

Transcriptional regulation of leptin gene promoter in rat

Hitomi Fukuda, Nobuko Iritani*

Faculty of Human and Cultural Studies, Tezukayama Gakuin University, 4-2-2, Harumidai, Sakai, Osaka 590-0113, Japan

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Abstract To investigate the DNA regulatory sequences required for stimulation and suppression of leptin gene expression, primary cultured hepatocytes and adipocytes of rats were transfected with plasmids containing the 5'-flanking sequences of the rat leptin gene fused to the luciferase gene. When two copies of the sequences spanning nucleotides -101 to -83 of the leptin promoter were used for transfection, the reporter activity significantly increased in the presence of glucose/insulin in comparison with glucose alone. The glucose/insulin stimulation of the transcription was inhibited by addition of polyunsaturated fatty acids. These results were similar to those found earlier for the transcription of the fatty acid synthase, FAS(-57/-35) and ATP citrate-lyase, ACL(-64/-41) genes. Cotransfection studies in the cells with a Sp1 expression vector and leptin (-101/-83) constructs showed the inactivation of the leptin promoter by Sp1. Gel mobility shift assays using an end-labeled leptin(-101/-83) construct as a probe revealed that nuclear factor(s) from rat liver or adipose tissue specifically formed complexes with the sequence. The DNA-protein complexes were common to the glucose/insulin-responsive regions of the leptin, ACL and FAS genes, suggesting that these genes are coordinately regulated. In addition, by antibody supershift assays, the transcription factor Sp1 was found to bind the GC-rich region located between nucleotides -101 and -83 of the leptin gene. Mutational analysis of this region showed that the sequence of the region was critical for glucose/insulin stimulation of transcription. Thus, we postulated that the region from -101 to -83 of the leptin gene is responsible for glucose/insulin stimulation of transcription, and that Sp1 is somehow involved in this regulation.

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Key words: Leptin; Response element; Glucose/insulin; Polyunsaturated fatty acid; Sp1

1. Introduction

Leptin is a hormone produced in adipose cells that is important in the regulation of energy expenditure, food intake and adiposity [1,2]. Leptin expression is regulated by glucocorticoids [3,4], insulin [5,6], β -adrenergic agonist [3], thiozolidinediones [7–9] and fasting [10,11]. Sp1 has been shown to be a transcription factor of leptin gene expression, binding to

nucleotides -101 to -83, and mutation in this region abolishes transcription factor binding and reduces promoter activity [12].

We previously mapped the sequences responsible to glucose/insulin stimulation in the proximal promoter region from -57 to -35 of fatty acid synthase (FAS) (EC 2.3.1.85) and -64 to -41 of ATP citrate lyase (ACL) (EC 4.1.3.8) of rat liver [13,14]. When two copies of a synthetic nucleotide probe of region -57 to -35 of the FAS gene were linked to a reporter gene and the resultant construct was used for transfection, the reporter gene activity was significantly increased in hepatocytes in response to glucose/insulin treatment and the insulin stimulation was suppressed by polyunsaturated fatty acid (PUFA), Sp1 and leptin [15]. The same results were observed with the -64 to -41 region of the ACL gene [16]. Nucleotides -101 to -83 of the leptin gene, -57 to -35 of the FAS gene and -64 to -41 of the ACL gene contain overlapping binding sites for Sp1 proteins [12,15,16]. It is thus possible that nucleotides -101 to -83 of leptin may be a glucose/insulin response element and Sp1 may be involved in the transcriptional regulation. Recent work in cultured adipose cells [17,18] and hepatocytes [19] has suggested that leptin may antagonize insulin action in these cells. In the present study, in order to make clear these possibilities, we have investigated the effects of glucose/insulin and PUFA on the transcription of the leptin gene, using leptin(-101/-83) linked to a reporter gene in primary cultured hepatocytes and adipocytes of rats.

2. Materials and methods

2.1. Materials

Restriction endonucleases, other enzymes and a sequencing kit were purchased from Takara Shuzo (Kyoto). The PGL3 promoter vector and dual luciferase assay system were purchased from Promega (Heidelberg). The luciferase assay kit was from Toyo Ink (Tokyo). Williams' medium E was purchased from Flow Laboratories (Costa Mesa, CA) and other culture media from Nissui Seiyaku (Tokyo). [^{14}C]Chloramphenicol (2.22 GBq/mmol) and [$\gamma\text{-}^{32}\text{P}$]ATP (110 GBq/mmol) were from ICN (Costa, CA). Antibodies against Sp1 and Sp3 were obtained from Santa Cruz Biotech (Santa Cruz, CA), lipofectin reagent from Life Technologies (Palo Alto, CA) and recombinant mouse leptin from R&D Systems Inc. (Minneapolis, MN).

2.2. Plasmid constructs

Plasmid pcatL, a luciferase vector containing β -actin enhancer and promoter, and PRL-SV40 were used as an internal control to normalize for variations in chloramphenicol acetyltransferase (CAT) and luciferase transfection efficiency [20]. Plasmid PL1cat, which contains a fragment spanning -94 to +37 of the L-type pyruvate kinase (LPK) gene, was produced from LPKcat [21]. Plasmid pRSVSp1 [22] was a generous gift from Prof. Y. Fujii-Kuriyama (Tohoku University, Japan). Plasmid pRCMVSp3 [23] was a generous gift from Prof. G. Suske (Philipps Universität Marburg). The following single-stranded oligonucleotides were synthesized by Gibco Ltd (Grand Island, NY).

*Corresponding author. Fax: (81) (722) 92-2135.
E-mail: iritani@hcs.tezuka-gu.ac.jp

Abbreviations: ACL, ATP citrate-lyase; FAS, fatty acid synthase; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; PUFA, polyunsaturated fatty acids

Leptin(–101/–83): 5'-AGCTGGGGCGGGAGTTGGCGCTC-3'
 Leptin(–101/–83)M: 5'-AGCTGAAGCTTGGAGTTGGCGCTC-3'
 FAS(–57/–35): 5'-GATCGTGGCCGCGCGGGGATGGCC-
 GCG-3'
 ACL(–64/–41): 5'-GATCTGATGGGGGCGGGGAGGAG-
 CCCG-3'
 Sp1: 5'-ATTCGATCGGGGCGGGGCGAGC-3'

Underlined sequences are mutations consisting of substituted bases. Double-stranded oligonucleotides of leptin(–101/–83) or leptin(–101/–83)M were inserted into the PGL3 promoter. Double-stranded oligonucleotides of FAS(–57/–35) were inserted into the *Bam*HI sites of PL1cat [15]. The sequence of these inserts was verified by dideoxy sequencing, using a sequencing kit [24]. Two copies of these sequences were linked to the reporter gene.

2.3. Primary cell culture and transfection

Male Wistar rats (200–250 g) maintained on a standard diet (Oriental Koubo, MF, Tokyo) were fasted for 16 h before experiments. The rat hepatocytes and adipocytes were isolated by collagenase digestion, and the DNA reporter constructs were transfected into the cells, as described previously [25]. Subsequently, the cells were cultured for 48 h in experimental medium (with 100 µg/ml streptomycin and 100 U/ml penicillin) containing 20 mM glucose with or without 0.1 µM insulin. To some cultures, 0.1 mM arachidonic acid or 0.3 µM recombinant mouse leptin was added. All transfections were performed at least three times in duplicate.

2.4. Reporter gene assay

The cells were incubated for 48 h after transfection, harvested and lysed by sonication. The extracts were heated at 60°C for 10 min to inactivate endogenous acetylase [26,27]. Prior to the heating step, aliquots were removed for luciferase assays [28]. The heated extracts were centrifuged and the supernatants were assayed for CAT activities. Amounts of the cell extracts normalized by the luciferase activity were used for CAT assays, and the acetylated and non-acetylated forms of [¹⁴C]chloramphenicol were determined using a scintillation counter. The percentages of the acetylated forms were calculated. The luciferase reporter activity was assayed by the dual luciferase assay system from Promega according to the manufacturer's instructions and measured with a Turner Design TD-20/20 luminometer [29]. The luciferase activity driven by the –101 to –83 region of the leptin promoter was expressed as a percentage of the SV40 promoter-driven luciferase activity, which served as a positive control in every transfection experiment.

2.5. Gel mobility shift assay

Nuclear extracts from rat livers and adipocytes were prepared as described by Gorski et al. [30]. The end-labeled double-stranded oligonucleotide corresponding to leptin nucleotides –101/–83 was incubated with the indicated amount of nuclear proteins as described previously [15]. The reaction mixture was incubated for 1 h at room temperature and then loaded onto a 4% non-denaturing polyacrylamide gel and separated by electrophoresis at 200 V for 1 h [21]. In competition studies, the indicated amounts of double-stranded oligonucleotide were added to the reaction mixtures. The nucleotide sequences of FAS(–57/–35) [15], ACL(–64/–41) [16] and Sp1 [31] are shown above. For antibody supershift assays, Sp1 or Sp3 antibody was added to the binding reaction mixture and incubated for 1 h at room temperature prior to adding the labeled probes. The gel was then fixed in 10% methanol/10% acetic acid, dried, and autoradiographed.

3. Results and discussion

3.1. Effects of glucose/insulin, PUFA, Sp1 family and leptin on leptin transcription

To explore possible regulatory elements in the 5'-flanking region of the leptin gene, double-stranded oligonucleotides corresponding to nucleotides –101 to –83 of the leptin gene were synthesized and linked the PGL3 promoter. The plasmid DNA was then transfected into rat hepatocytes and

adipocytes. The cells were cultured in the presence of glucose with or without insulin for 48 h, and then used to prepare extracts which were assayed for luciferase activity, as a reporter activity for leptin transcription. As shown in Table 1A, in the presence of glucose/insulin, the reporter activity of leptin gene transcription was markedly increased (by 1.5-fold) above that in the presence of glucose alone in rat hepatocytes. The stimulation by glucose/insulin was reduced by 44% in arachidonic acid-treated hepatocytes and by 36% in leptin-treated hepatocytes. In adipocytes, the reporter activity was also stimulated by glucose/insulin and suppressed by PUFA or leptin, although the degrees of stimulation and suppression were smaller than in hepatocytes.

To test the binding of Sp1 to the DNA, rat hepatocytes and adipocytes were cotransfected with the leptin gene region (–101/–83) linked to a reporter gene (PGL3 promoter) and the Sp1 expression vector. The leptin gene reporter activity was reduced in the presence of the Sp1 expression vector. It is suggested that Sp1 can bind to the Sp1 consensus sequence located between nucleotides –101 and –83 of the leptin gene, and that the bound Sp1 inactivates transcription. To further define the sequence involved in the glucose/insulin responsiveness of the leptin promoter, mutagenesis was performed in the region from –100 to –95 of the leptin gene. The mutation resulted in a loss of responsiveness to glucose/insulin (data not shown). The leptin gene reporter activity was not significantly changed in the presence of the Sp3 expression vector.

As a positive control, the transcriptional activities of FAS(–57/–35) linked to PL1cat or ACL(–64/–41) linked to ACLcat20 were measured in the same hepatocytes and adipocytes used for leptin transcription studies. The transcriptional activities of the FAS and ACL genes were stimulated by glucose/insulin, and the stimulation was suppressed by

Table 1
Effects of glucose/insulin, PUFA, leptin and Sp1 family on transcriptional activities of leptin gene and fatty acid synthase gene

	Hepatocytes	Adipocytes
(A) Relative reporter activity of Leptin(–101/–83)		
Glu	0.69 ± 0.06 ^b	0.47 ± 0.03 ^c
Glu+In	1.00 ± 0.09 ^a	0.87 ± 0.05 ^a
Glu+In+20:4	0.56 ± 0.20 ^{b,c}	0.65 ± 0.04 ^b
Glu+In+leptin	0.64 ± 0.13 ^b	0.73 ± 0.06 ^b
Glu+In+Sp1 vector	0.65 ± 0.16 ^b	0.58 ± 0.08 ^{b,c}
Glu+In+Sp3 vector	0.86 ± 0.12 ^{a,b}	0.86 ± 0.12 ^{a,b}
(B) Relative reporter activity of FAS(–57/–35)		
Glu	0.35 ± 0.04 ^c	0.39 ± 0.04 ^c
Glu+In	1.00 ± 0.12 ^a	0.63 ± 0.09 ^b
Glu+In+20:4	0.28 ± 0.10 ^c	0.43 ± 0.05 ^c
Glu+In+leptin	0.53 ± 0.17 ^{b,c}	0.39 ± 0.15 ^c

A: The plasmid construct containing leptin(–101/–83) linked to PGL3 promoter was introduced into primary cultured hepatocytes and adipocytes. The cells were cultured for 48 h to measure the reporter activities in Williams' E medium containing 20 mM glucose (Glu) with or without 0.1 µM insulin (In). When included, 0.1 mM arachidonic acid (20:4) or 0.3 µM leptin was added. To some cultures, leptin(–101/–83) linked to PGL3 promoter was cotransfected into cells with or without the Sp1 or Sp3 expression vector. B: The construct of FAS(–57/–35) linked to PL1cat was introduced into the same cells as used for A. The percentages of acetylated forms of [¹⁴C]chloramphenicol were determined as the CAT activities for each sample. In A and B, the relative reporter activities are normalized to Glu+In in hepatocytes. Means with different superscript letters in each item are significantly different at $P < 0.05$ (by ANOVA). Means ± S.D. of four experiments are shown.

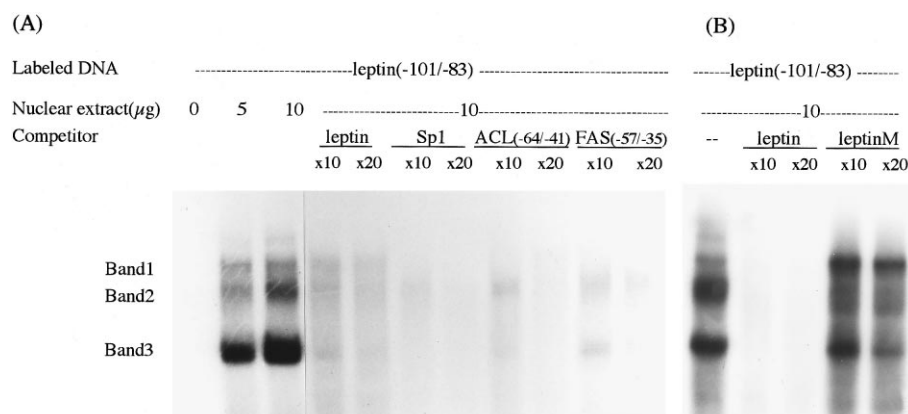


Fig. 1. Gel mobility shift assays using liver nuclear extracts with end-labeled leptin(–101/–83). Increasing amounts of rat liver nuclear extracts were incubated in the presence of end-labeled oligonucleotide leptin(–101/–83) and then loaded onto a 4% non-denaturing polyacrylamide gel. A: Competitor DNA [leptin(–101/–83), Sp1, ACL(–64/–41) or FAS(–57/–35)] was added in 10- and 20-fold molar excess relative to labeled DNA. B: Competitor DNA [leptin(–101/–83) or mutant leptin(–101/–83)] was added in 10- and 20-fold molar excess relative to labeled DNA. The species and molar ratios of competitor DNAs are indicated at the top.

PUFA or leptin in a manner similar to the leptin gene. The results for FAS transcription are shown in Table 1B, and the results for the ACL gene were very similar (data not shown). These results confirmed those of previous studies [15,16]. The transcriptional activities of the FAS and ACL genes stimulated by glucose/insulin were suppressed in the presence of Sp1 expression vector [15,16]. Leptin(–101/–83), FAS(–57/–35) and ACL(–64/–41) contain similar GC-rich sequences in which an Sp1 consensus sequence is found.

3.2. Gel mobility shift assays

To identify and characterize potential protein-binding activity associated with the leptin(–101/–83) region, an electrophoretic mobility shift assay was used. End-labeled oligonucleotide leptin(–101/–83) was incubated with nuclear extracts of rat liver and subjected to non-denaturing polyacrylamide gel electrophoresis. Three bands of DNA-protein complexes were observed and the formation of these complexes was competed against by increasing amounts of unlabeled leptin(–101/–83) oligonucleotide (Fig. 1A). These results demonstrated the specificity of the DNA-protein complex formation. In the presence of excess unlabeled leptin(–101/–83)M (mutated oligonucleotide), no competition was observed (Fig. 1B). This suggests that sequences between –101 and –83 of the leptin gene are essential for the binding of nuclear factor(s) to this putative glucose/insulin response region.

The similarities between nucleotides –54 to –43 of FAS and –60 to –49 of ACL were 9 out of 12, those between nucleotides –51 to –40 of FAS and –100 to –89 of leptin were 8 out of 12 and those between nucleotides –58 to –51 of ACL and –101 to –94 of leptin were 8 out of 8. Therefore, we tested whether these FAS and ACL sequences could compete for the nuclear factors binding to leptin(–101/–83). As shown in Fig. 1A, FAS(–57/–35) and ACL(–64/–41) oligonucleotides as well as leptin(–101/–83) itself could effectively compete against the formation of the three bands of DNA-protein complexes at 10- and 20-fold molar excess relative to the labeled leptin probe. Leptin(–101/–83), FAS(–57/–35) and ACL(–64/–41) contain GC-rich sequences in which a Sp1 consensus sequence is found. Addition of a given amount of Sp1 probe to the gel mobility shift reactions effectively

competed against the labeled leptin probe (Fig. 1A). These results suggested that Sp1 can bind to the glucose/insulin response elements of leptin(–101/–83) as well as to those of FAS(–57/–35) and ACL(–64/–41).

The results for nuclear extracts of adipose tissue are shown in Fig. 2. Only one band of DNA-protein complex (band 3) was seen in the fluorogram. Addition of Sp1 probe to the gel mobility shift reactions competed against the labeled leptin probe, although the competition was not as strong as in the liver nuclear extracts. After incubation of nuclear extracts of adipose tissue with the leptin probe in the presence of Sp1 antibody, the supershift band disappeared (Fig. 3). Therefore, any other binding protein than Sp1 may exist for DNA-protein complex formation in adipose tissue. The physiological significance of these findings awaits further investigation.

Since Sp1 and other related proteins, particularly Sp3, are known to bind to the Sp1 consensus sequence, we attempted to further demonstrate binding of the Sp1 family to the leptin gene. After incubation of nuclear extracts with the leptin probe in the presence of Sp1 antibody, band 1, band 2 and band 3 were supershifted, as shown in Fig. 3. The formation

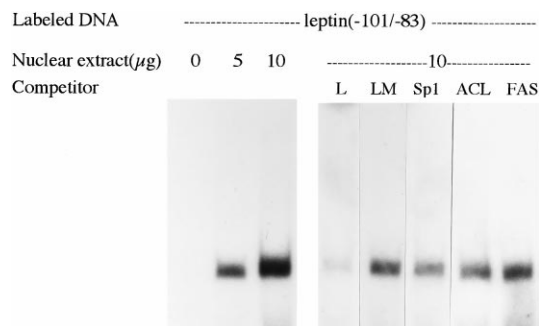


Fig. 2. Gel mobility shift assays using adipose tissue nuclear extracts with end-labeled leptin(–101/–83). Increasing amounts of rat adipose tissue nuclear extracts were incubated in the presence of end-labeled oligonucleotide leptin(–101/–83). Competitor DNA was added in 10-fold molar excess relative to labeled DNA. The species of competitor DNAs are indicated at the top. L: leptin(–101/–83), LM: mutant leptin(–101/–83), ACL: ACL(–64/–41), FAS: FAS(–57/–35).

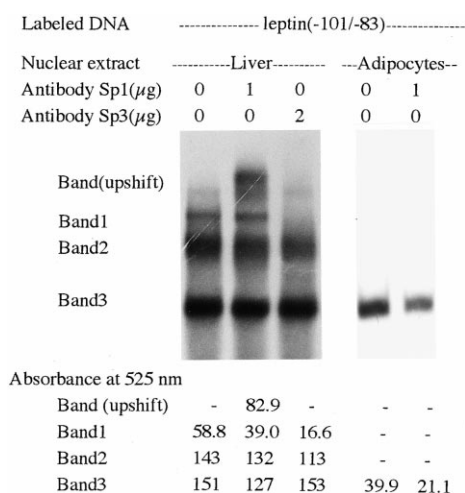


Fig. 3. DNA mobility supershift assay. 5 μg of nuclear extracts from rat liver or adipose tissue were incubated with or without Sp1 and Sp3 antibody (1 μg or 2 μg, respectively) before addition to end-labelled oligonucleotide leptin(-101/-83) and then loaded onto a 4% non-denaturing polyacrylamide gel. To measure the DNA binding the exposed film was scanned at 525 nm and the optical densities are shown below each fluorogram.

of band 1 and band 2 was efficiently inhibited in the presence of Sp3 antibody. These results show that band 1 and band 2 represent specific leptin(-101/-83)-containing protein-DNA complex(es) which contain Sp3 and Sp1. Band 3 represents specific leptin(-101/-83)-protein-DNA complex(es) which contain Sp1 but not Sp3. It has been reported that the glucose activation of acetyl-CoA carboxylase gene promoter II was mediated by a member of the Sp1 family of transcription factors [32], and moreover, Sp1, Sp3 and Sp4 have similar structural characteristics and identical DNA binding specificities [33,34].

Daniel and Kim [32] reported that promoter II of the acetyl-CoA carboxylase gene (-340 to -249) was activated by high concentrations of glucose, and that the effects of glucose were mediated by Sp1. On the other hand, Rolland et al. [35] reported that FAS promoter activity mainly depended on a region from -200 to -126, and this sequence exerted a strong negative effect on the FAS promoter in adipocytes from lean rats but not in those from obese rats. They demonstrated that Sp1 or Sp1-like proteins were bound to this DNA subregion.

The present studies demonstrated that Sp1 and Sp3 can bind to the sequences located between -101 and -83 of the leptin gene, and that the bound Sp1 inactivated the leptin transcription. Similarly, Sp1 and Sp3 bound to nucleotides -57 to -35 of the FAS gene and nucleotides -64 to -41 of the ACL gene, and the binding of Sp1 inactivated the transcription of the FAS(-57/-35) and ACL(-64/-41) genes whereas the binding of Sp3 stimulated the transcription of these genes (Fukuda et al., unpublished results). However, the binding of Sp3 did not stimulate the transcription of genes controlled by the leptin(-101/-83) region in the present experiments. Moreover, these three nucleotide regions have been postulated to be the response elements of glucose/insulin activation which are inactivated by PUFA, Sp1 or leptin. Thus, we postulated that Sp1 and Sp3 are somehow involved in the glucose/insulin activation and the PUFA or leptin inactivation of the leptin gene as well as the FAS and

ACL genes, suggesting that these genes are coordinately regulated.

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