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Efficient Production of Transgenic Cloned Calves Using Preimplantation Screening

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ABSTRACT

The genetic manipulation of donor cells before nuclear transfer (NT) enables prior selection for transgene integration. However, selection for genetically modified cells using antibiotic drugs often results in mixed populations, resulting in a mixture of transgenic and nontransgenic donor cells for NT. In this study, we attempted to develop efficient strategies for the generation of human bile salt-stimulated lipase (BSSL) transgenic cows. Preimplantation screening by either biopsy or green fluorescent protein (GFP) expression was used to detect NT-derived BSSL transgenic embryos to ensure that the calf born would be transgenic. We compared the development rates of NT-derived embryos from G418- and GFP-selected donor cells. There were no significant differences ($P < 0.001$) in cleavage rate (67.2% vs. 60.0%) and blastocyst formation rate (44.9% vs. 41.2%). We also compared the pregnancy rates of the G418/biopsy and GFP preimplantation screened NT-derived blastocysts. The Day 40 pregnancy rate of the G418/biopsy group (40%) was lower than that of the GFP group (57%), but the calf birth rate of the G418/biopsy group (40%) was higher than that of the GFP group (21%). Healthy BSSL transgenic calves were born after both screening processes. This is the first report of biopsy-screened cloned transgenic animals. The results suggest that both selection methods are useful for detecting transgenic NT embryos without negatively affecting their development into viable transgenic offspring.

assisted reproductive technology, early development, embryo, pregnancy

INTRODUCTION

Genetically modified animals have important applications in human medicine and agriculture. Nuclear transfer (NT) with transfected or gene-targeted cells is a more efficient method of producing transgenic livestock than pronuclear DNA microinjection [1–4]. The advantage of using NT to produce transgenic animals is the ability to use preselected genetically modified cells as donor nuclei. All of the animals created via NT from such selected cells should be but are not always transgenic. Several researchers [5–9] have observed a so-called bystander effect, where transgenic cells, which express the antibiotic-resistance gene, provide protection to nearby nontransgenic cells either by secretion of the gene product into the medium or by direct cell-to-cell contact. As the result of this bystander effect, many transfected colonies are mixed and contain both transgenic and nontransgenic cells. Unpublished results from our lab and studies from other groups [5–9] have shown that NT animals produced from drug-selected genetically modified donor cells are not always transgenic. To develop a more efficient and cost-effective system, we employed a preimplantation embryo screening system to ensure that all NT calves born would be transgenic. Biopsy of blastocyst-stage embryos has been used for preimplantation screening for genetic disease diagnosis, gender determination, and transgenic confirmation [10–13]. Preimplantation screening by biopsy and polymerase chain reaction (PCR) analysis of microinjected embryos has produced viable transgenic animals in both mice [14] and cattle [15]. However, the use of biopsy procedures in conjunction with somatic cell NT has not been reported, possibly because of concerns that these procedures might jeopardize subsequent embryo development and implantation. In addition to biopsy screening, another means of enhancing the efficiency of production of transgenic cloned cows involves the use of green fluorescent protein (GFP). GFP is a genetic reporter system derived from a bioluminescent jellyfish. When expressed in either eukaryotic or prokaryotic cells and illuminated by blue or ultraviolet light, GFP yields a bright green fluorescence [16]. GFP can be used as a genetic reporter for cell transfection and selection and for preimplantation embryo screening. It has been widely used as a marker in DNA microinjection embryos [17], ES cell-mediated chimeras [18], and cells for NT [19]. Both preselection screening approaches should allow only the selected, presumably transgenic embryos, to be transferred into recipients, thereby reducing the cost and increasing the efficiency of transgenic animal production.

The transgene used in these studies codes for a potentially therapeutic protein, bile salt-stimulated lipase (BSSL). BSSL is an orally active enzymatic protein that is normally produced by the human pancreas and is present in human breast milk and that helps break down fats to make them more available for use by the digestive system. This protein has significant potential for use in replacement therapy for patients with pancreatic insufficiency (including cystic fibrosis patients) and for premature infants who do not receive human breast milk. Transgenic BSSL cattle have the potential to produce large quantities of BSSL for this application. Here, we report the use of preimplantation screening by both biopsy and GFP expression methods to select transgenic embryos for efficient production of BSSL transgenic cloned calves.

MATERIALS AND METHODS

Construction of Human BSSL Constructs

Two constructs (Fig. 1) were made for transfection of bovine primary fetal fibloblasts. The pBSSLIII construct contains 4.2 kilobases (kb) of

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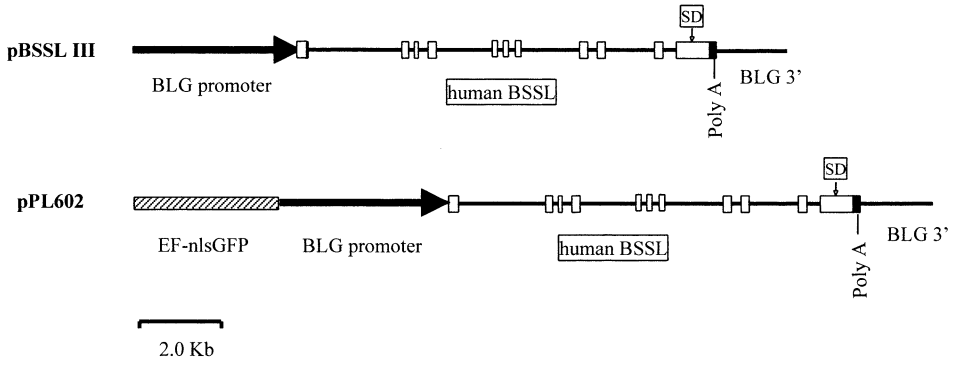

FIG. 1. pBSSLIII and pPL602 vectors. pBSSLIII consists of a 4-kb ovine BLG promoter (black arrow), 10 kb of human BSSL genomic DNA with 11 exons (open box), and 2.5 kb of ovine BLG poly(A) signal (black box) and downstream sequence. pPL602 has same structure as pBSSLIII except for a 3.9-kb EF-nlsGFP/pA cassette (hatched box) at the 5′ end of the BLG promoter. SD, Splice donor.

ovine BLG promoter, 10 kb of human BSSL genomic sequence, and 2.3 kb of ovine BLG poly(A) signal and 3′ flanking region. The pPL602 construct was made from pBSSLIII by inserting a 3.9-kb EF-nGFP fragment at the 5′ end of the ovine BLG promoter. To prepare the fragments for transfection, pBSSLIII was digested with *Mlu*I to remove the plasmid backbone and was purified twice in sucrose gradient (30 000 rpm for 18 h) to separate the 17-kb cassette from the plasmid backbone. The purified BLG/pBSSLIII fragment was mixed with a plasmid backbone-free pgk-neo/pA cassette at a 5:1 ratio before transfection. pPL602 was digested with *Mlu*I to remove the plasmid backbone and was purified twice in sucrose gradient (30 000 rpm for 18 h) before it was used for transfection. The contaminations of plasmid backbone in pBSSLIII, pPL602, and pgk-neo fragments were less than 1:500, as indicated by Southern analysis.

Isolation, Culture, and Transfection of Fibroblasts

Semen from Brown Swiss bulls was used to inseminate Holstein × Black Angus heifers. Fibroblast cells isolated from Day 70 fetuses as previously described [20] were designated BSFF1–BSFF5. Cells were cultured in Dulbecco modified Eagle medium, high glucose supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 0.1 mM nonessential amino acids (Invitrogen, Rockville, MD), and 2 ng/ml basic fibroblast growth factor (Roche, Indianapolis, IN) in a humidified incubator at 5% CO_2, 5% O_2 balanced with nitrogen. BSFF3 passage 1 cells in a T25 flask at 70% confluency were transfected with the pBSSLIII construct (5:1) using Geneporter (Gene Therapy Systems, San Diego, CA). Geneporter (10 μl) was mixed with 1.0 μg of backbone-free pBSSLIII and 0.2 μg of backbone-free PGK-neo in 1 ml of Optimem and exposed to cells for 4.5 h. Cells were harvested 24 h later and seeded into 10-cm plates. G418 selection (350 μg/ml) was applied 24 h after seeding. After 14 days, selected colonies were pooled for DNA analysis and cryopreserved. BSFF3 passage 2 cells (2.0×10^6) were electroporated with 5 μg backbone-free pPL602 vector in a 2-mm Gap cuvette for 1 msec at 275 V for three pulses. Cells were sorted by repeating flow cytometry for stable expression of GFP (Fig. 2) and used for NT.

Nuclear Transfer

The in vitro matured oocytes (Ovagenix, San Angelo, TX) were removed from the cumulus cells at 18 h postmaturation (hpm) by pipetting with a mouth pipette in handling medium FHM (Specialty Media, Phillipsburg, NJ) containing 0.3% hyaluronidase (Sigma, St. Louis, MO). The metaphase II-associated chromosomes were removed from oocytes at 18–22 hpm in FHM medium containing cytochalasin B (7.5 μg/ml; Sigma) and Hoechst 33342 (7.5 μg/ml; Sigma) by gentle aspiration of the polar body and the metaphase plate in a small amount of cytoplasm using a glass pipette. The process was performed under ultraviolet light to ensure removal of the oocyte chromatin. The donor cells were serum starved (0.5% serum) for 24–48 h before NT. Cells to be used as donors were picked up randomly in the G418/biopsy group. Only cells expressing GFP were picked for donors in the nGFP group. A single fibroblast cell was placed under the zona pellucida of the enucleated oocytes. The cell-cytoplast complexes were fused by application of an AC pulse of 5 V for 5 sec followed by two DC pulses of 1.25 kV/cm for 60 μsec using the ECM2001 Electrocell Manipulator (BTX Inc., San Diego, CA). The fusion rate was checked 30 min after the electrical pulse. The fused karyoplast-cytoplast complexes were activated by a 7-min incubation in 7% ethanol followed by 1 h of culture in cycloheximide (10 μg/ml; Sigma) and cytochalasin B (7.5 μg/ml) and then 3 h of culture in 10 μg/ml cycloheximide. The activated karyoplast-cytoplast complexes were cultured in G1 medium (G medium, Denver, CO) for 70–78 h followed by culture at 39°C in 5% CO_2, 5% O_2 and 90% N_2 for 96 h.

Screening of Transgenic Embryos

The blastocysts derived from the G418 group were biopsied to detect the transgenic embryos. Day 6 blastocysts were dissected with a microblade in Hepes-buffered G2 medium. A scratch was made with the microblade on the bottom of a plastic Petri dish to prevent the embryos from slipping during the biopsy process. The microblade was then used to cut away roughly ¼ of the embryo for use in a transgene-specific PCR. The remaining ¾ of the embryo was returned to the incubator. The biopsy sample was transferred directly into PCR tubes containing 4 μl embryo lysis buffer (40 mM Tris-HCl, pH 8.9, 0.9% Triton X-100, 0.9% Nonidet P40, 400 μg/ml proteinase K). Amplification was performed using Fisher (Pittsburgh, PA) brand buffer and *Taq* polymerase. Primers used to detect the presence of the transgene were BLG5′ (5′-CCTTCACCCA-AGGCCACGGTCACA) and BSSL3′ (5′-GGTGAGGCCCAACACAAC-CAGTTGC). Cycling conditions were 20 min at 65°C and 10 min at 95°C (hot start) then 35 cycles of denaturation for 45 sec at 94°C, annealing for 45 sec at 62°C, and extension for 1 min at 72°C, followed by a final elongation for 10 min at 72°C. Products were analyzed by agarose gel electrophoresis.

The blastocysts derived from the nGFP group were screened for the bright green fluorescent under ultraviolet light to detect the transgenic embryos.

Purification of Cattle DNA

Tissues were lysed overnight at 55°C in 50 mM Tris, pH 8.0, 0.15 M NaCl, 10 mM EDTA, 1% SDS, 1 M sodium perchlorate, 200 μg/ml proteinase K, and 1% 2-mercaptoethanol. Following lysis, DNA was purified by phenol-chloroform extraction, precipitated with isopropanol, and resuspended in Tris-EDTA buffer (TE). The DNA was then treated with Rnase and then proteinase K, and again extracted with phenol-chloroform, precipitated with ethanol, and resuspended in TE. DNA was isolated from blood samples using a mammalian blood DNA isolation kit (Roche).

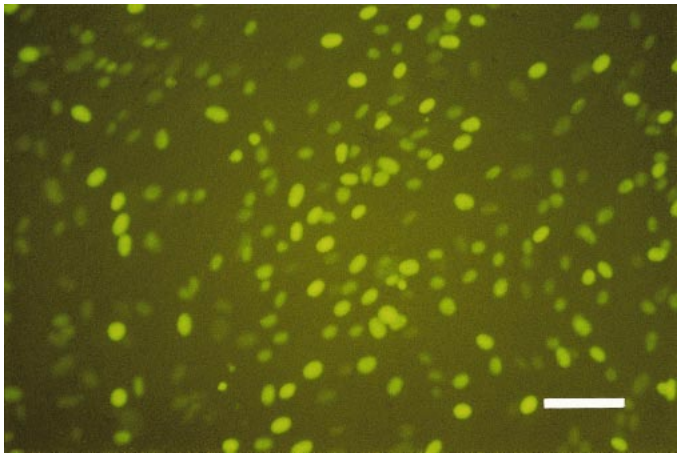

FIG. 2. nGFP expression in bovine fetal fibroblast cells. Bar = 50 μm.

TABLE 1. Development rates of NT-derived embryos from G418- and GFP-selected cell groups.

Donor	No couplets reconstructed	No. couplets fused (%)	No. embryos cleaved (%)	No. blastocysts (%)	No. embryos screened	No. transgenic blastocysts (%)
G418	443	198 (44.7)[a]	133 (67.2)[a]	89 (44.9)[a]	73	36 (47.9)[a]
GFP	331	165 (49.8)[a]	99 (60.0)[a]	68 (41.2)[a]	68	62 (91.0)[b]

[a,b] Values with different superscripts within the same column differ significantly ($P < 0.001$, chi-square test).

PCR Screening of Transgenic Cattle

One microliter of purified DNA at 30 μg/ml (in TE) was mixed with 4 μl lysis buffer (40 mM Tris, pH 8.9, 0.9% Triton X-100, 0.9% NP40, 0.4 mg/ml proteinase K), incubated for 15 min at 65°C, and then heated to 95°C for 10 min to deactivate the proteinase K. Fragments were amplified using *Taq* DNA polymerase (Fisher Scientific; 25 μl reaction volume) with the following parameters: 35 cycles of 94°C for 45 sec, 62°C for 45 sec, 72°C for 60 sec, 1 cycle of 72°C for 10 min, and a hold at 4°C. The primers used amplified a 487-base pair (bp) region across the junction between the ovine BLG promoter and the BSSL coding region. The 5′ primer was 5′-CCTTCACCCAAGGCCACGGTCACA-3′, and the 3′ end primer was 5′-GGTGAGGCCCAACACAACCAGTTGC-3′. Following amplification, the products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

Southern Blot Analysis of Transgenic Cattle

Purified genomic DNA (10 μg) was digested with *Pvu*II and separated on a 0.7% agarose gel. Following electrophoresis, the DNA was transferred to a nylon membrane and probed with a PCR-generated digoxigenin (Roche)-labeled probe specific for the promoter region of the construct (the 417-bp probe hybridizes with a 3016-bp fragment of *Pvu*II digested construct). The bands were detected using a chemiluminescent substrate system.

RESULTS

The development rates of NT-derived embryos from G418- and GFP-selected cells were compared. In four sessions, 443 matured metaphase II oocytes were enucleated and 443 total cell-cytoplast couplets were produced using G418-selected cells as donor cells. A total of 198 of 443 cell-cytoplast couplets were fused. In three sessions, 331 matured metaphase II oocytes were enucleated and 331 cell-cytoplast couplets were produced using nGFP-selected cells as donor cells. A total of 165 of 331 cell-cytoplast couplets were fused. The overall development rates from G418 and nGFP groups are presented in Table 1. Embryos from both groups showed very similar cleavage (67.2% vs. 60.0%) and blastocyst development (44.9% vs. 41.2%) rates when cultured in vitro. Preliminary experiments also demonstrated similar total cell numbers in Day 6 blastocysts from both groups (G418: 102 ± 21, n = 10; nGFP: 96 ± 16, n = 18).

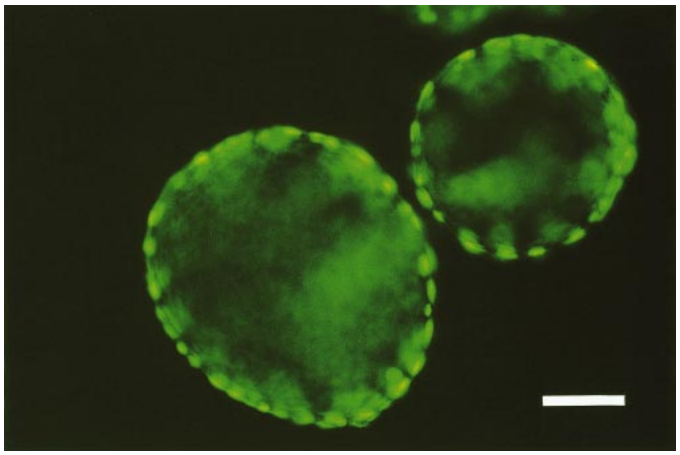

FIG. 3. nGFP expression in bovine blastocysts. Bar = 50 μm.

Because preliminary experiments indicated that colonies often contain a mixture of transfected cells and nontransfected bystander cells at the time of harvest, the cell pool was evaluated for the proportion of contaminating bystander cells. Limiting dilution of the pooled G418 cells was performed in microwells to produce single-cell-derived colonies. PCR analysis revealed that only 32.0% of the resulting colonies contained the transgene. These results confirmed that the pool of selected colonies was composed of a mixed population of transgenic and nontransgenic cells and illustrated the necessity of screening NT-derived preimplantation embryos.

To evaluate the use of embryo biopsy for the purpose of eliminating nontransgenic embryos, we screened Day 6 blastocysts produced from the G418-selected pool cells. A microblade was used to cut away roughly ¼ of the embryo for use in a transgene-specific PCR. The remaining ¾ of the embryo was returned to the incubator. Fifty-two of 73 embryos (70.1%) survived the biopsy process and reformed a blastocoele cavity by the next day, and 36 screened embryos were confirmed BSSL transgenic by PCR. Because of a limited number of recipient females, only 20 of the Day 7 transgenic embryos were shipped and transferred. As an alternative to embryo biopsy, a reporter gene encoding GFP was evaluated for screening of transgenic blastocysts. Of the 68 blastocysts produced by NT in the nGFP group, 62 (91%) expressed nGFP in the embryos (Fig. 3). Because of a limited number of recipient females, only 28 of the Day 7 transgenic embryos were shipped and transferred.

Pregnancy was assessed on a continuous basis throughout gestation. Recipients from both groups (G418/biopsy and nGFP) were examined on Days 40, 60, 90, and 240 by ultrasonography for the presence of a conceptus (Table 2). In the biopsy group, 4 of 10 pregnancies were established at Day 40, and there was no loss of pregnancy after Day 40. In the GFP group, 8 of 14 pregnancies were established at Day 40. Three of these eight pregnancies went to term, and five of them were lost after Day 40 (Table 2). Two pregnancies were lost between Days 40 and 60, one between Days 60 and 90, and one between Days 90 and 240, and one pregnancy (twins) was lost to induced abortion at Day 242 because of hydroallantois. In agreement with the sex of the BSSF-3 cell line used in this experiment, all

TABLE 2. Pregnancy rates of biopsy- and GFP-selected NT-derived BSSL transgenic embryos.

Selection	Recipients	Pregnant (%)				Calves born (%)
		Day 40	Day 60	Day 90	Day 240	
Biopsy	10	4 (40)	4 (40)	4 (40)	4 (40)	4 (40)
GFP	14	8 (57)	6 (43)	5 (36)	4 (29)[a]	3 (21)[b]

[a] For one recipient with twin fetuses, abortion was induced at Day 242 because of hydrallantois.
[b] One calf weighing 79.5 kg died 8 h after birth.

TABLE 3. Summary of cloned BSSL transgenic calves.

Calf no.	Construct/selection method	Birthweight (kg)	Transgenic status by PCR	BSSL copy no. by Southern blot
37	pBSSLIII/biopsy	43.2	+	2 or 3
38	pBSSLIII/biopsy	41.8	+	>10
39	pBSSLIII/biopsy	49.1	+	>10
40	pBSSLIII/biopsy	52.3	−	not transgenic
41	pPL602/FACS[a]	47.3	+	1
Twins	pPL602/FACS	NA[b]	+, +	NA
42	pPL602/FACS	79.5	+	NA
44	pPL602/FACS	43.6	+	1

[a] FACS, Fluorescence-activated cell sorting.
[b] NA, Not available.

calves were male. All seven calves (four from G418/biopsy and three from nGFP selection) were delivered by C-section 5–7 days before the due date. The four calves (numbers 37, 38, 39, and 40) born from the pBSSLIII-transfected G418/biopsy group weighed 95, 92, 108, and 115 pounds (43.2, 41.8, 49.1, and 52.3 kg) at birth. The three calves (numbers 41, 42, and 44) born from the pPL602-transfected GFP group weighed 104, 175, and 96 pounds (47.3, 79.5, and 43.6 kg) at birth (Table 3). The overly large 175-pound calf from the GFP group died 8 h after birth.

Tissue and blood samples from all newborn calves were collected for PCR screening and Southern blot analysis. Six of the seven newborn calves and both twins that were aborted at Day 242 were transgenic for BSSL as determined by PCR (data not shown). Delayed tissue sampling of the aborted twins and dead calf 42 resulted in poor DNA quality, thus preventing confirmation of transgenic status by Southern blot analysis. Southern blot analysis revealed that three of the four calves from the G418/biopsy group were BSSL transgenic, and both surviving calves from the GFP group were BSSL transgenic (Fig. 4).

BSSL copy numbers were determined by Southern analysis: calf 37, 2 or 3 copies; calves 38 and 39, >10 copies each; and calves 41 and 44, 1 copy each (Table 3).

DISCUSSION

In this study, neither biopsy nor nGFP preimplantation screening process inhibited the production of healthy cloned calves. Overall, pregnancy progressed to term in almost 30% of the recipients (7/24). Furthermore, the use of pooled cells may have increased the production efficiency of cloned transgenic cattle in both groups by avoiding expension of effort on a few colonies of unknown developmental potential. Each colony resulting from transfection and selection in G418 has widely different development potential when used for NT [21]. To reduce the chance of working with a colony with low developmental potential, we pooled the colonies after G418 selection and nGFP sorting. The use of early passage pools, compared with single colonies exposed to long-term culture during selection, has the added benefit of providing potentially healthier donor cells for NT. Compared with use of individual colonies, this strategy increases the number of possible integration events used to produce offspring, thus increasing the probability of obtaining one or more highly expressing lines. Some reports [22, 23] had indicated that nontransgenic cells have a higher cloning efficiency than that observed for drug-selected transgenic cells. This higher efficiency may be due to the processes of transfection and selection but may also be due to sampling errors related to the use of a single transgenic colony.

Preimplantation screening by biopsy is an adequate solution to the bystander problem. Although our biopsy group did not yield 100% accuracy, we did enrich the production of transgenic calves to 75% from a mixed-cell pool in which only 32% of the cells contained the transgene: 52 of 73 embryos (70.1%) survived the biopsy process. The pregnancy results suggested the that the biopsy process had no negative impact on the development of NT-derived embryos after they were transferred into recipients. Southern blot analysis revealed that three of the four calves from the G418/biopsy group were BSSL transgenic. The one nontransgenic calf obtained from the G418/biopsy group may have resulted from a labeling error during the biopsy process, microblade contamination, or a PCR artifact.

Preimplantation screening using nGFP has a number of advantages; no antibiotic resistance genes, no drug selection or extended time in culture, no additional embryo micromanipulation (biopsy), and no genetic screening (PCR) is required prior to transfer. The results with nGFP-selected embryos suggest there is no negative developmental impact associated with multiple rounds of GFP-based cell sorting, expression of the nGFP transgene in cells or embryos, or fluorescence assessment of the NT embryos when compared with the G418/biopsy method. The positive nGFP embryos demonstrated easily detectable bright fluorescence. Although only 9% of the nGFP blastocysts appeared negative, this percentage was higher than expected given that the cell population appeared to be virtually 100% GFP (+) at the time of NT. This observation may be explained by transient expression of nGFP in some fibroblasts, gene silencing in the embryo, or expression of nGFP below our visual detection limit. The GFP approach yielded a large number of transgenic blastocysts and produced transgenic calves at 100% efficiency (5/5 if the aborted Day 242 twins are included). Bondioli et al. [8] reported that 100% (2/2) of cloned pigs born from drug-selected cells were nontransgenic. In addition, Echelard et al. [9] reported that 4/6 (66.7%) cloned calves born from drug-selected CL53 cells

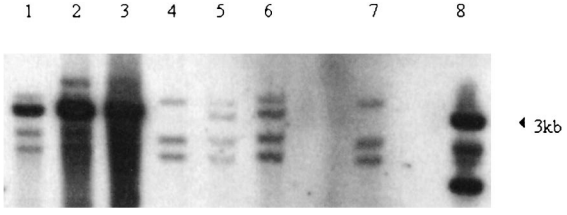

FIG. 4. Southern blot analysis of the cloned calves. Lanes 1–4: *Pvu*II-digested DNA of calves 37, 38, 39, and 40 from the G418/biopsy group; lanes 5 and 6: *Pvu*II-digested DNA of calves 41 and 44 from the GFP group; lane 7: *Pvu*II-digested DNA of nontransgenic cow for negative control; lane 8: plasmid DNA for positive control.

were nontransgenic. Preimplantation screening provides an opportunity to avoid this waste of animals and resources.

Because these animals were obtained from pools of transgenic cells, a variety of integration events were represented, as manifested by different transgene copy numbers in the clones. In general, animals derived from the BSSL-neo cotransfected pools had higher copy numbers than those obtained from GFP-transfected cells. The high ratio of BSSL transgene to selectable marker in the BSSL-neo selection pool may have selected for higher BSSL copy number as compared with nGFP selection. Transfection often results in multicopy integration of transgenes in a concatamer-type integration, whereas electroporation favors single-copy integration events, such as those observed in the GFP-selected animals [24].

We evaluated and compared methods for preimplantation screening of NT-derived embryos used to produce transgenic cattle. Both methods produced very similar in vitro development rates. However, subtle differences between the two protocols were observed. The biopsy group had a lower rate of pregnancy at Day 40 and a higher rate of live births with no pregnancies lost after Day 40. The GFP group had a higher rate of pregnancy at Day 40 and a lower rate of live births, with some pregnancies lost throughout gestation. These differences are subtle, and the numbers of pregnancies and live calves born in these experiments are too small to make significant distinctions when comparing overall efficiencies between groups. Both biopsy/PCR and GFP screening processes are acceptable approaches to increasing efficiency in the production of transgenic cloned calves.

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