The Mouse obese Gene

GENOMIC ORGANIZATION, PROMOTER ACTIVITY, AND ACTIVATION BY CCAAT/ENHANCER-BINDING PROTEIN α∗

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The obese gene product, leptin, regulates adiposity. Mice homozygous for a nonfunctional obese gene become massively obese and develop diabetes mellitus due to overeating and increased metabolic efficiency. The cDNA sequence of obese was recently reported (Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J. L. (1994) Nature 372, 425-432; Correction: (1995 Nature 374, 479). We have determined the genomic organization of the 5′ end of the mouse obese gene. The coding sequence is found upstream of exon 1. A minority (probably ~5%) of the obese mRNA contained an extra, untranslated exon between exons 1 and 2. Transcription of the obese gene was detected only in adipose cells. A 762-base pair obese gene promoter driving a luciferase gene yielded abundant activity in transiently transfected rat adipose cells in primary culture. The obese promoter was inactive in erythroid K562 cells. Deletion of bases from –762 downstream to –161 did not affect promoter activity in transfected adipose cells. The –161 minimal promoter contained consensus Sp1 and C/EBP enhancer-binding protein (C/EBP) motifs. Cotransfection with C/EBPα (a transcription factor important in adipose cell differentiation) caused 23-fold activation. These data suggest that the obese promoter is a natural target of C/EBPα.

Obesity is common in Western society, and the underlying molecular mechanisms are not well understood. Body weight is almost certainly regulated by a feedback control mechanism (see Ref. 1). Classic experiments with parabiotic rats showed that overeating by one animal caused its mate to starve (2). When animals subjected to forced over- or under-feeding were returned to ad libitum feeding, they adjusted their food intake appropriately to reach the same weight as control animals (3). If some adipose tissue is removed from a growing, chow-fed rat, the remaining adipose tissue enlarges, so that the rat attains the same total amount of body fat as an unoperated control (4). A recent study in humans demonstrated that upon weight gain or loss, the body's energy expenditure increases or decreases, respectively, suggesting an attempt to return to the original state (5). Taken together, these data suggest that individuals have a set point for body weight/adiposity and that a feedback control mechanism maintains the target weight.

In humans, obesity has been shown to have a large genetic component (6). However, little is known about the specific genes that are responsible. Insight into the regulation of obesity has come from the study of the ob/ob mouse (7). These mice grow massively obese and develop diabetes mellitus due to overeating and increased metabolic efficiency. Parabiotic animal experiments suggest that ob/ob animals are unable to make a satiety factor, but can respond to such a factor from a parabiotic mate. Similar experiments suggest that db/db mice make the factor missing in ob/ob mice, but cannot respond to it. Recently, Friedman's laboratory used positional cloning to isolate the obese gene encoding the leptin protein (8). The obese gene is expressed selectively in adipose tissue. Mature, secreted leptin is 146 amino acids long and is not similar in sequence to any known protein. Treatment of ob/ob mice with leptin caused reversal of the obese phenotype (9-11). In addition, leptin treatment caused slight weight loss in wild type mice (9-11).

Little is known about the regulation of the ob gene. The Ob(C57Bl1/ob/C57Bl1) mice have a R105term nonsense mutation and a 20-fold increased obese RNA level (8). This suggests that these mice have an intact mechanism to sense adiposity and to transcribe the ob gene, but do not make functional leptin. Thus, obes is probably subject to regulation at the level of transcription and/or RNA stability, and comprehension of the regulation of obese will increase our knowledge of the adiposity sensor. As a step toward understanding the regulation of the obese gene, we have cloned and sequenced the wild-type obese promoter and characterized its activity in transient expression assays in primary cultures of rat adipose cells.

MATERIALS AND METHODS

Primers—Primer sequences were: x200, 5'-GACTCTGGAGCCCG; x201, 5'-GGTAGCGACCGGCCG; x225, 5'-TTTCTATATGGGATGGTGTG; x226, 5'-TTTGTGACACCCACAACTGCT; x229, 5'-AGGCCCTG-GTGCAATTGCGCC; x230, 5'-TTGTTATAGCCTCGAGGCTGG; x247, 5'-CTCGCTACACGCTGAGCTGAGCG; x248, 5'-CGGAGAAAATGTCGCTGAGGCC; x249, 5'-CTGTTGCTGAATGTCACTTGC; x251, 5'-GAAATTTCAGCACAGGCCCTTGAAC; and x252, 5'-TGATTCTGGAGGAGC.

Cloning—P1 clones were obtained from Genome Systems (St. Louis, MO; done addresses 42 and 233) using PCR† primers x248 and x249, which yield a 141-bp product. Clone 42 was shotgun subcloned using BamHI, EcoRI, or HindIII into pBluescript SK+ (Stratagene) and subclones were selected by hybridization with oligonucleotide probes. A mouse epidydimal fat pad cDNA library (Clontech, ML035b) was

† The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); RSV, Rous sarcoma virus; kb, kilobase pair(s); RT-PCR, reverse transcriptase PCR.
screened to obtain the 5′ end of the obese mRNA. PCR was first carried out (0.5–1 µl of library in 50 µl, using 1.5 mm MgCl₂, 0.4 µm primers, 94 °C, 4 min followed by 25 cycles of 94 °C, 2 min, 61 °C, 1 min, 72 °C, 1 min) with primers for the obese coding region (x251) and the left (x225) or right (x226) arms. Nested PCR on 0.5 µl used primers x249 (obese coding) and x200 (left; ) or x201 (right; ), 30 cycles, and 55 °C annealing, otherwise as above. The PCR products were cloned (PCScript, Stratagene), and the clones containing the longest inserts were sequenced using an Applied Biosystems, Inc. model 373 DNA sequencer with the fluorescent primer or deoxykilo kits (Applied Biosystems). Sequence searches used BLASTN on the NCBI server (12) and FASTA (13).

RT-PCR—Reverse transcription was performed (14) on 1 µg of RNA with avian myeloblastosis virus reverse transcriptase, using x252 as the primer in a volume of 25 µl. PCR reactions (25 µl; 94 °C, 4 min then 24 or 30 cycles of 94 °C 1 min, 64 °C 1 min, and 72 °C 1 min) contained 1 µl of cDNA, 20 µM dNTPs, 2.5 µCi of [α-32P]CTP, 0.4 µM primers, 1.5 mm MgCl₂, and 1 unit of Taq polymerase. PCR primers x229/x249 generated a 270-bp product from the alternate exon/exon 2 splicing. PCR primers x247/x249 were predicted to yield a 195-bp band for exon 1/exon 2 splicing and a 288-bp band for exon 1/alternate exon/exon 2 splicing. PCR primers x247/x230 generated a 111-bp product from exon 1/alternate exon/exon 2 splicing. Product specificity was confirmed by digestion with restriction enzymes (exon 1/exon 2: FokI, 149; 46; BsaI, 55; 10; and AvalI, 90; 105; alternate exon/exon 2: FokI, 224; 46; BsaI, 130; 140; and AvalI, 165; 105; and exon 1/alternate exon, Rsal, 61; 50; Sfani, 74; 37).

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RESULTS AND DISCUSSION

Genomic Organization of obese—As a first step toward understanding the transcriptional regulation of the obese gene, we define its genomic organization. Using the reported coding sequence, a genomic P1 clone was obtained, mapped, subcloned, and partially sequenced (Fig. 1). The 5′ end of the mRNA was isolated using nested PCR on a mouse epididymal fat pad cdNA library (see “Materials and Methods”). Compar-

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contained 5, 9, 12, 20, and 21 bp of the 3' end of exon 1. The first exon was located −7.5 kb upstream of the 175-bp exon 2. Intron 2 was at least 2.5 kb in size; its 3' end was not mapped.

Evidence for Alternate Splicing—One clone of the 5' end of the mRNA had the last five bases of the first exon, followed by 93 bp of unrelated sequence and the second exon. These data suggested the existence of an alternate exon. This was confirmed by identification of the 93-bp sequence, flanked by splice acceptor and donor sites, in genomic DNA; 4 kb downstream of the first exon.

To further characterize the obese mRNAs, primer extension using an exon 2 primer was performed (Fig. 2A). In RNA from ob/ob adipose tissue, a single strong band was observed at −201 nucleotides, corresponding to a first exon size of −26 bp. A weak band (−20-fold less signal, but clearly visible on the original) was observed at −290 nucleotides, the size expected for mRNAs containing the alternate exon.

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Reverse transcriptase, although we cannot formally rule out another minor RNA specie.

We used a more sensitive RT-PCR assay for further characterization of the splicing products. RT-PCR with an exon 1/exon 2 primer set yielded the 195-bp product expected for direct splicing of exons 1 and 2 (Fig. 2B, center panel). Under the conditions used, the 288-bp product expected from RNAs containing the alternate exon was not observed. However, PCR reactions specific for splicing of exon 1 to the alternate exon and of the alternate exon to exon 2 detected the expected 111- and 270-bp products, respectively (Fig. 2B, left and right panels). Both types of obese mRNA were detected only in adipose tissue, and not in the brain, testes, liver, kidney, or adrenal samples. While the PCR assay was not strictly quantitative, the alternate exon products appeared less abundant, requiring six extra cycles to be amplified to a level comparable to the product without this exon. Thus, the alternative exon is also limited to adipose tissue and is present in a minor fraction of the obese mRNAs.

The alternate exon is the second example of alternate splicing in the obese gene, the first being the variable inclusion of the first exon (Fig. 3). Since the alternate exon is not contained in ATG, inclusion of this exon does not

**Fig. 3. Sequence of the mouse obese gene.** Genomic sequence surrounding the first exon is at the top, numbered relative to the start of transcription. Putative Sp1, C/EBP, and TATA sequences are underlined and so labeled. Below are the alternate exon and flanking sequence and exons 2 and 3. Exonic bases are uppercase, coding bases are italicized, and intronic bases are lowercase. The position and orientation of primers are indicated above the sequence. Restriction sites used in constructing the reporter plasmids are double underlined. GenBank accession numbers for exon 1 and the alternate exon are U36238 and U36239. The exon 2 and 3 sequences are from Ref. 8 (GenBank accession number U18812). Our intron 2 sequence agrees with GenBank accession number U22421.
The DNA sequence of the promoter is shown in Fig. 3. With the exception of a simple sequence repeat upstream of the promoter, none of the new DNA sequence showed statistically significant similarity to those already in GenBank. As predicted by the single strong start site, the promoter contained a TATA motif at −29 to −34. A match to the Sp1 consensus sequence (GGGCGG; Ref. 19) occurs at −95 to −100. A number of transcription factors of the Sp1-like zinc-finger family may bind to this motif. Between −49 and −58 is a short palindrome that is predicted to bind C/EBP (20).

Promoter Function in Adipose Cells and Erythroid Cells—Promoter function was tested using transient expression in primary rat adipose cells that express obese mRNA (data not shown; Ref. 21). The p(−762)ob-luc construct, containing 762 bp of promoter sequence produced reporter activity 100-fold greater than the assay background (Table I). In contrast, the p(−762rev)ob-luc plasmid, with the promoter region in the inverted orientation, showed only background activity, the same as the promoterless p(+)2ob-luc and pGL2-basic constructs. Induction of the RSV enhancer in the p(−762)ob-luc reporter plasmid increased transcription 15-fold, demonstrating that the obese promoter can interact with this enhancer.

The obese gene mRNA has been detected only in adipose tissues. We examined the tissue specificity of the obese promoter using transient expression in the erythroid K562 cell line (22). To allow comparison of the adipose cell and K562 data, the results were expressed relative to pCIS-luciferase, a highly expressed plasmid that is active in both types of cells (Table I). In K562 cells, the promoterless reporter plasmids expressed luciferase at −0.01% of the level of pCIS-luciferase. Addition of the obese promoter did not increase luciferase above this background. The level of expression of p(−762)ob-luc, after normalization to pCIS-luciferase, was 162-fold greater in adipose cells than in K562 cells. Thus, there is sufficient information contained in the 762-bp promoter to allow expression in adipose cells, but not in K562 cells.

To map its functional regions, various lengths of the obese promoter were tested for their ability to drive the expression of a luciferase reporter gene (Fig. 4A). Deletion of regions upstream of −161 did not reduce the promoter activity. We conclude that the region up to −161 functions as a promoter in adipose cells and that addition of another 600 bp of upstream DNA did not increase the promoter activity.

Transactivation by C/EBPα—The putative C/EBPα binding site in the proximal promoter is intriguing since C/EBPα promotes adipocyte differentiation (23, 24) and transactivates the promoters of many adipose-specific genes (25–27). Coexpression of a CMV-driven C/EBPα expression plasmid caused a 23-fold increase in obese reporter expression, with no change in expression of the cotransfected RSV-cat plasmid (Fig. 4B). These data demonstrate that C/EBPα can activate the promoter of the obese gene. Point mutations causing loss of C/EBP transactivation are needed for definitive identification of the C/EBP-responsive cis-element(s).

A number of adipose and hepatic genes are known to be regulated in response to hormones and metabolic state (28–30). For example, insulin and/or high glucose stimulate transcription of the genes for glyceraldehyde-3-phosphate dehydrogenase (31), fatty acid synthase (32), S14 (33), stearoyl-CoA desaturase I (34), and pyruvate kinase and decreases the transcription of the phosphoenolpyruvate carboxykinase (35). Recent evidence suggests that obese people have slightly elevated obese mRNA levels (36). The transcription factor binding motifs in the obese promoter, such as the putative C/EBP site, may play a role in mediating this regulation.
Given the postulated role of the obese gene as an adiposity sensor, transcription of the obese gene may be sensitive to lipid status. For example, polyunsaturated fatty acids decrease transcription of hepatic pyruvate kinase (37) and fatty acid synthase (38). With varying precision and certainty, candidate cis regulatory elements and their cognate trans factors have been identified as mediators of these events. Two different direct mechanisms are known to mediate the transcriptional response to intracellular hydrophobic ligands. The sterol regulatory element-binding proteins remain membrane-bound under sterol-replete conditions but are proteolytically cleaved to release an active transcription factor under low sterol conditions (39). Ligand binding to steroid hormone superfamily receptors causes the receptors to bind DNA and activate transcription. In particular, peroxisome proliferator-activated receptor γ is known to increase transcription in response to linoleic acid, oleic acid, and thiazolidinediones (40, 41) and has been proposed to regulate obese (40). The mouse obese promoter does not contain sequences that are identical to the reported sterol regulatory element or peroxisome proliferator-activated receptor regulatory element. However, obese may be controlled indirectly by these factors, or directly via distant elements and/or elements that do not conform to the classical sequence motifs. Elucidation of the regulatory mechanisms controlling expression of the obese gene and of the promoter elements that confer adipose specific expression will be important for understanding the regulation of body fat in the normal state and the pathogenesis of obesity.

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