Genetic Typing of the Mouse ob Mutation by PCR and Restriction Enzyme Analysis

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Abstract: A genetic typing method for the mouse obese (ob) mutation by PCR and restriction fragment length polymorphism (RFLP) analysis was developed. Three genotypes (ob/ob, ob/+ and +/+ ) of the ob mouse are rapidly differentiated with this assay. Since only a small biopsy specimen (tail end) is necessary for the genotyping, the ob mouse at any age is typable and can be used in subsequent breeding or research. Obese homozygotes (ob/ob) were efficiently produced by mating heterozygotes (ob/+ ) only, which were selected by using the PCR-RFLP assay.

Key words: genotyping, ob mouse, PCR-RFLP

The mouse obese (ob) mutation is an autosomal recessive mutation that occurred spontaneously in a non-inbred mouse stock at the Jackson Laboratory [8]. The mutation was then established, and has been maintained, in the C57BL/6J (B6) strain. Homozygous (ob/ob) animals develop profound and progressive obesity that is first recognizable at 4 weeks of age. At the same time, the obese animals manifest diabetic syndromes, whose progress is known to be strikingly dependent on the genetic background of the mouse strain; in B6-ob congenic mice, the disease is mild, and characterized by hyperphagia, transient hyperglycemia, marked hyperinsulinemia and islet hypertrophy [1, 4]. Since homozygous animals of both sexes are infertile, they are obtained by mating known heterozygotes (ob/+ ). However, genotypes of lean animals (ob/+ and +/+ ) are apparently indistinguishable, and heterozygotes can be discriminated from +/+ littermates by progeny test only. Breeding of the obese mouse is therefore laborious and time-consuming.

The ob mutation is a single gene mutation. The gene is mapped to proximal mouse chromosome 6 [5, 6], and has been recently cloned by Zhang et al. [9] by positional cloning. The essence of the mutation is a point (C →T) mutation that results in the change in an arginine residue at position 105 to a stop codon [9]. Based on the sequence data, we have developed a genetic typing method in this study. Three genotypes (ob/ob, ob/+ and +/+ ) are clearly and rapidly differentiated by the PCR amplification of genomic DNA and subsequent restriction fragment length polymorphism (RFLP) analysis.

The B6-ob congenic mouse strain was obtained from Wakayama Medical College in 1974, and has since then been maintained at our institute under specific pathogen-free conditions. Genotypes of the lean animals for DNA preparation were determined by progeny test. DNA samples for the PCR were prepared from tail biopsy samples.
specimens of B6-ob mice as well as four inbred strains (C3H/He, CTS, FLS and NOD) that are also maintained at our institute. About 0.5 cm of mouse tail was cut off and digested with proteinase K. Nucleic acids were extracted with a DNA isolation kit (QIAamp Tissue Kit; QIAGEN GmbH, Hilden, Germany). After the extraction and washing, they were eluted in 200 µl of distilled water. A two µl aliquot of the eluate (approximately 100–200 ng of genomic DNA) was used for the PCR.

Oligonucleotide primers for the PCR were synthesized by Japan Bioservice Inc. (Saitama, Japan). DNA amplification and restriction enzyme analysis were performed as described elsewhere [7]. A total volume of 20 µl reaction mixture in 0.5 ml microfuge tube was overlaid with one drop of mineral oil, and placed in a thermal cycler (Thermal Sequencer TSR-300; Iwaki Glass Co., Tokyo, Japan). The mixture was subjected to the PCR with an initial incubation at 94℃ for 1.5 min, 30 cycles of amplification (one cycle represents denature at 94 ℃ for 1 min, annealing at 55℃ for 1 min and extension at 73 ℃ for 1 min) and a final incubation at 73℃ for 5 min. Restriction enzymes used for RFLP analysis were BsoFI, Cfr131, Ddel, HaeIII, HinfI and SceFI. The PCR product (8 µl) was digested with 2–4 units of the enzymes for 1.5–2 hr. Digested and control PCR products were electrophoresed in 4% agarose gels, and photographed after ethidium bromide staining [7].

In a preliminary test, six primer pairs designed to amplify a portion (about 80–150 base pairs) of the ob gene containing the C→T mutation at nucleotide (nt) position 428 were used [9]. The sequences of primers were selected from the mouse ob cDNA sequence between nt positions 330 and 480. Then DNA samples prepared from B6-ob mice of three genotypes were amplified with these primers. In each amplification, though the exon-intron structure of the mouse ob gene has not been reported, the three samples yielded the PCR products of the same size that was expected from the nt sequence of mouse ob cDNA, suggesting that this region is included in a single exon. Of the six primer pairs tested, one pair (Fig. 1) that amplified the samples most efficiently was selected and used in subsequent studies. The concentration of MgCl₂ in the reaction mixture for this primer pair was adjusted to 1 mM.

Initially, DNA samples of B6-ob (three genotypes), C3H/He, CTS, FLS and NOD mice were amplified with this primer pair. All the PCR products were of the same size. After digestion with a restriction enzyme BsoFI, Cfr131, HaeIII, HinfI or SceFI (one or two restriction sites of each enzyme should be present in the amplified region), they generated migration patterns expected from the nt sequence of mouse ob cDNA. SceFI-digested products of B6-ob mice are shown in Fig. 2 as examples. After Ddel digestion, however, the PCR products for B6-ob mice of three genotypes generated different migration patterns (Fig. 2). The three patterns were easily distinguished from each other by the number of fragments, because only the mutant allele has a Ddel restriction site in the amplified region (Fig. 1). The migration pattern of PCR products for other mouse strains was the same as that of normal homozygote (+/+) as well as that of undigested controls.

Next, about 1,000 DNA samples of B6-ob mice, most of which were lean and younger than 10 weeks of age,
were tested to select heterozygotes for breeding. All the mice were typable by the PCR-RFLP assay with restriction enzyme DdeI, and the genotypes (ob/+ or ob/ob) were consistent with the breeding results or development of obesity, confirming the reliability of this assay. Although most of the homozygotes were detectable at 4 weeks of age, a small number of lean animals at 4–5 weeks of age were typed as ob/ob, and then they developed obesity at 5–6 weeks of age. For this reason, we select heterozygotes for breeding when lean animals are 6–7 weeks of age. The breeding method for the ob mouse was improved by mating heterozygous animals only, which were selected by means of the PCR-RFLP assay. Formerly, two or three lean females were mated with one heterozygous male. The females with no obese offspring in their first three litters were judged as possible normal homozygotes, and eliminated after their third birth. Since +/+ females were always used in breeding, the segregation ratio of the obese mouse was significantly lower than 1:3 ($\chi^2$ test, $P<0.001$). After the improvement, however, the ratio agrees with the expected ratio of 1:3 ($\chi^2$ test, 0.3<$P<0.5$) (Table 1).

![Fig. 2. Genotyping of B6-ob mice by means of the PCR-RFLP assay. The PCR products (lanes 1–4: undigested controls, lanes 5–7: DdeI digestion, lanes 8–10: ScaI digestion) were analyzed on 4% agarose gels. Lane M: DNA size marker; lanes 1, 5 and 8: normal homozygote (+/+); lanes 2, 6 and 9: obese homozygote (ob/ob); lanes 3, 7 and 10: heterozygote (ob/+); lane 4: negative control.](image)

| Table 1. Reproduction results for C57BL/6J-ob mice |
|---------------------------------|-------------------|-------------------|
|                                 | September, 1994–   | July–December,    |
|                                 | February, 1995b    | 1995b             |
| No. of births                  | 239               | 793               |
| No. of offspring               | 1035              | 3999              |
| Mean litter size               | 4.6               | 5.0               |
| No. of weanings (%)            | 892 (81.5)        | 3385 (84.6)       |
| No. of obese weanings (%)      | 141 (15.8)        | 769 (22.7)        |

a: Two or three females (ob/+ or +/) were mated with one male (ob/+).
b: Monogamous mating of heterozygotes. c: No. of obese weanings/No. of weanings.
Another significance in the development of the genetic typing method is probably its contribution to the research field. In the experiments with \textit{ob} mice, control animals for homozygotes are usually lean littermates. They are used as +/+ animals without genetic typing in most cases, because the progeny test is time-consuming and not suitable for young animals. It has already been reported, however, that heterozygotes and normal homozygotes (+/++) can give different results to each other in carefully designed experiments [2, 3], which fact indicates that incorrect conclusions may be obtained when the genotype of control animals remains +/+. In addition, genotyping itself is impossible until the animals are at least 4 weeks old, even for homozygotes. The experiments using neonatal and weanling \textit{ob} mice are therefore often difficult. Through the development of our typing method, however, genotyping of the \textit{ob} mouse at any age has become possible with only a small biopsy specimen. The three genotypes can be differentiated in 2–3 days with the PCR-RFLP assay, a simple genetic typing method. These advantages will greatly contribute to research on obese mice.

Note added in proof: While this paper was being reviewed, a PCR assay for the mouse \textit{ob} mutation, which is similar to our PCR-RFLP assay, was reported by Chehab \textit{et al.} (Nature Genetics 12: 318–320, 1996).

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\section*{References}