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(54) **Title:** An Obesity (Ob) Polypeptide Capable Of Modulating Body Weight, Nucleic Acids Therefor, And Diagnostic And Therapeutic Use Thereof

(57) **Abstract:** The present invention relates generally to the control of body weight of animals including mammals and humans, and more particularly to materials identified herein as modulators of weight, and to the diagnostic and therapeutic uses to which such modulators may be put. In its broadest aspect, the present invention relates to the elucidation and discovery of nucleotide sequences, and proteins putatively expressed by such nucleotides or degenerate variations thereof, that demonstrate the ability to participate in the control of mammalian body weight. The nucleotide sequences in object represent the genes corresponding to the murine and human *OB* gene, that have been postulated to play a critical role in the regulation of body weight and adiposity. Preliminary data, presented herein, suggests that the polypeptide product of the gene in question functions as a hormone. The present invention further provides nucleic acid molecules for use as molecular probes, or as primers for polymerase chain reaction (PCR) amplification, i.e., synthetic or natural oligonucleotides. In further aspects, the present invention provides a cloning vector, which comprises the nucleic acids of the invention; and a bacterial, insect, or a mammalian expression vector, which comprises the nucleic acid molecules of the invention, operatively associated with an expression control sequence. Accordingly, the invention further relates to a bacterial or a mammalian cell transfected or transformed with an appropriate expression vector, and correspondingly, to the use of the above-mentioned constructs in the preparation of the modulators of the invention. Also provided are antibodies to the OB polypeptide. Moreover, a method for modulating body weight of a mammal is provided. In specific examples, genes encoding two isoforms of both the murine and human OB polypeptides are provided.

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MODULATORS OF BODY WEIGHT, CORRESPONDING NUCLEIC ACIDS AND PROTEINS, AND DIAGNOSTIC AND THERAPEUTIC USES THEREOF

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to the control of body weight of mammals including animals and humans, and more particularly to materials identified herein as modulators of weight, and to the diagnostic and therapeutic uses to which such modulators may be put.

BACKGROUND OF THE INVENTION

Obesity, defined as an excess of body fat relative to lean body mass, is associated with important psychological and medical morbidities, the latter including hypertension, elevated blood lipids, and Type II or non-insulin-dependent diabetes melitis (NIDDM). There are 6-10 million individuals with NIDDM in the U.S., including 18% of the population of 65 years of age [Harris *et al.*, *Int. J. Obes.*, 11:275-283 (1987)]. Approximately 45% of males and 70% of females with NIDDM are obese, and their diabetes is substantially improved or eliminated by weight reduction [Harris, *Diabetes Care*, 14(3):639-648 (1991)]. As described below, both obesity and NIDDM are strongly heritable, though the predisposing genes have not been identified. The molecular genetic basis of these metabolically related disorders is an important, poorly understood problem.

The assimilation, storage, and utilization of nutrient energy constitute a complex homeostatic system central to survival of metazoa. Among land-dwelling mammals, storage in adipose tissue of large quantities of metabolic fuel as triglycerides is crucial for surviving periods of food deprivation. The need to maintain a fixed level of energy stores without continual alterations in the size and shape of the organism requires the achievement of a balance between energy intake and expenditure. However, the molecular mechanisms that regulate energy balance remain to be elucidated. The isolation of molecules that transduce nutritional information and

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control energy balance will be critical to an understanding of the regulation of body weight in health and disease.

An individual's level of adiposity is, to a large extent, genetically determined. Examination of the concordance rates of body weight and adiposity amongst mono- and dizygous twins or adoptees and their biological parents have suggested that the heritability of obesity (0.4-0.8) exceeds that of many other traits commonly thought to have a substantial genetic component, such as schizophrenia, alcoholism, and atherosclerosis [Stunkard *et al.*, *N. Engl. J. Med.*, 322:1483-1487 (1990)]. Familial similarities in rates of energy expenditure have also been reported [Bogardus *et al.*, *Diabetes*, 35:1-5 (1986)]. Genetic analysis in geographically delimited populations has suggested that a relatively small number of genes may account for the 30-50% of variance in body composition [Moll *et al.*, *Am. J. Hum. Genet.*, 49:1243-1255 (1991)]. However, none of the genes responsible for obesity in the general population have been genetically mapped to a definite chromosomal location.

Rodent models of obesity include seven apparently single-gene mutations. The most intensively studied mouse obesity mutations are the *ob* (obese) and *db* (diabetes) genes. When present on the same genetic strain background, *ob* and *db* result in indistinguishable metabolic and behavioral phenotypes, suggesting that these genes may function in the same physiologic pathway [Coleman *et al.*, *Diabetologia*, 14:141-148 (1978)]. Mice homozygous for either mutation are hyperphagic and hypometabolic, leading to an obese phenotype that is notable at one month of age. The weight of these animals tends to stabilize at 60-70 g (compared with 30-35 g in control mice). *ob* and *db* animals manifest a myriad of other hormonal and metabolic changes that have made it difficult to identify the primary defect attributable to the mutation [Bray *et al.*, *Am. J. Clin. Nutr.*, 50:891-902 (1989)].

Each of the rodent obesity models is accompanied by alterations in carbohydrate metabolism resembling those in Type II diabetes in man. In some cases, the severity of the diabetes depends in part on the background mouse strain [Leiter,

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Endocrinology, 124:912-922 (1989)]. For both *ob* and *db*, congenic C57BL/Ks mice develop a severe diabetes with ultimate β cell necrosis and islet atrophy, resulting in a relative insulinopenia. Conversely, congenic C57BL/6J *ob* and *db* mice develop a transient insulin-resistant diabetes that is eventually compensated by β cell hypertrophy resembling human Type II diabetes.

The phenotype of *ob* and *db* mice resembles human obesity in ways other than the development of diabetes - the mutant mice eat more and expend less energy than do lean controls (as do obese humans). This phenotype is also quite similar to that seen in animals with lesions of the ventromedial hypothalamus, which suggests that both mutations may interfere with the ability to properly integrate or respond to nutritional information within the central nervous system. Support for this hypothesis comes from the results of parabiosis experiments [Coleman, *Diabetologia*, 9:294-298 (1973)] that suggest *ob* mice are deficient in a circulating satiety factor and that *db* mice are resistant to the effects of the ob factor (possibly due to an ob receptor defect). These experiments have led to the conclusion that obesity in these mutant mice may result from different defects in an afferent loop and/or integrative center of the postulated feedback mechanism that controls body composition.

Using molecular and classical genetic markers, the *ob* and *db* genes have been mapped to proximal chromosome 6 and midchromosome 4, respectively [Bahary *et al.*, *Proc. Nat. Acad. Sci. USA*, 87:8642-8646 (1990); Friedman *et al.*, *Genomics*, 11:1054-1062 (1991)]. In both cases, the mutations map to regions of the mouse genome that are syntenic with human, suggesting that, if there are human homologs of *ob* and *db*, they are likely to map, respectively, to human chromosomes 7q and 1p. Defects in the *db* gene may result in obesity in other mammalian species: in genetic crosses between Zucker *fa/fa* rats and Brown Norway +/+ rats, the *fa* mutation (rat chromosome 5) is flanked by the same loci that flank *db* in mouse [Truett *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:7806-7809 (1991)].

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Because of the myriad factors that seem to impact body weight, it has not been possible to predict which factors and, more particularly, which homeostatic mechanisms, are primarily determinative of body weight. Thus, the principal problem underlying the present invention is to provide modulators of body weight which allow the control of adiposity and fat content of mammals.

SUMMARY OF THE INVENTION

According to the present invention the problem of control of adiposity and fat content of animals, particularly mammals, has been solved through the provision of obesity (OB) polypeptides and nucleic acid molecules coding for these polypeptides as disclosed herein. The present invention provides, for the first time, isolated polypeptides useful for modulation, *i.e.*, control and regulation, of body weight and adiposity as well as nucleic acid sequences encoding such polypeptides which not only allow for recombinant production of the OB polypeptides but are themselves useful in modulation of body weight.

Obesity (OB) polypeptides of the present invention have about 145 to about 167 amino acids, are capable of modulating body weight in an animal, particularly a mammal, and include allelic variants or analogs, including fragments, thereof having the same biological activity. The polypeptides can be prepared by recombinant or chemical synthetic methods. Presently preferred OB polypeptides include those having the amino acid sequence of SEQ ID NOS: 2, 4, 5 or 6, or allelic variants or analogs, including fragments, thereof.

Immunogenic fragments of OB polypeptides of the invention include: Val-Pro-Ile-Gln-Lys-Val-Gln-Asp-Asp-Thr-Lys-Thr-Leu-Ile-Lys-Thr (SEQ ID NO: 18); Leu-His-Pro-Ile-Leu-Ser-Leu-Ser-Lys-Met-Asp-Gln-Thr-Leu-Ala (SEQ ID NO: 19); Ser-Lys-Ser-Cys-Ser-Leu-Pro-Gln-Thr-Ser-Gly-Leu-Gln-Lys-Pro-Glu-Ser-Leu-Asp (SEQ ID NO: 20); and Ser-Arg-Leu-Gln-Gly-Ser-Leu-Gln-Asp-Ile-Leu-Gln-Gln-Leu-Asp-Val-Ser-Pro-Glu-Cys (SEQ ID NO: 21).

Human OB polypeptide analogs include those having the human amino acid sequences of SEQ ID NOS: 4 and 6, wherein one or more of amino acids 53, 56, 71, 85, 89, 92, 95, 98, 110, 118, 121, 122, 126, 127, 128, 129, 132, 139, 157, 159, 163, and 166 (according to the numbering of SEQ ID NO: 4) is substituted with another amino acid such as the divergent amino acid of the mouse OB polypeptide as set out in SEQ ID NO: 2, or an alanine. Such analogs also include those wherein: (a) the serine residue at position 53 is substituted with glycine, alanine, valine, cysteine, methionine, or threonine; (b) the serine residue at position 98 is substituted with glycine, alanine, valine, cysteine, methionine, or threonine; and (c) the arginine residue at position number 92 is substituted with asparagine, lysine, histidine, glutamine, glutamic acid, aspartic acid, serine, threonine, methionine, or cysteine. An OB polypeptide analog according to the invention preferably has 83 percent or greater amino acid sequence homology to the human OB polypeptide amino acid sequence set out in SEQ ID NO: 2, 4, 5 or 6.

Additional human OB polypeptide analogs according to the invention have the amino acid sequence of SEQ ID NOS: 4 and 6 and have: (a) one or more aspartic acid residues substituted with glutamic acid; (b) one or more isoleucine residues substituted with leucine; (c) one or more glycine or valine residues substituted with alanine; (d) one or more arginine residues substituted with histidine; (e) one or more tyrosine or phenylalanine residues substituted with tryptophan; (f) one or more of residues 121 through 128 (according to the numbering of SEQ ID NO:4) substituted with glycine or alanine; and (g) one or more residues at positions 54 through 60 or 118 through 166 (according to the number of SEQ ID NO: 4) substituted with lysine, glutamic acid, cysteine, or proline.

Presently preferred human OB polypeptide truncated analogs according to the invention include those wherein (according to the numbering of SEQ ID NO: 4): (a) one or more residues at positions 121 to 128 are deleted; (b) residues 1-116 are deleted; (c) residues 1-21 and 54 to 167 are deleted; (d) residues 1-60 and 117 to 167 are deleted; (e) residues 1-60 are deleted; (f) resides 1-53 are deleted; and, (g) an

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analog of subpart (a) wherein residues 1-21 are deleted. OB polypeptides and ob polypeptide analogs of the invention which lack the 21 amino acid "signal" sequence (*e.g.*, amino acids 1 through 21 of SEQ ID NO: 4)can have an N-terminal amino acid or amino acid sequence such as (1) methionine, (2) a glycine-serine-histidine-methionine sequence (SEQ ID NO: 38), (3) a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-histidine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-histidine-methionine sequence (SEQ ID NO: 98), (4) a leucine-glutamic acid-lysine-arginine-glutamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 26), (5) a glutamic acid-alanine-glutamic acid-alanine sequence (SEQ ID NO: 27), (6) a leucine-glutamic acid-lysine-arginine sequence (SEQ ID NO: 28); (7) a methionine-glycine-serine-serine-histidine-histidine-histidine-histidine-histidine-histidine-serine-serine-glycine-leucine-valine-proline-arginine-glycine-serine-proline sequence (SEQ ID NO: 99), and (8) a glycine-serine-proline sequence.

Derivatives of an OB polypeptide according to the invention have one or more chemical moieties attached thereto including water-soluble polymers such as polyethylene glycol. Polyethylene glycol derivatized derivatives can be mono-, di-, tri- or tetrapegylated *e.g.*, N-terminal monopegylated. Preferred N-terminal monopeglyated derivatives of ob polypeptides of the invention include OB polypeptides comprising the amino acid residues 22 through 167 of SEQ ID NO:4 or residues 22 through 166 of SEQ ID NO: 6, optionally having a (pegylated) methionine at position 21.

Isolated nucleic acid molecule provided by the present invention encode an OB polypeptide, allelic variant, or analog, including fragments, as described above. Specifically provided are DNA molecules for use in securing expression of an OB polypeptide having the biological activity of modulating body weight in a mammal, and selected from the group consisting of: (a) the DNA molecules set out in SEQ ID NOS: 1 and 3 or fragments thereof; (b) DNA molecules which hybridize to the DNA molecules defined in (a) or hybridizable fragments thereof; and (c) DNA molecules

that code on expression for the amino acid sequence encoded by any of the foregoing DNA molecules. Illustrative of such molecules is the human genomic DNA molecule of SEQ ID NOS: 22 and 24.

Preferred DNA molecules according to the invention encode a polypeptide having an amino acid sequence as set out in: (a) SEQ ID NO: 2; (b) amino acids 22 through 167 of SEQ ID NO: 2; (c) SEQ ID NO: 4; (d) amino acids 22 through 167 of SEQ ID NO: 4; (e) SEQ ID NO: 5; (f) amino acids 22 through 166 of SEQ ID NO: 5; and (g) SEQ ID NO: 6; and (h) amino acid 22 through 166 of SEQ ID NO: 6, as well as polypeptides which have an N-terminal amino acid or amino acid sequence as previously noted. Illustratively, a preferred DNA molecule has the sequence set out as the protein coding sequence of SEQ ID NO: 3 and particularly has the sequence set out as the sequence encoding amino acids 22 through 167.

Detectably labeled nucleic acid molecules hybridizable to a DNA molecule of the invention are also provided and include nucleic acid molecules hybridizable to a non-coding region of an OB nucleic acid, which non-coding region is selected from the group consisting of an intron, a 5´ non-coding region, and a 3´ non-coding region. The present invention also provides oligonucleotide primers for amplifying human genomic DNA encoding an ob polypeptide such as oligonucleotides set out in SEQ ID NOS: 29 through 32.

Vectors provided by the invention comprise a DNA molecule according to the invention as described above and preferably have the form of an expression vector which comprises the DNA molecule to operatively associated with an expression control sequence. Unicellular host cells of the invention are transformed or transfected with a DNA molecules of the invention or with a vector as described above. Preferred host cells include bacteria, yeast, mammalian cells, plant cells, insect cells, and human cells in tissue culture. Illustratively, such host cells are selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, yeast, CHO, R1.1, B-W, L-M, COS 1. COS 7, BSC1, BSC40, BMT10, and Sf9

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cells. Presently preferred yeast hosts include *Saccharomyces, Pichia, Candida, Hansenula* and *Torulopsis*. Also provided are mammalian cells containing an ob polypeptide encoding DNA sequence and modified *in vitro* to permit higher expression of ob polypeptide by means of a homologous recombinational event consisting of inserting an expression regulatory sequence in functional proximity to the ob polypeptide encoding sequence. The expression regulatory sequence can be an ob polypeptide expression or not and can replace a mutant ob polypeptide regulatory sequence in the cell.

The present invention provides methods for preparing an ob polypeptide comprising: (a) culturing a cell as described above under conditions that provide for expression of the ob polypeptide; and (b) recovering the expressed ob polypeptide. This procedure can also be accompanied by the steps of: (c) chromatographing the polypeptide on a Ni-chelation column; and (d) purifying the polypeptide by gel filtration. In a preferred embodiment, after step (c) and before step (d), the method includes chromatographing the ob polypeptide on a strong cation exchanger column.

The present invention also provides labeled and unlabeled monoclonal and polyclonal antibodies specific for ob polypeptides of the invention and immortal cell lines that produce a monoclonal antibody of the invention. Antibody preparation according to the invention involves: (a) conjugating an ob polypeptide to a carrier protein; (b) immunizing a host animal with the OB polypeptide fragment-carrier protein conjugate of step (a) admixed with an adjuvant; and (c) obtaining antibody from the immunized host animal.

The invention provides methods for measuring the presence of an OB polypeptide in a sample, comprising: (a) contacting a sample suspected of containing an OB polypeptide with an antibody (preferably bound to a solid support) that specifically binds to the OB polypeptide under conditions which allow for the formation of reaction complexes comprising the antibody and the OB polypeptide; and (b) detecting

the formation of reaction complexes comprising the antibody and ob polypeptide in the sample, wherein detection of the formation of reaction complexes indicates the presence of OB polypeptide in the sample. Correspondingly provided are *in vitro* methods for evaluating the level of OB polypeptide in a biological sample comprising: (a) detecting the formation of reaction complexes in a biological sample according to the method noted above; and (b) evaluating the amount of reaction complexes formed, which amount of reaction complexes corresponds to the level of OB polypeptide in the biological sample. When detecting or diagnosing the presence of a disease associated with elevated or decreased levels of OB polypeptide according to the invention, an evaluation as above is made and the level detected is compared to a level of OB polypeptide present in normal subjects or in the subject at an earlier time. An increase in the level of OB polypeptide as compared to normal or prior levels indicates a disease associated with elevated levels of OB polypeptide and a decreased level of OB polypeptide as compared to normal levels indicates a disease associated with decreased levels of OB polypeptide. Correspondingly provided are *in vitro* methods for monitoring therapeutic treatment of a disease associated with elevated or decreased levels of OB polypeptide in a mammalian subject comprising evaluating, as describe above, the levels of OB polypeptide in a series of biological samples obtained at different time points from a mammalian subject undergoing such therapeutic treatment.

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Pharmaceutical compositions according to the invention comprise an OB polypeptide as described above together with a pharmaceutically acceptable carrier and are useful in therapeutic methods for reducing the body weight of an animal. Additional pharmaceutical compositions of the invention for use in therapeutic methods for increasing the body weight of an animal comprise an antagonist of an OB polypeptide, preferably selected from the group consisting of an antibody that binds to and neutralizes the activity of the OB polypeptide, a fragment of the ob polypeptide that binds to but does not activate the OB polypeptide receptor, and a small molecule antagonist of the OB polypeptide. The present invention also provides corresponding body appearance improving cosmetic compositions for reducing or increasing the

body weight of an individual, which compositions are useful in cosmetic processes for improving the body appearance of an individual. Such cosmetic compositions are administered to the individual in a dose amount sufficient to modulate the individual's body weight to a desired level.

Also addressed by the present invention is the use of nucleic acid moles of the invention, as well as antisense nucleic acid molecules hybridizable to a nucleic acid encoding an OB polypeptide according to the invention, for manufacture of a medicament for (*e.g.*, gene therapy) modification body weight of an animal. Also provided is the use of an OB polypeptide or antagonist according to the invention for the manufacture of a medicament for modification of the body weight of an animal. Medicaments so developed can be employed for modification of the body weight of a mammal in treating a disorder selected from the group consisting of diabetes, high blood pressure and high cholesterol and as part of combinative therapy with a medicament for treating such disorders. Such medicaments can be employed in therapeutic methods involving intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, nasal, oral or pulmonary delivery systems.

Data presented herein show that the establish that the OB polypeptides of the invention in their form are secreted primarily from mammalian adipocytes and that the polypeptides function as hormones.

The Examples herein demonstrate that the OB polypeptide, alternatively termed herein "leptin," circulates in mouse, rat, and human plasma. Leptin is absent in plasma from *ob/ob* mice, and is present at ten-fold higher concentrations in plasma from *db/db* mice, and twenty-fold higher concentrations in *fa/fa* rats. Most significantly, daily injections of recombinant leptin dramatically reduces the body mass of *ob/ob* mice, significantly affects the body weight of wild-type mice, and has no effect on *db/db* mice.

In a further aspect, the ob polypeptide from one species is biologically active in another species. In particular, the human OB polypeptide is active in mice.

In a first instance, the modulators of the present invention comprise nucleic acid molecules, including recombinant DNA molecules (*e.g.*, cDNA or a vector containing the cDNA or isolated genomic DNA) or cloned genes (*i.e.*, isolated genomic DNA), or degenerate variants thereof, which encode polypeptides themselves serving as modulators of weight control as hereinafter defined, or conserved variants or fragments thereof, particularly such fragments lacking the signal peptide (alternatively referred to herein as mature OB polypeptide), which polypeptides possess amino acid sequences such as set forth in FIGURE 1A through E (SEQ ID NO:2), FIGURE 3 (SEQ ID NO:4), FIGURE 5 (SEQ ID NO:5) and FIGURE 6 (SEQ ID NO:6). In specific embodiments, amino acid sequences for two variants of murine and human ob polypeptides are provided. Both polypeptides are found in a form with glutamine 49 deleted, which may result from an mRNA splicing anomaly. The OB polypeptides from various species may be highly homologous; as shown in Figure 4, murine and human OB polypeptides are greater than 80% homologous.

The nucleic acid molecules, recombinant DNA molecules, or cloned genes, may have the nucleotide sequences or may be complementary to DNA coding sequences shown in FIGURE 1A through E (SEQ ID NO:1) and FIGURE 2A and B (SEQ ID NO:3). In particular, such DNA molecules can be cDNA or genomic DNA isolated from the chromosome. Nucleic acid molecules of the invention may also correspond to 5′ and 3′ flanking sequences of the DNA and intronic DNA sequences. Accordingly, the present invention also relates to the identification of a nucleic acid having a nucleotide sequence selected from the sequences of Figure 1A through E (SEQ ID NO:1) and Figure 2A and B (SEQ ID NO:3) herein, and degenerate variants, allelic variations, and like cognate molecules.

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A nucleic acid molecule of the invention can be DNA or RNA, including synthetic variants thereof having phosphate or phosphate analog, *e.g.*, thiophosphate, bonds. Both single-stranded and double-stranded sequences are contemplated herein.

The present invention further provides nucleic acid molecules for use as molecular probes, or as primers for polymerase chain reaction (PCR) amplification, *i.e.*, synthetic or natural oligonucleotides having a sequence corresponding to a portion of the sequences shown in Figure 1A through E (SEQ ID NO:1), Figure 2A and B (SEQ ID NO:3) and Figure 20A through C (SEQ ID NOs:22 and 24); or the 5′ and 3′ flanking sequences of the coding sequences; or intronic sequences of the genomic DNA. In particular, the invention contemplates a nucleic acid molecule having at least about 10 nucleotides, wherein a sequence of the nucleic acid molecule corresponds to a nucleotide sequence of the same number of nucleotides in the nucleotide sequences of Figure 1A through E (SEQ ID NO:1), Figure 2A and B (SEQ ID NO:3) and Figure 20A through C (SEQ ID NO:22), or a sequence complementary thereto. More preferably, the nucleic acid sequence of the molecule has at least 15 nucleotides. Most preferably, the nucleic acid sequence has at least 20 nucleotides. In an embodiment of the invention in which the oligonucleotide is a probe, the oligonucleotide is detectably labeled, *e.g.*, with a radionuclide (such as ^{32}P), or an enzyme.

In further aspects, the present invention provides a cloning vector, which comprises the nucleic acids of the invention that encode the ob polypeptide; and a bacterial, insect, or a mammalian expression vector, which comprises the nucleic acid molecules of the invention encoding the ob polypeptide, operatively associated with an expression control sequence. Accordingly, the invention further relates to a host cell, such as a bacterial cell, yeast cell, insect cell, or a mammalian cell, transfected or transformed with an appropriate expression vector, and correspondingly, to the use of the above mentioned constructs in the preparation of the modulators of the invention.

In yet a further aspect, the present invention relates to antibodies that bind to the ob polypeptide. Such antibodies may be generated against the full-length polypeptide, or antigenic fragments thereof. In one aspect, such antibodies inhibit the functional (*i.e.*, body weight and fat composition modulating) activity of the ob polypeptide. In another aspect, antibodies can be used to determine the level of circulating ob polypeptide in plasma or serum. In yet a further aspect, region-specific antibodies, particularly monoclonal antibodies, can be used as probes of OB polypeptide structure.

All of the foregoing materials are to be considered herein as modulators of body weight and fat composition, and as such, may be used in a variety of contexts. Specifically, the invention contemplates both diagnostic and therapeutic applications, as well as certain agricultural applications, all contingent upon the use of the modulators defined herein, including both nucleic acid molecules and peptides. Moreover, the modulation of body weight carries specific therapeutic implications and benefits, in that conditions where either obesity or, conversely, cachexia represent undesired bodily conditions, can be remedied by the administration of one or more of the modulators of the present invention.

Thus, a method for modulating body weight of a mammal is proposed that comprises controlling the expression of the protein encoded by a nucleic acid having a nucleotide sequence selected from the sequence of Figure 1A through E (SEQ ID NO:1), the sequence of Figure 2A and B (SEQ ID NO:3) and degenerate and allelic variants thereof. Such control may be effected by the introduction of the nucleotides in question by gene therapy into fat cells of the patient or host to control or reduce obesity. Conversely, the preparation and administration of antagonists to the nucleotides, such as anti-sense molecules, would be indicated and pursued in the instance where conditions involving excessive weight loss, such as anorexia nervosa, cancer, or AIDS are present and under treatment. Such constructs would be introduced in a similar fashion to the nucleotides, directly into fat cells to effect such changes.

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Correspondingly, the proteins defined by Figures 1A through E, 3, 5, and 6 (SEQ ID NO:1, SEQ ID NO:4, SEQ ID NO:5, and SEQ ID NO:6), conserved variants, active fragments thereof, and cognate small molecules could be formulated for direct administration for therapeutic purposes, to effect reduction or control of excessive body fat or weight gain. Correspondingly, antibodies and other antagonists to the stated protein materials, such as fragments thereof, could be prepared and similarly administered to achieve the converse effect. Accordingly, the invention is advantageously directed to a pharmaceutical composition comprising an OB polypeptide of the invention, or alternatively an antagonist thereof, in an admixture with a pharmaceutically acceptable carrier or excipient.

In addition, the OB polypeptide of the invention may be administered for its cosmetic effects, *e.g.*, to improve body appearance by reducing fat deposits. The OB polypeptide can be used independently or in conjunction with other cosmetic strategies, *e.g.*, surgery, for its cosmetic effects.

The diagnostic uses of the present nucleotides and corresponding peptides extend to the use of the nucleic acids to identify further mutations of allelic variations thereof, so as to develop a repertoire of active nucleotide materials useful in both diagnostic and therapeutic applications. In particular, both homozygous and heterozygous mutations of the nucleotides in question could be identified that would be postulated to more precisely quantitate the condition of patients, to determine the at-risk potential of individuals with regard to obesity. Specifically, heterozygous mutations are presently viewed as associated with mild to moderate obesity, while homozygous mutations would be associated with a more pronounced and severe obese condition. Corresponding DNA testing could then be conducted utilizing the aforementioned ascertained materials as benchmarks, to facilitate an accurate long term prognosis for particular tendencies, so as to be able to prescribe changes in either dietary or other personal habits, or direct therapeutic intervention, to avert such conditions.

The diagnostic utility of the present invention extends to methods for measuring the presence and extent of the modulators of the invention in cellular samples or biological extracts (or samples) taken from test subjects, so that both the nucleic acids (genomic DNA or mRNA) and/or the levels of protein in such test samples could be ascertained. Given that the increased activity of the nucleotide and presence of the resulting protein reflect the capability of the subject to inhibit obesity, the physician reviewing such results in an obese subject would determine that a factor other than dysfunction with respect to the presence and activity of the nucleotides of the present invention is a cause of the obese condition. Conversely, depressed levels of the nucleotide and/or the expressed protein would suggest that such levels must be increased to treat such obese condition, and an appropriate therapeutic regimen could then be implemented.

Further, the nucleotides discovered and presented in Figures 1A through E and 2A and B represent cDNA which, as stated briefly above, is useful in the measurement of corresponding RNA. Likewise, recombinant protein material corresponding to the polypeptides of Figures 1A through E and 3 may be prepared and appropriately labeled, for use, for example, in radioimmunoassays, for example, for the purpose of measuring fat and/or plasma levels of the OB protein, or for detecting the presence and level of a receptor for OB on tissues, such as the hypothalamus.

Yet further, the present invention contemplates not only the identification of the nucleotides and corresponding proteins presented herein, but the elucidation of the receptor to such materials. In such context, the polypeptides of Figures 1A through E, 3, 5, and/or 6 could be prepared and utilized to screen an appropriate expression library to isolate active receptors. The receptor could thereafter be cloned, and the receptor alone or in conjunction with the ligand could thereafter be utilized to screen for small molecules that may possess like activity to the modulators herein.

Yet further, the present invention relates to pharmaceutical compositions that include certain of the modulators hereof, preferably the polypeptides whose sequences are

presented in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5 and SEQ ID NO:6, their antibodies, corresponding small molecule agonists or antagonists thereof, or active fragments prepared in formulations for a variety of modes of administration, where such therapy is appropriate. Such formulations would include pharmaceutically acceptable carriers, or other adjuvants as needed, and would be prepared in effective dosage ranges to be determined by the clinician or the physician in each instance.

Accordingly, it is a principal object of the present invention to provide modulators of body weight as defined herein in purified form, that exhibit certain characteristics and activities associated with control and variation of adiposity and fat content of mammals.

It is a further object of the present invention to provide methods for the detection and measurement of the modulators of weight control as set forth herein, as a means of the effective diagnosis and monitoring of pathological conditions wherein the variation in level of such modulators is or may be a characterizing feature.

It is a still further object of the present invention to provide a method and associated assay system for the screening of substances, such as drugs, agents and the like, that are potentially effective to either mimic or inhibit the activity of the modulators of the invention in mammals.

It is a still further object of the present invention to provide a method for the treatment of mammals to control body weight and fat content in mammals, and/or to treat certain of the pathological conditions of which abnormal depression or elevation of body weight is a characterizing feature.

It is a still further object of the present invention to prepare genetic constructs for use in genetic therapeutic protocols and/or pharmaceutical compositions for comparable therapeutic methods, which comprise or are based upon one or more of the

modulators, binding partners, or agents that may control their production, or that may mimic or antagonize their activities.

Other objects and advantages will become apparent to those skilled in the art from a review of the ensuing description which proceeds with reference to the following illustrative drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 (A through E) depicts the nucleic acid sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) derived for the murine *OB* cDNA. A 39 base pair 5′ leader was followed by a predicted 167 amino acid open reading frame and an approximately 3.7 kb 3′ untranslated sequence. (In previously filed application Serial No. 08/347,563 filed November 30, 1994 and Serial No. 08/438,431, filed May 10, 1995, an additional 58-base 5′ non-coding sequence was determined subsequently, to be a cloning artifact. This artifact has no bearing on the coding region, the 39 base 5′ non-coding region presently depicted in FIGURE 1, or 3′ non-coding region of the gene.) A total of about 2500 base pairs of the 3′ untranslated sequence is shown. Analysis of the predicted protein sequence by observation and using the *SigSeq* computer program indicates the presence of a signal sequence (underlined). Microheterogeneity of the cDNA was noted in that approximately 70% of the cDNAs had a glutamine codon at codon 49 and 30% did not (see FIGURES 5 and 6, *infra*). This amino acid is underlined, as is the arginine codon that is mutated in C57BL/6J *ob/ob* mice (1J mice).

FIGURE 2 (A and B) depicts the nucleic acid sequence (SEQ ID NO:3) derived for the human *OB* cDNA. The nucleotides are numbered from 1 to 701 with a start site at nucleotide 46 and a termination at nucleotide 550.

FIGURE 3 depicts the full deduced amino acid sequence (SEQ ID NO:4) derived for the human *OB* gene corresponding to the nucleic acid sequence of FIGURE 2A and

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B. The amino acids are numbered from 1 to 167. A signal sequence cleavage site is located after amino acid 21 (Ala) so that the mature protein extends from amino acid 22 (Val) to amino acid 167 (Cys).

FIGURE 4 depicts the comparison between the murine (SEQ ID NO:2) and human (SEQ ID NO:4) deduced amino acid sequences. The sequence of the human *OB* deduced amino acid sequence was highly homologous to that of mouse. Conservative changes are noted by a dash, and non-conservative changes by an asterisk. The variable glutamine codon is underlined, as is the position of the nonsense mutation in C57BL/6J *ob/ob* (1J) mice. Overall, there is 83% identity at the amino acid level, although only eight substitutions were found between the valine at codon 22 (immediately downstream of the signal sequence overage) and the cysteine at position 117.

FIGURE 5 depicts the full length amino acid sequence (SEQ ID NO:5) derived for the murine *OB* gene as shown in FIGURE 3, but lacking glutamine at position 49. The amino acids are numbered from 1 to 166. A signal sequence cleavage site is located after amino acid 21 (Ala) (and thus, before the glutamine 49 deletion) so that the mature protein extends from amino acid 22 (Val) to amino acid 166 (Cys).

FIGURE 6 depicts the full deduced amino acid sequence (SEQ ID NO:6) derived for the human *OB* gene as shown in FIGURE 4, but lacking glutamine at position 49. The amino acids are numbered from 1 to 166. A signal sequence cleavage site is located after amino acid 21 (Ala) (and thus, before the glutamine 49 deletion) so that the mature protein extends from amino acid 22 (Val) to amino acid 166 (Cys).

FIGURE 7. (A) Physical map of the location of *ob* in the murine chromosome, and the YAC and P1 cloning maps. "M and N" corresponds to *Mul*I and *Not*I restriction sites. The numbers correspond to individual animals that were recombinant in the region of *ob* of the 1606 meioses that were scored. Met, Pax 4, D6Rck39, D6Rck13, and Cpa refer to locations in the region of *ob* that bind to the DNA probes. YACs

were isolated using D6Rck13 and Pax-4 as probes, and the ends were recovered using vectorette PCR and/or plasmid end rescue and used in turn to isolate new YACs. (B) The resulting YAC contig. One of the YACs in this contig, Y902A0925, was chimeric. Each of the probes used to genotype the recombinant animals is indicated in parentheses. (6) Corresponds to YAC 107; (5) corresponds to M16(+) (or M16(pLUS)); (4) corresponds to *adu*(+); (3) corresponds to *aad*(pICL); (2) corresponds to 53(pICL); and (1) corresponds to 53(+). (C) The P1 contig of bacteriophage P1 clones isolated with selected YAC end probes. The *ob* gene was isolated in a P1 clone isolated using the distal end of YAC YB6S2F12 (end (4)) (alternatively termed herein *adu*(+)).

FIGURE 8 presents a photograph of an ethidium bromide stain of 192 independent isolates of the fourth exon trapping experiment that were PCR amplified and characterized.

FIGURE 9 is a photograph of an ethidium bromide stain of PCR-amplified clones suspected of carrying *ob*. Each of the 7 clones that did not carry the artifact was reamplified using PCR and electrophoresed on a 1% agarose gel in TBE and stained with ethidium bromide. The size markers (far left unnumbered lane) are the commercially available "1 kB ladder". Lane 1 -- clone 1D12, containing an "HIV sequence." Lane 2 -- clone 1F1, a novel clone outside of the *ob* region. Lane 3 -- clone 1H3. Lane 4 -- clone 2B2, which is the identical to 1F1. Lane 5 -- clone 2G7, which contains an *ob* exon. Lane 6 -- clone 2G11, which is identical to 1F1. Lane 7 -- clone 2H1, which does not contain an insert.

FIGURE 10 presents the sequence of the 2G7 clone (SEQ ID NO:7), which includes an exon coding for a part of the *OB* gene. The primer sequences used to amplify this exon are boxed in the figure (SEQ ID NOS:8 and 9).

FIGURE 11 (A) Reverse transcription-PCR analysis of mRNA from different tissues of the same mouse with the 2G7 primers and actin primers. The RT-PCR

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reactions were performed using 100 ng of total RNA reverse transcribed with oligo dT as a primer for first strand cDNA synthesis. PCR amplification was performed for 35 cycles with 94° denaturation for 1'; 55° hybridization for 1'; and 72°C extensions for 2' with a 1' second autoextension per cycle. RT-PCR products were resolved in a 2% low melting point agarose gel run in 1x TBE buffer. (B) Northern blot of mRNA from different organs of the mouse using PCR labeled 2G7 as a probe. Ten μg of total RNA from each of the tissues was electrophoresed on an agarose gel with formaldehyde. The probe was hybridized at 65°C in Rapid Hybe (Amersham). Autoradiographic signals were apparent after 1 hour of exposure; the experiment shown was the result of a 24 hour exposure.

FIGURE 12 (A) An ethidium bromide stain from an RT-PCR reaction on fat cell (white adipose tissue) RNA from each of the mouse strains listed. Total RNA (100 ng) for each sample was reverse transcribed using oligo dT and reverse transcriptase, and the resulting single-stranded cDNA was PCR amplified with the 2G7 primers (lower bands) or actin primers (upper bands). Both the 2G7 and actin primers were included in the same PCR reaction. The products were run on a 1% agarose TBE gel. (B) Northern analysis corresponding to (A). Ten μg of fat cell (white adipose tissue) RNA from each of the strains indicated were run out and probed with the PCR labeled 2G7 probe as in Figure 11B, above. An approximately 20-fold increase in the level of 2G7 mRNA was apparent in white fat RNA from the C57BL/6J *ob/ob* (1J) strain relative to lean littermates. In both the RT-PCR and Northern experiments there was no detectable signal in 2G7 RNA from the SM/Ckc-+$^{Dac}ob^{2J}/ob^{2J}$ (2J) mice even after a 2 week exposure. A 24 hour autoradiographic exposure is shown. The same filter was hybridized to an actin probe (bottom portion of the panel).

FIGURE 13 is a Northern analysis of additional 2J animals and control animals that confirms the absence of the *ob* mRNA from 2J animals. The Northern analysis was performed as in Figures 11 and 12. In this case, the control RNA was ap2, a fat specific transcript. There is no significance to the varying density of the ap2 bands.

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FIGURE 14 compares the DNA sequence of the C57BL/6J (normal) and the C57BL/6J *ob/ob* (1J) mice in the region of the point mutation that leads to introduction of a premature stop codon (nonsense mutation) in the mutant strain cDNA. The *ob/ob* mice had a C→T mutation that changed an arginine residue at position 105. This base change is shown as the output from the automated DNA sequencer. RT-PCR was performed using white fat RNA from both strains (+/+ and *ob/ob*) using primers from the 5′ and 3′ untranslated regions. The PCR reaction products were gel purified and directly sequenced manually and using an Applied Biosystems, Inc. 373A automated sequencer with primers along both strands of the coding sequence.

FIGURE 15 (A) Southern blot of genomic DNA from each of the mouse strains listed. Approximately 5 μg of DNA (derived from genomic DNA prepared from liver, kidney or spleen) was restriction digested with the restriction enzyme indicated. The DNA was then electrophoresed in a 1% agarose TBE gel and probed with PCR labeled 2G7. Restriction digestion with *Bgl*II revealed an increase in the size of an approximately 9 kB (the largest) *Bgl*II fragment in SM/Ckc-+$^{Dac}ob^{2J}/ob^{2J}$ (2J) DNA. RFLPs were not detectable with any other restriction enzymes. Preliminary restriction mapping of genomic DNA indicated that the polymorphic *Bgl*II site is about 7 kB upstream of the transcription start site. None of the other enzymes tested extend past the mRNA start site. (B) Segregation of a *Bgl*II polymorphism in the SM/Ckc-+$^{Dac}ob^{2J}/ob^{2J}$ strain. Six obese and five lean progeny from the same generation of the coisogenic SM/Ckc-+$^{Dac}ob^{2J}/ob^{2J}$ (2J) colony were genotyped by scoring the *Bgl*II polymorphism as shown in (A). All of the phenotypically obese animals were homozygous for the larger allele of the polymorphic *Bgl*II fragment. The DNA in the "control" lane was prepared from an unrelated SM/Ckc-+^{Dac}+/+ mouse, bred separately from the SM/Ckc-+$^{Dac}ob^{2J}/ob^{2J}$ colony.

FIGURE 16 is a Southern blot of *Eco*RI digested genomic DNA from the species listed, using an *OB* cDNA as a probe (*i.e.*, a zoo blot). Hybridization signals were detectable in every vertebrate sample, even after a moderate stringency hybridization.

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The cat DNA in this experiment was slightly degraded. The restricted DNA was run on a 1% agarose TBE gel, and transferred to an imobilon membrane for probing. The filter was hybridized at 65°C and washed in 2X SSC/0.2% SDS at 65°C twice for twenty minutes and exposed for 3 days using Kodak (Rochester, N.Y.) X-OMAT film.

FIGURE 17 presents the expression cloning region of vector pET-15b (Novagen) (SEQ ID NOS.: 11 and 12).

FIGURE 18 presents analysis of the eluate from a His-binding resin (Ni) column for a recombinant mature murine ob fusion to a His-tag (A) and mature human OB fusion to a His-tag (B). Bacteria were transformed with vectors pETM9 and pETH14, respectively. Upon induction with 1 mM IPTG at optimal conditions, the transformed bacteria were able to produce 100-300 μg/ml of OB fusion protein, primarily in the inclusion bodies. The inclusion bodies were solubilized with 6M guanidine-HCl or urea, and fusion protein (present in the lysis supernatant) was loaded on the His-binding resin (Ni) column in 10 ml of 1x binding buffer with urea. The column was eluted stepwise with 5 ml aliquots of 20 μM, 60 μM, and 300 μM imidazole, and finally with strip buffer. The aliquots were analyzed for the presence of OB polypeptide fusion on a 15% acrylamide gel. Each lane contains the equivalent of 100 μl of bacterial extract.

FIGURE 19 (A) *In vitro* translation of *OB* RNA. A human *OB* cDNA was subcloned into the pGEM vector. The plasmid was linearized and plus strand RNA was synthesized using Sp6 polymerase. The *in vitro* synthesized RNA was translated in the presence or absence of canine pancreatic microsomal membranes. An approximately 18 kD primary translation product was seen after *in vitro* translation. The addition of microsomal membranes to the reaction led to the appearance of a second translation product about 2 kD smaller than the primary translation product. The size of the translation product of interleukin-1α RNA, which lacks an encoded signal sequence, was unchanged by the addition of microsomal membranes. These

data indicated the presence of a functional signal sequence. (B) *In vitro* translation in the presence or absence of proteinase K. Protease treatment resulted in complete proteolysis of the 18 kD primary translation product, while the 16 kD processed form was unaffected. Permeabilization of the microsome with 0.1% TRITON-X100 rendered the processed form protease sensitive. These results indicate that the product had translated into the lumen of the microsome.

FIGURE 20 (A through E) The sequence of the human *OB* gene (SEQ ID NOs:22 and 24). (F) A schematic diagram of the murine *OB* gene. (G) A schematic diagram of the human *OB* gene. In both (F) and (G), the start and stop codons are underlined. There is no evidence of a first intron homologous to the mouse first intron in the human gene, but its existence cannot be excluded.

FIGURE 21 presents a schematic drawing of one of the cloning strategies employed to achieve recombinant expression of *OB* in *Pichia* yeast. (A) Expression vector of *OB* with an α-mating factor signal sequence. (B) Schematic drawing of the structure of the recombinant fusion protein, including the amino acid sequence (SEQ ID NO:26) showing the *Xho*I site and putative KEX-2 and STE-13 cleavage sites, and the N-terminal surplus amino acids present after KEX-2 cleavage (SEQ ID NO:27). (C) An alternative strategy for producing mature OB involves preparing a construct with an amino acid sequence corresponding to a *Xho*I cleavage site and a KEX-2 cleavage site immediately upstream of the mature ob polypeptide sequence (SEQ ID NO:28).

FIGURE 22 Alternative expression strategy in *Pichia*. (A) Expression vector of an *OB* fusion with a His-tag adopted from the pET expression system under control of the α-mating factor signal sequence (SEQ ID NO:33). (B) Schematic drawing of the structure of the recombinant OB fusion protein containing a His-tag, which includes the α-mating factor signal sequence, putative KEX-2 and STE-13 cleavage sites, the His-tag, and a thrombin cleavage site, which would yield OB with three surplus N-terminal amino acid residues.

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FIGURE 23 (A) PAGE analysis of expression of murine *OB* (both the microheterogenous forms, *i.e.*, containing and missing Gln 49) in transformed pichia yeast. The expected band of approximately 16 kD is visible in the transformed yeast culture fluid (second and third lanes), but not in culture fluid from non-transformed yeast (first lane). (B) PAGE analysis of partially purified recombinant OB polypeptide on carboxymethyl cellulose, a weak cation exchanger. A band of about 16 kD is very visible in fractions 3 and 4 from the column, which was eluted with 250 mM NaCl. Lane 1 -- loaded sample; lane 2 -- flow through; lanes 3-5 -- fractions eluted with 250 mM NaCl.

FIGURE 24 shows that the OB protein circulates in mouse plasma. (A) Immunoprecipitations from mouse blood. 0.5 ml of mouse plasma was pre-cleared with unconjugated sepharose and incubated overnight with immunopurified anti-OB antibodies conjugated to sepharose 4B beads. The immunoprecipitate was separated on a 15% SDS-PAGE gel, transferred and Western blotted with an anti-OB antibody. The protein migrated with a molecular weight of approximately 16 kD, to the same position as the mature mouse ob protein expressed in yeast. The protein was absent in plasma from C57BL/6J *ob/ob* mice and increased ten-fold in plasma from C57BLB/Ks *db/db* mice relative to wild type mice. *db* mice have been suggested to overproduce the OB protein, secondary to resistance to its effects. (B) Increased levels of *OB* in fatty rats. The fatty rat is obese as a result of a recessive mutation on rat chromosome 5. Genetic data has suggested a defect in the same gene mutated in *db* mice. Plasma from fatty rats and lean littermates was immunoprecipitated and run on Western blots. A twenty-fold increase in the circulating level of OB is seen in the mutant animals. (C). Quantitation of the OB protein in mouse plasma. Increasing amounts of the recombinant mouse protein were added to 100 λ of plasma from *ob* mice and immunoprecipitated. The signal intensity on Western blots was compared to that from 100 λ of plasma from wild-type mice. A linear increase in signal intensity was seen with increasing amounts of recombinant protein demonstrating that the immunoprecipitations were performed under conditions of antibody excess. Similar signals were seen in the wild-type plasma sample and the

sample with 2 ng of recombinant protein indicating the circulating level in mouse plasma is approximately 20 ng/ml. (D) OB protein in adipose tissue extracts. Cytoplasmic extracts of mouse adipose tissue were prepared from *db* and wild-type mice. Western blots showed increased levels of the 16 kD protein in extracts prepared from *db* mice.

FIGURE 25 shows that the OB protein circulates at variable levels in human plasma. (A) Western blots of human plasma. Plasma samples were obtained from six lean volunteers. Immunoprecipitation and Western blotting revealed the presence of an immunoreactive 16 kD protein, identical in size to a recombinant 146 amino acid human protein expressed in yeast. Variable levels of the protein were seen in each of the six samples. (B) An ELISA (Enzyme Linked Immunoassay) for human ob. Microtiter plates were coated with immunopurified anti-human OB antibodies. Known amounts of recombinant protein were added to the plates and detected using immunopurified biotinylated anti-ob antibodies. Absorbance at 414 nm was plotted against known concentrations of OB to yield a standard curve. The resulting standard curve showed that the assay was capable of detecting 1 ng/ml or more of the human OB protein. (C) Quantitation of the OB protein in human plasma. An ELISA immunoassay was performed using 100 λ of plasma from the six lean volunteers and the standards used in panel B. Levels of the OB protein ranging from 2 ng/ml in HP1 to 15 ng/ml in HP6 were seen. These data correlated with the Western blot data in panel A.

FIGURE 26 shows that the OB protein forms inter- or intramolecular disulphide bonds. (A) Western blots under reducing and non-reducing conditions. The Western blots of mouse and human plasma were repeated with and without the addition of reducing agents to the sample buffer. When ß-mercaptoethanol is omitted from the sample buffer, immunoprecipitates from *db* plasma migrate with an apparent molecular mass of 16 kD and 32 kD. Addition of ß-mercaptoethanol to the buffer leads to the disappearance of the 32 kD moiety (see Figure 24). This result is recapitulated when the mouse protein is expressed in the yeast, *Pichia pastoris*. In

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this case, the mouse OB protein migrates to the position of a dimer. Under reducing conditions the purified recombinant mouse protein migrates with an apparent molecular weight of 16 kD, indicating that the 32 kD molecular form is the result of one or two intermolecular disuphide bonds. The human protein expressed *in vivo* and in *Pichia pastoris* migrates with a molecular mass of 16 kD under both reducing and non-reducing conditions (data not shown). (B) The human protein expressed in yeast contains an intramolecular disulphide bond. Secreted proteins generally assume their correct conformation when expressed in the *Pichia pastoris* expression system. The 146 amino acid mature human protein was expressed in *Pichia pastoris* and purified from the yeast media by a two-step purification protocol involving IMAC and gel filtration. The purified recombinant protein was subjected to mass spectrometry before and after cyanogen bromide cleavage. Cyanogen bromide cleaves at the carboxy terminus of methionine residues. The molecular mass of the recombinant yeast protein was 16,024±3 Da (calculated molecular mass = 16,024 Da). Cyanogen bromide cleaves after the three methionines in the protein sequence at amino acids 75, 89, and 157. The cyanogen bromide fragment with measured mass 8435.6 Da corresponds to amino acids 90-157 and 158-167 joined by a disulphide linkage between cys-117 and cys-167 (calculated molecular mass = 8434.5 Da). N.D. = note detected.

FIGURE 27 depicts the preparation of the bioactive recombinant protein. The nucleotide sequence corresponding to the 145 amino acid mature mouse OB protein was cloned into the pET·15b expression vector. This pET vector inserts a polyhistidine tract (His-tag) upstream of the cloned sequence which allows efficient purification using Immobilized Metal Affinity Chromatography (IMAC). The recombinant bacterial protein initially partitioned in the insoluble membrane fraction after bacterial lysis. The membrane fraction was solubilized using guanidium hydrochloride and loaded onto an IMAC column. The protein was eluted stepwise with increasing concentrations of imidazole as shown. The eluted protein was refolded and treated with thrombin to remove the His-tag, as described below. The final yield of soluble protein was 45 ng/ml of bacterial culture.

FIGURE 28 shows the biologic effects of the OB protein. Time course of food intake (panels A-C) and body weight (panels D-F). Groups of ten animals received either daily intraperitoneal injections of the OB protein at a dose of 5 mg/kg/day (solid squares), daily injections of PBS (solid circles) or no treatment (solid triangles). The treatment groups included C57Bl/6J *ob/ob* mice (panels A and D), C57Bl/Ks *db/db* mice (panels B and E) and CBA/J+/+ mice (panels C and F). The food intake of the mice was measured daily and the body weight was recorded at three to four day intervals as indicated. (The scale of the body weight in grams is different for the wild-type mice vs. the *ob* and *db* mice.) The food intake of the *ob* mice receiving protein was reduced after the first injection and stabilized after the fourth day at a level approximately 40% of that seen in the sham injected group ($p < .001$). The body weight of these animals decreased an average of 1.3 grams/day and stabilized after three weeks to a level approximately 60% of the starting weight ($p < .0001$). No effect of the protein was demonstrable in *db* mice. Small but significant effects on body weight were observed in CBA/J mice at two early time points ($p < .02$). The standard error of each measure is depicted by a bar and the statistical significance of these results is shown in Table 1.

FIGURE 29 shows the results of pair feeding of *ob* mice. (A) A group of four C57Bl/6J *ob/ob* mice were fed an amount of food equal to that consumed by the group of *ob* mice receiving recombinant protein. The weight loss for both groups was calculated after five, eight, and twelve days. The food-restricted mice lost (hatched bar) less weight than the *ob* mice receiving protein (solid bar) ($p < .02$). This result indicates that the weight-reducing effect of the OB protein is the result of effects on both food intake and energy expenditure. (B) Photograph of a treated *ob* mouse. Shown are two C57Bl/6J *ob/ob* mice. The mouse on the left received PBS and weighed 65 grams, which was the starting weight. The mouse on the right received daily injections of the recombinant OB protein. The starting weight of this animal was also 65 grams, and the weight after three weeks of protein treatment was 38 grams. (C) Livers from treated and untreated *ob* mice. Shown are livers from treated and untreated C57Bl/6J *ob/ob* mice. The liver from the mouse receiving PBS

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had the gross appearance of a fatty liver and weighed 5.04 grams. The liver from the mouse receiving the recombinant ob protein had a normal appearance and weighed 2.23 grams.

Figure 30 shows the *in situ* hybridization of *ob* to adipose tissue. Sense and antisense *ob* RNA was labeled *in vitro* using Sp6 and T7 polymerase and digoxigenin. The labeled RNAs were hybridized to paraffin embedded sections of adipose tissue from epididymal fat pads of eight week old C57Bl/Ks mice (labeled wild-type) and C57Bl/Ks *db/db* mice (labeled *db*). In the figure, the lipid droplets appear as unstained vacuoles within cells. The cytoplasm is a thin rim at the periphery of the cells and is indistinguishable from the cell membrane. Hybridization to all the adipocytes in the field was detected in the wild-type sections only using the antisense probe and greatly increased levels were seen in the tissue sections from the *db/db* animals.

FIGURE 31 shows that *OB* RNA is expressed in adipocytes *in vivo* and *in vitro*. Total RNA (10 micrograms) from several different sources was electophoresed on blotted and hybridized to an *ob* probe. Firstly, differences in cell buoyancy after collagenase digestion was used to purify adipocytes. *OB* RNA was present only in the adipocyte fraction. Lane S indicates the stromovascular fraction and A indicates the adipocyte fraction. In addition, *OB* RNA was not expressed in the undifferentiated 3T3-442 preadipocyte cells lane U. Differentiated adipocytes from these cell lines expressed clearly detectable levels of *OB* mRNA (lane D).

FIGURE 32 shows that *OB* RNA is expressed in all adipose tissue depots. All of the adipose tissue depots tested expressed *ob* RNA. The inguinal fat pad expressed somewhat lower RNA levels, although there was variability in the level of signals in different experiments. (Figure 31A) Lanes (1) epididymal (2) inguinal (3) abdominal (4) parametrial fat pads. Brown fat also expressed a low level of *OB* RNA. (Figure 31B) The level of *OB* expression in brown fat was unchanged in animals housed at