



A high resolution genetic mapping of the faded (*fe*) gene to a region between *D10mit156* and *D10mit193* on mouse chromosome 10

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The C57BL/6J-*fe/fe* mouse is a coat color mutant. The coat color of the homozygote mouse becomes progressively lighter with advancing age. The faded gene (*fe*) of C57BL/6J-*fe/fe* was mapped in a 2.0 cM distal to *D10mit191* by our group. To make a high-resolution map, we used the Korean wild mouse (KWHM) for a backcross panel, which was captured in 1995 and has been maintained as an inbred line by our laboratory. In the inter-specific backcross panel (N=400), the *fe* gene was mapped to 1.0 cM distal to *D10mit156*. The gene order was defined: centromere -*D10mit3/85* (1.3±0.6 cM)-*D10mit155* (1.3±0.6 cM)-*D10mit191* (2.0±0.7 cM)-*D10mit156* (1.0±0.5 cM)-*fe-D10mit193* (1.3±0.6 cM)-*D10mit54* (1.0±0.5 cM)-*D10mit44* (8.5±1.4 cM)-*D10mit42* (10.0±1.5 cM). The measured distance between *D10mit191* and *D10mit 44* differed in both inter-specific (DBA/2) and intra-specific (KWHM) backcross panels (14.2 vs 13.8 cM). Taken together, our high-resolution linkage map of the *fe* locus from an intra-specific backcross panel will provide a good entry point to isolate the *fe* gene.

Key words: Linkage analysis, hair color, faded mouse, haplotype analysis

Received 13 February 2013; Revised version received 28 February 2013; Accepted 2 March 2013

The mouse is a useful mammalian model for coat color research because of its high number of coat color mutations. Nearly 130 different genes with 1,000 different alleles in the mouse are known to affect coat color [1]. These coat color mutations are useful as visible genetic markers for genetic analysis and the generation of mouse knockout mutations. Variations of coat color are determined by combinations of four basic loci (A-D) in the mouse [2].

A number of coat color genes that correspond to human genetic diseases have now been cloned [3]. These mouse mutations have improved our knowledge of the mechanisms of pigmentary abnormalities in humans. Dominant spotting (W) [4], microphthalmia (mi) [5], piebald (s) [6], and lethal spotting (ls) [7] mutants are animal model for piebaldism [8], Waardenburg syndrome

[9], Hirschsprung disease [10], and Shah-Waardenburg disease [11] in humans, respectively. Mutations at albino (c) locus are responsible for Oculocutaneous albinism I [12] in humans. Brown (b) [13], pink-eyed dilution (p) [14], and recessive yellow (e) [15] mouse mutants are useful animal model for Oculocutaneous albinism III [16], Oculocutaneous albinism II [17], and red hair [18] respectively in humans.

The C57BL/6J-*fe/fe* mouse is a coat color mutant that was first described by Oh [19,20]. It occurred as a spontaneous mutation in the KSB inbred strain at Nagoya University. An autosomal recessive gene transmits this mutation with 100% penetrance. Homozygotes can be distinguished at 14 days of age by a slight dilution of coat color but are more easily classified at 30 days. The coat color appears faded (black becoming gray) and the

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under fur is white (the proximal region of hairs is white). Their eyes are black. Faded shows no different dilution effects in black and agouti background mice. Loss of pigment occurs progressively with age, so that homozygotes are almost white by 1 year of age [19].

The *fe* gene was mapped to a region between *D10mit191* and *D10mit44* on mouse chromosome 10 by our group [21]. The nearest marker, *D10mit191*, was located 2.1 cM proximal regions from the *fe* gene on chromosome 10. This distance between the *D10mit191* and *fe* genes makes it hard to identify the *fe* gene by chromosome walking.

In this study, we have established a high-resolution genetic map in the vicinity of the *fe* gene by generating a backcross panel penetrant for the faded mutation. We also were compared genetic distances between intra-specific and inter-specific backcross panels.

Materials and Methods

Animals

C57BL/6J-fe/fe, *C57BL/6J*, and the Korean wild mouse (KWHM) were used in this study. KWHM were captured in 1995, and subsequently maintained in our laboratory by brother sister mating. The 19th generation of KWHM was used for an inter-specific backcross panel. The animals were maintained at 22±2°C temperature, in 55±10% relative humidity, and under 12 hr artificial lighting from 08:30 to 20:30. The mice were

given commercial laboratory chow made for mouse (Jeiljedang, South Korea) and water *ad libitum*. The animal study was conducted in accordance with and the approval of the Institutional Animal Care and Use Committee of Hallym University.

Histology and hair phenotype

For histological characterization of hair shafts and hair bulbs, *C57BL/6J-fe/fe* and control (*C57BL/6J*) mice at 1 and 12 weeks of age were sacrificed under ether anesthesia. To investigate their hair bulbs, mid-dorsal skin was fixed in 10% neutral buffered formalin solution for 24 hr, and hair was removed with scissors. Then the skin tissue was fixed in 10, 20, and 30% sucrose phosphate buffer solutions over one day. The tissue was embedded in Tissue-tek (4583, Miles Inc., USA), quickly frozen, and cut into 10-µm sections using a cryostat-microtome. After rinsing in 0.1 M phosphate buffer for 10 min three times, they were stained with hematoxylin and eosin. Tissues were viewed under a Zeiss Axioskop light microscope (Carl Zeiss, Germany). Affected faded mice were carefully confirmed by their faded coat color at about 4 weeks of age.

Linkage analysis

C57BL/6J-fe/fe mice were crossed with the KWHM mice to produce F1. F1 mice were then backcrossed to *C57BL/6J-fe/fe* mice, and four hundred inter-specific progeny were analyzed. Linkage analyses were performed

Table 1. Sequences of the PCR primers used for genetic map construction

| Primers | Sequences (5' → 3') | Annealing temp. (°C) | MgCl ₂ conc. (mM) |
|------------------|---|----------------------|------------------------------|
| <i>D10mit3</i> | F: GTTGATAGTCCCACCTCACTCA R: TGAGAAATTCCATCTGTGGC | 62 | 1.5 |
| <i>D10mit85</i> | F: AAGAGAAAGTCATGTGCATGTG R: AAACAGTTGTCAGTTGTTGTTAACC | 62 | 1.5 |
| <i>D10mit155</i> | F: CTCCACCCAATGTACGCAC R: AAAGGCTGACACTGTATAGTGTGG | 62 | 1.5 |
| <i>D10mi191</i> | F: AGAAAGCAAACCTGGCATA GTCC R: TGTCTCAAGCAAGCATAGCA | 62 | 1.5 |
| <i>D10mit156</i> | F: ACCACAGATGAAAGAGAATTTGTG R: TCACTATAATTTCCAATTGCTTTATTT | 62 | 1.5 |
| <i>D10mit193</i> | F: TCCATTGGGATCAATGATACTG R: TCAAAGCACTGGGTTAGCA | 62 | 1.5 |
| <i>D10mit54</i> | F: TGTGGGACACACACGGAC R: GGCTAGACAGCGAGGAGATG | 62 | 1.5 |
| <i>D10mit44</i> | F: CCACCTTAGACAGTTTACATGGC R: CCACGCCAGCTTACTTCT | 62 | 1.5 |
| <i>D10mit42</i> | F: GCATT CAGAAGCTGGAAAAGG R: TGCCCAGCATATGTTTAAAGG | 62 | 1.5 |

using microsatellite markers by using a modification of a previously described method [22]. Six MIT markers over a 27-cM region between D10mit3/D10mit85 and D10mit42 were used for a high-resolution linkage test. Genomic DNA was prepared from the tail of N₂ progeny by standard SDS/proteinase K lysis and the phenol/chloroform extraction method.

PCR reactions using 400 ng of template DNA were carried out in a total volume of 25 μ L containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 2 pmol of each primer, and 1.75 U Taq polymerase (Takara, Japan). Target DNA was amplified in a Cyclogene Dri-Block[®] cyclor (Techne Cambridge Ltd., U.K.), with denaturation at 94°C for 30 s, annealing at 57-62°C for 1 min, extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min at the end of 30 cycles. Modifications in annealing temperature and/or magnesium concentration were made as needed to optimize the results with some primer pairs. The amplification products were separated on 15% polyacrylamide gel and detected by ethidium bromide staining. Percentage recombination and standard error were calculated according to Green [23]. Genetic distances are represented as cM \pm standard error, and order was established by minimizing putative double crossover events.

Results

Coat color change and skin histology of the C57BL/6J-*fe/fe* mouse

The coat color of a homozygote mouse (C57BL/6J-*fe/fe*) becomes progressively lighter with advancing age (Figure 1). The outer fur and under fur are white from the proximal region of the hair and show an age-related

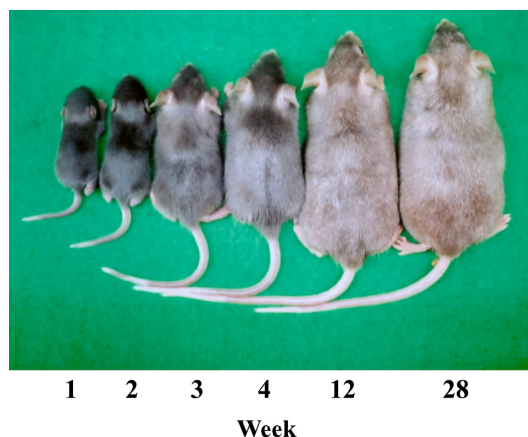


Figure 1. Age-related coat color change in C57BL/6J-*fe/fe* mice. The coat color of homozygous mice becomes progressively lighter with advancing age (black becoming gray).

pigment change toward white. However, these shafts appear normal in structure. Cleared hairs of a faded mouse display various clumps of pigment granules, which occur sporadically in the medulla of the shaft. The hair bulbs of C57BL/6J mouse show normal pigmentation (Figure 2A). Homozygotes of C57BL/6J-*fe/fe* can be distinguished at 2 weeks of age by a slight dilution of coat color. However, the hair bulbs of a C57BL/6J-*fe/fe* mouse are clearly abnormal from 1 week of age (Figure 2B). Melanin of the hair of a C57BL/6J-*fe/fe* mouse completely disappears by 12 weeks of age (Figure 2C).

A high-resolution genetic mapping of the faded gene (*fe*) using an intra-specific backcross panel

To create high-resolution mapping data from the analysis of the intra-specific backcross, we used the Korean wild mouse (KWHM) for a backcross panel. The KWHM mice were captured in 1995 and have been maintained in an inbred line by our laboratory. The

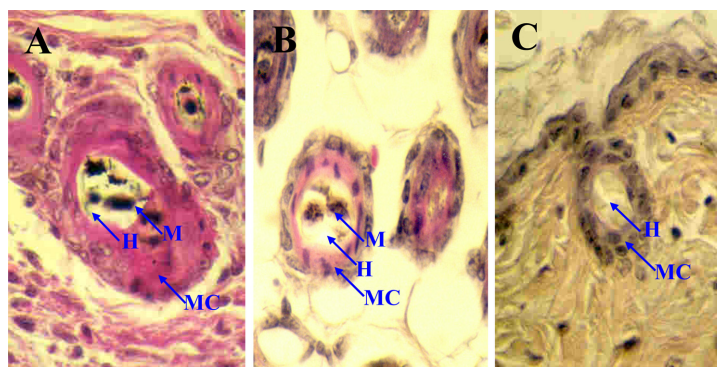


Figure 2. The skin of the C57BL/6J (A) and C57BL/6J-*fe/fe* mice (B,C). In faded mice, the reduction of melanin content in hair bulbs with age is shown. H&E staining (X 400). M, Melanin; MC, Melanocyte; H, Hair.

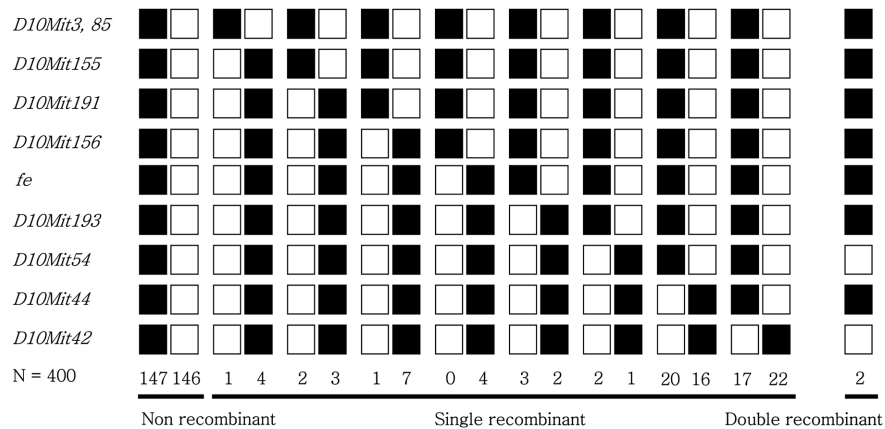


Figure 3. Haplotype analysis of an interspecific backcross between *fe* and KWHM mice (C57BL/6J-*fe/fe* X KWHM)F1 X C57BL/6J-*fe/fe*. Each column represents a chromosomal haplotype identified in the backcross and inherited from the F1 parent. The number of individuals is listed beneath each column. Black boxes represent *fe* alleles, and white boxes represent KWHM alleles as determined by SSCP scoring with markers indicated at the left.

KWHM strain was chosen over others because it is divergent enough to be distinguished from the inbred strains with microsatellite markers. Four hundred interspecific backcross progeny were analyzed at 6 MIT markers over a 27-cM region between *D10mit3/D10mit85* and *D10mit42*. Twenty-two mice showed a recombination between *fe* and *D10mit3/D10mit85* in the proximal region of the *fe* locus, yielding a genetic distance of 5.6 ± 1.1 cM between these two loci. Twelve mice exhibited a haplotype in which the recombination occurred between *fe* and *D10mit191*, indicating that *D10mit191* is 3.0 ± 0.9 cM proximal to *fe*. For the *D10mit156* marker, 4 of the 400 N2 animals showed recombination with *fe*, giving an estimated distance between *D10mit156* and *fe* of 1.0 ± 0.5 cM (Figure 3). This results show that *D10mit156* provides a useful anchor for the isolation of YACs containing the *fe* gene.

In the distal region of the *fe* locus, 83 and 44 mice showed a recombination between *fe* and *D10mit42* and between *fe* and *D10mit44*, respectively. Estimated distances between *fe* and *D10mit44*, and between *D10mit44* and *D10mit42* are 10.8 ± 1.6 and 10.0 ± 1.5 cM, respectively. In addition, 8 mice showed recombination between *fe* and *D10mit54*, placing these markers at a distance of 2.3 ± 0.7 cM. Two double recombination events between *fe* and *D10mit54* were found in the 400 N2 animals. Five mice exhibited a haplotype in which recombination occurred between *fe* and *D10mit193*, indicating that *D10mit193* is 1.3 ± 0.6 cM distal to *fe*. Since *D10mit3/D10mit85* has been shown to be proximal to *D10mit42*, the gene order was defined as follows: centromere-

D10mit3/D10mit85-D10mit155-D10mit191-D10mit156-fe-D10mit193-D10mit54-D10mit44-D10mit42 (Figure 4). The linkage maps from both backcrosses are shown in Figure 4. The order of the loci analyzed was identical between the two maps. The measured distance between *D10mit191* and *D10mit44* differed in these two crosses (14.2 vs. 13.8 cM).

Discussion

Because the C57BL-*fe/fe* mouse is a useful animal model for pigmentary abnormalities in humans, the identification of the mutated gene in C57BL-*fe/fe* mice will help in the identification of the mechanism of pigmentary abnormalities. The *fe* gene was mapped to a region between *D10mit191* and *D10mit44* on mouse chromosome 10 by our group [21]. The nearest marker, *D10mit191*, was located 2.1 cM proximal regions from the *fe* gene on chromosome 10. This distance between *D10mit191* and the *fe* genes makes it hard to identify the *fe* gene by chromosome walking. The gene product and function of the *fe* gene are not evident based on previous studies. For the first step to identify the *fe* gene, we have established a high-resolution genetic map in the vicinity of the *fe* gene by generating a backcross panel penetrant for the faded mutation. From the inter-specific backcross, (C57BL/6J-*fe/fe* X KWHM)F1 X C57BL/6J-*fe/fe*, 400 progenies were used for high-resolution mapping of the *fe* gene. The *fe* gene was mapped to 1.0 cM distal to *D10mit156*. Therefore, the gene order was defined: centromere-*D10mit3/85* (1.3 ± 0.6 cM)-*D10mit155* ($1.3 \pm$

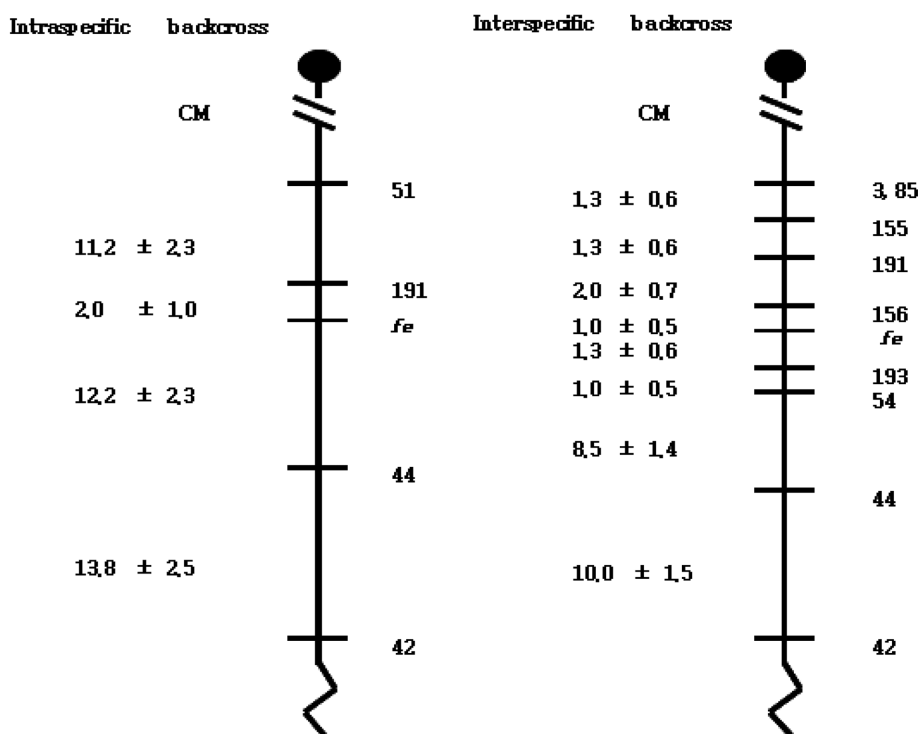


Figure 4. Genetic linkage maps of proximal chromosome 10 as derived from an interspecific backcross panel (C57BL/6J-*fe/fe* X KWHM)F1 X C57BL/6J-*fe/fe*) generated in this study. The distances of the intervals between loci and/or markers are not drawn to the same scale, and the locations of mapped human homologs are taken from the Mouse Genome Informatics (The Jackson Laboratory). Distances between intervals are shown in centimorgans ± standard error to the left of the map. The linkage map of intra-specific backcross panel came from Oh et al., 2011[21].

0.6 cM)-*D10mit191* (2.0±0.7 cM)-*D10mit156* (1.0±0.5 cM)-*fe*-*D10mit193* (1.3±0.6 cM)-*D10mit54* (1.0±0.5 cM)-*D10mit44* (8.5±1.4 cM)-*D10mit42* (10.0±1.5 cM).

In this study, intra-specific and inter-specific genetic distances were compared between each pair of adjacent markers common to both backcrosses. The measured distance between *D10mit191* and *D10mit44* differed in these two crosses (14.2 vs 13.8 cM). Although *Mus musculus* species originated from same ancestral *Mus musculus* one million years ago, the *Mus musculus domesticus* (C57BL/6J and CBA/J) and *Mus musculus molossinus* (Korean wild mouse) line diverged [24] almost 2,000 years ago. Because of this, structural rearrangements in the organization of genetic information probably represent an important mechanism for the establishment or maintenance of speciation. In a previous study it was concluded that the use of inter-specific crosses do not introduce any systemic bias into measurement of either linkage or gene order [24]. Our mapping results of the *fe* gene in intra- and inter-specific backcross panels, therefore, may be thought reasonable.

From mapping information obtained from the Mouse

Genome Informatics (The Jackson Laboratory), there are many genes between *D10mit156* and *D10mit193* on mouse chromosome 10. However, there is no gene related to coat color to our knowledge. The mouse map location of the *fe* locus appears to place it in a region homologous to human 6q21. No human candidate genes have yet been localized in this interval, which may potentially be the human homology of the *fe* gene.

The hair bulbs of the C57BL/6J-*fe/fe* mouse are abnormal from 1 week of age. Melanin of the hair of C57BL/6J-*fe/fe* mouse completely disappears by 12 weeks of age and shows a faded coat color. Mutation in the C57BL/6J-*fe/fe* mouse seems to differ with previously described mutations in mice that reduce the intensity of hair pigmentation or age-related pigment change (for example, d, ln, bg, p, Ga, rp, gl, gr, mh, Sl, si) [19,20].

In conclusion, we made a high-resolution map that flanks the *fe* gene region on mouse chromosome 10. The *fe* gene was mapped to 1.0 cM distal to *D10mit156*. Thus, the gene order was defined: centromere -*D10mit3/85* (1.3±0.6 cM)-*D10mit155* (1.3±0.6 cM)-*D10mit191* (2.0±0.7 cM)-*D10mit156* (1.0±0.5 cM)-*fe*-*D10mit193*

(1.3 ± 0.6 cM)-*D10mit54* (1.0 ± 0.5 cM)-*D10mit44* (8.5 ± 1.4 cM)-*D10mit42* (10.0 ± 1.5 cM). The nearest marker of the *fe* gene, *D10mit156*, is useful to isolate the *fe* gene using a candidate gene approach and chromosome walking. Taken together, our high-resolution linkage map of the *fe* locus from an intra-specific backcross panel will provide a good entry point to isolate the *fe* gene.

Acknowledgments

This work was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A6A1048184).

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