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

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Michiyo Amemiya-Kudo‡, Hitoshi Shimano§, Tomohiro Yoshikawa‡, Naoya Yahagi‡, Alyssa H. Hasty§, Hiroaki Okazaki‡, Yoshiaki Tamura‡, Futoshi Shionoiri‡, Yoko Iizuka‡, Ken Ohashi‡, Jun-ichi Osuga‡, Kenji Harada‡, Takanari Gotoda‡, Ryuichiro Sato**, Satoshi Kimura‡, Shun Ishibashi‡, and Nobuhiro Yamada§

From the ‡Department of Metabolic Diseases, Faculty of Medicine and the **Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8655, Japan and the §Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

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 leads to sequence and analyzes 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 overshot 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 administration 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

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‡ To whom correspondence should be addressed: Dept. of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Ibaraki 305-8575, Japan. Fax: 81-298-53-3053; E-mail: shimano-tky@umin.ac.jp.

§ Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8655, Japan and the Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

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; 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 NH2-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 cholesteroenic 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-

DMEM, Dulbecco’s minimally modified medium. This paper is available online at http://www.jbc.org
Mouse SREBP-1c promoter

**EXPERIMENTAL PROCEDURES**

**Materials**—Standard molecular biology techniques were used. We obtained cholesterol and 25-hydroxycholesterol from Sigma, Redivue [α-32P(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 Promotion and Construction of Luciferase Reporter Genes**—A SacI 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 BamHI-AvrII fragment (0.55 kb) extending from 5′-untranslated region to the promoter region was subcloned into Smal site of pGL2 basic vector (Promega) (pBP1c550-Luc). The adjacent upstream BamHI fragment (2 kb) was further cloned into pBPl550-Luc to generate a 2.6-kb fragment construct (pBPl2600Luc). 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′-TAAGAGCTCGG-TACCTCCCTACTGGGC-3′; 5′ primer, pBP1c400-Luc 5′-GGGCCTGTTACGGGAA-3′; pBP1c150-Luc, 5′-GGGAGAAACCCAGCTGCT-3′; pBP1c-90(NESS)Luc, 5′-CTGCTGATTGGCCATGTGCGCTACCCGA-3′; pBP1c-Essp-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-AvrII-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-NkI-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-mNkI-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-mNkII-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-mNkIII-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-mNkIV-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-mNkV-Luc, 5′-CCATGTGCGCTACCCGA-3′; pBP1c-mNkVI-Luc, 5′-CCATGTGCGCTACCCGA-3′. The DNA probe was prepared by annealing a complementary DNA sequence, and an SREBP target, designated SRE complex that is composed of an NF-Y site (inverted CCAAT), an E-box, an SRE3-like (31) E-boxes and a p300 site. At 25°C, the luciferase activity as measured by standard kits (Promega).

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

| SRE-Complex       | Luciferase Activity |
|-------------------|---------------------|
| NESSp-Luc         | 90 bp               |
| SREBP-1a          | Luc                 |
| 21                |
| ESSp-Luc          | Luc                 |
| 1.9               |
| SSp-Luc           | Luc                 |
| 2.0               |
| Sp-Luc            | Luc                 |
| 1.3               |
| mE-Luc            | Luc                 |
| 42                |
| mSRE-Luc          | Luc                 |
| 1.4               |
| mES-Luc           | Luc                 |
| 1.4               |
| mSp-Luc           | Luc                 |
| 35                |

Luc/β-gal (x10⁴ RLU/OD)

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.

| SRE-Complex       | Luciferase Activity |
|-------------------|---------------------|
| NESSp-Luc         | Luc                 |
| 3.2               |
| ESSp-Luc          | Luc                 |
| 1.2               |
| SSp-Luc           | Luc                 |
| 1.4               |
| Sp-Luc            | Luc                 |
| 1.1               |
| mE-Luc            | Luc                 |
| 2.9               |
| mSRE-Luc          | Luc                 |
| 1.3               |
| mES-Luc           | Luc                 |
| 1.2               |
| mSp-Luc           | Luc                 |
| 3.0               |

Luc/β-gal (x10⁴ RLU/OD)
SREBP to SRE complex, gel mobility shift assay was performed that SREBP-1a can activate the SREBP-1c promoter activity type construct.

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.

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 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-
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 2400 and 290 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 2400 and 2150 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 refeed 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
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 feed-back regulation of SREBP-1c expression through the SRE complex, whereas sterols can activate SREBP-1c expression through an unknown upstream sterol-inducible region.

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 oysterols. 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 SREBP-1c promoter may be inducibility of SREBP-1c by SREBPs rather than sterol regulation.

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