Leptin gene expression in human preadipocytes is switched on by maturation induced demethylation of distinct CpGs in its proximal promoter

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Running Title

Activation of leptin gene in preadipocytes
SUMMARY

The peptide hormone leptin plays a major role in the regulation of energy intake and expenditure and is predominantly expressed in mature adipocytes but not in preadipocytes. Using bisulfite genomic sequencing, we found that 32 CpGs, distributed within a 317 bp sequence of the proximal leptin promoter, were highly methylated in human preadipocytes (73.4% ± 9.0%). During maturation towards terminally differentiated adipocytes, this promoter region was extremely demethylated (9.4% ± 4.4%). CpG-methylation dependent transcriptional activity of the promoter fragment was determined in transfection experiments using a set of 5′-truncated mock-, HhaI- and SssI-methylated promoter-reporter constructs. While the methylated CpG within the C/EBPα recognition site down-regulated reporter expression, methylated CpGs proximal the TATA motif and/or in a further upstream region abrogated promoter activity completely. These distinct promoter CpG sequences were found unmethylated in leptin-expressing mature adipocytes. As evidenced by electrophoretic mobility shift assays, nuclear protein complexes were specifically formed on methylated oligonucleotide probes corresponding to the dedicated promoter sequences, indicating that methyl-CpG binding proteins participate in transcriptional repression and regulation of the human leptin gene.
INTRODUCTION

Terminal differentiation of precursor cells involves the co-ordinate expression of a subset of cell-type-specific genes that gives rise to a new morphological and functional phenotype. Fibroblast-like cells derived from adipose tissue, which have undergone commitment to the adipose lineage, can be induced to differentiate into adipocyte phenotype by treatment with insulin, insulin-like growth factor-1 (IGF-1), glucocorticoid, and triiodothyronine (T₃) (1). On exposure to differentiation inducers, preadipocytes undergo several rounds of cell division (clonal expansion), become growth-arrested and coordinately express adipocyte-specific genes (2-5). This process is regulated by a cascade of transcriptional activators, most notably CCAAT/enhancer binding proteins (C/EBPs) (6-8) and peroxisome proliferator-activated receptor γ2 (PPAR γ2) (9, 10). Maintenance of the terminally differentiated state is ensured by sustained expression of C/EBPα, which blocks mitosis (11, 12) and trans-activates a number of adipocyte-specific genes (13-15). One of these, the leptin gene, is transcriptionally highly activated by C/EBPα (16, 17). The sequence of the human leptin gene has been identified and spans about 18 kilobase pairs containing three exons. The 5’-flanking region includes a TATA motif, three GC boxes, an AP-2 binding site and a C/EBPα motif (18).

The gene product, leptin, is a multifunctional 16-kDa peptide hormone, mainly involved in energy homeostasis and regulation of body weight (19-21) as well as in reproduction (22), sympathetic nerve activation (23), hematopoiesis (24), angiogenesis (25) and immune response (26). In a previous study we have shown that in LiSa-2 cells, a human liposarcoma cell line, the adipocyte specific genes GLUT4, aP2, PEPCK, and SCD1 were inducibly expressed as the response to hormonal agents of adipocyte differentiation in the absence of leptin gene expression (27). However, treatment of these cells with DNA methyltransferase inhibitor 5-aza-deoxycytidine led to leptin gene expression. This observation argues for a role of DNA demethylation in leptin gene expression during the process of adipocyte
differentiation. The promoter regions of many eukaryotic genes contain stretches of CpG-rich sequences known as CpG islands. These are considered to be usually unmethylated in transcriptionally active genes (28). By contrast, the inactivation of the X-chromosome and genomic imprinting are established consequences of DNA methylation (29-31) Furthermore, it was shown that the extent of methylation of CpG islands in tissue-specific genes directly influenced their transcriptional activity (32-34). As first evidence of a developmentally regulated DNA demethylation in human adipocyte differentiation, we show that a highly methylated CpG island within the proximal promoter sequence of the silent leptin gene of human preadipocytes is unmethylated in terminally differentiated adipocytes expressing the leptin gene. We further show that distinct methylated CpG motifs in this proximal promoter sequence act as specific sites for methyl-CpG binding proteins which are involved in leptin gene repression.
EXPERIMENTAL PROCEDURES

Cell culture

Preadipocytes were prepared from human adipose tissue samples by collagenase digestion and cultured according to an established protocol (35). Pre-confluent cells were repeatedly sub-cultured in DMEM/Ham’s F12 (1:1) medium containing 10% FCS and antibiotics. LiSa-2 cells were cultured in IMDM/RPMI (4:1) medium containing 10% FCS and antibiotics (27). For adipose differentiation, cells were sub-cultured in serum-free DMEM/Ham’s F12 (1:1) medium supplemented with 10 µg/ml transferrin, 15 mM NaHCO3, 15 mM HEPES, 33 µM biotin, 17 µM pantothenate, 100 U/ml penicillin and 0.1 mg/ml streptomycin and further referred to as basal medium. Additionally, Lisa-2 cells were pre-incubated for 48 h in basal medium complemented with 0.5 µM 5-aza-dC. To induce adipose differentiation, basal medium of sub-confluent cultures was supplemented with 1nM insulin, 20 pM T3 and 1µM cortisol and further referred to as adipogenic medium. Media were changed twice a week. Cells were considered as differentiated adipocytes once their cytoplasm was loaded with lipid droplets.

Oligonucleotides

The oligonucleotides used in this study are listed in Table I. PCR primers were selected from the published human leptin gene promoter sequences (GenBank accession: U43589 and D62708) to amplify promoter fragments of different lengths. A sequence of 15 bases including the BglII restriction side (underlined) was attached to the 5’-end of the reverse primer, and 15 bases including the SacI restriction side were attached to the 5’-end of the forward primers to achieve suitable PCR products for sub-cloning into the promoter-less pGL3basic vector. Primer pairs +52-bsm and -258-bsm and 5’- deleted bsm primer pairs (deleted sequence is
DNA isolation and polymerase chain reaction (PCR)

Cells growing in the log phase were washed twice with phosphate-buffered saline (PBS) and lysed in digestion buffer, consisting of 10 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K at 50°C for 12 hours. Following phenol/chloroform extraction procedure, DNA was treated with DNase-free RNase (Boehringer), repeatedly phenol/chloroform extracted and ethanol precipitated. DNA was quantified by spectrophotometry.

PCR conditions were as follows: a mixture consisting of 100 ng DNA, forward and reverse primers, 25 pmol each, 10 pmol dNTP’s, 5 µl 10x PCR-buffer, 25 pmol MgCl2 and 2.5 U Taq-polymerase (Quiagen) at a total of 50 µl was hot started at 95°C and run for 5 minutes at 95°C, followed by 35 cycles of 95°C, 56°C and 72°C for one minute at each temperature, and a final elongation step for 10 minutes at 72°C. PCR products were resolved in a 1% low melting agarose gel with ethidium bromide staining and sized by comparison with a 100 bp DNA ladder. Expected bands were excised and eluted with the Wizard PCR Preps DNA Purification System (Promega).


**Bisulfite modifying of genomic DNA and sequencing**

Cellular DNA was treated with sodium bisulfite using the CpGenome DNA Modifikation Kit (Introgen) according the manufacturer’s protocol. PCRs were performed on modified DNA using the primers +52r-bsm and -258f-bsm. Nested PCRs were performed on 10 µl of the first PCR amplification product with the primers +47r-bsm and –253f-bsm. PCR products were purified by agarose gel elektrophorese and inserted for sequencing into a pGEM-T Easy vector (Promega). Plasmides were isolated with the Qiagen Plasmid Kit and custom sequenced using standard sequencing primers (MWG Biotech).

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA was isolated from cultured cells with TRIZol reagent according to the manufacturer’s instructions (Gibco/BRL) and treated with 1µg/µl RNase-free DNase (Boehringer). 5 µg RNA were reversely transcribed using random hexamer primers and SuperScript II reverse transcriptase according to the manufacturer’s protocol (Gibco/BRL). PCRs were performed as described on 1µl RT-reaction using primer pairs listed in Table I.

**Plasmid leptin-promoter constructs**

DNA fragments of the human leptin promoter corresponding to the region from +59 to -258 and a set of 5’- truncated promoter fragments were made via PCR using the primers listed in Table I. The agarose-gel-purified PCR products were cleaved with a mixture of 20 U each of SacI and BglII restriction enzymes in 50 µl restriction buffer A (Boehringer) for 6 hours at 37°C. The digests were purified by low melting agarose gel electrophoresis and ligated into the SacI / BglII site of the promoter-less luciferase-reporter vector pGL3Basic (Promega). The inserted promoter fragments were re-amplified by PCR using plasmid-specific PCR primers and the sequence confirmed on the specific patterns of different restriction enzymes.
digests (data not shown). Leptin promoter-pGL3Basic constructs were referred to as, for example, P-258, i.e., the insert is the PCR product amplified with primer pair –258f and +59r. An expressing plasmid for C/EBPα was made by inserting the coding sequence of the human C/EBPα gene into the cloning site of the pcDNA3.1/V5-Topo vector (Invitrogen).

**Plasmid CpG methylation**

20 µg plasmid DNA were incubated for 24 hours without (mock methylated) and with 20 U SssI - or HhaI-methylase (New England BioLabs) in 400 µl methylase buffer supplemented with 160 µM S-adenosylmethionine (SAM). After 4 hours of incubation, 2 µl SAM solution (32mM) were repeatedly added. Reaction was terminated by heating (60°C, 10 min) and methylated plasmids were purified by Wizard DNA Clean-Up System (Promega). The efficiency of CpG methylation of the inserted promoter constructs was tested as follows: 2 µg of plasmid DNA were digested with SacI / BglII. The inserts were separated by agarose gel electrophoresis and completeness of methylation was proven by the inhibition of the digest with 5-methyl-cytosine sensitive restriction enzymes (data not shown).

**Transient transfection of LiSa-2 cells**

Twenty-four hours prior transfection, each, 5 x 10⁴ LiSa-2 cells in 0.5 ml basal medium were seeded into 24-well tissue culture plates. For each well, 0.5 µg promoter-pGL3basic construct, 0.5 µg C/EBPα expression vector and 25 ng pRL-CMV vector were diluted in 25 µl serum-free DMEM medium and pre-complexed with 2 µl Lipofectamine Reagent (Invitrogen) in a total of 50 µl DMEM and incubated for 30 minutes at 20°C. The culture medium was replaced by 200 µl serum-free DMEM and the plasmid-lipofectamine complexes were added to the cells. Following 5 hours incubation at 37°C, 400 µl medium containing 20% (vol/vol) FCS were added. 48 hours after transfection, the cells were washed
once with PBS and lysed in Passive Lysis Buffer (100 µl/well). Luciferase activity was determined using the Dual-Luciferase Reporter Assay System according to the manufacturer’s instructions (Promega) in the Lumat LB 9507 luminometer (Berthold). Quadruple independent experiments were made for the transfections. Luciferase activity was measured in doublets. The relative promoter activity is expressed as the ratio of light units of leptin promoter driven firefly luciferase gene and CMV promoter driven renilla luciferase gene of the co-transfected pRL-CMV vector.

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear protein extract from rat liver (Geneka Biotechnology Inc.) or nuclear protein extract prepared according to the Dignam protocol from non-differentiated Lisa-2 cells was used in the protein binding reactions (36). Complementary oligonucleotide probes (Table 1) were annealed and end-labeled with $[^{32}P]dCTP$ using Klenow enzyme. The specific activity of the probes used in the assays was adjusted to 50,000 x cpm/0.1 pmol DNA. TATA probes were incubated at 20° C for 30 minutes with 1 µg rat liver nuclear protein extract in a buffer consisting of 10 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM dithiothreitol (DTT), 10 % glycerol, 0.2 µg/µl poly(dI-dC) and 1 µg/µl bovine serum albumin (BSA), in a total volume of 20 µl. For protein binding reactions on C/EBP probes, the binding buffer contained 20 mM HEPES pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll, 0.2 µg/µl poly(dI-dC) and 1 µg/µl BSA. Protein binding reactions on MeCP-200 probes were performed essentially as previously described (15), using nuclear extract from LiSa-2 cells. The methylated or unmethylated competitors were added in 10- or 100-fold molar excess. Samples were loaded on 4.5% polyacrylamide gels and run at 10V/cm for 2 hours in 0.5x TBE buffer. The gels were dried and exposed to X-ray film. Densitometric analysis of the DNA-protein complexes...
was performed on the captured images using the ImageMaster VDS software (Amersham Biosciences).
RESULTS

* A CpG island is located in the human proximal leptin promoter *

In this study, we used a 317 base pair DNA fragment, ranging from nt +59 to –258 of the human leptin promoter, to correlate the transcriptional activity of the leptin gene with the level of CpG methylation. Thirty-two CpG dinucleotides are distributed within this sequence, particularly located in the GC-boxes at –19, –95, and –100, proximal the TATA-box at –33, and –38, within the C/EBPα binding site at –51, and six CpG motifs at position –183, –186, –188, –200, –202 and –204. Figure 1 shows the result of a CpG-plot calculation. This program defines a CpG island as a region where, over an average of ten windows, the calculated % CG composition is over 50% and the calculated observed CpG distribution versus the expected is over 0.6 (37). The program recognizes in the leptin promoter fragment a CpG island, sized from nt +3 to –211 with a CG composition of 73.8%, the average observed-to-expected ratio of CpGs being 0.87.

* Adipose differentiation is essential for leptin expression *

Among other adipose tissue-specific proteins, leptin can be considered as a marker protein expressed in terminal differentiated adipocytes. To evaluate leptin mRNA expression and the presence of transcription factor mRNAs involved in the regulation of leptin promoter activity, RT-PCR experiments on preadipocyte and adipocyte RNA were carried out. The results are shown in Figure 2. In preadipocytes, leptin mRNA was undetectable, C/EBPα message was found in traces, while AP-2α and SP1 transcripts were found abundantly (gel image A). On culturing these cells for 20 days in adipogenic medium, the morphology changed to spheroid adipocytes loaded with lipid droplets, in which C/EBPα and leptin mRNAs were clearly detectable (gel image B).
Leptin promoter demethylation occurs during adipocyte differentiation

In a recent study, we postulated an association between DNA demethylation and leptin expression during hormone-induced adipocyte differentiation of LiSa-2 cells (27). Induction of leptin mRNA in LiSa-2 cells was only observed after pre-culturing the cells in basal medium supplemented with 5-aza-deoxycytosine (5-aza-dC). To confirm these findings, we determined the methylation pattern of the leptin promoter region of cultured human preadipocytes, in-vitro-differentiated adipocytes, and differentiated LiSa-2 cells with and without 5-aza-dC treatment. Cellular DNA was treated with bisulfite and the C to U converted leptin promoter sequence was PCR-amplified and sequenced. The promoter region investigated ranges from nt +47 to nt -253 and includes 32 CpG dinucleotides. As shown in Figure 3, following adipose differentiation, the level of CpG methylation in the leptin promoter DNA decreased greatly from 73.4% ±9.0% in leptin non-expressing preadipocytes to 9.4% ±4.4% in leptin expressing adipocytes. In LiSa-2 cells, the effect on demethylation caused by 5-aza-dC resembled that observed during preadipocyte differentiation. Upon 5-aza-dC treatment, promoter methylation declined in differentiated LiSa-2 cells from 48.1% ±12.5% to 11.5% ±11.0%.

Two informative examples of sequencing chromatograms are shown in Figure 4. Figure 4.1 represents the human leptin promoter region from nt -14 to nt -59, including the core promoter and the upstream C/EBPα site. To demonstrate the correctness of sequencing, the published sequence of the leptin gene is superimposed (line 4.1A) on the corresponding DNA sequence of preadipocytes (line 4.1B). In line 4.1C the sequencing chromatogram of bisulfite-modified preadipocyte DNA illustrates the CpG methylation status of this particular promoter region. The cytosines in the CpG dinucleotides at positions -19, -33, -38 and -51 remained and were therefore methylated in the original preadipocyte DNA. By contrast, in the modified DNA of differentiated adipocytes (line 4.1D) all cytosines were converted to uracils.
appearing after PCR as tymidines), indicating that the original promoter sequence was unmethylated. Figure 4.2 represents the leptin promoter region from nt -175 to nt –215, including two CpG triplets at nt -183 to nt -188 and at nt -200 to nt -204, all of which were originally methylated in preadipocyte-derived DNA as documented in line 4.2C. After terminal differentiation, these CpG motifs were unmethylated as evidenced in the sequencing chromatogram (line 4.2D).

**Methylated CpGs at distinct loci modulate or silence leptin gene promoter activity**

To evaluate the effect of CpG methylation on the activity of the human leptin promoter to drive the expression of the luciferase reporter gene, a set of 5’- truncated promoter fragments, ranging from nt +59 up to –258 of the leptin gene, were inserted upstream of the luciferase gene into pGL3Basic vectors. In addition to the mock methylated plasmid constructs, total CpG methylated constructs were made by the action of SssI methylase. GCGC sequences in the fragments at positions +44, +42, +11, -51, -74, -85, -186, -188, -202 and –204 were methylated with HhaI methylase. Subsequently, plasmid constructs were transiently transfected into LiSa-2 cells. Figure 5 shows leptin promoter activity in transfection experiments using construct P-258. Reporter expression increased two-fold in cells cultured after transfection in adipogenic medium compared to those cultured in basal medium. Co-transfection with a C/EBPα-expressing vector markedly increased the reporter expression rate to about thirty-fold, without an additional hormone effect. These results are in accordance with published data on co-transfection experiments to achieve leptin promoter expression in 3T3-L1 mouse preadipocytes (17) and indicate that in LiSa-2 cells ectopic C/EBPα expression is needed for stimulation of leptin promoter activity, too.

Figure 6A shows the results of transient transfection experiments with the mock-methylated promoter-plasmid constructs or after methylation by HhaI- or SssI-methyltransferase.
Construct P-106 containing the DNA binding sites for Sp1, C/EBPα, and the TATA-box, achieved the highest reporter activity of 103.9 relative light units. The additional AP-2 binding site in promoter fragment P-149 had no enhancing effect on promoter activity. In accordance with published data, the promoter fragment in construct P-106 can be considered as the minimal leptin promoter (17). A decrease in promoter activity was observed using constructs, which were extended in their upstream sequences. The activity declined to about one half of maximal activity when cells were transfected with P-174, and to about one quarter using P-199. After lengthening the promoter fragment sequence up to nt –258, a two-fold increase in reporter activity was observed compared to that of P-199, which approximated to the levels of P-174 or P-60, respectively. Excluding the AP-2 and Sp1 binding sites by shortening the fragment sequence to nt –60, the promoter activity dropped to one half of maximal activity. An additional, even more dramatic decrease in promoter activity was observed with construct P-35, which only consists of the TATA motif and downstream sequences. The decrease in promoter activity from 65.3 relative light units produced by P-60 compared to 15.9 produced by P-35 was a clear indication of the importance of the C/EBPα binding site for gene activation. The promoter activity of about 3 relative light units achieved by the TATA-less plasmid P-24, was just slightly above that produced by the promoter-less pGL3Basic vector (< 1.0 relative light units).

SssI methylation of the CpGs in the leptin promoter constructs P-258 and P-199 dramatically decreased the promoter activity to values of the promoter-less basic vector. This silencing effect of SssI methylation was less extensively achieved by plasmids P-174 up to P-60. The promoter activity increased from 0.3 relative light units obtained by P-199 to 8.8 obtained by P-60. SssI methylation of the core promoter fragment P-35 decreased the basal expression from 15.9 relative light units to 0.8, stressing the importance of the methylated CpG at –33 and –38 proximal the TATA motif in transcriptional regulation.
Except for promoter construct P-199, HhaI methylation of CpGs had a moderate effect on reporter gene expression. Using the construct P-258 and P-174 down to P-35, each methylated at the indicated CpGs of the leptin promoter region (Fig. 6C), the reporter expression declined to about one third compared to that obtained by unmethylated constructs. As these expression rates were approximately as high as that of the unmethylated construct P-35, in which the C/EBP\(\alpha\) DNA binding site is absent, the reduced promoter activity was most likely due to the methylated CpG dinucleotide at -51 in the C/EBP\(\alpha\) site. A striking response to HhaI-CpG methylation was observed by promoter construct P-199. While the unmethylated construct achieved 32.6 relative light units, HhaI methylation caused a dramatic decrease of reporter expression to 0.2 units, similar to the 0.3 units in case of SssI methylation. Surprisingly, reporter expression increased up to 10.1 light units, using the HhaI methylated promoter construct P–258, which contains two additional HhaI methylation sites at –202 and –204. However, methylation of P-258 at positions –186, –188, –201, –202, and –204 (SssI methylation) decreased the expression to 1.1 light units, i.e., to values comparable with the promoter-less vector. Collectively, the results of these transfection experiments indicate that methylation/demethylation of CpGs within three regions of the leptin promoter, i.e., at positions –33 and –38 proximal the TATA-box, at –51 within the C/EBP\(\alpha\) motif, and upstream at positions –186, –188 and –200 plays an important part in the regulation of leptin gene transcription.

**Methylated CpG sites in the leptin gene promoter are targets for protein binding**

To prove methylation-dependent protein-DNA interactions in the leptin promoter, we performed electrophoretic mobility shift assays (EMSA) with defined methylated DNA probes which correspond to the TATA-box region from position –19 to –42, including two CpGs at –33 and –38, the C/EBP\(\alpha\) binding motif from –39 to –64, including two CpGs at –51
and –62, and a CpG dense upstream region from position –178 to –210, including two CpG triplets at –183 to –188 and –200 to –204 (MBD-200, Table I). EMSA was performed on rat liver nuclear protein extract on TATA DNA probes, which were both unmethylated and methylated at positions –33 and –38. The result is shown in Fig. 7A. Complexes were formed on both methylated (M) and unmethylated (U) probes (lane 1, 2, asterisk). In competition experiments, total displacement of the unmethylated probe was achieved by 10-fold molar excess of unmethylated and methylated competitors (lane 7-10). The methylated probe was completely displaced from the complex by the corresponding competitor (lanes 5, 6). Even a 100-fold molar excess of unmethylated competitor led only to a partial displacement of the methylated probe (lanes 3, 4). Additional protein-DNA complexes were formed on the methylated probe, as shown in Figure 7A, lanes 2-4 (arrows). The methylation-specific binding of the probe was proven by total displacement with the methylated competitor (lanes 5, 6), whereas the unmethylated competitor was ineffective (lanes 3, 4). The result of an EMSA carried out with DNA probes corresponding to the binding region of C/EBPα and rat liver nuclear protein extract is shown in Figure 7B. DNA-protein complexes of virtually identical electrophoretic mobility, associated with C/EBPs-DNA complexes (7), were shown with both unmethylated and methylated probe (lanes 1, 2, arrows). In comparison to the protein binding affinity of the unmethylated C/EBPα probe, the methylated counterpart exhibited a markedly reduced binding affinity. Ten molar excess of both unmethylated and methylated competitors blocked probe binding. Confirming our results obtained in the transfection experiments, methylation of the CpG dinucleotide in the C/EBPα binding site obviously diminished binding affinity to its cognate element in the proximal leptin promoter. To investigate whether methylation of a CpG-dense region in the upstream promoter sequence of the human leptin gene affects binding of specific proteins, we performed EMSAs using nuclear protein extract derived from non-stimulated LiSa-2 cells and a probe (MBD-200).
corresponding to the promoter sequence from nt –178 to –210. In the methylated probe, cytosine was replaced by 5-methyl-cytosine at the positions –186, –188 and –200. As shown in Figure 7C, a strong protein complex is formed on both the unmethylated and methylated probe (lanes 1, 2, asterisk), which was only partly blocked by a 100-fold molar excess of unmethylated or methylated competitor. An additional complex was formed, which was restricted to the methylated probe (lane 2, arrow). Binding of the methylated probe was totally displaced by the methylated competitor but not by the unmethylated one. Thus, this shifted protein complex appeared to be specifically formed with the methylated probe. Against the background of our transfection experiments, these data suggest that methyl-CpG binding proteins are crucially involved in the regulation of the leptin gene expression.

**DISCUSSION**

**Demethylation at distinct CpG sites in the leptin gene promoter of human adipocytes is essential for its expression**

The connection between cellular differentiation and DNA methylation was established by Taylor and Jones, who converted 3T3 cells to new different phenotypes by treatment with 5-aza-dC and found cellular differentiation associated with specific demethylation on methylated DNA (38, 39). The role of DNA methylation in transcriptional regulation of tissue-specific genes has received considerable attention and appears to be the primary silencing mechanism for genes with a CpG-rich promoter (40, 41).

Terminal differentiation of preadipocytes induced by agents like insulin, glucocorticoids, and T₃ follows a precisely orchestrated program of transcriptional regulatory events at the promoters of ubiquitous and adipose tissue specific genes. Leptin, one example of a protein specifically expressed in adipose tissue, acts as a multifunctional hormone, and is
predominantly delivered by mature adipocytes. In a previous study we investigated the expression profile of adipocyte-specific genes of the liposarcoma cell line, LiSa-2, following terminal differentiation (27). We found that leptin expression was only induced after preventing DNA methylation by 5-aza-dC. The leptin gene promoter fragment used in this study is associated with a CpG island which includes the regulatory elements for transcriptional activation. DNA derived from cultured human preadipocytes and terminal differentiated adipocytes was bisulfite-modified, whereupon the pattern of CpG methylation in the leptin promoter region was determined by sequencing. 73.4% ± 9.0% of the included 32 CpG motifs were found to be methylated in preadipocytes. High frequency (> 90%) methylated CpG dinucleotides were observed in the leptin promoter region of preadipocytes and in other non-leptin-expressing cells (LiSa-2, human fibroblasts, data not shown) at positions -33 and -38 proximal the TATA motif, within the cognate element of C/EBPα at –51, in the SP1 sites at –19, –95 and –100, and at six CpG sites located in the promoter sequence from nt –183 to –204. Following terminal differentiation, the percentage of methyl-CpG in the promoter region dropped to 9.4% ± 4.4%. In addition, the CpG sites at the distinct promoter loci mentioned above, turned out to be unmethylated in adipocytes as well as in 5-aza-dC-treated and differentiated LiSa-2 cells. Recently, Yokomori et al. examined the methylation status of the mouse leptin promoter in 3T3-L1 preadipocytes and adipocytes from positions –54 to –159, including eight CpG dinucleotides (42). These authors found a variable degree of demethylation in seven CpG sites during differentiation, whereas the CpG at –54, located in the C/EBPα binding site of the mouse leptin promoter, remained highly methylated. The corresponding human leptin promoter fragment ranges from nt –51 to –160 and includes 13 CpG sites. At variance with the data reported by Yokomori et al., in our experiments all CpG sites were dramatically demethylated upon differentiation. In the leptin promoter of mature human adipocytes, the CpG dinucleotide within the C/EBPα motif and
the three CpGs within the SP1 sites were unmethylated. Additional evidence that DNA demethylation acts as an essential process in adipocytic differentiation was provided by showing that, in 3T3-L1 preadipocytes, the expression of the insulin-responsive glucose transporter GLUT4 gene was activated by demethylation of the promoter during further maturation to adipocytes (43).

**CpG methylation at the C/EBP\(\alpha\) motif down-modulates leptin promoter activity, whereas methylation at SP1 sites has no effect**

Methylation of CpG sites in the promoter region of tissue-specific genes is most crucial for their expression (41). One mechanism by which methylation may contribute to gene regulation is to reduce or inhibit the binding of transcription factors. However, a minority of known transcription factors have CpG sites in their recognition sequences. In the case of CREB, AP2, E2F, and c-Myc, methylated CpGs in their recognition site preclude the binding of the transcription factor, and thereby directly inhibit gene expression (44-47). SP1 binding at GC-rich elements was found to be unaffected by CpG methylation (48, 49). However, the contribution of methylated CpG in the SP1 recognition site to \(T1\alpha\) gene expression was also shown (50). We examined the effect of CpG methylation on transcription factor binding and leptin promoter activity indirectly by transfection experiments using 5'-truncated leptin promoter-reporter constructs, which were mock-, HhaI- or SssI-methylated. The sequence of the leptin promoter construct P-59 used in this study includes four HhaI methylation sites. One is present in the C/EBP\(\alpha\) binding site. Methylation at this CpG site diminished promoter activity by about 50%. Nuclear protein binding assays on unmethylated and at this CpG site methylated C/EBP\(\alpha\) probe yielded protein probe complexes with a diminished binding affinity to the methylated probe. These observations indicate that C/EBP\(\alpha\)-binding is affected by CpG methylation at its cognate element and is associated with regulation of gene
expression. The importance of the CpG sequence in the C/EBPα recognition site for factor
binding and leptin expression was recently shown (51). Base change from CG to AT in the
C/EBPα recognition sequence abolished factor binding and caused a sharp decrease in
promoter activity.

In transfection experiments using the promoter construct P-106, we here confirmed that
cooperative action of SP1 and C/EBPα achieved maximal promoter activity. The expression
markedly decreased to about 5% when the SssI methylated reporter construct was used. Thus,
we assume that the loss of promoter activity is not a consequence of inhibited SP1 binding to
methylated SP1 recognition sites, but is due to binding of methyl-CpG sensitive repressor
proteins on methylated CpGs proximal the TATA motif.

**Two highly methylated CpG motifs in the human preadipocyte leptin promoter recruit
methyl-CpG binding proteins which silence leptin gene expression**

We have shown that leptin gene promoter activity, as determined by transfection experiments,
is practically silenced using the SssI methylated promoter-reporter construct P-258, the SssI-
or HhaI methylated construct P-199, and even the SssI methylated core promoter construct P-35. In addition to the high-frequency methylated Sp1 and C/EBPα binding sites in the human
leptin promoter mentioned above, the CpGs at −33 and −38 proximal the TATA motif and a
region including the HhaI methylase-sensitive CpGs at −186, −188, −202 and −204 as well as
the CpG at −200, were highly methylated in preadipocytes. In sharp contrast, the
corresponding CpGs were found to be unmethylated in mature adipocytes. In nuclear protein
binding assays on unmethylated and specifically methylated DNA probes corresponding to
the two distinct promoter regions, we found proteins with specific binding affinity towards the
methylated promoter sites. These observations suggest that methyl-CpG binding proteins are
operative in leptin gene repression.
Considerable attention has been focused on proteins that bind to promoter-proximal methylated DNA and function as transcriptional repressors. Two chromosomal protein complexes with high affinity towards methylated DNA have been identified (52, 53). Methyl-CpG-binding protein-1 (MeCP1) is a protein complex composed of 10 major polypeptides. The MBD2 subunit binds specifically to a variety of methylated DNA sequences which contain at least 12 symmetrically methylated CpGs and represses transcription by recruiting nucleosome remodeling histone deacetylase (NuRD) (54, 55). Methyl-CpG-binding protein-2 (MeCP2) is reported to bind specifically to a single methylated CpG pair and to be concentrated in the centromeric heterochromatin (56, 57). It has been shown that the transcriptional repressing domain (TRD) of MeCP2 binds components of the mSin3A/HDAC complex and represses transcription via histone deacetylation and remodeling of the chromatin structure (58). An alternative pathway of transcriptional repression, aside from the recruitment of histone deacetylase, was reported, in which the TRD interacts with the TFIIB of the basal transcriptional machinery (59). This mechanism might also be operative in the repression of leptin promoter activity once CpGs proximal the TATA motif are methylated. Furthermore, the methylated motif, -CGCGGCG- was found in the Xist promoter which binds a methyl-CpG binding protein with high affinity (60). This motif is strikingly similar to the recognition site of Kaiso, a novel methylation-dependent transcriptional repressor. Kaiso is associated with MeCP1 and requires two symmetrically methylated CpG dinucleotids in its recognition sequence (61). A quite similar sequence, -CGCGCCG-, is located at –182 to –188 in the upstream region of the human leptin promoter, which we define as a binding site for a methyl-CpG binding protein. The biochemical characterization of the methyl-CpG binding proteins and histone-modifying proteins probably involved in transcriptional regulation of the leptin gene in the preadipocyte/adipocyte model will be addressed in future work.
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FOOTNOTES

1The abbreviations used are:

C/EBP, CCAAT/enhancer binding protein; PPARγ, peroxisome proliferator-activated receptor γ; GLUT4, insulin-responsive glucose transporter 4; aP2, fatty acid binding protein; PEPCK, phosphoenolpyruvate carboxykinase; SCD1, stearoyl-CoA desaturase; AP-2α, activating enhancer binding protein 2 α; SP1, transcription factor Sp1; CREB, cAMP response element-binding protein; TRD, transcriptional repressing domain; NuRD, nucleosome remodeling histone deacetylase; HDAC, histone deacetylase
FIGURE LEGENDS

Figure 1
CpG-plot of a 317 bp sequence of the human leptin promoter.
The average observed-versus-expected ratio of C plus G to CpG in a set of ten dinucleotides
is reported. The calculated probability of one CpG per ten dinucleotides is indicated (exp.
0.625). Program settings: Obs/Exp ratio > 0.60; %C + %G > 50.0; Length > 300.

Figure 2
RT-PCR analysis of C/EBPα, AP-2α, SP1 and leptin mRNA expression in human
preadipocytes (A) and adipocytes (B).
RT-PCR was performed on RNA extracted from human preadipocytes grown in adipogenic
medium on days 0 and 20 corresponding to undifferentiated cells (day 0) and differentiated
cells, termed as adipocytes (day 20) as described in methods.

Figure 3
Percentage of CpG methylation of the human leptin gene promoter fragment investigated.
DNA from indicated cultured cells was bisulfite-modified. A leptin gene promoter fragment
from nt +47 to nt –253 was amplified by PCR with primers specific for the C to U conversion
and sequenced. The 32 CpGs considered as 100%. Values are means ± standard deviation of
five independent sequencing reactions.

Figure 4
Methylation pattern of human leptin promoter regions.
Lines Figure 4.1A and 4.2A: Genbank sequences of the human leptin promoter region from base pair –14 to –59 and from –174 to –216. Regulatory elements are underlined. CpGs are in bold and numbered or indicated by asterisks. Lines Figure 4.1B and 4.2B: direct sequencing chromatography of the leptin promoter regions of DNA derived from preadipocytes, 4.1C and 4.2C: of bisulfite-modified DNA from preadipocytes, 4.1D and 4.2D: of bisulfite-modified DNA from adipocytes.

Figure 5
Activity of a leptin promoter-reporter construct in LiSa-2 cells.
LiSa-2 cells were transfected with the P-258 construct alone or together with a C/EBPα expressing vector. Reporter expression was measured after 48h culture in basal or adipogenic medium (c.f. methods). Values are means ± standard deviation of four independent transfection experiments and two luciferase activity determinations each.

Figure 6
A: Methylation-dependent leptin promoter activities of 5’-truncated leptin promoter-pGL3Basic constructs. Lisa 2 cells were transfected with the constructs, and relative light emission was determined 48h after transfection as described in methods.
Plasmids: mock-methylated   HhaI-methylated   SssI-methylated
Values are means ± standard deviation of four independent transfection experiments and two luciferase activity determinations each.
B: Size and positions of the leptin promoter-constructs sub-cloned upstream the luciferase reporter gene (LUC) into pGL3Basic vectors used for transfection experiments.
C: Schematic representation of exon1 and the proximal promoter region of the human leptin gene including the known regulatory elements. The DNA loci numbers of the CpG sites within the sequence, start codon in exon 1 is counted as +1 are placed above (19). SssI methylation sites (-*CG-) are ubiquitously, and HhaI methylation sites (-G*CGC-) are indicated by arrows (▼).

Figure 7
Details of electrophoretic mobility shift assays (EMSA) on methylated (M) and unmethylated (U) 32P-labeled DNA probes of human leptin promoter regions and corresponding competitors. Panel A: EMSA was performed using rat liver nuclear protein extract and probes range from nt –19 to –43 of the human leptin promoter TATA-box region, including two CpGs at –33 and –38. Specific complexes formed on U- and M-probes indicated by an asterisk, and specific complexes formed only on M-probe indicated by arrows. Panel B: EMSA was carried out using rat liver nuclear protein extract and probes range from –39 to –64 of the human leptin promoter including the C/EBPα recognition site and two CpGs at –51 and –62. Specific complexes formed on U- and M-probes indicated by arrows. Panel C: EMSA was carried out using nuclear protein extract derived from undifferentiated LiSa-2 cells and MBD-200 probes range from base pair –178 to –210, including six CpGs at –183, –186, –188, –200, –202 and –204. Specific complexes formed on U- and M-probes indicated by an asterisk, and specific complexes formed only on M probe indicated by an arrow.

Competitions were run with 10- or 100-fold molar excess of unlabeled U or M probe (competitor) in the reaction mixture.

Lanes 1, 7, 8, 9, 10: EMSA with U probe; lanes 7, 8 competition by adding U competitor; lanes 9, 10 competition by adding M competitor.
Lanes 2, 3, 4, 5, 6: EMSA with M probe; lanes 3, 4 competition by adding U competitor; lanes 5, 6 competition by adding M competitor.

Table legend

Table I

Sequences of oligonucleotides used in this study.
Details are given in the text.
| oligonucleotide | 5'-sequence-3' | template |
|----------------|----------------|----------|
| +59f           | TAATAGATCTACTGAGAGAAGGAGGAGGCGC DNA |          |
| -258f          | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| -199f          | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| -174f          | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| -149f          | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| -106f          | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| -60f           | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| -35f           | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| -24f           | TAATAGCTACTGAGAGAAGTCTCTGGCTCTCAC DNA |          |
| +52r-bsm, +47r-bsm | AAAACACRCRCRTAACCTTACTTTAC bsm-DNA |          |
| -258f-bsm, -253f-bsm | GGATT GGT YTGTTTTTATTAGTTATTTTTAA bsm-DNA |          |
| Leptin-f       | GCCCTATCTTTCTATGTGC c-DNA |          |
| Leptin-r       | TCTGTGGAAGTAGCTGTAAG c-DNA |          |
| C/EBP<sub>f</sub> | TTCCCTTACCAGCGCCGC c-DNA |          |
| C/EBP<sub>r</sub> | CATTGTCACCTGGTGATCCTCC c-DNA |          |
| Ap2-f          | ATTCGATCCAAATGACAGAAG c-DNA |          |
| Ap2-r          | CGGGGAGAGGAGGTGAAG c-DNA |          |
| Sp1-f          | ACTACAGTGATGACTGAGGAG c-DNA |          |
| Sp1-r          | CTGACAATGTGCTGCTTGGGA c-DNA |          |
| β-Actin-f      | TCTCTCACAACCTTCTTCC c-DNA |          |
| β-Actin-r      | CTGCTGATCCACATCTGGA c-DNA |          |
| TATA<sub>f</sub> | AGGTGGATCTGGGCCGCCTATAAAGAGGAGG c-DNA | nuclear extract |
| TATA<sub>r</sub> | AGGGGCTTCTTTATAGAGCTGCCTTATAAAGAGGAGG c-DNA | nuclear extract |
| C/EBP<sub>f</sub> | AGGTGGGATGGCGACTTGGACGAGTTGAT c-DNA | nuclear extract |
| C/EBP<sub>r</sub> | AGGTGGGATGGCGACTTGGACGAGTTGAT c-DNA | nuclear extract |
| MB-D-200<sub>f</sub> | AGGTGGGATGGCGACTTGGACGAGTTGAT c-DNA | nuclear extract |
| MB-D-200<sub>r</sub> | AGGTGGGATGGCGACTTGGACGAGTTGAT c-DNA | nuclear extract |

**Table I**
Figure 1
Figure 3
Figure 4.1

A

-31

G G G A B T T T G A T G A T C B G G G G

C E B P

-36

-35

G C T A A G A G G G G G G G G C A J

T A T A

-29

S p 1

B

-26

G G G C A T T G C C A A A G G T G T C C G G C A J

C E B P

-21

-20

G C T A A G A G G G G G G G G C A J

T A T A

-16

S p 1

C

-12

G G G C A T T G C C A A A G G T G T C C G G C A J

C E B P

-7

-6

G C T A A G A G G G G G G G G C A J

T A T A

2

S p 1

D

-1

G G G C A T T G C C A A A G G T G T C C G G C A J

C E B P

-1

-1

G C T A A G A G G G G G G G G C A J

T A T A

1

S p 1

Figure 4.2

A

-204 -202 -200

A T A C C C A A G G G T G G C C G C G C G G C C G

T A T A

-183 -182 -181

A T A C C C A A G G G T G G C C G C G C G G C C G

T A T A

-168 -166 -164

A T A C C C A A G G G T G G C C G C G C G G C C G

T A T A

Figure 4
Figure 5
Figure 6
Figure 7
Leptin gene expression in human preadipocytes is switched on by maturation induced demethylation of distinct CpGs in its proximal promoter
Ingo Melzner, Vanessa Scott, Karola Dorsch, Pamela Fischer, Martin Wabitsch, Silke Brüderlein, Cornelia Hasel and Peter Møller

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