in which the cells were cultured in suppressed and induced conditions for sterol regulation to see the effects of endogenous SREBPs in the cells. As shown in Fig. 4, the promoter activity of the basic 90-bp construct (NESSp-Luc) in induced condition was three times higher than that in suppressed condition. The same set of deletion and mutation analysis in this endogenous regulation gave essentially the same data as from SREBP-1a co-expression, confirming the importance of NF-Y and SRE3 for SREBP activation. The only exception was that mutated E-box construct gave the same fold activation in induced *versus* suppressed condition as the wild type construct.

Luciferase reporter assays for the 90-bp construct indicated that SREBP-1a can activate the SREBP-1c promoter activity through the SRE complex. To demonstrate the direct binding of SREBP to SRE complex, gel mobility shift assay was performed. Fig. 5 shows that the SRE complex probe was shifted after the addition of SREBP-1 protein (*lane 2*). The specificity of the binding was confirmed by its supershift by SREBP1 antibody (*lane 6*). In competition assays, the shifted band disappeared after addition of excess amount of the unlabeled SRE complex probe (*lane 3*) or its E-box mutant version (*lane 4*). In contrast, addition of the same excess amount of SRE complex probe with a mutation in SRE3 did not compete binding of SREBP-1 to native SRE complex probe (*lane 5*). Furthermore, labeled E-box mutated probe bound to SREBP-1 (*lane 7*), but the SRE3 mutated probe did not (*lane 8*). These data are consistent with the results of the luciferase assays and indicate that SREBPs activate the SREBP-1c promoter through binding to the SRE3 in the SRE complex.

Nuclear SREBP-1a, -1c, and -2 were compared in the transcriptional activities for the 90-bp construct of SREBP-1c promoter. Interestingly, all SREBP isoforms elicited similar activation when overexpressed in 293 cells (Fig. 6). Replacement of arginine for the conserved tyrosine residue in the basic region of SREBP family has been shown to abolish SREBP binding to SRE and not to E-box (5). The effect of this mutation on the 90-bp construct activity was estimated in each SREBP isoform. This mutation in each SREBP isoform essentially caused them to lose their activity, supporting that activation of native SREBP is mediated through binding to SRE3 and not to E-box (Fig. 6).

Upstream Region Responsible for Induction of the SREBP-1c Promoter by Sterols—The basic 90-bp construct contains an SRE and seems to be involved in activation of SREBP-1c expression by SREBPs and also in sterol regulation. To see whether the sterol regulation of this construct can be reflected in the longer and, thus, more physiological promoter, we estimated sterol regulation of longer versions of endogenous and exogenous SREBPs. Interestingly, in suppressed conditions with both cholesterol and 25-hydroxycholesterol in the medium, the longer promoter (400 bp to 2.6 kb) constructs showed 5-fold higher activity than the 90-bp construct in both SREBP-1a co-expression and induced/suppressed experiments (Fig. 7). This contrasts to the observation in the experiment done in just fetal bovine serum without cholesterol where the difference was only 2-fold (Fig. 2), suggesting that sequence upstream of the SRE complex (between -400 and -90 bp) contains some promoter activity that could be induced by ste-

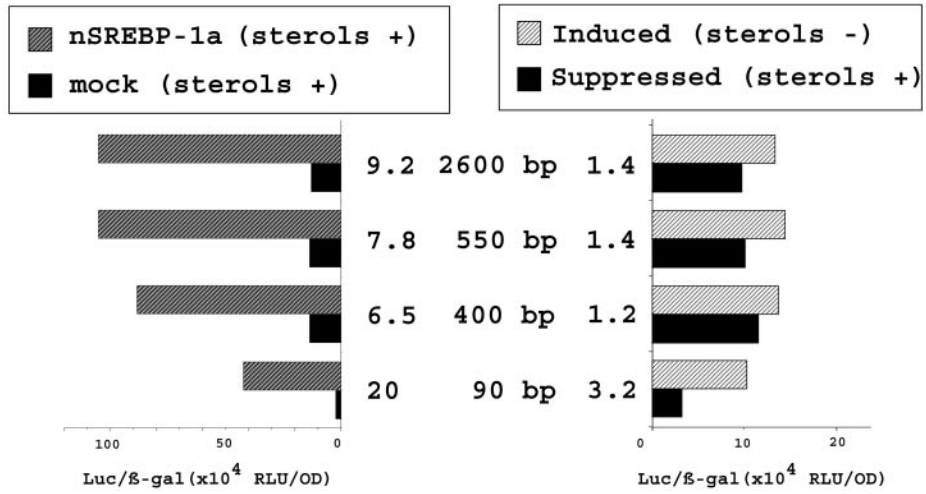

FIG. 7. **Dissociation of responses of the SREBP-1c gene promoter to nuclear SREBP and sterols.** The SREBP-1c promoter luciferase reporter plasmids (pBP1c2600, pBP1c550, pBP1c400, and pBP1c90) were transfected with a reference plasmid, pSV-β-gal, into 293 cells. The *left panel* shows the results from the experiments performed in cotransfection with pCMV-SREBP1a and incubation in suppressed condition as described in the legend to Fig. 3. The *right panel* contains the results from induced and suppressed conditions as described in Fig. 4. The values represent fold change relative to suppressed condition.

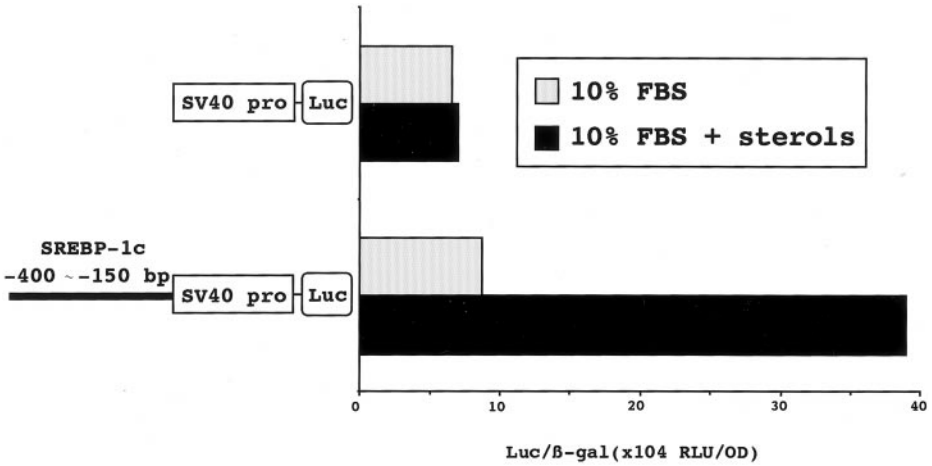

FIG. 8. **Presence of sterol-induced region upstream of the SRE complex in the SREBP-1c promoter.** The DNA fragment upstream of the SRE complex (between −150 and −400 bp) was subcloned into a luciferase reporter plasmid that contains SV40 promoter (pGL2 promoter vector). The plasmid was transfected with pSV-β-gal into 293 cells and cultured in DMEM with 10% fetal bovine serum in presence or absence of sterols (1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol). Empty pGL2 promoter vector was also transfected as negative control.

rols. Co-expression of SREBP-1a increased luciferase activity of the 90-bp construct approximately 20-fold. This fold increase was substantially reduced in the 2.6-, 0.5-, and 0.4-kb constructs (6–9). This tendency was more clear in induced *versus* suppressed conditions (Fig. 7, *right panel*). In suppressed condition, activity of the longer version was three times higher than that of the 90-bp construct, whereas there was no essential increase in the activity by addition of upstream sequences to the 90-bp construct in induced condition. These data suggest that there is a regulatory sequence between −400 and −90 bp (upstream of the 90-bp construct) that activates the promoter activity in suppressed condition and not in induced condition. To confirm this, the promoter DNA fragment between −400 and −150 bp was fused to SV40 promoter containing luciferase reporter gene (pGL2 promoter). As shown in Fig. 8, luciferase activity of this construct was increased 6-fold by addition of cholesterol and 25-hydroxycholesterol, demonstrating that the region contains an element(s) that confers sterol inducibility.

DISCUSSION

Positive Loop Activation of SREBP-1c—The current study clearly demonstrates that mouse SREBP-1c promoter contains a sterol regulatory element and can be induced by SREBPs and leads us to the speculation that nuclear SREBP-1c protein can autoregulate its own SREBP-1c precursor. In short, as long as cleavage of SREBP is active, it can further activate SREBP-1c expression to form a positive feedback loop (Fig. 9). This positive loop may partially explain the overshooting phenomenon of induction of lipogenic enzyme genes in the refed state. Refeeding with low fat/high carbohydrate diet after fasting causes a marked induction of SREBP-1 mRNA and active protein (17, 18), resulting in the activation of most hepatic lipogenic enzyme genes, often referred to overshooting. The molecular identity of the initial lipogenic signal is currently unknown, although it is likely to be some glucose metabolite. Once it starts induction of SREBP-1c, the autoregulatory loop of SREBP-1c induction could cause the overshooting. It is also possible that another factor or system could be responsible for the overshooting of SREBP-1c and downstream lipogenic genes. However, even if so, this positive loop should contribute to the supply of precursor protein to maintain the overshooting of SREBP-1 and thus, lipogenesis. Supporting this idea, SREBP-1 knockout mice, in which induction of hepatic lipogenic genes is severely impaired, also exhibit substantially low level of hepatic aberrant SREBP-1 mRNA, which is derived from an intact SREBP-1c promoter of the disrupted SREBP-1 gene (12). Studies with a knock-in animals in which only the SRE3 in the promoter is disrupted would be required to test this hypothesis. An autoregulation mechanism has been known in the modulation of gene expression of C/EBP family members (38), which play an important role in adipogenesis where SREBP-1c might be also involved (5).

Concomitant Presence of Sterol Inducible and Regulatory Elements in SREBP-1c Promoter—The presence of an SRE in the promoter of SREBP-1c places SREBP-1c expression at least partially under sterol regulation. The current transfection studies with the 90-bp construct in induced and suppressed conditions for sterol regulation supports this. However, the

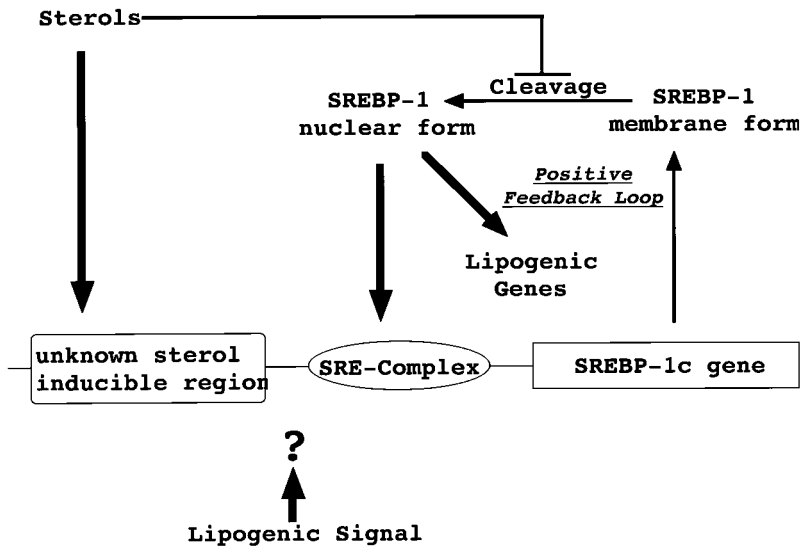

FIG. 9. **Autoloop effect by SREBPs and dual opposite effects by sterols on the mouse SREBP-1c gene.** The presence of the SRE complex in the SREBP-1c promoter makes it possible to form a positive loop expression of SREBP-1c as long as SREBP cleavage is active, possibly playing a role in a nutritional regulation of its target lipogenic genes. Sterols should have a feedback regulation of SREBP-1c expression through the SRE complex, whereas sterols can activate SREBP-1c expression through an unknown upstream sterol-inducible region.

extent of sterol regulation for SREBP-1c expression seems limited *in vivo*. The longer versions that reflect more physiological regulation showed much less sterol regulation (Fig. 7) because of the concomitant upstream sterol-inducible element. Consistent with these complex results from the current promoter analysis, hepatic SREBP-1 mRNA is not decreased (or even increased) by feeding mice a diet enriched with cholesterol and cholic acid, which causes huge accumulation of cholesterol in the livers, a situation that mimics a sterol suppressed condition in cultured cells and abolishes the SREBP-2 mature form. Therefore, the physiological relevance of the SRE complex in the SREBP-1c promoter may be inducibility of SREBP-1c by SREBPs rather than sterol regulation.

Current transfection studies comparing sterol regulation of the 90-bp construct and longer versions suggest that upstream of the SRE complex in the SREBP-1c promoter, there is a *cis*-element that induces SREBP-1c expression in suppressive conditions, presumably by cholesterol and/or oxysterols. The region somewhere between −400 and −150 bp seems responsible. This would explain why sterol regulation of SREBP-1c expression *in vivo* is obscure, because there are both sterol-suppressive and -inducible elements in the promoter. Currently, the physiological relevance of two elements in the same promoter that have opposite ways of cholesterol regulation is unknown. It can be speculated that sterol regulation by the SREBP cleavage system, which potentially inhibits lipogenesis, can be counterbalanced by this element and ensures lipogenesis even in the presence of sterols. Currently, studies are underway to specify the exact sequence of the *cis*-element and transcription factors responsible for this new regulation of SREBP-1c. This will help in understanding of the mechanism by which both sterol and fatty acid regulation are linked through SREBP-1c transcription.

Carbohydrate Response Elements in the SREBP-1c Promoter—An important question remains to be answered about the high carbohydrate diet-responsive element in the SREBP-1c promoter. It has been shown that hepatic SREBP-1c mRNA is induced by high carbohydrate diet and refeeding regiment after fasting. Although our finding of possible feed-positive loop of SREBP1c induction might partially explain the robust and prolonged induction of SREBP-1c *in vivo*, there must be a mechanism of transcription that responds to the initial lipogenic signal. We could not detect a significant induction of SREBP-1c promoter activity by increased glucose concentration in the medium in HepG2 cells and saw only a 3-fold activation in 293 cells, which hampered further analysis of possible carbohydrate response elements. There are some possible reasons why we could not reproduce the dietary *in vivo* observation in reporter gene assays in cultured cells. The 2.6-kb promoter that we currently used might not contain the responsible element that could reside far upstream or downstream such as in an intron or the 3′-flanking region, or the dietary response of SREBP-1c might be partially post-transcriptional. It is also possible that in luciferase assays, the considerable amount of transfected DNA containing SRE complex consumed endogenous SREBP and prevented the autoloop formation, which would indicate that reporter gene assays are intrinsically inappropriate to detect this kind of autoloop phenomenon. To address this question, further studies including transgenic mice harboring different longer SREBP-1c promoter reporter genes or finding a better cell culture system are required.

The current study revealed that the mouse SREBP-1c promoter contains multiple and complex regulatory elements through which both cholesterol and carbohydrates might be involved in the gene regulation of SREBP-1c. Further analysis on these elements might open up the new aspect of linkage between cholesterol, fatty acids, and fuel metabolism.

Acknowledgments—We are grateful to Drs. Joseph L. Goldstein and Michael S. Brown for continuous support of the project.

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1997) *Cell* **89,** 331–340
2. Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11041–11048
3. Sakai, J., Duncan, E. A., Rawson, R. B., Hua, X., Brown, M. S., and Goldstein, J. L. (1996) *Cell* **85,** 1037–1046
4. Wang, X., Briggs, M., Hua, X., Yokoyama, C., Goldstein, J., and Brown, M. (1993) *J. Biol. Chem.* **268,** 14497–14504
5. Kim, J. B., Spotts, G. D., Halvorsen, Y. D., Shih, H. M., Ellenberger, T., Towle, H. C., and Spiegelman, B. M. (1995) *Mol. Cell. Biol.* **15,** 2582–2588
6. Shimano, H., Horton, J. D., Shimomura, I., Hammer, R. E., Brown, M. S., and Goldstein, J. L. (1997) *J. Clin. Invest.* **99,** 846–854
7. Shimano, H., Horton, J. D., Hammer, R. E., Shimomura, I., Brown, M. S., and Goldstein, J. L. (1996) *J. Clin. Invest.* **98,** 1575–1584
8. Horton, J. D., Shimomura, I., Brown, M. S., Hammer, R. E., Goldstein, J. L., and Shimano, H. (1998) *J. Clin. Invest.* **101,** 2331–2339
9. Shimomura, I., Shimano, H., Horton, J. D., Goldstein, J. L., and Brown, M. S. (1997) *J. Clin. Invest.* **99,** 838–845
10. Goodridge, A. G. (1987) *Annu. Rev. Nutr.* **7,** 157–185
11. Hillgartner, F. B., Salati, L. M., and Goodridge, A. G. (1995) *Physiol. Rev.* **75,** 47–76
12. Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A. H., Osuga, J., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35832–35839
13. Kim, H. J., Takahashi, M., and Ezaki, O. (1999) *J. Biol. Chem.* **274,** 25892–25898
14. Worgall, T. S., Sturley, S. L., Seo, T., Osborne, T. F., and Deckelbaum, R. J. (1998) *J. Biol. Chem.* **273,** 25537–25540
15. Thewke, D. P., Panini, S. R., and Sinensky, M. (1998) *J. Biol. Chem.* **273,**

21402–21407
16. Yahagi, N., Shimano, H., Hasty, A. H., Amemiya-Kudo, M., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35840–35844
17. Horton, J. D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 5987–5992
18. Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B., and Spiegelman, B. M. (1998) *J. Clin. Invest.* **101,** 1–9
19. Kim, J. B., and Spiegelman, B. M. (1996) *Genes Dev.* **10,** 1096–1107
20. Shimomura, I., Hammer, R. E., Richardson, J. A., Ikemoto, S., Bashmakov, Y., Goldstein, J. L., and Brown, M. S. (1998) *Genes Dev.* **12,** 3182–3194
21. Foretz, M., Guichard, C., Ferre, P., and Foufelle, F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12737–12742
22. Foretz, M., Pacot, C., Dugail, I., Lemarchand, P., Guichard, C., Le Liepvre, X., Berthelier-Lubrano, C., Spiegelman, B., Kim, J. B., Ferre, P., and Foufelle, F. (1999) *Mol. Cell. Biol.* **19,** 3760–3768
23. Koo, S.-H., and Towle, H. C. (2000) *J. Biol. Chem.* **275,** 5200–5207
24. Hasty, A. H., Shimano, H., Yahagi, N., Amemiya-Kudo M., Perrey S., Yoshikawa, T., Osuga, J., Okazaki, H., Tamura, Y., Iizuka, Y., Shionoiri, F., Ohashi, K., Harada, K., Gotoda, T., Nagai, R., Ishibashi, S., and Yamada, N. (2000) *J. Biol. Chem.* **275,** 31069–31077
25. Shimomura, I., Bashmakov, Y., Ikemoto, S., Horton, J. D., Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 13656–13661
26. Soukas, A., Cohen, P., Socci, N. D., and Friedman, J. M. (2000) *Genes Dev.* **14,** 963–980
27. Xu, J., Nakamura, M. T., Cho, H. P., and Clarke, S. D. (1999) *J. Biol. Chem.* **274,** 23577–23583
28. Shimano, H., Shimomura, I., Hammer, R. E., Herz, J., Goldstein, J. L., Brown, M. S., and Horton, J. D. (1997) *J. Clin. Invest.* **100,** 2115–2124
29. Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264,** 8222–8229
30. Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A. E., Kel, O. V., Ignatieva, E. V., Ananko, E. A., Podkolodnaya, O. A., Kolpakov, F. A., L., P. N., and Kolchanov, N. A. (1999) *Nucleic Acids Res.* **26,** 364–370
31. Ericsson, J., Jackson, S. M., Lee, B. C., and Edwards, P. A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 945–950
32. Ericsson, J., Jackson, S. M., and Edwards, P. A. (1996) *J. Biol. Chem.* **271,** 24359–24364
33. Magana, M. M., and Osborne, T. F. (1996) *J. Biol. Chem.* **271,** 32689–32694
34. Sanchez, H. B., Yieh, L., and Osborne, T. F. (1995) *J. Biol. Chem.* **270,** 1161–1169
35. Jackson, S. M., Ericsson, J., Osborne, T. F., and Edwards, P. A. (1995) *J. Biol. Chem.* **270,** 21445–21448
36. Xiong, S., Chirala, S. S., and Wakil, S. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3948–3953
37. Magana, M. M., Koo, S. H., Towle, H. C., and Osborne, T. F. (2000) *J. Biol. Chem.* **275,** 4726–4733
38. Timchenko, N., Wilson, D. R., Taylor, L. R., Abdelsayed, S., Wilde, M., Sawadogo, M., and Darlington, G. J. (1995) *Mol. Cell. Biol.* **15,** 1192–1202