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Use of a Rat cDNA Probe Specific for the Y Chromosome to Detect Male-Derived Cells

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ABSTRACT: A cDNA probe that exhibits specificity for the rat Y chromosome was generated by using a set of primers specific to the murine *Sry* gene, the sex-determining region of the Y chromosome. A 459-base pair (bp) DNA fragment was obtained by polymerase chain reaction (PCR) amplification from male, but not female, rat genomic DNA (EMBL Nucleotide Sequence Database accession number X89730). This DNA fragment was purified, cloned using a vector, and digested with *EcoR1* to yield a 270-bp DNA fragment. This 270-bp cDNA fragment, when used as a probe in Southern blot analysis of rat DNA, was observed to bind to three separate bands of approximately 2.3, 5.0, and 7.0 kb in size. The binding was demonstrated with male, but not female, genomic DNA. Another set of primers was generated to sequences within the 270-bp fragment that produced a PCR product of 104 bp. This DNA fragment, when used as a probe in Southern blot analysis, enabled PCR detection of at least 0.1% male cells in a mixed population of female cells. These cDNA probes should prove useful in studies designed to track cell populations (e.g., tumor metastasis and hemopoietic cells after bone marrow transplantation) in syngeneic male/female pairs. In addition, a cDNA probe that is specific for the rat *Sry* gene might be valuable in studies of fetal male sexual development or the study of spermiogenesis.

Key words: *Sry* gene, polymerase chain reaction.

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The *Sry* gene, located on the short arm of the Y chromosome, encodes for a putative factor that contains a high-mobility group (HMG) region believed to bend its DNA target and modulate transcription (King and Weiss, 1993). The SRY protein presumably acts on one or more genes downstream to *Sry* to initiate a cascade of events that induces sexual development in the male mammal (Berta et al, 1990; Gubbay et al, 1990; Haqq et al, 1993). Most studies on this process, however, have used either murine (Lovell-Badge, 1993) or human (McElreavey et al, 1993) tissue, and little or no information has been published on the rat, a frequently used animal model in many other areas of research.

Experiments on bone marrow transplantation, immune cell function, and tumor growth often involve tracking donor cells within a recipient. One strategy, which permits the use of animals with identical major histocompatibility antigens and obviates the need to introduce foreign marker genes, is to use male–female combinations. With markers that can detect DNA specific for the Y chromosome, it is possible to trace the progeny of male cells within a female recipient (Van Dekken et al, 1989; Agematsu et al, 1990; Lawler et al, 1991; Przepiorka et al, 1991; Santucci et al, 1992; Landman-Parker et al, 1993; Gerritsen et al, 1994). Although this approach has been used with both mice and human subjects, analogous markers have not been available for the rat, a model commonly used for such studies. Here, we describe the derivation of a DNA probe specific for the rat Y chromosome and illustrate its use in the detection of male-derived cells by both Southern blotting and the polymerase chain reaction (PCR). This marker ought to prove useful in studies designed to identify donor cells as well as to investigate the role of SRY in male sexual development.

Materials and Methods

In summary, oligonucleotide probes were prepared as follows (details below): First, a 459- base pair (bp) product was prepared by PCR from genomic DNA of a male rat using primers selected from the analogous sequence published for the murine *Sry* gene; after plasmid expansion, an *EcoR1* digest resulted in a 270-bp fragment that was labeled and used for Southern blotting. From the sequence of this subclone, a second set of primers amplifying a 104-bp fragment was generated for use in subsequent PCR assays.

Preparation of DNA

Nucleated cells obtained from marrow or thymus of male rats were collected from the interface after Ficoll-Hypaque centrif-

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Table 1. *Optimal conditions for* Sry *primer set*

Cycles	$MgCl_2$ concentration 1.5 mM	2.5 mM	3.0 mM
Annealing temperature: 54°C			
20	+	+++	++
30	+	++	++
35	+	++	++
Annealing temperature: 58°C			
20	–	–	–
30	++	+++*	++
35	+++	++	++
Annealing temperature: 62°C			
20	+	+	–
30	++	++	++
35	++	++	++

–, No amplification; +, amplification detected, very weak signal; ++, amplification with a faint signal or multiple bands; +++, amplification with a clear strong signal.
* Considered optimal by authors.

ugation, washed 3× with PBS, and 10^7 cells were resuspended in 400 µl TNE buffer (10 mM Tris, pH 7.9; 10 mM ethylenediamine tetraacetic acid [EDTA]; 10 mM NaCl) plus 400 µl 20% sodium dodecyl sulfate (SDS) and 40 µl of pronase K (20 mg/ml). The suspension was agitated with a vortex mixer and then incubated at 37°C for 12–15 hours. DNA was phenol-extracted, ethanol-precipitated, and then dissolved in TE buffer (1 mM Tris, pH 7.9 with 0.1 mM EDTA).

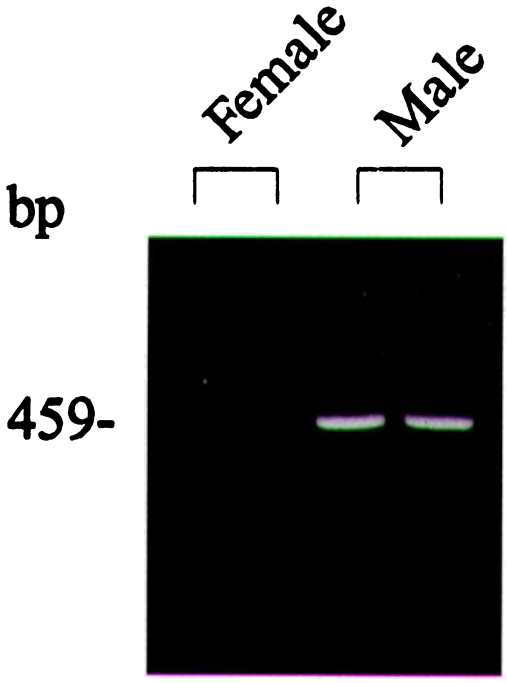

FIG. 1. The polymerase chain reaction (PCR) product obtained from reaction of primer set #1 with male and female rat genomic DNA. Two left lanes: female rat DNA; two right lanes: male rat DNA; samples obtained as described in Materials and Methods.

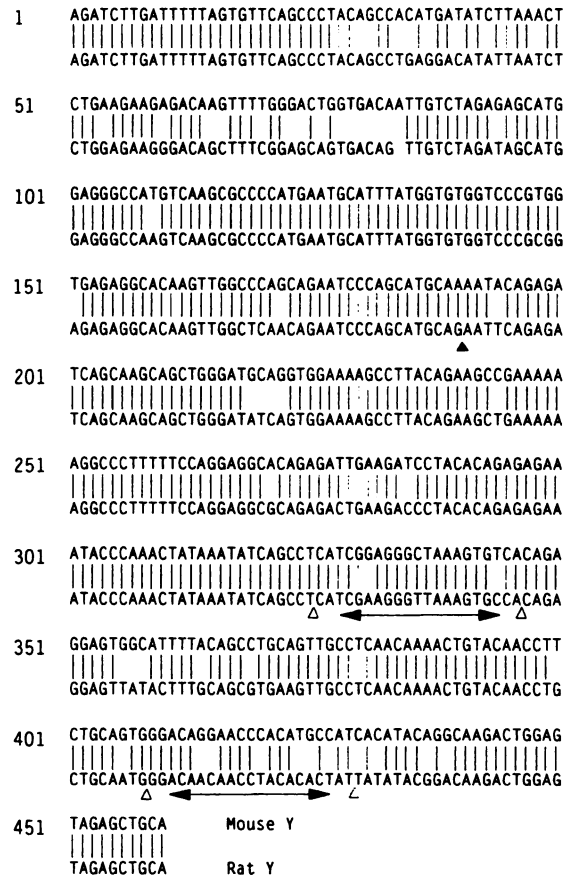

FIG. 2. Comparison of the nucleotide sequence of the 459-base pair (bp) PCR product obtained from rat DNA with that published for the homologous sequence within the murine *Sry* gene. The solid triangle denotes the *EcoR1* cleavage site, 3′ to which lies a 270-bp fragment. The open triangles indicate the primers used in the PCR assay to detect to the 104-bp sequence. Note that base #84, present in the mouse, was not identified in rat DNA.

Generation of the Initial Probe by PCR

The first PCR product (459 bp) was obtained using primer set #1 with the following sequence: 5′ primer, 5′-AGATCTTGATTTTTAGTGTTC-3′ and 3′ primer, 5′-TGCAGCTCTACTCCAGTCTTG-3′ to regions of the *Sry* gene of the murine "sex determining region" carried on the Y chromosome (Gubbay et al, 1990; Tucker and Lundrigan, 1993; Whitfield et al, 1993). The reaction mixture contained 1 µg genomic DNA, 1 µM of each primer, 200 µM dNTPs, 1× PCR buffer, and two units Taq polymerase (all from Perkin Elmer) in a final volume of 100 µl. Thirty amplification cycles were performed, each cycle consisting of a denaturation step at 94°C for 1 minute, an annealing step at 50°C for 1 minute, and an extension step at 72°C for 2 minutes. Following these 30 cycles, an additional extension was performed at 72°C for 7 minutes. The PCR products were separated by electrophoresis in 0.8% agarose gel and stained with ethidium bromide to assess their size.

Cloning and Sequencing of the Y Chromosome-Related PCR Product

The 459-bp DNA fragment, which was amplified by male (but not female) rat genomic DNA, was eluted from agarose gel,

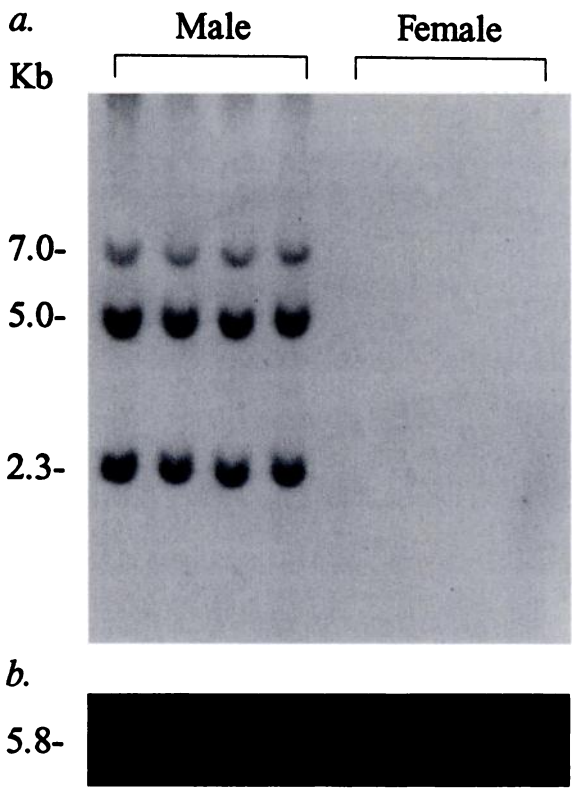

FIG. 3. Southern blot analysis of the *EcoR1*-digested genomic fragments. **(a)** Blot of genomic DNA prepared from thymi of four male (the four left lanes) and four female (the four right lanes) rats and hybridized with the ^{32}P-labeled 270-bp cDNA probe; **(b)** Hybridization of the same membrane with a labeled cDNA probe for actin as a control for gel loading.

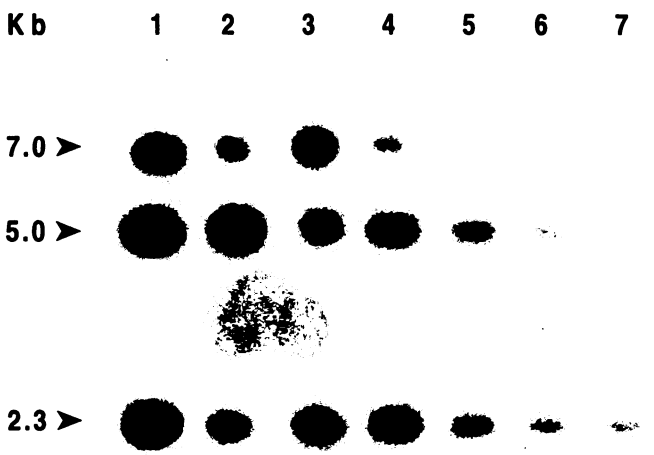

FIG. 4. Detection of male thymocytes in a mixture of female bone marrow cells by Southern blotting. A DNA mixture was made with the following ratios of male thymocytes added to female bone marrow cells: lane 1, 100/0; lane 2, 50/50; lane 3, 45/55; lane 4, 40/60; lane 5, 30/70; lane 6, 10/90; lane 7, 5/95.

purified, and cloned using the TA cloning™ vector (Invitrogen, San Diego, California; #K2000-01). Plasmid DNA was extracted following the "miniprep" protocol as described by Sambrook et al (1989). The inserts were then sequenced according to the dideoxy chain termination technique of Sanger et al (1977). The sequence of the 459-bp fragment is available through the EMBL Nucleotide Sequence Database under accession number X89730.

Preparation of the Probe for Hybridization

The plasmid DNA containing the insert was digested with *EcoR1* (5 units/μg) and then electrophoresed in low-melting-temperature agarose gel. A 270-bp fragment was isolated and labeled by random priming with [α-^{32}P]-dATP according to the procedure of Feinberg and Vogelstein (1983).

Southern Blot Analysis

Genomic DNA (3–5 μg), previously digested with *EcoR1*, was electrophoresed on a 0.8% agarose gel and transferred to a Gene Screen Plus™ membrane (#NEF-976; Dupont). After transfer, the membrane was subjected to prehybridization for 4 hours at 42°C in a solution containing 50% deionized formamide, 5× SSPE (1× SSPE = 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA), 100 μg denatured salmon sperm DNA/ml, and 1% SDS. Hybridization was performed for 12–16 hours at 42°C in prehybridization solution containing 10% dextran sulphate, 1× Denhardt's solution, and 1× 10^6 cpm/ml of the [α-^{32}P]-dATP-labeled 270-bp DNA probe. As a control for loading, the membrane also was reacted with 1 × 10^5 cpm/ml of [α-^{32}P]-dATP-labeled probe that binds to common sequences in the chicken actin gene (Cleveland et al, 1980). The membrane was washed twice in 2× standard saline citrate (SSC) for 20 minutes at room temperature, washed once in 0.1× SSC/0.1% SDS for 30 minutes at 65°C, and then exposed to autoradiographic film for 3–5 days.

Detection of Male-Derived Cells by PCR

The second PCR product (104 bp) was obtained using primer set #2 with the following sequences: 5′ primer, 5′-CATC GAAGGGTTAAAGTGCCA-3′ and 3′ primer, 5′-ATAGTG TGTAGGTTGTTGTCC-3′ to a region toward the 3′ end of the 459-bp DNA fragment. These primers were selected using Primer Analysis Software (Oligo Version 4.1 published by National Biosciences Inc., Plymouth, Minnesota). Optimal conditions were sought under a range of $MgCl_2$ concentrations, annealing temperatures, and number of cycles, and the results are shown in Table 1. The PCR product was separated by electrophoresis in 2.5% agarose gel and stained with ethidium bromide to assess size.

To assay for the presence of male cells in a mixture of male and female cells, the two populations were mixed in the proportions stated in "Results," and genomic DNA was prepared. The PCR reaction mixture (100 μl total volume) consisted of genomic DNA (0.5 ng/μl; 50 ng total), 1 μM of each primer, 200 μM of each dNTP, 1× PCR buffer containing 2.5 mM $MgCl_2$, and 0.01 u/μl of Taq DNA polymerase (all from Perkin Elmer). Thirty amplification cycles were performed, each cycle consisting of a denaturation step at 94°C for 1.25 minutes, an annealing step at 58°C for 2.5 minutes, and an extension step at 72°C for 2.5 minutes. Following these 30 cycles, the PCR products were separated by electrophoresis in 2.0% agarose gel and stained with ethidium bromide to assess their size. To minimize

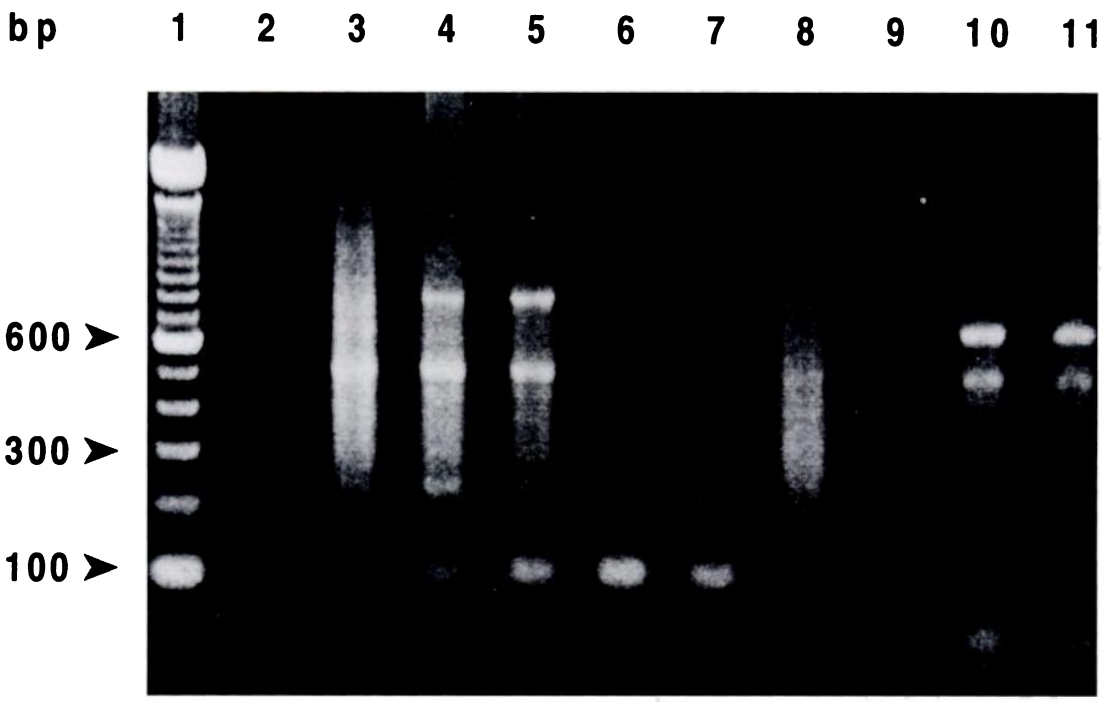

FIG. 5. Use of PCR to detect male thymocyte DNA in a suspension of female bone marrow cells. Male thymocytes were added to female nucleated bone marrow cells and the suspension was prepared for PCR analysis as described in Materials and Methods: Lane 1, standards/100 bp ladder; lane 2, empty; lane 3, 100% female rat DNA; lane 4, 1/1,000 male/female rat cells; lane 5, 1/100 male/female rat cells; lane 6, 100% male rat DNA; lane 7, 100% male mouse DNA; lane 8, 100% female mouse DNA; lane 9, blank; lane 10, 100% female human DNA; lane 11, 100% male human DNA.

the occurrence of false-positive signals, the recommendations of Kwok and Higuchi (1989) were followed.

Animals

Inbred male and female Brown Norway rats, age 6–10 weeks, were purchased from Charles River Canada (St. Constant, Québec, Canada) and received standard laboratory chow and water *ad libitum*. Animal care was in accordance with approved institutional guidelines.

Results

Following the strategies outlined above that used primers derived from the mouse *Sry* sequence, we first obtained a 459-bp fragment that was amplified from male but not female DNA (as presented in Fig. 1). Determination of the nucleotide sequence of this product enabled comparison with that published for the analogous DNA fragment from murine DNA. Five independent clones were sequenced, and the results are shown in Figure 2. Overall, comparison of the two fragments from rat and mouse indicated they were 88% homologous, with the greatest degree of overlap in the central region. From bases 1–100 there were 22 differences, but from bases 101–219 there were only three; similarly, from bases 229–350 there were only five differences, but from bases 351–451 there were 16 differences.

A 270-bp fragment, obtained by *EcoR1* digestion of plasmid DNA containing the 459-bp product (Fig. 2), was used as a probe to detect male-derived genomic DNA by Southern blotting at high stringency. As shown in Figure 3a, the probe reacted with bands of 2.3, 5.0, and 7.0 kb from male but not female DNA. By contrast, the control, a chicken actin probe, bound equally well to both male and female DNA (Fig. 3b). Use of this probe in conjunction with Southern blotting was adequate to detect 5% male-derived thymocytes added to a suspension of female bone marrow cells (Fig. 4) without having reached the lower limit of detection.

Additional primers were generated to the sequences described in Figure 2 (shown by the open triangles) and were used with PCR to amplify DNA to the level of detection from at least 0.1% male cells in a mixture of female cells (Fig. 5). The absence of a 104-bp band, when the reaction mixture was 100% female rat DNA, indicates male specificity. These primers also detected the presence of male murine cells, but further species restriction was demonstrated by the lack of a 104-bp band when human-derived male leukocyte DNA was used. Considering the sensitivity of this second set of primers to detect and amplify male-derived DNA, the optimum assay conditions were determined (Table 1). These conditions included addition of 2.5 mM $MgCl_2$, an annealing temperature of 58°C, and 30–35 cycles in the PCR.

Discussion

Using primers designed from the published sequence of the mouse *Sry* gene, a 459-bp sequence was amplified from male rat DNA. The specificity of these primers for the Y chromosome was demonstrated by the fact that they did not amplify female rat DNA (Fig. 1). Furthermore, the 270-bp cDNA probe derived from the 459-bp sequence could be used to detect male DNA in a mixture of a female cells by Southern blotting. The three bands observed using the 270-bp probe (Fig. 3) are consistent with the fact that the rat carries multiple copies of the *Sry* gene, in contrast to European-derived mice, which carry only one (Nagamine, 1994). Proof of this, however, would require sequence data from the appropriate flanking regions.

Comparison of the initial rat Y-linked PCR product of 459 bp with that of the mouse *Sry* gene shows that the two regions are approximately 88% homologous. The lower concentrations of 5% for Southern blotting and 0.1% for PCR detection were shown to illustrate the approximate range of activity of the probes and do not indicate the lower limits that might be attained by further refining the experimental conditions.

Subsequently, we have used this tool to confirm engraftment of male bone marrow cells injected into female recipient rats (An et al, 1996). In addition, the primers for the 104-bp cDNA have been used to distinguish male and female rat fetuses during mid- to late gestation. These primers also yielded a cDNA product when used against DNA samples from adult male tissues but did not yield any product when tested with various female tissues. Finally, because the probes selected to the 104-bp fragment amplify male DNA from both rats and mice, they may have wider application than a reagent reactive with only one species.

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