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DNA demethylation modulates mouse leptin promoter activity during the differentiation of 3T3-L1 cells

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Abstract

Aims/hypothesis. The mouse *leptin* gene, a major hormonal regulator of appetite and fat cell mass, expresses during the differentiation of 3T3-L1 preadipocytes to adipocytes. To determine if DNA methylation is involved in regulating the expression of the leptin gene, we examined the methylation status and methylation-sensitive transcription factors during 3T3-L1 differentiation.
Methods. DNase I footprinting, electrophoretic mobility-shift assays, and a Southwestern analysis were carried out using nuclear extracts from preadipocytes and adipocytes. Promoter activity was measured by luciferase assays. The CpG methylation pattern was determined.
Results. Transient transfection of reporter constructs with the leptin promoter showed that preadipocytes that do not transcribe the leptin gene show enough transactivation, suggesting the presence of an additional regulatory mechanism. We identified eight CpG sites in the promoter up to nt –161, all of which were highly methylated (> 92 %) in preadipocytes. Seven of these sites showed a varying degree of demethylation during differentiation, while the site at nt –54 remained methylated. In electrophoretic mobility-shift assays, DNA fragments from nt –115 to nt –70 generated a methylation-sensitive band with nuclear extracts from preadipocytes when the CpG sites were methylated. Southwestern analysis identified a 52 kM_r protein that binds strongly to the methylated probes. Promoter activity was reduced by methylation of the CpG sites up to nt –115, but not up to nt –70.
Conclusion/interpretation. These results suggest that methylation of specific CpG sites between nt –115 and nt –70 and a methylation-sensitive protein could contribute to leptin gene expression during adipocyte differentiation in 3T3-L1 cells. [Diabetologia (2002) 45: 140–148]

Keywords Leptin, methylation, CpG site, 3T3-L1 cells, methyl-CpG-binding proteins.

During the differentiation of 3T3-L1 preadipocytes to adipocytes, transcriptional activators and repressors regulate the expression of many adipocyte-specific genes [1–4]. In addition, DNA methylation could also play an important role during the process of adipocyte differentiation. We recently showed that methylation of specific CpG sites of the *GLUT4* gene and a methylation sensitive transcription factor, contribute to *GLUT4* gene regulation during 3T3-L1 differentiation [5]. Similar mechanisms could also regulate the expression of other genes induced during adipocyte differentiation.

Leptin, a major hormonal regulator of appetite and fat cell mass, is one of these adipocyte-specific genes [6, 7]. It is secreted by adipose tissue in response to a high content of body fat. Thus, mice lacking a functional leptin gene develop hyperphagia, hy-

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Abbreviations: EMSA, Electrophoretic mobility-shift assays; C/EBP, CCAAT/enhancer-binding protein; CED, β-cyanoethyl-N, N-diisopropylamino; DTT, dithiothreitol; FBS, fetal bovine serum

perglycemia, hyperinsulinemia, and obesity [8]. Since circulating leptin concentrations correlate with the amount of body fat, the regulation of the leptin gene expression is critical in maintaining normal body fat content. Expression of the leptin gene has been found to be induced during the differentiation of 3T3-L1 preadipocytes into adipocytes [9]. Like other adipocyte-specific genes, the leptin gene is regulated by CCAAT/enhancer binding protein α (C/EBPα) [10, 11]. Recently, Sp1 and Lp1 also have been reported to be enhancers [12].

To determine if DNA methylation is involved in the regulation of the leptin gene expression, we examined DNA methylation and methylation-sensitive transcription factors during the differentiation of 3T3-L1 cells from preadipocytes to adipocytes.

Materials and methods

Cell culture. 3T3-L1 cells [American type culture collection (ATCC), Manassas, Va., No.CCL 92.1] were grown in high glucose DMEM supplemented with 10% calf serum. Confluent 3T3-L1 preadipocytes were induced to differentiate into adipocytes as described [13]. Briefly, 1 day after confluence, the cells were treated with medium containing 10% fetal bovine serum (FBS), insulin (10 μg/ml), dexamethasone (0.2 μg/ml), and isobuthylmethylxanthine (0.5 mmol/l) for 3 days. After 3 days, this medium was replaced by a medium supplemented with 10% FBS and insulin (10 μg/ml) and 2 days later, the medium was replaced with DMEM supplemented with 10% FBS. Cells were used for studies 0, 5, and 10 days after induction of differentiation. The cells started to store fat droplets at day 5 and almost all became adipocytes at day 10 after differentiation by microscopic observation using Sudan staining. We also confirm the differentiation with the expression of leptin mRNA by RT-PCR in each experiment as reported previously [6].

Plasmids. The 5'-flanking region of the mouse leptin gene (nt –161 to nt + 26) was amplified from 3T3-L1 DNA by polymerase chain reaction. The following primers were used to amplify the leptin gene. 5'-TGCACTCGAGGCGCCTAG AATGGAG and 5'-GGGGTCTAGAGCAGCTGCTGGAGCA were used as the 5' and 3' primers, respectively. The plasmids pLepLuc-161, pLepLuc-115, pLepLuc-97, pLepLuc-70, and pLepLuc-43, contained fragments of the 5'-flanking region of the mouse leptin gene beginning at nt –161, –115, –97, –70, and –43, respectively. The plasmids were fused upstream to the luciferase coding region of the pGL3-Basic vector (Promega, Madison, Ws.).

Nuclear extracts. Nuclear extracts were prepared from 3T3-L1 preadipocytes (day 0) and adipocytes (day 10). Cells were harvested, washed with Dulbecco's modified phosphate-buffered saline without Mg^{2+} and Ca^{2+} (pH 7.4), and, after centrifugation at 500 · g, resuspended in five pellet volumes of buffer A [10 mmol/l HEPES-KOH (pH 7.9), 10 mmol/l KCl, 1.5 mmol/l MgCl2, 0.1 mmol/l EGTA, 0.5 mmol/l dithiothreitol (DTT), 0.5 mmol/l phenylmethylsulfonyl fluoride (PMSF), leupeptin (2 μg/ml), and pepstatin A (2 μg/ml)] containing 0.3 mol/l sucrose and 2% (v/v) Tween 40. The cells were then frozen, thawed, and gently homogenized and the nuclei were isolated by centrifugation of the homogenate at 25000 · *g* through a 1.5 mol/l sucrose cushion prepared in the same buffer. Nuclei were lysed in buffer B [10 mmol/l HEPES-KOH (pH 7.9), 420 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 0.1 mmol/l EGTA, 10% (v/v) glycerol, 0.5 mmol/l PMSF, leupeptin (2 μg/ml), and pepstatin A (2 μg/ml)] and the lysate was centrifuged at 100000 · g for 1 h. The resulting supernatant was dialysed for use in Southwestern analysis or electrophoretic mobility-shift assays (EMSA).

DNase I footprint analysis. A DNase I footprint analysis was carried out with a sure track footprinting kit (Pharmacia Biotech, Uppsala, Sweden). The *Xho* I-*Hind* III (nt –161 to nt + 20) fragment of the mouse leptin gene was end-labelled with [γ-^{32}P]ATP (> 5000 Ci/mmol; Amersham, Buckinghamshire, England) and T4 polynucleotide kinase and purified by agarose gel electrophoresis. Labelled DNA fragments (30000 cpm) were incubated with nuclear extracts in 50 μl of 10 mmol/l Tris-HCl buffer (pH 7.5) containing 2.5 μg of poly (dI-dC), 50 mmol/l NaCl, 2.5 mmol/l $MgCl_2$, 1 mmol/l DTT, 0.5 mmol/l EDTA, and 5% glycerol for 30 min at room temperature. Then the DNAs were digested with one unit of DNase I for 30 s, extracted with phenol-chloroform, and precipitated with ethanol. As sequence markers, the corresponding DNA fragment was chemically cleaved at nucleotides G and A [14]. The digested DNA samples were electrophoresed on an 8% polyacrylamide –7 mol/l urea gel, and the gel was then dried, exposed to an imaging plate and analysed with a Bas 2000 image analyser (Fuji, Tokyo, Japan).

EMSA. Each oligonucleotide was annealed to its complement and labelled using [α-^{32}P]dATP (> 6000 Ci/mmol; Amersham) and DNA polymerase Klenow fragment. Methylated oligonucleotides were prepared by including 5-methyl deoxycytidine CED (*β*-cyanoethyl-N, N-diisopropylamino) phosphoamidite (Pharmacia Biotech) during the appropriate cycle of synthesis. Each radioactive probe was incubated with 5 μg of nuclear proteins from preadipocytes in 10 μl of 20 mmol/l Tris-HCl (pH 7.5) containing 1 μg of poly(dI-dC), 50 mmol/l NaCl, 0.1 mmol/l DTT and 10% glycerol at room temperature. The following oligonucleotides were used in the studies as the probes and competitors:

5'-gatc^{-115}CTGGCCCGCTGGGTGGGGCGGGAGTTGGCGCTCGCAGGGA CTGGGG^{-70} (W1),
5'-gatc^{-115}CTGGCCm^{5}CGCTGGGTGGGGm^{5}CGGGA GTTGGm^{5}CGCTm^{5}C GCAGGGACTGGGG^{-70} (M1),
5'-gatc^{-115}CTGGCCCGCTGGGTGGGGCGGGAGTT^{-90} (W2),
5'-gatc^{-115}CTGGCCm^{5}CGCTGGGTGGGGm^{5}CGGGAGTT^{-90} (M2),
5'-gatc^{-94}GAGTTGGCGCTCGCAGGGACTGGGG^{-70} (W3),
5'-gatc^{-94}GAGTTGGm^{5}CGCTm^{5}CGCAGGGACTGGGG^{-70} (M3)

An Sp1 consensus oligonucleotide, 5'-ATTCGATCGGGGCGGGGCGAGC, was also used as a competitor.

Southwestern analysis. Thirty micrograms of nuclear proteins from preadipocytes was dissolved in a SDS sample buffer (10% glycerol, 70 mmol/l SDS, 250 mmol/l Tris, 200 mmol/l DTT, pH 6.8) and boiled, then electrophoresed on a 10% SDS-polyacrylamide gel for 2 h at 25 mA. Then separated proteins were electroblotted onto a nitrocellulose filter (Schleicher and Schuell, Dassel, Germany) in blotting buffer (25 mmol/l Tris, 0.193 mol/l glycine) for 2 h at 120 mA. The filters were incubated for 2 h in blocking buffer (10 mmol/l

HEPES pH 7.4, 1 mmol/l EDTA, 60 mmol/l NaCl, 1 mmol/l DTT, 5% non-fat milk powder) and incubated for 12 h at 4°C with labelled unmethylated or methylated probes (nt –115 to nt –70) ($5 \cdot 10^7$ cpm/μg) in binding buffer [10 mmol/l HEPES pH 7.4, 1 mmol/l EDTA, 60 mmol/l NaCl, 1 mmol/l DTT, 0.25% non-fat milk powder, 5 μg/ml poly(dI-dC)]. This incubation was carried out with or without 200-fold molar excesses of competitors. The following oligonucleotides were used as probes and competitors: W1, W2, W3, M1, M2, and M3 that were identical to the probes and competitors used in EMSA. 5'-gatc^{-40}TTCAGCTCTCCGCATCTTTCCCCCTCAAGCG GGTCTCACT^{-1} for the unmethylated *GLUT4* probe, and 5'-gatc^{-40}TTCAGCTCTCm^{5}CGCATCTTTCCCCCTCAAGm^5-CGGGTCTCACT^{-1} for the methylated *GLUT4* probe. The latter two were identical to the probes used in the experiments of the analysis of the *GLUT4* gene promoter [5]. Filters were washed with several changes of washing buffer (10 mmol/l HEPES pH 7.4, 1 mmol/l EDTA, 60 mmol/l NaCl, 1 mmol/l DTT, 0.25% non-fat milk powder), then dried, exposed to an imaging plate and analysed with a Bas 2000 image analyzer (Fuji). Molecular weight was measured by a pre-stained molecular weight marker (Bio-Rad, Richmond, Calif., USA).

Sequencing of the sodium bisulfite-treated promoter. Genomic DNAs were prepared from 3T3-L1 cells on days 0, 5, and 10 after induction of differentiation, using the SDS and proteinase K method, digested with *Pst* I and then subjected to sequential reactions to determine CpG methylation pattern according to a previous study [15]. The oligonucleotide primers were synthesized based on the reported sequences of the mouse leptin gene [10]. The top strand of the promoter sequence (nt –181 to nt + 20) of the leptin gene was amplified using 10 μl of the bisulfite-reacted DNA as a template and the oligonucleotides, 5'-GGGGAAGCTTAGGGTGTGGTTGAAGTTTTTT and 5'-GGGGTCTAGAACTACTAAAACAAAAATCCCTCC-A, as the 5'- and 3'-primers, respectively. The underlined regions indicate a *Hind* III site and a *Xba* I site added at each end of the amplified DNAs. After the digestion of these enzymes, the amplified DNAs were cloned into M13mp19 vectors for DNA sequencing.

Methylation of reporter plasmid. Methylation of the reporter plasmid was done as described [16]. Briefly, the plasmid, pLepLuc-115 or pLepLuc-70, was digested with *Hind* III and *Xba* I, and the fragment was gel-purified. Each promoter fragment was methylated in vitro with three units of *Sss*I methylase (New England Biolabs, Beverly, Mass., USA)/μg of DNA in the presence of 160 μmol/l *S*-adenosylmethionine at 37°C for 3 h. As unmethylated control, the same fragment was incubated under the same condition without *Sss*I methylase. Complete methylation was verified by digesting the DNA with an excess of *Hpa* II restriction enzyme. The methylated or unmethylated promoter fragment was ligated into the *Hind* III and *Xba* I sites of pGL3-basic vector at 16°C for 30 min. The DNA was ethanol-precipitated, and 5 μg of ligated DNA was used for transfection.

Transient expression analysis. 3T3-L1 preadipocytes (day 0) and adipocytes (day 10) were transfected by electroporation (300 V; capacitance, 960 microfarad) (Gene Pulser; BioRad). The cells were harvested, washed, and resuspended at $1.5 \cdot 10^7$ cells/ml^{-1} in 0.8 ml PBS, and cotransfected with 10 μg of the pLepLuc plasmids and 200 ng of the pRL-CMV plasmids. To examine the effect of methylation on leptin gene transcription, 5 μg of the mock-methylated or *Sss* I methylated plasmids were used. The cells were pulsed, then plated, and cultured for 48 h. Preparation of cell lysates and luciferase assays were done using a dual-luciferase assay system (Promega). A p value of less than 0.05 was considered statistically significant.

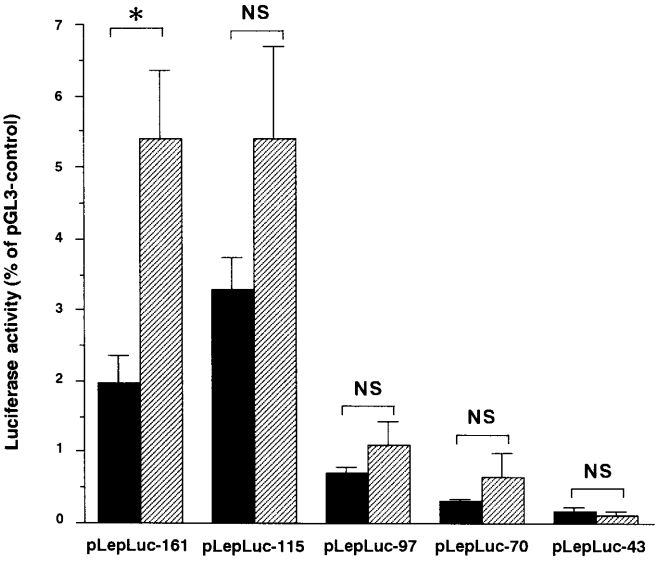

Fig. 1. Leptin promoter activity in preadipocytes and adipocytes. Deletion constructs of the leptin promoter were placed upstream of the luciferase gene and transiently transfected into preadipocytes and adipocytes. Luciferase activity, determined as described in Materials and methods, was normalized to pRL-CMV activity from a cotransfected plasmid in each extract, and expressed relative to the normalized luciferase activity of cells transfected with pGL3-Control. Standard errors were obtained from four independent experiments; Preadipocyte (■), Adipocyte (▨) *$p < 0.05$

Results

Promoter activity of the mouse leptin gene in preadipocytes and adipocytes. To determine the promoter activity of the mouse leptin gene, a series of 5'-deleted promoter fragments were fused to the luciferase gene in the plasmid, pGL3-basic vector, and transfected into 3T3-L1 preadipocytes and adipocytes. We found that the 161 bp mouse leptin promoter was 2.7-fold more active in adipocytes than in preadipocytes (Fig. 1). The activity of this promoter in preadipocytes was unexpected because these cells do not express leptin. The removal of sequences between nt –115 and nt –97 decreased the luciferase activity in both preadipocytes and adipocytes, suggesting the presence of an enhancer element in this region. The activity of the plasmid pLepLuc-161 above the empty vector (pGL3-basic) was 9.9-fold in the preadipocytes and 27-fold in the adipocytes.

Differential methylation of the leptin gene in 3T3-L1 cells during differentiation. To determine the methylation status of the eight CpG sites located at nt –159 (CpG/-159), nt –154 (CpG/-154), nt –109 (CpG/-109),

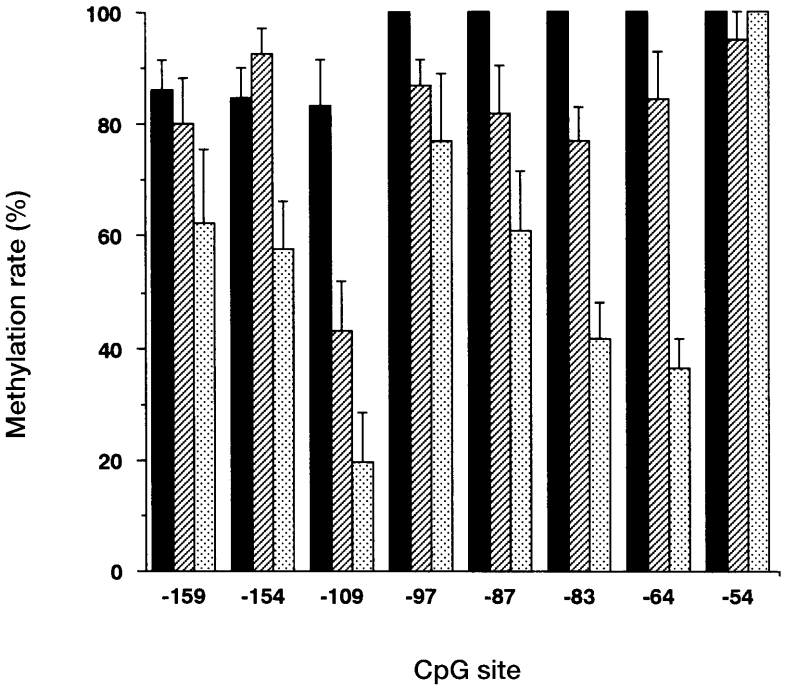

Fig. 2. The CpG methylation status in the leptin promoters during 3T3-L1 differentiation. The methylation status is shown as percentages of the total number of sequences analysed on days 0, 5, and 10 of 3T3-L1 preadipocyte differentiation. The values and standard errors were generated from four separate amplifications (two independent bisulfite-treatments and two separate amplifications from each bisulfite-treated DNA sample); Day 0 (■), Day 5 (▒), Day 10 (▤)

nt –97 (CpG/-97), nt –87 (CpG/-87), nt –83 (CpG/-83), nt –64 (CpG/-64), and nt –54 (CpG/-54), the leptin gene promoter was amplified and sequenced [15]. The methylation status at each site on days 0, 5, and 10 of differentiation are shown (Fig. 2). In 3T3-L1 cells, the expression of the leptin gene is initiated during differentiation from preadipocytes to adipocytes [9]. We also examined the time-course of leptin mRNA expression at day 0, 5, 7, and 10 of differentiation using RT-PCR. We could only find a faint signal at day 5 and an increased signal at day 7 and a maximum signal at day 10 (data not shown). Consistent with this differentiation-specific expression, we found that seven CpG sites, CpG/-159, CpG/-154, CpG/-109, CpG/-97, CpG/-87, CpG/-83 and CpG/-64 showed a varying degree of demethylation during differentiation. The eighth CpG site, CpG/-54 remained highly methylated in both preadipocytes and adipocytes.

Direct binding of nuclear protein from preadipocytes and adipocytes to the leptin gene promoter. Using DNase I footprinting analysis, we examined the binding of nuclear proteins to the leptin gene promoter. DNase I footprinting detected two protected regions within the 161 bp 5'-flanking sequence of the leptin gene (Fig. 3A, B). The proximal region (nt –63 to nt –45), which has been reported to be the C/EBPα binding site [10, 11], was protected only in adipocytes. In contrast, the distal region (nt –101 to nt –78) was protected in both preadipocytes and adipocytes. DNase I footprinting also indicated the positions of hypersensitive sites, nt –70, nt –71, nt –76, and nt –77 of the sense strand, and nt –80 of the antisense strand.

Methylation-sensitive protein binding to the 5'-flanking region of the leptin gene. The DNA fragment (nt –101 to nt –78) that was protected by DNase I footprinting analysis includes two known protein-binding sites, nt –100 to nt –95 for Sp1 and nt –89 to nt –82 for Lp1 [12]. We utilized EMSA to assay the effect of CpG methylation on protein binding to the promoter region. For these experiments, we used crude nuclear extracts of preadipocytes and eight probes; W1 (nt –115 to nt –70 without methylation), M1 (nt –115 to nt –70 with methylation), W2 (nt –115 to nt –90 without methylation), M2 (nt –115 to nt –90 with methylation), W3 (nt –94 to nt –70 without methylation) and M3 (nt –94 to nt –70 with methylation) (Fig. 4A). The W1 probe yielded several specific bands, all of which were excluded by the unlabelled W1 probe (Fig. 4B). In addition, the unlabelled Sp1 probe competed with all of these bands except one. The M1 probe yielded almost the identical bands as the W1 probe. All of these were effectively excluded by the unlabelled M1 probe and all but one band were excluded by the unlabelled W1 probe. This latter unchanged band could represent a methylation-dependent DNA binding protein (Fig. 4C). The M2 probe yielded several specific bands, all of which were excluded by the unlabelled, M2 probe and W2 probe. On the other hand, the M3 probe made three more bands compared with the W3 probe and these bands were competed out by the unlabelled M3 probe but not by the unlabelled W3 probe. These three bands could also represent methylation-dependent DNA binding proteins (Fig. 4D).

Southwestern analysis of protein binding to the 5'-flanking region of the leptin gene. Using Southwestern

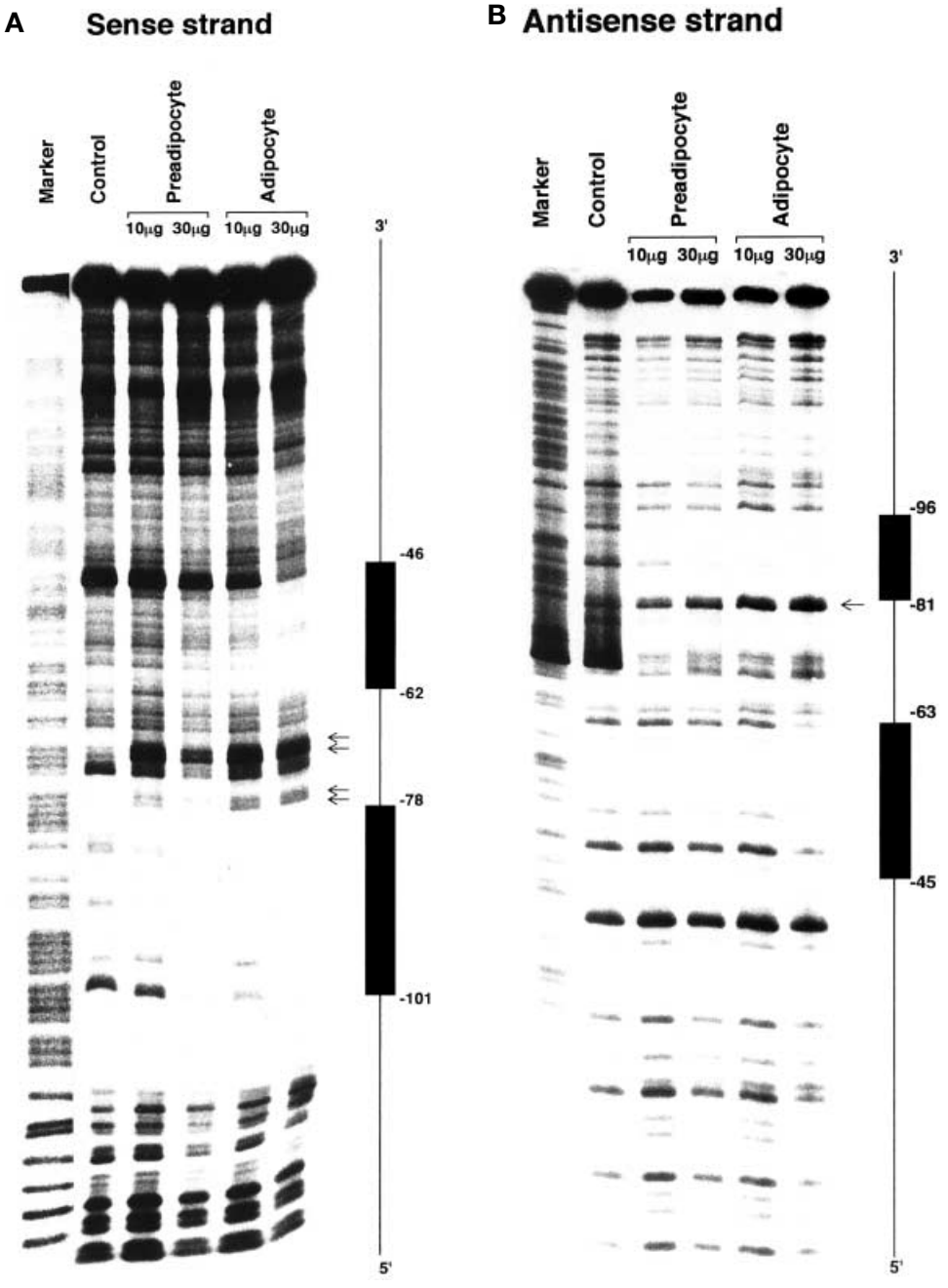

Fig. 3 (A, B). Direct binding of nuclear proteins from preadipocytes and adipocytes to the leptin gene promoter. DNase I footprints were obtained with nuclear proteins from preadipocytes or adipocytes by DNase I digestion of sense strand templates (**A**) and antisense strand templates (**B**) as described in Materials and methods. The protected regions are indicated by solid bars showing 5'- and 3'- positions. The arrows on the right indicate the hypersensitive sites

analysis, we found that the W1 probe bound to several proteins from preadipocytes. The M1 probe bound to these proteins, as well as another protein having apparent molecular weights of 52 kM_r. The 52 kM_r band was not observed by the W2 probe and the W3 probe but observed by the M2 probe and the M3 probe (Fig. 5A). These findings suggest that the 52 kM_r band could represent a methylation-dependent DNA binding protein. This protein competed with the unlabelled M1 probe, M2 probe, M3 probe and the methylated *GLUT4* probe but not with the unlabelled W1 probe and the unmethylated *GLUT4* probe (Fig. 5B). This 52 kM_r protein competed with the unlabelled M1 probe, M2 probe, M3 probe and the methylated *GLUT4* probe, but not with the unlabelled W2 probe (Fig. 5C).

Effect of methylation on leptin gene transcription. To determine the effect of methylation on leptin gene transcription, fragments of the leptin gene promoter

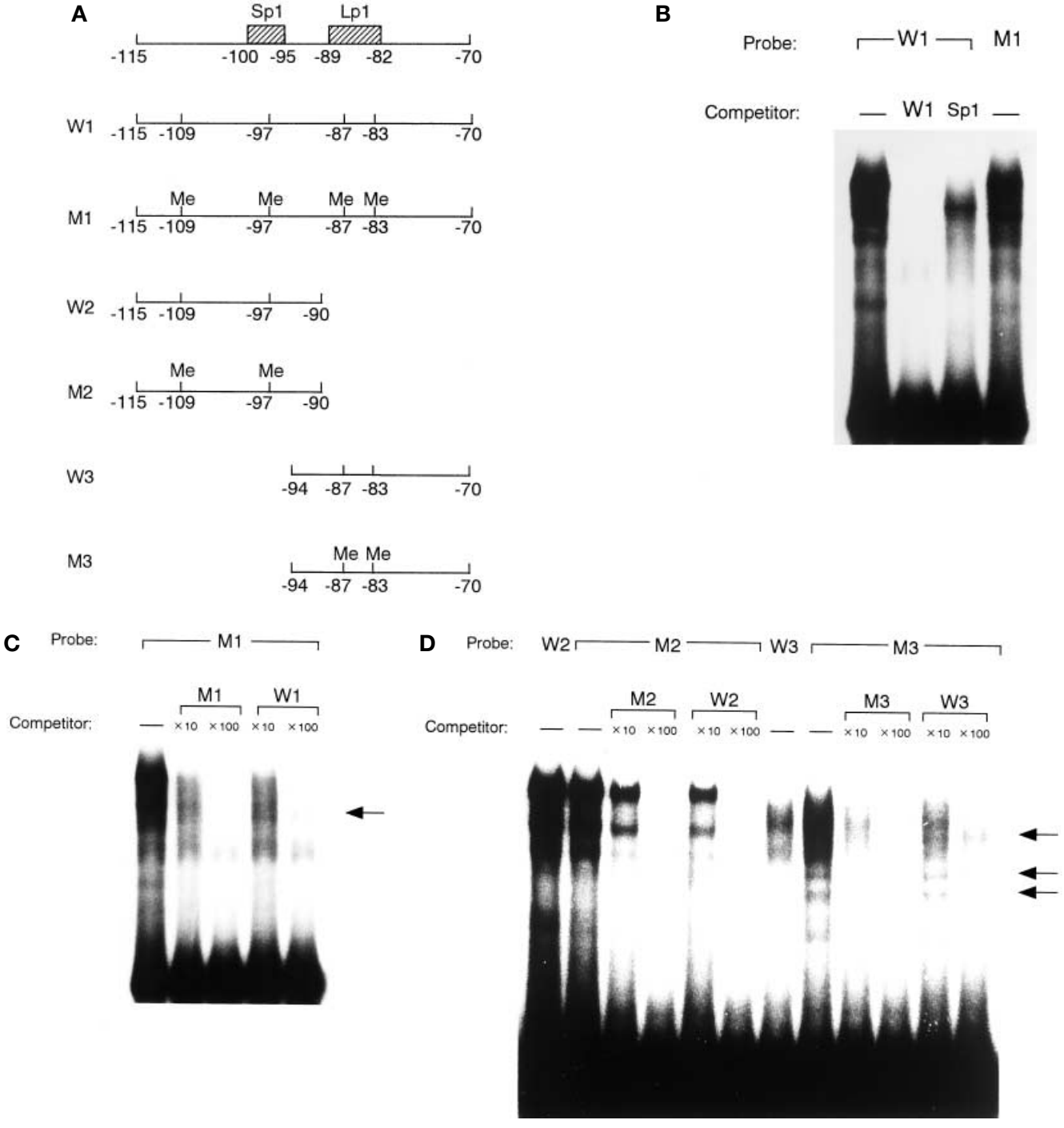

Fig. 4 (A–D). The binding to the 5'-flanking region of the leptin gene (nt –115 to nt –70) as calculated by EMSA. **B** EMSA of a reaction mixture containing nuclear proteins from preadipocytes with the W1 probe (nt –115 to nt –70) or the M1 probe in which all CpG sites had been methylated with the use of 5-methyl deoxycytidine CED phosphoamidite. A 100-fold molar excesses of the unlabelled, W1 probe or Sp1 probe was used as competitors. **C** EMSA of a reaction mixture containing the nuclear extracts of 3T3-L1 preadipocytes, the labelled M1 probe, and the indicated molar excesses of the unlabelled, W1 probe or M1 probe as competitors. The arrow indicates a methyl-CpG-binding protein. **D** EMSA of a reaction mixture containing the nuclear extracts of 3T3-L1 preadipocytes, the labelled W2 probe, M2 probe, W3 probe or M3 probe and the indicated molar excesses of the unlabelled W2 probe, M2 probe, W3 probe or M3 probe as competitors. The arrows indicate methyl-CpG-binding proteins

were methylated with *Sss*I methylase and ligated upstream of the luciferase gene in a reporter plasmid and the plasmids were transiently transfected into the 3T3-L1 preadipocytes. The activity of the methylated promoter extending to nt –115 (pLepLuc-115) was 57% lower than that of the corresponding mock-methylated promoter (Fig. 6). In contrast, the activity of a promoter extending to nt –70 (pLepLuc-70) was not affected by methylation. The activity of the mock methylated plasmid pLepLuc-115 above the empty vector (pGL3-basic) was 16.5-fold.

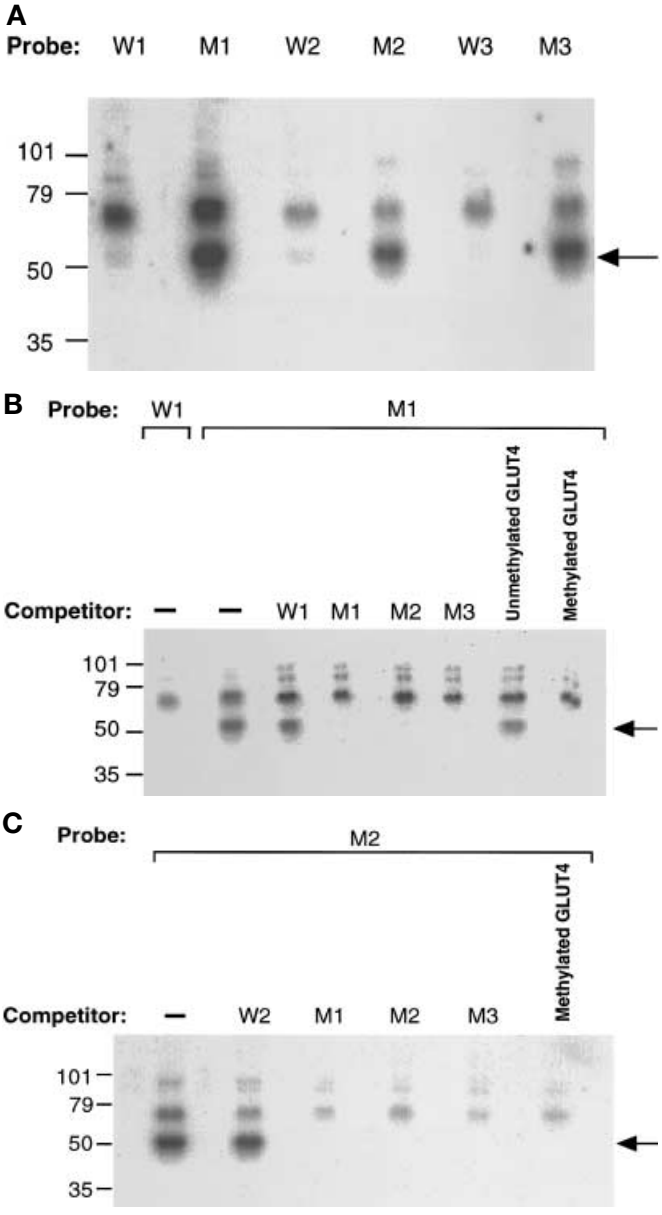

Fig. 5 (A–C). Methylation-dependent protein binding by Southwestern analysis. Preadipocyte nuclear proteins (30 μg) were electrophoresed on a 10% SDS-polyacrylamide gel. The separated proteins were electroblotted onto a nitrocellulose filter, and incubated with the labelled, W1 probe, W2 probe, W3 probe, M1 probe, M2 probe or M3 probe (**A**). The labelled W1 probe or M1 probe was used with 200-fold molar excesses of the unlabelled W1 probe, M1 probe, M2 probe, M3 probe, unmethylated *GLUT4* probe, or methylated *GLUT4* probe as competitors (**B**). The labelled M2 probe was used with 200-fold molar excesses of the unlabelled W2 probe, M1 probe, M2 probe, M3 probe or methylated GLUT4 probe as competitors (**C**). Bars and numbers on the left indicate the sizes of the marker proteins (Bio Rad). The arrow on the right indicates a methylation-dependent protein

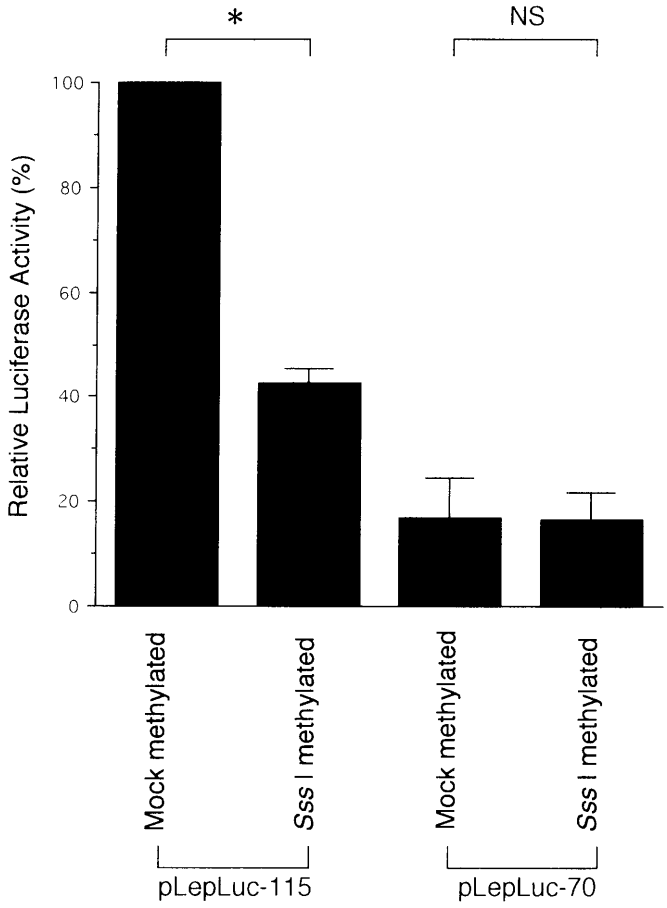

Fig. 6. Effect of methylation on leptin gene transcription. Preadipocytes were transfected with 5 μg of pLepLuc-115 or pLepLuc-70 after methylation with *Sss* I or mock-methylation. The luciferase activity of each cell lysate was normalized to the activity of pRL-CMV from a cotransfected plasmid, and the activity of each was expressed relative to that of cells transfected with mock-methylated pLepLuc-115. Standard errors were obtained from three independent experiments $*p < 0.01$

Discussion

Many genes are regulated by a combination of widely-expressed and tissue-specific transcription factors. In addition, *cis* element modifications, such as DNA methylation, could also be important in regulating gene expression during growth and development [17–19]. DNA methylation was recently shown to contribute to the cell-specific expression of the gene encoding L-histidine decarboxylase and cyclin D1 [16, 20]. We have also found that demethylation of specific CpG sites, as well as a methylation-sensitive transcription factor, contribute to the sex-specific expression of the *Cyp2d-9* gene and to the cell-specific expression of the thyrotropin receptor gene [21, 22].

The differentiation of 3T3-L1 preadipocytes to adipocytes is accompanied by the activation of numerous adipocyte-specific genes [23]. These genes are regulated by the transcription factors, such as C/EBPα, the proliferator-activated receptor-γ2, prea-

dipocyte repressor element binding protein, and the C/EBP undifferentiated protein [1–4]. We have also shown that *GLUT4* gene transcription during adipocyte differentiation is regulated by the 96 kM_r repressor protein [24]. When we examined the mechanism underlying *GLUT4* gene induction during the differentiation of 3T3-L1 cells, we also found that the methylation of specific CpG sites and the methylation-sensitive transcription factor contribute to *GLUT4* gene regulation [5]. In this study, we found that methylation of specific CpG sites also modulated the leptin promoter activity during adipocyte differentiation. A similar mechanism could regulate the expression of other genes induced during adipocyte differentiation.

In this study, we used the –161 bp promoter fragment of the mouse leptin gene, because the mouse leptin promoter up to –159 bp has been reported to be strongly transactivated by C/EBPα expression vector in the preadipocytes [11]. The region, however, could not bear all the important cis-acting regions for leptin induction during adipocytes differentiation as an example of the aP2 promoter [25].

The regulation of the leptin gene expression during 3T3-L1 differentiation has been reported to involve the nuclear DNA binding protein, C/EBPα [10, 11]. The DNA-binding proteins Sp1 and Lp1, also have been shown to contribute to leptin gene expression [12]. We also showed that the binding sites for Sp1 within the leptin gene promoter are located between nt –115 and nt –70. The in vitro binding of proteins to DNA does not always reflect binding in vivo [16, 26]. Indeed, Sp1 can bind to a promoter and activate transcription in vitro even when the CpG site within the Sp1 binding site is methylated. The methyl-CpG-binding protein, MeCP2, however, has been found to bind to DNA containing single methylated CpG site, and to repress Sp1-activated transcription of the human leukosialin gene when the promoter is methylated [27]. A methyl-CpG-binding protein, such as, MeCP2 could therefore recognize the methylated and demethylated leptin promoter and interact with Sp1 and/or other transcription factors such as Lp1 and contribute to the expression of the leptin gene during 3T3-L1 differentiation. Using EMSA, the methyl-binding proteins were observed not by the M2 probe but by the M1 and the M3 probe. Southwestern analysis, however, identified the methyl-binding protein that binds to all of the above three probes. The 52 kM_r band in the M2 lane is also specific. This discrepancy could come from the difference of the assay. Otherwise, the methyl-binding proteins that were observed by EMSA could be different from the 52 kM_r protein by Southwestern analysis which requires further study.

We have identified a 52 kM_r methyl-CpG-binding protein that binds to the promoter region between nt –115 and nt –70 of the leptin gene. The molecular weight of this protein differs from those of previously reported methyl-CpG-binding proteins, including MeCP2 [28–33] but is close to that of the 55 kM_r methyl-CpG-binding protein that binds to the *GLUT4* gene promoter [5]. The 55 kM_r protein was observed to recognize the demethylation of the *GLUT4* gene during the differentiation of 3T3-L1 preadipocytes to adipocytes. We found that the 52 kM_r protein competed with both the M1 probe (nt –115 to nt –70) and the methylated *GLUT4* probe. We also found that the 52 kM_r protein bound to both M2 probe (nt –115 to nt –90) and M3 probe (nt –94 to nt –70). These findings indicate that the 52 kM_r protein could be the same as the 55 kM_r protein and recognize methylated CpG sites regardless of their location in the DNA sequence. The 52 kM_r protein must not be a tissue-specific transcription factor, because this band was observed from both preadipocytes and adipocytes nuclear proteins. EMSA also showed that methylation-dependent DNA binding proteins were observed with crude nuclear extracts of preadipocytes and adipocytes (data not shown). Recently, MBD1, MBD2, MBD3, and MBD4 also have been reported as methyl-CpG-binding proteins [34, 35]. We also examined the supershift assay using M1 and M3 probes with antibodies against MeCP2. We could not detect any supershifted band (data not shown).

In conclusion, seven of the CpG sites in the leptin gene promoter, located between nt –159 and nt –64, were highly methylated in preadipocytes but showed a varying degree of demethylation in adipocytes. In addition, the promoter region extending from nt –115 to nt –70 bound to a 52 kM_r nuclear protein depending on the methylation status of these CpG sites. Since methylation of these CpG sites between nt –115 and nt –70 prevented the activation of the leptin gene promoter, our findings suggest that CpG methylation in the promoter region of the mouse leptin gene between nt –115 and nt –70 suppresses gene transcription in preadipocytes and that demethylation during adipocytes differentiation could contribute to the expression of this gene.

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