—Research Note—

Cassette DNA Fragment for Selection and Sexing of Preimplantation Bovine Transgenic Embryos

Koko KODAIRA, Kazumi ITOH, Masumi HIRABAYASHI, Kunihiro KODAIRA and Masatsugu UEDA

YS New Technology Institute, Inc., 519 Shimoishibashi, Ishibashi-machi, Shimotsugagun, Tochigi 329-0512, Japan

Abstract. A 369-bp cassette DNA fragment (LACFE) was constructed for simultaneous detection of exogenous gene integration and sexing of preimplantation bovine embryos. The LACFE consists of a part of the bovine lactoferrin gene, including three *Dpn* I sites and a pair of Y chromosome-specific primer sequences for polymerase chain reaction (PCR) of both ends. Bovine pronucleus embryos were microinjected with the LACFE modified by DNA adenine methylase. After 7 to 9 days culture *in vitro*, the blastocysts were bisected and the demi-embryos were analyzed by the PCR method following the nuclease treatment with *Dpn* I and *Bal* 31 nucleases (digestion-PCR) or without the nucleases (unmodified PCR), respectively. Results of sexing between a pair of demi-embryos agreed each other in 95 of 102 blastocysts. Ten of 102 demi-embryos were positive for the exogenous gene by unmodified PCR. On the other hand, 3 of 102 demi-embryos were positive by digestion-PCR, and included in the 10 demi-embryos. The digestion-PCR may exclude false-positive embryos, and prove to be an efficient selection of preimplantation bovine transgenic embryos. These results indicate that LACFE is useful for the simultaneous detection of the integration of exogenous genes and the sexing of preimplantation bovine embryos using only a single pair of primers. As the LACFE can be introduced into a construct of desirable transgene for microinjection, the LACFE is widely applicable for detection and sexing of transgenic embryos under the same analytical conditions.

Key words: Transgene, Bovine embryo, Sexing, Methylation, PCR.

(J. Reprod. Dev. 45: 105–110, 1999)

Transgenic farm animals are a powerful tool for the production of human protein in their milk [1–3]. In particular, cows are able to produce large quantities of milk and thus there is a great potential for cost-effective pharmaceutical production [4]. However, the low developmental rate of microinjected embryos and the long gestation period greatly increase the cost of establishing transgenic cattle. Selection of preimplantation embryos and multiple embryo implantation have been attempted to cut down the cost by reducing the number of

recipients required. If several unsexed embryos are transferred to a recipient, there is a high probability of producing a freemartin. In general, 90% of a twin cow of heterogeneous sex are sterile. To avoid the freemartin phenomenon, sexing of preimplantation embryos is necessary for the transfer of multiple embryos into a recipient. Furthermore, selection of preimplantation transgenic embryos can eliminate the unnecessary transfer of non-transgenic embryos into recipients. Therefore, detection of exogenous gene integration and sexing of microinjected embryos before transplantation is critical for cost-effective production of transgenic cattle.

Use of a polymerase chain reaction (PCR)-based method for the selection of transgenic embryos has been reported [5–8]. Direct PCR analysis (unmodified PCR), however, produces an unacceptably high rate of false positives because unmodified PCR can also detect unintegrated DNA in the embryo [5–8]. To eliminate false positives due to unintegrated DNA constructs in embryos, some researchers have demonstrated that transgene analysis can be accomplished with a combined use of methylated DNA constructs for microinjections and treatment with the adenine methylated DNA-sensitive endonuclease, *Dpn* I, and *Bal* 31 exonuclease prior to the PCR analysis [9–11]. This method is based on the fact that *Dpn* I restriction endonucleases cleave only N⁶-adenine-methylated 5'-GATC-3' DNA sequence by the DNA adenine methylation gene (*dam*). Mammalian embryos do not possess an adenine-specific methylation system during DNA replication [12].

In the present paper, we constructed of a 369-bp cassette DNA fragment (LACFE), including three *Dpn* I sites and a pair of Y chromosome-specific primer sequences at both ends and examined the preimplantation bovine embryos prepared by microinjection of the DNA fragment with the method of digestion-PCR [9]. The LACFE is suitable for simultaneous detection of exogenous gene integration and sex of microinjected preimplantation bovine embryos during a single PCR amplification.

Materials and Methods

Preparation of DNA for microinjection

Bovine genomic DNA was obtained from male bovine leukocytes according to the previously described method [13]. The specific DNA fragment of bovine lactoferrin (6th intron from 191 to 496; 377 bp; [14]) was amplified from the bovine genomic DNA by the method of PCR with a pair of oligonucleotide primers, SEX-LACFE2A (5'-AAG-TACTCTCATCCTACCTAATAGATTCCAGCTGCC GTGCCCCGCTATCT-3') and SEX-LACFE2B (5'-AG-TATACACAAATTCTGTCTCTGAAACAGATGAG CTGTCTACCGCTTTACGA-3') containing bovine Y-chromosome-specific sequences [15] in outside of primer sequences for the bovine lactoferrin. The DNA fragment was separated using 1% agarose gel electrophoresis, recovered with GENECLEAN (Bio101, La Jolla, CA, USA) and cloned into plasmid pT7Blue T-Vector (Novagen, Madison, WI, USA). The plasmid was propagated in *dam*⁺ *Escherichia coli* strain JM109 (TOYOBO, Osaka, Japan) or *dam*⁻ *Escherichia coli* strain DM1 (Life Technologies, Rockville, MD, USA). These plasmids were excised by treatment with *Sca* I (Takara Shuzo, Kyoto, Japan) and *Bst* 1107 I (Takara Shuzo), and the 369-bp DNA fragments were separated using 1% agarose gel electrophoresis, extracted, and recovered with GENECLEAN. The cassette DNA

Fig. 1. The structure of the cassette DNA fragment, LACFE. (A) Locations of the *Dpn* I cleavage sites. The solid and open boxes represent the bovine lactoferrin gene and the bovine male specific sequences for PCR, respectively. (B) The full sequence of LACFE. The positions of the *Dpn* I cleavage sites are indicated by a single underline and primer sequences are indicated by a double underline.

fragment, named LACFE (Fig. 1), has three *Dpn* I sites on a part of the lactoferrin between the primers for the bovine Y-chromosome-specific sequences. The purified DNA was dissolved at 5 µg/ml in 10 mM Tris-HCl (pH 8.0) containing 0.1 mM EDTA and stored at -20 °C until microinjection.

Microinjection and bisection of blastocysts

Bovine pronucleus embryos were prepared from oocytes obtained from a local slaughterhouse using the *in vitro*-maturing (IVM) and *in vitro*-fertilization (IVF) methods [14]. At 19 h after insemination, the embryos were stripped of cumulus cells by vortex-mixing and were centrifuged at 13,000 g for 10 min. Embryos with the least of one visible pronucleus were microinjected with the DNA solution under a Leitz inverted microscope with Nomarski differential interference contrast optics (×250 magnification). Successful DNA microinjection was conferred by full swelling of the pronucleus. Microinjected embryos of 30 to 40 were grouped in a 50 µl-microdrop of mSOF covered with mineral oil and cultured at 39 °C in a humidified CO₂-O₂ incubator conditioned in 5% CO₂-5% O₂-90% N₂ [14]. The day of IVF treatment was designated as Day 0. Blastocysts obtained from Day 7 to Day 9 were washed twice in phosphate buffered saline containing 0.2% (W/V) polyvinyl alcohol (PBS-PVA) and bisected so that both the trophectoderm and inner cell mass were halved. Bisected embryo samples were subjected to PCR analysis.

DNA prepared for PCR analysis

Each demi-embryo sample in 1 µl of PBS-PVA was transferred into a microcentrifuge tube containing 2 µl of lysate buffer [20 mM Tris-HCl (pH 7.5), 120 mM NaCl, 14 mM MgCl₂, 20 mM CaCl₂, 0.1% Triton X-100, 50 µg/ml acetylated BSA, 25 µg/ml Proteinase K (Merk, Rahway, NJ, USA)] and was stored at -20 °C until analysis. Light mineral oil was added to the thawed sample and embryonic DNA was released from nuclei by incubating the tube at 55 °C for 1 h. The digestion-PCR method described by Hyttinen *et al.* [9] was modified for this study. Briefly, the nuclease concentrations of *Dpn* I and *Bal* 31 in the digestion solution were increased 5 times and the reaction time was prolonged to 1 h. That is, after inactivation of Proteinase K at 94 °C for 10 min, 2 µl of nuclease

buffer [5 mM Tris-HCl (pH 7.5), 30 mM NaCl, 3.5 mM MgCl₂, 200 µg/ml acetylated BSA, 250 µM phenylmethylsulfonyl fluoride] with or without 150 mU of *Dpn* I restriction endonuclease (Promega, Madison, WI, USA) and 500 mU of *Bal* 31 exonuclease (Toyobo) was added to the tube and the tube were incubated at 37 °C for 1 h.

PCR amplification

PCR was performed in a 25 µl volume containing 10 pmol of each pair of primers, 1.25 units of LA *Taq* polymerase (Takara Shuzo), 250 mM each of dNTP, 2.5 mM MgCl₂, 0.1% Triton X-100, and Mg²⁺-free accessory buffer (Takara Shuzo) in a DNA thermal cycler (Perkin-Elmer, Foster City, CA, USA). After denaturation at 94 °C for 3 min, PCR amplification was performed for 45 cycles for 30 sec at 94 °C and 1 min at 68 °C. In the last cycle, the extension time was prolonged to 2 min. The primer pairs used were BOVSEX2A (5'-ACTCTCATCCTACCTAATAGATTCCAGCTG-3') and BOVSEX2B (5'-TACACAAATTCTGTCTCTGAAACAGATGAGCTG-3') for the transgene and bovine male specific sequences [15], and BOVSP2A (5'-TCATCTCGAGTGGAAGCAAAGAACCCCGCT-3') and BOVSP2B (5'-CCGTCTGACCTCGTGAGAAACCGCACACTG-3') for the bovine specific sequence as an internal control [17]. The amplified products were separated using 4% SeaKem GTG agarose (Takara Shuzo) gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. Analysis of the intensities of each band from the photographs was performed using the Molecular Analyst Version 2.0.1 (Bio-Rad, Hercules, CA, USA) program. Integration of LACFE in demi-embryos was judged on the basis of an integration index. The integration index was obtained by dividing the intensity of the LACFE band by that of the Y chromosome-specific band as both bands were amplified with common primer set. The integration index greater than 0.3 was decided to be the transgene positive, because the copy number of the Y chromosome-specific sequence per cell was estimated to be 3 as previously reported [14]. For female demi-embryos, the integration index was also calculated using the average intensity of the Y chromosome-specific band at the simultaneous analysis.

Results

Digestion-PCR for the detection of methylated DNA fragments

To determine the effectiveness of the nuclease treatment with *Dpn* I and *Bal* 31 under the condition in this study, the DNA cassette fragments of LACFE were prepared with *dam*⁺ *Escherichia coli* (JM109) and *dam*⁻ *Escherichia coli* (DM1), respectively. LACFE (10 fg), corresponding to the amount microinjected into an embryo, was mixed with male bovine genomic DNA (1 ng), corresponding to the amount derived from 100 cells, and digested with or without the nucleases before PCR. The result showed that *Dpn* I specifically recognized the adenine methylated sequence of GATC and the band of 369 bp was disappeared, while a slight band considered to be the cause of the polymerization was remained (Fig. 2; 2 and 6). *Bal* 31 digested the DNA fragment of LACFE completely but specific bands corresponding to bovine satellite DNA used as an internal control (236 bp) and the band corresponding to the Y chromosome (115 bp) were identified after the nuclease treatment (Fig. 2). When the DNA cassette fragment of *dam*-methylated LACFE was added to the bovine demi-embryo, the fragment was digested complete-

ly by the nuclease treatment with *Dpn* I and *Bal* 31 but the specific bands of 236 bp and 115 bp were also identified (Fig. 3). The detection limit for LACFE using PCR analysis was 0.025 fg. The Y chromosome-specific sequence could be detected in a single cell (data not shown).

Comparison of digestion-PCR with unmodified PCR

Methylated LACFE was microinjected into the pronuclei of 1018 zygotes and 106 blastocysts were obtained after 7 to 9 days of cultivation. The blastocysts were bisected and 103 pairs of well-bisected demi-embryos were analyzed for the integration of the injected DNA fragment and the sex of the embryos by the two PCR-based methods. A bovine specific 236-bp band, indicating completion of PCR, was detected in 102 pairs of demi-embryos. The sexes of the paired demi-embryos were the same in 95 of 102 pairs (93.1%).

While 10 of 102 demi-embryos were judged to be transgene-integrated embryos by the unmodified PCR method, only 3 were judged to be transgene positive. The 3 positive demi-embryos were included in the 10 positive demi-embryos (Table 1).

Fig. 2. PCR analysis after the nuclease treatment with *Dpn* I (D) and *Bal* 31 (B). 10 fg of *dam*-methylated LACFE (1–4) or unmethylated LACFE (5–8) were mixed with 1 ng of male bovine genomic DNA and used for the analysis. The LACFE specific PCR product is 369 bp, the internal control specific PCR product is 236 bp, and the Y chromosome-specific PCR product is 115 bp in size. M: 100 base-pair ladder (Pharmacia Biotech).

Fig. 3. PCR analysis with demi-embryos. 10 fg of *dam*-methylated LACFE was mixed with a demi-embryo and was analyzed after the nuclease treatment with (DB) or without (-) *Dpn* I and *Bal* 31. 1,2: A pair of female demi-embryos. 3,4: A pair of male demi-embryos. The LACFE specific PCR product is 369 bp, the internal control specific PCR product is 236 bp, and the Y chromosome-specific PCR product is 115 bp in size.

Table 1. Transgene detection and sexing of bovine demi-embryos using PCR with or without nuclease treatment

Injected	Blastocysts	Biopsied		Analyzed	TG (+) (% analyzed)	TG (-) (% analyzed)	coincided sex (% analyzed)
1018	106	106	digestion-PCR	102	3 (2.9)	99 (97.1)	95 (93.1)
			unmodified PCR	102	10 (9.8)	92 (90.2)	

Discussion

Although the recently developed cell-mediated method for producing transgenic livestock by nuclear transfer from stably transfected somatic cells to enucleated unfertilized egg will become a revolutionary technology [18, 19], microinjection of DNA into the pronuclei of fertilized embryos is one of the practical and reliable methods for the production of transgenic livestock. To cut down the cost for making transgenic cattles, reducing the number of recipient for an implantation of the microinjected embryo is very important. A powerful method for a selection of transgene-integrated embryo using a co-expressing marker gene of green fluorescent protein has been reported [20]. While a preimplantation selection of transgenic embryo is one of an efficient procedure to reduce a number of recipient for the embryo transfer, a transplantation of multiple blastocysts to a recipient without an occurrence of freemartin phenomenon is also effective for reducing a number of recipient. Therefore a simultaneous detection of transgene integration and sex of a preimplantation embryo with a single procedure of PCR is very effective for a preimplantation selection of transgenic embryos. We constructed a cassette DNA fragment named LACFE. The LACFE was designed to have three sites of *Dpn* I that helped the completion of eliminating unintegrated LACFE by the nuclease treatment with *Dpn* I and *Bal* 31, and to be detected by the PCR method with the same primer set for sexing. The fragment of LACFE can be introduced into a construct of desirable transgene for microinjection, and is very useful for the identification of transgene integration and sex of

the preimplantation bovine embryos with a single pair of primers.

We analyzed 102 pairs of demi-embryos derived from 1018 microinjected embryos, by the methods of digestion-PCR and unmodified PCR. The sexes determined by the two methods agreed with each other in 93% of analyzed embryos (95/102). This result was comparable with the result reported by Hyttinen *et al.* [9]. While 10 out of 102 blastocysts derived from the microinjected embryos were determined to be transgene-positive by the method of unmodified PCR, only 3 blastocysts were determined to be transgene-positive by the method of digestion-PCR. These 3 embryos were included in the 10 positive one, and the false positive embryos due to unintegrated transgene in the blastocysts were considered to be eliminated by the nuclease treatment prior to the PCR analysis [5–8]. The positive rate was lower than that in the previous report [9]. An integration index greater than 0.3 may provide the severe condition for detecting the transgenic blastocysts.

These results indicated that the cassette DNA fragment of LACFE is useful for the simultaneous detection of transgene-integration and sex of the blastocysts derived from microinjected embryos and can be widely applicable by combining with any desirable transgene for microinjection.

Acknowledgments

This work was supported by a grant from the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan.

References

1. Simons JP, Wilmut I, Clark AJ, Archibald AL, Bishop JO, Lathe R. Gene transfer into sheep. *Bio/technology* 1988; 6: 179–183.
2. Clark AJ, Bessos H, Bishop JO, Brown P, Harris

- S, Lathe R, McClenaghan M, Prowse C, Simons JP, Whitelaw CBA, Wilmut I. Expression of human anti-hemophilic factor IX in the milk of transgenic sheep. *Bio/technology* 1989; 7: 487-492.
3. Swanson ME, Martin MJ, O'Donnell JK, Hoover K, Lago W, Huntress V, Parsons CT, Pinkert CA, Pilder S, Logan JS. Production of functional human hemoglobin in transgenic swine. *Bio/technology* 1992; 10: 557-559.
 4. Bowen RA, Reed ML, Schnieke A, Seidel Jr GE, Stacey A, Thomas WK, Kajikawa O. Transgenic cattle resulting from biopsied embryos: expression of c-ski in a transgenic calf. *Biol Reprod* 1994; 50: 664-668.
 5. Ninomiya T, Hoshi M, Mizuno A, Nagao M, Yuki A. Selection of mouse preimplantation embryos carrying exogenous DNA by polymerase chain reaction. *Mol Reprod Dev* 1989; 1: 242-248.
 6. Behboodi E, Anderson GB, Horvat S, Medrano JF, Murray JD, Rowe JD. Microinjection of bovine embryos with a foreign gene and its detection at the blastocyst stage. *J Dairy Sci* 1993; 76: 3392-3399.
 7. Bowen RA, Reed M, Schnieke A, Seidel Jr. GE, Brink Z, Stacey A. Production of transgenic cattle from PCR-screened embryos. *Theriogenology* 1993; 39: 194.
 8. Horvat S, Medrano JF, Behboodi E, Anderson GB, Murray JD. Sexing and detection of gene construct in microinjected bovine blastocysts using the polymerase chain reaction. *Transgenic Res* 1993; 2: 134-140.
 9. Hyttinen J-M, Peura T, Tolvanen M, Aalto J, Alhonen L, Sinervirta R, Halmekytö M, Myöhänen S, Jänne J. Generation of transgenic dairy cattle from transgene-analyzed and sexed embryos produced *in vitro*. *Bio/technology* 1994; 12: 606-608.
 10. Hyttinen J-M, Peura T, Tolvanen M, Aalto J, Jänne J. Detection of microinjected genes in bovine preimplantation embryos with combined DNA digestion and polymerase chain reaction. *Mol Reprod Dev* 1996; 43: 150-157.
 11. Seo BB, Kim CH, Tojo H, Tanaka S, Yamanouchi K, Takahashi M, Sawasaki T, Tachi C. Efficient selection of preimplantation transgenic embryos by an improved procedure using *Dpn I-Bal 31* digestion and the polymerase chain reaction. *Reprod Fertil Dev* 1997; 9: 263-269.
 12. Peden KW, Pipas JM, Pearson-White S, Nathans D. Isolation of mutants of an animal virus in bacteria. *Science* 1980; 209: 1392-1396.
 13. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Press; 1989.
 14. Seyfert HM, Tuckoricz A, Interthal H, Koczan D, Hobom G. Structure of the bovine lactoferrin-encoding gene and its promoter. *Gene* 1994; 143: 265-269.
 15. Miller JR, Koopman M. Isolation and characterization of two male-specific DNA fragments from the bovine gene. *Anim Genet* 1990; 21: 77-82.
 16. Takahashi Y, First NL. *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* 1992; 37: 963-978.
 17. Taparowsky EJ, Gerbi SA. Sequence analysis of bovine satellite I DNA (1.715 gm/cm³). *Nucleic Acids Res* 1982; 10(4): 1271-1281.
 18. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; 385: 810-813.
 19. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KHS. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* 1997; 278: 2130-2133.
 20. Takada T, Iida K, Awaji T, Itoh K, Takahashi R, Shibui A, Yoshida K, Sugano S, Tsujimoto G. Selective production of transgenic mice using green fluorescent protein as a marker. *Nature Biotechnology* 1997; 15: 458-461.