Phenotypes of Mouse *diabetes* and Rat *fatty* Due to Mutations in the OB (Leptin) Receptor

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Mice harboring mutations in the *obese* (*ob*) and *diabetes* (*db*) genes display similar phenotypes, and it has been proposed that these genes encode the ligand and receptor, respectively, for a physiologic pathway that regulates body weight. The cloning of *ob*, and the demonstration that it encodes a secreted protein (leptin) that binds specifically to a receptor (OB-R) in the brain, have validated critical aspects of this hypothesis. Here it is shown by genetic mapping and genomic analysis that mouse *db*, rat *fatty* (a homolog of *db*), and the gene encoding the OB-R are the same gene.

The autosomal recessive *diabetes* mutation (*db*) was first detected in progeny of the C57BL/KsJ strain at the Jackson Laboratory (*1*) and mapped to the middle of chromosome 4. Subsequently, the mutation has been detected at least four more times in other mouse strains, including DW/Pas (*2*), as well as in two rat strains (*3*). The phenotype of *db/db* mice, which includes severe, early-onset obesity, extreme insulin resistance, and strain-specific susceptibility to diabetes, is identical to that of *ob/ob* mice on the same strain background (*4*). The *fatty* (*fa*) gene in rats (*5*, *6*) is thought to be a homolog of *db* because *fa/fa* mutants have an identical behavioral and metabolic phenotype as *db/db* mutants and because *fa* and *db* map to syntenic chromosomal regions (*3*).

The weight loss of *ob/ob* mice that are joined by parabiosis to *db/db* mice (*4*), the failure of *db/db* mice to respond to injection of OB protein (leptin) (*7–9*), the high levels of OB mRNA in adipose tissue (*10*, *11*), and the high serum levels of OB protein in *db/db* and *fa/fa* animals (*12*) together provide compelling evidence that the *db* gene product acts distally to the OB product in the same regulatory pathway for body fat. D. L. Coleman postulated that *db* encodes a receptor for OB (*13*). The recent molecular cloning of a transmembrane receptor (OB-R) (*14*) for OB (*15*) has made it possible to test the hypothesis that the mouse *diabetes* and rat *fatty* phenotypes are due to mutations in the receptor for OB.

We developed high-resolution genetic and physical maps of the regions containing the *db* and *fa* loci using a large genetic resource including these *db* alleles (*db*, *db^{3J}*, and *db^{Pas}*). The largest crosses segregated for the *db* BKs allele (*1*) against counter-

strains DBA/2J, SM/Ckc, SM/J, and MA/MyJ (obtained from the Jackson Laboratory). We also generated crosses with *db^{3J}* (129/J) (*16*) and *db^{Pas}* (DW/Pas) (*2*) against C57BL/6J and C57BL/KsJ, respectively. For the rat studies, we used outcrosses of *fa* [segregated on the 13M strain (*5*)] with Brown Norway (Charles River Labs) and WKY rats (from H. Ikeda of Kyoto University and maintained at Vassar College). The total number of meioses was 2724 in the mice and 1028 in the rats.

All obese mice were genotyped for close-flanking markers to identify the rare recombinants. The flanking markers were established with the use of a small subset of obese mice because the placement of *db* on chromosome 4 is a well-known landmark (*17*). The recombinants defined a ~0.5-centimorgan interval for *db* between D4Mit155 (telomeric) and D4Mit277 (centromeric) (Fig. 1). A physical map of this genetic interval (Fig. 2) was constructed by aligning contiguous genomic clones from D4MIT155 with the use of yeast artificial chromosome (YAC) (*18–20*), P1 (*21*, *22*), and bacterial artificial chromosome (BAC) (*23*) genomic clones.

The centromeric end of BAC 150L24 was used to identify YAC 21E6, which had one end (21E6-F) that contained a simple sequence repeat (SSR). This SSR was heterozygous in all seven centromeric (D4Mit277) *db* recombinants from the crosses of BKsJ with SM/Ckc or MA/MyJ (Fig. 1). This YAC includes BACs 43F6 and 54K10. BAC 43F6 contains a SSR that
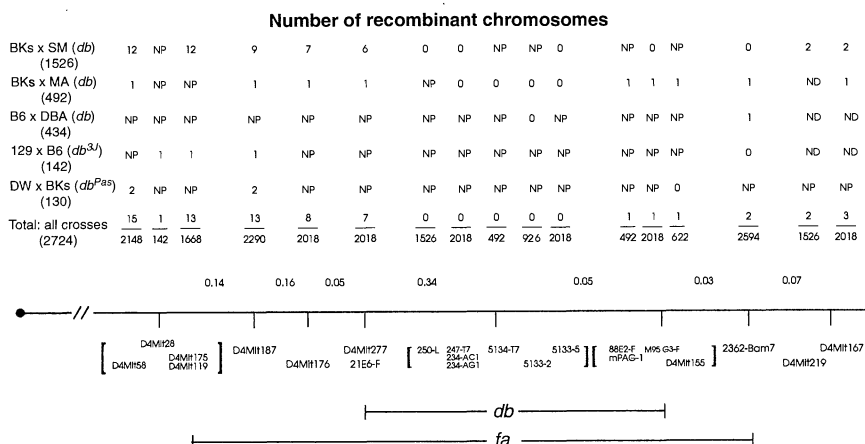
Number of recombinant chromosomes

BKs x SM (*db*) (1526)	12	NP	12		9	7	6	0	0	NP	NP	0	NP	0	NP	0	2	2
BKs x MA (*db*) (492)	1	NP	NP		1	1	1	NP	0	0	0	0	1	1	1	1	ND	1
B6 x DBA (*db*) (434)	NP	NP	NP		NP	NP	NP	NP	NP	NP	0	NP	NP	NP	NP	1	ND	ND
129 x B6 (*db^{3J}*) (142)	NP	1	1		1	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	0	ND	ND
DW x BKs (*db^{Pas}*) (130)	2	NP	NP		2	NP	NP	NP	NP	NP	NP	NP	NP	NP	0	NP	NP	NP
Total: all crosses (2724)	15 1 13				13	8	7	0	0	0	0	0	1	1	1	2	2	3
	2148	142	1668		2290	2018	2018	1526	2018	492	926	2018	492	2018	622	2594	1526	2018

Distances (centimorgans): 0.14 | 0.16 | 0.05 | 0.34 | 0.05 | 0.03 | 0.07

Markers: [D4Mit28 / D4Mit58] [D4Mit175 D4Mit119] D4Mit187 [D4Mit277 / D4Mit176 21E6-F] [250-L 247-T7 234-AC1 234-AG1] 5134-T7 [5133-5 / 5133-2] [88E2-F mPAG-1 / M95 G3-F D4Mit155] 2362-Bam7 [D4Mit167 / D4Mit219]

db interval and *fa* interval indicated below map.

Fig. 1. Genetic map of the mouse chromosome 4 region surrounding the *db* locus. The number of recombinant chromosomes is listed by F_1 intercross for each marker on the map and summed over the total number of meioses from all crosses in the bottom row. Total numbers of meioses from each cross are indicated in parentheses below the identity of the F_1 intercross. "NP" indicates that the marker was not polymorphic and therefore not informative for the cross. The order of the markers along chromosome 4 from centromere to telomere is indicated below the map and was determined by minimizing double recombinants. Markers unresolvable by genetic means are indicated with brackets. Distances between markers are indicated above the chromosome in centimorgans. The maximum interval containing *db* was determined on the basis of single recombination events. The maximum interval containing *fa* was similarly determined and is based on 8 recombinants with D4Mit119 and 12 with 2362-Bam7 in 1028 meioses. The breeding stocks of *db* on C57BL/KsJ mice, *db^{3J}* on 129/J mice, as well as on DBA/2J, C57BL/6J, MA/MyJ, SM/Ckc, and SM/J mice were obtained from the Jackson Laboratory. The colony of *db^{Pas}* on DW/Pas (*2*) was obtained from the Pasteur Institute (Paris). All mice were maintained on Purina Rodent Chow 5001 and fresh water under specific pathogen-free conditions. Obese mice were killed by CO_2 asphyxiation at 3 to 4 months of age, at which time their weights and lengths were recorded and blood was collected by cardiac puncture. The rat crosses have been described (*27*). Obesity was assessed by visual inspection by four experienced observers and was confirmed by calculation of body mass index (weight/nasoanal length^2). All genotypes were determined by PCR amplification of genomic DNA with the appropriate primer pairs (*27*). Alleles were detected by agarose or polyacrylamide gel electrophoresis of the amplified DNA directly or after restriction enzyme digestion. The DNA fragments were visualized by ethidium bromide. All obese mice were initially genotyped with informative flanking markers: D4Mit58 or D4Mit28 on the centromic side of *db* and D4Mit31, D4Mit12, or D4Mit43 on the telomeric side. All mice that were heterozygous at either side were subsequently genotyped for all informative markers.

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is nonrecombinant in the seven centromeric recombinants. The region defined by the set of 18 BACs and 1 YAC defines the chromosomal interval within which the *db* locus resides (Fig. 2). Furthermore, the YAC in this contig does not contain D4Mit277 (Fig. 1), the SSR that is closest to *db* on the centromeric side (*17*), which suggests that the apparent genetic distances in this interval may be inflated by a recombination hotspot. The *fa* mutation was flanked by D4Mit119 (8 recombinants in 1028 meioses) and by 2362-Bam7 (12 recombinants in 1028 meioses) and was nonrecombinant with 218-Sp6 (0 recombinants in 1028 meioses) (Fig. 2).

Polymerase chain reaction (PCR) primers for 5′ [3F (forward): 5′-GCTGCACTTAAC-CTGGC; 3R (reverse): 5′-GGATAACT-CAGGAACG] and 3′ untranslated region (UTR) (mapF: 5′-CACTATTTGCCCTTC-AG, mapR: 5′-GCCTGAGATAGGGGT-GC) portions of the mouse *Obr* gene were used to amplify DNA from selected BACs and YACs in the contig containing *db*. Products of the predicted size were generated from YAC 21E6 by both sets of primers. BACs 43F6 and 54K10 are fully contained within YAC 21E6 and generated products with the 5′ primers. BACs 242F3, 150L24, and 250K6 were positive for the 3′ UTR primers (see Fig. 2 for relative positions of these clones). Marker 250-L (obtained from BAC 250K6) is nonrecombinant with *db*. Marker 250-L is also contained in BACs 116P11, 242F3, and 150L24. Marker 247-T7 (an end of BAC 247C19) is nonrecombinant with *db* and is contained within three of the four BACs that are positive for 250-L (242F3, 150L24, and 250K6). The *Obr* 3′ UTR is positive for

these three BACs but not for 247C19. Therefore, the *Obr* 3′ UTR is between 250-L and 247-T7, two markers in the interval that is nonrecombinant with *db* (0 recombinants per 1526 meioses).

As constituent genomic clones were identified, we tested for the presence of large deletions or rearrangements in the DNA of the various *db* mutants by using entire BAC or P1 clones as probes on restriction digests (Bam HI, Bgl II, Eco RI, and Hind III) of genomic DNA. We suppressed the repetitive sequence hybridization by pre-annealing the labeled DNA probe with a 10,000- to 100,000-fold excess of mouse genomic DNA before hybridization to the filters. This method showed that there were no gross deletions in any of the mouse mutants, but the signal from rat DNA was too weak to interpret. The contiguous segment that was tested extends from BAC 29F3 to BAC 43F6. BAC 43F6 detected a 7.5-kb Bam HI fragment that was unique to db^{Pas}/db^{Pas}.

The genomic Southern (DNA) blots described above were probed with a mouse *Obr* complementary DNA (cDNA) corresponding to the first 837 of the 839 amino acids comprising the proposed extracellular domain of the receptor (*14*). The 7.5-kb fragment detected with BAC 43F6 was present in the Bam HI digest of db^{Pas}/db^{Pas} but not the DW+/+ (coisogenic) DNA. Thus, the alteration in the restriction fragment pattern of *Obr* in the db^{Pas}/db^{Pas} mutant must reflect the sequence variation in the region of the *Obr* gene that is mutated in db^{Pas} and produces the obese phenotype. Bgl II, Eco RI, and Hind III digests of genomic DNA generated identical numbers and sizes of fragments for db^{Pas}/db^{Pas} and DW+/+, but the relative intensities of the 5-kb Bgl II, 2-kb Eco RI, and 0.8-kb Hind III fragments were increased in the mutant (Fig. 3). In addition, the normal 3.5-kb band in the Bam HI digest was increased in intensity. We detected no restriction fragment length variations (RFLVs) or differences in hybridizing band intensities in *db* or db^{3J} DNA using this probe for the putative extracellular domain (*24*).

Comparison of genomic DNA from Zucker *fa/fa* rats and +/+ rats on Southern blots probed with the mouse OB-R cDNA also revealed different hybridization patterns. In the Bgl II digest, the *fa/fa* DNA showed loss of a 9.0-kb band and the appearance of a new 6.0-kb band; in the Bam HI digest, there was loss of a 15-kb band and the appearance of a new 14-kb band; and in the Hind III digest, there was loss of a 1.5-kb band and the appearance of a new 3.5-kb band (Fig. 3).

Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) of lung cDNA with primers 2 (nucleotides 1546 to 1576) and A (nucleotides 1268 to
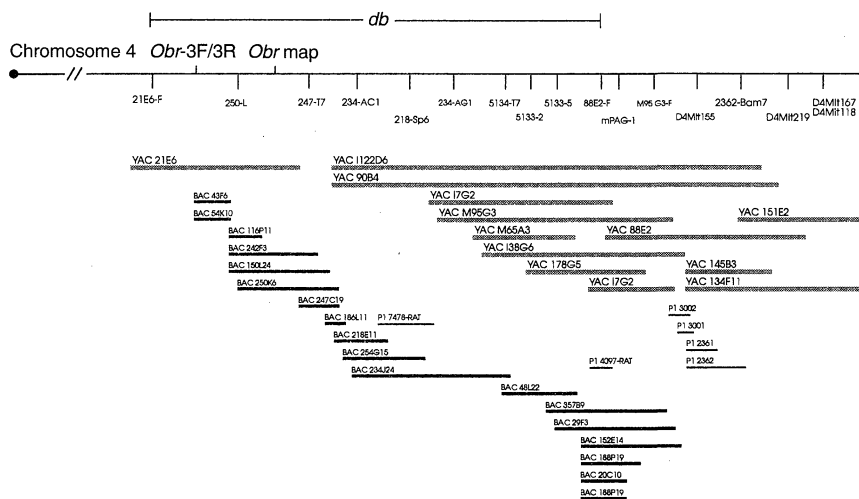

Fig. 2. Physical map of the mouse chromosome 4 region containing *db*. Genomic clones and markers were aligned by determination of sequence tagged site content. The contig is composed of YACs, BACs, and P1s. Two rat genomic clones are shown; all others are mouse clones. The maximum interval containing *db* is indicated above the physical map. Positions of the 5′ (*Obr*-3F/3R) and 3′ (*Obr* map) ends of *Obr* relative to genetic markers and within the interval containing *db* are indicated above the contig. All libraries were ordered or semi-ordered arrays that were screened by a top-down PCR approach. YAC clones (*18–20*) were identified by unique addresses. P1 (*21, 22*) and BAC (*23*) clones were identified by a secondary hybridization of a subpool of candidate clones. Positive clones were colony-purified and verified by colony PCR. Techniques for subcloning of the BAC and P1 clones as well as identification of SSR motifs, sequencing of plasmids, and development of primer pairs flanking SSRs have been described (*27*). End fragments were obtained by a modified ligation-mediated PCR technique (*28, 29*) involving four steps: (i) primer extension; (ii) restriction enzyme digestion; (iii) linker ligation; and (iv) amplification. Briefly, primer extension was performed by boiling DNA in the presence of the vector-specific primers (0.25 ng/μl) in Taq buffer, cooling to 94°C (at which point Taq DNA polymerase was added), annealing the primers at the appropriate temperature, and extending the primer at 72°C for 5 to 10 min. One microliter of the primer extension product was used for restriction enzyme digestion in a volume of 10 μl. Usually, six enzymes were used: Dra I, Hae III, Pvu II, Rsa I, Sca I, and Stu I. After 30 to 60 min at 37°C, 1 μl of the digest was used to ligate to pre-annealed double-stranded linkers (JRsa12 and JCla24 at concentrations of 0.5 ng/μl and 1.0 ng/μl) with T4 DNA ligase at room temperature (JRsa12 = ATGTTCATGGATAGT; JCla24 = CCGACGTCGACTATCCATGAACAT). After 30 to 60 min, 1 μl of the ligation reaction was used for PCR amplification for 35 cycles. For P1 and BAC clones, the vector primers were based on the Sp6 and T7 promoter sequences flanking the cloning sites. For YAC clones, a set of nested primers was used, with the initial primer extension primers based on the pBR322-derived sequence and the PCR primers based on the transfer RNA–derived sequence. When multiple fragments were amplified, an unrelated clone or host *Escherichia coli* or *Saccharomyces cerevisiae* DNA was used to identify fragments uniquely derived from the desired clone. Fragments were isolated by gel electrophoresis, reamplified, purified by gel electrophoresis and captured on diethylaminoethyl paper, and sequenced with Taq DNA polymerase.

995

1285) (*14*) indicated that the levels of OB-R mRNA in *db^Pas* mice were at most one-twentieth of that in DW/Pas mice (Fig. 4). Similar results were obtained with whole brain cDNA RT-PCR and Northern (DNA) blot analysis of lung, whole brain, liver, kidney, adrenal, hypothalamus, ovary, and heart tissue probed with the putative extracellular domain of the mouse OB-R cDNA (*24*).

The *db* and *fa* mutations and the *Obr* gene have all been mapped to a physical contig that encompasses only ~600 kb of DNA. The 3' UTR of the *Obr* gene maps to the nonrecombinant region of the contig because none of the seven meiotic recombinations of *db* with D4Mit277 is as close to the telomere as marker 250-L from BAC 250K6.

The increased intensity of a single band in *db^Pas* with each of four restriction enzymes in the Southern blots probed with OB-R cDNA suggests that this mutation is due to a partial duplication of the coding sequence for the putative extracellular domain of the *Obr* gene. The existence of the extra 7.5-kb fragment in the Bam HI digest of *db^Pas* DNA is consistent with this possibility. Because the *db^Pas* and DW/Pas mice are otherwise genetically identical, the detection of these differences in band pattern and intensity strongly suggests that the *db* phenotype results from disruption of the *Obr* gene. The reduction of OB-R mRNA in the *db^Pas* mice could be due to the effects of the apparent duplication on transcription or mRNA stability. The RFLV patterns of the Bgl II, Bam HI, and Hind III digests of *fa/fa* genomic DNA suggest that the *fa* phenotype is the result of a small insertion or deletion in the *Obr* gene.

On the basis of our mapping data and mutation analysis, we conclude that *db*, *fa*, and *Obr* are the same gene. The determination of the molecular genomic organization of the *Obr* gene, its expression characteristics, and the exact nature of the mutations in the various mouse and rat alleles are goals of ongoing analysis. The precise mechanisms by which the mutations described here—and those that must also be present in the *db*, *db^3J*, and *fa^k* animals—produce the apparent loss of function of OB-R remain to be defined. The absence of gross structural varia-tion in *db* or *db^3J* makes it more likely that these alleles are the result of point muta-tions. Such mutations may provide additional evidence about the domains of the receptor necessary to transduce the OB signal or the relevant tissues of expression if the mutation affects only a subset of the alternatively spliced forms. Likewise, it will be of great importance to assess the possible role of mutation and allelic variation at this locus in the regulation of body fat content in humans (*25*). Synteny relations between the rodent and human genomes suggest that the human homolog of *Obr* (*14*) is likely to reside on chromosome 1p31 (*26*).

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Fig. 3. Genomic Southern blot analysis of *db^Pas* and *fa*. Southern blots were probed with a cDNA encoding the proposed extracytoplasmic domain of the mouse OB-R. Samples from *db^Pas/db^Pas* mice and a lean littermate of the coisogenic strain, DW/Pas, are shown in the top panels, and samples from +/+ and *fa/fa* rats in the lower panels. Arrows indicate novel size bands or normal size bands of greater intensity for *db^Pas*, and the absence of normal size bands and their replacement with aberrantly sized bands for *fa*. A Southern blot with *db^Pas/db^Pas*, *db^Pas/+*, and +/+ DNAs replicated the results shown, making it unlikely that the restriction digestions were incomplete. Southern blots of YAC 21E6 and BAC 43F6, 54K10, 116P11, 242F3, 150L24, and 250K6 have the same sizes and number of bands observed in the mouse genomic Southern blots, which indicates that the hybridizing fragments map back to the interval containing *db*. DNA from mouse or rat (10 μg) was digested with the appropriate restriction enzyme for 8 hours and transferred to GeneScreen Plus membrane. A λ Hind III ladder was used to determine fragment sizes. The probe (10 to 50 ng) was labeled by random hexamer priming in the presence of [α-^{32}P]deoxycytosine triphosphate in a 25-μl reaction. The labeling reaction was heated to 65°C to inactivate DNA polymerase. The probe was added to pre-warmed hybridization solution containing 5× saline sodium citrate (SSC), 1% SDS, and 5% polyethylene glycol 8000. Hybridization was for at least 18 hours at 65°C. The filters were washed twice at 65°C in 0.4× SSC and 1% SDS and then exposed to film with intensifying screens at −70°C.
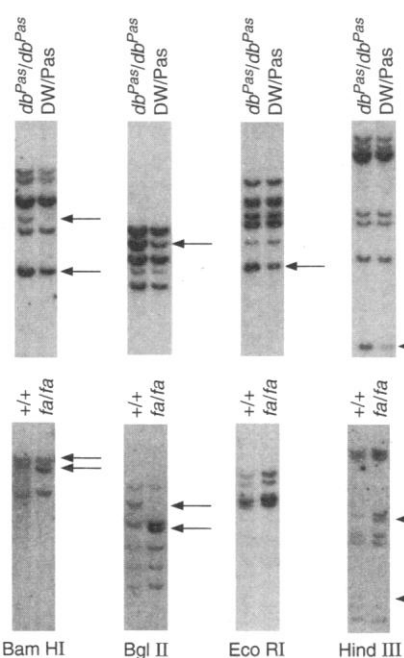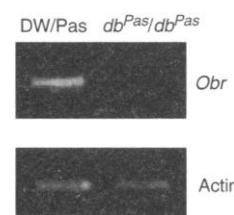
Fig. 4. Semi-quantitative RT-PCR of the *Obr* gene in *db^Pas* mice. After determining the linear range of amplification for each set of primers by varying the number of cycles six times between 15 and 30 cycles for *Obr* and between 15 and 25 cycles for actin, we amplified lung double-stranded cDNA for 25 cycles with *Obr* primers 2 (5'-CAGATAATAGAAAGATTG-GCTGGA) and A (5'-TATGACGCAGTGTACTGCTGCAAT), producing a 309-bp product, and for 20 cycles with actin primers, producing a 219-bp product. PCR products were subjected to electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Similar results were obtained from multiple preparations of whole brain RNA (*24*).