

SAFB1, an RBMX-binding protein, is a newly identified regulator of hepatic SREBP-1c gene

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Sterol regulatory element-binding protein (SREBP)-1c plays a crucial role in the regulation of lipogenic enzymes in the liver. We previously reported that an X-chromosome-linked RNA binding motif (RBMX) regulates the promoter activity of *Srebp-1c*. However, still unknown was how it regulates the gene expression. To elucidate this mechanism, we screened the cDNA library from mouse liver by yeast two-hybrid assay using RBMX as bait and identified scaffold attachment factor B1 (SAFB1). Immunoprecipitation assay demonstrated binding of SAFB1 to RBMX. Chromatin immunoprecipitation assay showed binding of both SAFB1 and RBMX to the upstream region of *Srebp-1c* gene. RNA interference of *Safb1* reduced the basal and RBMX-induced *Srebp-1c* promoter activities, resulting in reduced *Srebp-1c* gene expression. The effect of SAFB1 overexpression on *Srebp-1c* promoter was found only in the presence of RBMX. These results indicate a major role for SAFB1 in the activation of *Srebp-1c* through its interaction with RBMX. [BMB reports 2009; 42(4): 232-237]

INTRODUCTION

Sterol regulatory element-binding protein-1 (SREBP-1), a key transcription factor regulating the expression of lipogenic enzymes in the liver (1), consists of two isoforms, SREBP-1a and SREBP-1c (2, 3). The *Srebp-1c* gene is reported to be inducible, and its expression in the liver is transcriptionally regulated by nutrients such as glucose (4) and hormones such as insulin (5) or catecholamine (6), while the *Srebp-1a* gene is constitutively expressed.

We previously reported that the response of *Srebp-1c* gene expression after feeding in mouse liver is genetically determined (7). In addition, we identified an X-chromosome-linked RNA binding motif (RBMX), formerly called heterogenous nuclear ribonucleoprotein G, (hnRNP G) as a candidate molecule, ac-

counting at least partially for the mechanism of genetic alterations of *Srebp-1c* response after feeding, recognizing a single nucleotide polymorphism (-468 G to A) (8). Recently, the effect of RBMX on transcriptional regulation of genes other than *Srebp-1c* was also reported (9), but it remains unclear how RBMX regulates the promoter activities of genes. The *Rbmx* gene is located on the X chromosome and expressed in all primate nuclei ubiquitously. It binds to nascent pre-messenger RNA, and contributes to alternative splicing (10-13). It is not fully understood whether RBMX can recognize the particular DNA sequence and bind to it or whether it can interact with the RNA-polymerase complex. Since the ability of RBMX to associate with other proteins has already been reported (14), it is possible that it may regulate *Srebp-1c* promoter activity through interaction with other DNA-binding proteins or co-activators.

In this study, we examined whether RBMX acts as a co-activator associating with other proteins. To identify the proteins interacting with RBMX in mouse liver, we screened mouse cDNA library prepared from the liver by using two-hybrid assay with RBMX as bait. As described here, we identified scaffold attachment factor B1 (SAFB1) as an RBMX-binding protein, and we proposed that binding of SAFB1 to RBMX may participate in the mechanism by which RBMX regulates mRNA expression of *Srebp-1c* gene.

RESULTS

Identification of proteins directly binding to RBMX by yeast two-hybrid assay

To identify the proteins that interact with RBMX protein, we performed screening with yeast two-hybrid assay using RBMX as bait. We screened the cDNA library prepared from mouse liver with oligo (dT) primer. A total of 4.8×10^5 clones were plated onto 15-cm plates containing synthetic -His/-Leu/-Trp medium. Thirty transformants were isolated. After the fourth round of selection on synthetic -Ade/-His/-Leu/-Trp medium (high stringency) + X- α -gal plate, 18 colonies turned blue and grew robustly. Sequence analysis revealed that six clones among them had common sequence. Blast search revealed that this sequence is located in the *Safb1* gene (GenBank accession no. [XM001480312](#)), and we confirmed the specificity

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of its interaction with RBMX in yeast. SAFB1 has a DNA-binding motif, an RNA-binding motif, and a protein interaction motif. The six clones identified by two-hybrid assay screening correspond to the C-terminus of *Safb1*, which is reported to be a protein interaction domain (15, 16).

SAFB1 and RBMX bind to the upstream region of *Srebp-1c* promoter *in vivo*

To confirm binding of SAFB1 to the upstream region of the *Srebp-1c* gene in mouse liver, we performed chromatin immunoprecipitation (ChIP) assay. As shown in Fig. 1A, the ChIP assay clearly showed that both RBMX and SAFB1 bind to the upstream region of *Srebp-1c* gene promoter in mouse liver.

SAFB1 directly binds to RBMX in the liver

To confirm the binding of SAFB1 to RBMX in the liver, immunoprecipitation assay was performed. SAFB1 was detected in the immunoprecipitated proteins with RBMX antibody but was not detected with control IgG (Fig. 1B). Furthermore, RBMX was detected in the immunoprecipitated proteins with SAFB1 antibody but not with control IgG (Fig. 1C). These results demonstrate direct interaction between SAFB1 and RBMX in mouse liver.

Effects of RNA interference on the activity of *Srebp-1c* promoter and its gene expression

To examine whether SAFB1 functionally interacts with *Srebp-1c* promoter and its gene expression, we knocked down the *Safb1* expression using small interference RNA (siRNAs). Fig. 2A shows the results of immunoblotting at 48 h after transfection of *Safb1* siRNA in Fao cells. The SAFB1 expression decreased to 20% of

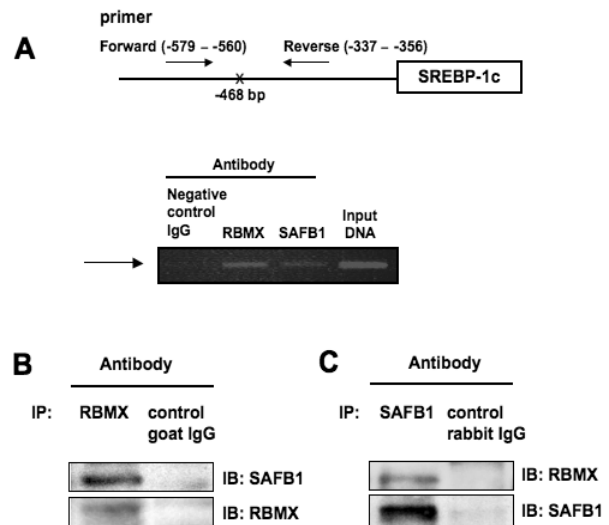


Fig. 1. The binding of SAFB1 at around -468 bp of *Srebp-1c* promoter and RBMX in mouse liver. (A) ChIP assay was performed using anti-SAFB1, anti-RBMX antibody, or negative control IgG. Input DNA or ChIP samples were used as a template for PCR using primer sets for the region containing the mouse *Srebp-1c* promoter around -468 bp. (B) The immunoprecipitation assay was performed using nuclear protein extracts prepared from mouse liver with anti-RBMX antibody or control goat IgG. Immunoblotting was performed with anti-SAFB1 antibody. (C) The immunoprecipitation assay was performed using nuclear protein extracts prepared from mouse liver with anti-SAFB1 antibody or control rabbit IgG. Immunoblotting was performed with anti-RBMX antibody. IP: immunoprecipitate; IB: immunoblot.

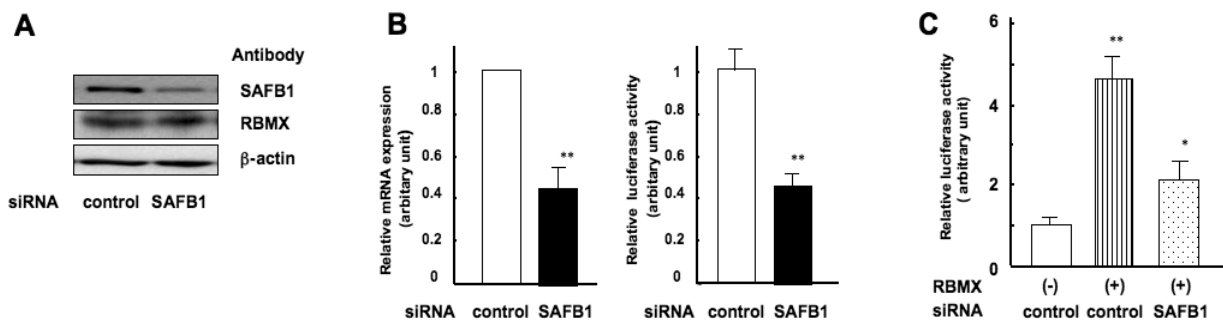


Fig. 2. Effects of *Safb1* RNA interference on the activity of *Srebp-1c* promoter and its gene expression. (A) The effects of *Safb1* siRNA on levels of protein in Fao cells. The whole cell lysates were extracted after 48-h transfection with control or *Safb1* siRNA (100 nM), and detected by using immunoblotting with anti-SAFB1 antibody, anti-RBMX antibody and anti- β -actin antibody as a loading control. (B) The effects of *Safb1* siRNA on *Srebp-1c* gene expression (left) and activity of *Srebp-1c* promoter (right). Fao cells were transfected with control or *Safb1* siRNA (100 nM), with (for luciferase assay) or without (for RT-PCR) 0.25 μ g/well of reporter plasmid (pGL3-CBA/JN), 0.2 μ g/well of reference plasmid (pCMV- β -gal) and analyzed after 48 h. Total RNA was isolated, and mRNA expressions of *Srebp-1c* were measured by RT-PCR. Analytical data were adjusted with internal control *Capdh*. The cells were lysed and analyzed by luciferase assay. All luciferase activities were normalized to the β -galactosidase activity. The data are expressed as means \pm SEM. ** $P < 0.01$ versus control siRNA. $n = 5$ for RT-PCR and $n = 6$ for luciferase assay. (C) The effects of *Safb1* siRNA on *Srebp-1c* promoter under overexpression of RBMX. Fao cells in a 12-well plate were transfected with reporter plasmid (0.25 μ g/well), reference plasmid (0.2 μ g/well), pcDNA-RBMX or control pcDNA plasmid (1 μ g/well), and control or *Safb1* siRNA (100 nM). Following 48-h incubation, the cells were lysed and analyzed by luciferase assay. All luciferase activities were normalized to the β -galactosidase activity. Data are expressed as means \pm SEM. ** $P < 0.01$ versus the control siRNA without overexpression of RBMX; * $P < 0.05$ versus the control siRNA with overexpression of RBMX. $n = 8$ in each group.

basal levels of protein. The effect of *Safb1* siRNA was sequence-specific, since control siRNA had no effect on levels of SAFB1 and *Safb1* siRNA did not affect levels of RBMX expression. As a result, mRNA expression of *Srebp-1c* gene and activity of the *Srebp-1c* promoter were both reduced by 50% in the cells co-transfected with *Safb1* siRNA, but not in those cotransfected with control siRNA (Fig. 2B). In addition, activity of *Srebp-1c* promoter induced by RBMX overexpression was also reduced by about 70% in the cells cotransfected with the *Safb1* siRNA, but not in those cotransfected with control siRNA (Fig. 2C).

Effects of RBMX and SAFB1 expression on *Srebp-1c* promoter activity

To investigate effects of increase in SAFB1 expression on activity of *Srebp-1c* promoter, we first transfected pcDNA-SAFB1 into Fao cells and confirmed expression of SAFB1 by immunoblotting. SAFB1 protein was enhanced in extracts from pcDNA-SAFB1-transfected cells compared to control pcDNA-transfected cells. The SAFB1 overexpression did not affect the levels of RBMX expression (Fig. 3A).

The activity of *Srebp-1c* promoter was remarkably increased when cells were transfected with pcDNA-RBMX, but not when they were transfected with pcDNA-SAFB1. However, co-transfection with pcDNA-SAFB1 and pcDNA-RBMX produced a

significant increase in the activity of *Srebp-1c* promoter ($P < 0.01$) (Fig. 3B). Thus, the effect of SAFB1 overexpression on *Srebp-1c* promoter was found only when RBMX was overexpressed.

DISCUSSION

Here, we identified SAFB1 as an RBMX-binding protein by using yeast two-hybrid assay. This interaction between SAFB1 and RBMX was also confirmed in mouse liver by assessment of immunoprecipitation with either SAFB1 or RBMX antibodies. According with our observation, binding of SAFB1 to RBMX was also recently reported for HEK 293 cells (17). These results suggest a functional role of SAFB1 binding in many types of cells or tissues. It has also been reported that SAFB1 is ubiquitously expressed in many adult tissues, including the testes, mammary glands, and brain (18). We also observed the expression of SAFB1 in mouse liver and in Fao cells, a rat hepatoma cell line. However, the physiological significance of SAFB1 binding to RBMX still remains unknown.

SAFB1 is also known as HAP (hnRNP A1-associated protein) (16) or HET (Hsp27 ERE-TATA-binding protein) (19). SAFB1 was originally identified based on its ability to bind to scaffold/matrix attachment regions and reported to be involved in the transcriptional regulation of small heat shock protein (19) and estrogen receptor (20). Thus, the transcriptional regulation of *Srebp-1c* through RBMX may be influenced by the interaction with SAFB1.

In fact, we observed that the reduction of SAFB1 expression in Fao cells using siRNA results in reduction of basal activity of *Srebp-1c* promoter by 50%. Particularly, the activity of *Srebp-1c* promoter induced by overexpression of RBMX was markedly inhibited by the transfection of *Safb1* siRNA, by 70%. Furthermore, ChIP assay indicates that the RBMX-SAFB1 complex recognizes the specific region of SREBP-1c promoter where we have found a single nucleotide polymorphism affecting *Srebp-1c* expression response to high-fructose diet in mouse liver (7). These results clearly indicate a major role for SAFB1 in activation of *Srebp-1c* promoter, especially in activation through RBMX. We investigated whether SAFB1 itself can directly bind to the promoter region of *Srebp-1c* using yeast one-hybrid assay and found no direct interaction between SAFB1 and *Srebp-1c* promoter (data not shown). Further studies are needed to identify other proteins which directly bind to both *Srebp-1c* promoter region and RBMX.

Based on the present results, however, we cannot delineate the precise molecular mechanism by which SAFB1 up-regulates the *Srebp-1c* promoter. Actually, it has been reported that SAFB1 acts as a corepressor (19, 20). However, it is reported that SAFB1 interacts with the C-terminal domain of RNA polymerase II, and it has been suggested to be involved in transcriptosome complex, coupling transcription and RNA processing (21). This interaction of SAFB1 and transcriptosome might be required for RBMX to activate the *Srebp-1c* promoter. In fact, a nuclear factor can show activation of one gene and

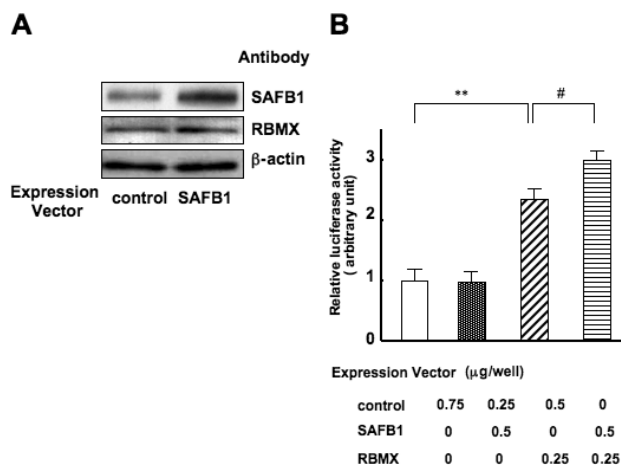


Fig. 3. Effects of SAFB1 overexpression on the activity of *Srebp-1c* promoter. (A) Effects of SAFB1 overexpression on levels of protein in Fao cells. Whole cell lysates were extracted after 48 h of transfection with control pcDNA or pcDNA-SAFB1 plasmid (2 $\mu\text{g}/\text{well}$ in a 6-well plate), and detected by using immunoblotting with anti-SAFB1 antibody, anti-RBMX antibody and anti- β -actin antibody as a loading control. (B) Effects of an increase in RBMX- or SAFB1-expression on activity of *Srebp-1c* promoter. Fao cells in a 12-well plate were transfected with each reporter plasmid (0.25 $\mu\text{g}/\text{well}$), reference plasmid (0.2 $\mu\text{g}/\text{well}$), and a total of 0.75 $\mu\text{g}/\text{well}$ of control pcDNA, pcDNA-SAFB1, or pcDNA-RBMX plasmid. Following 48-h incubation, cells were lysed and analyzed by luciferase assay. All luciferase activities were normalized to the β -galactosidase activity. Data are expressed as means \pm SEM. ** $P < 0.01$ versus transfection with control; # $P < 0.01$ versus transfection with RBMX. $n = 9$ in each group.

inhibition of another gene. For example, it has been reported that the peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α) increases transcription of genes related to mitochondrial biogenesis and fatty acid oxidation and decreases transcription of the GLUT4 gene (22).

Overexpression of SAFB1 does not affect the basal activity of *Srebp-1c* promoter in Fao cells. However, when RBMX is overexpressed, the effect of SAFB1 overexpression on the activity of *Srebp-1c* promoter becomes pronounced. Thus, we speculate that the level of SAFB1 is sufficient for maintaining the basal activity of *Srebp-1c* promoter in Fao cells and that the required amount of SAFB1 for the RBMX to stimulate the activity of *Srebp-1c* promoter depends on the level of RBMX expression. In other words, at the basal level, the level of RBMX expression is rate-limiting for the activity of *Srebp-1c* promoter and SAFB1 expression may be adequate; however when RBMX is overexpressed, the level of SAFB1 expression may become rate-limiting for the full activation of *Srebp-1c* promoter.

In conclusion, we identified SAFB1 as an RBMX-binding protein. SAFB1 binds to RBMX *in vivo*, and regulates the transcriptional activity of *Srebp-1c* promoter associating with RBMX.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were purchased from Invitrogen (Grand Island, NY), anti-RBMX (hnRNP G) antibody (G-17) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-SAFB1 antibody from Bethyl Laboratories (Montgomery, TX), and anti- β -actin antibody from SIGMA (St. Louis, MO).

Animals

CBA/JN male mice were purchased from Charles River Japan (Kanagawa, Japan). Under intraperitoneal pentobarbital anesthesia, the livers were excised, immediately frozen in liquid nitrogen, and stored at -80°C until further use. All experiments were approved by the Animal Care and Use Committee of Shiga University of Medical Science.

Cell culture

Fao (rat hepatoma) cells were kindly provided by Dr. C. R. Kahn (Joslin Diabetes Center, Boston, MA) and cultured at 37°C in 5% CO_2 atmosphere in DMEM supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 g/ml), and L-glutamine (2 mM).

Yeast two-hybrid assay

To identify the proteins that interact with RBMX, yeast two-hybrid assay was performed following the protocol outlined in the MATCHMAKER Two-Hybrid System (Clontech, Palo Alto, CA). Full-length mouse RBMX cDNA was ligated into pGBKT7 (pGBKT7-RBMX) as bait to identify cDNAs that encode putative binding domains capable of interacting with RBMX.

Double-strand cDNA library prepared from mouse liver, pGADT7-Rec vector (*Sma*I-linearized), and pGBKT7-RBMX plasmid were co-transformed into AH109, the yeast bait strain, and Ade⁺, His⁺, Leu⁺, Trp⁺ prototrophs were selected on minimal agar plates containing X- α -Gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside). Colonies that turned blue and grew robustly were prepared for polymerase chain reaction (PCR) using the BD Advantage 2 PCR Polymerase Mix (Clontech, Palo Alto, CA). Products of PCR were sequenced using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and screened with BLAST search.

Construction of RBMX and SAFB1 expression plasmids

The RBMX-expression plasmid, pcDNA-RBMX, was constructed as previously described (8). The 3.1-kb sequence of SAFB1 was amplified using cDNAs derived from mouse liver and was inserted into pT7Blue Blunt vector (Novagen, Madison, WI). After ligation into pT7Blue Blunt vector, the SAFB1 fragment was subcloned by performing long PCR with 5'-ACTGACCCCCGTTGTCCTCT-3' (sense primer) and 5'-CCTGTAGCACGCATGCCGTCCT-3' (antisense primer) and inserted into *Eco*RV-linearized pcDNA3.1(+) using a TaKaRa BKL kit (TaKaRa, Shiga, Japan) to form SAFB1-expressing plasmid (pcDNA-SAFB1). We confirmed that the full-length sequence of pcDNA-SAFB1 is identical to the *Safb1* gene in the NCBI gene database (GenBank accession no. **XM001480312**).

Cell transfection and luciferase assays

Transfections were performed using Superfect Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions with 0.25 $\mu\text{g}/\text{well}$ of the luciferase reporter plasmid carrying the *Srebp-1c* promoter of CBA/JN strain (pGL3-CBA/JN) (7), and 0.2 $\mu\text{g}/\text{well}$ of a β -galactosidase expression plasmid (pCMV- β -gal; Clontech, Palo Alto, CA) as reference plasmid. In the SAFB1 or RBMX expression experiments, a total of 0.75 $\mu\text{g}/\text{well}$ of control pcDNA, pcDNA-SAFB1 or pcDNA-RBMX plasmid was also transfected. The results were quantified with a luminometer and normalized to the β -galactosidase activity measured with the cell extracts.

Isolation of total RNA and Real-time quantitative PCR assay

Total RNAs were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time quantitative PCR (RT-PCR) was performed using the fluorescent dye SYBR Green I and a real-time quantitative PCR system (ABI Prism TM 7500 Sequence Detection System; Perkin-Elmer Applied Biosystems, Foster City, CA).

Primer sets were as follows: rat *Srebp-1c*, 5'-GGAGCCATG GATTGCACATT-3' and 5'-AGGAAGGCTCCAGAGAGGA-3'; and rat glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5'-TGCTGGTCTGAGTATGTGCG-3' and 5'-CAAGCAGTTGG TGGTACAGG-3'. Ct value was used to compute mRNA levels from the standard curve. Analytical data were adjusted with internal control *Gapdh*.

Immunoprecipitation and immunoblotting

Nuclear protein extracts from mouse liver or whole cell lysates from cultured cells were isolated as previously described (7, 23). Immunoprecipitation was performed by incubating 1 mg of the supernatants from liver samples in 1 ml of immunoprecipitation buffer (20 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 140 mM NaCl, 10 mM NaF, 1 mM sodium orthovanadate (Na₃VO₄), 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, aprotinin, and leupeptin, pH 7.5) containing primary antibody at 4°C overnight with agitation and with protein A/G agarose (Santa Cruz Biotechnology) for an additional 2 h. The immunoprecipitates were washed extensively with immunoprecipitation buffer and boiled with Laemmli sample buffer containing 100 mM dithiothreitol to elute for subsequent immunoblotting. Immunoblotting was performed as previously described (23).

RNA interference

siRNA oligonucleotides were designed by Dharmacon (Chicago, IL). The four designed siRNA sequences targeting rat *Safb1* (GenBank accession no. **NM022394**) were 5'-GCAUUAACCAU UUGCAUAA-3' (corresponding to nt 1550-1568 of the ORF), 5'-GCCGAGGCCCUAUGAAGUA-3' (nt 2295-2313), 5'-GUUAAA GAGUCUCGAAAGU-3' (nt 1984-2002), and 5'-ACAACGGGC UCGAGGAGAA-3' (nt 401-419). The siCONTROL Non-Targeting siRNA #1 (Dharmacon) was used as a negative control. We introduced 100 nM of siGENOME SMART pool reagent siRNA into Fao cells using DharmaFECT 1 Reagent (Dharmacon) and confirmed silencing effect by immunoblotting 48 h after transfection.

ChIP assay

ChIP assay was conducted using a commercially available kit (Active Motif, Carlsbad, CA). Briefly, mouse liver tissues were cross-linked for 10 min at room temperature in PBS with 1% formaldehyde. The reaction was stopped by addition of glycine to a final concentration of 0.125 M. Samples were washed twice with PBS, centrifuged, and resuspended in lysis buffer. After incubation for 30 min on ice, the samples were homogenized with a Dounce homogenizer. The chromatin was fragmented enzymatically for 5 min at 37°C. After centrifugation, the supernatant was divided into aliquots for 10-fold dilutions in dilution buffer. The antibodies and protein G-agarose were added and incubated for 18 h at 4°C. The precipitates were washed three times, and chromatin complexes were eluted. After reversal of cross-linking, the DNA was purified. Input DNA or ChIP samples were used as a template for PCR. Primers designed for detecting the regions containing the mouse *Sreb1c* promoter around -468 bp were: 5'-GGATCCAGAAGTGGATCATC-3' and 5'-TTCCCACCGCTG GTTCGTTCC-3'. Each reaction was cycled 36 times at 94°C for 20 seconds, 59°C for 30 seconds, and 72°C for 30 seconds.

Statistical analysis

Values are expressed as means ± SEM. Tukey's multiple com-

parison test was used to determine the significance of any differences among more than three groups. A P value of <0.05 was considered significant.

Acknowledgements

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