PCR Method for Genotyping and Zygosity-Testing of RasH2 Transgenic Mice

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Abstract: In short-term carcinogenicity testing using CB6F1-TgrasH2 mice, sibling nonTgrasH2 mice are used as a negative control. However, selection of TgrasH2 and nonTgrasH2 mice has been performed by PCR with only transgene specific primers by the conventional method. Therefore, the conventional method involves the risk of false negative results due to reaction failure, and contamination with TgrasH2 mice in the control mice group. Based on the nucleotide sequence information around the pre-integration site, we developed a genotyping method for distinguishing not only TgrasH2 mice (hemizygous for the Tg allele) but also nonTgrasH2 (homozygous for the nonTg allele) in a positive manner.

Key words: genotyping, PCR, rasH2 transgenic mouse

The hemizygous rasH2 transgenic mouse (TgrasH2) has three copies of the human c-Ha-ras gene arrayed in a head-to-tail configuration with its own promoter/enhancer in the chromosome 15E3 region [10]. The TgrasH2 line shows highly sensitive and facilitated carcinogenicity to various carcinogens [1, 4, 6, 8, 13]. Based on a preliminary study by Yamamoto et al. [14] and extensive validation studies in the International Life Science Institute Health and Environmental Science Institute (ILSI/HESI) project, the mice were suggested as a promising model for a short-term carcinogenicity testing system [5, 11].

CB6F1-Tg (c-Ha-ras) (CB6F1-TgrasH2) mice were obtained by breeding female BALB/cByJ and male C57BL/6J-Tg (c-Ha-ras) (C57BL/6J-TgrasH2) mice. This unique breeding system brings two advantages to the carcinogenicity testing model: one is the possibility of a wide range of responses to chemical compounds, and the other is the possibility of using sibling CB6F1-nonTg (CB6F1-nonTgrasH2) mice as a good examination control. However, selection and sorting of TgrasH2 and nonTgrasH2 mice have been performed by PCR genotyping with detection of only transgene specific sequences. It has not been possible to select and sort nonTgrasH2 mice in a positive manner because it has not been possible to know where and how the transgene was integrated into the host mouse genome, and what structural alterations in the host mouse genome occurred following transgene integration. The conventional method involves the risk of false negative results (no band for the transgene) due to reaction failure, and contamination with TgrasH2 mice in the control.
mice group. In carcinogenicity testing, the examination control plays an important role because, contamination of the examination control mice group with carcinogen hypersensitive TgrasH2 mice would affect the reliability of the carcinogenicity test results. Therefore, it is important to select nonTgrasH2 mice correctly, the same as when selecting TgrasH2 mice. To reduce the risk of false negatives, we developed a genotyping method for the correct selection of nonTgrasH2 mice simultaneous with the selection of TgrasH2 mice.

For production of the CB6F1-TgrasH2 and the CB6F1-nonTgrasH2 mice, C57BL/6J-TgrasH2 male mice were bred with BALB/cByJ female mice. All animals used were handled in accordance with the guidelines established by the Central Institute for Experimental Animals, Japan. Tail clips of C57BL/6J-TgrasH2, CB6F1-TgrasH2 and BALB/cByJ mice were obtained and digested with proteinase K by ordinary methods [9]. Genomic DNA was extracted using the MagExtractor System MFX-9600 Magnia R Plus (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. The multiplexing PCR mixture consisted of 9.4 µl of H2O, 1.5 µl of 10X AccuPrime™ PCR Buffer II, 0.3 µl of each primer (20 µM) and 0.3 µl of AccuPrime™ Taq DNA polymerase (Invitrogen life technologies, CA USA) together with 2 µl of genomic DNA (10 to 100 µg/µl). Thermal cycling conditions were as follows: 94°C, 3 min followed by 35 cycles of 94°C, 30 s; 65°C, 30 s; 68°C, 1 min; and 68°C, 3 min in a BioRad iCycler (Bio-Rad Laboratories, Inc., CA USA). For visualization purposes, 2 µl of the amplification product were subjected to electrophoresis on 3% Nusieve 3:1 agarose (Cambrex Bio Science Rockland, Inc., ME USA) gel/0.5X TAE and then stained with ethidium bromide (0.25 µg/ml).

Oligonucleotide primers used in this study and their sequences are listed in Table 1. We have cloned and sequenced around the integrated human c-Ha-ras genes and identified the 1,820-bp unique sequence in the nonTg allele at the corresponding pre-integration site in the rasH2 line [10]. Based on the nucleotide sequence information, we designed a specific primer set within the pre-integration site for the detection of the nonTg allele. To decrease the risk of false positives (faint non-specific bands), we designed two sets of primers for detection of the transgene (Tg allele) at two different sites. Figure 1 shows a schematic representation of the structure of integrated human c-Ha-ras genes and the corresponding region of the nonTg allele of the hemizygous TgrasH2 mouse. Three copies of the transgene on a transgenic allele are present in a head-to-tail tandem array. The hatched box on the nonTg allele indicates the region corresponding to the transgene integration site which is missing in the transgenic allele. Arrowheads depict both the position and direction of the oligonucleotides used, with the tip of the arrow representing the 3’ end of the oligonucleotide.

![Fig. 1. Schematic representation of the structure of integrated human c-Ha-ras genes and the corresponding region of the nonTg allele of the hemizygous TgrasH2 mouse. Three copies of the transgene on a transgenic allele are present in a head-to-tail tandem array. The hatched box on the nonTg allele indicates the region corresponding to the transgene integration site which is missing in the transgenic allele. Arrowheads depict both the position and direction of the oligonucleotides used, with the tip of the arrow representing the 3’ end of the oligonucleotide.](image)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of forward primer (5’–3’)</th>
<th>Name</th>
<th>Sequence of reverse primer (5’–3’)</th>
<th>Expected size (bp)</th>
<th>Target allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgF1</td>
<td>CACAAGCTCAGGACATGGAGGTG</td>
<td>TgR1</td>
<td>TCCCTATGACCTGCTCTTGTCCTC</td>
<td>510</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TgF2</td>
<td>TCCCTTAAGCCTTGTCCTGCCGA</td>
<td>TgR2</td>
<td>CCCACTTGAACCTTAAAGCCCCAGT</td>
<td>130</td>
<td>Transgenic</td>
</tr>
<tr>
<td>NonTgF</td>
<td>AATAGCAAACATCAGATCCTTGCG</td>
<td>NonTgR</td>
<td>ACTGCCCTGAACACATGCTCCTTG</td>
<td>260</td>
<td>Non-transgenic</td>
</tr>
</tbody>
</table>

![Table 1. List of primers, their sequences and expected PCR product size.](table)

![Table](image)
PCR METHOD FOR GENOTYPING OF TGRASH2 MICE

(NonTgF and NonTgR) PCR amplicon was observed at the expected size in BALB/cByJ female mouse DNA samples (Fig. 2, lane 1). This multiplexed PCR genotyping method was applied to genotyping of CB6F1 offspring. The offspring showing only 260-bp bands (Fig. 2, lanes 2 and 3) should be CB6F1-nonTgrasH2 genotypes, and the offspring showing three PCR amplicons consisting of 510-bp, 130-bp and 260-bp bands (Fig. 2, lanes 4 and 5) should be the CB6F1-TgrasH2 hemizygote genotype.

The 260-bp band created by the NonTgF and NonTgR primer set was seen not only in nonTgrasH2 but also in TgrasH2 samples. The NonTgF and NonTgR primer set recognizes the unique sequences in the nonTg allele (Fig. 1), and theoretically creates a specific PCR product from nonTg allele(s). However, it is difficult to demonstrate that the primers actually create a unique PCR product from nonTg allele(s) because the TgrasH2 mouse is a hemizygote and has only one nonTg allele. To ensure that the non-TgrasH2 mouse determined by this multiplexed PCR genotyping method is a nonTg homozygote, we checked their zygosity in the same way as genotyping for identification of homozygotes (wild and mutant type) and heterozygotes in knockout mice. The TgrasH2 homozygote has no unique sequences in the nonTg allele, and it creates only two PCR amplicons (510-bp and 130-bp bands) in the newly developed multiplexed PCR genotyping method. To produce the TgrasH2 homozygote, we bred male C57BL/6J-TgrasH2 and female C57BL/6J-TgrasH2 mice, and checked the zygosity of the offspring by this method and progeny testing. Unfortunately, we have never succeeded in obtaining the TgrasH2 homozygote (data not shown), which implies that the homozygous TgrasH2 mouse is embryonically lethal. It is unclear which stage is the critical point in normal development of the TgrasH2 homozygote. Therefore, we checked TgrasH2 zygosity in blastocyst samples created by an in vitro fertilization method. Ova and sperm of TgrasH2 (C57BL/6J-TgrasH2 x C57BL/6J-TgrasH2) were cultured in TYH medium for 6 h. Pronuclear formation of the eggs was assessed at 8 h after insemination and the fertilized eggs with a secondary polar body were transferred to modified Whitten’s medium and cultured at 37°C in an atmosphere of 5% CO₂ and 95% air for 4 additional days [3]. Template DNA from blastocyst samples was prepared by the following protocol [2]. A blastocyst was transferred to a thin-wall plastic PCR tube containing 20 μl of TPM buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 2 mM MgCl₂) and 40 μg/ml
proteinase K with a glass pipette and incubated at 55°C for 60 min. After incubation, proteinase K was inactivated by heating the samples at 95°C for 5 min. Part of the buffer (2 µl) was used as template DNA for PCR. A typical genotyping image is shown in Fig. 3. Three PCR amplicons, consisting of two Tg (510-bp and 130-bp bands) and one nonTg (260-bp band) derived amplicons, were observed in the C57BL/6J-TgrasH2 female and male mouse DNA samples (Fig. 3, lanes 1 and 5). Three different banding patterns were observed in the blastocyst samples. The banding pattern, which consisted of 510-bp, 130-bp and 260-bp bands (Fig. 3, lane 3), was the same as that of parental C57BL/6J-TgrasH2 hemizygous mice. Another banding pattern, which consisted of only a single 260-bp band (Fig. 3, lane 2), was the same pattern observed in the nonTg mouse sample (BALB/cByJ, Fig. 2, lane 1). The other banding pattern, which consisted of 510-bp and 130-bp bands (Fig. 3, lane 4), did not correspond to that of hemizygous TgrasH2 mice or nonTg mice. This result suggests that the newly developed multiplexed PCR genotyping method can clearly distinguish among the nonTgrasH2 homozygote, TgrasH2 hemizygote and TgrasH2 homozygote.

The 510-bp PCR product created with TgF1 and TgR1 primers in the TgrasH2 hemizygote was weaker than the other two (260 and 130-bp) bands (Fig. 3, lane 5). In general, it appears that larger targets tend to be more difficult to amplify in the multiplexing method. Therefore, we proposed the following criterion to ensure correct TgrasH2 genotyping: a sample that creates only two (510-bp and 260-bp and 260-bp and 130-bp) or only 510-bp or 130-bp PCR products should be classified as a “re-assay”.

Several methods, such as classical progeny testing, fluorescent in situ hybridization (FISH) [12], and quantitation of transgene DNA by real-time PCR [7], are known to determine transgene zygosity. The simplest way to determine transgene zygosity is PCR analysis, but the transgene/genome junction has to be identified. In TgrasH2 mice, we have clarified the genome structure around the integrated human c-Ha-ras transgene and identified a 1,820 bp deletion from the host mouse genome in the Tg allele [10]. Based on this information concerning the transgene/genome junction sequence, we developed a multiplexed PCR genotyping method for determining the transgene zygosity. This method allowed us to select nonTgrasH2 and TgrasH2 in a positive manner, and to prevent false negative and positive determinations. Furthermore, the new method will also be a useful tool for investigating the embryonic lethality of the TgrasH2 homozygote.

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References