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/Ckcc+Daw+ob/ob 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 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 (N₁ = G, A, or C, whereas N = G, A, C, or T): 1F, 5'-ATG CAT TGG GGA ACC CTC TG C TG G-3'; 140R, 5'-TTG CTA TGG TCA TTT-3'; 217R, 5'-GGGGTTTGG- GTTGCACTTTGGAC-3'; 562R, 5'-CCTGCTCAGGGC- CACACCTCTGTCG-3'; anchored-T, 5'-TTTCTAAGATT- CAGGCGCCGC(T)TG17N-3'; pdv34R, 5'-GCCACAGGAATTTCGGCAAGGGTTTCCCCATGC-3'; SMFOR, 5'-CGCAGCAT-

Abbreviation: RACE, rapid amplification of cDNA ends.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U48621).

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GCAAACGGTTGCAAGGC-3'; SMREV2, 5'-CGGGAGAAGCTTGCAATGCGCTGCG-3'; OB/S1, 5'-GCTTCTTTGGGCTTCAAACTGCAGG-3'; MUT1a, 5'-GAGCTTCTTGGGCCTTGCAACCGTTGGCGCTGCG-3'; MUT2a, 5'-TAAAAAAGTTGTGATCG-3'; with a MUTla, of Determination CAACTTAAAAACTGCCCCGCAGG-3'.

MUT2a, in detail. sequencing. amplification positive not performed 5'-RACE amplification cloning TA manufacturer. PCR from nucleotide electrophoresis. labeled reaction virus transcriptase was probe analysis. Tissue Primary rat tissue was obtained from adipose tissue that was used to maintain COS cells CV-1 (13), and COS cells (American Type Culture Collection). Cell Culture, obtained from the American Type Culture Collection (ATCC). For the human ob gene, the construct pdv34R was labeled 32P and used as control probes.

**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 another.

**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' (1R) extremes of the coding sequence. Most importantly, all three P1 clones also hybridized to the oligonucleotide SMFOR derived from 5'-RACE, and hence contain the transcription initiation site. The ob gene comprises three exons
Thirty-One basepairs gene box initiation site untranslated sequence, start translation site specific the second liver RNA human protection (short arrows), over are 2-kb implicated Spl a into kb to primary (TATAAGA; sequence region of the human ob promoter (Promega) inserted the pGL3-OB1 (Fig. 1B). The sequence immediately upstream of the transcription initiation 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 (TTGCACGA; Fig. 1B).

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. 2A). 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. 2B). 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
C/EBPa plays a role in adipocyte differentiation and expression of the ob gene. 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 demonstrated the role 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/EBPa as well as modestly decreasing the basal level of expression.

The observation that ob gene expression is subject to transcriptional control by C/EBPa is consistent with the important role of C/EBPa in adipocyte differentiation. Arguments in support of a role of C/EBPa in adipogenesis comes from the temporal activation of C/EBPa expression just before the coordinate expression of a group of adipocyte genes (20), the capacity of antisense C/EBPa RNA to inhibit adipocyte differentiation (26), and the fact that premature induction or overexpression of C/EBPa triggers adipocyte differentiation (24, 27). The important role of C/EBPa in adipogenesis was confirmed in mice in which the C/EBPa gene was disrupted by homologous recombination because the mutant mice failed to accumulate lipid in adipose tissue (28).

In addition to C/EBPa, 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,
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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