韓國受精卵移植學會誌(1995) 第 10 卷 第 1 號
Korean J. Emb. Trans. (1995) Vol. 10, No. 1, pp. 91~100

Sex Determination in Somatic and Embryonic Cells of the Pig by Cloned Male-Specific DNA Fragments†

Jin Tae Jeon, Sang Ho Lee, Ki Chang Hong and Sung Soo Park

Department of Animal Science, College of Natural Resources, Korea University, Seoul 136-701, Korea

클론된 웅성 특이 DNA절편에 의한 돼지의 성결정†

전진태 · 이상호 · 홍기창 · 박성수
고려대학교 자연자원대학 응용동물과학과

요 약

3.3 kb 웅성특이 DNA(pEM39 plasmid DNA)가 성특이 DNA 검색자로 활용되어질 수 있는가를 확인하기 위하여 구조적인 분석을 Southern blotting, DNA sequencing과 computer program 분석을 통하여 실시하였다. 전체 3.3 kb에서 유래된 약 1 kb 단위의 단편을 이용하여 표지된 짧은 DNA probe들은 Southern blot 분석에서 웅성특이성을 나타내었다. McGraw와 Jeon의 sequence에 대한 유사성 비교 자료로부터 여러 부분의 conserved region을 찾아내고 이것을 기초로 하여 5 개의 primer set들을 선발하였다. Conserved region에 존재하면서 computer program에 의해서 선발되어진 PMS1과 2의 primer set가 최종적으로 PCR 분석을 위하여 선정되었다. 이 primer set를 사용한 PCR 분석에서, 1 ng부터 10 pg까지의 웅성 genomic DNA에서 PCR 산물을 얻을 수 있었으며, 자성의 경우는 어떠한 산물도 찾을 수 없었다. PCR에 이용할 수정란의 시료는 2 세포기의 수정란에서 얻었으며 순수 분리된 genomic DNA에서 확립된 조건에서 PCR을 수행하였다. 8 개의 수정란을 분석한 결과 4 개의 웅성과 4 개의 자성 수정란을 확인하였다. 이러한 결과는 선정된 primer set가 돼지 수정란의 성을 조기감별하는데 효율적인 DNA probe로 사용될 수 있다는 것을 암시한다.

INTRODUCTION

Mammalian genome is composed of various families of repeated sequences although their biological roles is unclear at present (Davidson and Britten, 1979 ; Manuelidis and Ward, 1984). Many male- or Y-specific DNA fragments have been isolated and identified from different approaches, and it has been suggested that many repetititve DNAs are dispersed on Y chromosome in mammalian species (Cooke, 1976 ; Kunkel *et al.*, 1976 ; Miller and Koopman, 1989 ; McGraw *et al.*, 1988 ; Mileham *et al.*, 1988 ; Kudo *et al.*, 1993). In broad terms, there are three general categories of repeated DNA ; short, interspersed elements called SINEs (Singer, 1982) ; long interspersed elements called LINE (Singer, 1982) and tandem clustered repetitive elements(Smith *et al.*, 1987). This feature suggests that the cloned male- or Y-specific DNAs in mammalian species might

* 고려대학교 자연자원대학 유전공학과(Department of Genetic Engineering, College of Natural Resources, Korea University, Seoul 136-701, Korea)

have possibility of existence of gender neutral or autosomal sequences in the repeating unit. In human, it was identified that human *DYZ1* and *DYZ2* (Cooke, 1976) have both of Y-specific and autosomal repeat in the repeating unit (Smith *et al.*, 1987). It suggested that the cloned male- or Y-specific DNA should be examined whether the sequences have only Y-specific repeat or both Y-specific and autosomal repeats for the use of sex-specific DNA probes.

In pigs, although there are three male-specific DNAs reported (McGraw *et al.*, 1988 ; Mileham *et al.*, 1988 ; Jeon *et al.*, 1994), adequate information about their structures and a possible use as sex-specific DNA probes were not tested in detail. In this study, structural analysis of the cloned 3.3 kb male-specific DNA (pEM39 plasmid DNA ; Jeon *et al.*, 1994) was carried out to examine a possible use of the sequence as sex-specific DNA probes, and primer regions were selected for sex determination. Polymerase chain reaction(PCR) was also performed for purified genomic DNA from somatic cells and embryonic DNA from 2-cell stage embryos using one of the selected primer sets.

MATERIALS AND METHODS

1. Chemicals and enzymes

Restriction enzymes, other DNA modifying enzymes and λ DNA for molecular weight markers were purchased from Korea Steel Chemical Co. (Seoul, Korea) and Promega Co. (Madison, WI, USA). Enzymes were used according to the recommendation of the manufacturers. The hapten digoxygenin-dUTP(DIG-dUTP) for DNA labeling and chemiluminescent and colorimetric detection kit (Boehringer Mannheim, Germany) were used for Southern blotting.

IPTG, X-gal, EDTA and ampicillin (Ap) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tris(hydroxymethyl), KCl, BSA (Fraction V), $MgCl_2$ and Triton X-100 as molecular biology grade for PCR were also obtained from Sigma. Chemicals for electrophoresis were purchased from BRL (Gaithersburg, MD, USA) or Bio-Rad Laboratories (South Richmond, CA, USA).

2. Bacterial strains, plasmids and media

E. coli JM101 (Messing, 1979) strain was used as a host for the transformation and propagation of plasmids. pUC18 and 19 plasmids(Yanisch-Perron *et al.*, 1985) were used for subcloning of the cloned 3.3 kb male-specific DNA. Plasmid-harboring *E. coli* cells were cultured in LB medium supplemented with 50 μg of Ap per ml. M9 minimal medium plates were used for the storage of JM101.

3. Preparation of plasmid and genomic DNA

Plasmid DNA was isolated according to the method of Sambrook et al.(1989). Genomic DNA were prepared from the testes of male and the livers of female pigs by the methods described in Sambrook et al.(1988). Isolated genomic DNAs for the use of template DNA of PCR were dialyzed in 4 liters of TE(pH 8.0) buffer using a dialysis membrane (Dialysis tubing, Sigma) for 24 h at room temperature. Dialyzed DNAs were serially diluted to 10 ng to 0.1 pg as intervals of 10 times.

4. Recovery of DNA fragments from agarose gel

DNA fragments fractionated on an agarose gel for subcloning the 3.3 kb male-specific DNA and labeling DNAs with DIG-dUTP were recovered by electroelution in a dialysis membrane (Grivitz *et al.*, 1980) or using GENECLEAN kit (BIO 101 Inc., USA).

5. Subcloning of the cloned male-specific DNA

The plasmid DNA, pEM39 which contains a 3.3 kb DNA fragment of the male-specific DNA (Jeon *et al.*, 1994) was divided into four subclones for detecting the possibility whether the cloned male-specific DNA would have autosomal sequences through southern blot, and sequence analysis of the cloned male-specific DNA. Isolated DNA fragments were inserted into multiple cloning site (MCS) of the pUC18 or 19 plasmid DNA vector for subcloning(Sambrook *et al.*, 1988). Recovered DNAs(0.1 μg) and the plasmid DNA vector(0.01 μg) cut with corresponding enzymes were diluted with 30 μl of water. Four μl of 10 mM ATP, 0.5 weiss unit of T4 DNA ligase and 4 μl of 10x ligase buffer added to the mixture. The total mixture(40 μl) was incubated at 15 ℃ for 12 h.

$CaCl_2$-treated competent JM101 cells were transformed with the ligated plasmid DNA(Lederberg and Cohen, 1974). The transformed cells were spread on LB plates supplemented with 50 μg/ml of Ap, 0.5 mM IPTG and 0.004%(w/v) X-gal. The LB plates containing the transformed cells were incubated overnight at 37℃, and the positive clones were selected.

6. Southern blot analyses

Each male-specific DNA isolated and collected from 4 subclones was labeled with DIG-dUTP. Other procedures were carried out as described by Southern (1975) and Sambrook et al. (1989). Hybridization was performed at 45℃ in 5×SSC, 50% formamide, 2% blocking reagent (Boehringer Mannheim), 0.02% SDS, 0.1% N-lauroylsarcosine (Na-salt) and 100 μg/ml of denatured salmon sperm DNA. After hybridization, the filter was washed in 2×SSC containing 0.1% SDS for 30 min at room temperature and then 0.1x SSC containing 0.1% SDS at 68℃ for 1 h. The hybrids were directly detected with a chemiluminescent or a colorimetric detection kit.

7. DNA sequence analyses

The cloned 3.3 kb male-specific DNA was sequenced and this data was previously reported (Jeon *et al.*, 1994). With the sequence data, DNA sequence analyses such as homology search , secondary structure search and overlapping sequences, etc., were carried out using DNASIS (version 5.0) program (Hitachi, Co., Japan).

8. Selection of primer sets for PCR

The primer sets for PCR were selected by either homology data or Primer Detective program (Clontech Labs Inc., version 1.01, USA). In using the computer program, the selection condition was as follows (Innis and Gelfand, 1992) : 20 nt of primer length, 250 to 350 bp of PCR product, 50 to 60% of primer GC content and 55 to 80 ℃ of melting temperature.

9. PCR for purified genomic DNA from somatic cells

One of the selected primer sets was determined to be used. This primer set is composed of PMS1(5'-GGTCCCTTATGTGTAGGTGG-3': sense primer) and PMS2(5'-GTTGTCAAATCTCAGCCAGG-3': antisense primer). The oligonucleotides were synthesized by an automatic DNA synthesizer(Applied Biosystem, Model 380B, CA, USA) and purified through 18% of denatured polyacrylamide gel electrophoresis (Sambrook *et al.*, 1989).

To each tube containing the 5 μl of sample, 100 μl of reaction mixture consisting of 10 mM Tris-Cl (pH 8.9), 50 mM KCl, 0.1% Triton X-100, 0.01% BSA, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 0.2 μM of each sense and antisense primer and 5 units of *Taq* DNA polymerase (Korea Biotech. Inc., Taejon, Korea) were added and overlaid with 100 μl of mineral oil. The PCR amplification was performed in a DNA thermal

cycler (Hybaid, Model HB-TR1, Hybaid Ltd., Middlesex, UK) by 40 cycles of denaturation (94℃, 1 min), annealing (55℃, 1 min) and primer extension (72℃, 1 min) for 1 ng to 10 pg of genomic DNA sample (Kudo *et al.*, 1993). After last cycle, the samples were kept at 72℃ for a further 10 min.

The thermal condition for the embryonic sample was modified (Handyside *et al.*, 1990 ; Guido *et al.*, 1992 ; Kunidea *et al.*, 1992). After 20 cycles of denaturation (94℃, 1 min), annealing (55℃, 1 min) and primer extension (72℃, 1 min), then 1 *l* of the first amplified products was added into a freshly prepared PCR mixture. The second 40 cycles of PCR was performed under the same condition as that of the first amplification.

The 10 μl of the amplification products was electrophoresed in 1.5% agarose gel in the buffer of TBE. After electrophoresis at 50 V, the saparated fragments were visualized directly by ethidium bromide staining under UV illumination.

RESULTS AND DISCUSSION

1. Subcloning of the 3.3 kb male-specific DNA cloned

As shown in Fig. 1, pEM39 plasmid DNA which contains a 3.3 kb DNA fragment of the male-specific DNA sequence was divided into 4 subclones. Each subclones has about 1 kb size of fragment which is flanked with *Bam*HI sites and the size was adequate for sequencing DNA and obtaining general information of the cloned male-specific DNA. Three subclones were named pEM3905, pEM3907 and pEM3911 plasmid DNA according to their size, respectively, and a subclone was called as pEM3938 plasmid DNA according to its whole size to distinguish from

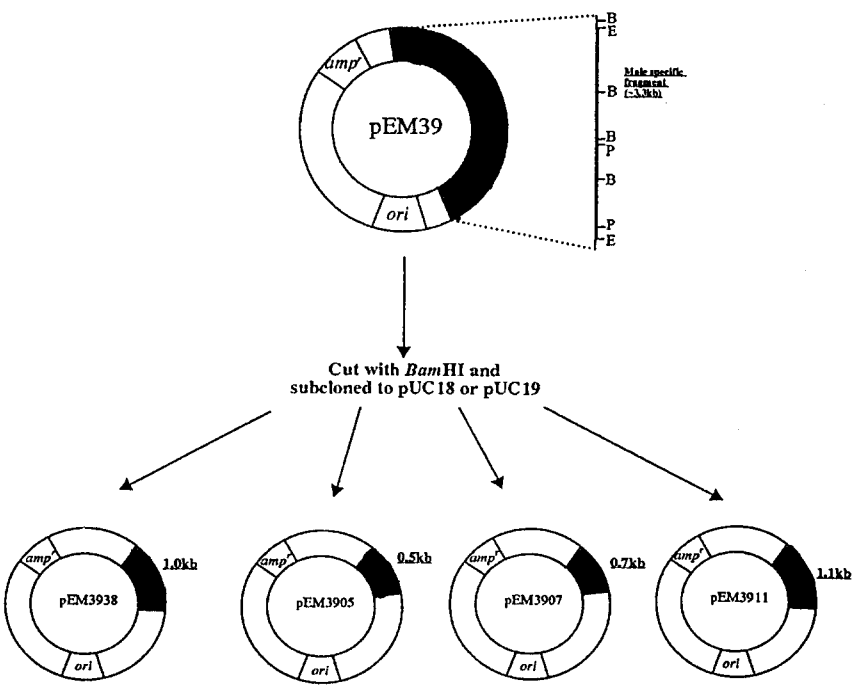

Fig. 1. Subcloning of pEM39 plsmid DNA. pEM39 was cut with *Bam*HI and isolated. Three (0.5 kb, 0.7 kb and 1.1 kb) of the four fragments are ligated into pUC18 or pUC19. An 1.0 kb fragment remained in pUC19 vector.

pEM3911 plasmid DNA.

2. Confirming male-specificity of the male-specific DNA through southern blot analyses

In human, there are two male-specific DNAs which was named *DYZ1* and *DYZ2* (Miller *et al.*, 1984). Both of the sequences identified from comparing the differences between male and female genomic DNA cut with Hae Ⅲ in agarose gel electrophoresis pattern (Cooke, 1976). In further experiments, it was suggested that the sequences locate on Yq region and homologous to DNA sequences present in autosomes (Bostock *et al.*, 1978). In detailed analyses, the sequences have several kinds of autosomal sequences flanked with Y-specific DNA in the repeating unit (Smith *et al.*, 1987). For this reason, when using the sequences as sex-specific DNA probes, the sequences located in Y-specific region should be selected. For eluding this troubles, several

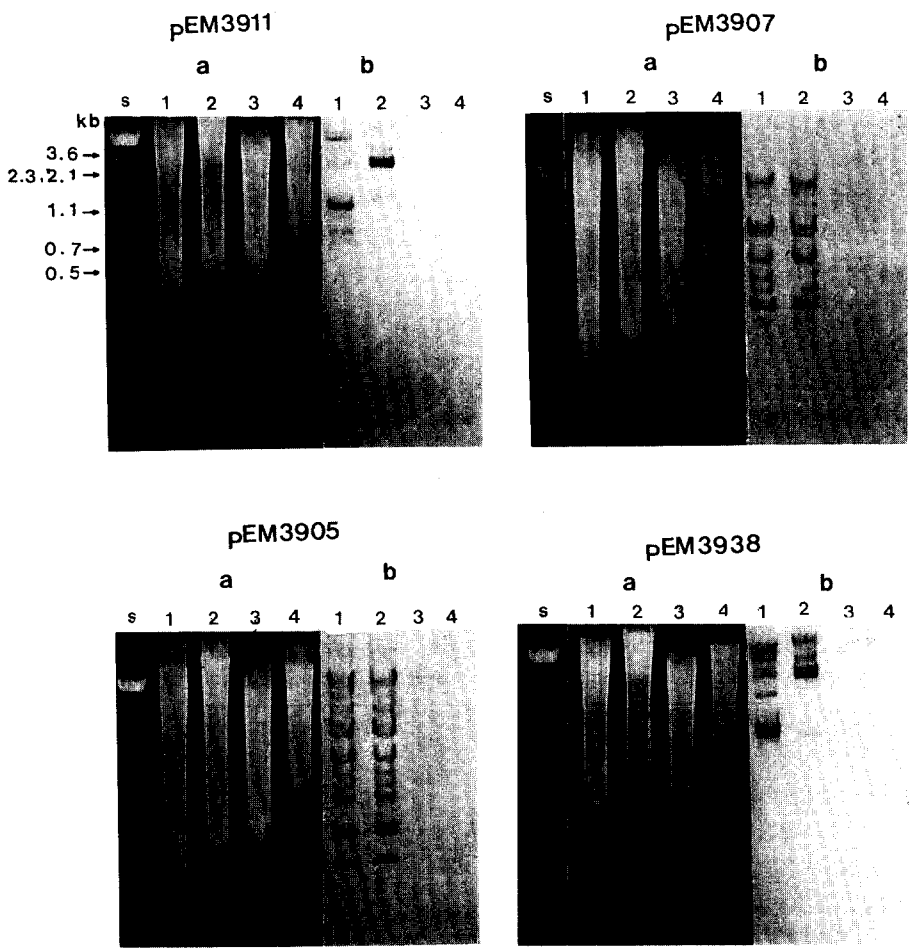

Fig. 2. Southern blot analyses of pEM3911, 3907, 3905 and 3938 plasmid DNA. About 10 μg of digested genomic DNA were loaded per well. The digested DNAs were separated by electrophoresis in 1% agarose gel for 5 h at 4 V cm^{-1} in 0.5 x TBE buffer. a, Ethidium bromide-stained gel ; b, Southern blot of gel a. Lane s, 0.5 μg of size marker (DNA cut with *Dra*I) ; 1, *Bam*HI and *Eco*RI ; 2, *Bam*HI digest of male DNA. Lane 3, *Bam*HI and *Eco*RI ; 4, *Bam*HI digest of female DNA.

results of early sex determination of embryos using DNA probes derived from Y-specific genes such as *SRY* and *ZFY*, etc., were recently reported (Guido *et al.*, 1992 ; Kunidea *et al.*, 1992). But, it is certain that the more repetitive copy is, the less embryonic DNA is required in sexing.

If the 3.3 kb male-specific DNA was Y-specific as a whole, we could use any regions of the sequence as sex-specific DNA probes. If not, we should be discriminate Y-specific regions from autosomal region just like *DYZ1* and *DYZ2* sequences. It was thought that a method to identify this feature might be southern hybridization using relatively short DNA probes derived from the cloned male-specific DNA. As shown in Fig. 2, although about 1 kb fragments were used as probes, male-specificity of them was still retained. If the male-specific DNA had autosomal sequences as a part, the signals in the Southern blot analyses might be identified in both male and female lanes. But, the positive signals were shown only in male lanes in spite of use of short probes prepared from 4 subclones. This result indicated that the 3.3 kb male-specific DNA might be Y-specific sequence which is not a portion but a whole. As it is not sufficient to certain that whole sequence of the cloned male-specific DNA are Y-specific, localization of the cloned sequence on the metaphase plates is now being under progress. Nevertheless, it was thought that any regions of the cloned 3.3 kb DNA fragment might be used as sex-specific probes due to the results of Southern analyses.

3. DNA sequence analyses

Sequence data of the 3.3 kb male-specific DNA was previously reported (Jeon *et al.*, 1994). Homology data were obtained by comparing Jeon's (Jeon *et al.*, 1994) to McGraw's sequence (McGraw *et al.*, 1988). As shown in Table 1, the most homologous region is the 1.1 kb fragment corresponding to pEM3911 plasmid DNA (89%) which is at 5' region of both McGraw's and Jeon's sequence, and it is thought that this region is highly conserved. We do not have any idea of whether this male-specific DNA is transcribed or not, since we do not have any data of mRNA and protein. But, the primer sequences for PCR would be selected from 1.1 kb region (pEM3911 plasmid DNA) based on the high homology between two sequences or from either 0.7 (pEM3907 plasmid DNA) or 0.5 kb region (pEM3905 plasmid DNA) based on the high repetitiveness and male-specificity as shown in Southern blot analyses.

4. Selection of primer sets for PCR

As shown in Table 2, we selected 5 sets of primer sequence by either homology data or a computer program. One set of them was finally chosen for PCR, because it was located in highly conserved region between Jeon's and McGraw's sequence, and was also selected by a computer program. As shown in Fig. 3, the primer set(PMS1 and 2) has synthesized 269 bp from 13 to 281 and Tm and GC percentage was 76.8 ℃ and 50(PMS1) and 55 %(PMS2), re-

Table 1. Homology between McGraw's and Jeon's sequence

	pEM3911	pEM3907	pEM3905	pEM3938	Total(pEM39)
(a)	89.0	88.6	42.2	79.6	72.7
(b)	89.0	88.6	86.1	88.3	88.0

(a) was percentage of homology containing the sequences of deleted region.

(b) was calculated without deleted region of both sequences.

Table 2. List of selected primers for PCR

No.	Sequence	Source	Direction	Position
PMS1	GGTCCCTTATGTGTAGGTGG	pEM3911	5 '→ 3'	13-32
PMS2	GTTGTCAAATCTCAGCCAGG	pEM3911	←	262-281
PMS3	CCTTGATTTCATTGTCCAGG	pEM3911	→	657-676
PMS4	ACCTCTCTGTGAGAAAAGAG	pEM3911	←	921-940
PMS5	TCAGGCTCCCAAGCTCAATT	pEM3907	→	1122-1141
PMS6	GGGCATTTTCAGCTCCACAC	pEM3907	←	1365-1384
PMS7	AGACTGTGTATTCCTGGGAA	pEM3905	→	2120-2139
PMS8	TGGGAACTCTCACCGTCACC	pEM3905	←	2346-2367
PMS9	ATCCCTTCCAGTCTTTTCCA	pEM3938	→	2578-2597
PMS10	GCTGGGATACGTCTGGTGGG	pEM3938	←	2825-2844

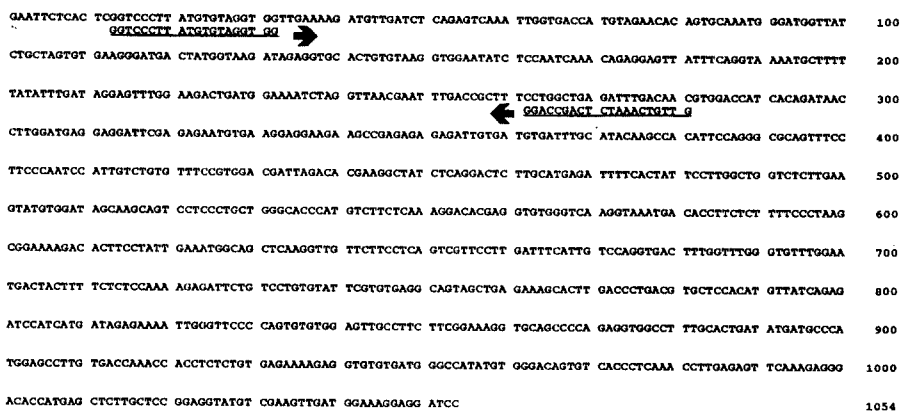

Fig. 3. Target region of PCR amplification.
The DNA sequences of the 1.1 kb male-specific DNA fragment corresponding to pEM3911 plasmid DNA were shown. Underlined characters between the lines are DNA sequences of PMS1 and 2 primer set. Arrows indicates the direction of PCR amplification.

spectively. Also, 3' end homology between two pairs was not found. The primer set was satisfied with the general condition of primers for PCR (Innis and Gelfand, 1992 ; Rychilk, 1993).

5. PCR for purified genomic DNA from somatic cells and embryonic DNA

For confirming male-specificity of target region, 300 bp *Taq*I fragment of the 1.1 kb fragment corresponding to pEM3911 plasmid DNA was subjected to Southern hybridization. As shown in Fig. 4, positive signals were found only in the male lanes. It enhanced that the target region of PCR is male-specific.

The primary condition of PCR such as annealing temperature, pH and concentration of $MgCl_2$, etc., using PMS1 and 2 primer set was defined using genomic DNA from somatic cells as a template(data not shown). Male and female genomic DNAs were diluted to 1 ng, 100 pg, 10 pg and 1 pg. Since 5.5 pg is equivalent to an average weight of genomic DNA contained in a diploid cell, 10 pg of genomic DNA has a quantity of genomic DNA from about 2 cell. If 10 pg of purified genomic DNA was sufficient as a template for PCR, products could be yielded from

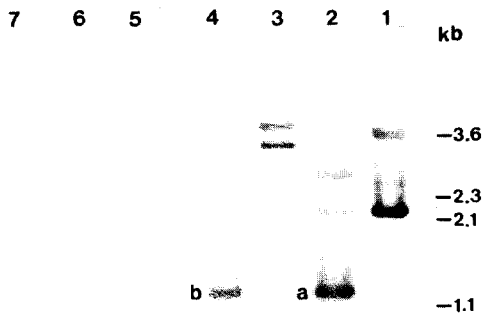

Fig. 4. Southern blot analysis of target region for PCR.
The 300 bp *Taq*I fragment of pEM3911 plasmid DNA was labeled with DIG-dUTP. Lane 1, 10 μg of male DNA cut with *Bam*HI ; 2, *Bam*HI and *Eco*RI ; 3, *Eco*RI ; 4, 1 ng of 1.1 kb fragment in pEM3911 ; 5, 10 μg of female DNA cut with *Bam*HI ; 6, *Bam*HI and *Eco*RI ; 7, *Eco*RI. a, 1.1 kb fragment in the 10 μg of male genomic DNA and b, 1.1 kb fragment of pEM3911 plasmid DNA.

genomic DNA of 2 or 4 cells. As shown in Fig. 5, after 40 cycles of amplification, PCR product was yielded from 1 ng to 10 pg of male genomic DNA and any products were not found in female sample. The result was similar to those of Kudo et al. (1993) using bovine male-specific DNA sequence. It suggested that products could be produced from genomic DNA in 1 or 2 cell by two step PCR.

The two setp PCR method has been used for the detection of Y-specific single copy genes (Guido *et al.*, 1992 ; Kunidea *et al.*, 1992) such as *SRY*, *ZFY*, etc., and for amplifying the signals of products (Handyside *et al.*, 1990). In this experiment with the embryonic sample, the two step PCR method was used for obtaining a strong signal. As shown in Fig. 6, four male and female signals were identified, respectively, among eight embryos tested. We believe that only

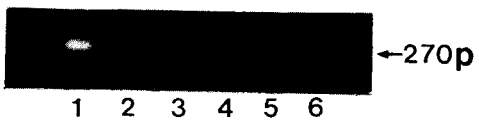

Fig. 5. PCR product of purified genomic DNA sample.
Lane 1, 1 ng of male DNA ; 2, 100 pg of male DNA ; 3, 10 pg of male DNA ; 4, 1 pg of male DNA ; 5, 1 ng of female DNA and 6, negative control (no DNA sample). 270p, 270 bp product.

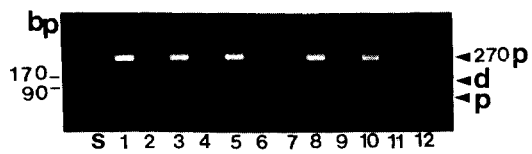

Fig. 6. PCR amplification of embryonic samples.
Lane s, size maker (DNA cut with *Dra*I) ; 1 to 8 , IVF 2 or 3 cell stage embryos ; 9, activated embryo (control) ; 10, 1 ng of purified male DNA ; 11, 1 ng of purified female DNA and 12, negative control (no DNA sample). 270 p, 270 bp product ; d, primer-dimer and p, primer bands. Sample 1, 3, 5 and 8 were male embryos.

male embryos should have produced PCR products, considering that the both positive and negative control lanes clearly showed the opposite results.

These results suggest that PMS1 and 2 primer set which were used in PCR should be an applicable probe for the predetermination of sex in porcine embryos.

SUMMARY

Structure of the cloned 3.3 kb male-specific DNA(pEM39 plasmid DNA) was analyzed through Southern blotting and computer program analysis to know whether it can be used as sex-specific DNA probes. Various DNA probes

(about 1 kb) prepared from the 3.3 kb male-specific DNA with the combined digestion of restriction enzymes still showed the male-specificity on Southern blotting. Five sets of primers were selected on the basis of conserved regions between Jeon's and McGraw's sequences. They were investigated by the analysis of computer program which determine the usefulness of primers for PCR analysis and one pair of primers (PMS1 and 2) was finally selected. The selected primers produced a PCR product only in male genomic DNA but not in female. Embryonic samples were collected from 2-cell stage embryos and PCR was performed under the difined condition using purified genomic DNA. Among eight embryos tested, four embryos were identified as males and four were females. The results suggest that the above primers could be useful as DNA probes for the early determination of sex in porcine embryos.

ACKNOWLEDGEMENT

This work was supported by a Genentic Engineering Research grant from the Minisitry of Education in 1991~1993.

REFERENCES

Bostock CJ, Gosden JR and Mitchell AR. 1978. Localisation of a male-specific DNA fragment to a sub-region of the human Y chromosome. Nature 272:288-292.

Cooke H. 1976. Repeated sequence specific to human males. Nature 262:182-186.

Davidson EH and Britten RJ. 1979. Regulation of gene expression : possible role of repetitive sequences. Science 204:1052-1059.

Grivitz SC, Bacchetti S, Rainbow AJ and Graham FI. 1980. A rapid and efficient procedure for the purification of DNA from agarose gels. Anal. Biochem. 106:492-496.

Guido DP, Giambiagi S, Mancardi S, De Luca L, Burrone O, Frasch ACC and Ugalde RA. 1992. Sex determination of bovine embryos by restriction fragment polymorphisms of PCR amplified *ZFX/ZFY* loci. Bio/Technology 10:805-807.

Handyside AH, Kontogianni EH, Hardy K and Winston RML. 1990. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature 344:768-770.

Innis MA and Gelfand DH. 1992. Optimization of PCRs. In : PCR protocols. Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. Academic Press. USA pp. 3-12.

Jeon JT, Lee SH, Hong KC and Park SS. 1994. Isolation and cloning of a male-specific DNA fragment from genomic DNA in pigs (*Sus scrofa*). Mol. Cells 4:301-307.

Kudo T, Sato S and Sutou S. 1993. Sexing of bovine embryos with male-specific repetitive DNA by polymerase chain reaction : cloning and characterization of bovine male-specific repetitive DNA. J. Reprod. Dev. 39:55-63.

Kunidea T, Xian M, Kobayashi E, Imamichi T, Moriwaki K and Toyoda Y. 1992. Sexing of mouse preimplantation embryos by detection of Y chromosome-specific sequences using polymerase chain reaction. Biol. Reprod. 46:692-697.

Kunkel L, Smith KD and Boyer SH. 1976. Human Y-chromosome-specific reiterated DNA. Science 191:1189-1190.

Lederberg EM and Cohen SN. 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. 119: 1072-1074.

Manuelidis L and Ward DC. 1984. Chromosomal and nuclear distribution of the *Hin*dⅢ 1.9

kb human DNA repeats segment. Chromosoma 91:28-38.

McGraw RA, Jacobson RJ and Akamatsu M. 1988. A male-specific repeated sequence in the domestic pig (*Sus scrofa*). Nucl. Acids Res. 16:10389 (Abstr.).

Messing J. 1979. A multipurpose cloning system based on single-stranded DNA bacteriophage M13. Recomb. DNA Tech. Bull. 2:43-48.

Mileham AJ, Siggens KW and Plastow GS. 1988. Isolation of a porcine male specific DNA sequence. Nucl. Acids Res. 16:11842 (Abstr.).

Miller JR and Koopman M. 1989. Isolation and characterization of two male-specific DNA fragments from the bovine genome. Anim. Genet. 21:77-82.

Miller OJ, Drayna D and Goodfellow P. 1984. Report of the Committee on the Genetic Constitution of the X and Y chromosomes. Human Gene Mapping 7. Los Angeles Conference (1983). Seventh International Workshop on Human Gene Mapping. Cytogenet. Cell Genet. 37:176-205.

Rychilk W. 1993. Selection of primers for polymerase chain reaction. In : Methods in molecular biology. Vol.15. PCR protocols. White, B. A. Human Press. USA pp. 31-40.

Sambrook J, Fritsch EF and Maniatis T. 1989. Molecular cloning : A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press. USA.

Singer MF. 1982. Highly repeated sequences in mammalian genomes. Int. Rev. Cytol. 76:67-112.

Smith KD, Young KE, Talbot CC and Schmeckpeper BJ. 1987. Repeated DNA of the human Y chromosome. Development Suppl. 101:77-92.

Southern EM. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Yanisch-Perron CJ, Vieira CJ and Messing J. 1985. Improved M13 phage cloning vectors and host strains : nucleotide sequence of the M13mp18 and pUC19 vectors. Gene 33:103-119.