Exp. Anim. 62(3), 267–273, 2013

—Original—

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 Skh: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 Skh: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 Skh: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 Skh:HR-1

(Received 18 February 2013 / Accepted 30 March 2013)

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

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^{hr}) 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^{hr}/Hr^{hr}) 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^{hr} 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* mutation 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^{x}") is the same as that (Hr^{hr}) 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/Hr^{x}) and homozygous males (Hr^{x}/Hr^{x}). Wild-type HR mice (Hr/Hr), which had no mutated HR (Hr^{x}) 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^{x}/Hr^{x}) and wild-type (Hr/Hr) genomes 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).

Table 1. List of primers used in this study

Name	Sequence (5'- to 3')
For genome analysis (Fig. 1)	
Int6-F1290	ACCACCCTGGAATCTTCCGTGAAAAA
Int6-R1458	CATGCTTGCTGTGGAGAGTGCGTGCAT
Int6-R539	CACACACGCAGACAAAACTCACTCGT
Int6-R642	TGGCAGTTTATAGCTGTCTGTCACTCTGG
Int6-R850	TGGGCCACCATGGCCAGATTTAACACA
Int6-R979	CACGTGCATGTGTGGACATGTCTGCCTTA
F1843	CGGCTGTGTGTAGCCTGTGGTCGCATA
F1913	AGCACACAGATGACTGCGCCCAGGAG
F193	TTCCTCCAAGGCCCCAAGGACACACTC
F2052	CTGTTTCTGCCAGGTTGATGCCCGTGT
F224	AGAGCGCTGAGCAGAAAGCGGGAGAAC
F2463	GGGCCTGAGCCTTCCATTGTCACCAGT
R1151	TGGCGTGTGAGCCAGGTCTTTTTCAGC
R1873	CGGCTATGCGACCACAGGCTACACACA
R1972	GGGTCAGGATCAGGGAACAGGCAGCAT
R2078	ACACGGGCATCAACCTGGCAGAAACAG
R2433	TGGCCCCAGGGCTTTCTCTTGGATCTT
R3455	AGGCTGGCTCCCTGGTGGTAGAGCTGA
For determining genome sequence in homozygous HR mice (Fig. 2)	
mHR-int6-F514	ACGAGTGAGTTTTGTCTGCGTGT
mHR-int6-R806	CGTAGGTCCTCCTGTTTGCTTGGTCATCA
For genotyping (Fig. 3)	
mHR-mut-S776	GGTCTCGCTGGTCCTTGA
mHR-int6-S607	TCTGGAACCAGAGTGACAGACAGCTA
mHR-int6-R850	TGGGCCACCATGGCCAGATTTAACACA

Table 2. DNA polymerase and elongation time for primer sets in Fig. 1

Primer set in Fig. 1	DNA polymerase*	Elongation time
1	HotStarTaq	5 min
2	KOD FX	3 min
3	KOD FX	6 min
4–7	KOD FX	3 min
8, 9	HotStarTaq	2 min
10–12	HotStarTaq	1 min
13–15	HotStarTaq	30 s

*See Materials and Methods for detailed PCR conditions.

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 × l DNA Analyzer (Life Technologies). The obtained sequence was compared to genome databases at the NCBI using a BLAST search.

Genotyping PCR

Primers 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^x allele contained an insertion mutation in intron 6 (Fig. 1).

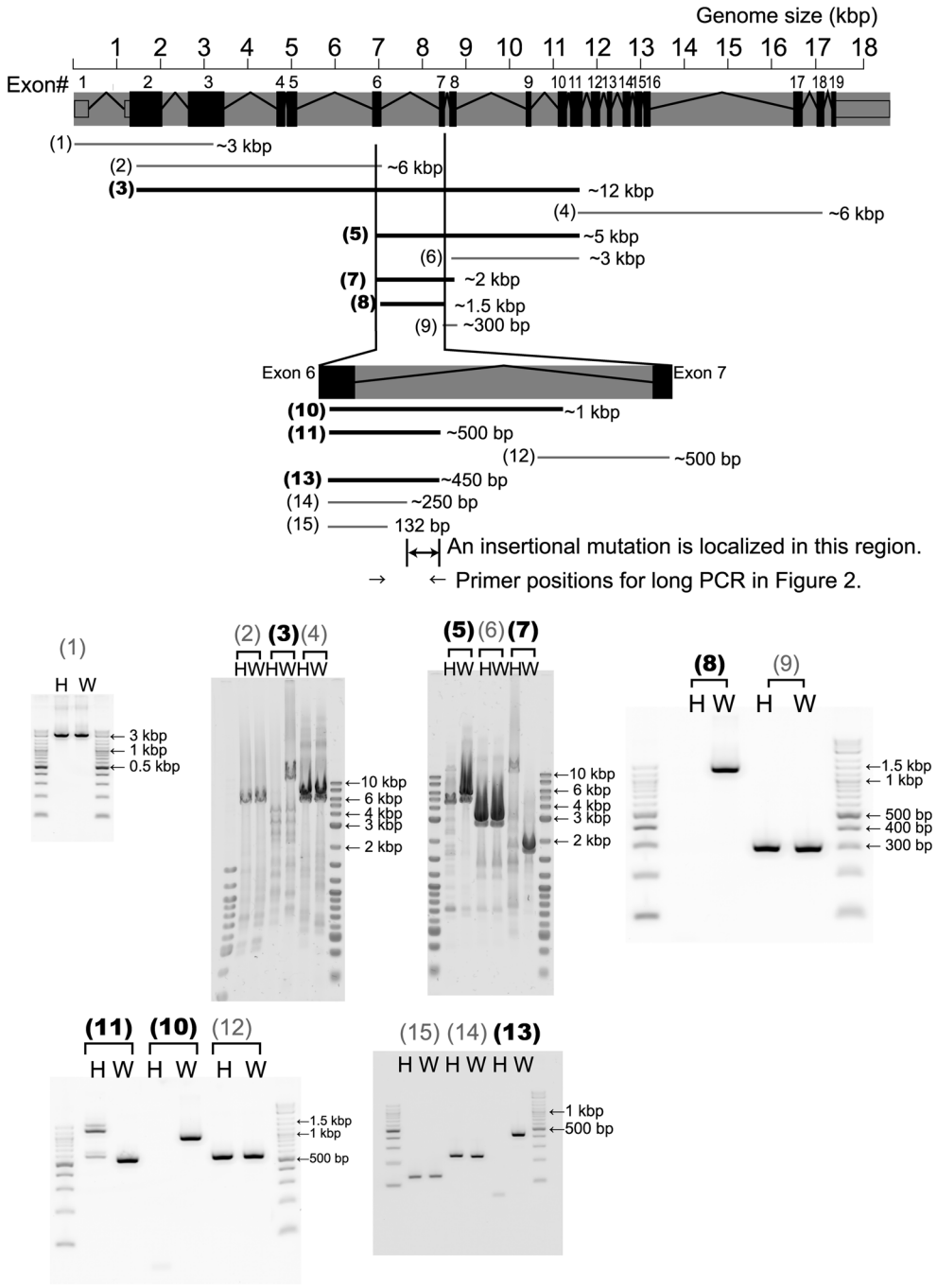

Fig. 1. Analysis of *Hr* alleles in homozygous (H) and wild-type (W) HR mice. The normal (wild-type)*Hr* allele is ~19 kbp in length and consists of 19 exons. A total of 15 overlapping PCRs covering the entire *Hr* coding sequence revealed that an insertion mutation was localized in intron 6 of the Hr^x 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 R1972, (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.

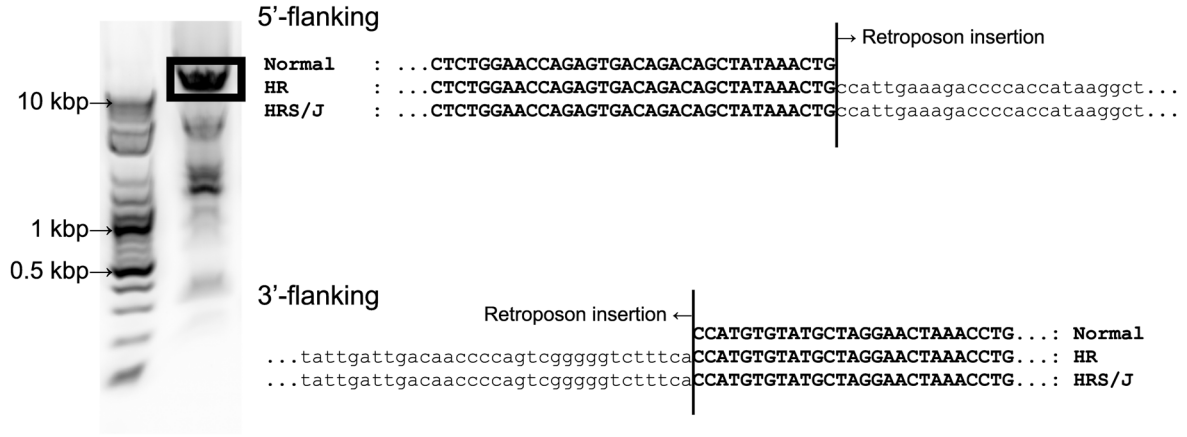

Fig. 2. Determination of DNA sequences flanking the insertion mutation in HR mice. PCR with two primers, mHR-int6-F514 and mHR-int6-R806 (positions and sequences are shown in Fig. 1 and Table 1, respectively) produced an ~13-kb-long amplicon containing insertion mutations in homozygous HR mice. Sequencing and BLAST searches indicated that the HR mice share the same insertion mutation as HRS/J mice. The sequences of the 5' and 3' regions flanking the insertion mutation of HRS/J mice were retrieved from GenBank (accession numbers M20235 and M20236, respectively).

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^{x} turned out to be Hr^{hr}.

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^{hr}) 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^{hr} 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^{hr} 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^{hr} 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^{hr}.

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^{hr} alleles. Our three primers flanking the insertional mutation in the Hr^{hr} 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

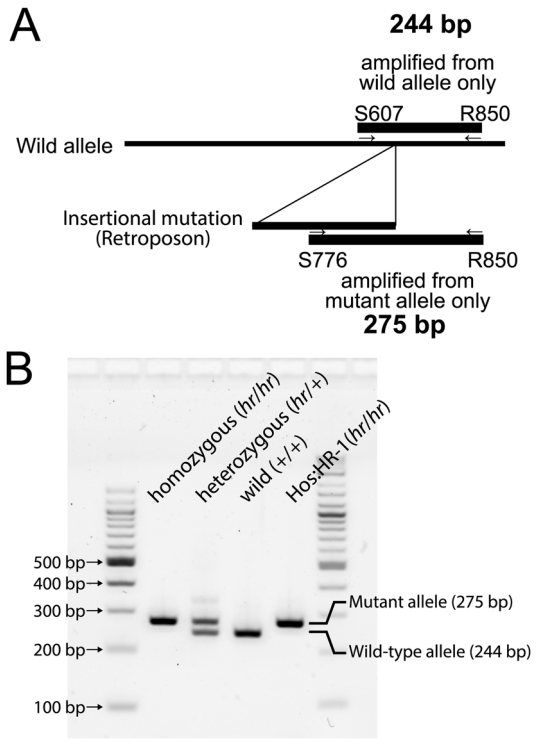

Fig. 3. PCR for genotyping *Hr* alleles in HR and Hos:HR-1 mice. (A) Primer positions (primer sequences are shown in Table 1). PCR using the primers mHR-int6-S607 (S607) and mHR-int6-R850 (R850) produces 244-bp amplicons from wild-type alleles (*Hr*, + in Fig. 3B) only. PCR using the primers mHR-int6-S776 (S776) and mHR-int6-R850 (R850) produces 275-bp amplicons from mutant alleles (Hr^{hr}, *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}/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^{hr}). Electropherograms of PCR products indicate the zygosities of homozygous, heterozygous, and wild-type HR mice as well as homozygous Hos:HR-1 mice.

often difficult to compare precisely. In contrast, the flanking 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 hybridization 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 addition, 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 zygosities.

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.

References

1. Ahmad, W., Panteleyev, A.A., Henson-Apollonio, V., Sundberg, J.P., and Christiano, A.M. 1998. Molecular basis of a novel rhino (hr^{rhChr}) phenotype: a nonsense mutation in the mouse hairless gene. *Exp. Dermatol.* 7: 298–301. [Medline] [CrossRef]
2. Ahmad, W., Panteleyev, A.A., Sundberg, J.P., and Christiano, A.M. 1998. Molecular basis for the rhino ($hr^{rh\text{-}8J}$) phenotype: a nonsense mutation in the mouse *hairless* gene. *Genomics* 53: 383–386. [Medline] [CrossRef]
3. Benavides, F., Oberyszyn, T.M., VanBuskirk, A.M., Reeve, V.E., and Kusewitt, D.F. 2009. The hairless mouse in skin research. *J. Dermatol. Sci.* 53: 10–18. [Medline] [CrossRef]
4. Brooke, H.C. 1926. Hairless mice. *J. Hered.* 17: 173–174.
5. Cachon-Gonzalez, M.B., Fenner, S., Coffin, J.M., Moran, C., Best, S., and Stoye, J.P. 1994. Structure and expression of the hairless gene of mice. *Proc. Natl. Acad. Sci. USA* 91: 7717–7721. [Medline] [CrossRef]
6. Garber, E. 1951. hr^{br} − bald. *Mouse News Lett.* 5: 10.
7. Hogan, B., Beddington, R., Constantini, F., and Lacy, E. 1994. Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Woodbury.
8. Howard, A. 1940. "Rhino," an allele of hairless in the house

mouse. *J. Hered.* 31: 467–470.

9. Noguchi, A., Takekawa, N., Einarsdottir, T., Koura, M., Noguchi, Y., Takano, K., Yamamoto, Y., Matsuda, J., and Suzuki, O. 2004. Chromosomal mapping and zygosity check of transgenes based on flanking genome sequences determined by genomic walking. *Exp. Anim.* 53: 103–111. [Medline] [CrossRef]
10. Schaffer, B.S., Grayson, M.H., Wortham, J.M., Kubicek, C.B., McCleish, A.T., Prajapati, S.I., Nelon, L.D., Brady, M.M., Jung, I., Hosoyama, T., Sarro, L.M., Hanes, M.A., Rubin, B.P., Michalek, J.E., Clifford, C.B., Infante, A.J., and Keller, C. 2010. Immune competency of a hairless mouse strain for improved preclinical studies in genetically engineered mice. *Mol. Cancer Ther.* 9: 2354–2364. [Medline] [CrossRef]
11. Stoye, J.P., Fenner, S., Greenoak, G.E., Moran, C., and Coffin, J.M. 1988. Role of endogenous retroviruses as mutagens: the hairless mutation of mice. *Cell* 54: 383–391. [Medline] [CrossRef]
12. Sundberg, J.P. 1994. Inbred laboratory mice as animal models and biomedical tools: general concepts. pp. 9–19. *In*: Handbook of Mouse Mutations with Skin and Hair Abnormalities: Animal Models and Biomedical Tools. (Sundberg, J.P. ed.) CRC Press, Boca Raton.
13. Sundberg, J.P. and King, L.E. Jr. 2001. Morphology of hair in normal and mutant laboratory mice. *Eur. J. Dermatol.* 11: 357–361. [Medline]