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Mouse Elovl-6 promoter is an SREBP target

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Abstract

Elovl-6, a long fatty acid elongase, contributes to de novo synthesis of fatty acids and regulates hepatic insulin sensitivity. Hepatic regulation of Elovl-6 gene expression in various nutritional conditions suggested that, like other lipogenic enzyme genes, Elovl-6 is a target of SREBP-1, a transcription factor governing fatty acid synthesis. Supportively, adenoviral RNAi knockdown of SREBP-1 in mouse liver suppressed Elovl-6 mRNA and fatty acid synthase levels. Therefore, we analyzed mouse Elovl-6 gene promoter to determine its role as an SREBP-1 target. Luciferase reporter assays of 1.4-kb 5′ flanking region of mouse Elovl-6 gene in HepG2 cells demonstrated that nuclear SREBPs activated the Elovl-6 promoter, highlighting two SREBP binding sites: proximal SRE-1 and distal SRE-2. EMSA indicated that SRE-1 had higher affinity than SRE-2 for SREBP. ChIP assays confirmed in vivo binding of hepatic nuclear SREBP-1c protein. These data demonstrated that Elovl-6 is regulated directly and primarily by SREBP-1c.

Keywords: Fatty acids; Elongase; Sterol regulatory element-binding protein; Lipogenic enzymes; Transcription factor

Sterol regulatory element-binding proteins (SREBPs) are members of the basic helix–loop–helix-leucine zipper family of transcription factors [1–3] that regulate biosynthesis of both cholesterol and fatty acids. SREBP is initially bound to the rough endoplasmic reticulum membrane, and is cleaved in a sterol-dependent manner to liberate the amino-terminal portion containing a basic helix–loop–helix-leucine zipper domain (nuclear SREBP), which enters the nucleus where it can bind to specific sterol response elements (SREs) in the promoters of target genes. Three isoforms of SREBP, SREBP-1a and -1c (also known as ADD1), and SREBP-2 have been characterized [4–6]. SREBP-2 plays a crucial role in the regulation of cholesterol biosynthesis, whereas SREBP-1 mainly controls the gene expression of lipogenic enzymes [7,8]. Nuclear SREBP-2 has high affinity for classic SREs, which are usually found in the promoters of cholesterogenic genes and the LDL receptor gene. Nuclear SREBP-1 also has a broad binding capacity for SRE-like sequences, including E-boxes that are occasionally found in the promoters of lipogenic genes [9].

Elovl-6 (also known as LCE/FACE) is a lipogenic enzyme and was identified as an SREBP-1 target by microarray analysis of SREBP-1 transgenic mice. Elovl-6 exhibits fatty acyl-CoA elongase activity specific for long chains (C12–C16 saturated and monosaturated fatty acids) and is important for tissue fatty acid composition. Recently, analysis of Elovl-6-deficient mice demonstrated that hepatic Elovl-6 plays a crucial role in obesity-induced insulin resistance [10–12].

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Hepatic expression of Elovl-6 is regulated by nutritional factors and liver X receptor (LXR), both of which are important regulators of SREBP-1c. Moreover, the nutritional regulation of hepatic Elovl-6 is significantly suppressed in SREBP-1-deficient mice [11]. These data indicate that Elovl-6 is regulated by SREBP-1 in mouse liver. Although one potential SRE:SREBP binding site in the Elovl-6 promoter was mentioned on the basis of sequence alignment analysis [10], the mouse Elovl-6 promoter has not been fully analyzed and has yet to be confirmed as a direct target of SREBPs. In this study, a detailed promoter analysis of mouse Elovl-6 gene was performed. Our results confirmed that the Elovl-6 promoter is an SREBP target. Two SREBP binding sites were found and were required for maximal activation by SREBP-1, but the downstream SRE was more crucial than the upstream SRE.

Materials and methods

Materials. T0901317 and eicosapentaenoic acid (EPA) sodium salt were obtained from Sigma Chemicals. Redivue [α-^{32}P] dCTP was purchased from GE Healthcare Bio-Sciences KK. Restriction enzymes were obtained from Takara Bio Inc. and plasmid DNAs for transfection were prepared with the QIAGEN Plasmid Midi Kit (Qiagen).

Animal experiments. All animal studies were approved by the Animal Care Committee of the University of Tsukuba. Male C57BL/6J mice were purchased from Clea Japan. The mice were housed in colony cages, maintained on a 12-h light/dark cycle, and given free access to water and a standard chow diet (MF, Oriental Yeast) or a high-fat/high-sucrose diet [13]. After adenovirus injection, the mice were sacrificed in the light phase in a 4 h-fasted state. Tissues were isolated immediately, weighed, and stored in liquid nitrogen.

Preparation of recombinant adenovirus. SREBP-1-specific RNA interference (RNAi) constructs were subcloned into U6 entry vector (Invitrogen) using the SREBP-1 coding sequences 5′-tagagcgagcgttgaactgtatt-3′, and a recombinant adenoviral plasmid was generated by homologous recombination with pAd promoterless vector (Invitrogen). We produced recombinant adenovirus in 293 cells and purified them by CsCl gradient centrifugation as described previously [14,15].

Total RNA preparation and Northern blotting. Total RNA was isolated from mouse livers and H2.35 cells using Sepazol RNA I Super reagent (Nacalai Tesque Inc.). Northern blot analysis was performed using a ^{32}P-labeled probe as described previously [11,12].

Expression plasmids and reporter plasmids. All expression vectors have been described previously [16,17]. The reporter plasmid Elovl-6-1399-Luc was engineered to contain a fragment of the mouse Elovl-6 promoter from −1399 to ±0 bp cloned into the MluI/XhoI sites of the pGL3 basic vector (Promega) containing the coding sequences of firefly luciferase cDNA. Other constructs were produced by PCR using this construct as a DNA template, and the PCR products were inserted into the pGL3 basic vector. The primers used in PCR were as follows: 5′-primer Elovl-6-473-Luc, 5′-cttacgcgtagggtgctgcgggat-3′; Elovl-6-273-Luc, 5′-cttacgcgtccaagaggtggcgaatg-3′; Elovl-6-173-Luc, 5′-cttacgcgtaacaaaactatcccccagggc-3′; Elovl-6-103-Luc, 5′-ccgacgcgt- cccgatcgcacgaggggga-3′; and Elovl-6-88-Luc, 5′-cttacgcgtgggaggagatttccctgtaga-3′, 3′-primer 5′-gggctcgagaccgtaggaggaaatgaatg-3′. The site-directed mutagenesis constructs, SRE-1(m) and SRE-2(m)-Luc, were produced by PCR with the following primers: SRE-1(m)-5′, 5′-gggttaattttcccgatataaagaggggaggagatttc-3′; SRE-1(m)-3′, 5′-gaaatctcctcccctctttatatcgggaaaattaaccc-3′; SRE-2(m)-5′, 5′-catttatttgcctggagttctacg-gacagagaacctggc-3′; and SRE-2(m)-3′, 5′-gccaggttctctgtccgtagaactccaggcaaataaatg-3′. The double-mutagenesis construct SRE-1 2(m)-Luc was produced by ligation of SRE-1(m) and SRE-2(m) PCR products digested with HindIII. Ligation was performed using the Quick Ligation Kit (New England Biolabs Inc.).

Cell cultures. HepG2 cells were cultured in minimum essential medium Eagle (Sigma) supplemented with 1 mM sodium pyruvate (GIBCO), 1% non-essential amino acids (GIBCO), 10% FBS, and 1% penicillin–streptomycin (Sigma). For luciferase assay, HepG2 cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well. H2.35 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 200 nM dexamethasone (Sigma), 4% FCS, and 1% penicillin–streptomycin. For chromatin immunoprecipitation assay (ChIP), H2.35 cells were seeded in 15-cm plates and the indicated chemicals were added to the confluent cells.

Transfection and luciferase assays. Each expression plasmid (200 ng), luciferase reporter plasmid (200 ng), and pSV40-*Renilla* plasmid (50 ng) was cotransfected into HepG2 cells using the FuGENE6 reagent (Roche Applied Science) according to the manufacturer's instructions. After 24 h of transfection, cells were washed with phosphate buffer saline (PBS) and harvested. Luciferase assays were performed according to the manufacturer's instructions (Promega), and luciferase activity was quantified using the ARVO SX 1420 multilabel counter (Perkin Elmer Life Sciences). As the internal standard, SV40 *Renilla* luciferase control vector was also cotransfected to normalize for transfection efficiency.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as described previously [17,18]. In brief, a recombinant SREBP-1a protein was produced using the TNT T7 quick-coupled transcription/translation system (Promega). The reaction mixture was incubated with in vitro synthetic protein lysate and then analyzed on 5% polyacrylamide gels in 0.25 × TBE at room temperature. The SREBP-1 antibody (anti-SREBP-1, sc-8984; Santa Cruz) was used to confirm specific binding. The wild-type (wt) and mutant (mut) probes for EMSA were as follows: Elovl-6 SRE-1 (wt), gttaattttcccgatcgcacgaggggaggac; Elovl-6 SRE-1 (mut), gttaattttcccgatATAaAgaggggaggac; Elovl-6 SRE-2 (wt), gcctggagttctcatgccagagaacctggc; and Elovl-6 SRE-2 (mut), gcctggagttctACGgAcagagaacctggc. Each probe contains the indicated SRE (underlined). Mutations in these SREs are shown in capital letters.

ChIP assay. ChIP assays were performed as previously described with some modifications [19]. In brief, we immunoprecipitated the complexes of genomic DNA and SREBP-1 with antibodies against SREBP-1 (rabbit polyclonal; No. 931 for mouse SREBP-1 [20]) from H2.35 cells. The extracted genomic DNA was then subjected to PCR amplification. To amplify the Elovl-6 promoter region containing SRE-1 and SRE-2, the following primer sets were used: for SRE-1, 5′-ccagtccaccaaccaccttc-3′ (sense), and 5′-aatgaatgccttgcggtctt-3′ (antisense); and for SRE-2, 5′-cgggatcgaggtagtgagga-3′ (sense), and 5′-gcctggcctttgaatgaagt-3′ (antisense).

Results

To evaluate the short-term effect of SREBP-1c deletion on Elovl-6 gene expression, normal C57BL6 mice were infected with adenoviruses containing SREBP-1 RNAi. Northern blot analysis revealed that knockdown of SREBP-1 was complete and was accompanied by a strong suppression of fatty acid synthase levels, a representative SREBP-1 target gene. Elovl-6 gene expression was also severely inhibited, indicating that Elovl-6 could be a potential target of SREBP-1 (Fig. 1A). Subsequently, the mouse Elovl-6 promoter was tested as an SREBP target. The 1.4-kb 5′ region upstream of the mouse Elovl-6 gene was cloned into a luciferase vector and used for luciferase reporter assay after the transfection of expression plasmids for various transcription factors into cells. The Elovl-6 promoter was strongly activated by coexpression of SREBP-1a, -1c, and -2. The reporter was weakly activated by HNF-4α and Sp-1, but not LXR, RXR, and PPARs (Fig. 1B).

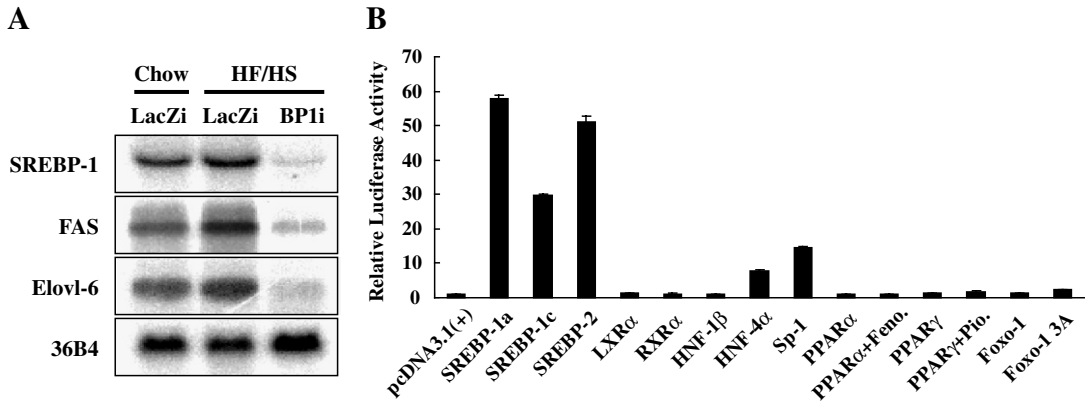

Fig. 1. Regulation of Elovl-6 promoter by SREBP-1. (A) Northern blot analysis of 36B4, SREBP-1, FAS, and Elovl-6 gene expression in the liver of SREBP-1 knockdown mice. Knockdown of hepatic SREBP-1 was performed by adenoviral expression of RNA interference (RNAi). Seven-week-old male C57BL/6J mice were fed a standard chow or high-fat/high-sucrose (HF/HS) diet for 15 weeks, followed by tail vein injection with adenovirus encoding RNAi targeting SREBP-1 (BP1i) or LacZ (LacZi) sequence (adenoviral dose of 1.0×10^{11} viral particles per mouse). After 6 days of HF/HS feeding, the mice were sacrificed in a 4-h-fasted state. (B) Activation of the Elovl-6 promoter by SREBPs. The mouse Elovl-6 promoter region (1399 bp) was fused to a luciferase reporter gene (Elovl-6-1399-Luc). HepG2 cells were cotransfected with Elovl-6-1399-Luc as a reporter gene, pSV40-*Renilla* as a reference, and the indicated expression plasmids or control vector (pcDNA3.1). At the same time, 100 μM fenofibrate (Sigma) or 10 μM pioglitazone (Cayman Chemical) was added to the indicated cells. The relative Luc activity was expressed as a fold change to that of the control vector (pcDNA3.1). The luciferase activity was normalized to *Renilla* luciferase activity and the assays were performed in triplicate. Results are expressed as means ± SEM. Feno, fenofibrate; Pio, pioglitazone.

As shown in Fig. 2A, the mouse Elovl-6 promoter sequence contains two putative SREs as potential SREBP binding sites, SRE-1 and SRE-2, and an E-box. Sequential deletion studies indicated that SRE-1 was mainly responsible for SREBP activation (Fig. 2B), whereas SRE-2 contributed weakly and E-box did not contribute at all. EMSA indicated that SREBP-1a protein binds strongly to SRE-1, but weakly to SRE-2 (Fig. 3A). The introduction

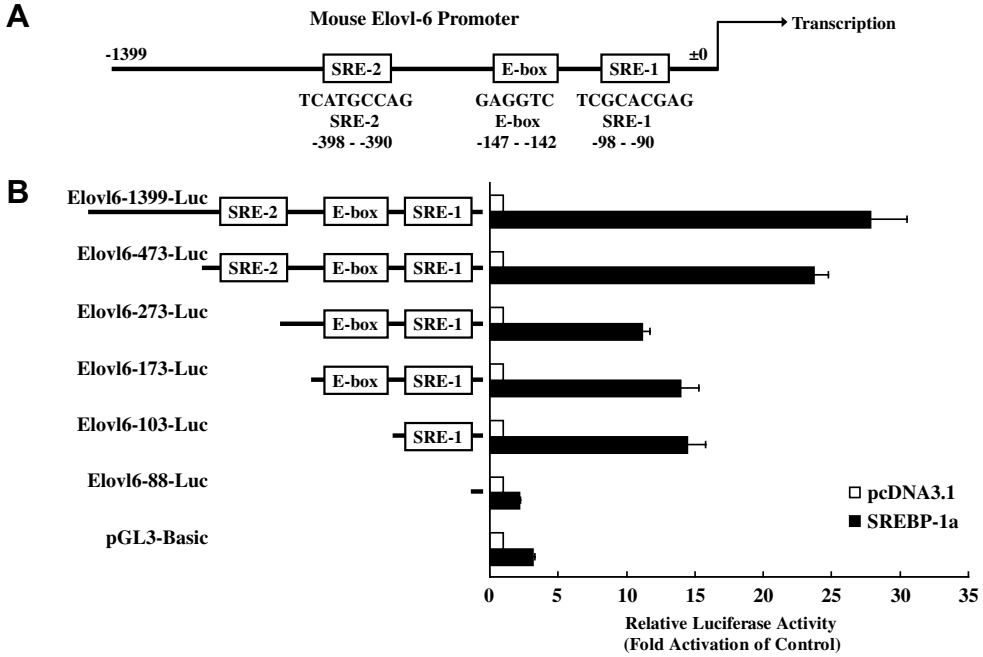

Fig. 2. Requirement of both SRE-1 and SRE-2 regions for SREBP-1a activation of mouse Elovl-6 promoter. (A) Schematic representation of mouse Elovl-6 promoter with the sequences and locations of potential SREBP binding sites (SRE-2, E-box, and SRE-1 as novel potential sites). (B) Deletion studies in SREBP-1a activation of the mouse Elovl-6 promoter. The schematic representation of mouse Elovl-6 promoter luciferase deletion constructs used in this study is shown. Various reporter gene plasmids (Elovl-6-1399, -473, -273, -173, -103, and -88-bp-Luc) were cotransfected into HepG2 cells with pSV40-*Renilla* as a reference and SREBP-1a expression plasmid or pcDNA3.1. The relative Luc activity was expressed as a fold change to that of the control vector (pcDNA3.1). The luciferase activity was normalized to *Renilla* luciferase activity, and the assays were performed in triplicate. Results are expressed as means ± SEM.

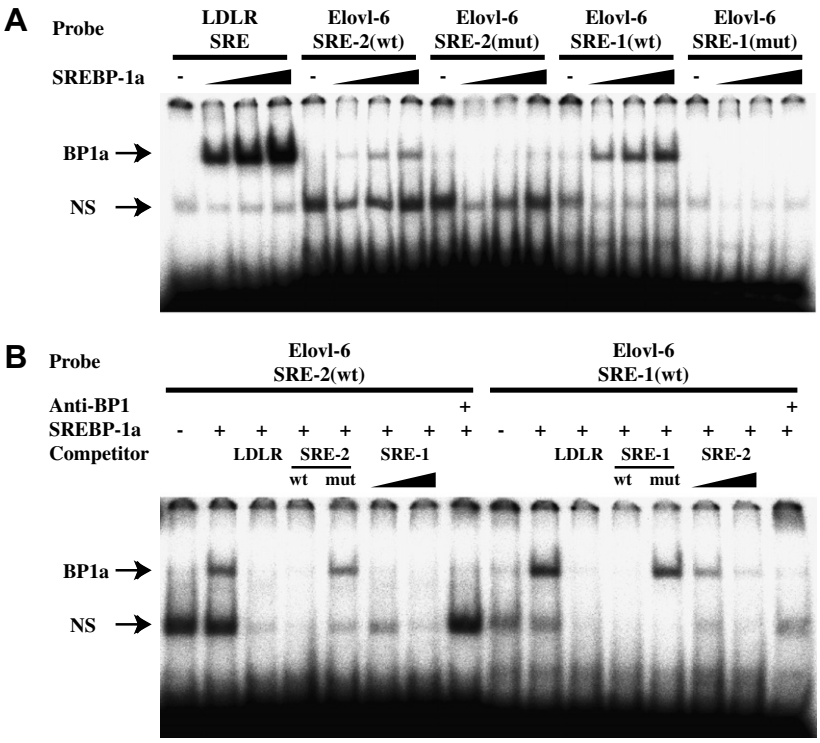

Fig. 3. SREBP-1a binding to SRE-1 and SRE-2 in mouse Elovl-6 promoter. (A and B) EMSA with radiolabeled probes for the authentic human LDL receptor SRE (LDLR SRE) and Elovl-6 SREs (wild-type (wt) and mutant (mut) as indicated in "Materials and methods"). In vitro translated SREBP-1a proteins were incubated with these labeled probes. After incubation, the binding reaction mixtures were subjected to EMSA on a nondenaturing polyacrylamide gel as described in "Materials and methods". (A) Various amounts of in vitro translated products were incubated with probes. (B) The competition was performed using unlabeled probes as competitors in 250- or 1000-fold molar excess. Specific binding was confirmed using a polyclonal antibody against SREBP-1 for competition. The bound SREBP-1a/DNA complex (BP1a) and a non-specific shifted complex (NS) are shown in Fig. 3.

of mutations into the sites SRE-1 and SRE-2 cancelled the binding. The signal was abolished by the addition of cold authentic SRE DNA probe from the LDL receptor promoter, and supershifted by the addition of SREBP-1 antibody, confirming the specificity of SREBP bindings to SRE-1 and SRE-2 (Fig. 3B). Mutual competition between SRE-1 and SRE-2 in EMSA demonstrated that SRE-1 has higher affinity for SREBP than SRE-2. This supports data from the deletion studies, which show that SRE-1 is more important for SREBP activation. Results from EMSA and the deletion studies were further supported by mutation analysis (Fig. 4A). The promoter construct containing both SRE-1 and SRE-2 was activated by SREBP. Mutation of SRE-1 and/or SRE-2 decreased this activation to the extents predicted by the deletion studies and EMSA. Finally, direct binding of SREBP-1 to SRE-1 and SRE-2 in vivo was estimated by ChIP assay. First, SREBP-1c in H2.35 cells was activated by T0901317, an LXR agonist, and suppressed by EPA (Fig. 4B). Concomitantly, Elovl-6 expression was activated by T0901317 and suppressed by EPA. ChIP assay of nuclei from these sets of treated H2.35 cells confirmed direct binding of SREBP-1 to SRE-1 in vivo (Fig. 4C). The signal from SRE-1 was consistently changed by T0901317 and EPA in an SREBP-1-regulated manner. In contrast, the positive signal showing direct binding to SRE-2 could not be detected.

Discussion

The present study clearly demonstrates that Elovl-6 is an SREBP target. As previously reported, expression of Elovl-6 was regulated in an SREBP-dependent manner in transgenic and knockout mice [11]. Nutritional regulation of Elovl-6 expression, such as fasting–refeeding, high-fat diet, polyunsaturated fatty acids, or in ob/ob mice, as well as activation by LXR could be mediated through SREBP-1c. Our data confirmed that SREBP-regulated Elovl-6 expression is mediated through the direct binding of SREBP to the Elovl-6 promoter. In luciferase reporter assays, SREBP-1a and SREBP-2 equally activated the Elovl-6 promoter, whereas SREBP-1c was less active. This pattern of transactivation, depending upon SREBP isoforms, was similarly observed in many typical SREBP target gene promoters [9]. Elovl-6 was a crucial step in the fatty acid synthesis pathway, suggesting that SREBP-1c is the major transcription factor for the physiological regulation of Elovl-6.

A series of promoter analyses demonstrated that there are two SREBP binding sites: SRE-1 and SRE-2. Although both could potentially contribute to the activation of the Elovl-6 promoter, proximal SRE-1 having higher affinity for SREBP is more important for SREBP activation. ChIP assay of our experimental settings demonstrated that

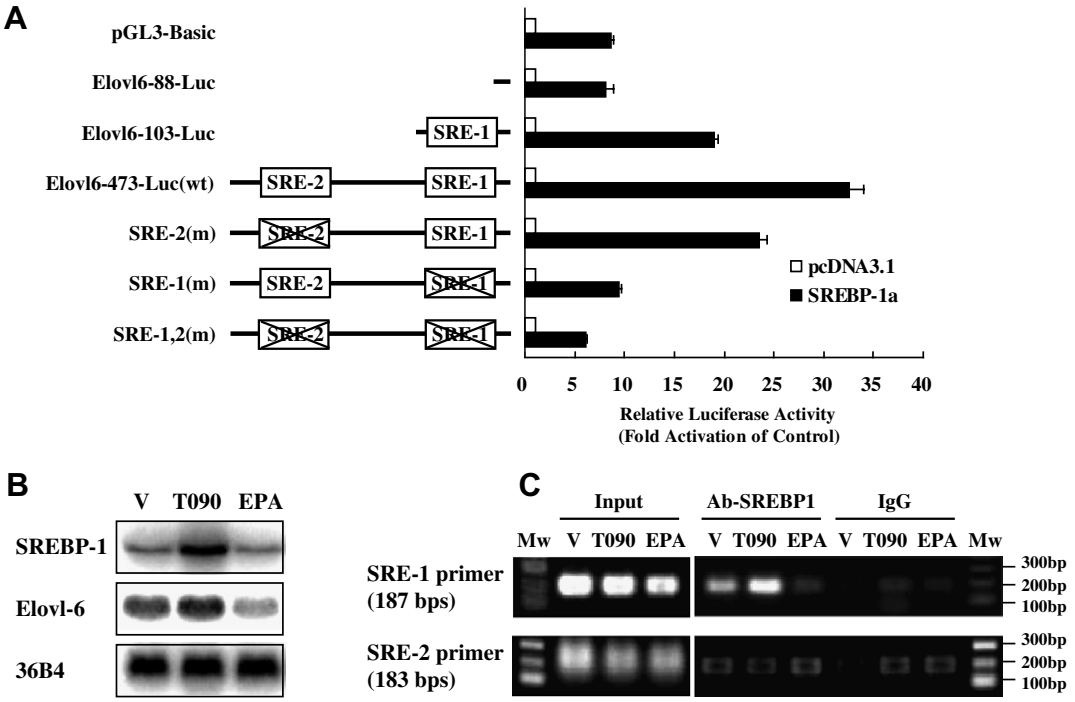

Fig. 4. SRE-1 as a novel SREBP binding site is stronger than SRE-2 in exogenous and endogenous SREBP-1 activation of Elovl-6 promoter, (A) Effects of mutation in SRE-1 and/or SRE-2 on SREBP-1a activation of Elovl-6 promoter. Native (Elovl-6-473, -103, and -88-bp-Luc), mutant-SRE-1 (SRE-1(m)), mutant-SRE-2 (SRE-2(m)), or combined mutant- SRE-1 and 2 (SRE-1, 2(m)) reporter gene plasmids were cotransfected into HepG2 cells with pSV40-*Renilla* and SREBP-1a expression plasmid or pcDNA3.1. These mutant plasmids have the same mutations in the indicated SREs as the EMSA probes. The relative Luc activity was expressed as a fold change to that of the control vector (pcDNA3.1). The luciferase activity was normalized to *Renilla* luciferase activity and the assays were performed in triplicate. Results are expressed as means ± SEM. (B) Northern blot analysis of 36B4, SREBP-1, and Elovl-6 gene expression in H2.35 cells. Confluent cells were incubated with T0901317 (1 μM) or EPA (50 μM) for 24 h. V, vehicle. T090, T0901317. (C) Chromatins prepared from H2.35 cells (under the same conditions as described in A) were subjected to chromatin immunoprecipitation assay with an anti-mouse SREBP-1 antibody and rabbit IgG as the negative control. Immunoprecipitate samples were subjected to PCR using primers to amplify the SRE-1- or SRE-2-containing region of the mouse Elovl-6 promoter. *M*w, molecular weight.

SREBP bound only to SRE-1 in vivo. Therefore, these results indicate that SRE-1 is the primary binding site of SREBP in the Elovl-6 promoter.

Regulatory mechanism(s) other than SREBPs may also exist. To elucidate such mechanisms, further studies on Elovl-6 regulation using regions far upstream or introns of this gene are required. However, presently it can be concluded that SREBPs are the primary transcription factors for the proximal promoter region of mouse Elovl-6 gene.

Acknowledgments

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