Transcriptional regulatory regions for expression of the rat fatty acid synthase

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Abstract **We previously mapped the sequences responsive to insulin/glucose stimulation and polyunsaturated fatty acid (PUFA) suppression in proximal promoter region from −57 to −35 of fatty acid synthase (*FAS*) gene of rat liver [Fukuda et al. (1996) *Biochem. Mol. Biol. Int.* 38, 987–996]. When two copies of the sequences spanning −57 to −35 were linked to a reporter gene containing heterologous promoter and were used for transfection, the reporter activity significantly increased in response to insulin/glucose treatment in hepetocytes. This increase was inhibited by addition of PUFA. Gel mobility shift assays using the sequence from −57 to −35 as a probe revealed nuclear factor(s) from rat liver that specifically complexed with the sequences. In addition, by antibody supershift assays, we have detected the binding of the transcriptional factor Sp1 at the GC-rich region located within −57 to −35 of the FAS promoter. Cotransfection studies in rat hepatocytes, with the Sp1 expression vector and FAScat constructs, showed the inactivation of the promoter. These results were similar to those for the region from −68 to −52 of *FAS* gene (an insulin response element). The region from −68 to −52 of *FAS* gene competed for the formation of DNA–protein complexes to the region from −57 to −35 in the gel shift assay. Mutational analysis showed that the overlapping region of these two sequences was essential for the binding of Sp1. It has been demonstrated that both the regions from −57 to −35 and from −68 to −52 of the *FAS* gene are responsible for regulation due to insulin/glucose and PUFA, and Sp1 may be involved in the regulation.**

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Key words: Fatty acid synthase; Response element; Insulin; Glucose; Polyunsaturated fatty acid

1. Introduction

Fatty acid synthase (FAS) [EC 2.3.1.85], one of a set of lipogenic enzymes, catalyzes the synthesis of long-chain fatty acids. We previously reported that *FAS* gene expression is very low in fasted rat liver and greatly increased by refeeding a high carbohydrate diet, in a similar manner of other lipogenic enzymes [1,2]. Polyunsaturated fatty acid (PUFA) feeding markedly decreased the gene expression. Moreover, the gene expression is very low in diabetic rat, and insulin treatment causes a marked and rapid increase [1,2]. We previously mapped the responsive region to insulin/glucose stimulation and PUFA suppression in proximal promoter region from position −57 to position −35 of FAS by using the transfection system of cultured primary hepatocytes [3]. Nucleotides −56 to −43 of the *FAS* gene have some sequence similarity to −136 to −123 of the PUFA responsive region of L-type pyruvate kinase (*LPK*) gene, and −60 to −49 of insulin/glucose and PUFA responsive region of ATP citrate-lyase (*ACL*) gene [3–5]. Moustaid et al. [6] found that insulin response sequences of *FAS* gene located in the region −68 to −52 in 3T3-L1 adipocytes. In order to further investigate the transcriptional regulation of *FAS* gene, we have attempted to identify the *cis*-acting DNA sequences responsible to glucose, insulin and polyunsaturated fatty acids by CAT assay, gel shift assay and antibody supershift assay. A synthetic nucleotide probe of −57 to −35 of *FAS* gene was used for transfection system in cultured hepatocytes of rat, comparing with a synthetic probe of −68 to −52 of *FAS* gene.

2. Materials and methods

2.1. Materials

Restriction endonuclease and other enzymes were purchased from Takara Shuzo (Kyoto). The sequence kit, luciferase assay kit and acetyl-CoA were from Applied Biosystems, Toyo Ink (Tokyo) and Sigma, respectively. William's medium E was purchased from Flow Laboratories. Other culture media were obtained from Nissui Seiyaku (Tokyo). [^{14}C]Chloramphenicol (2.22 GBq/mmol) and [γ-^{32}P]ATP (110 GBq/mmol) was from ICN. Antibody against Sp1 was from Santa Cruz Biotec. Lipofectin reagent was from Life Technologies.

2.2. Plasmid constructs

Plasmid pUC0cat, a promoter-less CAT vector, and pUC2cat, a CAT vector with simian virus 40 early enhancer and promoter were used as negative and positive controls, respectively [7]. Plasmid pactL, a luciferase vector containing β-actin enhancer and promoter, was used as an internal control to normalize for variations in transfection efficiency [8]. Plasmid FAScat57, which contains fragment of −57 to +79 of *FAS* gene, was produced from FAScat1604 (contain fragment −1604 to +79) by 5′-deletion using exonuclease III [3]. Plasmid PL1cat, which contains fragment −94 to +37 of *LPK* gene, was produced from LPKcat [9]. Plasmid pRSV(Xho) and pRSVSp1 [10] were generously gifted by Prof. Y. Fujii-Kuriyama (Touhoku University). The following single-stranded oligonucleotides were synthesized by GIBCO.

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FAS(-57/-35):  5′-GATCGTGGCCGCGCGGGGATGGCCGCG-3′
                    3′-CACCGGCGCGCCCCTACCGGCGCCTAG-5′
Mutant FAS(-57/-35):5′-GATCGTAATTACGCGGGGATGGCCGCG-3′
                    3′-CATTAATGCGCCCCTACCGGCGCCTAG-5′

FAS(-68/-52):  5′-GATCGCCCATGTGGCGTGGCC-3′
                    3′-CGGGTACACCGCACCGGCTAG-5′
ACL(-64/-41):  5′-GATCTGATGGGGGGCGGGGAGGAGCCCG-3′
                    3′-ACTACCCCCCGCCCCTCCTCGGGCCCTAG-5′
Sp1:           5′-ATTCGATCGGGGCGGGGCGAGC-3′
               3′-TAAGCTAGCCCCGCCCCGCTCG-5′
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Double-stranded oligonucleotides of FAS(−57/−35) or FAS(−68/−52) were inserted into the *Bam*HI sites of PL1cat. The sequence of these inserts was verified by dideoxy sequencing, using a sequence kit [11]. Two copies of the sequences spanning −57 to −35 or −68 to −52 were linked to PL1cat.

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Abbreviations: ACL, ATP citrate-lyase; FAS, fatty acid synthase; LPK, L-type pyruvate kinase; CAT, chloramphenicol acetyltransferase; RSV, Rousarcoma virus; PUFA, polyunsaturated fatty acid

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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 experiments. The hepatocytes were isolated by the collagenase perfusion method [12] and plated at a density of 3×10^6 cells/60 mm Primaria culture dish (Falcon). After a 6 h attachment period, medium was replaced with modified William's E media (lacking methyl linoleate) supplemented with 5 mM glucose, 26 mM sodium bicarbonate, 2 mM glutamine and 1 μM dexamethasone, and then mixtures of 2 μg of pactL and 18 μg each of CAT constructs were transfected into hepatocytes using lipofectin for 16 h [13]. To explore the function of Sp1, fixed amount (8 μg) of FAS(−57/−35) or FAS(−68/−52) linked to PL1cat and 2 μg of pactL were mixed with various amounts of pRSVSp1. Total amount of DNA was kept constant in a series of transfection experiments by adding pRSV(Xho) as a carrier. Subsequently, cells were cultured for 48 h in experimental media (with 100 μg/ml streptomycin and 100 U/ml penicillin) containing 20 mM glucose or 20 mM pyruvate with or without 0.1 μM insulin. When included, 0.1 mM arachidonic acid was added. All transfections were performed at least 3 times in duplicate.

2.4. CAT and luciferase assay

The cells were incubated for 48 h after transfection, harvested and lysed by sonication. Then 20 μl of the supernatant from each sample were assayed for luciferase using a kit [14]. Normalized amounts of the cell extracts by luciferase activity were used for CAT assays after heating at 60°C for 10 min [15,16]. Acetylated and nonacetylated forms of [^{14}C]chloramphenicol were determined by scintillation counter and the percentages of the acetylated forms were calculated.

2.5. Gel mobility shift assay

Nuclear extracts were prepared as described by Gorski et al. [17]. End-labeled double-stranded oligonucleotide of FAS(−57/−35), FAS (−68/−52) or Sp1 was incubated with the indicated amount of nuclear proteins in 10 mM Hepes, pH 7.6, containing 75 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 10% glycerol, 1 μg of poly(dI-dC), 1×10^4 cpm ^{32}P-labeled oligonucleotide probe (0.1 ng). The reaction mixture was incubated for 1 h at room temperature and then loaded onto an 8% non-denaturing polyacrylamide gel in 45 mM Tris, 45 mM borate, pH 8.3, and separated by electrophoresis at 100 V for 4 h [9]. In competition studies, the indicated amounts of double-stranded oligonucleotide were added to the reaction mixture. The nucleotide sequences of FAS(−57/−35), FAS(−68/−52), ACL [5] and Sp1 [18] are shown above. For antibody supershift assays, 2 μl of Sp1 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. Identification of cis-active elements

To explore possible regulatory elements in the 5′-flanking region of *FAS* gene, double-stranded oligonucleotides −57 to −35 of the *FAS* gene were synthesized and linked into PL1cat, which CAT activity was very low. The plasmid DNA was transfected to rat hepatocytes. The cells were cultured in the presence of 20 mM glucose or 20 mM pyruvate with or without 0.1 μM insulin for 48 h, and the extracts were assayed for CAT activity. As shown in Table 1, the FAScat57 (−57/+79) and FAS(−57/−35) linked to PL1cat exhibited similar CAT activities in the presence of glucose and insulin. The result shows that the 23 bp sequence between −57 and −35 is sufficient to insulin/glucose responsive to the *FAS* gene. Moreover, pyruvate alone induced the CAT activity of FAS(−57/−35) linked to PL1 cat, in comparison with glucose alone. It is suggested that pyruvate stimulation did not necessarily require insulin to confer the expression, whereas glucose stimulation required insulin. The insulin/glucose stimulation of the CAT activity of FAS(−57/−35) linked to PL1cat was reduced in arachidonic acid-treated cells, representing a 60% decline in the activity. PUFA appeared to suppress the insulin stimulation. This suggests the region between −57 and −35 of the *FAS* gene is responsive to PUFA suppression as well as insulin/glucose stimulation of this gene.

Moustaid et al. [6] reported that insulin response sequences of the *FAS* gene may be located in the region from −68 to −52 in 3T3-L1 adipocytes, and sequences between −68 and −60 are essential for recognition and interaction with a *trans*-acting factors. Therefore, FAS(−68/−52) linked to PL1cat was transfected to rat hepatocytes and the extracts were assayed for CAT activity. The insulin/glucose stimulation of the CAT activity of FAS(−68/−52) linked to PL1cat was similar to those of FAS(−57/−35) linked to PL1cat and FAScat57 (−57/+79). However, pyruvate alone did not induce the CAT activity of FAS(−68/−52) linked to PL1cat, although pyruvate alone induced the activity of FAS(−57/−35) linked to PL1cat. The reason has not been elucidated. FAS(−57/−35) was at the region of GC-rich sequence in which Sp1 consensus sequence is found. This suggests the possible involvement of the Sp1 transcription factor in the insulin/glucose activation and/or the PUFA inactivation of promoter of FAS.

3.2. Regulation of the FAS gene by Sp1

To determine directly the binding of Sp1 to these sequences, rat hepatocytes were cotransfected with FAS(−57/−35) or FAS(−68/−52) linked to PL1cat construct and the Sp1 expression vector (pRSVSp1). pRSV(Xho) was lack Sp1 sequences, and served as the negative control. The CAT activity of FAS(−57/−35) linked to PL1cat was reduced in the presence of the Sp1 expression vector (Fig. 1). It is suggested that the Sp1 consensus sequences located between −57 and −35 of the *FAS* gene can bind to Sp1 and that the bound Sp1 inactivated transcription. The CAT activity of FAS(−68/−52) linked to PL1cat was also reduced in the presence of the Sp1 expression vector.

3.3. Gel mobility shift assays

We examined DNA–protein binding activity of FAS(−57/−35) or FAS(−68/−52) region by electrophoresis mobility shift assay. End-labeled oligonucleotide FAS(−57/−35) was incubated with nuclear extract of rat liver and subjected to

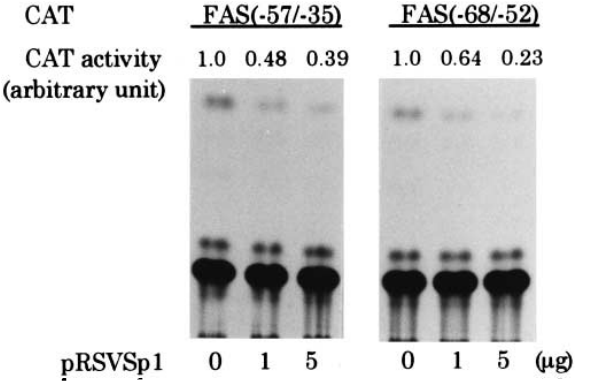

Fig. 1. Effect of Sp1 on the expression of CAT activity driven by promoter. FAS(−57/−35) or FAS(−68/−52) linked to PL1cat (8 μg) and pactL (2 μg) were cotransfected into rat hepatocytes with indicated amounts of pRSVSp1 (the Sp1 expression vector) in a total amount of DNA (15 μg) adjusted with pRSV(Xho) (the empty vector). One of the typical results is shown.

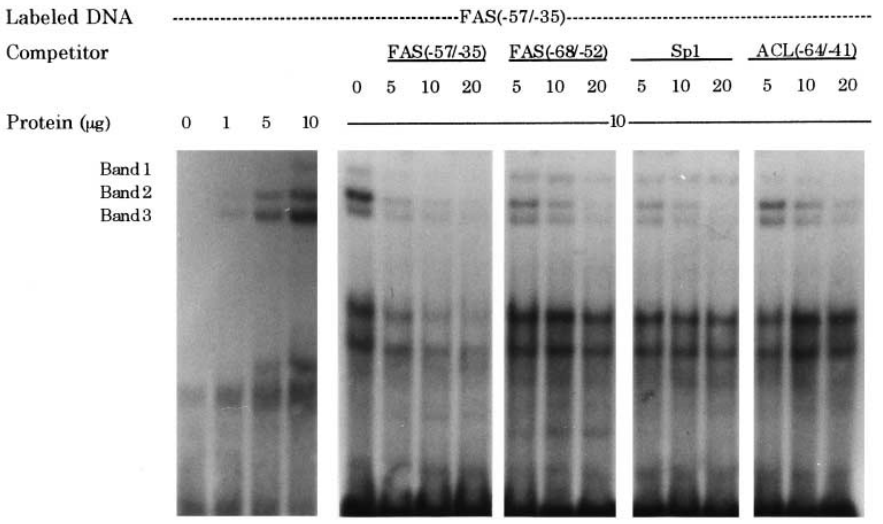

Fig. 2. Gel mobility shift assays using liver nuclear extracts with end-labeled FAS(−57/−35). Increasing amounts of rat liver nuclear extracts were incubated in the presence of end-labeled oligonucleotide FAS(−57/−35). The species and molar ratios of competitor DNAs are indicated at the top.

a non-denaturing polyacrylamide gel electrophoresis. Three bands of DNA–protein complexes were observed; the complexes were competed away by increasing amount of unlabeled oligonucleotide of FAS(−57/−35) (Fig. 2). Addition of the same amount of poly(dI-dC) or L-1 oligonucleotide [9], which contains hepatocyte nuclear factor 1 binding site of the *LPK* gene, to gel mobility shift reactions had no competition (data not shown). These results demonstrated specificity of the DNA–protein complex formation. Excess unlabeled FAS(−68/−52) also could effectively compete for the formation of three bands to the labeled FAS(−57/−35).

On the other hand, we showed previously that the *ACL* gene promoter segment −104 to −20 is the insulin/glucose stimulation and PUFA suppression response elements [5]. Since the similarity between nucleotides −54 to −43 of the *FAS* gene and −60 to −49 of the *ACL* gene was 9 out of 12, we tested whether this ACL sequence competes for the nuclear factors binding to FAS(−57/−35). As shown in Fig. 2,

Table 1
The CAT activities of the constructs of FAS gene

	Additions	Relative CAT activity (%)
FAScat57(−57/+79)	Glu	42.7 ± 7.50
	Glu+Ins	90.7 ± 16.7
	Glu+Ins+20:4	37.1 ± 7.42
	Pyr	82.7 ± 15.5
	Pyr+Ins	106 ± 21.2
	Pyr+Ins+20:4	68.0 ± 12.6
FAS(−57/−35) linked to PL1cat	Glu	60.2 ± 10.0
	Glu+Ins	103 ± 14.8
	Glu+20:4	64.1 ± 15.2
	Glu+Ins+20:4	61.1 ± 18.1
	Pyr	87.3 ± 12.5
	Pyr+Ins	91.4 ± 25.6
	Pyr+Ins+20:4	64.2 ± 20.0
FAS(−68/−52) linked to PL1cat	Glu	66.8 ± 14.3
	Glu+Ins	117 ± 14.8
	Glu+20:4	52.5 ± 19.4
	Glu+Ins+20:4	56.2 ± 19.3
	Pyr	50.0 ± 10.2
	Pyr+Ins	113 ± 20.7
	Pyr+Ins+20:4	60.9 ± 21.0
PL1cat	Glu	1.20 ± 0.05
	Glu+Ins	1.31 ± 0.07

The constructs of the *FAS* gene (FAScat57(−57/+79), FAS(−57/−35) or FAS(−68/−52) linked to PL1cat, and PL1cat) were introduced into primary hepatocytes. The hepatocytes were incubated with or without 0.1 μM insulin (Ins) for 48 h in the presence of 20 mM glucose (Glu) or 20 mM pyruvate (Pyr). When included, 0.1 mM arachidonic acid (20:4, n-6) was added. The percentage of acetylated forms of [^{14}C]chloramphenicol were determined as the CAT activities for each sample. The relative CAT activities are expressed as percentage of that of pUC2cat. Mean ± SD of 4 experiments are shown.

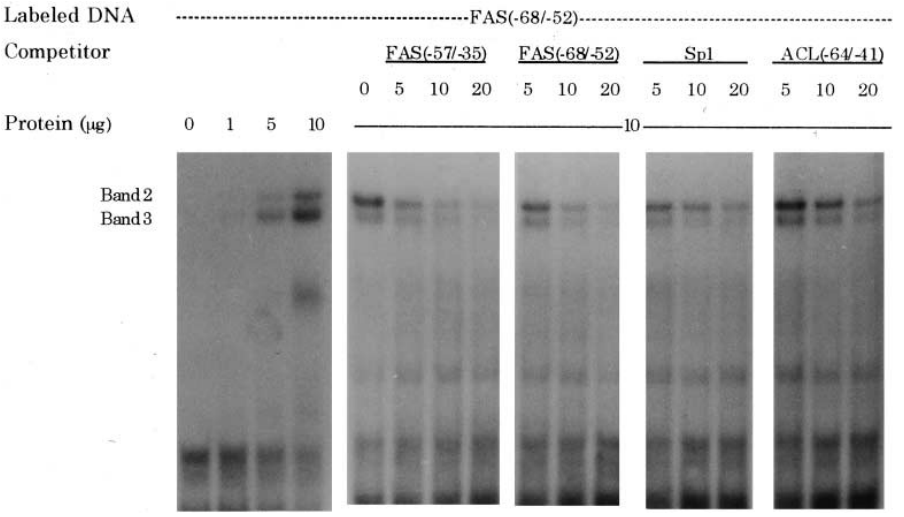

Fig. 3. Gel mobility shift assays using liver nuclear extracts with end-labeled FAS(−68/−52). Increasing amounts of rat liver nuclear extracts were incubated in the presence of end-labeled oligonucleotide FAS(−68/−52). The species and molar ratios of competitor DNAs are indicated at the top.

ACL(−64/−41) oligonucleotide as well as FAS(−57/−35) itself, could effectively compete for the formation of the three bands of DNA–protein complexes at 5, 10 and 20 molar excess (in ratio) to labeled FAS(−57/−35) probe. Addition of the same amount of Sp1 probe to the gel mobility shift reactions had effectively compete for the formation of band 2 and 3 to the labeled FAS(−57/−35) probe.

Moreover, end-labeled oligonucleotide FAS(−68/−52) was incubated with nuclear extracts of rat liver and subjected to a non-denaturing polyacrylamide gel electrophoresis. As shown in Fig. 3, two bands of DNA–protein were observed; the migration pattern of band 2 and band 3 due to end-labeled FAS (−68/−52) was very similar to band 2 and band 3 due to end-labeled FAS(−57/−35). Band 1 due to FAS(−57/−35) was missing in the migration pattern due to (FAS−68/−52). The two bands were competed away by increasing amounts of unlabeled oligonucleotide of FAS(−68/−52). FAS(−57/−35), ACL(−64/−41) and Sp1 oligonucleotide as well as FAS(−68/−52) itself, could effectively compete for the formation of the two bands of DNA–protein complexes. Finally, end-labeled oligonucleotide Sp1 was incubated with nuclear extracts of rat liver and subjected to a non-denaturing polyacrylamide gel electrophoresis. The migration patterns of band 2 and band 3 of the labeled FAS(−57/−35) and also the labeled FAS(−68/−52) were very similar to those formed by using end-labeled Sp1 (Fig. 4). FAS(−57/−35) and FAS (−68/−52) as well as Sp1 itself, could effectively compete for the formation of bands to the labeled Sp1 probe. These results

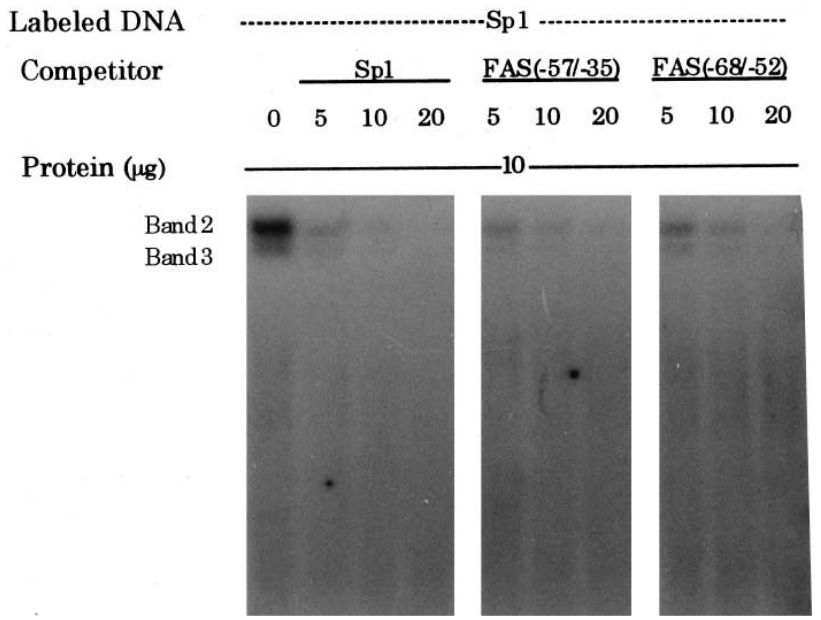

Fig. 4. Gel mobility shift assays using liver nuclear extracts with end-labeled Sp1. The species and molar ratios of competitor DNAs are indicated at the top.

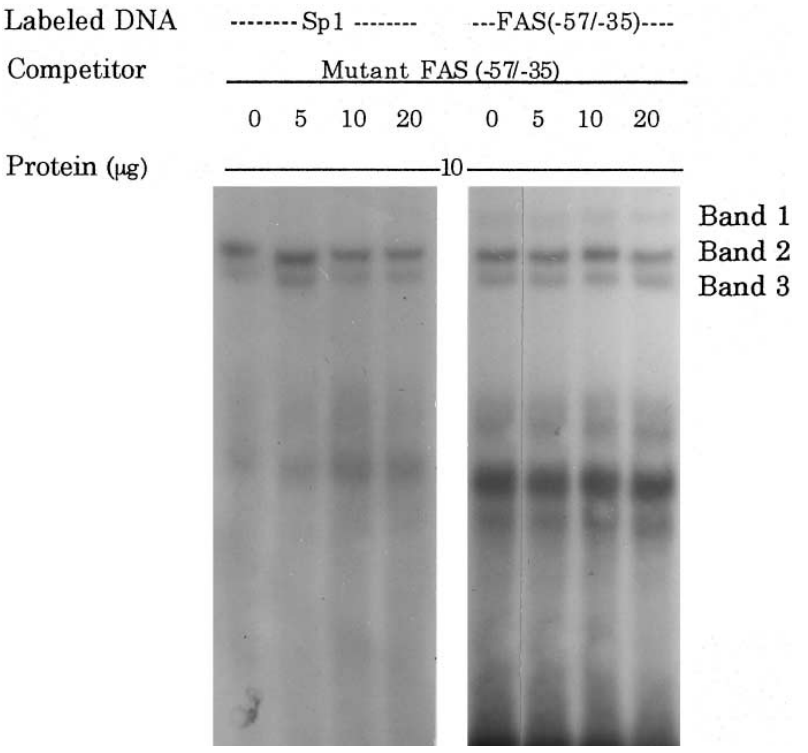

Fig. 5. Gel mobility shift assays with mutant oligonucleotide of FAS(−57/−35) using liver nuclear extracts with end-labeled Sp1 or FAS(−57/−35). The species and molar ratios of competitor DNAs are indicated at the top.

suggested that Sp1 binds to FAS(−57/−35) as well as FAS(−68/−52).

Sp1 was shown to bind to $^{G}/_{T}{}^{G}/_{A}GGCG^{G}/_{T}{}^{G}/_{A}{}^{G}/_{A}{}^{C}/_{T}$ decanucleotides, and multiple binding sites appear to be a common feature of promoters that are Sp1 responsive [20–24]. *FAS* gene promoter region from −35 to −68 contains two potential overlapping binding sites for Sp1 family, which are nucleotides −57 to −48 and −62 to −53. The former region is included in FAS(−57/−35) oligonucleotides and the latter in FAS (−68/−52).

We next tested this possibility using mutant oligonucleotide, mutated at the Sp1 site (−55 to −51) of FAS(−57/−35), as competitor in DNA mobility shift assay. When oligonucleotide of FAS (−57/−35) or Sp1 was end-labeled, it was not competed off with the excess unlabeled mutant of *FAS* gene (Fig. 5), suggesting the overlapping region in nucleotides of

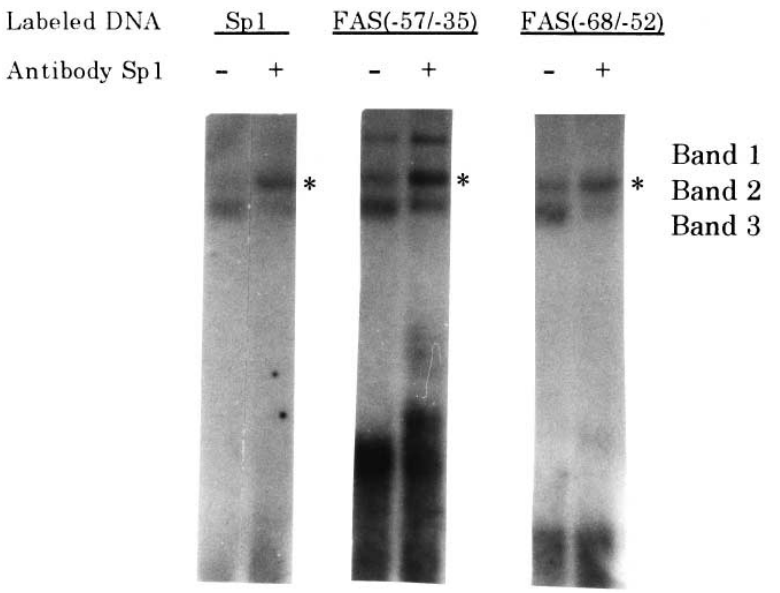

Fig. 6. DNA mobility supershift assay. Mobility shift assays were performed with or without Sp1 antibody. Labeled FAS(−57/−35), FAS(−68/−52) and the consensus Sp1 were used as probes in the binding assay.

FAS(−57/−35) and (−68/−52) is essential for the binding of Sp1.

To further demonstrate that Sp1 binding to the *FAS* gene, Sp1-specific antibody was added to the gel mobility shift assay. After incubation of nuclear extract with FAS(−57/−35) or FAS(−68/−52) probe in the presence of Sp1 antibodies, band 3 resulted in supershift, as shown in Fig. 6. The supershift band above band 3 coincided with band 2. The result shows that band 3 represents an FAS(−57/−35) or FAS (−68/−52) specific protein–DNA complex, which contains Sp1. It was reported that the glucose activation of *acetyl-CoA carboxylase* gene promoter II was mediated by a member of the Sp1 family of transcription factor [19], as members of a gene family, Sp1, Sp3 and Sp4 show similar structural characteristics and identical DNA binding specificity [20,21]. It is possible that band 2 represent a complex due to other Sp1 family proteins, such as Sp3 and Sp4.

Daniel and Kim [19] 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 Sp1. Moreover, they have shown that nuclear extracts from glucose-treated cells exhibit increased Sp1 binding activity [25]. 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 so that it binds more effectively to the promoter sequence. On the other hand, Rolland et al. [26] reported that FAS promoter activity mainly depended on a region from −200 to −126 and this sequence exerted a strong negative effect on 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 the sequences located between −57 and −35 of the *FAS* gene can bind to Sp1 and that the bound Sp1 inactivated transcription. Similarly, the nucleotide −68 to −52 of *FAS* gene and the nucleotide −64 to −41 of *ACL* gene [Fukuda et al., submitted] bound to Sp1 and the bound Sp1 inactivated its own transcription. On the other hand, these three nucleotides have been demonstrated to be the response elements of the insulin/glucose activation and the PUFA inactivation. Thus, we postulated that Sp1 is somehow involved in the PUFA inactivation of the *FAS* genes and *ACL* gene. Further studies are required to address this question.

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