

A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction

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The adipocyte-specific hormone leptin, the product of the *obese* (*ob*) gene, regulates adipose-tissue mass through hypothalamic effects on satiety and energy expenditure¹⁻⁴. Leptin acts through the leptin receptor, a single-transmembrane-domain receptor of the cytokine-receptor family⁵⁻⁷. In rodents, homozygous mutations in genes encoding leptin¹ or the leptin receptor⁶ cause early-onset morbid obesity, hyperphagia and reduced energy expenditure. These rodents also show hypercortisolaemia, alterations in glucose homeostasis, dyslipidaemia, and infertility due to hypogonadotropic hypogonadism⁸. In humans, leptin deficiency due to a mutation in the leptin gene is associated with early-onset obesity⁹. Here we describe a homozygous mutation in the human leptin receptor gene that results in a truncated leptin receptor lacking both the transmembrane and the intracellular domains. In addition to their early-onset morbid obesity, patients homozygous for this mutation have no pubertal development and their secretion of growth hormone and thyrotropin is reduced. These results indicate that leptin is an important physiological regulator of several endocrine functions in humans.

We studied a family (HD) with a strong prevalence of morbid obesity occurring early in life. The 19-year-old proband (HD416) is the fourth child of nine siblings. Her parents are second cousins in a consanguineous family of Kabilian origin. She shared her phenotype with two other morbidly obese sisters (HD413 and HD417, who died at age 19) (Fig. 1a). None of the siblings had features of Prader-Willi or Bardet-Biedel syndromes, or had abnormalities in karyotype or in brain computerized-tomography scans. Parents and other siblings do not share the same severe obese phenotype (Fig. 1a). Serum leptin concentrations (Table 1) of the three sisters were much higher than those measured in 842 consecutive subjects consulting in our department ($20 < \text{body mass index (BMI)} < 80 \text{ kg per m}^2$, leptin range $2-160 \text{ ng ml}^{-1}$). Serum leptin levels of both parents and three of the siblings (HD414, HD418 and HD419) were half those of the affected sisters. Two additional siblings (HD412 and HD415) had leptin levels in the normal range for their BMI.

To determine whether the genes encoding leptin or the leptin receptor (Ob-R) were involved in the extreme obesity of the affected sisters, we studied the genotype of all family members at microsatellite markers at these loci. Four markers located at the *ob* locus showed no segregation of any haplotype with the disease. In contrast, markers (D1S203, D1S220 and D1S198) located at the Ob-R gene locus cosegregated with the morbidly obese phenotype.

We scanned the 18 coding exons of the entire long leptin-receptor isoform for mutations, using single-stranded conformation-dependent polymorphism (SSCP). An abnormal conformer of exon 16 was found in a homozygous state in HD416 and in a heterozygous state in her mother (Fig. 2a). Direct nucleotide sequencing of exon 16 of HD416 identified a G → A base substitution in the splice donor site of exon 16 (Fig. 2b). Both parents and four of the six non-affected siblings were heterozygous for this variant, whereas the other two non-affected siblings were homozygous for the normal allele (Fig. 1b). The lod score for linkage between the G → A mutation and morbid obesity is 3.53, at a recombination fraction of 0, under a recessive model with complete penetrance. No mutation of exon 16 was detected in 402 French Caucasians who were of normal weight or obese¹⁰, confirming that this mutation is not a common one.

We investigated the effect of the Ob-R gene mutation on the encoded messenger RNA. Amplification of a 275-base-pair (bp) complementary DNA region spanning exon 16 (exons 15-17, a

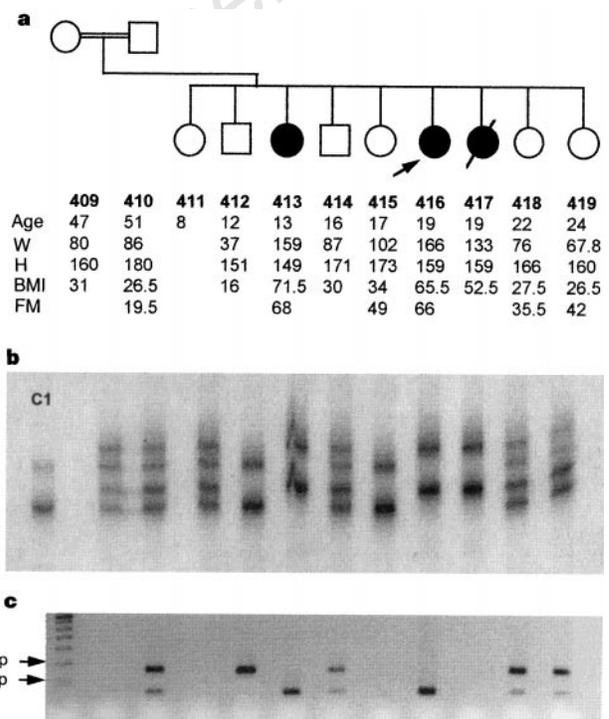


Figure 1 Pedigree and clinical characteristics of the HD family, and analysis of exon 16 of the leptin receptor in this family. **a**, Pedigree and clinical characteristics of the HD family. Filled symbols denote the three affected girls, with an arrow indicating the proband. Identification numbers (HD409, HD410, etc) and available clinical data are shown. Age, years; W, weight in kg; H, height in cm; BMI, kg per m². FM is the percentage of total body fat measured in the father and five siblings using biphotonic absorptiometry (Hologic Device QDR 1000/W, Watham, MA). The father, HD410, is a diabetic who is treated by diet and with an α -glucosidase inhibitor. No clinical data except for age were available for the non-obese youngest sibling, HD411. **b**, SSCP analysis of exon 16 of the human leptin receptor gene in the HD family. SSCP analysis on a 5%, non-denaturing, glycerol-free polyacrylamide gel (electrophoresed at 6-20W for 4-7 h at 4°C) shows a mutation, resulting in a mobility shift, seen in homozygous form in HD413, HD416 and HD417. Of the clinically unaffected siblings, four (HD411, HD414, HD418 and HD419) were heterozygous and two (HD412 and HD415) were homozygous for the wild-type sequence. C1 is a control obese subject who is unrelated to the HD family. **c**, RT-PCR of the region of the human leptin receptor gene spanning exon 16. For clinically unaffected individuals, a PCR product with an expected size of 276 bp was seen. For affected sisters HD416 and HD417 there was a unique band of 170 bp that lacked exon 16. The father and the heterozygous sibling express both mRNA species. mRNA was not available for HD409, HD411, HD414 and HD417.

region common to all transmembrane-domain-containing isoforms) shows that an abnormal Ob-R mRNA is expressed in the heterozygous subjects. This mRNA is not expressed in the normal individuals but is the only form expressed in the affected sisters (Fig. 1c). Direct nucleotide sequencing of the amplified product produced from reverse transcription and polymerase chain reaction (RT-PCR) confirms that the abnormal mRNA results from skipping of exon 16. The receptor mRNA lacking exon 16 potentially encodes a protein of 831 amino acids (Ob-Rhd), which would contain the first 830 amino-terminal amino acids of the normal receptor extracellular domain and extra glutamine at the carboxy terminus. This protein would lack both the transmembrane and the intracellular domains of the Ob-R. The expression of the 824 amino acids of the human Ob-R extracellular domain in COS cells leads to the secretion of a leptin-binding protein that is able to dimerize¹¹. To determine whether Ob-Rhd has similar properties, we assessed how leptin is carried in the serum of the patients by radioimmunoassay quantification of leptin in eluted fractions after gel filtration chromatography. In normal and obese controls, a low proportion (5–20%) of serum leptin circulates in a high-molecular-mass complex, as described^{12,13}. Its apparent relative mass (M_r) is 440K, which is close to that found with similar techniques¹³. In contrast, in the serum of the homozygous and heterozygous mutant patients 80% of leptin circulates as a complex of similar size. Recombinant human leptin displaces ¹²⁵I-labelled leptin from the high-molecular-mass complex, confirming the presence of a specific leptin binding factor. The M_r of the complex could indicate dimerization of Ob-Rhd and also the presence of short circulating forms of Ob-R.

The affected sisters were studied for obesity and short stature during childhood. Their birthweight was normal (3.60, 4.00 and

3.620 kg for HD416, HD413 and HD417, respectively), but severe obesity rapidly developed within the first months of life (Fig. 3). They showed abnormal eating behaviour resembling that seen in Prader-Willi syndrome and in individuals with anatomical damage of the hypothalamic area; behaviour included fighting with other children for food and acting with impulsivity and stubbornness. Repeated psychological evaluations showed emotional lability and social disability but no mental retardation. The resting metabolic rates (RMRs) of HD416 and HD413, evaluated by indirect calorimetry, were 2,510 and 2,181 kcal per 24 h, respectively (the RMR predicted by the Harris and Benedict formula is similar: 2,370 and 2,453 kcal per 24 h in HD416 and HD413, respectively). Their core temperature was normal. Other HD family members are moderately obese but none has a phenotype comparable to that of the affected sisters. HD415, who has the wild-type Ob-R genotype, has the highest BMI of the unaffected siblings (Fig. 1a). The absence of morbid obesity in the heterozygotes indicates that the lack of one normal allele for Ob-R and secretion of an abnormal leptin-binding protein does not affect body-weight regulation by leptin. The leptin levels of the heterozygotes are about half those of the homozygous patients, indicating that the Ob-R mutation might have a codominant effect on the hormone (Table 1). These high leptin concentrations might be related to leptin trapping by the serum leptin-binding factor, resulting in a prolonged half-life of leptin (assuming that the bound leptin is inactive). In homozygotes, the elevated leptin levels might be related to the same mechanism but, in the absence of a functional Ob-R (that is, leptin resistance), an inappropriate oversecretion of leptin is also possible.

The homozygous patients did not spontaneously develop puberty. They had no mammary glands, sparse pubic hair and no

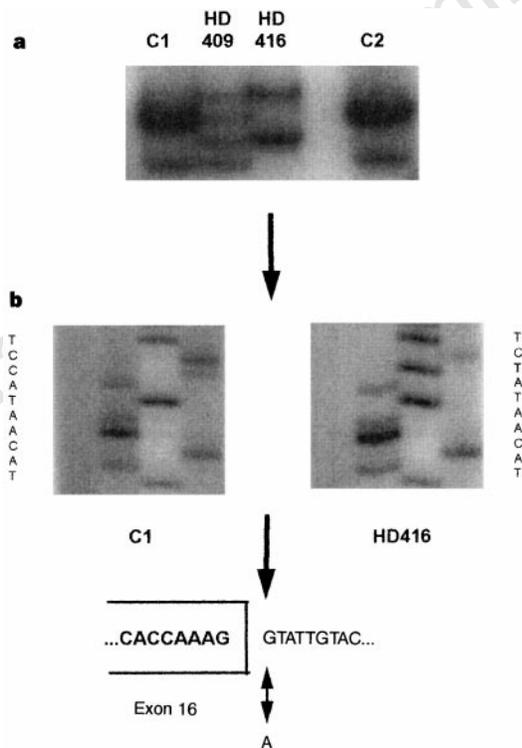


Figure 2 SSCP scanning and DNA-sequence analysis of exon 16 of the human leptin receptor. **a**, An homozygous mobility shift in exon 16 was seen for the proband HD416 as compared with two control obese subjects (C1 and C2) unrelated to the family. Here mother, HD409, was heterozygous for the same variant conformer. **b**, Sequence analysis (reverse complementary strand shown) of the exon 16/intron 16 junction of HD416 and of a control subject. There is a G → A base substitution in the splice donor site of exon 16 in patient HD416.

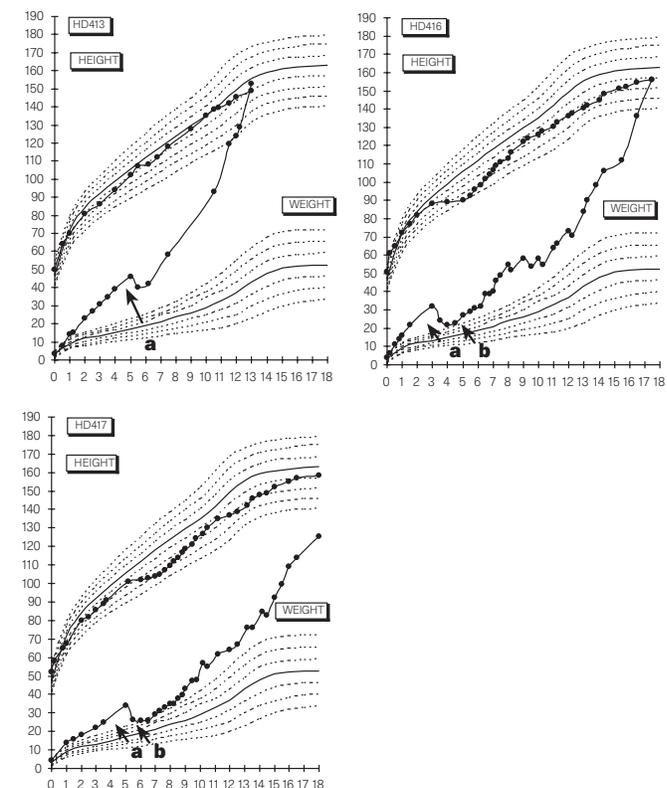


Figure 3 Height and weight curves for the three affected sisters from birth to adult age. The letter **a** indicates a period of food of food-intake restriction. The restrictive diet of 500 kcal per day resulted in weight loss and in a dramatic decrease in growth velocity, which persisted even after food-intake increase and weight regain. The letter **b** indicates the introduction of treatment with levothyroxine and the start of treatment with exogenous growth hormone. The x-axis indicates age in years; the y-axis indicates height in cm of weight in kg.

axillary hair. At age 13.5 (HD413) and 19 (HD416 and HD417), the patients were amenorrhoeic. Basal concentrations of oestradiol were low, and levels of luteinizing hormone and follicle-stimulating hormone (basal and in response to gonadotropin-releasing hormone) remained low or in the normal range (Tables 1 and 2). These features characterize hypogonadism of central origin.

Leptin-deficient *ob/ob* and Ob-R-deficient *db/db* mice are infertile, and leptin is involved in the onset of puberty in rodents¹⁴⁻¹⁶. In humans, longitudinal and transversal studies show increasing leptin levels before the onset of puberty^{17,18}, but the leptin-deficient children were too young to confirm an effect of leptin deficiency on gonadotropin secretion⁹. The hypogonadotropic hypogonadism of the sisters with mutated Ob-R shows that signalling through Ob-R is necessary for sexual maturation in women. One functional copy of the human Ob-R gene seems to be enough to allow normal reproductive functions in the heterozygous members of the family. Two heterozygous girls (HD418 and HD419) passed through puberty normally, with onset of menstruation at 13 and 15 years, respectively. The heterozygous males had normal sexual maturation.

The secretion of growth hormone in response to stimulation is generally decreased in obese children¹⁹, and the effects of growth-hormone deficiency are mimicked, except that obese children have normal or slightly increased insulin-like growth factor-I (IGF-I) levels²⁰ and, often, accelerated growth²¹. The affected sisters here showed a mild but significant growth delay during early childhood (Figs 1 and 3), despite normal parental height. No overnight burst of growth hormone was observed and growth-hormone secretion remained low in response to stimulation tests (<5 ng ml⁻¹) (Table 2). Unexpectedly²⁰, concentrations of IGF-I and IGF-binding protein 3 (IGF-BP3) were decreased (Table 2). Levels of IGF-I and IGF-BP3 increased after administration of growth hormone, as also observed in patients lacking growth hormone (Table 2). This inadequate secretion of growth hormone in the patients with the Ob-R mutation does not occur in leptin-deficient children, who appear to have normal linear growth⁹. In rodents, levels of growth hormone in the pituitary and of growth-hormone-releasing hormone in the hypothalamus are decreased in young *db/db* mice²². Both *ob/ob* and *db/db* mice show stunted growth curves²², and leptin-receptor-deficient obese Zucker rats secrete low levels of growth hormone²³. Administration of leptin antiserum in rodents decreases spontaneous growth-hormone secretion²⁴, indicating a direct effect of leptin on growth.

Low levels of free thyroxine, normal levels of basal thyroid-stimulating hormone (TSH) and a sustained TSH response to

Table 2 Endocrinological data for the three sisters with homozygously mutated Ob-R genes*

	HD416	HD413	HD417	Reference values
GnRH test	(13.5)	(13)	(13.5)	girls Tanner P2
LH (mU ml ⁻¹) basal	<0.7	<0.2	<0.8	1.0-4.0
LH peak	1		0.9	6.0-60
FSH (mU ml ⁻¹) basal	1	<0.1	1.20	1.0-4.0
FSH peak	4.3		3.2	3.0-20
Oestradiol (pg ml ⁻¹)	10	17	13	8.0-50
.....				
GH peak (ng ml ⁻¹) after orthithine and insulin test†				
Normal diet	1.6 & 0.1	0.4 (10.5)	3.5 & 1.4	>10
Overnight Restriction diet	<1	3	1.6	
	1.4		2.6	
.....				
IGF (ng ml ⁻¹)				
Basal	(5) 47	(2) 52	(7) 50	130 ± 50 at 2 years 190 ± 60 at 5 years 210 ± 60 at 7 years
After GH stimulation	378	182	135	
.....				
‡IGF-BP3				
Basal (WLB)	(5) 30%			84.5 ± 1.4%
After GH stimulation	70%			
.....				
Thyroid hormones				
FT4 (pg ml ⁻¹)	(5) 7	(5) 7	(7) 5	15.8 ± 3.3
TSH (μU ml ⁻¹)	3	1	5	<5
.....				
TRH test				
TSH basal	(5) 3	(5) 1.4	(7) 2	<5
TSH peak	25	28	15	17 ± 5
TSH 120'	16	13	10	<5
.....				
Glucocorticosteroids				
FLU (μg per 24 h)	(11.5) 89	(10.5) 60	(7) 64	<40 prepubertal girls
	(19) 107			<80 pubertal girls
	(19) 115	(13.5) 127		107 ± 5.2
Cortisol (ng ml ⁻¹), 8:00	79	78		
Cortisol, 16:00	68	32		6.0-50
ACTH (pg ml ⁻¹), 8:00	68	26		
ACTH, 16:00				
.....				
OGTT				
Glycaemia (mmol l ⁻¹) basal	(3) 4.8		(5) 4.9	3.7-5.9
Peak	5.5		7.4	
120'			4.7	<10
Insulinaemia (ng ml ⁻¹) basal	5		<5	<15
Peak	7		49	
120'			8	30-90

* The three sisters were studied several times during infancy at different ages. Ages (in years) at which the sisters were studied are shown in parentheses.

† Normal diet means *ad libitum* diet and restrictive diet means a 700 kcal (25% glucose) diet. ‡ IGF-BP3 in HD416 (measured by WLB, western ligand blot) is given as a percentage of total. IGF-BP3 levels were measured in basal conditions and after three days of administration of growth hormone (GH). LH, luteinizing hormone; FSH, follicle-stimulating hormone; free thyroxine; TRH, thyrotropin-releasing hormone; FLU, free urinary cortisol; ACTH, adrenocorticotropic hormone; OGTT, oral glucose tolerance test.

Table 1 Recent biological and hormonal characteristics in the HD family

HD	Genotype	Leptin (ng ml ⁻¹)	Glycaemia (mmol l ⁻¹)	Triglycerides (mmol l ⁻¹)	Cholesterol (mmol l ⁻¹)	Insulin (μU l ⁻¹)	FSH (IU l ⁻¹)	LH (IU l ⁻¹)	Oestradiol (pg ml ⁻¹)	Testosterone (ng ml ⁻¹)	IGF-I (ng ml ⁻¹)
409	MN	362	3.92	0.62	5.54	10	26.6	17.2	7	0.16	164
410	MN	145	6.66	2.25	2.55	14	1.65	1.51	13	3.95	(221 ± 84)
412	NN	5.6	4.31	0.5	5.0	8	1.25	0.63	<5	0.41	164
413	MM	670	6.4	1.0	4.26	14	<0.2	<0.1	17	0.54	(211 ± 67)
414	MN	212	4.08	0.75	4.13	16	3.89	1.56	16	3.46	385
415	NN	88	4.5	0.87	4.26	30	4.11	12.3	216	0.48	(313 ± 52)
416	MM	600	4.53	1.12	4.40	41	5.9	4.00	21	0.19	154
417	MM	526	6.0	-	-	-	-	-	13	0.22	(303 ± 50)
418	MN	240	4.08	0.94	5.80	6	3.44	4.44	100	0.32	203
419	MN	294	3.75	0.69	4.0	17	5.47	6.52	44	0.50	(303 ± 50)

The family (except HD411) was studied recently. MM and MN are homozygous and heterozygous, respectively, for the Ob-R gene mutation. NN are subjects with the wild-type genotype. IGF-I reference range depends on age, sex and maturation. The other hormonal reference ranges are as follows. Adult men: follicle-stimulating hormone (FSH), 1.0-6.0; luteinizing hormone (LH), 0.8-6.0; oestradiol, 10.0-40.0; testosterone, 3.4-10. Follicular phase (19-40 years): FSH, 2.0-6.0; LH, 1.1-4.5; oestradiol, 19-94; testosterone, 0.10-0.70. Postmenopausal (<60 years): FSH, 25-90; LH, 12-50; oestradiol, 5-50; testosterone, 0.15-0.50. Puberal boys, stage II: FSH, 0.4-2.8; LH, 0.4-1.9; oestradiol, 6-27; testosterone, 0.20-3.0. Puberal girls, stage II: FSH, 0.8-4.2; LH, 0.3-2.5; oestradiol, <5-46; testosterone, <0.05-0.35.

stimulation with thyrotropin-releasing hormone indicate hypothalamic hypothyroidism in the affected sisters here (Table 2). The leptin-deficient children⁹ present mild elevation of TSH levels and normal levels of free thyroxin, which can be seen in thyroid dysfunction of central origin. Leptin effects on the thyrotrope axis have been shown in normal mice²⁵, but impairment in the thyrotrope axis is not clearly demonstrated in leptin- or Ob-R-deficient rodents.

Morning levels of adrenocorticotrophic hormone and cortisol and tests for dexamethasone and metyrapone were normal in the affected girls, indicating no gross defect in the hypothalamic pituitary adrenal axis. Urine-free cortisol excretions were slightly elevated (Table 2). This is common in obesity²⁶, and may not be caused by the Ob-R mutation.

The sisters homozygous for the Ob-R mutation repeatedly showed normal fasting glycaemia and normal oral glucose tolerance (Table 2) despite their extreme obesity (Fig. 1). Post-glucose-load insulinaemia was normal during childhood. At 13.5 and 19 years, respectively, insulin levels of HD413 and HD416 were similar to those of their moderately obese sister (HD415, with the wild-type genotype) and of French morbidly obese patients¹⁰ (mean insulin levels: 20 ± 12 mIU per ml). Ob-R-deficient patients are similar to leptin-deficient children in showing normal glucose homeostasis. In *ob/ob* and *db/db* mice, the importance of the diabetic phenotype depends on the background. Severe hyperglycaemia is seen on the C57BL/KsJ background^{27,28} and mild diabetes with transient hyperglycaemia and marked hyperinsulinaemia are seen on the C57BL/6J background^{27,28}. All these data indicate that leptin deficiency may not necessarily lead to diabetes. Contrasting with *db/db* and *ob/ob* mice, which are characterized by hypertriglyceridaemia and hypercholesterolaemia, fasting triglyceridaemia and cholesterolaemia are normal in the patients with the Ob-R mutation (Table 1).

We have described a mutation in the human leptin receptor and have analysed the clinical and biological data available for the affected patients. Our results indicate that a functional leptin receptor is required not only for the regulation of body weight but also for sexual maturation and for secretion of growth and thyrotropic-hormones. Leptin is therefore a critical link between energy stores and hypothalamic pituitary functions in humans. □

Methods

Genotyping. Genotypes were determined using microsatellite polymorphisms located at the Ob or at the Ob-R gene loci using standard techniques.

SSCP. Ob-R-coding exons from the HD family and from exon 16 of the controls were amplified in the presence of [α -³²P]-dATP using described primers²⁹ or the following primers: forward exon 1, 5'-TAA ATT TAG AGA CTT ATC TAT AAT CCC-3'; reverse exon 1, 5'-TAA CTA GAA ATA GGA AAT TCT GTT AGC-3'; forward exon 3, 5'-TTT TTT TTA AAT TCA GAT GCA AAC TGG A-3'; reverse 4 I, 5'-ACA TAA GGA GAG TGT CGT; forward exon 6, 5'-AGT AAC GGT TCC ACA TCA ACT TG-3'; forward exon 7I, 5'-CAG AAT GTT TGT CTT CAT CTG ATA TCC-3'; reverse exon 7II, 5'-ATT CAA GTT GTG GAA CAA AAT GAA CA-3'; reverse exon 8, 5'-ATT TTT ATC TTC ACT GTG CCC AC-3'; forward exon 11, 5'-GTA CTT CAG GGC CCC TTT AGA TAC ATA-3'; reverse exon 11, 5'-TTT GAA GAA ATA CTT TTC AGC CAT A-3'. (Coding exons 2, 4, 7/8 and 18 were amplified using 2, 2, 2 and 4 primer pairs, respectively.) The radiolabelled PCR products were electrophoresed overnight in acrylamide gel (with 0% and 10% glycerol) and autoradiographed according to standard SSCP techniques (Fig. 1b).

RT-PCR amplification of human Ob-R cDNA. Total RNA from lymphocytes was prepared using the RNAlastepure Kit (Eurogentec, Seraing, Belgium). RNAs were reverse-transcribed using AMV reverse transcriptase (Gibco BRL, Oxford, England). PCR (1.25 mM MgCl₂, annealing temperature 60 °C) was performed for exons 15–17 using the following primers (forward 15, 5'-CAT TTT ATC CCC ATT GAG AAG TA-3'; reverse 17, 5'-CTG AAA ATT AAG TCC TTG TGC CCA G-3').

Sequencing. PCR products were sequenced by AmpliTaq cycle sequencing (Promega, Lyon, France) using [γ -³²P]-end-labelled primers.

Gel-filtration analysis of serum leptin and displacement of bound ¹²⁵I-labelled leptin. Serum (1 ml) was applied to an Ultragel AcA 44 column (Biosepra, Villeneuve La Garenne, France). Immunoreactive leptin was measured by radioimmunoassay in 0.5-ml fractions. Displacement of ¹²⁵I-labelled leptin was measured after incubation of serum plus 15,000 c.p.m. of ¹²⁵I-labelled leptin, with or without 10 μ g of human recombinant leptin (Peninsula, UK).

Determination of molecular mass of leptin-binding factor. Serum (100 μ l) was incubated with 15,000 c.p.m. ¹²⁵I-labelled leptin applied to a Sephacryl S300 HR 6/2/98column, and calibrated with a gel-filtration kit (Pharmacia, St Quentin-Yvelines, France).

Hormonal assays. Hormonal measurements were made using commercially available kits. IGF1 and IGF-BP levels were measured as described³⁰.

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