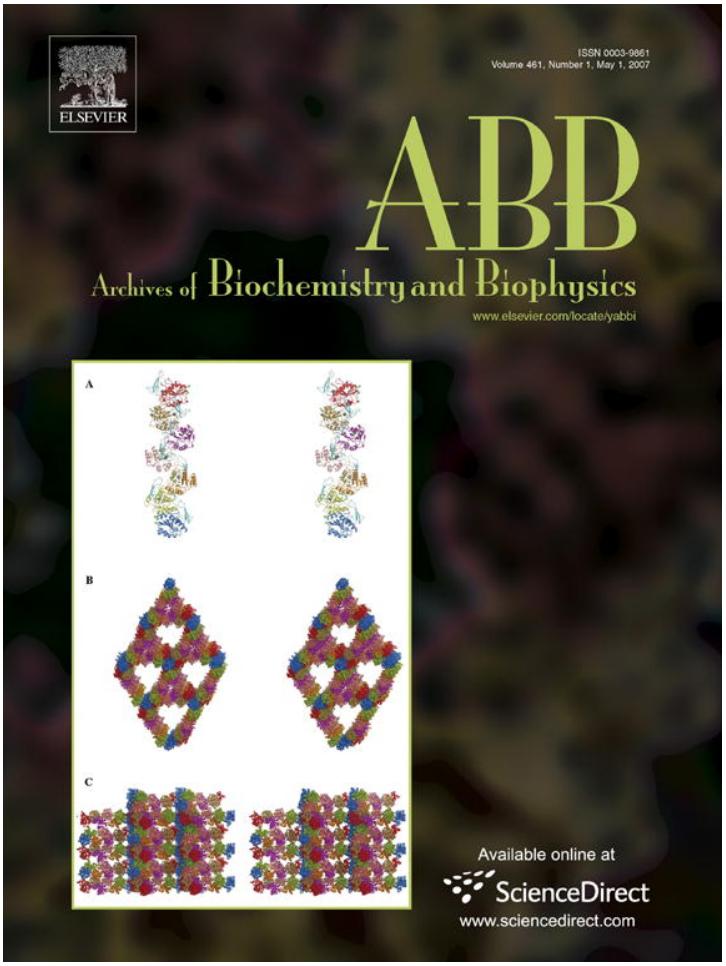

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Identification of *cis*-regulatory elements and *trans*-acting proteins of the rat *carbohydrate response element binding protein* gene ☆

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Received 11 December 2006, and in revised form 26 February 2007
Available online 16 March 2007

Abstract

Carbohydrate response element binding protein (ChREBP) is a transcription factor that activates liver glycolytic and lipogenetic enzyme genes in response to high carbohydrate diet. Here we report the transcriptional regulatory mechanisms for the rat *ChREBP* gene. Firstly, we determined the transcription initiation site and the nucleotide sequences of the rat *ChREBP* promoter region encompassing approximately 900 bp from the ATG initiation codon. Reporter gene assays demonstrated that the major positive regulatory region exists in the nucleotide sequence between −163 and −32 of the *ChREBP* gene. This region contains a cluster of putative transcription factor binding elements that consist of two specificity protein 1 (Sp1) binding sites (−66 to −50 and −93 to −78), a sterol regulatory element (−101 to −110), and two nuclear factor-Y (NF-Y) binding sites (−23 to −19 and −131 to −127). Mutations introduced into these sites caused marked reduction of *ChREBP* promoter activities. Functional synergisms were observed between Sp1/NF-Y and Sp1/sterol regulatory element-binding protein. Additionally, electrophoretic mobility shift assays and chromatin immunoprecipitation assays demonstrated that these factors bound to these elements. Thus, we conclude that functional synergisms between these transcription factors are critical for *ChREBP* gene transcription.

Keywords: ChREBP; Sp1; NF-Y; SREBP; Transcription

An important function of the liver is to convert excess amount of carbohydrate into triacylglycerols for the energy storage. Insulin has been well documented to stimulate this process through the regulation of genes involved in fatty acids synthesis. Sterol regulatory element-binding protein (SREBP)[1]-1c, a basic helix-loop-helix/leucine zipper (bHLH/LZ) transcription factor, is known to be involved in insulin signaling [1,2]. Three isoforms of SREBPs have been identified and amongst them, SREBP-1c is the major isoform expressed in tissues involved in energy homeostasis such as the liver [3,4]. Lipogenic genes such as acetyl-CoA carboxylase (ACC) and ATP citrate-lyase are reported to be targets of SREBP-1c. SREBPs are synthesized as precursor proteins that require proteolytic processing to become an active form. Sterol depletion has been found to enhance this processing of SREBPs [5–7]. However, proteolysis of SREBP-1c is not stimulated by sterol depletion

☆ The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and Genbank nucleotide sequence databases with Accession No. AB270602.

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[1] *Abbreviations used:* SREBP, sterol regulatory element-binding protein; bHLH/LZ, basic helix-loop-helix/leucine zipper; ACC, acetyl-CoA carboxylase; PK, pyruvate kinase; FAS, fatty acid synthase; S_{14}, spot 14; ChoRE, carbohydrate response element; ChREBP, ChoRE binding protein; FBS, fetal bovine serum; SL2, Schneider line 2; Sp, specificity protein; NF, nuclear factor; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; HNF, hepatocyte nuclear factor; LXR, liver X receptor.

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doi:10.1016/j.abb.2007.02.028

and instead, insulin is required for this processing in the liver [8,9]. Insulin also increases the SREBP-1c mRNA, which regulates lipogenic genes transcription through the activation of SREBP-1c in the liver [1].

Recent studies revealed that insulin alone cannot account for the full induction of lipogenic genes in response to high carbohydrate diet. In primary cultures of hepatocytes, not only insulin but also rising glucose concentration is required for the induction of some lipogenic genes such as L-type pyruvate kinase (PK), fatty acid synthase (FAS), spot 14 (S_{14}) and ACC [10]. These lipogenic genes are identified to contain another sequence that responses to high carbohydrate diet. This sequence termed the carbohydrate response element (ChoRE) and composes of two E-box sequences that are separated by 5 bp [11–14]. Although several transcription factors are reported to interact with this element, recent studies revealed that ChoRE binding protein (ChREBP) is an important physiological factor [15]. Thus, both SREBPs and ChREBP are considered to be essential transcription factors for full induction of lipogenetic enzyme gene transcription in response to high carbohydrate diet.

ChREBP is expressed mainly in the liver, adipose tissue, kidney, and small intestine, but many other tissues also express this protein in small amounts [15,16]. The rat ChREBP is composed of 865 amino acids and contains several functional regions including N-terminal nuclear localization signal, proline-rich domain, bHLH/LZ domain and multiple phosphorylation sites for cAMP dependent protein kinase or AMP-activated protein kinase. Uyeda et al. explained how glucose controls the activity of ChREBP through these phosphorylation sites. Phosphorylation of Ser196 located beside the nuclear localization signal blocks ChREBP translocation to the nucleus and phosphorylation of Thr666 or Ser568 results in loss of ChoRE binding activity [17]. On the other hand, dephosphorylation of ChREBP induced under the high glucose conditions by protein phosphatase 2A allows translocation to the nucleus and bind to ChoRE of the lipogenic genes. This protein phosphatase is activated by a glucose metabolite, xylulose 5-phosphate [15,18]. In addition, ChREBP does not act alone, but it functions in a heterodimeric complex with the bHLH/LZ transcription factor Max-like protein X. This dimerization with Max-like protein X is important for carbohydrate responsiveness [19,20].

While functional importance of ChREBP has become apparent, transcriptional regulation of the *ChREBP* gene is poorly understood. Recent studies suggested that high carbohydrate diet caused marked induction of the ChREBP mRNA in the liver as well as its target lipogenetic genes [21]. In cultured hepatocytes, expression of *ChREBP* is stimulated by high glucose in the presence of insulin but inhibited by AMP-activated protein kinase. These data suggest that stimulation of ChREBP by high carbohydrate diet occurs not only at a qualitative level of protein but also at a quantitative level. However, the underlying mechanisms for regulation of the *ChREBP* gene transcription are little known. Here, we examined *cis*-regulatory elements and *trans*-acting proteins of the rat *ChREBP* gene in order to elucidate its transcriptional regulatory mechanisms.

Materials and methods

Materials

Dye terminator cycle sequencing kit was obtained from PE Applied Biosystems. Dual luciferase reporter gene assay kit, pGL3-Basic, pRL-CMV and pRL-SV40 were obtained from Promega. The Qiagen plasmid and Qiaex gel extraction kits were purchased from Qiagen. [α-^{32}P]-dCTP (111 TBq/mmol), [γ-^{32}P]ATP (111 TBq/mmol) were obtained from Amersham Biosciences. 5′-Full Race Core Set, T4 polynucleotide kinase, restriction endonucleases and Ex Taq DNA polymerase were purchased from Takara Bio. Fetal bovine serum (FBS), amphotericin B and T0901317 were obtained from Sigma. Flyfectin lipofection kit and KOD+DNA polymerase were purchased from OzBio and Toyobo, respectively.

Animals and treatment

Six weeks old male Sprague-Dawley rats were used. All animal experiments were approved by the Animal Experiment Committee, Nagoya University, and rats were treated in accordance with the guidelines.

Cell culture

HepG2, HeLa, and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Nissui Pharmaceuticals). Hepatocytes were isolated from Sprague-Dawley rats by the collagease perfusion method [22] and cultured in Williams' medium E or M199 (Gibco BRL) as previously described [23]. Schneider line 2 (SL2) cells were maintained in Schneider's medium (Invitrogen). These mediums were supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen), 0.4% (v/v) amphotericin B and 10% (v/v) FBS, and all cell lines except for SL2 were maintained in a 37 °C/5% CO_2 humidified incubator. SL2 was maintained in a 25 °C incubator.

Determination of the 5′-terminal cDNA sequence

Five microgram of DNase I-treated total liver RNA was reverse transcribed with AMV reverse transcriptase and 5′ phosphorylated RT primer (5′-CTGCTTTGCAGACTGGATCTTGAGAGTGGA-3′) by using 5′-Full Race Core Set, according to the manufacturer's instructions. cDNA was concatemerized according to the instructions. Then the product was amplified by PCR using 1 μl of concatemerized cDNA as template, ChREBP-1s (5′-AGCATCGATCCGACACTCAC-3′) and ChREBP-1as (ACCTGGCAAGTTCACGGATAG-3′) primer set, and Ex Taq DNA polymerase for 25 cycles. PCR product was cloned into pST-Blue-1 vector (Novagen).

Plasmids

Reporter plasmids of pChREBPluc874, 163, and 133 were constructed as follows: The following oligonucleotides were used as primers for PCR to amplify promoter DNA fragments of the rat *ChREBP* gene. ChREBP–874/*Mlu*I (5′-AATACGCGTTAGGTTTCAGGGTCAGTGAG-3′); ChREBP–163/*Mlu*I (5′-TATACGCGTAAGCCAGGACCAGTGGTTTC-3′); ChREBP–133/*Mlu*I (5′-TATACGCGTAACATCACCTGCCAACCAAG-3′); and ChREBP+7*Xho*I (5′-AGACTCGAGACACTCTGGATAGCAGTTTG-3′). The fragment ChREBP (–874 to +7) was obtained by PCR using the rat DNA, ChREBP–874Mlu and ChREBP+7Xho primers, and KOD+ DNA polymerase. Similarly the fragment ChREBP

(–163 to +7) and (–113 to +7) were obtained by PCR using ChREBP–163Mlu and ChREBP+7Xho, and ChREBP–133Mlu and ChREBP+7Xho primers, respectively. These amplified PCR products were digested by *Mlu*I/*Xho*I and then ligated into the same sites of pGL3-Basic vector to produce pChREBPluc163 and pChREBPluc133, respectively. pChREBPluc395 was generated by digestion of pChREBPluc874 with *Mlu*I/*Nhe*I, and ligated into the same sites of pGL3-Basic vector. Similarly pChREBPluc32 was generated by digestion of pChREBPluc163 with *Pvu*II, blunt-ended with *Klenow* fragment, and then digested with *Mlu*I before ligating into *Mlu*I/*Sma*I sites of pGL3-Basic vector. *Drosophila* expression plasmids of specificity protein (Sp) 1, pPac-Sp1, and nuclear factor (NF)-Y, pPacNF-YA, pPacNF-YB and pPacNF-YC, were described previously [24]. Mammalian expression vector of SREBP-1a active form, pSREBP1a, and bacterial expression plasmid of SREBP-1a active form, pET-SREBP-1a [25], were generously provided by Dr. Ryuichiro Sato (The University of Tokyo, Tokyo, Japan). *Drosophila* expression plasmids, pPac-RL and pPac-SREBP-1a were constructed as follows. Renilla luciferase (amino acids 2–312) and SREBP-1a (amino acids 2–487) cDNAs were amplified with Renilla+2/*Bam*HI (5′-CTAGGATCCACT TCGAAAGTTTATGATCCA-3′) and Renilla+312/Xho (5′-GCGCTC GAGTTATTGTTGATTTTTGAGAAC-3′), or SREBP+2/*Bam*HI (5′-T TAGGATCCGACGAGCCACCCTTCAGCGAGGCG-3′) and SREBP+487/TTA/*Xho*I (5′-GGCCTCGAGTAACGAGTCACTGC CACTTGCC ACC-3′) primer sets, and pRL-SV40 or pET-SREBP-1a as template. PCR products were digested with *Bam*HI/*Xho*I and then ligated into pPac-Sp1 digested by the same endonucleases to remove Sp1 cDNA. The sequences of all plasmids were confirmed by DNA sequencing.

Mutagenesis

Site directed mutagenesis was performed as described previously [26]. The mutations were introduced into pChREBPluc395 using the following oligonucleotides: mS-1 (5′-GTCCTTGTGGTGGGCGTGGTCTTG-3′), mS-2 (5′-TTACCATGGCCACGCCTCTGCCCCA-3′), mN-1 (5′-ATT AGCATAAGTACTGAGCCTGGTG-3′), mN-2 (5′-AGTTTGAGAGC GGTACCTTGCCAGC-3′), mSRE (5′-TGGTTGGCAGAATTCGTTT AAATTA-3′).

Reporter gene assays

HepG2, HeLa, and COS-7 cells, and hepatocytes were transfected with 600 ng of reporter plasmid and the indicated amount of pRL-SV40 or pRL-CMV. In the case of overexpression assay of HepG2 cells, SREBP-1a expression plasmid was co-transfected and the total amount of DNA was adjusted by the addition of the empty vector. The transfection conditions and luciferase reporter assays have been described previously [24]. SL2 cells were co-transfected with 800 ng of reporter plasmid, 1 ng of pPac-RL, and 20 ng of the indicated expression plasmids. All plasmids used for transfection were prepared using the Qiagen plasmid Midi kit.

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from rat liver [27]. Recombinant SREBP-1a was prepared as described previously [25]. EMSAs are performed as described previously [25,27]. When N-2 probe was used, 100-fold molecular excess of an internal competitor N-2 upstream was used to reduce non-specific complex. When S-1 and S-2 probes were used, 0.5 μg of salmon sperm DNA were added to the reaction mixture. The oligonucleotides used in EMSAs are listed in Table 1. Competition experiments included a 200-fold molar excess of unlabeled competitor oligonucleotides. Supershift assays were carried out by preincubating the nuclear extract or purified His-tagged SREBP-1a with anti-NF-Y antibody (Rockland), anti-Sp1 antibody (Santa Cruz), or anti-SREBP-1 antibody (MBL) for 30 min at room temperature before the addition of labeled oligonucleotide. Anti-M_1-type PK antibody was used as a control. After the binding reaction, the mixture was subjected to electrophoresis on a 4.5% polyacrylamide gel in 0.5 × Tris–borate/EDTA buffer at 200 V for 1 h. The gel was dried and analyzed by FUJIX BAS-2500.

Table 1
Oligonucleotide sequences used in EMSAs

Oligonucleotides	Nucleotide sequences
S-1	5′-agctCAAGACCACGCCCACCACAAGGAC-3′ 3′-GTTCTGGTGCGGGTGGTGTTCCTG-5′
S-2	5′-agctTGGGGCAGAGGCGTGGCCATGGTAA-3′ 3′-ACCCCGTCTCCGCACCGGTACCATT-5′
N-1	5′-agctCACCAGGCTCATTGGTTATGCTAAT-3′ 3′-GTGGTCCGAGTAACCAATACGATTA-5′
N-2	5′-agctGCTGGCAAGGATTGGCTCTCAAACT-3′ 3′-CGACCGTTCCTAACCGAGAGTTTGA-5′
Ch-SRE	5′-agctTAATTTAAACATCACCTGCCAACCA-3′ 3′-ATTAAATTTGTAGTGGACGGTTGGT-5′
mS-1	5′-agctCAAGACCAC<u>GATATC</u>CACAAGGAC-3′ 3′-GTTCTGGTG<u>CTATAG</u>GTGTTCCTG-5′
mS-2	5′-agctTGGGGCAGAG<u>ACTAGT</u>CCATGGTAA-3′ 3′-ACCCCGTCTC<u>TGATCA</u>GGTACCATT -5′
mN-1	5′-agctCACCAGGCTC<u>AGATCT</u>TATGCTAAT-3′ 3′-GTGGTCCGAG<u>TCTAGA</u>ATACGATTA-5′
mN-2	5′-agctGCTGGCAAGG<u>TACCG</u>CTCTCAAACT-3′ 3′-CGACCGTTCC<u>ATGGC</u>GAGAGTTTGA-5′
mCh-SRE	5′-agctTAATTTAAAC<u>GAATT</u>CTGCCAACCA-3′ 3′-ATTAAATTTG<u>CTTAA</u>GACGGTTGGT-5′
GC box	5′-ATTCGATCGGGGCGGGGCGAGC-3′ 3′-TAAGCTAGCCCCGCCCCGCTCG-5′
Y-box	5′-gatcGTGCTGATTGGTTTTCCG-3′ 3′-CACGACTAACCAAAAGGC-5′
LDLR	5′- agctGATCCTGATCACCCCACTGAGGAGGATC-3′ 3′-CTAGGACTAGTGGGGTGACTCCTCCTAG-5′
N-2 upstream	5′-GCTGGCAAGG-3′ 3′-CGTCCGTTCC-5′

Mutated sequences are underlined.

Chromatin immunoprecipitation (ChIP)

Rat hepatocytes were cultured in the basal medium for 16 h. Then, hepatocytes were treated with 25 mM glucose and 100 nM insulin for 16 h or 100 nM insulin and 10 μM TO-0901317 for 12 h. The cells were fixed by adding formaldehyde (1.0% final concentration) directly to the culture medium and incubated at room temperature for 10 min. ChIP assays were performed with anti-Sp1 antibody, anti-NF-Y antibody, anti-SREBP-1 antibody (Santa Cruz) or normal IgG as described previously [28]. Precipitated DNA fragments were resuspended in 20 μl of double distilled water. DNA samples (2 μl) were used as template for the PCR. Primers for PCR amplification of the *ChREBP* promoter (–163 to +7) are described above.

Statistics

Statistical analysis was performed by Bonferroni multiple comparisons test or Student's *t*-test. Statistical significance was defined as $p < 0.05$.

Results

Determination of the transcription initiation site

To investigate transcriptional regulatory mechanisms of the *ChREBP* gene, we isolated 5′ flanking region of the *ChREBP* gene from rat kidney genomic library using

standard procedures. The nucleotide sequences of the promoter region encompassing approximately 900 bp from the ATG initiation codon were determined by DNA sequencing (Fig. 1). Then, we determined the transcription initiation site by 5′ rapid amplification of cDNA end method and two major sites were identified.

Identification of promoter region of the rat ChREBP gene

To investigate the transcriptional regulatory region of the rat *ChREBP* gene, we constructed pChREBPluc873, in which a DNA fragment of –873 to +7 relative to the major transcription initiation site of the rat *ChREBP* gene was linked to upstream of the firefly luciferase reporter gene. pGL3-Basic, a promoter-less luciferase reporter vector and pChREBPluc873 were transiently transfected into various cells and their luciferase activities were then determined. As shown in Fig. 2, pChREBPluc873 exhibited a 19- to 24-fold higher activity than that of pGL3-basic vector in all cells tested. 5′-Deletion mutant up to –395 (pChREBPluc395) resulted in significant decrease in luciferase activity in only hepatocytes. Deletion up to –163 (pChREBPluc163) caused significant increase in activity in hepatocytes and COS-7 cells, and deletion up to –113 (pChREBPluc113) resulted in decrease in activity by 34 to 53% in all cells. Further deletion up to –32 (pChREBPluc32) essentially eliminated the promoter activity in all cells. Thus, it is suggested that the nucleotide sequence between –163 and –32 of the rat *ChREBP* gene is required for the promoter activity in all cells tested and that ubiquitous transcription factors are mainly involved in this activity. Therefore, we further examined this region in detail.

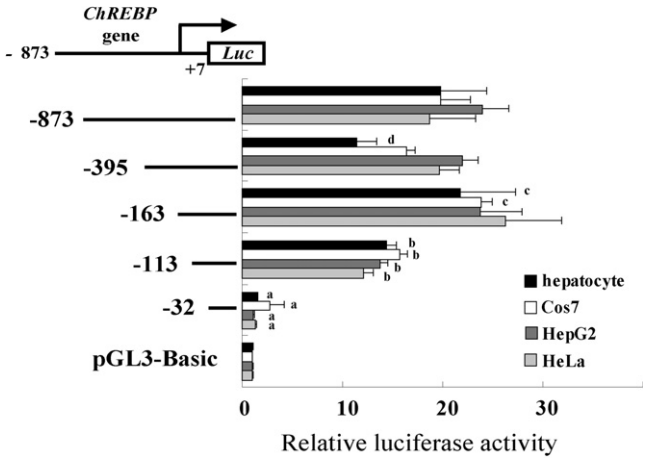

Fig. 2. Deletion analysis of promoter activity of rat *ChREBP* gene. Schematic diagrams of reporter constructs are shown on the left. Various mammalian cells were transiently transfected with 600 ng of luciferase reporter plasmid and 10 ng of pRL-SV40. The luciferase activities of the deletion constructs are expressed relative to cells transfected with pGL3-Basic. Data are the means ± SD of at least three transfection experiments. [a] $p < 0.01$ compared to –113. [b] $p < 0.01$ compared to –163. [c] $p < 0.01$ compared to –395. [d] $p < 0.01$ compared to –873.

pChREBPluc873
-873 taggtttcagggtcagtgagagaacttcctgagcanaaanaaaaggccggtgagtgtcggatcg atgctgggg
-800 gtgggggtggggtcgcgtctcctgggacagggaggtgatctagagagggtgaattctcagaatc ccctctgaactttgctatacaggtgtaggacaatc
-700 ataagagcacctgggtgcaagggtcaaccacacaggaccctgggaaagtgaagaaaggcaggtg agcgatttactaaacacactgtgaagcagagaatga
-600 ccctgaatttctgatattctctctctctctctctctctctctctctctctctctctctctctctct ctctctctctcgtgtttatgtgtctaggaacactta
-500 tggggctcagaggtcaaattttaaaaatcgagtattttcttccaacatgcgggacctagcactc gaactcatgtcctcaatcctggcggcaaacgctttt
pChREBPluc395
-400 atcagctagccatcttgacggctggaacttctgatctttttctgcctccacctccgagtgttg cacttaacagaatcgccccatgttaagtttctgtg
-300 gtacggaggatggaacccaagccttcgtcatgcgaggcaaacactgttccaaccgagttgcagc cccagccccagccagccttttctgaaacctgaaaa
pChREBPluc163
-200 GTAGGGACTAAAAATTGCTCACAGCAGACAGAAAGGTTAAGCCAGGACCA
pChREBPluc113
Y- box (N-1)
SRE (Ch-SRE)
-150 GTGGTTTCACACCAGGCTCATTGGTTATGCTAATTTAAACATCACCTGCC
GC box (S-1)
GC box (S-2)
-100 AACCAAGACCACGCCCACCACAAGGACAAATAGTGGGGCAGAGGCGTGGC
pChREBPluc32
Y- box (N-2)
-50 CATGGTAATTAGGCTCAGCTGGCAAGGATTGGCTCTCAAACTGCTATCCA
+1
GAGTGTCCGGGAGTTGGCGACAATAGTGGCCATG

Fig. 1. Sequence of the promoter region of rat *ChREBP* gene. Two transcriptional initiation sites were identified. The downstream initiation site is designated as +1 and the upstream site is indicated by arrow head. Putative *cis*-acting elements are underlined. The 5′ end of the promoter region in each reporter plasmid is shown by arrows.

Identification of trans-acting proteins for the promoter of ChREBP gene

A computer search (the TFSEARCH program) revealed that the promoter region from –162 to –32 of the rat *ChREBP* gene contains a cluster of putative transcription factor binding elements, consisting two Sp1 binding sites (GC boxes, –66 to –50 and –93 to –78), sterol regulatory element (SRE, –101 to –110), and two NF-Y binding sites (Y-boxes, –23 to –19 and –131 to –127). Hereafter, we refer to these putative binding sites as S-1 and S-2, Ch-SRE, and N-1 and N-2, respectively (Fig. 1).

To determine whether these sites are involved in transcriptional regulation of the *ChREBP* gene, we introduced mutations into these sites of pChREBPluc392 independently or simultaneously and analyzed their promoter activities in HepG2 cells. As shown in Fig. 3a, the reporter activity significantly decreased by mutation of S-2, N-1, or N-2, but not of S-1 or Ch-SRE. When mutations were introduced into both S-1 and S-2, additional reduction of the activity was observed compared with mutation of S-2 alone. Mutation of both NF-Y sites caused no further change in activity when compared with mutation of N-1 or N-2 alone. Although we could not detect reduction in activity when mutation was introduced into Ch-SRE, we considered that SREBPs are present mostly in an inactive state under culture conditions used. Thus, we co-transfected the expression plasmid of SREBP-1a active form with pChREBPluc395 into HepG2 cells. About 20-fold enhancement of the promoter activity by SREBP-1a was observed. On the other hand, this activation by SREBP-1a was greatly reduced when Ch-SRE element was mutated (Fig. 3b). Similar results were obtained in HeLa cells (data not shown). Given this result, we performed reporter gene assays in the sterol-depleted medium or sterol-supplemented medium to alter endogenous active SREBP levels. The luciferase activity of pChREBPluc395 was elevated by 4.6-fold in sterol-depleted medium compared with sterol-supplemented medium (Fig. 3c). However, the reporter with mutated Ch-SRE was hardly activated by sterol depletion. These results suggested that endogenous SREBP is involved in transcription of the *ChREBP* (Fig. 3c). Thus, we conclude that all putative *cis*-acting elements mentioned above are involved in the *ChREBP* gene transcription.

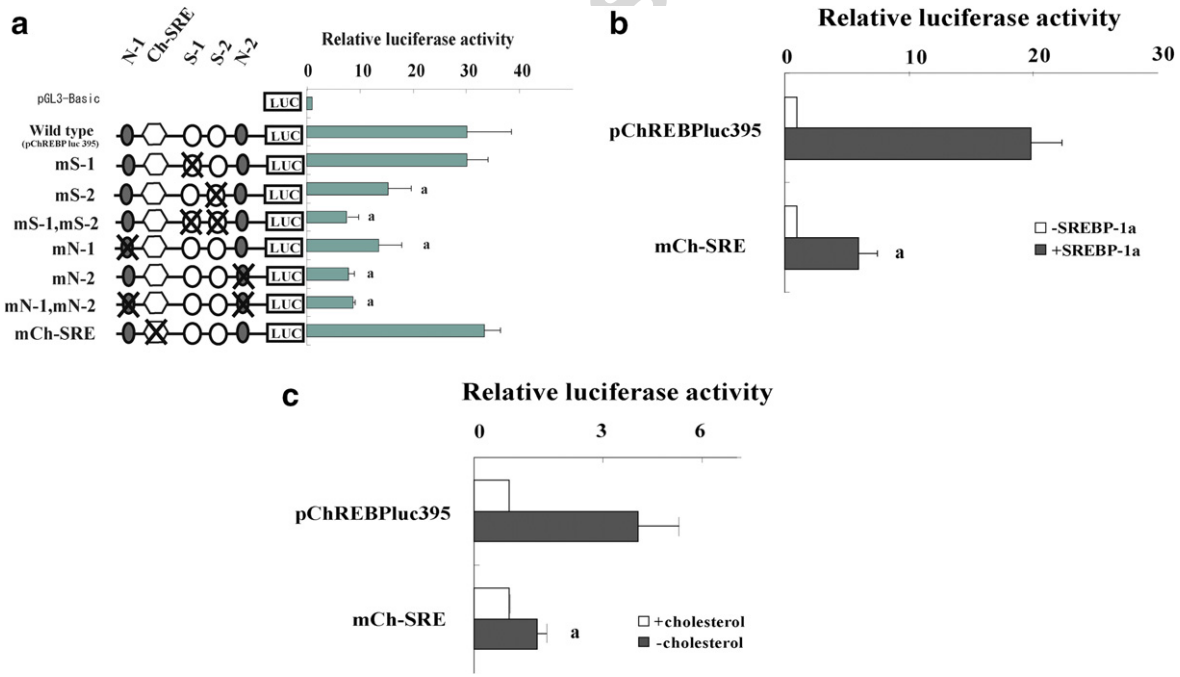

Fig. 3. Effects of mutation at the Sp1, NF-Y and SREBP putative binding sites of *ChREBP* promoter in HepG2. (a) Schematic diagrams of reporter constructs are shown on the left. The putative *cis*-acting elements were indicated and *cis*-elements containing mutated sequences are shown by crosses. HepG2 cells were transfected with 600 ng of wild type or mutated reporter plasmid and 1 ng of pRL-CMV. The luciferase activities are expressed relative to cells transfected with pGL3-Basic. Data are the means ± SD of at least three experiments. [a] $p < 0.01$ compared to wild type. (b) HepG2 cells were transfected with 600 ng of luciferase reporter plasmid shown on the left, 10 ng of pRL-SV40 and 20 ng of SREBP-1a expression vector or empty vector. The luciferase activities are expressed relative to cells transfected with empty vector. Data are the means ± SD of at least three experiments. [a] $p < 0.01$ compared to pChREBPluc395. (c) HepG2 cells were transfected 600 ng of reporter plasmid shown on the left and 10 ng of pRL-SV40 for 6 h. Then, the cells were incubated under the SREBP-1a induced or suppressed conditions. For the induced condition (−cholesterol), medium contained 10% lipoprotein deficient serum, 50 μM pravastatin and 50 μM mevalonate, while the suppressed condition (+cholesterol) consisted of DMEM supplemented with 10% FBS, 1 μg/ml 25-hydroxycholesterol and 10 μg/ml cholesterol [25]. The luciferase activities are expressed relative to cells incubated under the suppressed condition. Data are the means ± SD of three experiments. [a] $p < 0.05$ compared to pChREBPluc395.

Sp family members, NF-Y and SREBP-1a cooperatively stimulate the transcription of the ChREBP gene in Drosophila SL2 cells

To examine the direct effect of Sp family proteins, NF-Y, and SREBP-1a on the transcriptional activity of the *ChREBP* gene, we performed transient transfection assays using *Drosophila* SL2 cell line, since this cell line is devoid of endogenous Sp family proteins and NF-Y [29,30]. We used pChREBPluc395 plasmid containing all of putative Sp1, NF-Y and SREBP binding sites as a reporter. Although co-transfection of either Sp1 or NF-Y with the reporter had only a marginal effect on the luciferase activity, co-transfection of both Sp1 and NF-Y expression vectors caused about 66-fold enhancement in activity (Fig. 4a). The synergistic effect with NF-Y was also observed using Sp3 instead of Sp1. To determine whether Sp1 and NF-Y transactivate the ChREBP promoter through S-1, S-2, N-1, and N-2 sites, we performed co-transfection assays with the mutant reporters, and Sp1 and NF-Y expression vectors. Overexpression of Sp1 and NF-Y resulted in about 150-fold stimulation of the activity of pChREBPluc395 in SL2 cells. On the other hand, all mutations resulted in significant reduction of the transcriptional activity (Fig. 4b). The S-2 and N-1 mutations were more effective than the S-1 and N-2 mutations, respectively. These results suggested that all putative Sp1 and NF-Y binding sites are required for full synergistic interaction between Sp1 and NF-Y in regulation of *ChREBP* gene transcription.

Next, we determined the effect of SREBP-1a on the *ChREBP* promoter in SL2 cells. When SL2 cells were co-transfected with SREBP-1a expression vector alone, no significant change in activity was observed. However, co-transfection of SREBP-1a with Sp1 caused significant increase in activity compared to each effector alone, whereas co-transfection with NF-Y resulted in no significant change in activity compared to NF-Y alone (Fig. 4c). To investigate which Sp1 binding site is essential for functional synergism between Sp1 and SREBP-1a, we performed reporter gene assays using mutated reporters.

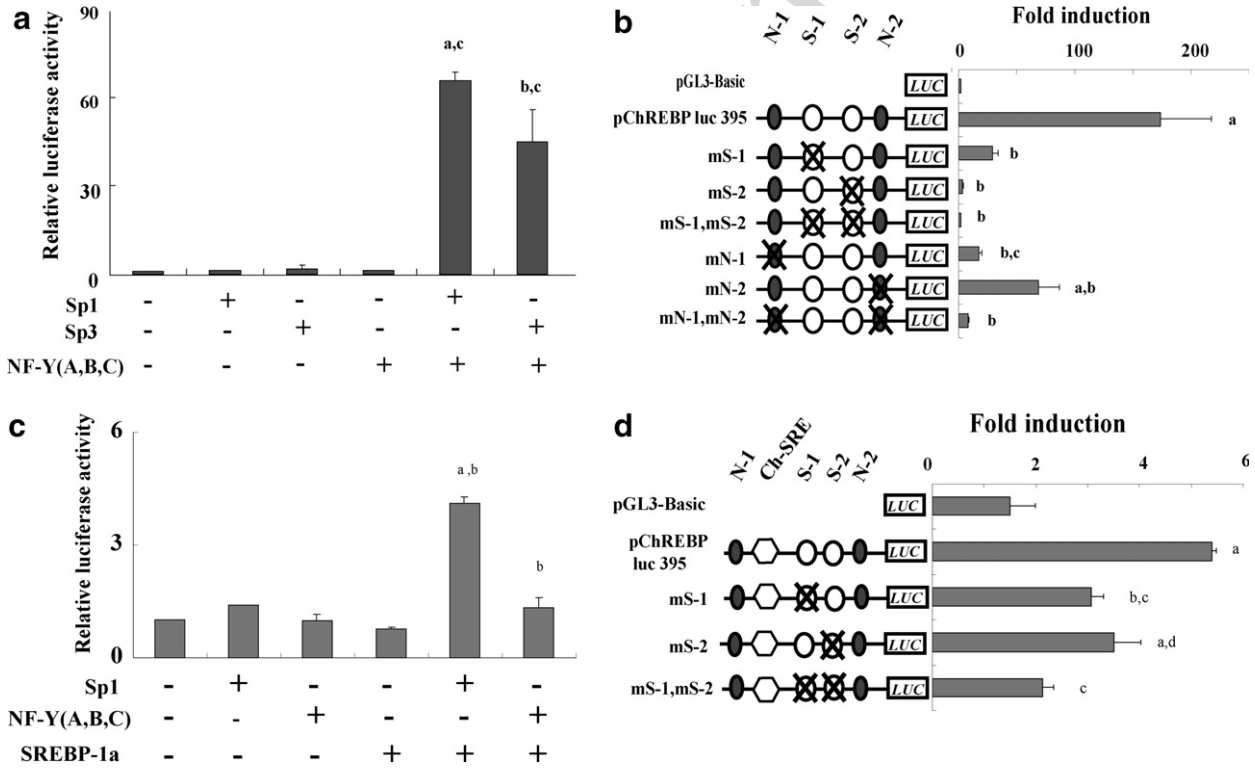

Fig. 4. Synergistic activation by Sp1/Sp3 and NF-Y, and by Sp1 and SREBP in SL2 cells. (a) *Drosophila* SL2 cells were co-transfected with 800 ng of pChREBPluc395, 5 ng of pPac-Renilla and 20 ng of pPac, 20 ng of pPac-Sp1or 20 ng of pPac-Sp3 with or without 7 μg each of NF-YA, NF-YB and NF-YC expression vectors. The luciferase activities are expressed relative to the cells transfected with empty vector. Data are the means ± SD of at least three experiments. [a] $p < 0.01$ compared to Sp1. [b] $p < 0.01$ compared to Sp3. [c] $p <$ compared to NF-Y. (b) SL2 cells were co-transfected with 800 ng of various reporter plasmids shown on the left, 5 ng of pPac-Renilla, 20 ng of pPac-Sp1 and 7 ng each of NF-YA, NF-YB, and NF-YC expression vectors or corresponding amount of empty vector. The luciferase activities were expressed relative to the cells transfected with empty vector. Data are the means ± SD of at least three experiments. [a] $p < 0.01$ compared to pGL3-Basic. [b] $p < 0.01$ compared to pChREBPluc395. [c] $p < 0.05$ compared to mN-2 (c) SL2 cells were co-transfected with 800 ng of pChREBPluc395, 5 ng of pPac-Renilla and 20 ng of pPac, 20 ng of pPac-Sp1 or 7 ng each of NF-YA, NF-YB and NF-YC expression vectors with or without of 20 ng of pPac-SREBP-1a. The luciferase activities are expressed relative to the cells transfected with empty vector. Data are the means ± SD of at least three experiments. [a] $p < 0.01$ compared to Sp1. [b] $p < 0.01$ compared to SREBP-1a (d) SL2 cells were co-transfected with 800 ng of reporter plasmids shown on the left, 5 ng of pPac-Renilla and 20 ng each of Sp1 and SREBP-1a expression vectors or empty vector. The luciferase activities were expressed relative to the cells transfected with empty vector. Data are the means ± SD of three experiments. [a] $p < 0.01$ compared to pGL3-Basic. [b] $p < 0.05$ compared to pGL3-Basic. [c] $p < 0.01$ compared to pChREBPluc395. [d] $p < 0.05$ compared to pChREBP395.

The S-1 and S-2 mutations caused 43 and 35% reduction in activity, respectively (Fig. 4d). When mutations were introduced into both of Sp1 binding sites, additional reduction in the activity was observed and synergistic activation of SREBP and Sp1 disappeared to levels comparable with pGL-3 Basic vector. These results suggested that functional synergisms between Sp1 and NF-Y, and between SREBP-1a and Sp1 are critical for the *ChREBP* gene promoter activity.

Sp1, NF-Y, and SREBP interacted with the ChREBP gene promoter

EMSAs using rat liver nuclear extract or recombinant SREBP-1a were performed to determine whether Sp1, NF-Y, and SREBP-1a can recognize the sites tested above. When the end-labeled S-1, S-2, N-1, or N-2 probes were incubated with 5 μg of the rat liver nuclear extract, specific complexes were observed (Fig. 5a and b).

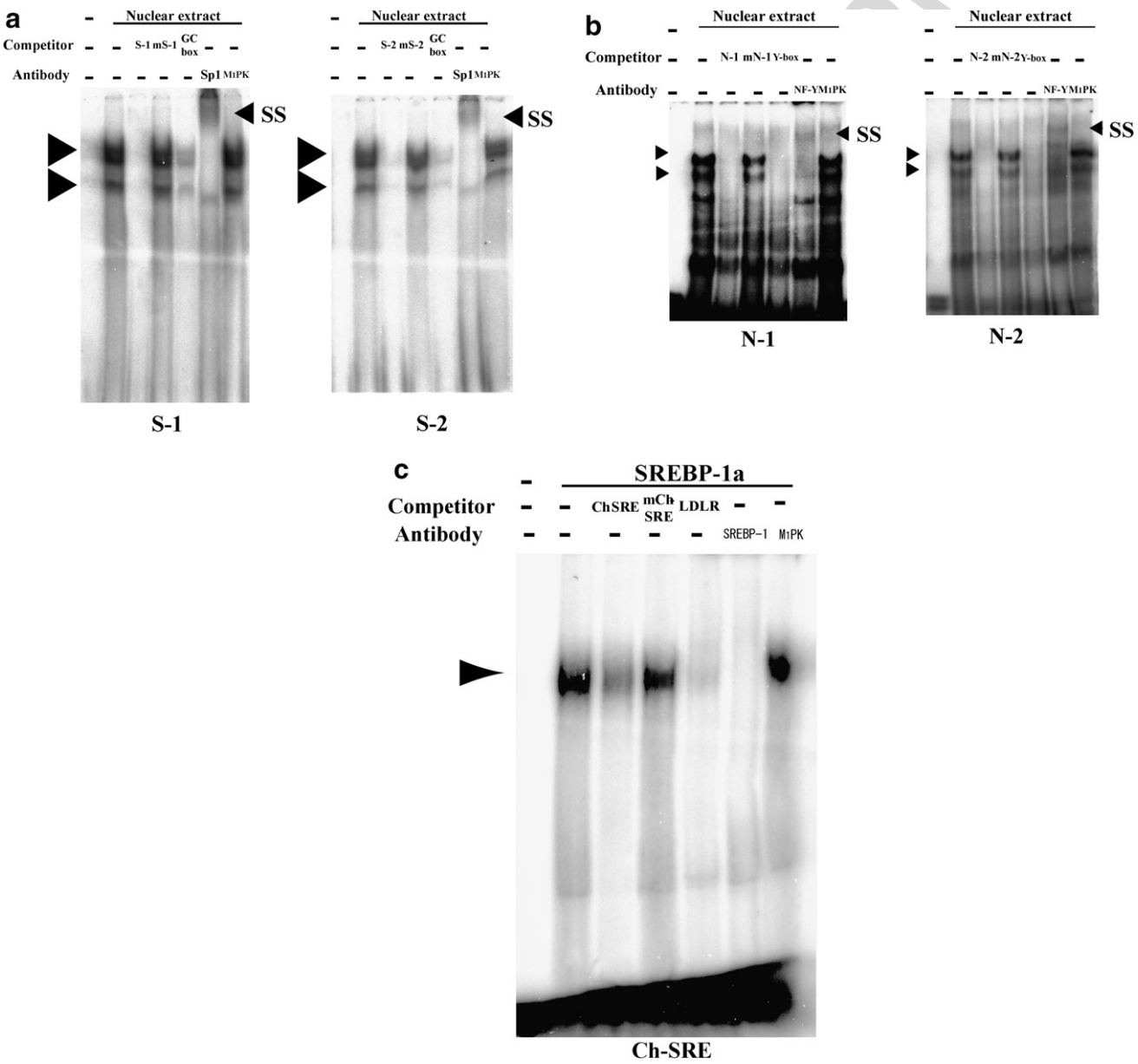

Fig. 5. EMSAs of *cis*-acting elements of the *ChREBP* gene. (a) End-labeled S-1 (left) or S-2 (right) oligonucleotides were incubated with 5 μg of rat liver nuclear extract. Unlabeled oligonucleotides were added as competitors at 200-fold molar excess where indicated. Anti-Sp1 antibody or anti-M_1-Type PK (M_1PK) antibody used are shown at the top. The arrowheads on the left indicate specific DNA/protein complexes. SS, supershifted complex. (b) End-labeled N-1 (left) or N-2 (right) oligonucleotides were incubated with 5 μg of rat liver nuclear extract. Unlabeled oligonucleotides were added as competitors at 200-fold molar excess where indicated. Anti-NF-Y antibody and anti-M_1-type PK (M_1PK) antibody used are shown at the top. The arrowheads on the left indicate specific DNA/protein complexes. SS, supershifted complex. (c) End-labeled Ch-SRE oligonucleotides were incubated with bacterially expressed recombinant SREBP-1a. Unlabeled oligonucleotides were added as competitors at 100-fold molar excess where indicated. Anti-SREBP-1 antibody and anti-M_1PK antibody used are shown at the top. The arrowhead on the left indicates specific DNA/protein complex.

The complexes with S-1 or S-2 were reduced by addition of 200-fold molar excess of unlabeled S-1, S-2, or consensus Sp1 binding sequence (GC box) but not by addition of mutated oligonucleotide (mS-1 or mS-2). A supershifted band was observed by addition of anti-Sp1 antibody but not by addition of anti-M_1-type PK antibody (Fig. 5a). Similarly, the complexes with N-1 or N-2 were decreased by addition of unlabeled N-1, N-2 or consensus NF-Y binding sequence (Y-box) and were supershifted by addition of anti-NF-Y antibody (Fig. 5b). Addition of mN-1, mN-2 or anti-M_1-type PK antibody failed to affect formation of the complexes. Incubation of the end-labeled Ch-SRE with recombinant SREBP-1a generated a specific complex (Fig. 5c). This complex was markedly reduced by addition of 100-fold molar excess of unlabelled Ch-SRE or SREBP binding sequence of low density lipoprotein receptor (LDLR), but not by addition of mutated Ch-SRE. Addition of anti-SREBP-1 antibody resulted in disappearance of the complex but addition of anti-M_1PK antibody did not. These results indicated that Sp1, NF-Y and SREBP-1 can bind to S-1/S-2, N-1/N-2 and Ch-SRE sites, respectively.

Sp1, NF-Y, and SREBP-1 are associated with the ChREBP promoter in vivo

To confirm the association of Sp1, NF-Y, and SREBP-1 with the promoter of the *ChREBP* gene *in vivo*, we performed ChIP analysis with rat hepatocytes. A specific primer set for PCR was used to produce a 171 bp DNA fragment of the *ChREBP* gene promoter region (–163 to +7) that contains S-1, S-2, N-1, N-2, and Ch-SRE. A PCR product specific to *ChREBP* promoter was obtained from immunoprecipitates with anti-Sp1, anti-NF-Y, (Fig. 6a) and anti-SREBP-1 antibodies (Fig. 6b). In contrast, there was essentially no amplified band form immnoprecipitates with normal IgG. These results confirmed association of Sp1, NF-Y, and SREBP-1 to the *ChREBP* gene promoter *in vivo*.

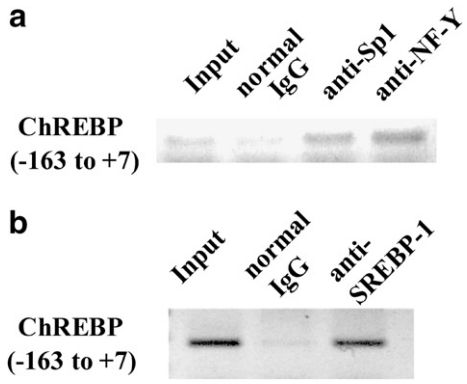

Fig. 6. ChIP analysis of ChREBP gene. (a) Rat hepatocytes were cultured in M199 medium supplemented 25 mM glucose and 100 nM insulin for 16 h. Then, the hepatocytes were subjected to formaldehyde cross-linking followed by immunoprecipitation with anti-Sp1 antibody, anti-NF-Y antibody or normal IgG. Precipitated DNA was analyzed by PCR with a primer set specific for the promoter of *ChREBP* gene. Input corresponds to 1/250 of the amount of DNA used in the assay. (b) Rat hepatocytes were cultured in M199 medium supplemented 1 μM of T0901317 and 100 nM insulin for 16 h. Then, the hepatocytes were subjected to formaldehyde cross-linking followed by immunoprecipitation with anti-SREBP-1 antibody or normal IgG. Precipitated DNA was analyzed by PCR with a primer set specific for the promoter of *ChREBP* gene. Input corresponds to 1/2000 of the amount of DNA used in the assay.

Discussion

In this study, we isolated 5′ flanking region of the rat *ChREBP* gene and determined its sequence and transcription initiation sites. This gene did not contain TATA box. Instead, multiple GC box motifs were found in the upstream region. There were no evident initiator element and no downstream element. These features are consistent with the multiple transcription start sites of the *ChREBP* gene [40].

Reporter gene assays using 5′-deletion constructs suggested the presence of multiple *cis*-acting elements in promoter region of the rat *ChREBP* gene. The region from –873 to –396 may contain a positive regulatory element specific to hepatocytes and the region from –395 to –164 may contain a negative regulatory element effective in hepatocytes and COS-7 cells. However, we have not further examined these regions in this study, since multiple regulatory elements acting in all cells examined were suggested to be present in the region between –163 and –32.

We identified the positive regulatory elements and *trans*-acting proteins of the *ChREBP* gene. The regulatory elements contain two GC boxes (S-1 and S-2), two Y-boxes (N-1 and N-2) and an SRE (Ch-SRE). Our results indicated that Sp family proteins, NF-Y, and SREBP recognized and bound to a S-1/S-2, N-1/N-2, and Ch-SRE, respectively. Although the S-1 mutation had no independent effect in HepG2 cells, simultaneous mutation of S-1 and S-2 caused additional reduction of the promoter activity. This may be due to the difference in binding affinity among Sp family proteins or in synergistic interaction between transcriptional components in HepG2 cells. Involvement of S-1 region in the transcription of the *ChREBP* gene was confirmed by reporter assays in SL2.

Reporter gene assays in SL2 demonstrated importance of synergistic interaction among these transcription factors in the *ChREBP* transcription. As indicated in Fig. 4, Sp1, NF-Y and SREBP did not show any independent effect on the *ChREBP* promoter, but they functionally interacted to activate transcription of the *ChREBP* gene. Functional and physical interactions between Sp1 and NF-Y have been reported in other genes [24,31]. Like these genes, this synergism is important for the *ChREBP* transcription.

Here we propose that the *ChREBP* transcription is regulated by SREBPs. The several genes regulated by SREBPs require binding sites for co-regulatory transcription factors such as Sp1 and NF-Y, nearby SRE sequences [32]. Like these genes, Sp1 and NF-Y binding motifs are located near

the SRE in the *ChREBP* promoter. Functional synergism between SREBP-1a and Sp1 but not between SREBP-1a and NF-Y was observed in SL2. Difference in synergism between SREBPs/Sp1 and SREBPs/NF-Y is recently reported [33,34]. In the *FAS* gene promoter, there are both binding sites for Sp1 and NF-Y near the SRE. The NF-Y-binding site is necessary for sterol regulation, while the Sp1-binding site is dispensable. In cultured hepatocytes, however, the Sp1-binding site is also required for glucose/insulin activation of the *FAS* promoter. Osborn and co-workers reported that the different SREBP isoforms utilize distinct co-regulatory factors to activate target gene, because the major SREBP isoforms in sterol-depleted cultured cells are SREBP-1a and SREBP-2 but the major SREBP isoform in hepatocytes is SREBP-1c [33]. The other report showed that although there are both binding sites for Sp1 and NF-Y in the *acetyl-CoA synthetase 1* promoter, SREBP synergistically activated this promoter with Sp1, but not with NF-Y [34]. Thus, not only isoforms of SREBPs but also the binding sites for co-regulator such as Sp1 and NF-Y are critical for the transcriptional regulation of the genes by SREBPs. The context of *cis*-elemets near SRE may define the efficiency for gene transcription.

SREBP-1c is considered as a master regulator of lipogenesis in response to insulin [35]. Hepatic *SREBP-1c* expression is depressed during fasting but increases markedly when animals are refed a high carbohydrate diet [36]. Some of SREBP-1c target genes such as *ACC*, *FAS*, and S_{14} are also demonstrated to be the target of ChREBP [16,20]. These genes have both of SRE and ChoRE and are not fully activated by only insulin without rising glucose concentration. Previous study demonstrated that SREBP-1c could not induce the *ChREBP* gene expression in glucokinase knockout hepatocytes, suggesting that activation of *ChREBP* gene transcription by SREBP-1c is not direct [21]. In this study, however, we observed activation of *ChREBP* promoter though Ch-SRE. Thus, we speculate that the *ChREBP* gene contain ChoRE or other enhancer sequence to support SREBP-1c activity in addition to SRE like *ACC*, *FAS* and S_{14}. During preparation of this manuscript, it has been reported that mouse *ChREBP* gene promoter contains functional liver X receptor (LXR)-binding site in the upstream of our reported region [37]. LXR is also reported to activate *SREBP-1c* gene transcription in response to insulin [38]. In addition, *SREBP-1c* gene promoter contains SRE [39]. Thus, both LXR and SREBP-1c may be required for full induction of *ChREBP* gene in response to carbohydrate diet.

Acknowledgements

This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, and Technology of Japan. We thank Dr. R. Sato for providing plasmids. We also thank Dr. V. W. Keng for critical reading of the manuscript.

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