J. Biochem. 126, 437-444 (1999)

Regulation of ATP Citrate-Lyase Gene Expression in Hepatocytes and Adipocytes in Normal and Genetically Obese Rats[1]

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Received January 20, 1999; accepted June 9, 1999

Transcriptional regulation of ATP citrate-lyase (ACL, one of the lipogenic enzymes) gene by glucose/insulin, polyunsaturated fatty acid (PUFA), and leptin has been investigated in hepatocytes and adipocytes of obese Wistar fatty rats and their lean littermates. The sequence spanning nucleotides −64 to −41 of the ACL gene, which is responsive to glucose/insulin stimulation [*Eur. J. Biochem.* 247, 497-502, 1997], was linked to a reporter gene and transfected into rat hepatocytes or adipocytes. The chloramphenicol acetyltransferase (CAT) activities in the presence of glucose alone were similar in primary cultured cells from both obese and lean rats. In the presence of glucose/insulin, however, the CAT activities were markedly increased in the hepatocytes of lean rats, but were not significantly increased in those of obese rats. The stimulation by glucose/insulin was reduced in PUFA-treated cells of lean rats. The stimulation was also reduced in leptin-treated cells or *ob* gene expression vector-containing cells. However, PUFA- or leptin-treated cells from obese rats did not show a significant reduction in insulin stimulation. The same effects were observed at the endogenous mRNA and enzyme levels. Similar results were seen in adipocytes, although the stimulation and suppression levels were much smaller than in hepatocytes. The expression of endogenous insulin receptor in hepatocytes and adipocytes was reduced in the presence of leptin or PUFA. We previously found that insulin-binding capacities are also reduced in the presence of leptin or PUFA and are very low in obese rats in comparison with lean. Moreover, gel mobility shift assays using end-labeled ACL-(−64/−41) revealed that nuclear factor(s) including Sp1 bind specifically to the sequence, and DNA-protein complex formation is reduced in the obese rats. Thus, the reductions in the insulin-stimulated ACL transcription may be ascribed in part to reductions in insulin binding to receptors and DNA-protein complex formation.

Key words: ATP citrate-lyase gene expression, insulin, leptin, insulin receptors, obese rat.

We previously mapped the sequences responsible for glucose/insulin stimulation and polyunsaturated fatty acids (PUFA) suppression in the proximal region, nucleotides −64 to −41, of the ATP citrate-lyase (ACL) [EC 4.1.3.8] gene of rat liver (*1*). When two copies of a synthetic nucleotide probe for nucleotides −64 to −41 of the ACL gene were linked to a reporter gene and the resultant construct used for transfection, the reporter activity was significantly increased in response to glucose/insulin treatment in hepatocytes. This increase was suppressed by the addition of PUFA (*2*).

In addition, we demonstrated the effects of glucose/insulin, PUFA, and leptin on the transcription of fatty acid synthase (FAS) [EC 2.3.1.85] in cultured hepatocytes using the synthetic nucleotide probe for nucleotides −57 to −35 of the FAS gene linked to a reporter gene (*3*). Leptin, the product of the *ob* gene, controls food-intake and body weight through the brain (*4-6*). On the other hand, Kim *et al.* reported that *ob* gene expression in 30A5 preadipocytes suppresses acetyl-CoA carboxylase mRNA and lipid synthesis, which are induced by hormone treatment (*7*). Moreover, recent work in cultured adipose cells (*7, 8*) and hepatocytes (*9*) has suggested that leptin may antagonize insulin action in these cells.

The Wistar fatty rat with non-insulin-dependent diabetes mellitus (NIDDM) is obese, hyperphagic, hyperinsulinemic, and hypertriglyceridemic (*10*). They also show decreased glucose tolerance (*10*) and a glycemic response to exogenous insulin (*11*). We previously found that the gene expressions of hepatic lipogenic enzymes (ACL, FAS, acetyl-CoA carboxylase [EC 6.4.1.2], malic enzyme [EC 1.1.1.40], and glucose-6-phosphate dehydrogenase [EC 1.1.1.49]) are similar in obese rats and their lean littermates fed a diet lacking PUFA (*12*). When the rats were fed a diet containing a source of PUFA, such as corn oil or fish oil, however, gene expression was reduced in the lean rats, but the effect was much less in obese rats. Consequently, when the rats were fed a polyunsaturated fat, contained in ordinary diets, lipogenic enzyme gene expression was higher in obese rats than in lean rats. Thus, the higher gene

[1] This work was supported by Tezukayama Gakuin College, and Japan Private school promotion Funds (Japan).

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Abbreviations: ACL, ATP citrate-lyase; CAT, chloramphenicol acetyltransferase; PUFA, polyunsaturated fatty acids.

expression of lipogenic enzymes in obese rats can be ascribed to defects in the PUFA-mediated suppression of the gene expression. Moreover, PUFA suppresses the insulin stimulation of lipogenic enzyme gene expression (*13*). However, the results in whole animals are influenced by so many factors that it is hard to draw definite conclusions as to the mechanisms of action of hormones and nutrients. In particular, it is difficult to analyze the interactions of hormones and nutrients. Thus, it is advantageous to use primary cultures of cells for further studies on the mechanisms regulating gene expression. Liver and white adipose tissue are the major tissues in which lipogenic enzyme genes are expressed. In the present study, in order to investigate the regulation of lipogenic enzyme gene expression in obese rats, we compared the effects of glucose/insulin, PUFA, and leptin on the transcription of ACL, using a synthetic probe containing nucleotides −64 to −41 of the ACL gene linked to a reporter gene, in cultured hepatocytes and adipocytes of obese Wistar fatty rats and their lean littermates.

MATERIALS AND METHODS

Materials—[α-^{32}P]dCTP (111 TBq/mmol), [γ-^{32}P]ATP (111 GBq/mmol), and [^{14}C]chloramphenicol (2.22 GBq/mmol) were purchased from ICN Pharmaceuticals. Restriction endonuclease and other enzymes were purchased from Takara Shuzo. The sequence kit and luciferase assay kit were from Takara Shuzo and Toyo Ink, respectively. Williams' medium E was purchased from GIBCO, Ltd. Other culture media were obtained from Nissui Seiyaku. Lipofectin reagent was from Life Technologies. Nylon filters (Hybond N) were purchased from Amersham. Recombinant mouse leptin was obtained from R&D Systems. Most other reagents were obtained from Sigma and Wako. Antibodies against Sp1 and Sp3 were from Santa Cruz and a part of the antibody against Sp3 was a generous gift from Professor G. Suske, Philipps Universitat Marburg, Germany. Human insulin receptor cDNA was a generous gift from Professor Y. Ebina, Institute for Enzyme Research, University of Tokushima, Tokushima.

Animals—Female Wistar fatty rats (*fa/fa, fa/Fa*) and their lean littermates (*Fa/Fa*) (*10*), 10-11 weeks old, were a generous gift from Takeda Chemical Industries, Tokyo. Rats were housed in wire-bottomed cages kept in a temperature-controlled room (24°C) under an automatic lighting schedule (0800 h to 2000 h), and had free access to water and commercially available stock diet (No. MF, Oriental Shiryou). The care and treatment of experimental animals were in accordance with the *Guide for the Care and Use of Laboratory Animals* (*14*).

Plasmid Constructs—Plasmid pactL, a luciferase vector containing β-actin enhancer and promoter, was used as an internal control to normalize variations in transfection efficiency (*15*). The following single-stranded oligonucleotides were synthesized by GIBCO.

ACL(−64/−41): 5′-GATCTGATGGGGGGCGGGGA-GGAGCCCG-3′
ACL(−64/−41)M: 5′-GATCTGATGGGGGGATATC-AGGAGCCCG-3′
Sp1: 5′-ATTCGATCGGGGCGGGGCGAGC-3′
Sp1M: 5′-ATTCGATCGGTTCGGGGCGAGC-3′
NF1: 5′-GATCTTTTGGCTTGAAGCCAATATGAG-3′

Underlined sequences are the mutations from the substituted bases. Double-stranded oligonucleotides of ACL-(−64/−41) were inserted into the *Bam*HI sites of ACL-20cat, which is the CAT plasmid containing a −20 to +128 nucleotide fragment of the ACL gene and shows no response to glucose/insulin (*2*). Plasmid pRSV*ob*, which contains fragment −59 to +540 of the *ob* gene, was inserted into the *Bam*HI and *Hin*dIII sites of pBK-RSV (*16*). The sequences of these inserts were verified by dideoxy sequencing using a sequence kit (*17*).

Cell Culture and Transfection—Wistar fatty rats and their lean littermates maintained on a stock diet were fasted for 16 h before sacrifice. Hepatocytes were isolated by the collagenase perfusion method (*18*) 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 μM dexamethasone; then mixtures of 13 μg ACL(−64/−41) linked to ACLcat20 and 2 μg pactL were transfected into the hepatocytes using lipofectin for 16 h (*19*). To explore the effects of *ob* gene expression, 13 μg of ACL(−64/−41) linked to ACLcat20 and 2 μg pactL were mixed with 5 μg pRSV*ob* (*ob* gene expression vector) or pBK-RSV (the empty vector), and the mixtures were transfected into hepatocytes.

Isolated adipocytes were prepared from epididymal fat pads of rats by collagenase digestion (*20*). Adipose cells (5×10^5 cells) were mixed with Dulbecco's PBS and placed in electroporation cuvette (Bio-Rad) along with 13 μg ACL-(−64/−41) linked to ACLcat20, 2 μg pactL, and pRSV*ob* or pBK-RSV. Electroporation was performed using a Gene Pulser Transfection Apparatus (Bio-Rad) for transfection (*21*). After electroporation, the cells were transfered into polystyrene tubes (Falcon). Subsequently, the cells were cultured for 48 h in experimental media (with 100 μg/ml streptomycin and 100 units/ml penicillin) containing 20 mM glucose with or without 0.1 μM insulin. When included, 0.1 mM arachidonic acid or 0.3 μM recombinant mouse leptin was added. The cells were cultured in a humidified chamber at 37°C under 5% CO_2 in air. All transfections were performed four times in duplicate.

CAT and Luciferase Assays—The cells were incubated for 48 h after transfection, harvested, and lysed by sonication. Then 20 μl of the supernatant from each sample was assayed for luciferase using a kit (*22*). Amounts of the cell extracts normalized by luciferase activity were used for CAT assays after heating at 60°C for 10 min (*23, 24*). Acetylated and nonacetylated forms of [^{14}C]chloramphenicol were determined by scintillation counting and the percentages of the acetylated forms were calculated.

Preparation of RNA and Dot Blot Hybridization—Total cellular RNA was isolated from the cell cultures by the acid guanidinium thiocyanate-phenol-chloroform extraction method (*25*). Dot blot hybridization of total cellular RNA was performed as described previously (*26*). ACL cDNA species were cloned as described in our previous report (*27*). A rat *ob* cDNA fragment spanning nucleotides −59 to +540 was cloned from rat adipose tissue by reverse transcription and polymerase chain reaction amplification

according to Ref. *14*. Human insulin receptor cDNA (*28*) was a generous gift from Y. Ebina (Institute for Enzyme Research, University of Tokushima). The genomic clone of rat rRNA was obtained from the Japanese Cancer Research Resources Bank. A *Bam*HI/*Eco*RI fragment of about 1 kb was isolated from this clone and used as a probe for 18S rRNA. The probes were labeled by a multiprime DNA-labeling system kit (Amersham) using [α-^{32}P]dCTP. To measure the level of mRNA expression, the total RNA (20 μg) was denatured with formamide, spotted on a nylon filter, and then hybridized with ^{32}P-labeled cDNA as described previously (*29*). Relative densities of the hybridization signals were determined by scanning the autoradiograms at 525 nm (Model CS-9000, Shimadzu) and normalizing to the values of the 18S rRNA.

Gel Mobility Shift Assay—Nuclear extracts were prepared from obese or lean rat liver and adipose tissue as described by Gorski *et al.* (*30*). An end-labeled double stranded oligonucleotide of ACL(−64/−41) was incubated with the 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), and 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 buffer, pH 8.3, and separated by electrophoresis at 100 V for 4 h (*31*). In competition studies, the indicated amounts of double-stranded oligonucleotide were added to the reaction mixture. The nucleotide sequences of ACL(−64/−41), ACL(−64/−41)M, Sp1, Sp1M, and NF1 are shown above. For antibody supershift assays, Sp1 or Sp3 antibody was added to the binding reaction mixture, and the mixtures were incubated for 1 h at room temperature prior to adding the labeled probes. Samples were then loaded onto 4% non-denaturing polyacrylamide gels. The gels were then fixed in a solution of 10% methanol/10% acetic acid, dried, and autoradiographed.

Western Blot—Nuclear proteins from obese and lean rat livers were separated by 8% SDS-polyacrylamide gel electrophoresis according to Laemmli (*32*) and blotted onto nitrocellulose using a Bio-Rad semidry transfer apparatus according to the manufacturer's instructions. The membranes were blocked with 5% Halmein Casein in Tris-buffer and saline containing 0.1% Tween 20 (blocking solution), and then incubated with anti-Sp1 or Sp3 followed by horseradish peroxidase-conjugated anti-rabbit IgG antibody (Santa Cruz) in blocking solution. Bound antibody was detected by enhanced chemiluminescence following the manufacturer's directions (Amersham).

Enzyme Activities—The cells were homogenized and centrifuged for 30 min at $105{,}000\times g$ at 4°C, and supernatant fractions were collected for the measurement of enzyme activities. ACL activity was assayed according to Takeda *et al.* (*33*). The enzyme activities in the supernatants of cell homogenates are shown as mU/mg protein, where 1 mU is the amount catalyzing the formation of 1 nmol product per min at 37°C for ACL. Protein contents were determined by the method of Lowry *et al.* (*34*).

Statistical Analysis—Two- or three-way ANOVA was followed by inspection of all differences between pairs of means by the least significant difference test (*35*). Differences were considered significant at $p<0.05$.

RESULTS

Glucose/Insulin and PUFA Regulation of ACL Gene Expression in Hepatocytes and Adipocytes from Obese and Lean Rats—We previously reported that the region from −64 to −41 of the ACL gene is responsible for the stimulation of ACL gene expression by glucose/insulin, and that the stimulation is suppressed by PUFA in hepatocytes of normal Wistar rats (*1*). The sequence spanning nucleotides −64 to −41 of the ACL gene was linked to a reporter gene, and the effects of glucose/insulin and PUFA on the CAT activities were examined in hepatocytes and adipocytes of obese and lean rats. The relative CAT activities are shown in Fig. 1. In primary cell cultures, the CAT activities in the presence of glucose alone were similar in cells from obese and lean rats. In the presence of glucose/insulin, however, the CAT activities were markedly increased (by 2-fold) above those in the presence of glucose alone in the hepatocytes from lean rats, but were not significantly increased in the hepatocytes from obese rats. The stimulation by glucose/insulin was reduced by 70% in arachidonic acid-treated hepatocytes from lean rats. In hepatocytes from obese rats, however, the stimulation by glucose/insulin was small, and no significant suppression by PUFA was observed. The levels of mRNA expression and the enzyme activities of endogenous ACL in hepatocytes changed similarly to the CAT activities (Fig. 1).

In adipocytes from lean rats, the expression of ACL(−64/−41)-driven CAT activity was stimulated significantly by glucose/insulin. However, the stimulation was small in comparison with that in hepatocytes. The stimulation by glucose/insulin was reduced in arachidonic acid-treated adipocytes. In adipocytes from obese rats, however, the stimulation by glucose/insulin was very small in comparison with that in adipocytes from lean rats and there was no significant suppression by PUFA. Results similar to these were seen for the levels of mRNA expression and enzyme activities of endogenous ACL in adipocytes.

Effect of Leptin or ob Gene Expression Vector on ACL Gene Expression in Hepatocytes and Adipocytes from Obese and Lean Rats—To test whether leptin itself affects ACL gene expression, recombinant mouse leptin was used to examine the regulation of ACL transcription in hepatocytes and adipocytes from obese and lean rats (Table I). In the presence of leptin, the CAT activity stimulated by glucose/insulin was significantly decreased in hepatocytes from lean rats, but not in those from obese rats. However, leptin had little effect on the transcription of ACL in adipocytes, regardless of genotype. A similar result was seen for the levels of mRNA expression and enzyme activities of endogenous ACL.

To examine the effects of *ob* gene expression on ACL gene expression, hepatocytes were cotransfected with the plasmid construct containing ACL(−64/−41) linked to ACLcat20 and with the *ob* gene expression vector (pRSV-*ob*). The CAT activity expressed by hepatocytes transfected with ACL(−64/−41) linked to ACL20cat was reduced in the presence of the *ob* gene expression vector, as it was in hepatocytes treated with leptin (Table I).

Effect of Leptin or PUFA on the Levels of Insulin Receptor mRNA Expression in Hepatocytes and Adipocytes from Normal Rats—The effect of leptin or PUFA on the

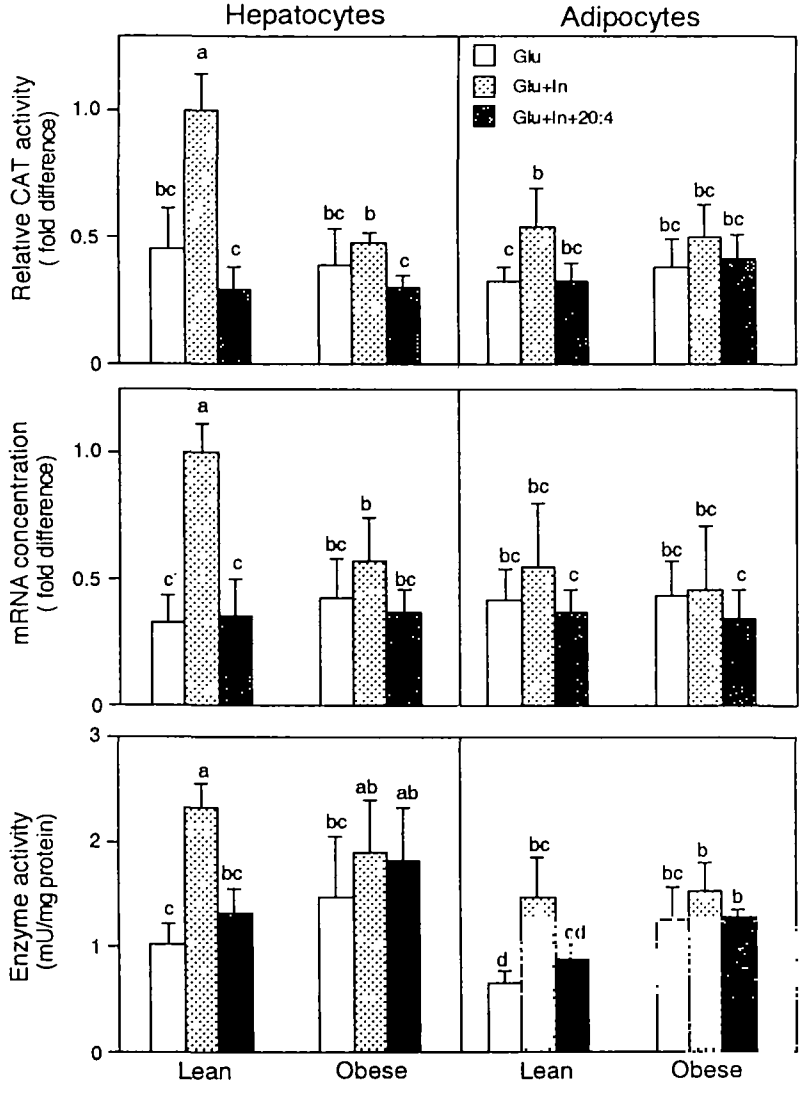

Fig. 1. **ACL gene expression in hepatocytes and adipocytes of the obese and lean rats.** The construct of ACL(−64/−41) linked to ACL20cat was introduced into primary hepatocytes or adipocytes. The cells were incubated with or without 0.1 μM insulin (In) for 48 h in the presence of 20 mM glucose (Glu). In some cases, 0.1 mM arachidonic acid (20:4) was added. The percentage of acetylated forms of [^{14}C]chloramphenicol was determined as the CAT activities for each sample. The relative CAT activities (top figures) and mRNA expression levels (middle figures) were normalized to the value for glucose/insulin (Glu+In) in hepatocytes from lean rats. To measure the levels of mRNA expression, the total RNA (20 μg) from hepatocytes and adipocytes was used. The levels of mRNA expression and the enzyme activities of endogenous ACL in the cells are shown. Enzyme activities (bottom figures) in the supernatant 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 product at 37°C. Means with different superscript letters are significantly different at $p<0.05$. Results are mean±SD of four experiments.

TABLE I. **Effect of leptin or *ob* gene expression vector on ACL gene expression in hepatocytes and adipocytes from obese and lean rats.** A plasmid construct containing ACL (−64/−41) linked to ACLcat20 was introduced into primary hepatocytes or adipocytes. The cells were incubated in William'E medium containing 20 mM glucose (Glu) with or without 0.1 μM insulin (In). In some cases, 0.3 μM leptin was added. To some cultures, ACL (−64/−41) linked to ACLcat20 was cotransfected into cells with or without pRSV*ob*, which is the *ob* expression vector (*ob* vector). The percentage of acetylated forms of [^{14}C]chloramphenicol was determined as the CAT activity for each sample. To measure the levels of mRNA expression, total RNA (20 μg) from hepatocytes and adipocytes was used according to "MATERIALS AND METHODS." The relative CAT activities and the levels of mRNA expression are normalized to the value for Glu+In for hepatocytes from lean rats. The levels of mRNA expression and the enzyme activities of endogenous ACL in the cells are shown. The enzyme activities in the supernatants of cell homogenates are shown as mU/mg protein, where 1 mU is the amount of enzyme catalyzing the formation of 1 nmol/min product at 37°C. Means with different superscript letters within each item are significantly different at $p<0.05$. Results are mean±SD of four experiments.

	Hepatocytes		Adipocytes	
	Lean	Obese	Lean	Obese
CAT activity (fold difference)				
Glu+In	1.00±0.14^{a}	0.48±0.04^{b}	0.54±0.15^{b}	0.50±0.13^{bc}
Glu+In+leptin	0.38±0.07^{bc}	0.38±0.09^{bc}	0.42±0.12^{bc}	0.44±0.13^{bc}
Glu+In+*ob* vector	0.29±0.11^{c}	0.33±0.02^{c}	0.38±0.10^{bc}	0.39±0.10^{bc}
ACL mRNA concentration (fold difference)				
Glu+In	1.00±0.11^{a}	0.57±0.17^{b}	0.55±0.12^{b}	0.46±0.15^{bc}
Glu+In+leptin	0.32±0.12^{c}	0.44±0.20^{bc}	0.41±0.19^{bc}	0.35±0.07^{bc}
Glu+In+*ob* vector	0.55±0.13^{bc}	0.46±0.13^{bc}	0.40±0.10^{bc}	0.38±0.10^{bc}
ACL enzyme activity (mU/mg)				
Glu+In	2.32±0.23^{a}	1.90±0.49^{ab}	1.48±0.34^{bc}	1.53±0.26^{b}
Glu+In+leptin	1.18±0.30^{bc}	1.90±0.23^{ab}	1.07±0.12^{c}	1.25±0.19^{bc}
Glu+In+*ob* vector	0.97±0.22^{bc}	1.93±0.35^{ab}	0.95±0.16^{c}	1.16±0.19^{bc}

endogenous insulin receptor levels of mRNA expression was examined in primary cultured hepatocytes and adipocytes from normal rats (Table II). In the presence of glucose/insulin, the levels of mRNA expression were markedly increased above those in the presence of glucose alone in hepatocytes. The stimulation by glucose/insulin was reduced by leptin or arachidonic acid. A similar result was seen in adipocytes.

Gel Mobility Shift Assays—The DNA-protein binding activity of ACL(−64/−41) was examined by electrophoresis mobility shift assay. ^{32}P-labeled oligonucleotide ACL(−64/−41) was incubated with nuclear extracts from obese or lean rat liver and subjected to non-denaturing polyacrylamide gel electrophoresis (Fig. 2). Three DNA-protein complex bands were observed in both obese and lean rats. The intensities of the bands in the nuclear extract from obese rats were low and showed a decrease in the formation of DNA-protein complexes in obese rats in comparison with lean rats. The complexes were competed away by unlabeled oligonucleotides of ACL(−64/−41). In the presence of excess unlabeled mutant ACL(−64/−41), no competition was observed. These results demonstrate the specificity of the DNA-protein complex formation in obese and lean rats. ACL(−64/−41) is a G+C-rich region in which the Sp1 and Sp1 family consensus sequence is found. As shown in Fig. 2, Sp1 oligonucleotide could effectively compete for the formation of band 1 and band 2 of DNA-protein complexes in both rat phenotypes. The addition of the same amount of mutant Sp1 probe to the gel mobility shift reactions yielded no competition. These results suggest that Sp1 binds to ACL(−64/−41). Furthermore, when ^{32}P-labeled NF1 (containing the nuclear factor 1 binding site), as a control, was added to the gel mobility shift assay reactions, the formation of DNA-protein complexes was similar in obese and lean rats. Results similar to these were obtained for adipocytes (Fig. 3).

To demonstrate further that Sp1and Sp1 family members bind to the ACL gene, Sp1- or Sp3-specific antibody was added to the gel mobility shift assay. Using a polyclonal antibody against Sp1, band1 was upshifted to a higher molecular weight (Fig. 4). When anti-Sp3 was added, the intensity of band1 diminished markedly. The result shows that band1 represents an ACL(−64/−41)-specific DNA-protein complex containing Sp1 and Sp3.

Western Blot Analysis—The levels of Sp1 and Sp3 in liver nuclear extracts were compared in obese and lean rats. Figure 5 shows Western immunoblotting experiments in which nuclear extracts were probed with a polyclonal antibody against Sp1. This antibody detected two major immunoreactive bands in liver nuclear extracts. The intensities of the bands were markedly higher in nuclear proteins prepared from lean rat liver than from obese rat liver. Sp3 antibody detected three immunoreactive bands. The intensities of the bands were barely detectable in nuclear

TABLE II. **Effects of leptin or PUFA on the levels of insulin receptor mRNA expression in hepatocytes and adipocytes from normal rats.** Cells were incubated in William'E medium containing 20 mM glucose (Glu) with or without 0.1 μM insulin (In). In some cases, 0.1 mM arachidonic acid (20:4) or 0.3 μM leptin was added. To measure the levels of mRNA expression, the total RNA (20 μg) from hepatocytes or adipocytes was used. The levels of mRNA expression are normalized to Glu+In in hepatocytes. Means with different superscript letters are significantly different at $p<0.05$. Results are mean±SD of four experiments.

Addition	Levels of insulin receptor mRNA expression (fold difference)	
	Hepatocytes	Adipocytes
Glu	0.45 ± 0.04^{a}	1.60 ± 0.23^{b}
Glu+In	1.00 ± 0.15^{c}	2.29 ± 0.40^{a}
Glu+In+20:4	0.55 ± 0.18^{e}	1.39 ± 0.37^{bc}
Glu+In+leptin	0.77 ± 0.19^{d}	1.31 ± 0.31^{bc}

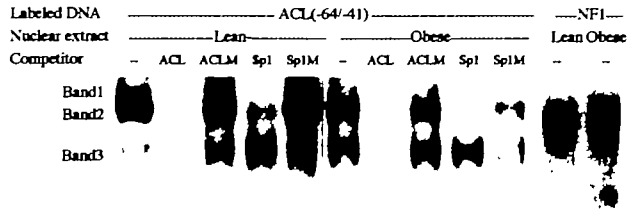

Fig. 2. **DNA mobility assay using obese and lean rat liver nuclear extracts with end-labeled ACL (−64/−41) or nuclear factor 1 (NF1).** Ten micrograms of rat liver nuclear extract from obese and lean rats was incubated and then loaded onto an 8% non-denaturing polyacrylamide gel. Competitor DNA (ACL; ACL-(−64/−41), ACLM; mutant ACL(−64/−41), Sp1 and Sp1M; mutant Sp1) was added in a 10-fold molar excess relative to labeled DNA.

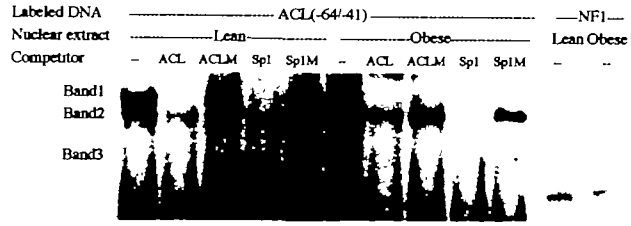

Fig. 3. **DNA mobility assay using obese and lean rat adipose tissue nuclear extracts with end-labeled ACL (−64/−41) or nuclear factor 1 (NF1).** Ten micrograms of rat adipose tissue nuclear extracts from obese and lean rats was incubated and then loaded onto an 8% non-denaturing polyacrylamide gel. Competitor DNA (ACL; ACL(−64/−41), ACLM; mutant ACL(−64/−41), Sp1 and Sp1M; mutant Sp1) was added in a 10-fold molar excess relative to labeled DNA.

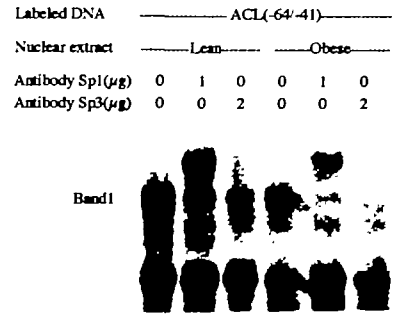

Fig. 4. **DNA mobility supershift assay using obese and lean rat liver nuclear extracts with end-labeled ACL (−64/−41) with or without Sp1 or Sp3 antibody.** Five micrograms of nuclear extracts from obese and lean rat liver was incubated with or without Sp1 or Sp3 antibody (1 or 2 μg, respectively) before the addition of ^{32}P-labeled ACL(−64/−41), and then loaded onto a 4% non-denaturing polyacrylamide gel.

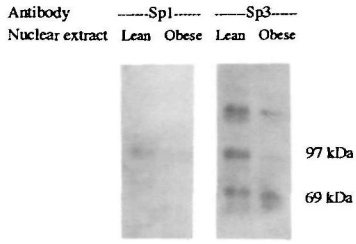

Fig. 5. **Western blot analysis of Sp1 and Sp3 in nuclear extracts from obese and lean rat liver.** One hundred microgram of nuclear protein extract prepared from obese and lean rat livers was separated by 8% SDS-polyacrylamide gel electrophoresis and probed with antibody against Sp1 and Sp3.

extracts from obese rats, although they were very clear in lean rats. This demonstrates that transcription factors Sp1 and Sp3 are present at lower levels in liver extracts of obese rats than in those of lean rats.

DISCUSSION

Fasting produces a rapid decrease in *ob* mRNA levels in rat adipose tissue, an effect that is probably mediated by a decrease in plasma insulin (*36*). We previously found that plasma insulin and leptin concentrations, as well as the levels of *ob* mRNA expression, in adipose tissue in obese Wistar fatty rats and their littermates are markedly decreased by fasting compared with the levels in fed rats (*3*). In both the fasted and fed states, plasma insulin concentrations are markedly higher in obese than in lean rats, while glucose concentrations are slightly higher. Plasma leptin concentrations are also significantly higher in obese than in lean rats. To eliminate the effects of endogenous *ob* gene expression in adipose tissue, in the present experiments, primary cultures of hepatocytes and adipocytes were obtained from fasted rats. The plasma insulin concentrations were (nmol/liter) 1.06 ± 0.22 and 0.09 ± 0.01 (mean $\pm$ SD, $n=6$) in the fasted obese and lean rats, respectively. Plasma leptin concentrations were (μg/liter) 20.0 ± 2.40 and 0.94 ± 0.15 (mean $\pm$ SD, $n=6$) in the fasted obese and lean rats, respectively. These data and more were shown in a previous report about fatty acid synthase (*3*). The same animals were used in the present experiments.

The sequence from -64 to -41 of the ACL gene, which is responsible for the stimulation by glucose/insulin and suppression by PUFA in hepatocytes (*2*), was linked to a reporter gene and transfected into hepatocytes or adipocytes from obese and lean rats. Liver and white adipose tissue are the major tissues in which lipogenic enzyme genes are expressed. The CAT activities expressed in the presence of glucose alone were similar in cells obtained from obese and lean rats. In the presence of glucose/insulin, however, the CAT activities were markedly increased in cells from lean rats, but not in cells from obese rats. A similar result was previously obtained for the CAT activities of the sequence from -57 to -35 of the FAS gene (*3*). These can be ascribed to the insulin resistance of obese rats. We previously observed that the insulin-binding capacities of receptors are very low in livers from obese Wistar fatty rats compared with their lean littermates (*37*).

The stimulation of CAT activities by glucose/insulin was reduced in PUFA-treated adipocytes and hepatocytes from lean rats. The stimulation was also reduced in leptin-treated cells or in *ob* gene expression vector-containing cells from lean rats. In the presence of arachidonic acid or leptin, the insulin-binding capacities to partially purified insulin receptors from the liver and isolated adipocytes of normal rats were decreased (*3*). Walder *et al.* (*38*) also reported that leptin decreases the maximal insulin binding in a dose-dependent manner. Moreover, in the present experiment, we found that the levels of insulin receptor mRNA expression in hepatocytes or adipocytes are also decreased in the presence of arachidonic acid or leptin. Leptin and PUFA appear to suppress the insulin stimulation of ACL transcription, possibly due to a reduction in insulin action. This may be due, at least in part, to the reduction in the insulin binding capacities to receptors and also to insulin receptor gene expression. In obese rats, however, the stimulation by glucose/insulin is weak, and no significant suppression by PUFA or leptin was observed. We previously observed that the insulin-binding capacities of receptors in livers from obese rats are too low to permit detection of any reduction caused by PUFA (*37*).

Choen *et al.* (*8*) also demonstrated that leptin may attenuate insulin activity in isolated hepatocytes. Specific receptors for leptin have been shown to be expressed in various tissues apart from the hypothalamus, including liver, pancreas, and adipose tissue (*39*). Therefore, exogenous leptin can act in hepatocytes, although leptin is not expressed. Kim *et al.* (*7*) reported that *ob* gene expression in 30A5 preadipocytes suppresses the acetyl-CoA carboxylase mRNA level and lipid synthesis that are induced by insulin treatment. They observed that leptin suppresses the accumulation of lipid droplets that occurs in adipocytes. Therefore, leptin may suppress lipid synthesis and lipid levels by regulating insulin action. In the present study, leptin was found to suppress the transcription of a reporter gene linked to the sequence spanning nucleotides -64 to -41 of the ACL gene in primary cultured hepatocytes and adipocytes. We previously found a similar effect of leptin on the CAT activities of the sequence from -57 to -35 of the FAS gene linked to a reporter gene containing a heterologous promoter (*3*). Thus, leptin and PUFA suppress the insulin stimulation of lipogenic enzyme transcription without the participation of the brain.

Endogenous insulin receptor expression in hepatocytes and adipocytes is reduced in the presence of leptin or PUFA. We previously found that the insulin-binding capacities are also reduced in the presence of leptin or PUFA in adipose tissues and partially-purified insulin receptors from the livers of normal rats (*3*). The insulin-binding capacities are very low in obese rats in comparison with lean (*37*). It is suggested that the reduction in insulin receptor expression and insulin-binding capacities to receptors may be caused, in part, by the reduction of insulin action in ACL transcription.

Moreover, in the present experiments, gel mobility shift assays using labeled ACL($-64/-41$) revealed that nuclear factor(s) specifically bound to the sequence and DNA-protein complex formation are much less in obese rats than in lean. ACL($-64/-41$) is a G+C-rich region containing the Sp1 and Sp1 family consensus sequence. Using antibodies against Sp1 or Sp3, it was demonstrated that the

DNA-protein complex contains Sp1 and Sp3. Western blot analysis demonstrated that transcription factors Sp1 and Sp3 are present at lower levels in liver extracts from obese rats than in liver extracts from lean. On the other hand, Rolland *et al.* (*40*) reported that FAS promoter activity depends mainly on the region from −200 to −126, and that this sequence exerts a strong negative effect on FAS promoters in adipocytes from lean but not obese rats. They demonstrated that Sp1 or Sp1-like proteins are bound to this DNA subregion.

Araki *et al.* (*41*) demonstrated that transcription factor Sp1 can bind to the cluster of four G-C boxes in the promoter of the insulin receptor gene, suggesting that the efficient expression of the insulin receptor gene possibly requires the binding of transcriptional factor Sp1. However, transcription factor Sp1 is present at lower levels in liver extracts from obese rats. Thus, the reduction in insulin-stimulated ACL transcription in obese rats may be ascribed, in part, to the reduction in DNA-protein complex formation and insulin binding to receptors. Sp1 appears to be an important transcription factor for lipogenic enzymes. Some other mechanisms for leptin and PUFA suppression and for the reduction in the insulin stimulation of ACL transcription in obese rats remain unclear and will be studied in the future.

We thank Dr. H. Ikeda of the Pharmaceutical Research Division, Takeda Chemical Industries, Osaka, for useful discussion and the generous gift of Wistar fatty rats.

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