

Transcriptional regulation of fatty acid synthase gene and ATP citrate-lyase gene by Sp1 and Sp3 in rat hepatocytes¹

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Abstract When two copies of the sequences spanning -57 to -35 of the fatty acid synthase (FAS) or -64 to -41 of the ATP citrate-lyase (ACL) gene linked to a reporter gene were transfected into primary cultured hepatocytes, the reporter activities significantly increased in response to insulin/glucose treatment. In cotransfection experiments of the FAS($-57/-35$) with the Sp1 or Sp3 expression vector, the reporter activities of transcription were suppressed by Sp1 and stimulated by Sp3. In the cotransfection experiments of ACL($-64/-41$), the activities were suppressed by Sp1 but were unchanged by Sp3. A similar effect of Sp1 and Sp3 on transcription was seen in mRNA concentrations and enzyme activities of endogenous FAS and ACL. Moreover, the mRNA concentrations and enzyme activities of endogenous acetyl-CoA carboxylase were suppressed by Sp1 and greatly increased by Sp3. Gel mobility super shift assays using antibodies against Sp1 or Sp3 revealed the binding of the transcription factors Sp1 and Sp3 with the GC rich regions located within FAS($-57/-35$) and ACL($-64/-41$) genes. The formation of DNA-protein complexes was decreased in rats fed a high-carbohydrate diet in comparison with that in fasted rats, but feeding the corn oil diet inhibited this decrease. In Western immunoblotting assay, however, the amount of Sp1 and Sp3 remained unchanged in the dietary conditions. Therefore, the binding of DNA-protein complexes was not due to changes in the amount of Sp1 and Sp3 but to changes in the binding activity, suggesting that these transcription factors may be an important determinant of lipogenic enzyme expression.

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Key words: Fatty acid synthase; ATP citrate-lyase; Response region; Insulin/glucose; Sp1; Sp3

1. Introduction

The gene expression of lipogenic enzymes in the rat liver was increased by a high-carbohydrate diet and decreased by feeding polyunsaturated fat, fasting or a diabetic state [1]. We previously mapped the responsive sequences to insulin/glucose-stimulation and PUFA-suppression in proximal promoter regions from position -57 to position -35 of the fatty acid synthase (FAS) gene and -64 to -41 of the ATP citrate-

lyase (ACL) gene by using a transfection system of cultured primary hepatocytes [2,3]. The nucleotides -54 to -43 of the FAS gene have some sequence similarity (9 of 12) to those from -60 to -49 of the ACL gene which are insulin/glucose responsive. Transcriptional regulation is exerted by the combined action of proteins binding to distinct promoter and enhancer elements. It has become evident that a limited number of *cis*-acting DNA elements are recognized not only by single factors, but also by a set of different families of transcription factors, thereby acting positively or negatively on transcription [4].

Daniel and Kim [5] reported that the promoter II of acetyl-CoA carboxylase gene (-340 to -249) was activated by high concentrations of glucose and the effect of glucose was mediated by the transcription factor Sp1. Moreover, they have shown that nuclear extracts from glucose-treated cells exhibit increased Sp1 binding activity [6]. However, Rolland et al. [7] 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. We demonstrated that the GC rich regions located from -57 to -35 of FAS and from -64 to -41 of ACL can bind to Sp1 and that the bound Sp1 inactivated transcription [8,9]. To further investigate the transcriptional regulation of FAS and ACL genes, we attempted to identify the participation of Sp1 and its family.

2. Materials and methods

2.1. Materials

Restriction endonuclease and other enzymes were purchased from Takara Shuzo. The luciferase assay kit was from Toyo Ink. Williams' medium E was purchased from Flow Laboratories. Other culture media were obtained from Nissui Seiyaku. [¹⁴C]Chloramphenicol (2.22 GBq/mmol), [α -³²P]dCTP (111 TBq/mmol) and [γ -³²P]ATP (110 GBq/mmol) were purchased from ICN. Nylon filters (Hybond N) were purchased from Amersham. Antibodies against Sp1 and Sp3 were from Santa Cruz Biotech. Lipofectin reagent was from Life Technologies. Most other reagents were obtained from Sigma and Wako.

2.2. Plasmid constructs

Plasmid pactL, a luciferase vector containing β -actin enhancer and promoter, was used as an internal control to normalize for variations in transfection efficiency [10]. Plasmid PL1cat, which contains fragment -94 to $+37$ of the L-type pyruvate kinase (LPK) gene, was produced from LPKcat [11]. Plasmid ACLcat20, which contains fragment -20 to $+128$ of the ACL gene, was produced from ACLcat2394 [3]. Plasmid pCH110, encoding β -galactosidase activator, was used to measure a transfection efficiency [12]. Prof. Y. Fujii-Kuriyama (Tohoku University, Japan) generously donated plasmid pRSVSp1 [13]. Plasmid pRCMVSp1 was obtained by cloning the rat Sp1 cDNA as

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¹ Enzymes: fatty acid synthase (EC 2.3. 1.85), ATP citrate-lyase (EC 4.1.3.8) and acetyl-CoA carboxylase (EC 6.4.1.2).

Abbreviations: ACL, ATP citrate-lyase; FAS, fatty acid synthase; CAT, chloramphenicol acetyltransferase

*Xho*I fragment from pRSVSp1 into the *Hind*III site of plasmid pRCMV via *Hind*III linker. Plasmid pRCMVSp3 [14] was generously donated by Dr. G. Suske (Institute für Molekularbiologie und Tumorforschung, Germany). The following single-stranded oligonucleotides were synthesized by GIBCO.

FAS(–57/–35): 5'-GATCGTGGCCGCGCGGGGATGGCCGCG-3'
 ACL(–64/–41): 5'-GATCTGATGGGGGCGGGAGAGCCCCG-3'
 Sp1: 5'-ATTCGATCGGGGCGGGGCGAGC-3'

Double-stranded oligonucleotides of FAS(–57/–35) or ACL(–64/–41) were inserted into the *Bam*HI sites of PL1cat or ACLcat20, respectively. Two copies of the sequences spanning –57 to –35 of FAS or –64 to –41 of ACL were linked to reporter genes.

2.3. Primary hepatocyte culture and transfection

Male Wistar rats (200–250 g) maintained on a stock diet (Oriental Koubo, MF) were fasted for 16 h before killing. Hepatocytes were isolated by the collagenase perfusion method [15] and plated at a density of 3×10^6 cells/60 mm Primaria culture dish (Falcon). After a 6 h attachment period, the medium was replaced with modified Williams' E media (lacking methyl linoleate) supplemented with 5 mM glucose, 26 mM sodium bicarbonate, 2 mM glutamine and 1 μ M dexamethasone; then mixtures of 8 μ g of FAS(–57/–35) linked to PL1cat or ACL(–64/–41) linked to ACLcat20 and 2 μ g of pactL were mixed with 5 μ g of pRCMVSp1, pRCMVSp3 or pRCMV (as control) were transfected into hepatocytes using lipofectin for 16 h [16]. To measure the transfection efficiency, 5 μ g plasmid pCH110 were transfected into hepatocytes. Subsequently, the cells were cultured for 48 h in experimental media (with 100 μ g/ml streptomycin and 100 units/ml penicillin) containing 20 mM glucose with 0.1 μ M insulin. All transfections were performed at least three times in duplicate.

2.4. Chloramphenicol acetyltransferase (CAT) and luciferase assay

The cells were incubated for 48 h after transfection, harvested and lysed by sonication. Then the supernatant from each sample was assayed for luciferase using a standard kit [17]. The amounts of cell extracts normalized by luciferase activity were used for CAT assays after heating at 60°C for 10 min [18,19]. Acetylated and non-acetylated forms of [¹⁴C]chloramphenicol were determined by a scintillation counter and the percentages of the acetylated forms were calculated.

2.5. Preparation of RNA and dot blot hybridization

Total cellular RNA was isolated from cell cultures by the acid guanidinium thiocyanate-phenol-chloroform extraction method [20]. Dot blot hybridization of total cellular RNA was performed as described previously [21]. Acetyl-CoA carboxylase, ACL and FAS cDNA species were cloned as described previously [21–23]. The genomic clone of rat rRNA was obtained from the Japanese Cancer Research Resources Bank. A *Bam*HI/*Eco*RI fragment of approximately 1 kb was isolated from this clone and used as a probe for 18S rRNA. The probes were labeled using a multiprimer DNA-labeling system kit (Amersham) with [α -³²P]dCTP. Relative densities of hybridization signals were determined by scanning the autoradiograms at 525 nm (Model CS-9000, Shimadzu) and normalized to the values of the 18S rRNA.

2.6. Enzyme activities

The cells were homogenized and centrifuged for 30 min at 105000 \times g at 4°C, and supernatant fractions were collected for measurement of enzyme activities. FAS, ACL and acetyl-CoA carboxylase activities were assayed as described previously [24].

2.7. Gel mobility supershift and Western blot assays

Nuclear extracts were prepared from rat livers as described by Gorski et al. [25]. Male Wistar rats were fasted for 2 days and then refed a high-carbohydrate/fat-free diet or a 10% corn oil diet for 5 h. The high-carbohydrate diet contained 67% sucrose and the amount of 10% sucrose was substituted by corn oil in the 10% corn oil diet. Antibody against Sp1 or Sp3 was added to the reaction mixture and this was incubated for 1 h at room temperature prior to adding the labeled probes as described previously [8]. The nucleotide sequences of ACL(–64/–41), FAS(–57/–35) and Sp1 are shown above. Western blot assays using anti-Sp1 or anti-Sp3 were performed as described previously [26].

3. Results

3.1. Regulation of the FAS gene by Sp1 and Sp3

We previously mapped the sequence responsible for insulin/glucose-stimulation in the proximal promoter region from –57 to –35 of FAS of rat hepatocytes [8]. The sequence from –57 to –35 of the FAS gene contains potential overlapping binding sites for the Sp1 family. To determine directly the binding of Sp1 or Sp3 to these sequences, rat hepatocytes were cotransfected with FAS(–57/–35) linked to the PL1cat construct and the Sp1 expression vector (pRCMVSp1) or the Sp3 expression vector (pRCMVSp3). pRCMV lacked Sp1 or Sp3 sequences and served as the control. The CAT activity of FAS(–57/–35) linked to PL1cat was reduced in the presence of the Sp1 expression vector, whereas the activity was markedly increased in the presence of the Sp3 expression vector (Fig. 1A, top). It is suggested that the Sp1 consensus sequences, located between –57 and –35 of the FAS gene, can bind

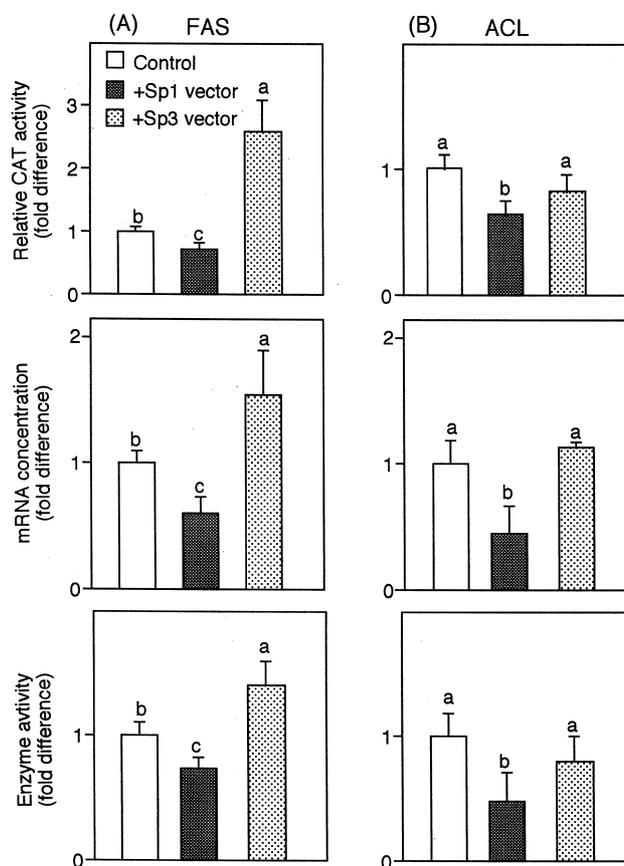


Fig. 1. Effects of Sp1 or Sp3 on the expression of (A) FAS and (B) ACL genes. FAS(–57/–35) linked to PL1cat, ACL(–64/–41) linked to ACLcat20 (8 μ g) or pactL (2 μ g) was cotransfected into rat hepatocytes with 5 μ g of pRCMVSp1 (the Sp1 expression vector), pRCMVSp3 (the Sp3 expression vector) or pRCMV (the empty vector for control). The cells were incubated in Williams' E medium containing 20 mM glucose and 0.1 μ M insulin. The percentage of acetylated forms of [¹⁴C]chloramphenicol was determined as the CAT activity for each sample. The relative CAT activities, the mRNA concentrations and enzyme activities are normalized to the value for the control vector. The mRNA concentrations and enzyme activities of endogenous ACL or FAS in the cells are shown. Means with different superscript letters in each square are significantly different at $P < 0.05$ (by ANOVA). Results are mean \pm S.D. of four experiments.

to Sp1 or Sp3, and that the bound Sp1 inactivated transcription and the bound Sp3 activated transcription.

The effects of the Sp1 or Sp3 gene expression vector on the endogenous FAS mRNA concentrations and enzyme activities were examined. The FAS mRNA concentrations were reduced to half in hepatocytes treated with the Sp1 gene expression vector (Fig. 1A, middle). In hepatocytes treated with Sp3 expression vector, however, the mRNA concentrations were increased 2.5-fold. A similar finding was observed in the enzyme activities (Fig. 1A, bottom). When rat hepatocytes were cotransfected with the Sp1 or Sp3 expression vectors, galactosidase expression vector was added, the cells were stained with X-Gal, and the transfection efficiency was $48 \pm 12\%$ ($n = 5$).

3.2. Regulation of the ACL gene by Sp1 and Sp3

We previously reported that the sequences responsible for insulin/glucose-stimulation were in the proximal promoter region from -64 to -41 of ACL of the rat liver [9]. The CAT activity of ACL($-64/-41$) linked to ACLcat20 was reduced in hepatocytes cotransfected with the Sp1 expression vector. The findings were similar to the cotransfection studies of FAS gene expression. However, the activity was not changed in hepatocytes cotransfected with the Sp3 expression vector (Fig. 1B, top). A similar finding to the transcription was seen in endogenous mRNA concentrations and enzyme activities of ACL (Fig. 1B, middle and bottom).

3.3. Regulation of acetyl-CoA carboxylase mRNA concentration and enzyme activity by Sp1 and Sp3

The effects of Sp1 and Sp3 on endogenous acetyl-CoA carboxylase mRNA concentration and enzyme activity were examined in the hepatocytes obtained from the experiments described above. The mRNA concentration was reduced to half in hepatocytes cotransfected with Sp1 expression vector (Table 1). In hepatocytes cotransfected with Sp3 expression vector, however, the mRNA concentrations were markedly increased. A similar finding was observed in the enzyme activities.

3.4. Gel mobility super shift assays

We examined the DNA-protein binding activity of the ACL($-64/-41$) or the FAS($-57/-35$) region by electrophoresis mobility shift assay. 32 P-labeled oligonucleotide ACL($-64/-41$) was incubated with nuclear extract of rat liver and subjected to a non-denaturing polyacrylamide gel electrophoresis. One of the typical autoradiograms is shown in Fig. 2A. One band of the DNA-protein complex was observed; the complex was competed away in increasing amounts of unlabeled oligonucleotides of ACL($-64/-41$) [26]. The intensities

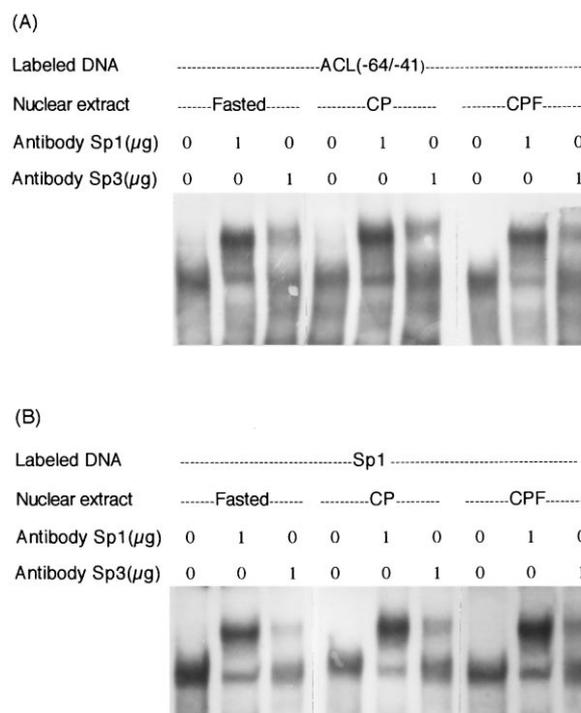


Fig. 2. DNA mobility shift assay. Gel mobility shift assays were performed with or without antibody against Sp1 or Sp3. (A) Labeled ACL($-64/-41$) or (B) labeled consensus Sp1 was used as a probe in the binding assay. Rats were fasted for 2 days and then refed a high-carbohydrate diet or a 10% corn oil diet for 5 h before being killed. Two micrograms of nuclear extracts from rat livers were incubated with or without the antibody (1 μg) before addition of the labeled oligonucleotides. The intensities of the bands for labeled ACL were 90.6, 70.3 and 91.9 (arbitrary units) for liver nuclear extracts of rats fasted, refed a high-carbohydrate diet and refed a corn oil diet, respectively (A). Those for labeled consensus Sp1 were 73.5, 43.6 and 61.4, respectively (B). The mean values \pm S.D. of four rats were shown in Section 3.

(arbitrary units) of the bands for ACL($-64/-41$) are written in the legend of Fig. 2A. Moreover, the relative intensities of the bands were 1.00 ± 0.19 , 0.73 ± 0.21 and 0.96 ± 0.18 (mean \pm S.D., $n = 4$) for liver nuclear extracts of rats fasted, refed a high-carbohydrate diet and refed a corn oil diet, respectively. Those for labeled FAS($-57/-35$) were 1.00 ± 0.09 , 0.75 ± 0.18 and 1.15 ± 0.20 , respectively. The autoradiograms for labeled FAS($-57/-35$) are not shown. Similar results were seen in FAS($-57/-35$) and ACL($-64/-41$). The intensities for the bands were decreased in rats fed the high-carbohydrate diet in comparison with that in fasted rats, but this decrease was inhibited by feeding the corn oil diet.

Table 1
Effects of transfection of the Sp1 or Sp3 gene expression vectors on acetyl-CoA carboxylase gene expression in rat hepatocytes

| | mRNA concentration (fold difference) | Enzyme activity (mU/mg) |
|-----------------------|--------------------------------------|-------------------------|
| Control vector | 1.00 ± 0.12^b | 2.62 ± 0.96^b |
| Sp1 expression vector | 0.51 ± 0.12^c | 1.34 ± 0.18^c |
| Sp3 expression vector | 3.51 ± 0.25^a | 6.66 ± 0.83^a |

5 μg of pRCMVSp1 (the Sp1 expression vector), pRCMVSp3 (the Sp3 expression vector) or pRCMV (control vector) were transfected into rat hepatocytes. The cells were incubated with 0.1 μM insulin and 20 mM glucose. The mRNA concentrations and enzyme activities of endogenous acetyl-CoA carboxylase in the cells are shown. The relative mRNA concentrations are normalized to the values for control. The enzyme activities in the supernatants of the cell homogenates are shown as mU/mg protein, where 1 mU is the amount of enzyme catalyzing the formation of 1 nmol/min at 37°C. Means with different superscript letters in each column are significantly different at $P < 0.05$ (by ANOVA). Results are mean \pm S.D. of four experiments.

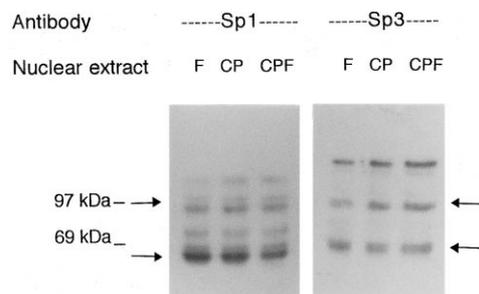


Fig. 3. Western blot analysis of Sp1 and Sp3 in nuclear extracts of rat livers. One hundred micrograms of nuclear protein extract prepared from rat livers which were fasted (F) 2 days and then refed a high-carbohydrate diet (CP) or a 10% corn oil diet (CPF) for 5 h before being killed, were separated by 8% SDS-polyacrylamide gel electrophoresis and probed with antibody against Sp1 and Sp3. Molecular weight markers are indicated on the right.

When ^{32}P -labeled Sp1 was incubated with nuclear extract of rat liver, one band of DNA-protein complex was observed; the complex was competed away in an increasing amount of unlabeled oligonucleotide of Sp1, FAS(–57/–35) or ACL(–64/–41) [8,9]. One of the typical autoradiograms was shown in Fig. 2B. The intensities (arbitrary units) of the bands for consensus Sp1 are written in the legend of Fig. 2B. Moreover, the relative intensities of the bands were 1.00 ± 0.21 , 0.65 ± 0.18 and 0.98 ± 0.14 (mean \pm S.D., $n = 4$) for liver nuclear extracts of rats fasted, refed a high-carbohydrate diet and refed a corn oil diet, respectively. Similar results to labeled ACL and FAS were seen in labeled Sp1.

To demonstrate further that Sp family binds to the ACL, specific antibody against Sp1 or Sp3 was added to the gel mobility shift assay. After incubation of nuclear extract with ^{32}P -labeled oligonucleotide ACL(–64/–41) probe in the presence of anti-SP1, the band of ACL(–64/–41) was upshifted to a higher molecular weight. When anti-Sp3 was added, the band was also upshifted and migrated slightly higher than that of the Sp1-antibody complex. Similar findings were observed in the labeled oligonucleotide FAS(–57/–35). Only the findings of ACL(–64/–41) are shown in Fig. 2A. These findings show that the band represents an ACL(–64/–41)- or FAS(–57/–35)-specific protein-DNA complex, which contains Sp1 and a little Sp3. The migration pattern of the band was very similar to that formed using ^{32}P -labeled Sp1 (Fig. 2B).

3.5. Western blot analysis

The concentrations of Sp1 and Sp3 in liver nuclear extracts were compared in rats fasted and then refed a high-carbohydrate diet or 10% corn oil diet for 5 h (Fig. 3). Western immunoblot experiments in which nuclear extracts were probed with a polyclonal antibody against Sp1. Antibody against Sp1 detected two major immunoreactive bands, a doublet at approximately 97 kDa and a single band at 66 kDa in liver nuclear extracts. These bands are similar to the pattern previously reported [27]. Antibody against Sp3 detected three major immunoreactive bands. The bands of approximately 97 kDa and 65 kDa were consistent with previous observations [14]. The intensities of the bands for Sp1 and Sp3 were not significantly different in fasted, high-carbohydrate-fed or corn oil-fed rats. This demonstrated that transcription factors, Sp1

and Sp3 are present at similar levels in the liver extracts of rats fasted, fed a high-carbohydrate diet or a corn oil diet.

4. Discussion

In this study we showed that Sp1 and Sp3 have distinct effects on FAS or ACL promoter activities in rat hepatocytes. When rat hepatocytes were transfected with FAS(–57/–35) or ACL(–64/–41) linked to a reporter gene, the reporter activities were reduced in the hepatocytes cotransfected with Sp1 expression vector. In hepatocytes cotransfected with the Sp3 expression vector, however, the reporter activity of FAS was increased but the activity of ACL was not changed. A similar effect to each transcription was seen in mRNA concentrations and enzyme activities of endogenous FAS and ACL. Moreover, mRNA concentrations and enzyme activities of endogenous acetyl-CoA carboxylase were reduced in the hepatocytes cotransfected with Sp1 expression vector, but markedly increased in the those cotransfected with Sp3 expression vector. Sp1 coordinately reduced gene expressions of FAS, ACL and acetyl-CoA carboxylase. Sp3 increased gene expressions of FAS and acetyl-CoA carboxylase, but the effect of Sp3 on ACL gene expression was not clear. The effect of Sp3 was not always similar in lipogenic enzyme gene expression. Rajakumar et al. [28] demonstrated that the binding of nuclear factors Sp1 and Sp3 to the mouse glucose transporter isoform 3 (Glut 3) gene with Sp1 mediated suppression, and with Sp3 mediated activation of Glut 3 transcription.

Gel mobility shift assays were used to determine whether protein-DNA binding to the GC element correlated with the function of the promoter. Since a number of proteins bind the GC element, including other members of the Sp1 family, specific antibodies against Sp1 and Sp3 were used and identified individual components in the complexes. When ^{32}P -labeled ACL, FAS or Sp1 was incubated with nuclear extract of rat livers, the DNA-protein complex formation was decreased in rats fed a high-carbohydrate diet in comparison with that in fasted rats, and this decrease was inhibited by addition of corn oil to the high-carbohydrate diet. In Western immunoblotting experiments using anti-Sp1 or Sp3 antibody, however, the intensities of the bands were not significantly different in the dietary groups. Therefore, it is suggested that the protein amounts of Sp1 and Sp3 were not different but the binding activities of these proteins were different. Daniel et al. [6] reported that glucose treatment of the cells (mouse 30A5 preadipocytes) increased Sp1 binding to two GC rich glucose response elements in promoter II of acetyl-CoA carboxylase, and nuclear extracts from glucose-treated cells exhibit increased Sp1 binding activity. This increase in the binding activity is not due to glucose-mediated changes in the amount of Sp1 in the nucleus but to an increase in the activity that modifies Sp1. It was concluded that Sp1 exhibits enhanced binding activity to the promoter II and transcriptional activation is the result of glucose-induced dephosphorylation by type I phosphatase. Therefore, the binding activity of DNA-Sp1 as well as Sp3 binding appeared to be changed by the nutritional conditions in the present experiment.

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