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The adipocyte specific transcription factor C/EBPα modulates human *ob* gene expression

(3T3-L1/preadipocytes and adipocytes/leptin/obesity/C/EBP)

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ABSTRACT **The *ob* gene product, leptin, apparently exclusively expressed in adipose tissue, is a signaling factor regulating body weight homeostasis and energy balance. *ob* gene expression is increased in obese rodents and regulated by feeding, insulin, and glucocorticoids, which supports the concept that *ob* gene expression is under hormonal control, which is expected for a key factor controlling body weight homeostasis and energy balance. In humans, *ob* mRNA expression is increased in gross obesity; however, the effects of the above factors on human *ob* expression are unknown. We describe the structure of the human *ob* gene and initial functional analysis of its promoter. The human *ob* gene's three exons cover ≈15 kb of genomic DNA. The entire coding region is contained in exons 2 and 3, which are separated by a 2-kb intron. The first small 30-bp untranslated exon is located >10.5 kb upstream of the initiator ATG codon. Three kilobases of DNA upstream of the transcription start site has been cloned and characterized. Only 217 bp of 5′ sequence are required for basal adipose tissue-specific expression of the *ob* gene as well as enhanced expression by C/EBPα. Mutation of the single C/EBPα site in this region abolished inducibility of the promoter by C/EBPα in cotransfection assays. The gene structure will facilitate our analysis of *ob* mutations in human obesity, whereas knowledge of sequence elements and factors regulating *ob* gene expression should be of major importance in the prevention and treatment of obesity.**

Obesity, a disorder of energy balance, is a major health problem in Western societies, linked to cardiovascular disease, diabetes and an increased mortality rate (1). The description of six single-gene mutations resulting in obesity in mice is consistent with the implication of genetic factors in the etiology of obesity (2). In the obese (*ob*) mouse, a single mutation results in profound obesity which is often accompanied by diabetes (3). This phenotype results from both a marked hyperphagia and a decrease in energy expenditure. Parabiosis experiments have suggested that *ob* mice are deficient of a blood-borne factor regulating nutrient intake and energy metabolism (4). By using positional cloning techniques, the mouse *ob* gene, and its human homologue, which is highly similar to the mouse gene, have been cloned (5). The mutant SM/Ckc-+^{Dac}*ob*^{2J}/*ob*^{2J} mouse carries a genomic alteration that results in the complete absence of *ob* mRNA, whereas in C57BL/6J-*ob*/*ob* mice a nonsense mutation results in a truncated, nonfunctional protein. The *ob* gene is expressed in adipose tissue, and its mRNA contains a signal sequence. Studies with specific antibodies confirmed the presence of the *ob* gene product, leptin, in the plasma of normal mice and its absence in the plasma of *ob*/*ob* mutants (6). Three groups (6–8) independently confirmed that leptin reduces food intake, increases energy expenditure, induces weight loss, and normalizes metabolic parameters such as insulin and glucose when injected in wild-type, diet-induced obese mice or C57BL/6J *ob*/*ob* mice. Recent studies demonstrated that the expression of the *ob* gene itself is controlled by the nutritional status of the animal. Fasting reduces expression, whereas food intake increases expression (9–12), an effect that is accounted for by changes in insulin levels (10, 11). Glucocorticoids have also been shown to regulate *ob* gene expression (13, 14).

Because *ob* is exclusively expressed in adipocytes, we have initiated a series of studies to examine the role of adipogenic factors in the expression and regulation of the *ob* gene. The expression of two important adipocyte transcription factors, PPARγ and C/EBPα, is induced during adipocyte differentiation, and these factors are maintained in the mature adipocyte. Several adipocyte specific genes have binding sites for these factors and have been shown to be transcriptionally responsive to chemical modulators of these factors (reviewed in ref. 15).

To better understand the role of leptin in the development of human obesity, it is crucial that we gain insight into the regulation of *ob* gene expression in humans. Therefore, we have determined the structure of the human *ob* gene and studied its regulation in two different adipocyte model systems. In these studies we used primary rat adipocytes as a model of fully differentiated adipocytes (16) and the mouse 3T3-L1 cell line as a preadipocyte model. Our results demonstrate that 217 bp of DNA upstream of the transcription start site controls basal and tissue-specific *ob* gene expression. Furthermore, this region contains a sequence element which responds to C/EBPα, a key transcription factor implicated in the determination of the adipocyte phenotype, as well as other potential regulatory sites.

MATERIALS AND METHODS

Oligonucleotides. The oligonucleotides used for various aspects in this manuscript are as follows (N_1 = G, A, or C, whereas N = G, A, C, or T): 1F, 5′-ATG CAT TGG GGA ACC CTG TGC GG-3′; 140R, 5′-TGT GAA ATG TCA TTG ATC CTG GTG ACA ATT-3′; 217R, 5′-GAGGGTTTTG-GTGTCATCTTGGAC-3′; 562R, 5′-CCTGCTCAGGGC-CACCACCTCTGTCG-3′; anchored-T, 5′-TTCTAGAATT-CAGCGGCCGC(T)$_{30}$N$_1$N-3′; pdv34R, 5′-GCCACAAGAA-TCCGCACAGGGTTCCCCATGC-3′; SMFOR, 5′-CGCAGC-

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Abbreviation: RACE, rapid amplification of cDNA ends.
Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U48621).
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GCCAACGGTTGCAAGGC-3′; SMREV2, 5′-CGCGGGAA-GCTTGCCTTGCAACCGTTGGCGCTGCG-3′; OB/S1, 5′-GCTTCTTGGGCCTTGCAACCGTTGGCGCTGC-GATTCCTACGGGGCTCCATGCCTGC-3′; MUT1a, 5′-GA-GCCTCTGGAGGGACATCA-3′; MUT2a, 5′- TGGCGTCT-TCCATGGGGTCT-3′; CEBPfor, 5′-GCCTGCGGGGCAGT-TAAAAAAGTTGTGATCG-3′; and CEBPrev, 5′-CGATCA-CAACTTAAAAAACTGCCCCGCAGG-3′.

Isolation of the Human *ob* Gene, Restriction Mapping, Determination of Intron/Exon Boundaries, and DNA Sequencing. A human adipose tissue λgt11 library was screened with a fragment of mouse *ob* cDNA clone pmob1 (11, 13). Several positive clones were obtained, and clone ob6.1 was characterized in detail. This sequence was used to screen a λDASHII human genomic library. The two clones obtained did not contain the promoter. To isolate genomic P1 clones containing the entire human *ob* gene, we used the primer pair 1F and 140R to amplify a 140-bp probe with the ob6.1 plasmid as a template. Three positive clones were isolated: 5135, 5136, and 5137.

Determination of the Transcription Initiation Site. *5′ Rapid amplification of cDNA ends (RACE).* The Marathon cDNA amplification kit (Clontech) was used for 5′-RACE. The 5′-RACE was performed on total RNA (1 μg) from several independent human white adipose tissue samples and on double-stranded cDNA derived from human adipose tissue (Clontech). The anchored T primer included in the kit or the *ob*-specific primer 562R were used to prime first strand synthesis on adipose tissue RNA, and second strand synthesis was performed according to the instructions provided by the manufacturer. PCR products were recovered, ligated into the TA cloning vector pCRII (InVitrogen), and sequenced.

Primer extension. The oligonucleotide pdv34R was ^{32}P-labeled to a specific activity of 10^7 dpm/50 ng and purified by gel electrophoresis. For primer extension, 10^5 dpm of oligonucleotide was added to 50 μg of adipose tissue total RNA from different patients in a final volume of 100 μl. Primer extension analysis was performed following standard protocols utilizing a mixture of 1.25 units of avian myeloblastosis virus reverse transcriptase (BRL) and 100 units of Moloney murine leukemia virus reverse transcriptase (BRL). A sequencing reaction and molecular mass standards were used to map the 5′ end of the extension products.

S1 analysis. A 5′ ^{32}P-labeled, gel-purified oligonucleotide probe OB/S1 (50,000 cpm) was annealed (14 hr, 30°C) to 50 μg adipose tissue total RNA from different subjects in 20 μl of hybridization buffer. A 10 μl aliquot of the annealing reaction was subjected to S1 nuclease digestion according to the manufacturer's protocol (Ambion, Austin, TX), and the reaction products were analyzed on a 10% denaturing gel. A ^{35}S-labeled 10-nucleotide ladder was used as a size standard.

Tissue Samples, Cell Culture, and mRNA Analysis. Omental adipose tissue was obtained from overnight fasted nonobese adult French subjects undergoing elective surgery. They had given informed consent, and the project was approved by the Ethics Committee of the University of Lille. Adipose tissue was immediately frozen in liquid nitrogen until RNA preparation.

Primary rat adipocytes were obtained exactly as described by Hajduch *et al.* (17). Standard cell culture conditions were used to maintain 3T3-L1 (American Type Culture Collection), CV-1 (a kind gift from R. Evans, Salk Institute, La Jolla, CA), and COS cells (American Type Culture Collection). RNA preparation, Northern blot hybridizations, and quantification of total cellular RNA were performed as described (11). A mouse *ob* cDNA fragment spanning nucleotides +50 to +659 (13), a human GAPDH cDNA clone (18), and a human γ-actin (19) cDNA clone were used as control probes.

Analysis of Promoter Activity. To test the activity of the human *ob* promoter, several reporter constructs were made. A 7-kb *Hin*dIII fragment of P1 clone 5135 that hybridized to oligonucleotide SMFOR, was subcloned into the *Hin*dIII site of pBluescript (Stratagene). From this construct a 3-kb fragment, containing sequences from −3 kb (5′ *Hin*dIII site) to +30 relative to the transcription start site, was amplified by PCR with the T7 primer and SMREV2 (containing a *Hin*dIII site).

The PCR product was digested with *Hin*dIII and ligated into the *Hin*dIII site of the promoterless luciferase reporter vector pGL3-Basic (Promega) to generate pGL3-OB1 and sequenced to confirm orientation. The reporter construct pGL3-OB2 contains a *Kpn*I–*Hin*dIII subfragment, spanning from positions −217 to +30. The C/EBPα mutant construct pGL3-KOB1 was constructed using the mismatch PCR technique. Briefly, an oligonucleotide corresponding to sequences 5′ to the Asp-718 in pGL3-OB1 (MUT1a) and a 3′ oligonucleotide outside the multiple cloning site (MUT2a) and two additional oligonucleotides encompassing the C/EBP site (CEBPfor and CEBPrev) were synthesized. The first PCR step involved amplification with pGL3-OB1 as template and the primer pairs CEBPrev plus MUT1a in one reaction and CEBPfor plus MUT2a in a second reaction. The gel-isolated products were pooled and reamplified with MUT1a plus MUT2a primers. The resultant PCR product was digested with *Nco*I and Asp-718 and subcloned into *Nco*I and Asp-718 digested pGL3-OB1 and sequenced to confirm the mutant sequence. The pMSVC/EBPα expression vector is described elsewhere (20). Transfections were performed by using either standard calcium phosphate precipitation techniques (21) or electroporation for primary adipocytes (16). Luciferase assays were carried out exactly as described (21). pGL3-Basic and pGL3-Control (Promega) were used as transfection controls for comparison across the cell lines. Relative expression of the pGL3-OB plasmids in pre-adipocytes was several fold lower than the primary adipocytes (raw luciferase values of ≈5000 versus ≈25,000 in a comparable assay) and the data are presented as relative levels within a given cell type.

RESULTS

Characterization of the Transcription Initiation Site of the Human *ob* Gene. Recent work has characterized the cDNA for the human *ob* gene (22). To unambiguously map the 5′ end of the cDNA, several approaches were undertaken. First, 5′-RACE was performed, which generated four independent clones using two different sources of human adipose tissue RNA as starting material. Three additional independent 5′-RACE products were obtained with adipose tissue cDNA purchased from Clontech. All seven 5′-RACE products contained an identical sequence that extended up to 46 bp 5′ to the ATG codon, whereas a single clone was 2 bp longer (Fig. 1). Next, primer extension experiments were performed by using four independent human adipose tissue RNA samples. Two major extension products of 94 and 91 bp were observed with the pdv34R primer. The longest extension product was 9 nucleotides longer than the longest 5′-RACE clone (Fig. 1). Finally, we mapped the start site of the mRNA by using the S1 nuclease protection assay. This method confirmed the most 5′ start site as identified by primer extension assay (Fig. 1). The relative positions of the transcription initiation sites as determined by the different techniques are in agreement with one other.

Structural Organization of the Human *ob* Gene. A P1 human genomic library was screened by using the human *ob* cDNA. Three positive clones (5135, 5136, and 5137), each spanning >80 kb of genomic sequence hybridizing to the human *ob* cDNA, were obtained. All three clones were next shown to hybridize with oligonucleotides from the 5′ (1F) and 3′ (562R) extremes of the coding sequence. More important, all three P1 clones also hybridize to the oligonucleotide SMFOR derived from 5′-RACE, and hence contain the transcription initiation site. The *ob* gene comprises three exons

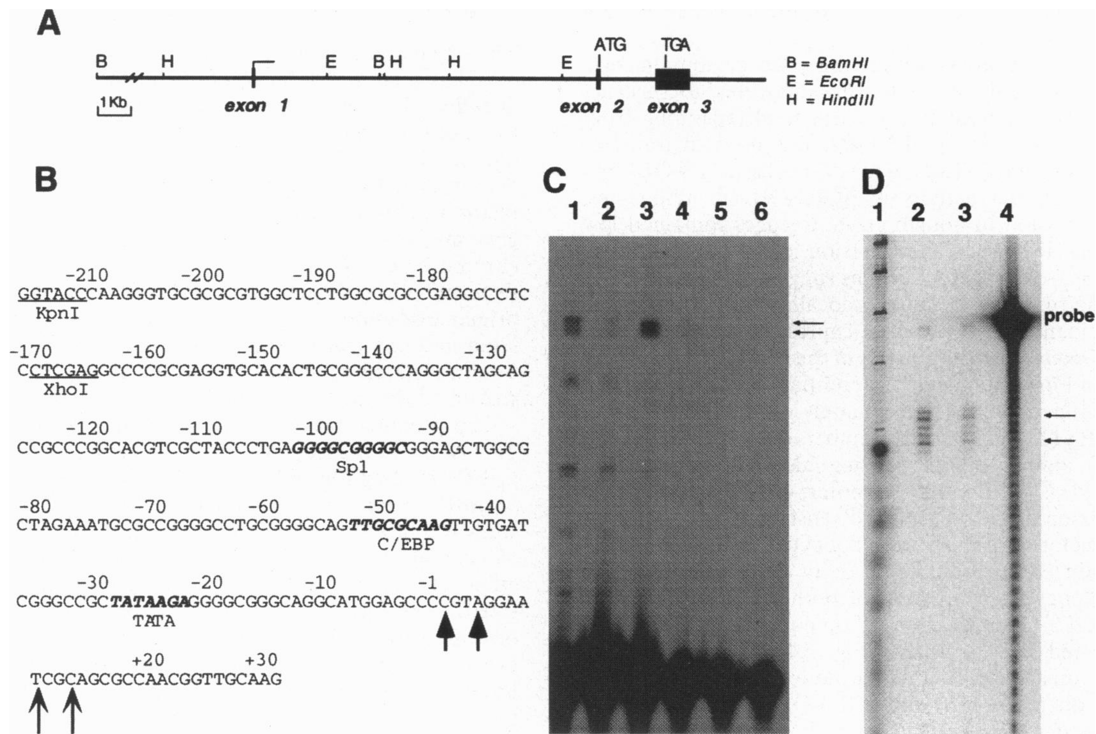

FIG. 1. Determination of transcription initiation site. (*A*) Genomic structure of the human *ob* gene. Three exons with transcription and translation start sites are identified and relevant restriction enzyme sites are indicated. (*B*) DNA sequences of the proximal promoter and exon 1 of the human *ob* gene are shown. Transcription initiation sites, as determined by 5′-RACE (long arrows) and primer extension and S1 nuclease protection (short arrows), are indicated. (*C*) Primer extension. Human adipose tissue RNA from four different subjects (lanes 1–4) as well as a human liver RNA sample (negative control, lane 5) were used in primer extension. Lane 6 is the primer by itself. The major extension products are indicated by arrows. (*D*) S1 nuclease analysis. Molecular weight standards (lane 1) and the S1 probe (lane 4) bracket two different human adipose RNA samples (lanes 2 and 3) with the 5′ end of the mRNA identified by arrows.

distributed over ≈15 kb of genomic DNA. The entire coding sequence is contained in exons 2 and 3, which are separated by a 2-kb intron. The first exon, comprising only the 5′-untranslated sequence, is 30 bp (located 10.5 kb upstream of the second exon), which contains the initiator ATG (Fig. 1).

Tissue-Specific Determinants of the Human *ob* Promoter. Previous studies suggested that the expression of the *ob* mRNA is specific for adipose tissue and strongly regulated in rodents (9–14). We subcloned the region 5′ to the transcription initiation site and sequenced the proximal promoter (Fig. 1*B*). Thirty-one basepairs upstream of the transcription initiation site an AT-rich sequence is observed, which might serve as a TATA box (TATAAGA; Fig. 1*B*). The sequence immediately upstream of the transcription initation site is extremely GC-rich, including several consensus Sp1 binding sites, which implicates Sp1 in the expression of this gene. Whether these factors are important for the regulation of adipocyte-specific *ob* gene expression awaits further study. Sequence analysis identified a CAAT-like consensus C/EBP protein binding site at position −45 (TTGCGCAAG; Fig. 1*B*).

In experiments designed to evaluate the tissue specificity of the *ob* promoter, a DNA fragment extending from about −3 kb to +30 bp relative to the transcription initiation site was inserted into the pGL3-Basic luciferase vector (Promega) to generate pGL3-OB1 (Fig. 2*A*). This vector was then transfected into primary rat adipocytes, mouse 3T3-L1, CV-1, and COS cells. Transfection efficiency of the various cell lines was monitored by evaluation of the activity of control vectors. Relative to the promoterless parent vector, the human *ob* promoter fragment stimulated luciferase expression up to 15-fold in primary rat adipocytes. In the 3T3-L1 cells maintained under nondifferentiating conditions, luciferase expression was 10- to 15-fold higher in the pGL3-OB1-transfected cells relative to the pGL3-Basic vector. In CV-1 cells, the same *ob* promoter fragment induced luciferase expression <2.5-fold (Fig. 2*B*). Similar results were obtained with COS cells (data not shown). These results are consistent with the observation that *ob* mRNA expression is primarily observed in adipocytes and preadipocytes and suggest that the sequences necessary for

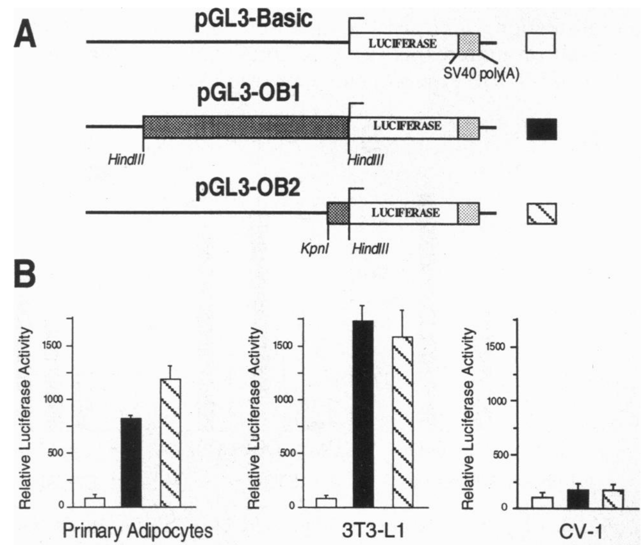

FIG. 2. Tissue-specific activity of the *ob* promoter. (*A*) Schematic representation of the different reporter constructs (pGL3-Basic, pGL3-OB1, and pGL3-OB2) used in transfection assays. Transfections were performed as described. (*B*) Luciferase activity of the pGL3-OB1 construct (solid bars) and pGL3-OB2 (hatched bars) after transfection in rat primary adipocytes, 3T3-L1 and CV-1 cells. Data (mean ± SD) are normalized to the promoterless pGL3-Basic control vector (open bars). Each experiment was performed at least three times in triplicate.

adipocyte lineage-specific expression of the *ob* gene are contained within these 3 kb.

To further define areas within this 3-kb region that are important for *ob* gene expression in adipocytes, a construct containing a 247-bp *Hin*dIII/*Kpn*I fragment (spanning from positions −217 to +30), pGL3-OB2, was used to transfect primary rat adipocytes, 3T3-L1 and CV-1 cells. pGL3-OB2 had comparable promoter activity to pGL3-OB1 in adipocytes, while the expression in nonadipocyte lineages remained low (Fig. 2*B*). The difference in expression level between adipocytes and cells from different origins suggests the existence of tissue-specific regulatory elements localized to the proximal 217 bp as evidenced by the robust expression of pGL3-OB2.

C/EBPα Determines the Activity of the Human *ob* Promoter *in Vivo* and *in Vitro*. Adipocyte differentiation has been shown to be determined by the coordinately acting transcription factors PPARγ (23) and various members of the C/EBP family (21, 23, 24) among others. Having identified a potential binding site for C/EBP in the promoter, we examined its role in the expression of the *ob* gene. Cotransfection of a C/EBPα expression vector in primary rat adipocytes and 3T3-L1 preadipocytes induced *ob* promoter activity significantly. In primary rat adipocytes, expression of both the 3-kb promoter construct, pGL3-OB1, and the 217-bp construct, pGL3-OB2, were stimulated 2.5- to 4-fold (Fig. 3*A*). In 3T3-L1 cells, C/EBPα cotransfection stimulated the expression of the two reporter vectors pGL3-OB1 and pGL3-OB2 by about 2.5-fold (Fig. 3*B*). No significant effect was seen on the promoterless control. The upregulation of the pGL3-OB1 reporter vector in primary rat adipocytes by C/EBPα was concentration-dependent (Fig. 3*C*). The fact that stimulation was seen with both the pGL3-OB1 and pGL3-OB2 constructs indicated that the consensus sequence contained within the 217 bp adjacent to the transcription initiation site was likely to be responsible for the increased luciferase expression. To further test this hypothesis, we mutated the consensus C/EBP site at nucleotide positions −53 to −45 from TTGCGCAAG to TTAAAAAAG (mutant nucleotides underlined) in the pGL3-OB1 vector. When the mutant C/EBP construct pGL3-KOB1 was introduced into primary rat adipocytes, basal luciferase expression was reduced by more than 30% and the 2-fold stimulation of the wild-type promoter construct seen upon cotransfection with C/EBPα was absent, demonstrating that in the 3-kb promoter, this site was functional in mediating the effect of C/EBPα on *ob* gene expression (Fig. 4).

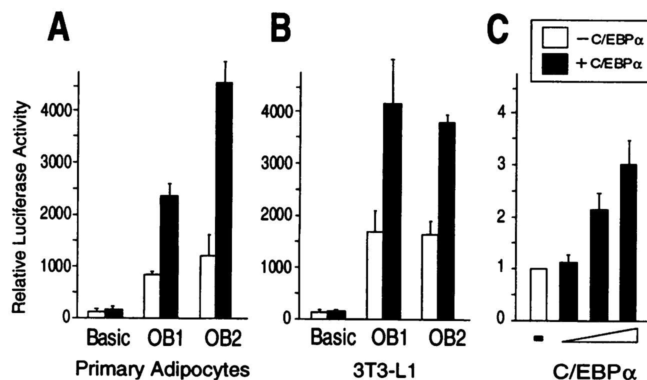

FIG. 3. The *ob* promoter is regulated by C/EBPα levels. (*A*) Cotransfection of 8 μg C/EBPα (solid bars) induces *ob* promoter activity of both the pGL3-OB1 and pGL3-OB2 constructs in primary rat adipocytes. Open bars are without cotransfected C/EBPα. (*B*) Cotransfection of C/EBPα (2 μg) induces *ob* promoter activity of both the pGLB3-OB1 and pGL3-OB2 constructs in 3T3-L1 preadipocytes. (*C*) The luciferase activity of the pGL3-OB1 reporter vector is induced by C/EBPα cotransfection in a dose-dependent fashion in primary rat adipocytes. The amounts of C/EBPα expression vector cotransfected were 0 (-), 2, 4, and 8 μg.

DISCUSSION

The association of murine obesity with mutations in the *ob* gene has generated intense interest in molecular studies aimed at delineating factors potentially involved in human obesity. In this study, we determined the structure of the human *ob* gene, identified potential regulatory elements in the promoter and demonstrated the role of an important adipocyte transcription factor, C/EBP in the expression of the *ob* gene. The human *ob* gene covers ≈15 kb of genomic DNA, previously mapped to chromosome 7p32 (22, 25), and consists of three exons. Determination of the transcription initiation site by 5′-RACE, primer extension and S1 nuclease protection are well correlated and confirm that the first small exon, containing only 5′-untranslated sequence, is located more than 10.5 kb upstream of the initiator codon.

The proximal promoter of the human *ob* gene contains a TATA-like sequence, GC-rich Sp1-like sequences, as well as a C/EBP binding site and possible PPAR binding sites (PPREs). *ob* mRNA expression is restricted to the adipocyte. In this study we have demonstrated that as little as 217 bp of human *ob* gene upstream sequences are sufficient to drive high level, adipocyte-specific expression observed for the *ob* gene. The C/EBP site located within the proximal promoter was demonstrated to be functional and important for high level expression of the *ob* gene in preadipocytes and adipocytes. Indeed, mutation of the C/EBP site abolished the induction of the *ob* gene observed upon cotransfection of C/EBPα as well as modestly decreasing the basal level of expression.

The observation that *ob* gene expression is subject to transcriptional control by C/EBPα is consistent with the important role of C/EBPα in adipocyte differentiation. Arguments in support of a role of C/EBPα in adipogenesis comes from the temporal activation of C/EBPα expression just before the coordinate expression of a group of adipocyte genes (20), the capacity of antisense C/EBPα RNA to inhibit adipocyte differentiation (26), and the fact that premature induction or overexpression of C/EBPα triggers adipocyte differentiation (24, 27). The important role of C/EBPα in adipogenesis was confirmed in mice in which the C/EBPα gene was disrupted by homologous recombination because the mutant mice failed to accumulate lipid in adipose tissue (28). In addition to C/EBPα, C/EBPβ and δ are suggested to play an important transient role in early adipocyte differentiation by relaying the effects of hormonal stimulants such as glucocorticoids, insulin, and stimulators of the cAMP signaling pathways (29, 30). In fact, it was recently demonstrated that C/EBPβ induces PPARγ expression in the preadipocyte,

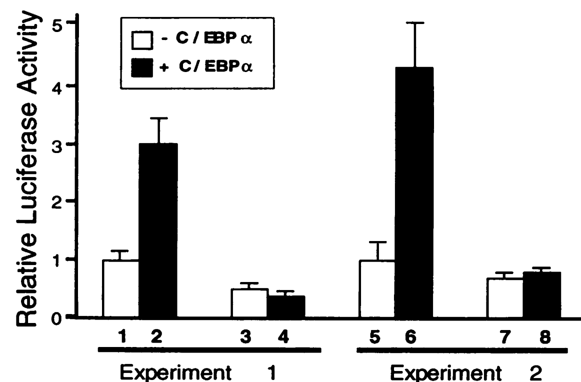

FIG. 4. C/EBP binding site mutant abolishes C/EBP stimulation of *ob* reporter constructs in primary rat adipocytes. Analysis of transfected wild-type pGL3-OB1 luciferase reporter versus the C/EBP mutant pGL3-KOB1 ± cotransfected C/EBPα. Results are presented as the mean of three replicates in two identical experiments for the wild-type plasmid lanes 1, 2, 5, and 6 or the C/EBP mutant in lanes 3, 4, 7, and 8.

subsequently triggering differentiation (31). Because C/EBPα enhances the expression of the satiety factor leptin, one can hypothesize that this will ultimately lead to a limitation in caloric uptake. This might have an important feedback regulatory function to block excessive energy storage in adipocytes and consequently limit excessive adipogenesis. The expression of the *ob* gene and its promoter is not only influenced by adipocyte transcription factors such as C/EBPα, but appears also to be under hormonal control. The cloning of the human *ob* promoter provides us with the necessary tools to analyze the mechanisms underlying such hormonal control and the relationship to satiety.

In conclusion, the structure of the human *ob* gene was determined and we have demonstrated physiologically relevant mechanisms by which the *ob* gene expression may be influenced, particularly by modulation of C/EBP levels or activity. The elucidation of the structure of the human *ob* gene will be useful for studies of mutations in this gene that may predispose to certain forms of obesity. Knowledge of sequence elements and factors regulating *ob* gene expression should furthermore be of major importance in the prevention and treatment of obesity.

Note: After submission of this manuscript, a report identifying C/EBPα sites in the mouse *ob* gene promoter was published (32).

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