Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9034–9037, September 1995
Biochemistry

Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes

(obesity/protein secretion/*ob* locus/CCAAT/enhancer-binding protein/preadipocyte differentiation)

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Contributed by M. Daniel Lane, June 22, 1995

ABSTRACT **A mutation within the obese gene was recently identified as the genetic basis for obesity in the *ob/ob* mouse. The obese gene product, leptin, is a 16-kDa protein expressed predominantly in adipose tissue. Consistent with leptin's postulated role as an extracellular signaling protein, human embryonic kidney 293 cells transfected with the obese gene secreted leptin with minimal intracellular accumulation. Upon differentiation of 3T3-L1 preadipocytes into adipocytes, the leptin mRNA was expressed concomitant with mRNAs encoding adipocyte marker proteins. A factor(s) present in calf serum markedly activated expression of leptin by fully differentiated 3T3-L1 adipocytes. A 16-hr fast decreased (by ≈85%) the leptin mRNA level of adipose tissue of lean (*ob/+* or *+/+*) mice but had no effect on the ≈4-fold higher level in obese (*ob/ob*) littermates. Since the mutation at the *ob* locus fails to produce the functional protein, yet its cognate mRNA is overproduced, it appears that leptin is necessary for its own downregulation. Leptin mRNA was also suppressed in adipose tissue of rats during a 16-hr fast and was rapidly induced during a 4-hr refeeding period. Insulin deficiency provoked by streptozotocin also markedly down-regulated leptin mRNA and this suppression was rapidly reversed by insulin. These results suggest that insulin may regulate the expression of leptin.**

The *ob/ob* mouse has been widely investigated as a model of genetic obesity (1, 2) since the discovery of the *ob* mutation in 1950 (3). The phenotype of mice that are homozygous for the *ob* mutation is characterized by hyperinsulinemia, hyperglucocorticoidemia, hyperglycemia, insulin resistance, altered central nervous system activity, hyperphagia, reduced metabolic rate of brown adipose tissue, and a massive increase in white adipose tissue (reviewed in refs. 1, 2, and 4). As many of these phenotypic characteristics, including adipocyte hypertrophy and hyperplasia, develop in *ob/ob* mice before the onset of hyperphagia, it is evident that initiation of obesity in this model does not result solely from elevated energy intake. Indeed, obesity occurs even in *ob/ob* mice pair-fed to the level of energy intake of their lean littermates. Parabiosis experiments with *ob/ob* mice indicate that a factor circulating in the bloodstream of lean (*ob/ob* littermates) or *db/db* mice reverses the effects of the *ob* mutation (5, 6). More recently, Friedman's group (7) mapped the *ob* locus, cloned the *ob* gene, and showed that the gene is primarily, if not exclusively, expressed in adipose tissue of normal rodents. The deduced amino acid sequence of the gene product, referred to hereafter as leptin (8), specifies a 16-kDa protein that possesses a leader sequence and presumably is secreted. The apparent secretory nature of leptin is consistent with a large body of evidence linking the dysfunction (or dysregulation) in *ob/ob* mice, including feeding behavior, to the hypothalamus (2, 4). As the effects of mutations at the *ob* locus are pleiotropic, it is possible that leptin also interacts directly with other target tissues. Most importantly, Friedman and colleagues (8) recently demonstrated that injection of purified recombinant leptin dramatically reduces body weight of *ob/ob* and wild-type mice.

In view of the apparent role of leptin in regulating body weight and composition and the fact that adipose tissue is the primary tissue site in which leptin mRNA is produced (7), we investigated factors that regulate its expression by adipocytes both in cell culture and *in vivo*. We show that expression of leptin is induced during differentiation of 3T3-L1 preadipocytes into adipocytes with a time course comparable to that of other adipocyte markers. Moreover, expression of leptin mRNA is regulated in white adipose tissue by changes in physiological state (i.e., fasting/feeding and diabetes) in which the concentration of circulating insulin is altered *in vivo*.

EXPERIMENTAL PROCEDURES

Leptin cDNA and Expression Vector. Based on the published sequence of the leptin cDNA, primer oligodeoxynucleotides (5′-GGAATTCAGGAAAATGTGCTGGAGA-3′ and 5′-GGAATTCTCAGCATTCAGGGCTAAC-3′) were used in a polymerase chain reaction (PCR) to amplify the coding region of the leptin cDNA from reverse-transcribed RNA from 3T3-L1 adipocytes. The PCR product was subcloned into pBluescript (Stratagene). The nucleotide sequence of the insert of several clones was found to be identical to that reported for the leptin cDNA (7). To create a eukaryotic expression vector, the coding region of leptin cDNA was cloned into pcDNAI/AMP (Invitrogen) in the sense (pOb1) and antisense (pOb2) orientations.

Antibodies. A peptide corresponding to amino acids 21–38 (VPIQKVQDDTKTLIKTIV) of leptin was synthesized with an additional carboxyl-terminal cysteine, which was used to crosslink the peptide to keyhole limpet hemocyanin. Immunization of rabbits was performed by HRP (Denver, PA). For immunoprecipitation of leptin, the antibody was crosslinked to protein A-Sepharose with dimethyl pimelimidate (9).

Cell Lysates and Immunoblotting. Cell lysis and immunoblot analysis were performed as described (10, 11) with 15% acrylamide gels. Prestained low molecular weight markers (BRL) were used as standards.

Cell Culture. Mouse 3T3-L1 preadipocytes were maintained and differentiated into adipocytes as described (11). Transfection of the human embryonic kidney cell line 293 was by calcium phosphate coprecipitation (12). Clonal cell lines were obtained by cotransfecting expression vectors (22.5 μg) with pSV2neo (2.5 μg) and selecting cells with G418 at 200 μg/ml.

Isolation and Analyses of RNA. Total RNA was isolated from cells and tissues by either guanidine thiocyanate extraction followed by ultracentrifugation through CsCl (13) or RNA Stat-60 (Tel-Test, Friendswood, TX). RNase protection analysis (14, 15) used antisense probes for leptin (nucleotides +110 to +619) and CCAAT/enhancer-binding protein α (C/EBPα) (nucleotides +229 to +446) mRNA. Results were quantified with a phosphor imager. Northern blot analysis was performed as described (10, 11).

Abbreviation: C/EBPα, CCAAT/enhancer-binding protein α.

Animals. *ob/ob* mice (C57BL/6J) and their lean littermates were obtained from The Jackson Laboratories. Male Sprague–Dawley rats were obtained from Harlan–Sprague–Dawley.

RESULTS AND DISCUSSION

Expression and Secretion of Leptin. The deduced amino acid sequence of leptin includes a putative amino-terminal leader sequence but lacks an internal membrane-spanning domain (7). Thus, leptin appears to be a secretory protein. To ascertain whether leptin is, in fact, secreted, the coding region of mouse leptin cDNA was inserted into the pcDNA1/AMP expression vector in the sense and antisense orientations, and the vectors were transiently transfected into 293 cells. After a 48-hr incubation in serum-containing medium the cell monolayers were shifted to serum-free medium for 16 hr, after which leptin levels in cell lysates and medium were assessed by SDS/PAGE and immunoblot analysis with antibody against leptin (Fig. 1*A*). Leptin was detected in both the cell lysate and the medium from cells transfected with the sense leptin expression vector, but not from control cells or cells transfected with the antisense vector. Consistent with the predicted molecular weight of leptin, the immunoreactive protein exhibited a mobility by SDS/PAGE corresponding to a molecular mass of ≈15 kDa. Secretion of leptin was verified by following the time course of secretion from 293 cells stably transfected with the sense leptin expression vector. Leptin steadily accumulated in the medium over a 10-h period, while that in the cells remained at a relatively low steady-steady level (Fig. 1*B*). Furthermore, treatment of the transfected cells with brefeldin A, an inhibitor of translocation of secretory proteins from the endoplasmic reticulum to the Golgi apparatus (reviewed in ref. 16), blocked secretion of leptin into the medium, leading to massive intracellular accumulation (Fig. 1*C*). Together, these results show that leptin has the characteristics of a secretory protein, which would be required if, as postulated, this molecule functions in transmitting a signal to a distal end-organ such as the hypothalamus.

Expression of Leptin by 3T3-L1 Adipocytes. As leptin mRNA appears to be present only in adipose tissue* (ref. 7 and Fig. 2*A*), it was of interest to determine whether expression of the mRNA was induced during preadipocyte differentiation. Mouse 3T3-L1 preadipocytes were selected as a cell culture model with which to address this question, since the 3T3-L1 system has been shown to faithfully mimic the *in vivo* differentiation process and has been extensively characterized (reviewed in refs. 18 and 19). Quantification of leptin mRNA by RNase protection analysis revealed that fully differentiated 3T3-L1 adipocytes express leptin mRNA whereas preadipocytes do not (Fig. 2*A*). However, the level of expression by 3T3-L1 adipocytes is quite low (≈1%) relative to that in mouse adipose tissue (Fig 2*A*; compare lanes 10–12 with lanes 13–15), raising the possibility that adipocytes in the tissue context have access to humoral or other factors that upregulate expression of leptin mRNA.

During adipose conversion of 3T3-L1 preadipocytes, the leptin mRNA first appears 3 days after the cells are subjected to the differentiation protocol and then increases progressively as the cells acquire the terminally differentiated phenotype (Fig. 2*B*). The kinetics of expression of the leptin mRNA parallels that of the stearoyl-CoA desaturase 1 (SCD1; Fig. 2*B*) and insulin-responsive glucose transporter (GLUT4; ref. 11) mRNAs, which encode adipocyte marker proteins (20, 21), with expression of these mRNAs occurring about 1 day after the appearance of the C/EBPα mRNA. C/EBPα is known to be a pleiotropic transcriptional activator of a number of adipocyte genes, including the SCD1 and GLUT4 genes, during differentiation of 3T3-L1 preadipocytes (22–25). It will be of interest to determine whether C/EBPα is capable of transactivating expression of the leptin promoter, since the kinetics of expression of the C/EBPα and leptin mRNAs are consistent with a transactivation mechanism. The expression of all three mRNAs oscillates on an alternate-day cycle corresponding to the alternate-day pattern of feeding the cells with fresh serum-containing medium.

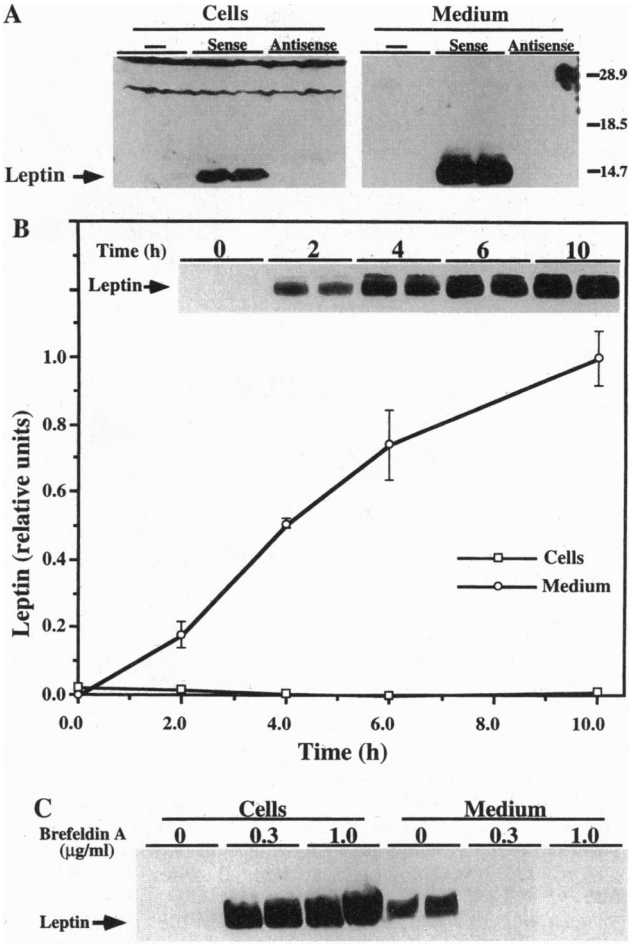

FIG. 1. Expression and secretion of leptin by 293 cells transfected with a leptin expression vector. (*A*) Human embryonic kidney 293 cells were mock transfected or were transiently transfected with sense (pOb1) or antisense (pOb2) leptin expression vectors. After 48 hr in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, cell monolayers were washed once with phosphate-buffered saline and then incubated in serum-free DMEM for an additional 16 hr. Proteins in the medium were precipitated with 12.5% trichloroacetic acid and centrifuged. Pellets were washed with ice-cold acetone and dissolved in 1% SDS/60 mM Tris, pH 6.8. Cell monolayers were lysed in the same buffer. After SDS/PAGE, gels were immunoblotted with anti-leptin antibody. The apparent molecular mass of cellular and secreted leptin was ≈15 kDa. (*B*) Stably pOb1-transfected 293 cells were shifted to serum-free medium (as in *A*), and cell lysates and medium were collected at the indicated times. After SDS/PAGE and immunoblot analysis, results were quantified and the level of intracellular expression normalized to the level in the medium. Immunoblot results for leptin in medium are shown in the *Inset*. (*C*) pOb1-transfected 293 cells (as in *B*) were washed once with phosphate-buffered saline and incubated for 6 hr in medium with brefeldin A at 0, 0.3, or 1.0 μg/ml. Cell lysates and medium were subjected to immunoblot analysis as in *A*.

*The low level of leptin mRNA detected in skeletal muscle is probably due to infiltration of this tissue with adipocytes (17), since C/EBPα mRNA is also detected by RNase protection analysis (results not shown). Leptin appears to be expressed at low levels in brown adipose tissue of mice at 6 days of age.

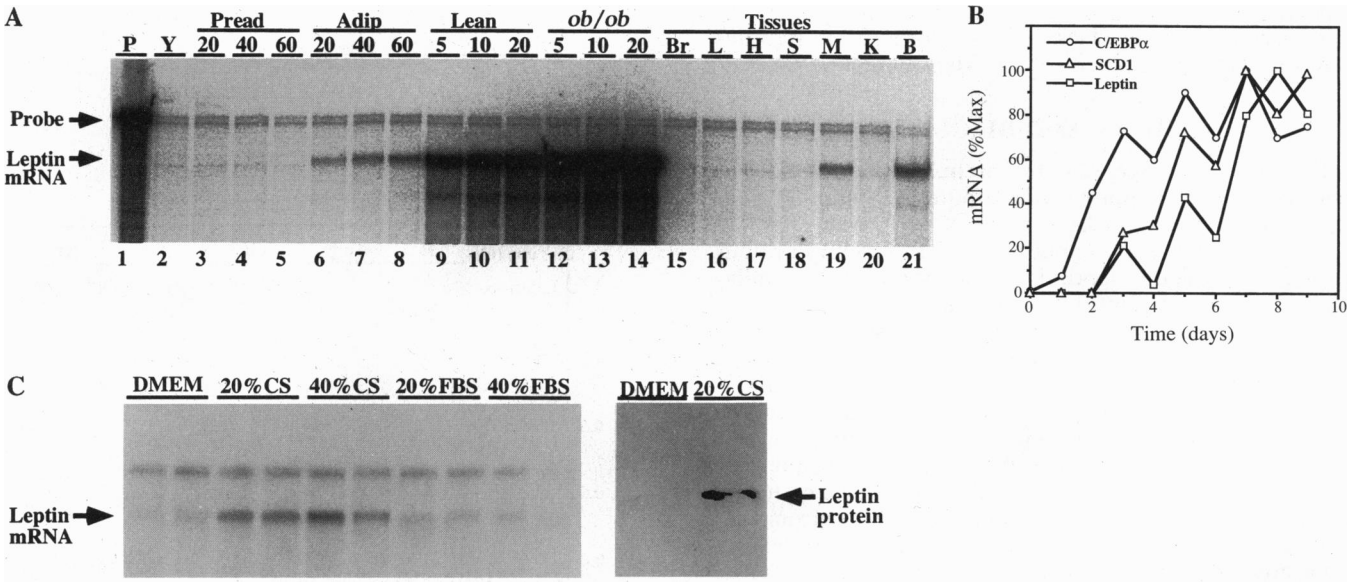

FIG. 2. Expression of leptin mRNA by 3T3-L1 cells and tissues from lean and genetically obese (*ob/ob*) mice. (*A*) ^{32}P-labeled sense and antisense RNA probes corresponding to an ≈500-nucleotide segment of the coding region of the leptin cDNA were generated for RNase protection analysis. Control hybridizations of the sense leptin probe to 20 μg of yeast tRNA or white adipose tissue RNA did not generate a protected fragment (results not shown). The antisense leptin probe was hybridized alone (lane 1) or to 20 μg of yeast tRNA (lane 2); 20, 40, or 60 μg 3T3-L1 preadipocyte RNA (lanes 3–5); 20, 40, or 60 μg of 3T3-L1 adipocyte RNA (lanes 6–8); 5, 10, or 20 μg of lean-littermate mouse adipose tissue RNA (lanes 9–11); 5, 10, or 20 μg of *ob/ob* mouse adipose tissue RNA (lanes 12–14); or 20 μg of lean mouse brain (Br), liver (L), heart (H), spleen (S), skeletal muscle (M), kidney (K), or brown adipose tissue (B) RNA (lanes 15–21) and then digested with RNases A and T_1. (*B*) RNA was isolated from 3T3-L1 cells 2 days after confluence (day 0) and then daily for 9 days after subjection of the cells to the differentiation protocol. RNase protection analysis was used to assess C/EBPα and leptin mRNA levels and Northern analysis to evaluate the SCD1 mRNA level. Data were quantified with a phosphor imager and normalized to the maximal level of expression for each mRNA. (*C*) Fully differentiated 3T3-L1 adipocytes (11 days after induction of differentiation) were fed hourly for 6 hr with DMEM, or DMEM supplemented with 20% calf serum (CS), 40% CS, 20% fetal bovine serum (FBS), or 40% FBS. Two hours after the final feeding, total RNA was isolated and the level of leptin mRNA expression was evaluated by RNase protection analysis. Six 10-cm monolayers of 3T3-L1 adipocytes (day 11) were fed at 2-hr intervals over 6 hr with either DMEM or DMEM supplemented with 20% CS. Sixteen hours later, the medium was harvested and brought to 1% (vol/vol) Nonidet P-40 prior to a 4-hr incubation at 4°C with Sepharose beads crosslinked to leptin antiserum. After the beads were washed five times with 1% Nonidet P-40, proteins were eluted with SDS at 100°C and subjected to SDS/PAGE and immunoblot analysis with anti-leptin antibody.

Since a tissue environment, which allows exposure to factors present in plasma, may be necessary for high-level expression of the leptin mRNA by adipocytes, an attempt was made to supply such factors to 3T3-L1 adipocytes with various types of serum. While serum from rabbits, goats, and fasted or fed rats failed to increase expression of leptin mRNA in 3T3-L1 adipocytes (results not shown), expression was increased 3- to 4-fold by a relatively short exposure (8 hr) to high levels of bovine calf serum (Fig. 2*C*). Fetal bovine serum had a negligible effect. The increased expression of leptin mRNA promoted by calf serum led to a corresponding increase in the expression and secretion of leptin protein (Fig. 2*C*). Further studies are needed to identify and characterize blood-borne and/or tissue factors that activate expression of the leptin gene to a level comparable to the level expressed by adipocytes in a tissue context.

Regulated Expression of the Leptin mRNA *in Vivo*. Given that mutations at the *ob* locus drastically alter energy metabolism and appetite (reviewed in ref. 1), it might be expected that changes in the global energy status of the animal would affect expression of leptin. To determine whether fasting alters expression of the leptin mRNA in "normal" lean and leptin-deficient obese animals, *ob/ob* mice and their lean littermates either were fed a standard laboratory chow diet or were fasted overnight (≈16 hr). Fasting of lean mice suppressed expression of the leptin mRNA in white adipose tissue by ≈85% (Fig. 3*A*). This finding was verified in similar experiments with normal rats in which an overnight fast caused a 70% reduction of leptin mRNA in white adipose tissue (Fig. 3*B*) that was then rapidly reversed by a 4-hr refeeding period. In contrast, the level of leptin mRNA in adipose tissue of *ob/ob* mice, which was ≈4-fold higher than in lean fed mice, was unaffected by fasting (Fig. 3). While a longer fast may have suppressed the expression of leptin mRNA in *ob/ob* mice, the overexpression of leptin mRNA and its failure to downregulate after a 16-hr fast suggests that the functional gene product is required for downregulation of leptin mRNA.

As the concentration of circulating insulin is known to decrease in the fasted state, this change may be responsible for the reduced expression of the leptin mRNA caused by fasting. To further explore this possibility, adult male rats were rendered diabetic—i.e., insulin-deficient—with streptozotocin. Within 5 days, their blood glucose levels reached >350 mg/dl, verifying the diabetic state. The levels of leptin mRNA in white adipose tissue of the diabetic animals plummeted ≈90% (Fig. 4). Diabetes-induced suppression of leptin mRNA expression can be rapidly reversed. Within 4 hr of insulin administration, the levels of leptin mRNA in adipose tissue increased to ≈60% of the nondiabetic control animals.

Accumulating evidence suggests that regulation of leptin expression by adipose tissue is complex and rigorous. Mutations at the *ob* locus that block the expression of functional leptin cause upregulation of leptin mRNA in adipose tissue (Fig. 3; ref. 7). Similarly, *db/db* mutant mice and Zucker *fa/fa* rats, which appear to lack functional leptin receptors (6, 26), overproduce leptin mRNA and protein (ref. 8 and results not shown). The simplest interpretation of these observations is that leptin and its putative receptor are required for the generation of a negative feedback signal that downregulates the expression of leptin mRNA and protein. Whether the signal is blood-borne or transmitted via the central nervous system is not known.

The present study suggests an additional mechanism for the control of expression of leptin mRNA. Both fasting (Fig. 3)

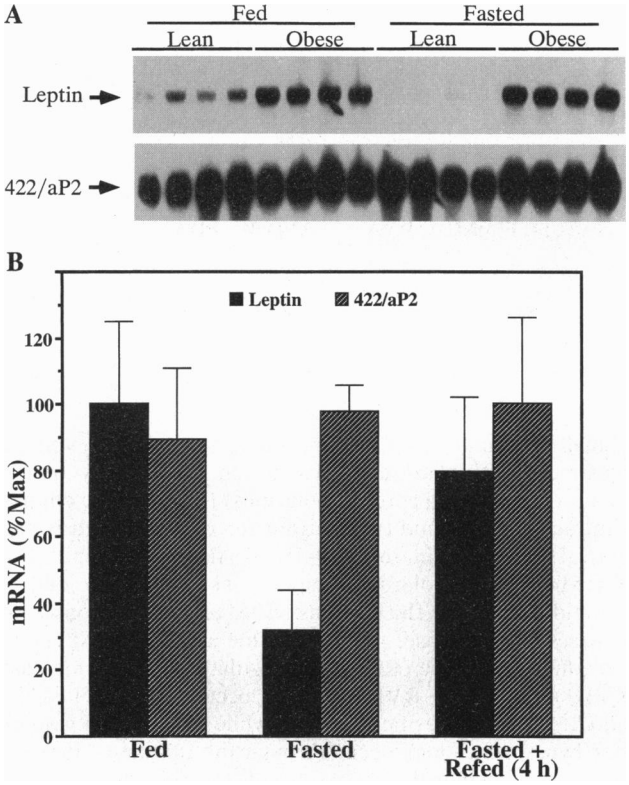

FIG. 3. Effect of fasting on the expression of leptin mRNA in white adipose tissue from lean and obese mice (*A*) and rats (*B*). (*A*) Four *ob/ob* mice (45 ± 2 g) and four lean littermates (26 ± 2 g) were fasted for 16 hr; the same number of *ob/ob* and lean mice were given access to a laboratory chow diet. Epididymal adipose tissue was isolated and subjected to Northern blot analysis with leptin and 422/aP2 probes. (*B*) Six male rats (≈300 g) were fasted for 16 hr, while another three had access to a chow diet. After fasting, three rats were given access to the chow diet for an additional 4 hr. RNA was isolated and analyzed as above. Leptin and 422/aP2 mRNAs were quantified with a phosphor imager; bars show mean and SD relative to the maximal expression.

and streptozotocin-induced diabetes (Fig. 4) downregulate the level of leptin mRNA in adipose tissue. Moreover, the decrease in leptin mRNA level in the fasted or diabetic state is rapidly (<4 hr) reversed by refeeding (Fig. 3*B*) or insulin administration (Fig. 4), respectively. A common feature of both of these altered physiological states is a reduced level of circulating insulin. It is of interest in this connection that fasting down-regulates expression of the leptin mRNA in adipose tissue of lean mice, but not *ob/ob* mice. This failure to downregulate may be due to the hyperinsulinemia that accompanies obesity in the *ob/ob* mouse, which persists even in the fasted state (27). The effect of insulin on leptin expression by the adipocyte may be indirect, since insulin alone does not cause upregulation of leptin mRNA in fully differentiated 3T3-L1 adipocytes in culture (unpublished observations).

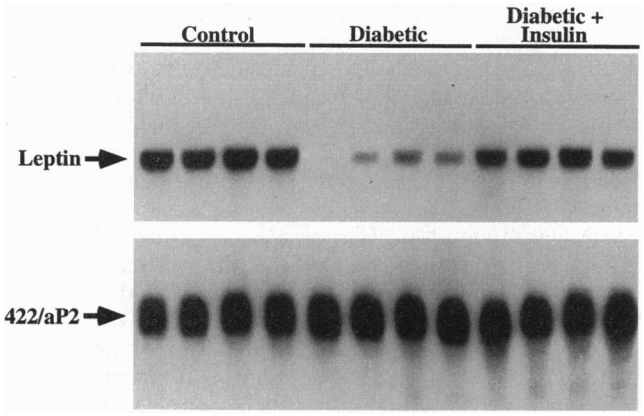

FIG. 4. Effect of streptozotocin-induced diabetes on the expression of leptin mRNA in white adipose tissue. Eight rats were injected with 65 mg of streptozotocin per 100 g of body weight while four rats were left untreated (Control). After 5 days, blood glucose levels in all streptozotocin-treated animals were >350 mg/dl (Diabetic). Four diabetic rats were injected with 15 units of insulin (Iletin I) per kg of body weight, which suppressed blood glucose to 39 mg/dl within 4 hr (Diabetic + Insulin). Epididymal adipose tissue RNA was subjected to Northern blot analysis with [^{32}P]-labeled leptin and 422/aP2 probes.

Note Added in Proof. Results similar to those of Halaas *et al.* (8) were reported by Pelleymounter *et al.* (28) and Campfield *et al.* (29).

We thank Drs. Dale Romsos, David Cooke, and Suzanne Mandrup for reviewing the manuscript. This work was supported by a grant from the National Institute of Diabetes and Digestive and Kidney Diseases. O.A.M. is supported by a National Research Service Award from the National Institute of Diabetes and Digestive and Kidney Diseases.

1. Bray, G. A. & York, D. A. (1979) *Physiol. Rev.* **59,** 719–809.
2. Bray, G. A., York, D. A. & Fisler, J. S. (1989) *Vitam. Horm.* **45,** 1–125.
3. Ingalls, A. M., Dickie, M. M. & Snell, G. D. (1950) *J. Hered.* **41,** 317–318.
4. Friedman, J. M. & Leibel, R. L. (1992) *Cell* **69,** 217–220.
5. Coleman, D. L. (1973) *Diabetologia* **9,** 294–298.
6. Coleman, D. L. (1978) *Diabetologia* **14,** 141–148.
7. Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J. M. (1994) *Nature (London)* **372,** 425–432.
8. Halaas, J. L., Gajiwala, K. S., Maffei, M., Cohen, S. L., Chait, B. T., Rabinowitz, D., Lallone, R. L., Burley, S. K. & Friedman, J. M. (1995) *Science* **269,** 543–546.
9. Schneider, C., Newman, R. A., Sutherland, R., Asser, U. & Greaves, M. F. (1982) *J. Biol. Chem.* **257,** 10766–10769.
10. MacDougald, O. A., Cornelius, P., Lin, F.-T., Chen, S. S. & Lane, M. D. (1994) *J. Biol. Chem.* **269,** 19041–19047.
11. MacDougald, O. A., Cornelius, P., Liu, R. & Lane, M. D. (1995) *J. Biol. Chem.* **270,** 647–654.
12. Graham, F. L. & van der Ebb, A. J. (1973) *Virology* **52,** 456–457.
13. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
14. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zin, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12,** 7035–7056.
15. Kaestner, K. H., Ntambi, J. M., Kelly, T. J. & Lane, M. D. (1989) *J. Biol. Chem.* **264,** 14755–14761.
16. Klausner, R. D., Donaldson, J. G. & Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116,** 1071–1080.
17. Bernlohr, D. A., Doering, T. L., Kelly, T. J. & Lane, M. D. (1985) *Biochem. Biophys. Res. Commun.* **132,** 850–855.
18. MacDougald, O. A. & Lane, M. D. (1995) *Annu. Rev. Biochem.* **64,** 345–373.
19. MacDougald, O. A. & Lane, M. D. (1995) *Curr. Biol.* **5,** 618–621.
20. Ntambi, J. M., Buhrow, S. A., Kaestner, K. H., Christy, R. J., Sibley, E., Kelly, T. J. & Lane, M. D. (1988) *J. Biol. Chem.* **263,** 17291–17300.
21. Kaestner, K. H., Christy, R. J., McLenithan, J. C., Braiterman, L. T., Cornelius, P., Pekala, P. H. & Lane, M. D. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3150–3154.
22. Christy, R. J., Yang, V. W., Ntambi, J. M., Geiman, D. E., Landschulz, W. H., Friedman, A. D., Nakabeppu, Y., Kelly, T. J. & Lane, M. D. (1989) *Genes Dev.* **3,** 1323–1335.
23. Kaestner, K. H., Christy, R. J. & Lane, M. D. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 251–255.
24. Lin, F.-T. & Lane, M. D. (1992) *Genes Dev.* **6,** 533–544.
25. Lin, F.-T. & Lane, M. D. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8757–8761.
26. Truett, G. E., Bahary, N., Friedman, J. F. & Leibel, R. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7806–7809.
27. Cuendet, G. S., Loten, E. G., Cameron, D. P., Renold, A. E. & Marliss, E. B. (1975) *Am. J. Physiol.* **228,** 276–283.
28. Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T. & Collins, F. (1995) *Science* **269,** 540–543.
29. Campfield, L. A., Smith, F. J., Guisez, Y., Devos, R. & Burn, P. (1995) *Science* **269,** 546–549.