Zygosity Determination in Hairless Mice by PCR Based on \( Hr^{hr} \) Gene Analysis

Osamu SUZUKI, Minako KOURA, Yoko NOGUCHI, Kozue UCHIO-YAMADA, and Junichiro MATSUDA

Laboratory of Animal Models for Human Diseases, National Institute of Biomedical Innovation, 7–6–8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

Abstract: We analyzed the \( Hr \) gene of a hairless mouse strain of unknown origin (HR strain, http://animal.nibio.go.jp/e_hr.html) to determine whether the strain shares a mutation with other hairless strains, such as HRS/J and Shk:HR-1, both of which have an \( Hr^{hr} \) allele. Using PCR with multiple pairs of primers designed to amplify multiple overlapping regions covering the entire \( Hr \) gene, we found an insertion mutation in intron 6 of mutant \( Hr \) genes in HR mice. The DNA sequence flanking the mutation indicated that the mutation in HR mice was the same as that of \( Hr^{hr} \) in the HRS/J strain. Based on the sequence, we developed a genotyping method using PCR to determine zygosities. Three primers were designed: S776 (GGTCTCGCTGGTCCTTGA), S607 (TCTGGAACCAGAGTGACAGACAGCTA), and R850 (TGGGCCACCATGGCCAGATTTAACACA). The S776 and R850 primers detected the \( Hr^{hr} \) allele (275-bp amplicon), and S607 and R850 identified the wild-type \( Hr \) allele (244-bp amplicon). Applying PCR using these three primers, we confirmed that it is possible to differentiate among homozygous \( Hr^{hr} \) (longer amplicons only), homozygous wild-type \( Hr \) (shorter amplicons only), and heterozygous (both amplicons) in HR and Hos:HR-1 mice. Our genomic analysis indicated that the HR, HRS/J, and Hos:HR-1 strains, and possibly Shk:HR-1 (an ancestor of Hos:HR-1) strain share the same \( Hr^{hr} \) gene mutation. Our genotyping method will facilitate further research using hairless mice, and especially immature mice, because pups can be genotyped before their phenotype (hair coat loss) appears at about 2 weeks of age.

Key words: hairless, genome, genotyping, mice, zygosity

Introduction

Many hairless mouse strains such as HRS/J and Shk:HR-1 are often used in studies of skin, cancer, and immunology by Benavides et al. [3] and Sundberg [12]. At our institute, we have been maintaining a hairless mouse strain of unknown origin called HR (http://animal.nibio.go.jp/e_hr.html). It was introduced from a University in California (there is no precise university name in our records) to Yokohama City University in 1964. The strain was then introduced in 1965 to the Institute of Medical Science of the University of Tokyo, where a mutated Hairless gene from this strain was transferred into a BALB/c background. The strain was introduced to our institute (National Institutes of Health, at the time of introduction) in 1981. The HRS/J strain was established in 1964 by inbreeding mice obtained by crossing offspring of the hairless mice first found in London [4] with BALB/c mice at the Jackson Laboratory (http://jaxmice.jax.org/strain/000673.html). In addition, the Hos:HR-1 strain was established in 1987 at Hoshino Laboratory Animals Inc. by inbreeding the Shk:HR-1
outbred strain, which had been established at Temple University by crossing the CBA strain (http://www.hoshino-lab-animals.co.jp/english/products/HR1-en.html) with hairless mice of unknown origin from Sandra Biological Supply. It remains unknown whether HR mice carry the same mutation as other hairless strains, such as HRS/J and Skh:HR-1 (Hos:HR-1), even though the three strains show the same phenotype.

The hairless mutation was first found in a mouse in 1924 [4]. This mutation is an autosomal recessive mutation (Hr<sup>hr</sup>) in the Hr gene [11]. Murine Hr localizes to the 70-Mb position of mouse chromosome 14, and contains 19 exons [5]. The hr mutation is caused by an insertion of the murine leukemia virus into intron 6 [11]. Both HRS/J and Skh:HR-1 (Hos:HR-1) carry this mutation [10]. Homozygous mutants (Hr<sup>hr</sup>/Hr<sup>hr</sup>) show normal development of the first hair coat (first hair cycle). Starting at 2 weeks of age, they lose their hair coat rapidly and completely due to an abnormal second hair cycle [4, 13]. At weaning (~3 weeks of age), they are completely hairless. In general, females homozygous for Hr<sup>hr</sup> often fail to nurse their litters due to abnormal lactation (except Hos:HR-1 homozygous females, which show normal lactation; thus, this low nursing activity is thought to depend on the genetic background, not the Hr<sup>mutation</sup> itself). Therefore, most hairless strains have been maintained by mating heterozygous females (normal hair coat) and homozygous males (no hair coat). In this case, pups are a mixture of heterozygous and homozygous mutants. Homozygous mutants cannot be distinguished from heterozygous ones based on appearance alone because they both have coats before 2 weeks of age. Hence, a genotyping method is also needed if younger mice are to be used.

We analyzed the Hr gene of the HR strain maintained at our institute to determine if its Hr mutation (tentatively called “Hr<sup>mut</sup>”) is the same as that (Hr<sup>hr</sup>) of other hairless strains (such as HRS/J). In addition, we developed a PCR method to determine the genotypes of pups before the phenotype (hair coat loss) appears (~2 weeks of age) based on the sequence information of HR mice.

### Materials and Methods

**Hairless mice**

At our institute, HR mice (nbio#: nbio003) have been maintained by crossing heterozygous females (Hr<sup>Hr</sup>) and homozygous males (Hr<sup>Hr</sup>/Hr<sup>Hr</sup>). Wild-type HR mice (Hr<sup>Hr</sup>/Hr<sup>Hr</sup>), which had no mutated HR (Hr<sup>mut</sup>) alleles, were produced by crossing heterozygous females and males. Hos:HR-1 mice homozygous for hairless genes were purchased from Hoshino Laboratory Animals, Inc. (Bando, Japan) through Japan SLC, Inc. (Hamamatsu, Japan) and used for genotyping tests. All mice were housed under specific pathogen-free conditions with food (CMF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water provided ad libitum. All animal experiments were conducted in accordance with the guidelines for animal experiments of the National Institute of Infectious Diseases, Tokyo, Japan, and the National Institute of Biomedical Innovation, Osaka, Japan.

**Genomic PCR**

Hepatic DNA was extracted from homozygous, heterozygous, and wild-type HR mice using an AllPrep DNA/RNA/Protein Extraction Mini Kit (#80004, Qiagen, Hilden, Germany). Primers (Table 1) for PCRs amplifying 15 regions (Fig. 1) in the Hr gene were designed based on the Hr gene sequence retrieved from the Ensemble database (http://www.ensembl.org). The difference between homozygous (Hr<sup>Hr</sup>/Hr<sup>Hr</sup>) and wild-type (Hr<sup>Hr</sup>/Hr<sup>Hr</sup>) genotypes was determined using multiple PCRs with HotStarTaq (#203443, Qiagen; regions 1, 8–13) or KOD FX neo (KFX-201, TOYOBO, Osaka, Japan; regions 2–7) DNA polymerases. All PCRs were conducted using a Hybaid Sprint thermal cycler (Thermo Scientific, Waltham, MA, USA) in active-tube control mode. Thermal conditions were as follows: for HotStarTaq, 94°C for 15 min (denaturation and enzyme activation), 40 cycles of 94°C for 10 s, 60°C for 10 s, and 72°C for the appropriate amount of time (see Table 2 for elongation time), and then 72°C for 5 min; for KOD FX neo, 95°C for 2 min, 40 cycles of 98.5°C for 10 s and 68°C for the appropriate amount of time (see Table 2 for elongation time), and then 68°C for 5 min. PCR products were separated in agarose gels: for regions 1 and 8–13, 2% E-Gel EX containing SYBR Safe (G4020-02, Life Technologies, Grand Island, NY, USA); for regions 2–7, 0.5% SeaKem LE agarose gels (#50001, Lonza, Basel, Switzerland) with SB buffer (#SB20-1, Faster Better Media LLC, Hunt Valley, MD, USA) and subsequently stained with GelGreen (#41004, Biotium, Inc., Hayward, CA, USA). Stained gels were photographed with a laser scanner (FX Pro, Bio-Rad, Hercules, CA, USA).
Determination of DNA sequences flanking the insertion site

The genomic region containing the insertion mutation site was amplified by PCR from genomic DNA from homozygous HR mice and a set of two primers, mHR-int6-F514 and mHR-int6-R806 (see Table 1), and KOD-FX neo under the following thermal conditions: 95°C for 2 min, 40 cycles of 98.5°C for 10 s and 68°C for 3 min, and then 68°C for 5 min. PCR products, approximately 13 kbp in length, were gel-purified on a 1% agarose gel, and both the 5’ and 3’ ends were sequenced using an Applied Biosystems 3730 × 1 DNA Analyzer (Life Technologies). The obtained sequence was compared to genome databases at the NCBI using a BLAST search.

Genotyping PCR

Primer sets for genotyping Hr alleles were designed according to the sequence information of the alleles (Table 1 for primer sequences; Fig. 3A for primer positions). All three primers were used simultaneously to determine the genotypes of HR and Hos:HR-1 mice. PCRs were conducted using a Hybaid Sprint thermal cycler and HotStarTaq DNA polymerase under the following thermal cycling conditions: 94°C for 15 min, 40 cycles of 94°C for 10 s, 60°C for 10 s, and 72°C for 30 s, and then 72°C for 5 min. PCR products were separated in 2% agarose gels (E-gel EX, G4020-02) and photographed with a laser scanner.

Results

PCR analysis of the Hr allele in HR mice

Analysis of Hr alleles in homozygous (H) and wild-type (W) HR mice by means of 15 multiple overlapping PCRs indicated that the Hr^2 allele contained an insertion mutation in intron 6 (Fig. 1).
Fig. 1. Analysis of Hr alleles in homozygous (H) and wild-type (W) HR mice. The normal (wild-type) Hr allele is ~19 kbp in length and consists of 19 exons. A total of 15 overlapping PCRs covering the entire Hr coding sequence revealed that an insertion mutation was localized in intron 6 of the Hr allele. KOD FX neo was used for PCR amplification of regions 2–7, and HotStarTaq was used for PCR of other regions. PCRs shown in gray typeface (1, 2, 4, 6, 9, 12, 14, 15), no difference between homozygous and wild-type hr mice. PCRs shown in black typeface (3, 5, 7, 8, 10, 11, 13), no band or different bands were obtained in homozygous hr mice. Six agarose gel electropherograms show the band patterns of all PCRs. The primer sets used were: (1) F224 and r1151, (2) F193 and R1873, (3) F193 and R2433, (4) F2463 and R3455, (5) F1843 and R2433, (6) F2052 and R2433, (7) F1843 and R2078, (8) F1843 and R2078, (9) F1913 and R2078, (10) F1843 and Int6-R1458, (11) F1843 and Int6-R979, (12) Int6-F1290 and R1972, (13) F1843 and Int6-R850, (14) F1843 and Int6-R642, and (15) F1843 and Int6-R539. The primer sequences and elongation time are shown in Tables 1 and 2, respectively. The primer positions for long PCR shown in Fig. 2 are also indicated in this figure.
Sequencing the mutated region in HR mice

Long PCR for amplifying genomic regions containing the insertional mutation (Fig. 1 for the primer positions) produced an ~13-kb-long amplicon (Fig. 2). Both the 5’ and 3’ ends of the amplicon were sequenced. Analysis of both sequences using BLAST search revealed that HR mice carried the same insertional mutation as HRS/J mice; i.e., $Hr^a$ turned out to be $Hr^{br}$.

Genotyping of $Hr$ alleles in HR and Hos:HR-1 mice

Primers for genotyping $Hr$ alleles were designed according to their sequence information (see Fig. 3A for primer positions). All three primers were used simultaneously for genotyping PCR. The zygosities of HR and Hos:HR-1 mice were determined using amplicons from both mutant and wild alleles with the following primer sets: S776 and R850 (275 bp, longer bands), and S607 and R850 (244 bp, shorter bands), respectively (Fig. 3B).

Discussion

Our genomic analysis revealed that the HR mice at our institute share the same hairless mutation ($Hr^{br}$) as HRS/J and Skh:HR-1 (an ancestor of Hos:HR-1) mice. This indicates that the HR strain is a descendent of the original hairless mice found in London in 1924 [4]. This possibility was also suggested by the fact that the phenotype of HR mice is identical to that of other hairless mice carrying $Hr^{br}$ alleles. Our genomic analyses confirmed this possibility. Although other mutations of the $Hr$ gene, such as rhino ($Hr^{rh}$) [8] and bald ($Hr^{ba}$) [6] lead to hairlessness, their phenotypes differ from that of HR mice. Rhino mice become completely hairless by 35 days of age, like HRS/J mice, but older rhino mice have a different phenotype: their skin becomes progressively looser and redundant, forming folds, flaps, and ridges [12]. Rhino alleles contain various types of mutations, different from $Hr^{br}$ alleles [1, 2]. These mutations result in a truncation of hairless proteins. On the other hand, bald mice are phenotypically intermediate between the hairless and rhino strains [6]. The similarities between the $Hr^{br}$ and $Hr^{ba}$ alleles are unclear because the bald gene has not yet been sequenced. Thus, HR mice are genetically and phenotypically hairless mice that carry $Hr^{br}$.

Based on the genomic sequence around the insertional mutation, we developed a PCR genotyping method. The method was confirmed to be useful for zygosity checks of both HR and Hos:HR-1 strains, and possibly more strains carrying $Hr^{br}$ alleles. Our three primers flanking the insertional mutation in the $Hr^{br}$ gene distinguished the zygosities of hairless strains in a single PCR assay. Flanking primer methods [7], often used for the genotyping of transgenes [9], are simple and precise for zygosity determination. Other methods, such as Southern blots and quantitative real-time PCR, are also used for zygosity checks, but are challenging in practice. Both methods use quantitative tests, the results of which are
often difficult to compare precisely. In contrast, the flank-
ing primer method is based on qualitative tests (presence or absence of target amplicons) and is easy to perform
with no need for complicated procedures such as hybrid-
ization of radioactive probes, precise adjustment of
template concentration, and so forth. Our primers are
different from those of Schaffer et al. [10]. Their primers
targeted similar positions but had a lower Tm than our
primers. We believe that our primers have an advantage
because a higher Tm often leads to better results. In ad-
dition, PCR results are highly dependent on thermal
controls, such as the block-temperature (i.e., based on
the temperature of blocks, not that within PCR tubes)
and active-tube controls (i.e., based on the temperature
within PCR tubes). PCR using a thermal cycler with
block temperature control needs a longer reaction time
than PCR with active-tube control. In the present study,
we used 10 s for the denature and annealing times in a
thermal cycler with active-tube control. If a thermal
cycler using block-temperature control is used, a longer
period, e.g., 30 s, should be used.

Immature hairless mice can be precisely genotyped
using our PCR method before their phenotype (hair coat
loss) appears. This would enable the use of such mice
for research that requires knowledge of precise zygos-
ities.

In summary, the HR strain at our institute carries the
same Hr/hr alleles as HRS/J and Skh:HR-1 (Hos:HR-1).
Our genotyping method could be used for zygosity
checks of various hairless mouse strains that carry Hr/hr
alleles. This method will facilitate the study of hairless
mice, and especially immature mice, the zygosities of
which cannot be determined based on appearance alone.

Acknowledgment

This work was supported by a grant from the Ministry
of Health, Labour, and Welfare, Japan.

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Fig. 3. PCR for genotyping Hr alleles in HR and Hos:HR-1
mice. (A) Primer positions (primer sequences are shown
in Table 1). PCR using the primers mHR-int6-S607
(S607) and mHR-int6-R850 (R850) produces 244-bp
amplicons from wild-type alleles (Hr, + in Fig. 3B) only.
PCR using the primers mHR-int6-S776 (S776) and
mHR-int6-R850 (R850) produces 275-bp amplicons
from mutant alleles (Hr/hr, in Fig. 3B) only. (B) Zygosity
determination by PCR. Zygosities of Hr alleles
were determined by PCR using three primers (S607,
S776, and R850) simultaneously. If only 275-bp amplicons
were produced, the mice were taken to be homozygous
(Hr/hr). If only 244-bp amplicons were produced, the mice were wild-type
(Hr/Hr). If both amplicons were produced, the mice were heterozygous
(Hr/hr). Electropherograms of PCR products indicate
the zygosities of homozygous, heterozygous, and wild-
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