Novel PCR Assay for Determining the Genetic Sex of Mice

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Key Words

Genetic sex · Mouse · PCR · Sex determination · X chromosome · Y chromosome

Abstract

A number of studies require the determination of the genetic sex of mouse embryos before sexual differentiation and/or of mutant mice that display partial or complete sex reversal. The majority of current methods for sexing by PCR involve multiplexing of 2 primer pairs. We have developed a novel sexing PCR using a single primer pair that amplifies fragments from the X and the Y chromosome with a clear size difference between the respective amplicons. This assay provides a rapid and reliable method to identify the genetic sex of mice across different mouse strains.

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In mice, like in most mammals, genetic sex is determined at the time of fertilization when the X chromosome-bearing oocyte receives an X or a Y chromosome from the sperm. XX and XY individuals will normally develop into females and males, respectively. Sexual differentiation, however, does not start until approximately 11.5 days post coitum, when the male-determining gene *Sry* is expressed from the Y chromosome in the bipotential genital ridge, the embryonic anlage of the gonads. The expression of *Sry* induces a complex network of testis-spe-

cific gene expression, regulation and interaction that directs the differentiation of the genital ridge into a testis. If no functional *Sry* is expressed, or its expression is delayed, female-determining gene pathways are activated resulting in the development of an ovary [Wilhelm et al., 2007]. The testes and ovaries then produce hormones that will drive the differentiation of most, if not all, other secondary sexual characteristics. Until these differentiation processes take place, XX and XY embryos are morphologically and anatomically indistinguishable. Any experiments utilising samples before such a stage require sexing by other means.

Methods to determine the genetic sex are also necessary to study mice with partial or complete sex reversal. These mutations have been found in a number of genes involved in sex determination and sex differentiation. They encode factors expressed in the bipotential genital ridge prior to *Sry* expression such as the insulin receptors [Nef et al., 2003], primary sex determining genes like *Sox9* in XY and *Cttnb1* in XX animals [Chaboissier et al., 2004; Barrionuevo et al., 2006; Liu et al., 2009] as well as downstream genes such as *Fgf9* and its receptor *Fgfr2* [Schmahl et al., 2004; Kim et al., 2007; Bagheri-Fam et al., 2008]. Similarly, transgenic mice with ectopic gene expression in the developing gonads display sex reversal phenotypes such as the expression of *Sox3* or *Sox10* in an ovary resulting in XX female-to-male sex reversal [Polanco et al., 2010; Sutton et al., 2011]. In order to fully characterize the phenotypes of these mice, it is critical to unequivocally determine their genetic sex.

Over the years, a variety of methods have been developed to identify the genetic sex of mouse embryos. These range from enzyme assays detecting the activity of the X-linked enzyme glucose-6-phosphate dehydrogenase [Williams, 1986] to cytogenetic techniques such as chromosome C-banding [Gimenez et al., 1993] or amnion staining to visualize the Barr body [Capel and Batchvarov, 2008], which are of limited use because they require experience or have low accuracy. More recently, PCR amplification of Y-chromosomal DNA that detects the male-determining gene *Sry* [Kunieda et al., 1992; Greenlee et al., 1998; Lavrovsky et al., 1998; Lambert et al., 2000; McClive and Sinclair, 2001] and/or the Y-chromosomal gene *Zfy* [Kunieda et al., 1992; Greenlee et al., 1998; Beverdam et al., 2010] has been used to determine male genetic sex. However, in these assays, female genetic sex is concluded based on the absence of a PCR amplicon. To overcome this limitation, several PCR assays have been developed that combine detection of *Sry* and/or *Zfy* with the amplification of an X-chromosomal gene or an autosomal gene as internal control. Examples of these internal controls include *Dxnds3* [Kunieda et al., 1992; Greenlee et al., 1998], the genes encoding the β -subunit of the thyroid-stimulating hormone (*TSH β*) [Lavrovsky et al., 1998], myogenin (*Myog*) [McClive and Sinclair, 2001], and interleukin 3 (*Il3*) [Lambert et al., 2000]. Owing to the potential confounding problems with multiplex PCR assays [Markoulatos et al., 2002], the development of a simplex PCR assay for determining genetic sex is an important goal.

There are currently only 2 methods described that use a simplex PCR assay for the determination of the genetic sex in mice [Chuma and Nakatsuji, 2001; Clapcote and Roder, 2005]. The first of these uses PCR to amplify the homologous genes *Uba1* and *Ube1y1* on the X and Y chromosome, respectively. Here, the primers were designed to cover deleted regions within the *Ube1y1* gene, resulting in 2 amplification products in males, but only the larger product in females [Chuma and Nakatsuji, 2001]. The alternative simplex method uses a single set of primers to amplify the X-chromosomal gene *Kdm5c* (formerly *Jarid1c*) and the Y-chromosomal gene *Kdm5d* (formerly *Jarid1d*; [Clapcote and Roder, 2005]). However, in both cases, the size differences of the amplicons are relatively small, 19 bp for *Uba1/Ube1y1* and 29 bp for *Kdm5c/d*, presenting some difficulties in accurately assessing the results by gel electrophoresis. Here, we present a novel PCR assay using 1 pair of primers that results in a much larger size difference for products generated from the X and the Y chromosome, respectively.

Materials and Methods

Mouse Strains

Tail tissue was collected from the outbred CD1 and Quackenbush, the inbred C57BL/6, Balb/c, FVB, and 129 mouse strains as well as from mice on a mixed background (CBA $\times$ C57BL/6). Protocols and use of animals were approved by the Animal Welfare Unit of the University of Queensland (approval # IMB/131/09/ARC), which is registered as an institution that uses animals for scientific purposes under the Queensland Animal Care and Protection Act (2001).

DNA Extraction

Pieces of mouse tissue were placed in 200 μ l of PBND buffer (PCR buffer with non-ionic detergents, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% (v/v) Nonidet P-40, 0.45% (v/v) Tween 20; autoclaved and stored frozen) supplemented with 100 μ g/ml proteinase K. Samples were incubated at 55°C with occasional vortexing for 15 min up to overnight, followed by heat-inactivation of the proteinase K at 95°C for 5 min. One microliter of each sample was added directly to a 25 μ l PCR reaction.

Primer Design

Genomic sequences for the X-chromosomal *Xlr* (X-linked lymphocyte-regulated complex, NM_011725) and the Y-chromosomal *Sly* (*Sycp3*-like Y-linked, BC049626) genes were obtained from the UCSC server [Kent et al., 2002] and compared for sequence identity using BLASTN (v2.2.23) [Altschul et al., 1997]. Genomic regions with high sequence identity were re-aligned using the CLUSTAL X Multiple Sequence Alignment Program (v2.0.12) (<http://www.clustal.org>) and analysed using the Jalview (v2.6) sequence alignment editor [Clamp et al., 2004]. Candidate primer pairs were analysed using NetPrimer (<http://www.premierbiosoft.com/netprimer/index.html>) and screened for specificity using NCBI Primer-BLAST [Ye et al., 2012] and UCSC in silico PCR [Zweig et al., 2008].

PCR Reactions

Genomic DNA was amplified with the following primer pairs: SX_F, 5'-GATGATTTGAGTGGAATGTGAGGTA-3'; SX_R, 5'-CTTATGTTTATAGGCATGCACCATGTA-3'; Zfy_F, 5'-GACTAGACATGTCTTAACATCTGTCC-3'; Zfy_R, 5'-CCTATTGCATGGACTGCAGCTTATG-3' [Beverdam et al., 2010]; Ube_F, 5'-TGGTCTGGACCCAAACGCTGTCCACA-3'; Ube_R, 5'-GGCAGCAGCCATCACATAATCCAGATG-3' [Chuma and Nakatsuji, 2001]. PCR reactions were performed in a final volume of 25 μ l with 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 μ M primers, and 1 unit Taq polymerase (New England Biolabs, Mass., USA) and the following PCR parameters: initial denaturation at 94°C for 2 min, 35 cycles with 94°C for 30 s, 57°C (SX and Ube primers) and 55°C (Zfy primers) for 30 s, and 72°C for 30 s, followed by final elongation at 72°C for 5 min. PCR products were electrophoresed together with a DNA ladder (100 bp, New England Biolabs) on 2% agarose gels and visualized with ethidium bromide under UV-illumination.

Fig. 1. Comparison of the pseudoautosomal genes *Sly* and *Xlr*. **A** Schematic representation (not to scale) of the genomic arrangements of *Sly* and *Xlr*. Homologous exons are marked in orange. Primers are indicated with arrows. **B** Sequence alignment of *Sly* intron 8 and *Xlr* intron 6. The alignment of the homologous in-

trons of the Y-chromosomal gene *Sly* (intron 8) and the X-chromosomal gene *Xlr* (intron 6) revealed a 405-bp deletion in *Sly* intron 8. Primers to amplify these regions from the sex chromosomes are indicated with forward (SX_F) and reverse (SX_R) arrows. Identical regions between the 2 genes are marked by yellow boxes.

Results and Discussion

To identify suitable genes for the determination of the genetic sex of mouse embryos, we selected the pseudoautosomal genes *Xlr* and *Sly*. These genes belong to highly multicopy gene families with homologues on the X and Y chromosomes. The Y-chromosomal *Sly* gene family consists of at least 70 copies with additional 30 noncoding copies identified [Toures et al., 2005; Cocquet et al., 2009]. The encoded protein is specifically expressed in round spermatids and is thought to play a role in postmeiotic sex chromosome repression [Cocquet et al., 2009]. In contrast, the *Xlr* gene family encodes RNA transcripts that are stage-specifically expressed in lymphoid cells, predominantly late-stage T and B cells [Cohen et al., 1985; Siegel et al., 1987]. Like *Sly*, the *Xlr* gene family consists of numerous copies, with estimates of 50–70 copies per haploid genome, localized in at least 2 different areas of the mouse X chromosome [Garchon et al., 1989]. To date, only 1 potential protein-coding transcript has been identified, with all others predicted to be noncoding [Siegel et al., 1987; Garchon et al., 1989].

Interestingly, *Sly* arose as a chimeric gene fusion of the X-linked genes *Xmr* (also known as *Slx*) and *Xlr*. *Sly* exons 1–4 are derived from *Xmr* exons 1–4, followed by a duplication of *Xmr* exons 3–4. The 3'-end of *Sly*, exons 7–10, matches *Xlr* exons 5–8 (fig. 1A) [Ellis et al., 2007]. The alignment of the homologous regions of *Sly* (exon 7–10) and *Xlr* (exon 5–8) identified a 405-bp deletion within intron 8 of *Sly* by comparison to intron 6 of *Xlr* (fig. 1B). This suggested that PCR amplification across this region should result in amplicons with a large enough size difference to be easily distinguished by gel electrophoresis. We designed primers to regions upstream and downstream of the deletion that were almost identical between the 2 genes (fig. 1, arrows) and would yield a 280-bp and a 685-bp PCR product for *Sly* and *Xlr*, respectively.

We next performed PCR in a blinded assay with genomic DNA from 3 XX and 3 XY mice from each of the following mouse strains, CD1 (fig. 2A), C57BL/6 (fig. 2B) and mixed background (CBA×C57BL6; fig. 2C). Each genomic DNA was used in a PCR reaction with the primers for *Sly/Xlr* as well as primer pairs for *Zfy* [Beverdam et al.,

Fig. 2. Determination of the sex of mice using *Sly/Xlr* PCR. XX and XY mouse genomic DNA from CD1 (A), C57BL/6 (B6) (B) and mixed background (C) mice was used as template to amplify *Sly* intron 8 from the Y chromosome and *Xlr* intron 6 from the X chromosome (SX, right panels). As controls, the same genomic DNAs were used as template to amplify *Zfy* (left panels) and *Uba1/Ube1y1* (Ube, middle panels). L = 100-bp DNA ladder.

2010] and *Uba1/Ube1y1* [Chuma and Nakatsuji, 2001] as controls. Sexing PCR using *Zfy* primers confirmed the presence of the Y chromosome in 3 out of the 6 samples for each mouse strain (fig. 2, left panels). Similarly, using the primers amplifying *Uba1* from the X chromosome and *Ube1y1* from the Y chromosome, we obtained the expected 217-bp *Uba1* in XX samples and the 198-bp *Ube1y1*, but rarely detected the 217-bp product in XY samples (fig. 2, middle panels).

As predicted, amplification using the primers for *Sly/Xlr* resulted in a 280-bp product from the Y chromosome. However, amplification from the X chromosome not only yielded the expected 685-bp fragments, but also products of approximately 480 and 660 bp (fig. 2, left panels). Similar to the *Uba1/Ube1y1* assay, the larger PCR products from the X chromosome were often only faintly detected using XY genomic DNA. This is most likely due to the preferential amplification of shorter products. Sequencing of some of the X-chromosomal PCR products revealed that they arise from different copies of the *Xlr* gene family (data not shown). The same amplification products were obtained for XX and XY mice from the in-

bred mouse strains 129, Balb/c and FVB as well as for the outbred Quackenbush mice (data not shown), showing that this PCR is a reliable method to identify the genetic sex of mice across different mouse strains.

To determine the optimal annealing temperature for the primer pair, we repeated the PCR using different annealing temperatures ranging from 45 to 65°C. We obtained the expected PCR products at all temperatures tested with the highest yield for both sexes at 52.7°C and 57.6°C (data not shown). In addition, we shortened the incubation steps during the PCR for rapid genotyping, which is essential, for example, for experiments involving the culture of live tissues. Sufficient amplification was still achieved with initial denaturation at 94°C for 3 min, followed by 35 cycles of 15 s each of 94, 57 and 72°C, and a final elongation for 5 min at 72°C. A further reduction in time was achieved by the short amount of time needed for the gel electrophoreses. Due to the considerable size difference of the amplicons, 15 min at 100 V/cm was sufficient for their separation, making it a fast and reliable method to determine the genetic sex of mice and mouse embryos.

Our new sexing PCR method not only has the advantage that it uses a single primer pair to amplify sequences from both the X and the Y chromosome simultaneously, but also it is easy to interpret the results based on the large size differences in the fragments obtained from the X and Y chromosome, respectively. In addition, our assay is robust, can be used for different mouse strains, saves time and material, and is therefore less expensive than other methods.

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