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Genetics

Increased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the *db* locus

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ABSTRACT The gene product of the recently cloned mouse obese gene (*ob*) is important in regulating adipose tissue mass. ob RNA is expressed specifically by mouse adipocytes *in vivo* in each of several different fat cell depots, including brown fat. ob RNA is also expressed in cultured 3T3-442A preadipocyte cells that have been induced to differentiate. Mice with lesions of the hypothalamus, as well as mice mutant at the *db* locus, express a 20-fold higher level of ob RNA in adipose tissue. These data suggest that both the *db* gene and the hypothalamus are downstream of the *ob* gene in the pathway that regulates adipose tissue mass and are consistent with previous experiments suggesting that the *db* locus encodes the ob receptor. In *db/db* and lesioned mice, quantitative differences in expression level of ob RNA correlated with adipocyte lipid content. The molecules that regulate expression level of the *ob* gene in adipocytes probably are important in determining body weight, as are the molecules that mediate the effects of ob at its site of action.

The lipostasis theory postulates that the size of the body fat depot is regulated by a feedback loop (1). Body weight is lightly regulated *in vivo*, and the original fat cell mass is precisely reconstituted after lipectomy in adults (2). These findings suggest that the feedback loop operates at a set level in each individual.

The recently cloned mouse obese gene (*ob*) appears to encode a fat cell signal in this feedback loop (3). Adipocytes transplanted from genetically obese C57BJ/6J *ob/ob* mice into wild-type animals (or from wild-type mice into *ob/ob* mice) ultimately achieve the same lipid content as adipocytes from the recipient animal (4, 5). This result suggests that the function of *ob* is not autonomous to fat cells.

If the *ob* gene encodes a signal that acts at a distant site to regulate the overall size of the body's lipid stores, (*i*) this signal should be made in adipocytes, the principal site of lipid storage, (*ii*) this signal should be made in all adipose tissue depots, and (*iii*) a secondary increase in *ob* expression level should be associated with defects downstream of *ob* in the pathway(s) that control adiposity. In this paper, we present data consonant with these predictions.

MATERIALS AND METHODS

***In Situ* Hybridization.** White fat tissues from identical abdominal regions of wild-type (wt) and *db* mice were processed simultaneously according to the modified method described by Richardson *et al.* (6). Briefly, tissues were fixed in Bouins' solution for 2 hr at 4°C. They were then dehydrated by serial treatment of increased ethanol concentrations from 10% to 100%, each for 5 min at 4°C. Further incubation of tissues with xylene (1 hr) and paraffin (2 hr) was done at 65°C. Embedded wt and *db/db* fat tissues were sectioned and mounted on to the same conditions later. Sections were baked at 65°C for 1 hr and treated with xylene and serial dilutions of ethanol from 100 to 50%, each for 3 min at room temperature. Antisense RNA probe of *ob* gene was synthesized by *in vitro* transcription of linearized *ob* gene-coding sequence upstream of a Sp6 RNA polymerase promoter. *In situ* hybridization was done exactly as described by Schaeren and Gerfin–Moser (7).

RNA Preparation and Cell Culture. Total RNA and Northern blots were prepared as described (3). Stromal vascular cells and adipocytes were prepared according to Rodbell (8) and RNA from both fractions was prepared according to Dani *et al.* (9, 10). After subcloning, 3T3-F442 cells were grown in Dulbecco's modified Eagle medium/10% fetal bovine serum (standard medium) (10). At confluence, cells were treated in standard medium supplemented with 2 nM triiodothyronine (T_3) and 17 nM insulin. Twelve days later, RNA was prepared as above.

Gold Thioglucose (GTG) Treatment. One-month-old female CBA/J mice were treated with a single i.p. injection of aurothioglucose (Sigma A0632) at a dose of 2.0 mg/g in normal saline. Control animals were injected with normal saline. Mice were weighed 1 month after treatment. Adipose tissue RNA was isolated from those treated animals whose weight had increased >20 g after GTG treatment. Experimental animals were used in accordance with The Rockefeller University guidelines.

RESULTS

The *ob* gene was recently found to be expressed in adipose tissue (3). As adipose tissue is composed of many cell types including adipocytes, preadipocytes, fibroblasts, and vascular cells, *in situ* hybridization was performed to sections of epididymal fat pads from normal animals with sense and antisense ob RNA (6, 11). When the antisense probe was used, positive signals were detectable in all adipocytes in the section (Fig. 1, labeled wt). Signals were not noted when the antisense probe was hybridized to brain sections (data not shown). Hybridization of the antisense probe to sections of adipose tissue from C57BL/Ks *db/db* mice was greatly increased, confirming the adipocyte-specific expression of ob RNA and demonstrating a large increase in the level of ob RNA per adipocyte in these animals (Fig. 1, labeled db/db). Mice mutant at the *db* locus are massively obese as part of a syndrome that is phenotypically identical to that seen in C57BL/6J *ob/ob* mice (12).

ob RNA was not synthesized by adipose tissue stromal cells separated from adipocytes. As expected, cells in the adipocyte fraction expressed ob RNA using Northern blots (Fig. 2). The same result was obtained using reverse-transcription-PCR (data not shown). These data support the conclusion that only adipocytes express the *ob* gene. Data from cultured adipocytes

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Abbreviations: GTG, gold thioglucose; wt, wild type.
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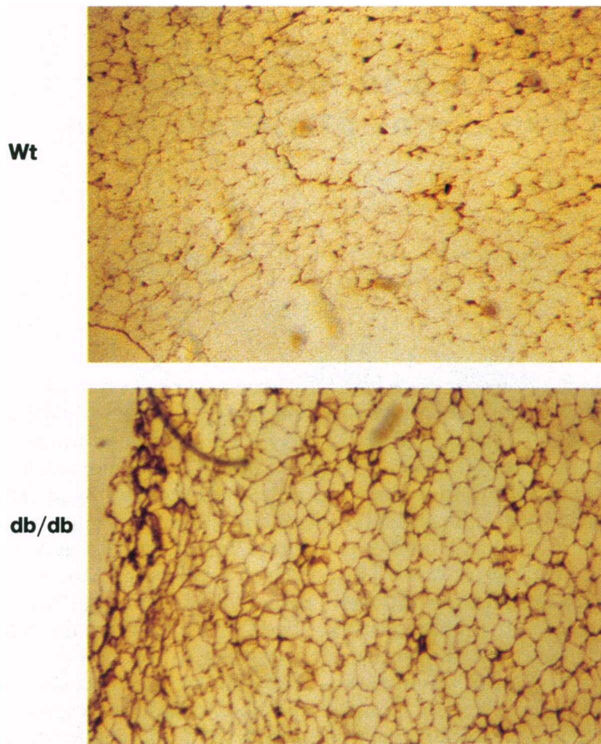

FIG. 1. *In situ* hybridization of ob to adipose tissue. Antisense ob RNA was labeled *in vitro* using Sp6 polymerase and digoxigenin. The labeled RNAs were hybridized to paraffin-embedded sections of adipose tissue from epididymal fat pads of 8-week-old C57BL/Ks mice (labeled wt) and C57BL/Ks *db/db* mice (labeled db). 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 cell membrane. Hybridization to all adipocytes in the field was detected in the wt sections with the antisense probe, and greatly increased levels were seen in tissue sections from the *db/db* animals. (×45.)

confirm this conclusion. In these studies, 3T3-F442A cells were cultured under conditions that lead to lipid accumulation, as part of a cellular program leading to differentiation into adipocytes. ob RNA was expressed neither in exponentially growing cells nor in confluent 3T3-F442A preadipocyte cells that express early markers, whereas differentiation of these cells into adipocytes led to the expression of detectable levels of ob RNA (Fig. 2) (13). The level of ob RNA is extremely sensitive to the culture conditions, as no message was observed in late postconfluent cells not exposed to insulin (unpublished observation).

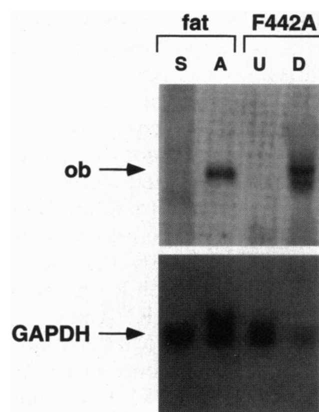

FIG. 2. ob RNA is expressed in adipocytes *in vivo* and *in vitro*. Total RNA (10 μg) from several different sources was electrophoresed on Northern blots and hybridized to an ob probe. Differences in cell buoyancy after collagenase digestion were used to purify adipocytes before preparation of RNA. ob RNA was present in only the adipocyte fraction (lane S, stromovascular fraction; lane A, 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).

Hybridization studies showed that ob RNA is expressed *in vivo* in several different fat depots including that epididymal, parametrial, abdominal, perirenal, and inguinal fat pads (Fig. 3 *Left*). The precise level of expression in each of the depots was somewhat variable, with inguinal and parametrial fat expressing lower levels of ob RNA. ob RNA is also expressed in brown adipose tissue, although the level of expression is ≈50-fold lower in brown fat relative to the other adipose tissue depots. These quantitative differences correlated loosely with reported differences in cell size among the different fat cell depots (14). The amount of ob RNA in brown fat is unaffected by cold exposure (Fig. 3 *Right*). In this experiment, the level of uncoupling protein RNA increased in brown fat after cold exposure, whereas the level of ob RNA did not change (15). In aggregate, these data confirm that all adipocytes are capable of producing ob RNA and demonstrate a variable level of expression in different fat depots. These data support the possibility that the level of the encoded protein correlates with the total adipose tissue mass.

We next measured the levels of ob RNA in *db/db* mice and mice with lesions of the hypothalamus. Lesions of the ventromedial hypothalamus result in obesity as part of a syndrome resembling that seen in *ob/ob* and *db/db* mice (16). Parabiosis experiments suggest such lesions result in over-expression of a blood-borne factor that suppresses food intake and body weight (17). Similar results are noted when mice mutant at the *db* locus are joined by parabiosis to normal mice, suggesting the ob receptor may be encoded by the *db* locus (18). Thus, obesity resulting from ventromedial-hypothalamus lesions and the *db* mutation may be the result of resistance to the effects of the ob protein. If so, a secondary increase in the levels of ob RNA in adipose tissue would be predicted.

Hypothalamic lesions were induced in female CBA mice using the chemical GTG (19). This treatment results in specific hypothalamic lesions, principally in the ventromedial hypothalamus, with the subsequent development of obesity within several weeks (unpublished work). In our experience, a single i.p. injection of GTG of 2 mg/g of body weight results in the development of obesity within 4 weeks. One-month-old female CBA/J mice (20–25 g) were treated with GTG, and the subsequent weight gain of treated and control animals is shown

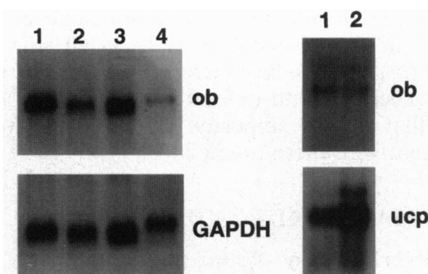

FIG. 3. ob RNA is expressed in all adipose tissue depots. All adipose tissue depots tested expressed ob RNA. The inguinal fat pad expressed somewhat lower RNA levels, although there was variability in the levels of signals in different experiments. (*Left*) Lanes: 1, epididymal; 2, inguinal; 3, abdominal; 4, parametrial fat pads. Brown fat also expressed a low level of ob RNA. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (*Right*) Lanes: 1, brown fat RNA/reverse transcription; 2, brown fat at 4°C. The level of ob expression in brown fat was unchanged in animals housed at 4°C for 1 week, whereas the abundance of the brown fat-specific uncoupling protein RNA, known to be cold-inducible, increased 3-fold.

Table 1. Weight gain in GTG-treated mice

Weight gain, g	Control, no. (%)	GTG-treated, no. (%)
<10	41 (100%)	4 (4%)
10–20	0 (0%)	15 (16%)
>20	0 (0%)	74 (80%)

One-month-old female CBA/J mice were treated with GTG. GTG (Sigma A0632) was administered i.p. in normal saline solution at 2.0 mg/g. Body weight of control (n = 41) and injected (n = 93) animals was recorded before and 1 month after injection. Animals were housed five to a cage and were fed ad libitum. The amount of weight gained 1 month after injection is shown. Animals with a body weight gain >20 g 1 month after injection were selected for further study.

(Table 1). Adipose tissue RNA was prepared from *db/db* mice and from those GTG-treated animals that gained >20 g. Northern blots showed a 20-fold increase in the level of ob RNA in 2-month-old *db/db* and GTG-treated mice compared with normal animals (Fig. 4).

DISCUSSION

The gene product of the mouse *ob* gene circulates in mouse and human plasma, where it may act to regulate the adipose tissue mass (unpublished work). Further studies on the regulation of expression and mechanism of action of *ob* will have important implications for our understanding of the physiologic pathway that regulates body weight.

In this report we show that the ob gene product is expressed exclusively by adipocytes in all adipose tissue depots. This result is consistent with the possibility that the protein product of the *ob* gene correlates with the body's lipid stores. Moreover ob RNA is up-regulated 20-fold in *db* mice and mice with hypothalamic lesions. In these animals, the actual increase in the level of ob RNA per cell is likely to be even higher than 20-fold because the adipocyte cell size is increased ≈5-fold in these animals (see Fig. 1) (14). These data position the *db* gene and the hypothalamus downstream of *ob* in the pathway that controls body weight and is consistent with the hypothesis that the ob receptor is encoded at the *db* locus (18). The molecular cloning of the ob receptor and/or the *db* gene will resolve this issue. The increase in the level of ob RNA in GTG-treated mice also suggests a non-cell-autonomous function of the ob gene product in fat cells (4, 5). Thus, if the encoded protein acted directly on fat cells to inhibit growth or differentiation, the overexpression of the wt *ob* gene in GTG-treated mice would result in a lean phenotype.

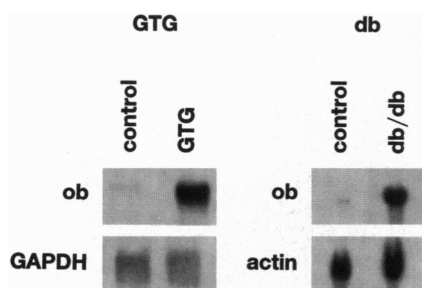

FIG. 4. Expression of ob RNA in *db/db* and GTG-treated mice. Total RNA from the parametrial fat pads of *db/db* and GTG-treated mice was electrophoresed on a Northern blot. GTG administered as a single dose causes obesity by inducing specific hypothalamic lesions. One-month-old CBA female mice were treated with GTG (2.0 mg/g) with a resulting increase of >20 g in treated animals relative to control animals (<5 g). Hybridization of an ob probe in RNA from *db/db* and GTG-treated mice revealed a 20-fold increase in the abundance of ob RNA relative to control RNA [actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)].

The most parsimonious explanation of these data is that the ob protein functions as an endocrine signaling molecule that is secreted by adipocytes and acts, directly or indirectly, on the hypothalamus. Direct effects on the hypothalamus would require that mechanisms exist to allow passage of the ob gene product across the blood–brain barrier. Mechanisms involving the circumventricular organ and/or specific transporters could permit brain access of a molecule the size of that encoded by the *ob* gene (20–22). However, this hypothesis must be considered with caution until the means by which the protein might cross the blood–brain barrier have been identified. Moreover, possible effects on other target organs will need evaluation.

The fat cell signal(s) that are responsible for the quantitative variation in the expression level of the *ob* gene is not yet known but correlates with differences in adipocyte cell size. Adipocytes from *db/db* mice are five times as large as those from normal mice, with a cell size of ≈1.0 μg of lipid per cell (14). Prior evidence has indicated that fat cell lipid content and/or size is an important parameter in determining body weight (23, 24). Conceivably each fat cell could express a low level of ob RNA that further increases in proportion to cell size. It is also possible that cell size is not the sensed parameter and merely correlates with the intracellular signal that increases expression of the *ob* gene in adipocytes from *db/db* and ventrolmedial-hypothalamus-lesioned mice. In any case, the components of the signal-transduction pathway regulating the synthesis of ob RNA are likely to be important in determining body weight. Genetic and environmental influences that reduce the expression level of ob would act to increase body weight, as would influences that decreased sensitivity to the encoded protein. The specific molecules that regulate expression levels of the *ob* gene are as yet unknown and await determination of the level(s) of gene control that leads to quantitative variation in the level of ob RNA and an examination of the regulatory elements of the *ob* gene. Identification of the molecules that regulate expression of the *ob* gene in adipocytes and those that mediate the effects of the encoded protein at its site(s) of action will greatly enhance our understanding of the physiologic mechanisms that regulate body weight.

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