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Phenotype-Linked Amino Acid Alteration in Leptin Receptor cDNA from Zucker Fatty *(fa/fa)* Rat

Mitsuru Iida,*,† Takashi Murakami,*,[1] Kaori Ishida,* Akira Mizuno,* Masamichi Kuwajima,* and Kenji Shima*

**Department of Laboratory Medicine, School of Medicine, University of Tokushima, Kuramotocho 3-chome, Tokushima 770; and †Diagnostic Division, Otsuka Pharmaceutical Co., Ltd., Aza Ebisuno Hiraishi, Kawauchi-cho, Tokushima 771-01, Japan*

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The mouse *obese (ob)* gene product (leptin), expressed specifically in adipose cells, regulates energy balance in mice. Both mouse *diabetes (db)* and rat *fatty (fa)* gene products are thought to play major roles in leptin signaling pathways in the hypothalamic area. Mutations of these genes in murines result in marked obesity and type II diabetes as part of a syndrome that resembles morbid obesity in humans. Reported herein are the cloning and sequencing of one of spliced variant forms of rat leptin receptor (OB-R) cDNA with a short intracellular domain. In the Zucker *(fa/fa)* rat, no changes in either the gene structure or the expression levels were observed. However, phenotype-linked nucleotide alteration exists in the cDNA from Zucker *(fa/fa)* rat, which results in an amino acid substitution.

Obesity represents a common human health problem in industrialized societies, and is associated with diabetes, hypertension and hyperlipidaemia. For many years, the molecular pathogenesis of obesity was obscure, and the identification by Y. Zhang *et al.* of a gene, *obese (ob),* whose recessive mutation causes severe hereditary obesity in mice, represents a major finding in this area (1). A number of studies have shown that recombinant *ob* gene product (leptin), purified from *Escherichia coli,* can cause weight reduction in mice when exogeneously administered (2–5). Another well-characterized recessive obesity mutation in mice is *diabetes (db).* Mice which are homozygous for the *db* mutation exhibit an obesity phenotype nearly identical to the phenotype of *ob/ob* mice (6). Parabiosis studies with *ob/ob* and *db/db* mice indicate that *db/db* mice may be defective with respect to reception of the *ob* gene product signal (7).

In late 1995, mouse leptin receptor (OB-R) cDNA was cloned by screening a cDNA expression library, prepared from mouse choroid plexus (8). The OB-R appears to be a single membrane-spanning receptor which is most closely related to gp130, the signal-transduction component of the interleukin-6 receptor, and to the receptor for granulocyte colony-stimulating factor and for the leukemia-inhibitory factor. The intracellular domain is short (composed of only 34 amino acids) compared to that of the related receptors, and to its human homologue, which is composed of 304 amino acids. Genetic mapping showed that the OB-R gene lies quite close to the *db* gene, but, at that time, no mutation was identified in the gene of *db/db* mouse.

Recently, several spliced variant forms of mouse OB-R cDNA were reported, and an abnormally spliced variant was found for the *db/db* mouse (9,10). By the abnormal splicing, *db/db* mouse expresses very little, if any, levels of one of spliced variant forms which possesses a long intracellular domain.

In this laboratory, we have successfully cloned rat *ob* cDNA (11). The adipose tissues of the

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession Nos. D84125 and D84126.

[1] To whom reprint requests should be addressed. Fax: +81-886-31-9495.

Abbreviations: OB-R, leptin receptor; *ob, obese; db, diabetes; fa, fatty;* SD, Sprague-Dawley; bp, base pairs; kb, kilobases; RT, reverse transcription; PCR, polymerase chain reaction.

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Zucker (*fa/fa*) rat expressed *ob* mRNA at high levels, suggesting a mutation or an abnormal expression of the OB-R. This finding is consistent with a report in that the *fa* gene maps to rat chromosome 5 in a region of conserved synteny with the mouse chromosome 4 region which contains the *db* gene (12,13).

In this paper, we report the cloning of rat OB-R cDNA by the reverse transcription-polymerase chain reaction (RT-PCR) (14–16), using two sets of primers. For each set of primers, one is specific for the sequence corresponding to the 5′ or 3′-noncoding region of mouse OB-R cDNA and the other is specific for its coding region sequence. We also cloned OB-R cDNA from the Zucker (*fa/fa*) rat (17) and found phenotype-linked nucleotide alteration in the cDNA from the Zucker (*fa/fa*) rat, which results in an amino acid substitution, glutamine to proline.

MATERIALS AND METHODS

Animals. A male Zucker (*fa/fa*) rat and its male lean littermates (*Fa/?*) were obtained from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan). Male Sprague-Dawley (SD) rat, Wistar rat and BALB/c mouse were purchased from Japan SLC, Inc. (Hamamatsu, Japan). A male and a female Wistar (*Fa/fa*) rats (18) were kindly provided by the Takeda Chemical Industries, Ltd. (Osaka, Japan), and were maintained and bred in our animal facilities under specific pathogen-free conditions (Institute for Animal Experimentation, The University of Tokushima). The *fa/fa* rats were distinguished visually from their lean littermates (*Fa/?*) by their obese phenotype.

DNA analysis. DNA was extracted from rat liver by successive proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation (19). DNAs (5 μg) were digested to completion with *Eco*R I, *Hin*d III, *Bam*H I or *Pst* I, separated in 0.7% agarose gel, and transferred to a Hybond-N nylon hybridization membrane (Amersham International plc, Buckinghamshire, England) (20). The membrane was hybridized with the [α-^{32}P]dCTP random-priming labeled (21) rat OB-R cDNA probe ("Cloning of rat OB-R cDNA using RT-PCR", below), washed at a stringency of 0.5 × standard saline citrate (SSC; 1 × SSC is 150 mM NaCl, 15 mM sodium citrate), 0.1% sodium dodecyl sulfate (SDS) at 68 °C, and exposed to X-ray film, as described elsewhere (22).

RNA analysis. Total RNA was prepared by homogenizing tissues in solution containing 4.4 M guanidine thiocyanate, 0.1 M β-mercaptoethanol, and 25 mM sodium citrate (pH 7.0), followed by centrifugation through 5.7 M CsCl (23,24). RNAs (13 μg) were denatured in 50% formamide, 2.2 M formaldehyde at 65°C for 10 min, and electrophoresed in 1% agarose gel containing 2.2 M formaldehyde. The gel was blotted onto to a Hybond-N nylon hybridization membrane. The membrane was hybridized, washed and exposed to X-ray film, as described under "DNA Analysis". The amount of intact RNA in each lane of the gel was judged to be constant by ethidium bromide fluorescence, identifying specific bands of 18S and 28S RNA directly in the gel and after transfer of the RNA to the nylon hybridization membrane. A Bio-image analyzer BAS2000 (Fuji Film Institution, Tokyo, Japan) (25) was used for quantification.

Cloning of rat OB-R cDNA using RT–PCR. RT-PCR (14–16) was performed using total RNAs from lungs. For cDNA synthesis, RNA (10 μg) was incubated for 2 h at 42 °C in a reaction mixture (10 μl) for reverse transcription (RT), which contained 50 mM Tris (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, 5 mM DTT, 20 mM each of dNTPs, 50 units of RNase inhibitor (Promega Corporation, Madison, Wisconsin, U.S.A.), 50 pmol of oligo$(dT)_{17}$, and 17 units of avian myeloblastosis virus-reverse transcriptase XL (Life Science inc., St. Petersburg, Florida, U.S.A.). After the incubation, the mixture was heated at 98 °C for 10 min. For amplification of OB-R cDNA, the polymerase chain reaction (PCR) was carried out in a reaction mixture (50 μl; provided by the manufacturer) with the above RT reaction mixture (1 μl), 200 μM each of dNTPs, 0.5 units of Perfect Match PCR Enhancer (Stratagene Cloning Systems, La Jolla, California, U.S.A.), 2.5 units of *TaKaRa LA Taq* DNA polymerase (TAKARA SHUZO CO., LTD., Otsu, Shiga, Japan) and 17 pmol each of the sense and antisense kinased primers. For cloning OB-R cDNA from SD rat, we used two sets of primers, S1-A3 and S2-A4 (S1-A3; sense primer S1 and antisense primer A3, S2-A4; sense primer S2 and antisense primer A4). The primers S1 and A4 are specific for the sequence of the 5′ or 3′-noncoding region of mouse OB-R cDNA (8) and the primers S2 and A3 are specific for the sequence of its coding region (see Fig. 1A). The sense primer S1 is 30 bases long and is comprised of residues −30 to −1 from the adenine of the translation initiation site, GCAAATCCAGGTGTACACCTCTGAAGAAAG. The sense primer S2 is 30 bases long and is comprised of residues 1139 to 1168, GCATTGTGAGTGACCGAGTTAGCAAAGTTA. The antisense primer A3 is 30 bases long and comprises residues 1242 to 1213, CTGCTCATTGCAGCAGTACACTGCGTCATA. The antisense primer A4 is 30 bases long, comprising residues 2716 to 2687, TTGGGTTCATCTGTAGTGGTCATGAGAGAC. Conditions for the PCR were 96°C for 40 s, 65 °C for 40 s and 72 °C for 100 s (35 cycles) using a GeneAmp PCR System 9600 (Perkin-Elmer Corp., Norwalk, Connecticut, U.S.A.). The PCR products were fractionated by electrophoresis in agarose gels. cDNA products of approximately 1.3 kilobases (kb) (S1-A3) and 1.6 kb (S2-A4) were isolated from the gels using a SUPREC (TAKARA SHUZO CO., LTD.), and cloned into a *Hinc* II digest of plasmid pUC19. The nucleotide sequences were determined by the dideoxynucleotide chain-termination method, using synthetic oligonucleotide primers which were complementary to the vector or to rat OB-R cDNA sequence and ABI 373A automated DNA Sequencing System (Perkin-Elmer Corp.) (26,27). These cDNA fragments were recovered by restriction enzyme digestion, mixed, and used as a probe for DNA and RNA analyses.

Restriction enzyme Hpa II digestion of rat OB-R cDNAs. RT-PCR was performed using primers S15 and A3. The sense primer S15 is 21 bases long, and is specific for the sequence from residue 639 to 659 of rat OB-R cDNA. The sequence of this primer is AATCACATCTGCTGGTGTGAG. Conditions for the PCR were 96 °C for 30 s, 57 °C for 30 s and 72°C for 60 s (30 cycles). After the PCR, the products were subjected to successive phenol/chloroform extraction and ethanol precipitation. The samples were digested by a restriction enzyme *Hpa* II or *Msp* I, and fractionated by electrophoresis in agarose gels.

RESULTS

Cloning of rat OB-R cDNA using RT–PCR. For cloning OB-R cDNA, RT-PCR was performed using total RNAs from the lungs of SD and Zucker (*fa/fa*) rats, and BALB/c mouse. Figure 1B shows the electrophoretic patterns of some of the reaction products. The cDNA products of approximately 1.3 kb and 1.6 kb were isolated from the gels, and cloned into a *Hinc* II digest of pUC19. The nucleotide sequences of cDNA clones from SD and Zucker (*fa/fa*) rats were determined. Nearly all the OB-R cDNA sequences were the same for these two strains of rat (sequences of some clones had several altered sequences, possibly due to misreading during the RT-PCR), with the exception that all of the clones from the Zucker (*fa/fa*) rat contained cytosine nucleotide at residue 806 rather than adenine (Fig. 2, 3A,B). As a result of this nucleotide difference for residue 806, the codon 269 becomes glutamine in SD rat and proline in Zucker (*fa/fa*) rat, respectively. In the vicinity of this area (approximately from codon 263 to 277), both nucleotide and amino acid sequences are well conserved for the SD rat, mouse and human (Fig. 3A). By this change in nucleotide sequence, the restriction enzyme *Hpa* II site is newly created in the cDNA sequence from Zucker *fa/fa* rat (Fig. 3B). To confirm that this nucleotide change actually exists, RT-PCR of lung RNAs from several rat strains was performed using primers S15 and A3 and the products were subjected to *Hpa* II digestion (Fig. 3C). If the RT-PCR products (approximately 600 bp) contain *Hpa* II site at around the residue 806, segmented fragments of approximately 430 bp and 170 bp

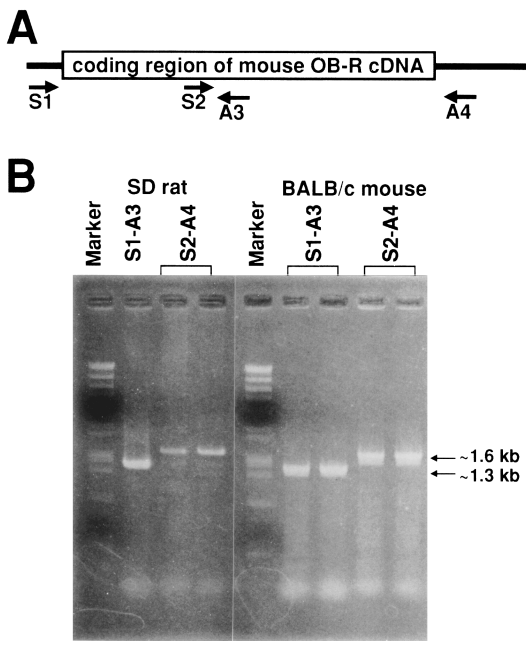

FIG. 1. Schematic representation of the cloning strategy of rat OB-R cDNA (A) and electrophoretic patterns of RT–PCR products of total RNAs from lungs of SD rat and BALB/c mouse: A; Open box represents coding region of mouse OB-R cDNA. Thick lines represent its 5′ and 3′ noncoding regions. Arrows represent primers used for RT–PCR. B; Arrows indicate amplified OB-R cDNAs (approximately 1.3 kb and 1.6 kb), which were isolated from the gels, and cloned into a *Hinc*II digest of pUC19.

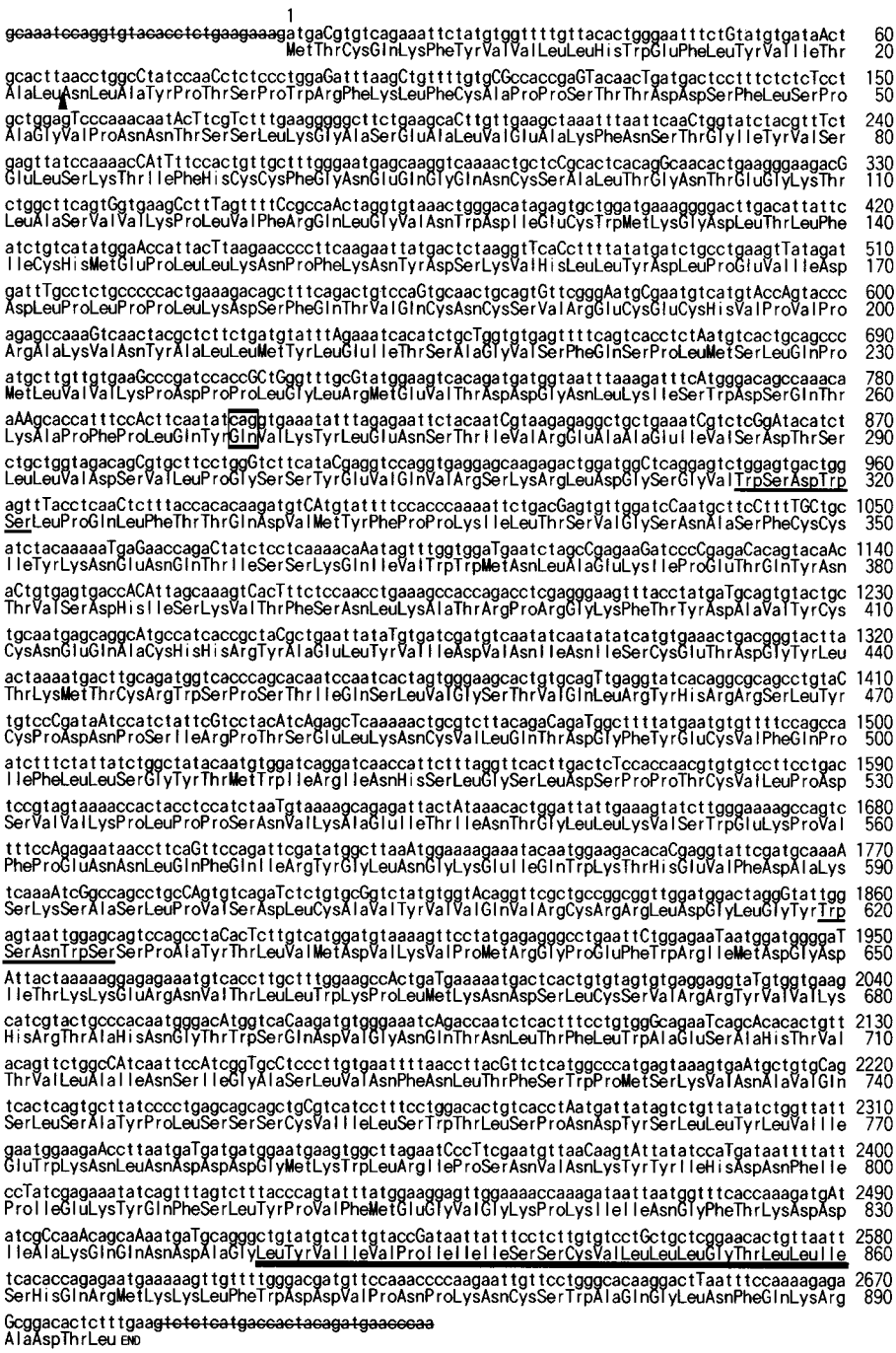

FIG. 2. Composite sequences derived from the OB-R cDNAs from SD rat: The predicted amino acid sequence is shown beneath the nucleotide sequence. The adenine of the translation initiation codon (nucleotide sequence) or the first Met (amino acid sequence) is numbered +1. Nucleotides which differ from those of mouse are shown in upper-case letters. The predicted signal peptidase cleavage site is marked with a vertical arrow head. The putative transmembrane domain is heavily underlined. Two Trp-Ser-X-Trp-Ser motifs are thinly underlined. The glutamine codon at codon 269 which changes to proline in Zucker (*fa/fa*) rat is boxed. Struck through nucleotide sequences in noncoding regions, which indicate the primers used for RT-PCR, are unreliable.

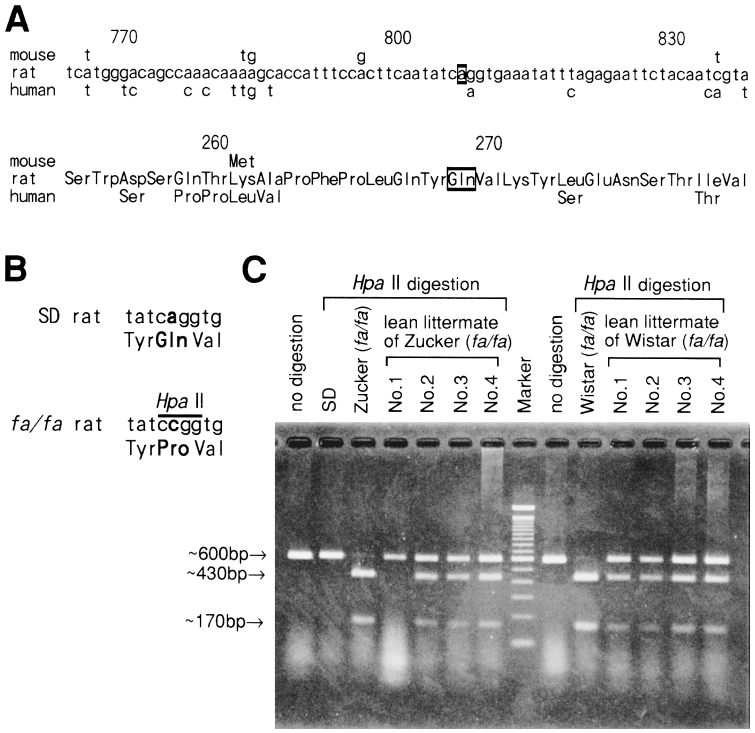

FIG. 3. Nucleotide and predicted amino acid sequences of rat OB-R cDNA in the vicinity of codon 269 (A, B) and electrophoretic patterns of restriction enzyme digestion of RT-PCR products from several rat strains (C): A; Nucleotide (top) and predicted amino acid (bottom) sequences of SD rat are aligned with those of mouse (upper) and human (lower). In the mouse and human sequences, only altered sequences are noted. Altered sequences in Zucker (*fa/fa*) rat are boxed. Positions of the sequences are noted by numbers. B; Sequences of SD and Zucker (*fa/fa*) rats are shown separately. Sequences which differ between two rats are indicated by bold letters. Newly created restriction enzyme *Hpa*II site in Zucker (*fa/fa*) rat is indicated by a line. C; RT–PCR products of lung RNAs from various strains of rats were subjected to *Hpa*II digestion. Arrows indicate unsegmented (approximately 600 bp) and segmented (approximately 430 bp and 170 bp) RT–PCR products by digestion of restriction enzyme *Hpa*II.

would be expected as a result of *Hpa* II digestion. The RT-PCR products from the Zucker (*fa/fa*) and the Wistar (*fa/fa*) rats were almost completely fragmented by *Hpa* II digestion. The RT-PCR products from SD rat and the lean littermate No. 1 of the Zucker (*fa/fa*) rat were resistant to *Hpa* II digestion. The products from the other lean littermates of the Zucker (*fa/fa*) and the Wistar (*fa/fa*) rats were partially fragmented. By a digestion of *Msp* I, which recognizes the same sequence as the *Hpa* II, the same results were obtained. These results indicate that the residue 806 is a cytosine nucleotide but not adenine in *fa/fa* rats. This suggests that the genotype of the lean littermate No. 1 of the Zucker (*fa/fa*) rat is *Fa/Fa,* and those of the other lean littermates are *Fa/fa.* Two copies of the Trp-Ser-X-Trp-Ser motif, which is conserved among the class I cytokine receptors, such as a gp130, also exist in the rat OB-R cDNA (Fig. 2).

Blot hybridization of genomic DNAs of several strains of rats. Rat OB-R cDNA fragments were excised from the vector and used as a probe for blot hybridization. DNAs from several strains of rats, including Wistar and SD rats, and the Zucker (*fa/fa*) rat and its lean littermates, were digested to completion with *Eco*R I, *Hin*d III, *Bam*H I or *Pst* I, electrophoresed, and transferred to a nylon hybridization membrane. The membrane was then hybridized with the [α-^{32}P]dCTP labeled probe. As shown in Fig. 4, genomic DNA fragments of approximately 6 kb, 4 kb and 0.7 kb in the *Eco*R I digest, approximately 25 kb and 10 kb in the *Bam*H I digest, more than six DNA fragments

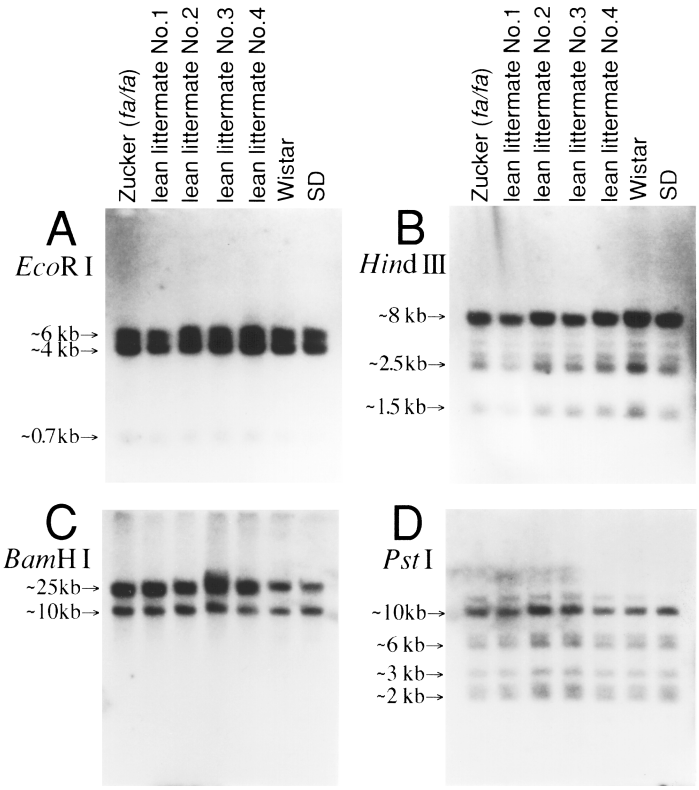

FIG. 4. Blot hybridization of genomic DNAs of various strains of rats: The rat OB-R cDNA probe was hybridized to digested genomic DNA (5μg) of each rat strain. The restriction enzymes used for digestion were *Eco*RI (A), *Hin*dIII (B), *Bam*HI (C) and *Pst*I (D).

including approximately 8 kb, 2.5 kb and 1.5 kb in the *Hin*d III digest, and, more than seven DNA fragments including approximately 10 kb, 6 kb, 3 kb and 2 kb in the *Pst* I digest hybridized with the rat OB-R cDNA probe. There was no difference in the patterns of the hybridized bands for the seven rats, and no obvious structural changes of the OB-R genes were observed in these rat strains.

Expressions levels of OB-R mRNA in various tissues of rats. Total RNAs from various rat tissues were electrophoresed, transferred to a nylon hybridization membrane, and then hybridized with the rat OB-R cDNA probe. A number of organs and tissues showed hybridization signals (Fig. 5A). Hybridization signals were clearly observed for RNAs from the brain, lung, spleen, adipose tissue, small intestine and liver. Of these, the spleen expressed OB-R mRNA at the highest level.

Expression levels of OB-R mRNA in some organs of SD and Zucker (*fa/fa*) rats and the lean littermate No. 4 of the Zucker (*fa/fa*) rat were also examined (Fig. 5B). No differences in the expression levels of OB-R mRNA were observed among brains or lungs of these rats.

DISCUSSION

One of spliced variant forms of rat OB-R cDNA with a short intracellular domain was cloned, and a difference in the nucleotide sequence between SD and Zucker (*fa/fa*) rats was found. As a result of this, the codon 269 is glutamine in SD rat and proline in Zucker (*fa/fa*) rat, respectively. This Zucker (*fa/fa*) type of amino acid sequence is linked to its obese phenotype. Obese *fa/fa* rats of both Zucker and Wistar express OB-R whose codon 269 is proline alone. The lean littermates of Zucker (*fa/fa*) and Wistar (*fa/fa*) rats express OB-R whose codon 269 is glutamine alone or glutamine/proline depending on their genotypes. We cannot presently attribute the obese phenotype

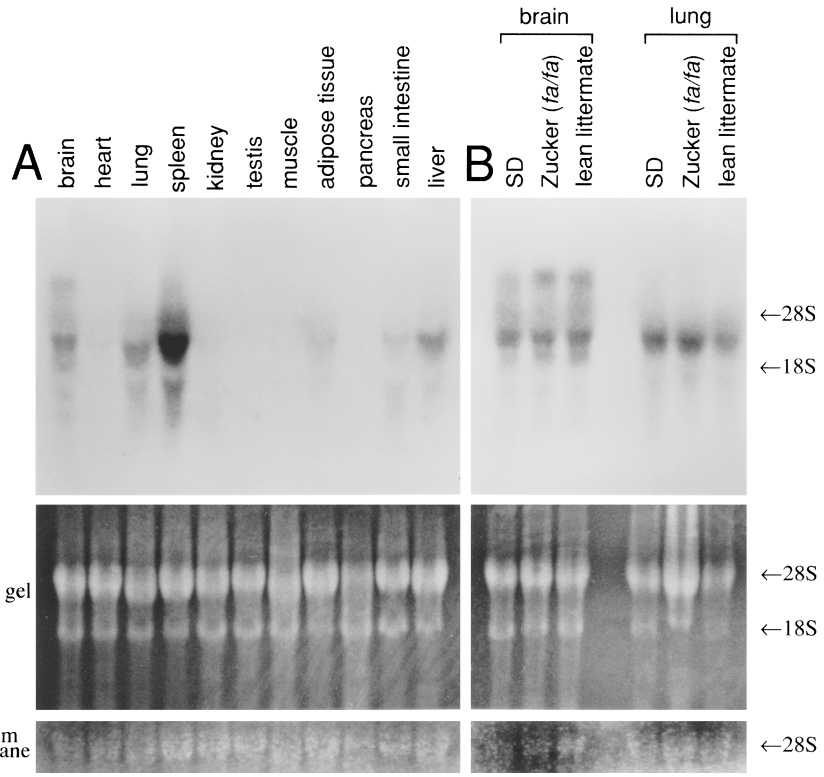

FIG. 5. Expression levels of OB-R mRNA in various rat tissues (A), and in brains and lungs of some rat strains (B): The rat OB-R cDNA probe was hybridized to electrophoresed total RNAs (13 μg) isolated from various tissues of the lean littermate No. 4 of the Zucker (*fa/fa*) rat (A) and to those from brains and lungs of SD and Zucker (*fa/fa*) rats and the lean littermate No. 4 of the Zucker (*fa/fa*) rat (B). Photographs of the ethidium bromide fluorescence of gels (gel) and nylon hybridization membranes (membrane) are shown with the autoradiograms.

of the *fa/fa* rat to this change in amino acid sequence, until expression studies of this "proline type" OB-R are performed and/or OB-R cDNAs of all the spliced variant forms, especially those with a long intracellular domain, and its chromosomal gene from the *fa/fa* rat are cloned. Because the amino acid sequences in the vicinity of codon 269 of OB-R cDNA are well conserved among rat, mouse and human, there is a possibility that this amino acid change disrupts OB-R function such as a leptin binding.

For the case of the Zucker (*fa/fa*) rat, no changes in either the gene structure or the expression levels of OB-R were found. S. C. Chua Jr. *et al*. detected a structural change in the OB-R gene of Zucker (*fa/fa*) rat by DNA blot analysis using mouse OB-R cDNA (13). Whether this contradiction is due to strain differences of the Zucker (*fa/fa*) rat is not presently known. In any case, a restriction fragment length polymorphism (RFLP) by *Hpa* II (or *Msp* I) digestion is useful to determine the genotypes of Zucker rat prior to weaning.

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