Amplification of Bovine Y-Chromosome DNA by Microdissection and Polymerase Chain Reaction

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Abstract: *In order to establish a strategy to construct a DNA library from a specific region of a chromosome, bovine Y-chromosome DNA was amplified by the microdissection of whole chromosomes and polymerase chain reaction (PCR). Intact Y-chromosomes were microdissected from metaphase lymphocyte chromosomes and were digested with Proteinase K, followed by DNA extraction with phenol/chloroform. Purified DNA was cleaved with the restriction enzyme Sau3AI and the fragments were ligated to a synthetic linker-primer into which EcoRI site composite was introduced. The resultant Y-chromosome nucleotide sequences were amplified by PCR and cloned with vector DNA (EcoRI-digested pUC19 DNA). Sau3AI-digested pUC19 DNA served as a positive control and could be amplified by PCR using at least 0.2 pg of DNA. The Y-chromosome was successfully microdissected under an inverted microscope (400 ×), DNA was amplified by the PCR method and a smear of DNA (0.15–1.0 kb) was obtained. Two of sixty-five clones (0.05–1.2 kb) were male-specific by dot hybridization to male and female genomic DNA but no clone was found to be Y-chromosome specific by Southern hybridization.*

Key words: *Microdissection, Microcloning, PCR, Bovine, Y-chromosome, Sexing.*

Recently DNA probes derived from Y-chromosome specific DNA sequences have been used to determine the sex of bovine embryos or sperm by slot blot hybridization [1], polymerase chain reaction (PCR) [2, 3] or fluorescent *in situ* hybridization (FISH) [4]. These male-specific DNA sequences, which were enriched by heterologous hybridization of male and female genomic DNA fragments, have been patented [5–7] or restricted to research and not to be used for industrial use [8]. Consequently, new primer sequences for sexing of bovine embryos should be developed by ourselves. Kudo *et al.* [9] cloned the new male-specific repetitive DNAs by phenol emulsion reassociation and succeeded in applying them for sexing embryos [10].

On the other hand, Scalenghe *et al.* [11] dissected fragments from section 3 of the salivary gland X-chromosome of *D. melanogaster* and obtained region-specific clones by cloning to a vector. Bishop *et al.* [12] applied the microdissection and microcloning method to the Y-chromosome and obtained 4 unique or low copy number probes mapping to the Sxr region of mice. Lüdecke *et al.* [13] succeeded in isolating a region-specific probe for Langer-Giedion syndrome by microdissection of banded chromosomes and universal enzymatic amplification instead of *in-vitro* packaging. Wesley *et al.* [14] succeeded in amplifying genomic DNA fragments by the PCR method with a nonspecific primer. Furthermore, Meltzer *et al.* [15] generated region-specific DNA probes by chromosome microdissection and PCR amplification with a degenerate primer sequence and then used inserts for FISH.

In the present study, at first we amplified a Y-chromosome genomic DNA by PCR of microdissected and extracted DNA by improved methods in order to establish a strategy to construct a DNA library from a specific region of the chromosome. Secondly we characterized the cloned bovine Y-chromosome DNA by dot and Southern hybridization.

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Materials and Methods

Chromosome preparation

Bovine (Holstein) male chromosome squashes were prepared by fixing lymphocytes. Briefly, male calf blood was collected from the jugular vein and cultured in HEPES-buffered TCM199 with Earl's salt (GIBCO, USA) supplemented with 10% fetal bovine serum and phytohemagglutinin (GIBCO, 13 μg/ml) for 70 h at 38°C. Then 0.4 μg/ml of vinblastine sulfate (Sigma, USA) was added to the medium and further cultured for 6 h. Chromosome squashes were prepared by the fixation with acetic acid-methanol (1:3) after the hypotonic treatment with 0.9% sodium citrate. They were preserved at −20°C until microdissection.

Microdissection of bovine Y-chromosome

All micromanipulations were performed under a Nikon inverted microscope (Tokyo, Japan) with a Narishige micromanipulator (Tokyo, Japan). Each position of the Y-chromosome was recorded (400 ×) and about 20 intact Y-chromosomes were microdissected from metaphase lymphocyte chromosomes with a siliconized glass needle followed by transfer to a glass slide with a hole (10 mm in diameter). They were covered with Serum tube (11 mm in diameter, Sumitomo Berkrite) containing wet Kim wipe for 2 min to make a small water drop including chromosome fragments and then covered with paraffin oil.

DNA extraction and Sau3AI digestion

DNA extraction and digestion with restriction enzyme were carried out under the microscope. About 1–5 nl of Proteinase K solution (500 μg/ml, 10 mM Tris-HCl: pH 7.5, 10 mM NaCl, 0.1% SDS) was added to the water drop containing chromosome fragments and incubated for 2 h at 37°C. A five-fold volume of water saturated phenol was added to this mixture and phenol was removed with a micropipette after 10 min. This step was repeated three times. Two-μl of chloroform was then added to the drop and the paraffin oil was changed followed by incubation for 1 h. More than a 10-fold volume of *Sau*3AI reaction mixture [10% *Sau*3AI (50 U/μl, Takara) in 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$] was added to the DNA solution and incubated for 4 h at 37°C. Distilled water was added to make 1 μl and this drop was transferred to a 0.5 ml Eppendorf tube.

Amplification of Y-chromosome derived DNA fragments by PCR

The DNA fragments from the Y-chromosome were amplified by the method of Jinno *et al.* [16] with minor modification of restriction as shown in Fig. 1. Briefly, pUC19 DNA/*Sau*3AI fragments were ligated with synthetic primer (5'-CGGGAATTCTGGCTCTGCGACAAC-3') with introduced *Eco*RI site composites mediated through a synthetic linker (5'-GATCGTTG-3') as a pilot experiment. The DNA fragments (5, 1 and 0.2 pg) were added to a final volume of 5 μl in the tube of reaction mixture (7.0 μM linker, 29 μM primer, 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 10 mM dithiothreitol, 0.1 mg/ml bovine serum albumin and 14 U/μl T_4 DNA ligase) and incubated at 15°C, for 30 min. The DNA ligates (5 μl) were amplified by PCR in a final volume of 50 μl composed of 200 μM each of dNTP, 20 mM Tris-HCl pH 8.5, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Tween-20 and 2.5 U of Taq DNA polymerase (Takara, Japan). Amplifications were performed in a program temperature control system (Astec, Tokyo, Japan). After one cycle at 73°C for 2 min; 94°C for 3 min; 60°C for 2.5 min and 73°C for 2 min for end filling, Taq DNA polymerase was added and 25 cycles at 94°C for 2 min for denaturing; 60°C for 2.5 min for anealing; 73°C for 2 min for extension; and one cycle at 73°C for 10 min were carried out.

The *Sau*3AI-digested DNA fragments of bovine Y-chromosome were ligated with primer and linker, and amplified by PCR as described above. PCR products

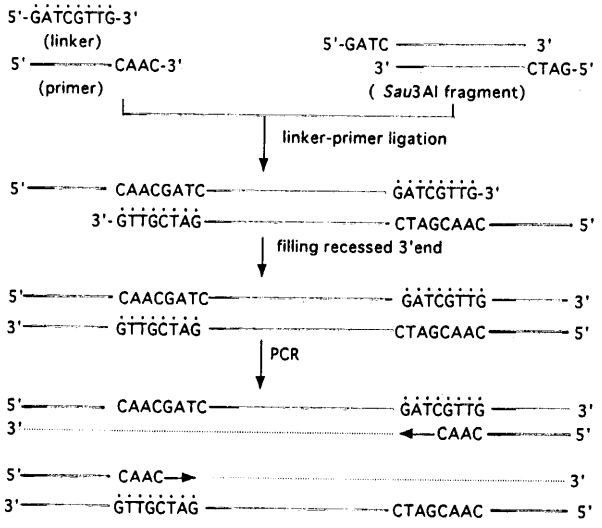

Fig. 1. Alternative amplification of DNA fragments from dissected chromosome by ligation with primer through linker.

were identified by electrophoresis on 3% agarose gel.

Cloning of EcoRI DNA fragments

We have employed the α-complementation system [17] of β-galactosidase to descriminate recombinant clones by using pUC19 vectors. Briefly, PCR products of DNA fragments from the Y-chromosome were digested with *Eco*RI overnight at 37°C and ligated to *Eco*RI-digested dephosphorylated pUC19 DNA at 15°C overnight. Two-microliters of the ligation mixture was added to 210 μl of competent bacteria (JM 109 cells). Plating of the bacteria on LB agar containing ampicillin (LB-Amp agar) with 25 μl each of 4% 5-bromo-4-chloro-3-indolyl-β-D-galactoside and 0.1 M isopropyl-β-D-thiogalactopyranoside resulted an average 200 recombinant colonies per 2 μl ligation mixture. These white colonies indicating recombinant colonies were replated on LB-Amp agar and served as a source for plasmid DNA isolation. Preparation of plasmid DNA from culture of bacteria was carried out by the alkaline lysis method [18]. Insert DNA was released by *Eco*RI digestion and isolated on 3% agarose gels. Bacteria with recombinant plasmid were cultured in LB-Amp solution overnight and stored with 20% glycerol at −80°C.

Dot hybridization

Dot hybridization was carried out by means of a non-radioactive DNA labeling and a detection kit (Boehringer-Mannheim Yamanouchi) based on digoxigenin (DIG), anti-DIG and ELISA under supplier-recommended conditions. The principle of DIG-labeled DNA probe detection is as follows. That is, at first, the DIG-11-dUTP labeled DNA probe is located at the anti-DIG-antibody and alkaline phosphatase (ALP) conjugate. Then this location is visualized by antibody-ALP linked color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt. Briefly, 1 μl of *Eco*RI-digested clone DNA was spotted on a nylon filter (Hybond-N+, Amersham, UK) and was dried in an oven at 80°C for 2 h. *Eco*RI-digested genomic DNA from phenol-chloroform extracted bovine male and female liver DNA was labeled with DIG-11-dUTP by random oligonucleotide priming and hybridized to the denatured clone DNA at 65°C overnight. After washing with 2xSSC-0.1% SDS and 0.1xSSC-0.1% SDS, the DIG-labeled DNA probe was detected by color reaction with anti-DIG and ALP under the supplier-recommended conditions.

Southern hybridization

Y-Positive clones from the dot hybridization were verified by Southern hybridization to genomic DNA digested with some restriction enzymes [19]. Briefly, 90 and 900 ng of *Bam*HI-, *Eco*RI-, *Hind*III- and *Sau*3AI-digested male and female genomic DNA were electrophoresed in 1.1% agarose gel and transferred to a nylon filter. Recombinant plasmid DNA was digested with *Eco*RI and the insert DNA was recovered from a DEAE-cellulose filter after the agarose gel electrophoresis [18]. Each insert DNA was labeled with DIG-11-dUTP, hybridized to a nylon filter and the signal was detected immunologically as mentioned above.

Results

Y-Chromosomes were successfully isolated from the preparation by microdissection (Fig. 2-a, b), and 20–25 Y-chromosomes were pooled on a glass slide with a hole.

Defined *Sau*3AI-digested pUC19 DNA fragments were successfully amplified at more than 0.2 pg DNA as shown

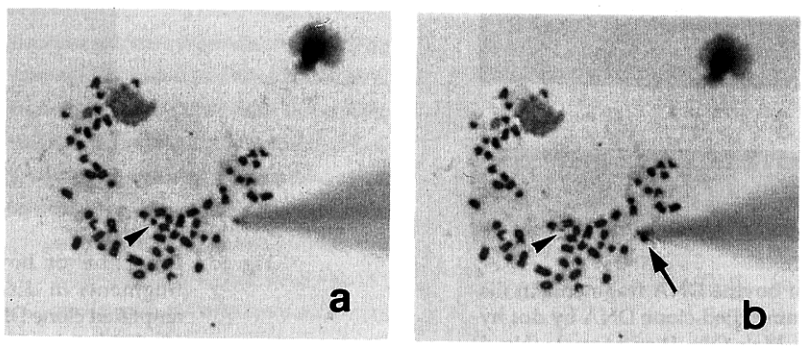

Fig. 2. Microdissection of bovine Y-chromosome (400 ×). (a) Chromosome preparation (arrow head: Y) and (b) microdissection of Y-chromosome (arrow: dissected Y).

in Fig. 3 (Left). These amplified DNA bands were shifted to the cathode because of the binding of double linker-primer at both sides of the cohesive ends. DNA fragments were successfully amplified at the size of 0.15 to 1.0 kb by PCR (Fig. 3, Right). These amplified DNA fragments were digested with *Eco*RI and ligated to *Eco*RI-digested pUC19 vector DNA. Sixty-five (62.5%) of 104 clones investigated included the *Eco*RI inserts and their sizes averaged 0.47 kb with a maximum insert of 1.2 kb and a minimum of 50 bp.

All of these clones were tested by dot hybridization to biotinylated male and female liver DNA. Among these, only two clones (300 bp and 500 bp) hybridized specifically to male DNA (Fig. 4). These clones were labeled with DIG-11-dUTP and further tested by Southern hybridization to male and female liver DNA. As shown in Fig. 5, the microclone insert hybridized to two bands on Southern blots of both *Sau*3AI-digested male and female liver DNA when used as a DIG-labeled hybridization probe. In the present study, no clone was found to be Y-chromosome specific by Southern hybridization.

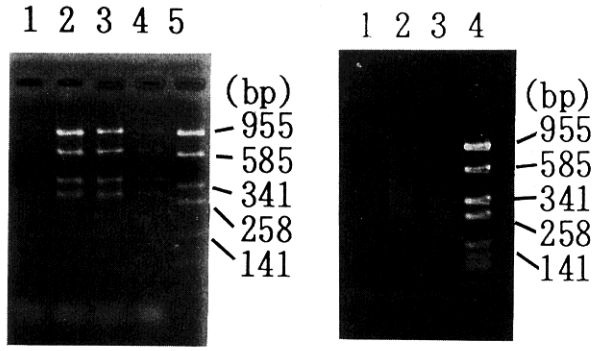

Fig. 3. (Left) PCR products from *Sau*3AI-digested pUC19 DNA fragments. Lane 1, PCR with no added DNA; lanes 2–4, PCR products of pUC19 DNA (5, 1, 0.2 pg); lane 5, no amplified pUC19 DNA (0.25 μg). (Right) PCR products from microdissected bovine Y-chromosome. Lane 1, PCR with no added DNA; lanes 2 and 3, PCR products of Y-chromosome derived DNA/*Sau*3AI fragments, lane 4, Size marker.

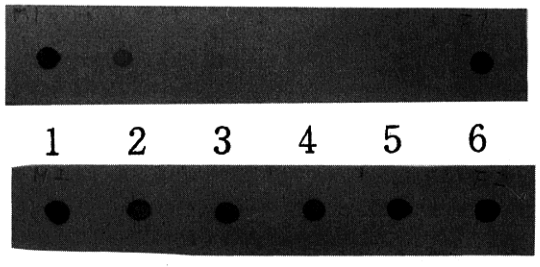

Fig. 4. Detection of bovine DNA fragments in dissected and amplified clone DNA by dot hybridization. Male (No. 1) and female (No. 6) liver DNA and clone DNA (Nos. 2–5) were hybridized to DIG-11-dUTP labeled female (upper filter) and male (lower filter) liver DNA.

Discussion

In the bovine case, only X- and Y-chromosomes are metacentric and the other autosomal chromosomes are all telocentric. It is very easy to identify the Y-chromosome in the preparations without special staining. Unless using PCR, one needs to prepare more chromosome fragments or much DNA for amplification of microdissected chromosome fragments. In the present

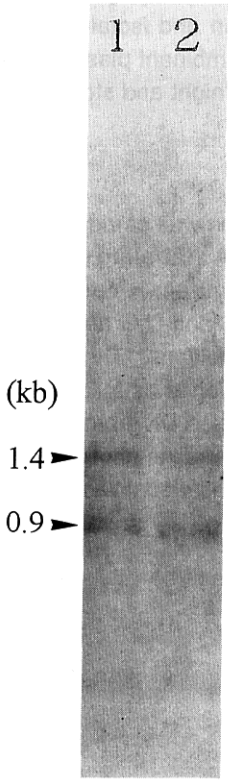

Fig. 5. Detection of bovine DNA fragments in dissected and amplified clone DNA (BDYC-62, 300 bp) by Southern hybridization. Lane 1, *Sau*3AI-digested male liver DNA (900 ng), lane 2, *Sau*3AI-digested female liver DNA (900 ng).

study, amplification of plasmid DNA fragments showed that the minimum DNA content was 0.2 pg and similar results to laboratories using the PCR method were obtained.

There are two types of primers (specific or random) for DNA amplification from microdissected chromosomes or fragments. Lüdecke *et al.* [13] demonstrated that chromosomal *Rsa*I fragments were ligated to *Sma*I vector with symmetric polylinker sequence *Eco*RI-*Sma*I-*Eco*RI and they were successfully amplified by PCR. In our strategy, *Sau*3AI-digested DNA fragments extracted from bovine Y-chromosomes were ligated to a synthetic set of linker (8 mer) and this resulted in a 5' cohesive end. Simultaneously, these ligates were further ligated with synthetic primer (24 mer) with an *Eco*RI-cloning site introduced at positions 4 to 9 from the 5' end. Then the recessed 3' end was filled at 72°C, 10 min and ordinary PCR was performed.

Only four fragments (258–955 bp) of *Sau*3AI-digested pUC19 DNA could be successfully amplified by PCR although 11 other smaller fragments (less than 141 bp) were not amplified. This size is thought to be due to a limitation in this primer-linker ligation system.

The proportions of insert clones in investigated clones varied from 22% to 88% [11, 20, 21]. The size of insert DNA also varied from 0.15 to 8.0 kb [11, 20–22]. These proportions and sizes of DNA inserts depend on the kinds of cloning vector and cloning efficiency. In the present study, the averaged size of inserts was 0.47 kb (0.05–1.2 kb). Lüdecke *et al.* [23] have used pUC vectors and *E. coli* (DH5) in cloning the amplified DNA from dissected chromosome fragments by universal enzymatic amplification. They also obtained smaller (average 0.15–0.22 bp) cloned DNA fragments.

We intended to isolate bovine Y-chromosome specific DNA fragments by a direct chromosome dissection and PCR amplification because it is quite easy to distinguish Y-chromosome from other chromosomes in cattle, but none of 65 microclones from amplified DNA of microdissected bovine Y-chromosome have been shown to hybridize specifically to sequences carried on the Y-chromosome. We had already prepared DNA probes for a centromere region of the translocated chromosome by this technique as well and it has been proven to hybridize to the centromere by FISH (in preparation). Accordingly, the clones isolated in the present study were probably shown to be homologous to other chromosomes because it is well known that some human Y-chromosome sequences have homology with the X-chromosome or autosomes, e.g., pseudoautosomal region [24] and telomere [25]. It can be used for the preparation of region specific DNA probe from other chromosomes as mentioned above although it was not possible to get Y-chromosome specific DNA fragments by means of this strategy because of these structural features of the Y-chromosome.

Acknowledgments

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