DNA Demethylation During the Differentiation of 3T3-L1 Cells Affects the Expression of the Mouse GLUT4 Gene

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GLUT4 is the major glucose transporter in adipose tissue and skeletal and cardiac muscles. We examined the mechanisms underlying GLUT4 gene expression in 3T3-L1 cells, which express the gene during their differentiation from preadipocytes to adipocytes. In transient transfections, the activity of a mouse GLUT4 promoter extending to –100 bp in the 5′-flanking region did not differ significantly between 3T3-L1 preadipocytes and adipocytes. Promoter activity up to –590 bp in preadipocytes and adipocytes showed a 70% lower and 228% higher activity, respectively, than promoter activity extending to –100 bp. We also examined methylation status of the GLUT4 promoter. Up to –100 bp, there were five CpG sites at –11, –30, –58, –63, and –75 bp. Two CpG sites at –11 and –30 bp were highly methylated in preadipocytes (60 and 92%, respectively) and highly demethylated in adipocytes (28.6 and 25%, respectively). Conversely, three CpG sites at –58, –63, and –75 bp were highly demethylated in both preadipocytes and adipocytes (<12%). In gel mobility-shift assays, a fragment extending from –40 to –1 bp generated a methylation-sensitive band with nuclear extracts from both preadipocytes and adipocytes when the CpG sites were methylated. Southwestern analysis identified a protein of ~55 kDa that bound strongly to the methylated probe. Furthermore, methylation of the CpG sites inhibited promoters extending to –50 or –70 bp. These results suggest that in addition to cell type–specific transcription factor, methylation of specific CpG sites and the methylation-sensitive transcription factor contribute to GLUT4 gene regulation during 3T3-L1 differentiation. *Diabetes* 48:685–690, 1999

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CAT, chloramphenicol acetyl transferase; C/EBP, CCAAT/enhancer-binding protein; CED, β-cyanoethyl-*N*, *N*-diisopropylamino; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; GABP, GA-binding protein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TSHR, thyrotropin receptor.

Glucose uptake, a rate-limiting step in glucose utilization, is mediated by facilitated diffusion via glucose transporters located in a plasma membrane (1,2). Tissue-specific expression of the genes that encode these membrane transport proteins regulates glucose uptake by different cell types (3). The insulin-responsive glucose transporter GLUT4 is expressed in adipose tissue and in cardiac and skeletal muscles, all of which exhibit insulin-stimulated glucose transport activity (3–6). The expression of GLUTs in adipose and muscle tissues is developmentally regulated (7,8). Similarly, GLUT4 is expressed in C2C12 or L6 cells during the differentiation from myoblasts to myotubes (9–11) and in 3T3-L1 cells during the differentiation from preadipocytes to adipocytes (4,6,12). Although the mechanism underlying GLUT4 gene expression in these cells during differentiation is not well known, binding sites for the transcription factors myocyte enhancer factor 2 and CCAAT/enhancer-binding protein (C/EBP) have been reported to be important for GLUT4 gene expression in C2C12 cells and 3T3-L1 cells, respectively (10–12).

DNA methylation is also an important factor in regulatory gene expression during growth and development (13–15). In general, DNA methylation is associated with the inhibition of gene expression. We recently reported that both the binding of the transcription factor GABP (GA-binding protein) and the activation of the thyrotropin receptor (TSHR) gene promoter were prevented by methylation of CpG sites at –93 and –85 bp in the 5′-flanking region of the gene. These CpG sites were highly methylated in FRT cells and completely demethylated in FRTL-5 cells, consistent with TSHR gene expression in the latter, but not in the former (16). These results suggested that methylation of specific CpG sites and the presence of methylation-sensitive transcription factors contribute to gene regulation. In the present study, we examined the mechanism underlying GLUT4 gene induction during the differentiation of 3T3-L1 cells from preadipocytes to adipocytes at the level of DNA methylation.

RESEARCH DESIGN AND METHODS

Cell culture. 3T3-L1 cells (American Type Culture Collection, Rockville, MD; CCL 92.1) were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Confluent 3T3-L1 preadipocytes were induced to differentiation into adipocytes, as previously described (17). Briefly, 1 day after confluence, 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

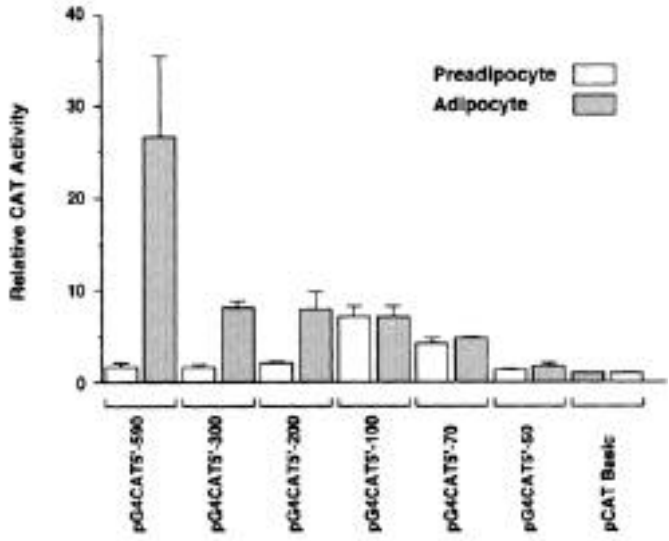

FIG. 1. GLUT4 promoter activity in preadipocytes and adipocytes. Deletion constructs of the GLUT4 promoter were placed upstream of the CAT gene and transiently transfected into preadipocytes and adipocytes. Promoter activity was measured as the conversion rate of chloramphenicol to its acetylated forms as described in METHODS. CAT activity was normalized to the amount of β-galactosidase activity from a cotransfected plasmid in each extract and expressed relative to the normalized CAT activity of cells transfected with pCAT-Basic. SEs were obtained from four independent experiments.

replaced by 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, 4, and 8 days after induction of differentiation.

Plasmids. The 5′-flanking region of the mouse GLUT4 gene (–590 to +20) was obtained from 3T3-L1 cells by polymerase chain reaction. The following primers were used to amplify the GLUT4 gene: 5′-GATACACTAGGAACGGAAGT and 5′-ACCAAGGCTCTCCGGGATCT were used as the 5′ and 3′ primers, respectively; the plasmids pG4CAT5′-590, pG4CAT5′-300, pG4CAT5′-200, pG4CAT5′-100, pG4CAT5′-70, and pG4CAT5′-50, containing –590, –300, –200, –100, –70, and –50 bp of the 5′-flanking region of the mouse GLUT4 gene, respectively, were fused to the chloramphenicol acetyl transferase (CAT) coding region of pCAT-Basic plasmid (Promega, Madison, WI). The in vitro methylation of plasmid DNA was performed using *Sss*I methylase according to the instructions of the supplier (New England Biolabs, Beverly, MA). Unmethylated control plasmids (mock-methylated plasmids) were prepared identically without addition of methylase. Before transfection, the methylated and mock-methylated plasmids were phenol-extracted and ethanol-precipitated.

Sequencing of the sodium bisulfite–treated promoter. Genomic DNAs were prepared from 3T3-L1 cells on days 0, 4, and 8 after induction of differentiation, using the SDS/proteinase K method, digested with *Pst*I, and then subjected to sequential reactions to determine the CpG methylation pattern according to Frommer et al. (18). The oligonucleotide primers were synthesized based on the reported sequences of the GLUT4 genes (12). The top strand of promoter sequence (–325 / +20) of the GLUT4 gene was amplified using 10 μl of the bisulfite-reacted DNA as a template and the oligonucleotides 5′-GGGGAAGCTTTAAG TATATGTAGTTTATAT and 5′-GGGGTCTAGAACCAAAACTCTCCAAAATCT as the 5′- and 3′-primers, respectively. The underlined regions indicate a *Hin*dIII site and an *Xba*I site added at each end of the amplified DNAs. Amplified DNAs were digested with *Hin*dIII and *Xba*I and then cloned into M13mp19 vectors for DNA sequencing.

Nuclear extracts. Nuclear extracts were prepared from 3T3-L1 preadipocytes (day 0) and adipocytes (day 8). Cells were harvested, washed with Dulbecco's modified phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+} (pH 7.4), and, after centrifugation at 500*g*, suspended in a 5-pellet vol of buffer A (10 mmol/l HEPES-KOH [pH 7.9], 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 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% (vol/vol) Tween-40. Cells were then frozen, thawed, and gently homogenized, and nuclei were isolated by centrifugation of the homogenate at 25,000*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% [vol/vol] glycerol, 0.5 mmol/l PMSF, leupeptin [2 μg/ml], pepstatin A [2 μg/ml]), and the lysate was centrifuged at 100,000*g* for 1 h. The resulting supernatant was dialyzed for use in Southwestern analysis or gel mobility-shift assays.

Gel mobility-shift assay. Each oligonucleotide was annealed to its complement and labeled by using [α-^{32}P]dATP (>6,000 Ci/mmol; Amersham, Amersham, U.K.) and DNA polymerase Klenow fragment. Methylated oligonucleotides were prepared by including 5-methyl deoxycytidine β-cyanoethyl-*N*, *N*-diisopropylamino (CED) phosphoamidite (Pharmacia Biotech, Uppsala, Sweden) during the appropriate cycle of synthesis or with the use of *Hpa*II methylase. Each radioactive probe was incubated with 5 μg of nuclear proteins in 10 μl of 20 mmol/l Tris-HCl (pH 7.5) containing 1 μg of copolymer dI-dC, 50 mmol/l NaCl, 0.1 mmol/l DTT, and 10% glycerol at room temperature. The following oligonucleotides were used as probes in the studies: gatc^{-40}TTCAGCTCTCCGCATCTTTCCCCCTCAAGCGGGTCTCACT^{-1}, and gatc^{-40}TTCAGCTCTCm^{5}CGCATCTTTCCCCCTCAAGm^{5}CGGGTCTCACT^{-1}. A Sp1 consensus oligonucleotide, 5′-ATTCGATCGGGGCGGGGCGAGC, and a methylated Sp1 consensus oligonucleotide, 5′-ATTm^{5}CGATm^{5}CGGGGm^{5}CG GGGm^{5}CGAGC, were also used as competitors.

Southwestern analysis. Nuclear proteins (30 μg) from adipocytes were dissolved in SDS sample buffer (10% glycerol, 70 mmol/l SDS, 250 mmol/l Tris, 200 mmol/l DTT, pH 6.8), boiled, and then electrophoresed on a 10% SDS-polyacrylamide gel for 2 h at 25 mA. Separated proteins were then electroblotted onto a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) in blotting buffer (25 mmol/l Tris, 0.193 mol/l glycine) for 2 h at 120 mA. 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% nonfat milk powder) and incubated for 12 h at 4°C with labeled unmethylated or methylated probes (5×10^7 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% nonfat milk powder). Filters were washed with several changes of binding buffer (10 mmol/l HEPES, pH 7.4, 1 mmol/l EDTA, 60 mmol/l NaCl, 1 mmol/l DTT), then dried, exposed to an imaging plate, and analyzed with a Bas 2000 image analyzer (Fuji, Tokyo). Molecular weight was determined with prestained molecular weight marker (Bio-Rad, Richmond, CA).

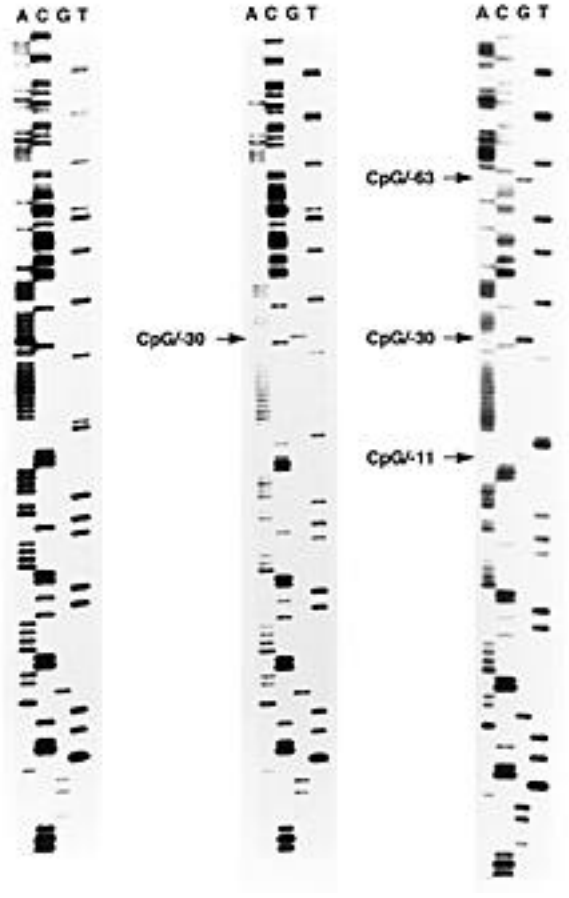

FIG. 2. Sample DNA sequences of bisulfite-treated GLUT4 promoters. Promoter sequences with different methylation patterns obtained from 3T3-L1 cells (days 0, 4, and 8) are shown. Arrows indicate CpG sites located at –11 (CpG/–11), –30 (CpG/–30), and –63 bp (CpG/–63) in the 5′-flanking region of the GLUT4 gene.

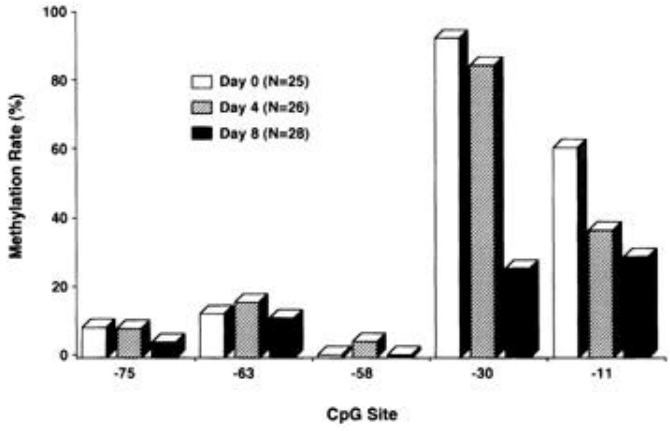

FIG. 3. Level of CpG methylation in GLUT4 promoters at various stages of 3T3-L1 differentiation. The levels of methylation are shown as percentages of the total number of sequences (indicated by N) analyzed on days 0, 4, and 8 of 3T3-L1 preadipocyte differentiation. The values were generated from two independent bisulfite-treatments and two separate amplifications from each bisulfite-treated DNA sample.

Transient expression analysis. 3T3-L1 preadipocytes (day 0) and adipocytes (day 8) were transfected by electroporation (300 V; capacitance, 960 μF) (Gene Pulser; Bio-Rad). Cells were harvested, washed, suspended at 1.5×10^7 cells/ml in 0.8 ml PBS, and cotransfected with 20 μg of the pG4CAT plasmids or pCAT-Basic plasmids and 5 μg of the β-galactosidase expression plasmid pCH110. To examine the effect of methylation on GLUT4 gene transcription, 10 μg of the mock-methylated or *Sss*I- methylated plasmids were used. Cells were pulsed, then plated and cultured for 72 h. To measure CAT activity, cells were lysed by freezing and thawing, and the lysate (30 μg of protein) was incubated with [^{14}C]chloramphenicol according to the method of Gorman et al. (19).

RESULTS

Promoter activity of the mouse GLUT4 gene in 3T3-L1 preadipocytes and adipocytes. As shown in Fig. 1, the activities of promoters extending up to –100 bp (pG4CAT5′-50, pG4CAT5′-70, and pG4CAT5′-100) did not differ significantly between preadipocytes and adipocytes. The activity of a promoter extending to –590 bp (pG4CAT5′-590) was increased by 228% compared with that of a promoter extending to –300 bp (pG4CAT5′-300) in adipocytes. In preadipocytes, however, no significant difference in promoter activities existed between the two plasmids. Conversely, the activity of a promoter extending to –200 bp (pG4CAT5′-200) was 70% lower than that of pG4CAT5′-100 in preadipocytes, but not in adipocytes.

Differential methylation of the GLUT4 gene in 3T3-L1 cells during differentiation. To determine the methylation status of five CpG sites located at –11 bp (CpG/–11), –30 bp (CpG/–30), –58 bp (CpG/–58), –63 bp (CpG/–63), and –75 bp (CpG/-75), the GLUT4 gene promoter was amplified and sequenced according to the method of Frommer et al. (18). Some examples of sequences are depicted in Fig. 2, and Fig. 3 summarizes the methylation levels at each site on days 0, 4, and 8 of differentiation. In 3T3-L1 cells, GLUT4 gene expression is initiated during the differentiation from preadipocytes to adipocytes. Consistent with this differentiation-specific expression pattern, we found that CpG/–11 and CpG/–30 were highly methylated in preadipocytes (day 0), with methylation levels of 60% and 92%, respectively. In contrast, in adipocytes (day 8), both of those CpG sites were highly demethylated with methylation levels of 28.6% and 25%, respectively. CpG/–58, CpG/–63, and CpG/–75 were highly demethylated (<12%) in both preadipocytes and adipocytes.

Methylation-sensitive protein binding to the 5′-flanking region of the GLUT4 gene. With both nuclear extracts of 3T3-L1 preadipocytes and adipocytes, a strong band was observed when CpG/–11 and CpG/–30 were methylated on the sense strand or on both strands (Fig. 4*A*). Furthermore, an unlabeled probe that had been methylated using 5-methyl

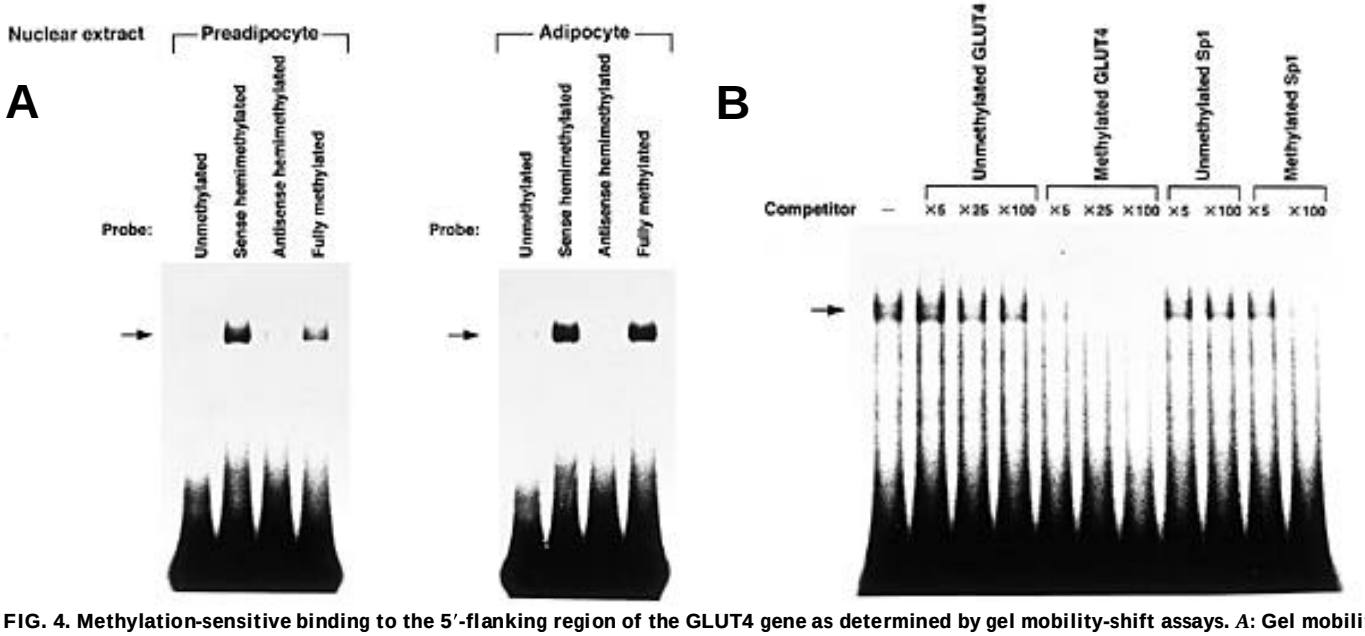

FIG. 4. Methylation-sensitive binding to the 5′-flanking region of the GLUT4 gene as determined by gel mobility-shift assays. *A*: Gel mobility-shift assay of a probe encompassing –40 to –1 bp of the GLUT4 promoter that was either unmethylated, fully methylated, or hemimethylated at the sense or antisense strand using 5-methyl deoxycytidine CED phosphoamidite at CpG/–11 and CpG/–30. The probe was incubated with nuclear extracts of 3T3-L1 preadipocytes and adipocytes. Arrows indicate specific complexes. *B*: Gel mobility-shift assay of reaction mixtures containing the nuclear extracts of 3T3-L1 preadipocytes, ^{32}P-labeled methylated GLUT4 probe (–40 to –1 bp of the GLUT4 promoter), and the indicated molar excesses of unlabeled unmethylated GLUT4, methylated GLUT4, unmethylated Sp1, or methylated Sp1 competitors. The arrow indicates a specific complex.

deoxycytidine CED phosphoamidite effectively competed with the labeled methylated probe for protein binding. An unlabeled unmethylated probe, in contrast, was a less effective competitor for the labeled methylated probe (Fig. 4*B*). To determine whether this methylation-sensitive protein binding is sequence-specific, we used Sp1 consensus oligonucleotide as a CpG-enriched DNA. As shown in Fig. 4*B*, a methylated Sp1 consensus oligonucleotide effectively competed. An unmethylated Sp1 consensus oligonucleotide, in contrast, did not compete at all.

Southwestern analysis of protein binding to the 5′-flanking region of the GLUT4 gene. Southwestern analysis demonstrated that the unmethylated probe bound to three proteins with an apparent molecular weight of 114, 85, and 80 kDa, respectively (Fig. 5). The methylated probe bound to the above three proteins and another protein (55 kDa). These results suggest that the band at 55 kDa may represent a methylation-dependent DNA binding protein.

Effect of methylation on GLUT4 gene transcription. 3T3-L1 adipocytes were transiently transfected with either mock-methylated or *Sss*I-methylated plasmids (Fig. 6). The activity of *Sss*I-methylated pG4CAT5′-70 was 60% lower than that of the corresponding mock-methylated plasmid. Furthermore, the activity of *Sss*I-methylated pG4CAT5′-50 was also reduced to that of the promoterless plasmid pCAT-Basic. The basal activity of the promoterless plasmid pCAT-Basic was not affected by methylation.

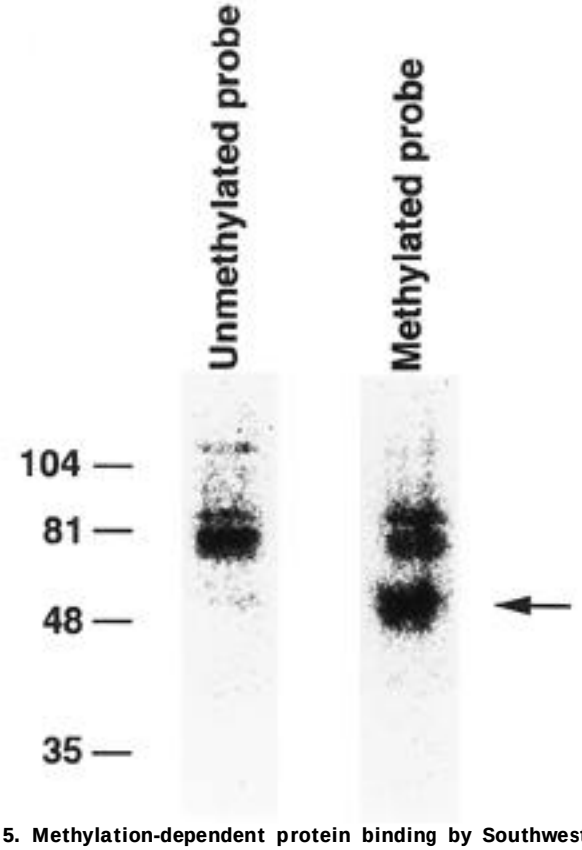

FIG. 5. Methylation-dependent protein binding by Southwestern analysis. Adipocyte nuclear proteins (30 µg) were electrophoresed on a 10% SDS-polyacrylamide gel. The separated proteins were electroblotted onto a nitrocellulose filter and incubated with labeled unmethylated or methylated probes (–40 to –1 bp of the GLUT4 promoter) as described in METHODS. Arrows and numbers on the left indicate the sizes of the marker proteins (Bio-Rad). The arrow on the right indicates a methylation-dependent protein.

DISCUSSION

The differentiation of 3T3-L1 preadipocytes to adipocytes is accompanied by the activation of many adipocyte-specific genes (20), including the GLUT4 gene. The regulation of GLUT4 gene expression during differentiation has been reported to involve the nuclear DNA binding protein C/EBP (12). C/EBP is known to bind to several viral and mammalian enhancers and CCAAT boxes (21,22). Moreover, C/EBP activates several genes, including the stearoyl-CoA desaturase I and aP2 genes, that are induced during differentiation of 3T3-L1 preadipocytes (21). C/EBP itself is also expressed during differentiation, and it may therefore play a central role in adipocyte differentiation (23). In addition to C/EBP, transcriptional activators, such as peroxisomal proliferator–activated receptor-γ2 and fatty acid–activated receptor as well as transcriptional repressors, such as preadipocyte repressor element binding protein and C/EBP undifferentiated protein, have been reported to regulate adipocyte differentiation (24–27). In the present study, we have demonstrated that the 5′-flanking region of the GLUT4 gene between –590 and –300 bp contains sequences that act as an enhancer in adipocytes, whereas sequences between –200 and –100 bp act as a repressor in preadipocytes. These enhancer and repressor sequences may contribute to the tissue-specific regulation of GLUT4 gene expression.

Disruption of the mouse DNA methyltransferase gene has shown that DNA methylation plays a crucial regulatory role during development (13–15). For instance, cell transformation results in a high degree of DNA methylation (28), whereas demethylation of the *myo* D gene or of another regulatory gene leads to myogenesis (29). Furthermore, the parental imprinting of the H19 and Igf 2 receptor genes is regulated by allele-specific methylation: the promoters of these genes are methylated in the inactive paternal genes and demethylated in the active maternal copies (30). We previ-

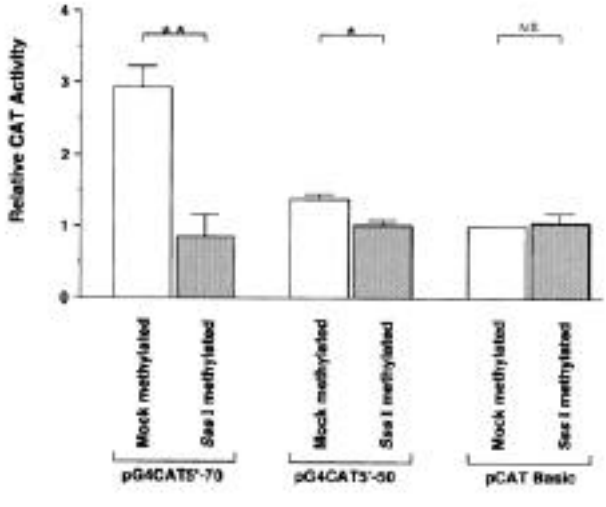

FIG. 6. Effect of methylation on GLUT4 gene transcription. Adipocytes were transfected with 20 µg of pG4CAT5′-70 or pG4CAT5′-50 after they had been subjected to methylation with *Sss*I or to a mock-methylation reaction. CAT activity of the cell lysates was measured, normalized to the amount of β-galactosidase activity from a cotransfected plasmid in each extract, and expressed relative to the normalized CAT activity of cells transfected with mock-methylated pCAT-Basic. SEs were obtained from four independent experiments. *P < 0.05, **P < 0.01.

ously reported that DNA demethylation and a methylation-sensitive transcription factor, GABP, are responsible for the sex-specific transcription of the sex-limited protein (*Slp*) and *Cyp* 2d-9 genes (31,32). GABP also regulates the transcription of the TSHR gene in a methylation-dependent manner, and methylation of specific CpG sites that interact with GABP and the methylation sensitivity of GABP contribute to the failure of FRT cells to express the endogenous TSHR gene (16). The present study demonstrated that specific CpG sites of the GLUT4 promoter (CpG/–11 and CpG/–30) are demethylated during the differentiation of 3T3-L1 preadipocytes to adipocytes and that the methylation of these CpG sites reduces the activity of the GLUT4 promoter. It appears, therefore, that a methyl-binding protein binds to and represses the GLUT4 gene promoter in preadipocyte, in which these CpG positions are methylated. Thus, in addition to C/EBP, DNA methylation and a methylation-sensitive transcription factor may be involved in the regulation of GLUT4 gene expression during differentiation. Further studies should clarify whether a similar mechanism may regulate the expression of other genes that are induced during adipocyte differentiation.

Two mechanisms have been proposed through which DNA methylation may regulate gene transcription. First, methylation may directly prevent the binding of transcription factors to promoters. Various methylation-sensitive transcription factors have been described, including activator protein-2, activating transcription factor/cAMP regulatory element (CRE)-binding protein, nuclear factor-κB, and GABP (15,31,32). For example, the activity of activator protein-2 in the regulation of cAMP-dependent transcription of the human proenkephalin gene depends on the demethylation of the cAMP regulatory element (33). Similarly, myeloid-specific transcription of the mouse M lysozyme gene is regulated by a single CpG methylation site within the enhancer (34). Second, DNA methylation may regulate gene transcription through proteins that bind to methylated DNA. Several proteins have been reported to bind to methylated CpG sequences (35–41). Among these proteins, MeCP1, MeCP2, and MDBP2 recognize methylated CpG sites regardless of their location in the DNA sequence and inhibit the transcription of associated genes (35–37). In contrast, MMBP-1, MMBP-2, MMBP-3, and MDP bind to methylated CpGs in a sequence-specific manner (38–40). Recently, a novel protein has been identified that binds to a specific region of the mouse *Xist* gene promoter in a methylation-dependent manner (41). In the present study, we identified a 55-kDa nuclear protein that bound to the GLUT4 promoter only when certain CpG sites were methylated on the sense strand or on both strands. This 55-kDa protein may be one of the members that recognize methylated CpG sites regardless of their location in the DNA sequence. It remains to be elucidated whether this protein is identical to any of the known methyl-CpG–binding proteins.

In conclusion, both CpG/–11 and CpG/–30 were highly methylated in preadipocytes and highly demethylated in adipocytes. In addition, the promoter region extending from –40 to –1 bp bound to a 55-kDa nuclear protein depending on the methylation status of these CpG sites. Methylation of these CpG sites prevented the activation of the GLUT4 promoter. These results suggest that in addition to cell type–specific transcription factor, methylation of specific CpG sites as well as the methylation-sensitive transcription factor contribute to the regulation of GLUT4 gene expression during the differentiation of 3T3-L1 preadipocytes to adipocytes.

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