Typing of X Chromosomes Bearing Tabby Allele in Mouse Preimplantation Embryos by Detection of a Microsatellite Marker

Ruby P.A. VALDIVIA^{1, 2)}, Tetsuo KUNIEDA³⁾,
Masahira HATTORI⁴⁾, and Yutaka TOYODA^{1, 5)}

¹⁾Department of Reproductive and Developmental Biology, and ⁴⁾Laboratory of Molecular Medicine, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan, ²⁾Laboratory of Molecular Biology, Faculty of Odontology, University of Chile, Santiago, Chile, ³⁾Faculty of Agriculture, Okayama University, Okayama 700, Japan, ⁵⁾Present address: The Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan.

Abstract: Tabby (*Ta*) is a semidominant allele of the locus on the mouse X chromosome, which causes a characteristic coat pattern and developmental defects in endocrine glands. To establish a method for identifying the X chromosome bearing the *Ta* allele in early preimplantation embryos, we performed PCR amplification of an X chromosomal microsatellite marker locus from preimplantation embryos obtained from mating between XO female and XY male mice. The microsatellite marker locus was shown to be polymorphic between X chromosomes bearing the *Ta* and wild-type alleles. The amplification of the marker locus from early preimplantation embryos demonstrated that the *Ta* locus can be correctly typed by this method from embryos as little as 2 cells.

Key words: microsatellite, Tabby, XO

Identification of genotypes of mutant loci in early developmental stages is required to investigate the development of mutant animals that have particular developmental defects. Detection of a particular polymorphic marker locus which is closely linked to the mutant locus is an effective method for typing of the mutant locus in which no molecular defect has been identified. Variation in the length of simple sequence repeats such as (CA)_n and (GA)_n, which is highly polymorphic in mammalian genome [13] and can be easily typed by the polymerase chain reaction (PCR), has proved a useful marker for typing the mutant locus in

preimplantation embryos. In the present study, we established an effective method to identify embryos having the Tabby (*Ta*) mutation in preimplantation stages by detecting the polymorphic marker locus of the X chromosome and using the XO-XY mating system. *Ta* is the semidominant allele of the locus on the X chromosome, which affects a variety of tissues of ectodermal origin [3–5]. Hemizygous males and homozygous females are characterized by the absence of guard hairs, bald patches behind the ears, and defects in the endocrine glands. Heterozygous females have a characteristic striped coat pattern as a result of random

(Received 16 January 1996 / Accepted 31 May 1996)

Address corresponding: T. Kunieda, Faculty of Agriculture, Okayama University, Tsushima-naka, Okayama 700, Japan

X chromosome inactivation.

The Tabby colony used in the present study has been maintained by repeating of reciprocal crosses: +/O females are mated with *Ta/Y* male siblings, and the next generation is made by mating *Ta/O* females with their +/Y male siblings. Because only XO female mice were used for mating in this system, no recombination between loci on X chromosomes occurs through the generations. This indicates that any polymorphic marker loci on the X chromosome can be used for identifying the X chromosomes bearing the *Ta* and wild-type alleles in this mating system. Based on the genetic background of the Tabby mice, we chose the *DXMit25* microsatellite locus [7] as such a marker for identification of the *Ta* locus.

To determine whether the *DXMit25* locus can be used to distinguish X chromosomes of the Tabby mice, genomic DNAs prepared from *Ta/+*, *Ta/O*, *Ta/Y*, +/O, and +/Y mice were subjected to the PCR amplification by using the set of primers for the *DXMit25* locus (5'CCCTACAAGCATTTGGCTTGT3' and 5'CCAAATGCCCAAGCAAGCACA3'). Twenty nanograms of genomic DNAs were subjected to the PCR amplification in a 50- μ l PCR reaction containing 1 unit of *Taq* polymerase (Boehringer Mannheim Yamanouchi, Tokyo, Japan) and 0.1 mM each of the primers. The PCR reaction was performed with initial denaturation at 94°C for 3 min, 40 cycles of each consisting 94°C for 30 sec, 55°C for 2 min and 72°C for 3 min, and a final incubation at 72°C for 7 min by TSR-300 Thermal Sequencer (Iwaki). Ten microliters of PCR products were resolved by electrophoresis through 3% agarose gels in TBE buffer and stained with ethidium bromide.

As shown in Fig. 1, heterozygous females (*Ta/+*) gave doublet bands visible on agarose gels, while the males (+/Y or *Ta/Y*) and hemizygous females (+/O and *Ta/O*) gave a single band. The amplified fragments in mice with the *Ta* allele (*Ta/O* and *Ta/Y*) were shorter than those with the wild-type allele (+/O and +/Y). Direct sequencing of the PCR products was performed to determine the exact length of the amplified fragments. The PCR products were purified by precipitation with 60 μ l of 2.5 M NaCl/20% PEG. Nucleotide sequence of the PCR products was determined with a DNA sequencer (Pharmacia LKB) using *Taq* microcycle sequencing method [6]. The sequence analysis revealed that the shorter fragment is 299 bp in length and con-

Fig. 1. PCR products amplified from DNAs extracted from mice in the Tabby colony using sets of primers for the *DXMit25* and *Zfy* loci. Fragments of 309 or 299 bp corresponding to the *DXMit25* locus are amplified in all mice, and *Ta/+* mice gave doublet bands. A fragment of 617 bp corresponding to the *Zfy* locus is amplified in male mice. Lanes on the sides show *Hae*III digested fragments of Φ X174 DNA as molecular weight markers.

tains 10 CA-dinucleotide repeats, while the longer fragment is 309 bp in length and contains 15 CA-dinucleotide repeats. The upper band of the doublet band visible in the *Ta/+* mice, which migrated slower than the fragments corresponding to either of the *Ta* and wild-type allele, is likely to be a heteroduplex fragment formed by the annealing of strands of the longer and shorter fragments [10], because the PCR amplification of the *DXMit25* locus from a mixture of DNAs from the *Ta/O* and +/O mice gave the same band pattern as that of the *Ta/+* mouse (data not shown). The *Ta/+* mice is, therefore, likely to have both shorter and longer fragments. Typing of the *DXMit25* locus in more than 150 mice of the Tabby mouse colony indicated the perfect correspondence between the Tabby phenotypes and the amplified fragments of the *DXMit25* locus, indicating that the genotypes of the *Ta* locus were correctly identified by the typing of the *DXMit25* locus. To confirm the presence of the Y chromosome, PCR amplification of the Y-chromosomal sequence, the *Zfy* gene, was simultaneously performed with mouse *Zfy* gene specific primers [9]. As shown in Fig. 1, only male mice (*Ta/Y* and +/Y) gave a fragment of expected size (627 bp) for the *Zfy* locus.

Fig. 2. PCR products amplified from blastocyst stage embryos with sets of primers for *DXMit25* and *Zfy* loci. Fragments of 309 or 209 bp corresponding to the *DXMit25* locus are amplified in all embryos, and the *Ta/+* embryo gave doublet bands. A fragment of 617 bp corresponding to the *Zfy* locus is amplified in male embryos. Lanes on the both sides show *Hae*III digested fragments of ϕ X174 DNA as molecular weight markers.

In vitro fertilization and culture of mouse preimplantation embryos were carried out as reported [8]. Sets of *in vitro* fertilization (*Ta*/O $\times$ +/Y and +/O $\times$ *Ta*/Y) were prepared. Oocytes collected from superovulated females were inseminated with preincubated epididymal spermatozoa in TYH medium [11]. Six hr after the *in vitro* insemination, all oocytes at the pronuclear stage were washed twice by passages through 400- μ l drops of Whitten's medium (WM). Each group of embryos was cultured in a 100- μ l drop of WM. At 2-cell, 4-cell, 8-cell, morula and blastocyst-stages, the embryos were treated with 0.5% pronase and each embryo was collected into a 0.5 ml micro centrifuge tube containing phosphate-buffered saline (PBS) as described by Valdivia *et al.* [12]. The genomic DNAs from preimplantation embryos were prepared by heating the embryos 3 times at 95°C for 8 min with 2 min cooling intervals at 60°C in the micro centrifuge tubes containing PBS.

Figure 2 shows the detection of the *DXMit25* and *Zfy* loci in the blastocyst stage embryos obtained from the matings between the Tabby mice. Band patterns corre-

sponding to *Ta*/+, +/O, and *Ta*/Y were observed in 11, 2, and 15 embryos, respectively, obtained from the mating of *Ta*/O with +/Y, and those corresponding to *Ta*/+ and +/Y were observed in 3 and 1 embryos, respectively, obtained from the mating of +/O with *Ta*/Y. Because *in vitro* fertilization of +/O with *Ta*/Y gave low fertilization rate (data not shown), few embryos obtained from the mating of *Ta*/O with +/Y were genotyped in each stage of the development. The band patterns of the PCR products were not contradictory to the expected genotypes of the embryos except that the *Ta*/O genotype was not detected in the embryos obtained from the mating between +/O and *Ta*/Y. All embryos examined were identified as either of the genotypes. The genotypes of the *Ta* locus in the blastocyst stage embryos, as well as the sex of the embryos, therefore appeared to be correctly identified by this method.

Two step PCR amplification was applied to genotype the embryos at earlier stages of the development (from 2-cell to morula stages), because the single step PCR amplification failed to amplify the target fragments from the embryos at these stages. Embryos at these stages were subjected to first PCR amplification in 50- μ l PCR reaction containing the primers for the *Zfy* and *DXMit25* loci, then 2 μ l of the first PCR products were subjected to second PCR amplification in 50- μ l PCR reaction containing inner sets of primers for the *Zfy* locus [9] or *DXMit25* locus (5'TTCCC-AAGCTGCTGTTTCTT3' and 5'TGGCAGCACTTT-AAGCATTG3') as described [9]. The PCR amplifications were performed in 20 cycles under the conditions described above. Figure 3 shows a representative of the fragments amplified from the 2-cell stage embryos by the inner set of primers for the *DXMit25* locus. Band patterns corresponding to *Ta*/+, +/O, and *Ta*/Y were observed in 8, 6, and 14 embryos, respectively, obtained from the mating of *Ta*/O with +/Y, and those corresponding to *Ta*/+, *Ta*/O, and +/Y were observed in 7, 2, and 6 embryos, respectively, obtained from the mating of +/O with *Ta*/Y. The band patterns of the PCR products were not contradictory to the expected genotypes of the embryos. All embryos examined were identified as either of the genotypes. These findings indicate that it is possible to genotype the *Ta* locus in preimplantation embryos with a small number of blastomeres obtained by microbiopsy of the embryos. The remaining portion of each embryo can be subjected to

Fig. 3. PCR products amplified from 2-cell stage embryos by the two step PCR method with sets of primers for the *DXMit25* locus. Fragments of 174 or 164 bp are amplified in all embryos, and *Ta/+* embryos gave doublet bands. The lane on the left side shows *HaeIII* digested fragments of ϕ X174 DNA as molecular weight markers.

subsequent experiments including implantation of the embryos to observe later developmental stages of the Tabby mice.

In the present study, we established an effective method for identifying embryos with the *Ta* allele in preimplantation stages by detecting the polymorphic marker locus of the X chromosome and the XO-XY mating system. *DXMit25* localized to position 28 on the mouse X chromosome and *Ta* localized to 37 [1, 7]. Although the distance between these two loci seems enough to generate recombination between the X chromosomes in several generations, it is not possible to recombine two loci of the X chromosomes in the XO-XY mating system. In fact, typing of the *DXMit25* locus in more than 150 mice of the Tabby mouse colony indicated perfect correspondence between the Tabby phenotype and the amplified fragments of the *DXMit25* locus. We therefore concluded that the present method is an accurate and effective method for identifying embryos with the *Ta* allele in the XO-XY mating system.

The *Ta* mutation affects a variety of tissues of ectodermal origin including the endocrine glands [3–5] and is believed to be homologous to the human X linked disease locus for hypohidrotic ectodermal dysplasia (XHED) [2]. Analysis of the development of the *Ta* embryos by using the present method will provide valuable information about this syndrome. A disadvantage of the present method, however, is that it could not be used in a normal (XX-XY) mating system. Marker loci on the mouse X chromosome that localize close to the *Ta* locus and demonstrate polymorphism between the X chromosome bearing *Ta* and wild-type alleles are required to establish a method for identifying of *Ta* genotypes in the normal mating system.

We thank Dr. N. Takagi for kindly providing the Tabby mice and Dr. A. Weaver for providing the sequence data on the *DXMit25* locus. This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (Y.T.).

References

1. Brockdorff, N., Kay, G., Smith, S., Keer, J.T., Brown, S.D.M., and Rastan, S. 1991. *Genomics* 10: 17–22.
2. Davisson, M.T. 1987. *Genomics* 1: 213–227.
3. Falconer, D.S. 1953. *Z Indukt Abstammungs-Vererbungst* 85: 210–219.
4. Gruneberg, H. 1971. *Proc. R. Soc. Lond. Biol.* 179: 139–156.
5. Gruneberg, H. 1971. *J. Embryol. Exp. Morphol.* 25: 1–19.
6. Hattori, M., Yoshioka, K., and Sakaki, Y. 1992. *Electrophoresis* 13: 560–565.
7. Herman, G.E., Boyd, Y., Chapman, V., Chatterjee, A., and Brown, S.D.M. 1994. *Mammalian Genome* 5: S276–S288.
8. Hoshi, M. and Toyoda, Y. 1985. *Jpn. J. Zootech. Sci.* 56: 913–937.
9. Kunieda, T., Xian, M., Kobayashi, E., Imamichi, T., Moriwaki, K., and Toyoda, Y. 1992. *Biol. Reprod.* 46: 692–697.
10. Nagamine, C.M., Chan, K., and Low, Y-F.C. 1989. *Am. J. Hum. Genet.* 45: 337–339.
11. Toyoda, Y., Yokoyama, M., and Hoshi, T. 1971. *Jpn. J. Anim. Reprod.* 16: 147–151.
12. Valdivia, R.P.A., Kunieda, T., Azuma, S., and Toyoda, Y. 1993. *Mol. Reprod. Dev.* 35: 121–126.
13. Weber, J.L. and May, P.E. 1989. *Am. J. Hum. Genet.* 44: 388–396.