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methods

A rapid, microplate SNP genotype assay for the *leptin*^{ob} allele[S]

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Abstract **A nonsense mutation in the mouse *leptin* gene causes genetic obesity. As a result of extensive research in the field of obesity, the use of *leptin*^{ob} mice is widespread. This mutation renders mice sterile, creating the need to breed heterozygous mice. For this reason, *leptin*^{ob} genotyping is necessary. To date, gel-based assays have been used for genotyping. Using the Invader Plus® assay for single nucleotide polymorphism (SNP) detection, we have developed a gel-free microplate SNP assay for genotyping *leptin*^{wt} and *leptin*^{ob} alleles.—**Oler, A. T. and A. D. Attie. **A rapid, microplate SNP genotype assay for the *leptin*^{ob} allele. *J. Lipid Res.* 2008.** 49: **1126–1129.**

Supplementary key words obesity • obese • single nucleotide polymorphism

Leptin is a 167 amino acid peptide hormone produced primarily in adipose tissue. It binds to a cell surface receptor, where it elicits a variety of physiological responses. It is a potent suppressor of appetite when it signals in the hypothalamus.

Leptin was discovered by positional cloning of a locus underlying the morbid obesity of the *obese* mutant mouse (1), a spontaneously occurring mutant that was discovered at the Jackson Laboratory in 1950 (2). This mouse has a nonsense mutation in the *leptin* gene, leading to a null allele. The *obese* mouse is widely used in studies of obesity and diabetes. In fact, it is the most widely used animal model of obesity in the entire Jackson Laboratory collection.

The pioneering studies of Coleman and others (3–5) showed that certain mouse strains become diabetic when made obese through the introgression of the *leptin*^{ob} allele. These studies have inspired numerous investigators to combine the *leptin*^{ob} allele with other mutations to gain new insights into the molecular, genetic, and physiological processes underlying resistance and susceptibility to obesity-induced type 2 diabetes (6, 7). This strain is also used extensively in nutritional and pharmacological studies aimed at the treatment of obesity and diabetes.

Manuscript received 2 January 2008.

Published, JLR Papers in Press, February 13, 2008.
DOI 10.1194/jlr.D800002-JLR200

The *leptin*^{ob} allele has a C→T mutation that changes an arginine codon to a stop codon at position 105 (1). At present, the wild-type and mutant alleles are most commonly genotyped by restriction endonuclease digestion of a PCR-amplified sample of genomic DNA, followed by agarose gel electrophoresis (8, 9). This procedure takes ~8 h to perform, of which ~1 h involves hands-on time. Because of the need for gel electrophoresis, the length of this procedure increases considerably with the number of samples. An alternative method, single-stranded conformational polymorphism analysis, also involves gel electrophoresis and is quite labor-intensive (10).

We have developed a genotyping assay for *leptin*^{wt} and *leptin*^{ob} alleles that eliminates the need for gel electrophoresis, using the Invader Plus® assay for single nucleotide polymorphism (SNP) detection (11). The presence of the targeted SNP yields a fluorescent signal that can be quantitated in a standard plate reader. The entire process takes 0.5 h hands-on and a total of 2.5 h and can be scaled to hundreds of samples with minimal additional time.

MATERIALS AND METHODS

Genomic DNA extraction

One centimeter tail clips from mice were digested overnight at 55°C in 300 μl of tail buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 μg/μl proteinase K). The digests were brought to room temperature, and 100 μl of Gentra Puregene Protein Precipitation Solution (Qiagen) was added. Samples were vortexed for 5 s and incubated on ice for 20 min. Samples were spun in a microcentrifuge at 4°C for 5 min at maximum speed. Supernatants were poured into a new tube containing 300 μl of isopropanol. Samples were inverted 20 times, or until a string of precipitated DNA was visible. Samples were spun again at room temperature for 2 min. Supernatants were poured off, and 300 μl of 70% ethanol was added. Tubes were gently inverted once and spun again at room tem-

Abbreviations: FAM, fluorescein; FRET, fluorescence resonant energy transfer; RFU, relative fluorescence units; SNP, single nucleotide polymorphism.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data.

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perature for 2 min at maximum speed. Again, the supernatants were poured off, with care to not lose the DNA pellets. Samples were spun at room temperature for 2 min at maximum speed. The supernatants were removed with a pipette tip, and the samples were air-dried for 30 min at room temperature. After 30 min, 200 μl of 1× Tris-EDTA was added to each pellet, and samples were stored overnight at 4°C before use. In preparation for the Invader Plus® assay, genomic DNA samples were first diluted on a 96-well plate: 4 μl (~200 ng to 1 μg of DNA) of genomic DNA into 120 μl of water (final volume, 30×). Samples were carefully mixed by pipetting to avoiding cross-contamination of wells.

DNA sequences

All sequences are shown 5′ to 3′. The target DNA sequences for *leptin* (NM_008493) surrounding the SNP are GATGGAGGAGGTCTCgGAGATTCTCCAGGTCATTGGT for *leptin*^{wt} and GATGGAGGAGGTCTCaGAGATTCTCCAGGTCATTGGT for *leptin*^{ob}, with the SNP nucleotide in lowercase. Primer sequences used to amplify this target region are Forward (CAGGTCCTCACCAGCCTGCCTTCC) and Reverse (CAGGGAGCAGCTCTTGGAGAAGGCC). The Invader® oligonucleotide sequence is AATGACCTGGAGAATCTCA. The Invader® probe sequences are as follows: for *leptin*^{wt}, gacgcggagCGAGACCTCCT; for *leptin*^{ob}, cgccgaggTGAGACCTCCT, with the 5′ flap region in lowercase.

Invader Plus® Assay

A *Leptin* probe/primer mix was made with 2.0 μM each of Forward and Reverse primers, 2.5 μM each of *leptin*^{wt} and *leptin*^{ob} probes, and 0.25 μM Invader® oligonucleotide. An Invader Plus® core kit containing fluorescence resonant energy transfer (FRET) mix, Invader Plus® $MgCl_2$, Cleavase® VIII, and no-target control was purchased from Third Wave Technologies, Inc. For the Invader Plus® assay, hard-shell skirted 96-well plates with conical bottoms were used (Bio-Rad). To each well, 7.7 μl of cocktail was added. This cocktail contains 3 μl of *Leptin* probe/primer mix, 3 μl of FRET mix, 0.375 μl of Invader Plus® $MgCl_2$, 0.3 μl of 1 mM deoxynucleoside triphosphates, 1 μl of Cleavase® VIII, and 0.06 μl of Hot Start *Taq* DNA polymerase (Fermentas) per sample. Then, 7.3 μl of each genomic DNA sample that was diluted and mixed on the 96-well plate was added to each well. The 15 μl cocktail/genomic DNA mixture was overlaid with 15 μl of mineral oil. To each plate, one sample each of *leptin*$^{+/+}$,

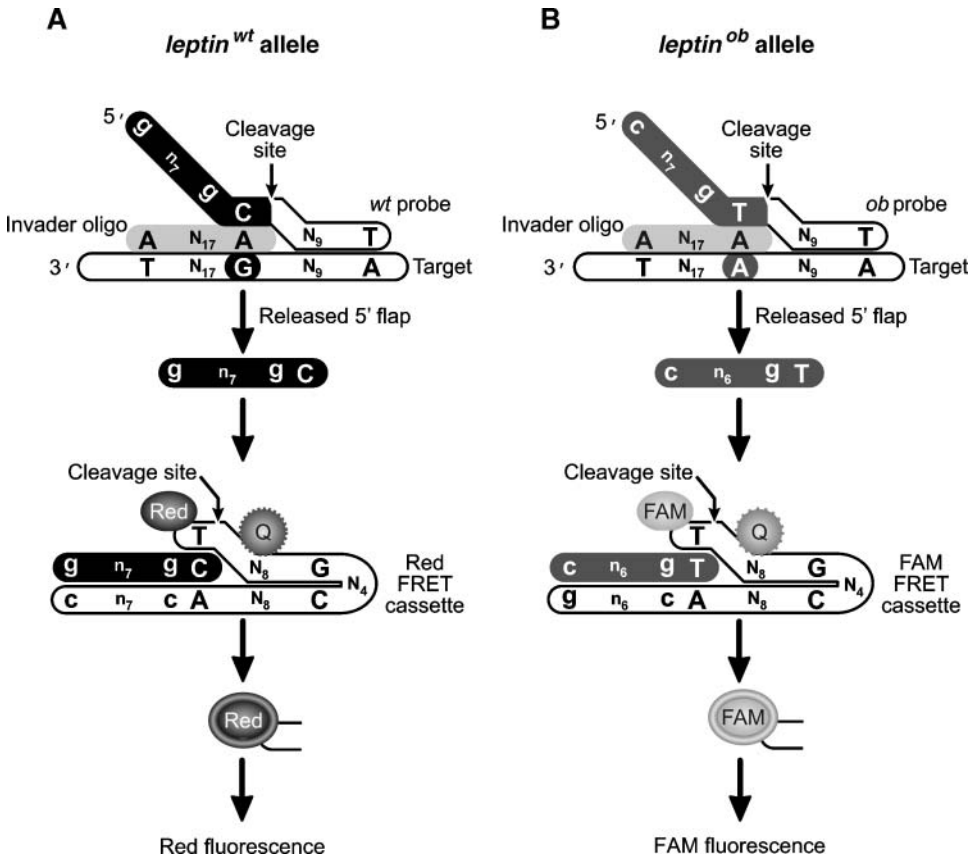

Fig. 1. Invader Plus® assay for *leptin* single nucleotide polymorphism (SNP) genotyping. After amplification of the target region for the *leptin*^{wt} allele (A) and for the *leptin*^{ob} allele (B), the Invader® oligonucleotide anneals to the target DNA. The respective probes anneal to the target DNA. This creates a mismatch in a DNA triplex (CAG and TAA for *leptin*^{wt} and *leptin*^{ob}, respectively), which allows cleavage by Cleavase®. The 5′ flap of the probe is released and anneals to its specific fluorescence resonant energy transfer (FRET) cassette. This creates a new DNA triplex (TCA and TTA for *leptin*^{wt} and *leptin*^{ob}, respectively), which is also a substrate for cleavage. This cleavage reaction removes the quench from the fluorophore, resulting in a fluorescent signal. Bases in lowercase represent DNA sequence not complementary to the *leptin* target DNA, and bases in uppercase not involved in the DNA triplexes are for the orientation of DNA binding. N_x and n_x represent the number of bases in between. FAM, fluorescein.

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leptin$^{ob/ob}$, and *leptin*$^{ob/+}$ known genomic DNA and three no-target controls were added. The plate was run in a thermal cycler with the following protocol: 2 cycles of 95°C for 2 min, 65°C for 1 min, and 72°C for 1 min; 33 cycles of 95°C for 15 s, 65°C for 1 min, and 72°C for 30 s; and one cycle of 99°C for 10 min, 50°C for 15 min, and 10°C hold.

The plate was read on a Tecan Ultra 384 fluorescent plate reader with multilabeling capability. To detect the *leptin*^{ob} allele, a fluorescein (FAM) excitation/emission wavelength of 485/535 nm was used, and for the *leptin*^{wt} allele, a Red excitation/emission wavelength of 570/620 nm was used. Data are represented as relative fluorescence units (RFU). A *z* optimization was initially done to determine the best *z* position and mirror setup for each excitation/emission wavelength.

Calculations

Data were analyzed according to the following calculation:

$$\text{FAM/Red ratio} = \frac{\dfrac{\text{RFU}_{\text{FAMsample}}}{\text{RFU}_{\text{FAMaveragenotargetcontrol}}} - 1}{\dfrac{\text{RFU}_{\text{Redsample}}}{\text{RFU}_{\text{Redavergenotargetcontrol}}} - 1}$$

If the FAM/Red ratio is <0.5, the genotype is *leptin*$^{+/+}$. If the ratio is >0.5 but <15, the genotype is *leptin*$^{ob/+}$. If the ratio is >15, the genotype is *leptin*$^{ob/ob}$.

RESULTS

We developed two genotyping assays, for the *leptin*^{wt} and the *leptin*^{ob} alleles. Two primers were designed to amplify the target region containing the SNP. One Invader® oligonucleotide was made that is complementary to the target region 5′ of the SNP while containing a mismatch at the terminal 3′ end. Two Invader® probe oligonucleotides were made, one containing the *leptin*^{wt} nucleotide and one containing the *leptin*^{ob} nucleotide. These probe oligonucleotides are complementary to the target region at the SNP nucleotide and 3′ of the SNP, but they are not complementary 5′ of the SNP. Instead, this 5′ flap is complementary to a specific FRET cassette. During the 50°C incubation at the end of thermal cycling, the Invader® oligonucleotide and the probe oligonucleotide anneal to their complementary target DNA, creating a mismatch triplex of DNA at the SNP nucleotide (**Fig. 1**). This DNA triplex is recognized and cleaved by Cleavase® 3′ of the SNP nucleotide, releasing the 5′ flap of the probe oligonucleotide containing the SNP nucleotide at the terminal 3′ end. Allele-specific 5′ flaps will be released for each *leptin*^{wt} or *leptin*^{ob} allele. These 5′ flaps will anneal to their specific FRET cassettes, creating another mismatch triplex of DNA, creating a substrate for Cleavase®. Cleavage releases the quencher from the fluorophore. Applying thermal cycling, the reaction and signal are amplified. We used a FRET cassette with Red fluorophore for the C nucleotide (*leptin*^{wt} allele; Fig. 1A) and a FRET cassette with FAM fluorophore for the T nucleotide (*leptin*^{ob} allele; Fig. 1B). The assay measures all three possible genotypes: Red signal only (*leptin*$^{+/+}$), FAM signal only (*leptin*$^{ob/ob}$), and both Red and FAM signals (*leptin*$^{ob/+}$).

We obtained *leptin*$^{+/+}$, *leptin*$^{ob/ob}$, and *leptin*$^{ob/+}$ mice (n = 7 in each group) from our breeding colony. They were genotyped using single-stranded conformation polymorphism analysis. The samples were then subjected to the Invader Plus® assay, and the fluorescence was detected on a plate reader (**Fig. 2**). The FAM RFU corresponding to the *leptin*^{ob} allele are shown in Fig. 2A. The Red RFU corresponding to the *leptin*^{wt} allele are shown in Fig. 2B. The FAM/Red ratios corresponding to the genotype are shown in Fig. 2C.The mean FAM/Red ratios for the *leptin*$^{+/+}$, *leptin*$^{ob/+}$, and *leptin*$^{ob/ob}$ mice were 0.07 ± 0.02, 1.4 ± 0.2, and 41 ± 10 ($P < 0.0001$ by one-way ANOVA). Ratio thresholds were determined empirically by evaluat-

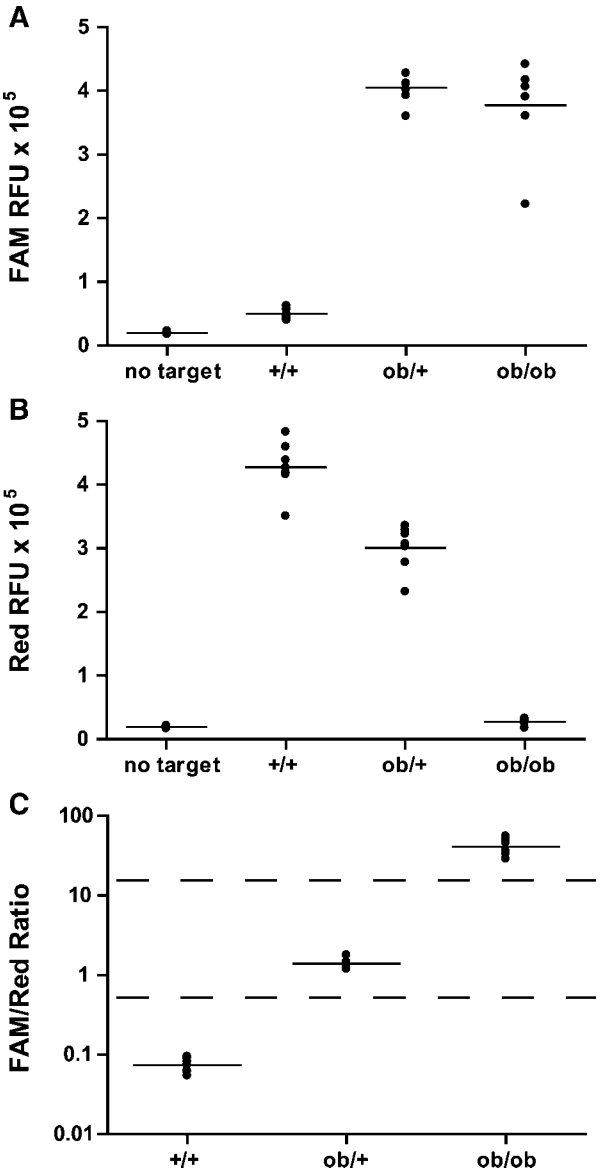

Fig. 2. Genotyping of *leptin* alleles. FAM relative fluorescence units (RFU) are shown in A and Red RFU are shown in B. The FAM/Red ratio is shown in C. In C, the thresholds used to distinguish the ob/ob from the ob/+ and the ob/+ from the +/+ are shown as dashed lines. All values are for seven different mice tested on seven individual assays, with a line indicating the mean. For the FAM/Red ratio in C, $P < 0.0001$ by one-way ANOVA.

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ing the lowest ratio for *leptin*$^{ob/ob}$ and the lowest ratio for *leptin*$^{ob/+}$ (Fig. 2C). There were no ambiguous results within the samples tested. These ratio thresholds can change if there is a differential decrease in fluorescence signal strength. This can occur with time and light exposure. An Excel template was used for ease in the analysis of genotypes. This template and a detailed protocol are available in the supplementary material.

DISCUSSION

We have developed and validated a gel-free, microplate fluorescence-based genotyping assay for the wild-type and *obese* alleles of *leptin*. This method is extremely reliable, is able to identify heterozygotes unambiguously, and is quite rapid. The method is amenable to automation and can easily be scaled to perform large numbers of assays in parallel. When this method was used to test our unknown samples, the failure rate was <1%. A failure will not result in a genotyping error because it results in the absence of both the FAM and Red signals. A single plate can handle 90 samples plus controls. Because of the extensive use of *leptin*^{ob} mice, this method is an invaluable tool.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-066369 and DK-058037.

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