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Obtaining transgenic bovine skin fibroblasts containing human interferon alpha gene

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The study aimed at obtaining the heifer's transgenic cell lines with integrated human interferon alpha gene *(IFNα)* having in view cloning of Polish Red cattle. The gene construct used (pbLGIFN-GFPBsd) contained the entire human interferon alpha gene under the bovine β-lactoglobulin promoter (bLG) and the green fluorescent protein gene (*gfp*) fused to the blasticidin resistance gene (*bsd*) under the human cytomegalovirus (CMV) immediate-early promoter. The gene construct was introduced by lipofection into heifer's ear fibroblasts. The GFP- and Bsd-positive cell lines have been obtained. The molecular analysis (PCR) and cytogenetic characterization (FISH) confirmed obtaining the stable heifer's transgenic cell lines with integrated human *IFNα* gene.

KEY WORDS: cattle / fibroblasts / interferon / lipofection / skin / transgenesis

Creation of transgenic farm animals is one of biotechniques which bring a benefit in animal breeding, medicine (xenotransplantation), pharmacology (bioreactors)

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and veterinary medicine (immunity). There are many methods used for creation of transgenic farm animals, such as microinjection of foreign DNA into pronuclei of zygote, sperm- mediated DNA transfer or transgenic somatic cell nuclear transfer [Wolf 2000, Wall *et al.* 2002, Robl *et al.* 2007]. Microinjection has predominantly been used to create the transgenic sheep, pig [Hammer *et al.* 1985] and cattle [Krimpenfort *et al.* 1991]. This technology works reliably, but is inefficient and results in a random integration and variable expression patterns in the transgenic offspring [Robl *et al.* 2007]. Recently, microinjection as a method of obtaining transgenic cattle has also been used by authors of the present study [Duszewska *et al.* 2004].

The most useful method for creation transgenic animals is somatic cell nuclear transfer employing the transgenic donor cells. Beside reduction of time, costs and a number of animals required, there are another advantages of transgenesis by the nuclear transfer using the transfected cells in comparison with the traditional DNA-microinjection technique. Firstly, male as well as female cell lines can be used depending on a particular project. Secondly, transfected cells can be stored frozen and the integration and expression of the transferred sequences can be investigated in cultured cells before the nuclear transfer procedure is conducted. After the nuclear transfer each cell of the transgenic animal contains the introduced transgene that eliminates mosaicism and ensures germline transmission [Wolf *et al.* 2000]. Transgenic somatic cell nuclear transfer (TSCNT) has been used for creation of transgenic sheep [Schnieke *et al*. 1997], cattle [Cibelli *et al*. 1998] and goat [Keefer *et al*. 2001]. TSCNT requires preparation of transgenic cells that are nuclear donors. The objective of this study was to obtain the heifer transgenic cell lines available for cloning Polish Red cattle.

Numerous approaches have been developed to facilitate the transfer of genes into cells, based on physical, chemical or viral strategies [Colosimo *et al*. 2000, Recillas-Targa 2006]. One of the chemical methods is lipofection which was also used in the present study. Liposome-mediated gene transfer was applied for DNA delivery into heifer's fibroblasts isolated from ear's skin. Lipofection is a relatively easy method of gene transfer. Liposomes have no limitations of the size of DNA to be delivered into cells [Lee and Jaenisch, 1996]. The DNA is coated by liposomes, which are then added to cells suspension. Liposomes are fused with the cellular membrane and release their content into the cytoplasm [Felgner *et al*. 1987].

The objective of this study was to obtain the heifer's transgenic cell lines with integrated human interpheron alpha gene (*IFNα*) for cloning Polish Red cattle. The pbLGIFN-GFPBsd gene construct was introduced by lipofection into bovine ear skin fibroblasts. The transgene containing the entire human interferon alpha gene *(IFNα*) under the bovine β-lactoglobulin promoter (bLG) was introduced into pTracer-EF/ Bsd A plasmid vector (INVITROGEN) containing *gfp* gene fused to the blasticidin resistance gene *(Bsd*).

Material and methods

Isolation and culture of fibroblasts from heifers' ear tissue

Bioptates from ears of three eight-months-old Polish Red heifers (ear-mark numbers 6335, 6349, 6350) were aseptically removed and washed several times in a phosphate- buffered saline (PBS) containing 50 µg/ml gentamycin sulfate, 100 IU penicillin, and 50 µg/ml streptomycin. The tissue samples were cut with scalpel blades and then digested in 5 ml of a trypsin-EDTA phosphate-buffered saline solution for 45 min at 37°C. The digests were centrifuged at 300 × g for 30 min. The sediment pellet was then resuspended and cultured for 7 days in 5 ml TCM 199Hepes with 20% FBS containing 50 µg/ml gentamycin sulfate, 100 IU penicillin, and 50 µg/ml streptomycin, in 38.5°C at 5% CO_2. The cells were passaged upon confluence. During that time the medium was replaced on day 2 and 5.

Preparation of pbLGIFN-GFPBsd gene construct

The pbLGIFN-GFPBsd construct contained a 1175 bp fragment of the bovine β-lactoglobulin promoter and a 973 bp human *IFNα* gene inserted in pTracer-EF/Bsd A plasmid vector (INVITROGEN) – Figure 1. In the first step the bovine β-lactoglobulin

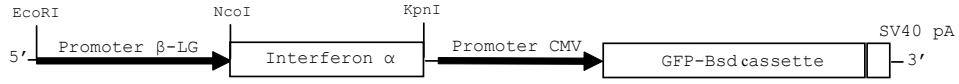

Fig. 1. Plasmid pbLGIFN-GFPBsd composed of the bovine β-lactoglobulin promoter fused to the human interferon alpha gene followed by the GFP-Bsd cassette containing cycle 3-GFP gene fused to the blasticidin resistance gene (*bsd*) under the human cytomegalovirus (CMV) immediate-early promoter.

promoter (GenBank Accession X14710) was amplified using genomic sequence as a template, and primers:

bLG-F (5'-TTGAATTCAGCCAGAGCTAGTCTAGGAG-3') and
bLG-R (5'- TTCCATGGCTGCAGCTGGGGT-3').

PCR products were digested with *EcoR*I and *EcoRV* and cloned into pBluescript SK+ plasmid vector. The 5' end of the insert was modified by addition of *EcoR*I restriction site. The 3' end contained *Nco*I restriction site. In this way ATG codon responsible for initiation of translation process was introduced. In the next step human genomic DNA isolated from peripheral blood lymphocytes was used as a template for interferon alpha gene IFN-F: (5'-ATCACCATCACCTGGTTCCGCGTGGATCTATGGCCTTGACCTTTGCTT-3') and IFN-R 5'-AGAAAGCTTGCAAAAGTTCAATGAACAAC-3' primers for interferon alpha gene amplification (GenBank Accession J00207). The 5' end of the gene was modified by addition of *Nco*I restriction site, sequence encoding six histidine residues and sequence recognized by thrombin. The 3' end of the gene was modified by addition of *Hind*III restriction site. PCR products were digested with restriction enzymes (*Nco*I and *Hind*III) and ligated with the vector containing the bovine β-

lactoglobulin promoter within *Nco*I and *Hind*III restriction sites. In the final step the sequence containing bovine β-lactoglobulin promoter and human interferon alpha gene was digested with *Eco*RI and *Kpn*I and recloned into 6.0 kb of pTracer-EF/Bsd A plasmid vector. The nucleotide sequence of the gene construct was determined using a cycling sequencing kit and ALFExpress sequencer (PHARMACIA BIOTECH).

Transfection of ear skin fibroblasts

The ear skin fibroblasts isolated from three heifers (no. 6335, 6349 and 6350) were transfected by both pbLGIFN-GFPBsd gene construct (Fig. 1) and pTracer-EF/Bsd A plasmid which was used as a control. In total, 36 experiments with transfection of *in vitro* cultured lines 6335, 6349, 6359 were conducted.

The pbLGIFN-GFPBsd gene construct and pTracer-EF/Bsd A vector were purified with UltraMobius Plasmid Kit (NOVAGEN), digested to linear form with *Sca*I and used for transfection by lipofection. Cells were plated at density of about 1×10^6 cells per 60 mm dish. For each transfection, sample of 8 μg of plasmid DNA was diluted in 500 μl serum-free DMEM. Simultaneously 20 μl of Lipofectamine 2000 (INVITROGEN) was diluted in 20 μl of serum-free DMEM and incubated for 5 min. The diluted Lipofectamine 2000 and the diluted DNA were mixed. After 20 min of incubation, DNA-Lipofectamine 2000 complexes were added to the flasks containing cells and medium and mixed gently by rocking. The cells were incubated for 24 h at 38.5°C in 5% CO_2. Then medium was replaced for continuing incubation. Two days later the medium was supplemented with 8 μg/ml blasticidine S for selection of the positively-transfected stable cell lines and cultured for 8 days. During that time the medium was replaced every 2-3 days. Following 8-day antibiotic-mediated selection, the generated cell clones were extracted and frozen in liquid nitrogen.

Screening for the presence of transgene

The screening procedure involved the isolation of genomic DNA from the ear skin fibroblast cells and the amplification of two PCR-fragments encompassing the promoter-gene junction. In the case of pbLGIFN-GFPBsd gene construct forward primers were located in the β-lactoglobulin promoter region and reverse primers in the region coding the interferon alpha. PCR product of 800 bp was amplified with:

bLGIFN-F1 (5'- GAAGCCACCCTCCAGGACA-3') and
bLGIFN-R1 (5'- CCCCACCCCCTGTATCACA-3') primers.

The 398 bp fragment was obtained with:

bLGIFN-F2 (5'- CGAATGGAAGAAGGCCTCCTA -3') and
bLGIFN-R2 (5'- ATGTCTGTCCTTCAAGCAGGA - 3') primers.

In the case of pTracer-EF/Bsd A control vector forward primers were located in the CMV-IE promoter region and reverse primers in the region coding the GFP protein. PCR product of 250 bp was amplified with:

CMV-F11 (5'-CACGCTGTTTTGACCTCCAT-3') and
GFP-R11 (5'-TCACCCTCTCCACTGACAGA-3') primers,

whereas the 393 bp fragment was obtained with:

CMV-F12 (5'- CGTGTACGGTGGGAGGTCTA - 3') and
GFP-R12 (5'-TGGCCATGGAACAGGTAGTT-3') primers.

The reaction mixture contained 100 ng DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.25 mM dNTP, 7.5 pmol of each primer and 0.7 U Taq polymerase (SIGMA-ALDRICH) in a final volume of 20 µl. PCR was performed as follows: denaturation at 94°C, 45 s; annealing at 55°C, 45 s; synthesis at 72°C, 90 s (30 cycles). PCR products were fractionated in 1.5% agarose gel containing 1 × TBE buffer.

Fluorescent *in situ* hybridization (FISH)

Fibroblasts were cultured in DMEM containing 50 µg/ml gentamycin sulfate, 100 IU penicillin, 50 µg/ml streptomycin, 1% amino acids and 20% foetal bovine serum (FBS). Cells were digested with 0.1% trypsin and 0.2% EDTA, in a hypotonic solution (0.075M KCl) and fixed with glacial acetic acid and methanol mixture (1:3). The gene construct pbLGIFN-GFPBsd and control vector pTracer-EF/Bsd A were used as a molecular probe for FISH after labelling reaction with biotin-16-dUTP. Slides with interphase nuclei were treated with RNase (100 µg/ml in 2 × SSC solution), and pepsin (100 µg/ml in 0.01 N HCl), and then dehydrated with 70%, 80% and 95% ethanol and denatured by incubation in 70% formamide in 2 × SSC (20 × SSC - 3 M NaCl, 0.3 M sodium citrate, pH 7.0) solution at 70°C for 3 min. After dehydration in ethanol the serial dilutions of denatured probe were placed onto slides and incubated for 16 h at 37°C. It was followed by post-hybridization washes on next day and a signal detection using the combined layer of FITC-avidin, goat anti-avidin and again FITC-avidin antibodies (VECTOR LABORATORIES). Slides were stained in DABCO/DAPI (VECTOR LABORATORIES) and analysed using fluorescence microscope (ZEISS, Axiovert 200).

Results and discussion

The objective of this study was to obtain the heifer's transgenic cell lines available for cloning. Aiming at this the pbLGIFN-GFPBsd gene construct was introduced by lipofection into bovine skin fibroblasts. The effectiveness and safety of lipofection as DNA delivery method has been shown by Nabel *et al*. [1993], Caplen *et al*. [1995] and Hui *et al*. [1997]. The transgene in question includes human interferon alpha gene and bovine β-lactoglobulin promoter and also selection gene as *bsd* and reporter gene as *gfp*.

The results of transfection of three *in vitro* cultured bovine skin fibroblast lines are presented in Table 1. All cell lines derived from heifers no. 6349 and 6350 survived the pbLGIFN-GFPBsd gene construct transfection. In case of heifer 6335 only four cell lines survived.

Table 1. Insertion of pbLGIFN-GFPBsd gene construct and pTracer-EF/Bsd A as a control vector into lines of heifers' ear skin fibroblasts

Cell line	Plazmid	No. of survival after selection/no. of experiments	GFP-positive	PCR-positive
Heifer's ear skin fibroblast line 6335	pbLGIFN-GFPBsd	4/6 (66.66%)	4	4
	pTracer-EF/Bsd A	4/6 (66.66%)	4	4
Heifer's ear skin fibroblast line 6349	pbLGIFN-GFPBsd	6/6 (100%)	6	6
	pTracer-EF/Bsd A	4/6 (66.66%)	4	4
Heifer's ear skin fibroblast line 6350	pbLGIFN-GFPBsd	6/6 (100%)	6	6
	pTracer-EF/Bsd A	5/6 (83.33%)	5	5

Blasticidin S deaminase gene (*bsd*) isolated from *Aspergillus terreus* was used for the selection of stable transfected fibroblast cells by Kimura *et al*. [1994]. The enzyme allowed to maintain the cell lines expressing the blasticidin-resistant gene, what means that the introduced transgene integrated with fibroblast's genome. All cells lines that survived were also GFP-positive (Photo 1). Green fluorescent protein gene (*gfp*) was isolated from *Aequorea victoria* by Prasher *et al*. [1992] and used, after pronuclear injection, in screening of embryos preimplantation-stage in mice [Takada *et al.* 1997, Kato *et al.* 1999] and cattle [Chan *et al*. 1999, 2002, Rosochacki *et al.* 2001] as well as in selecting cells for somatic cloning of mice [Ono *et al*. 2001], goats [Keefer *et al.* 2001], pigs [Park *et al.* 2002] and cattle [Chen *et al.* 2002, Bordignon *et al*. 2003].

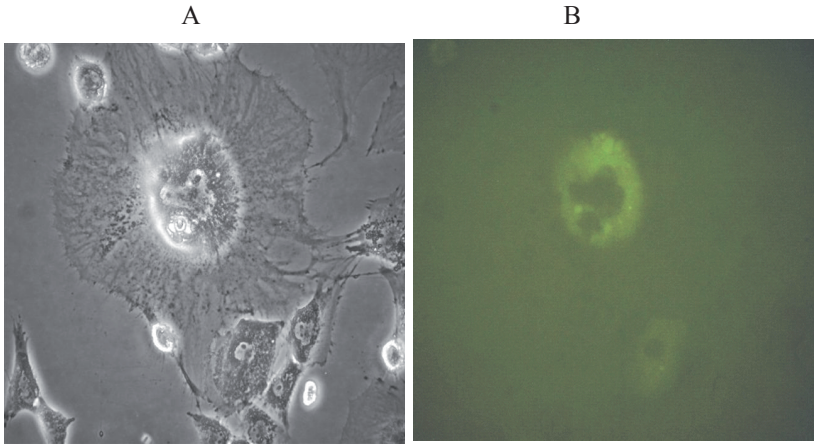

Photo 1. The heifer's ear skin fibroblasts transfected with pbLGIFN-GFPBsd construct; A – under light microscopy, B – under UV light microscopy (GFP-positive).

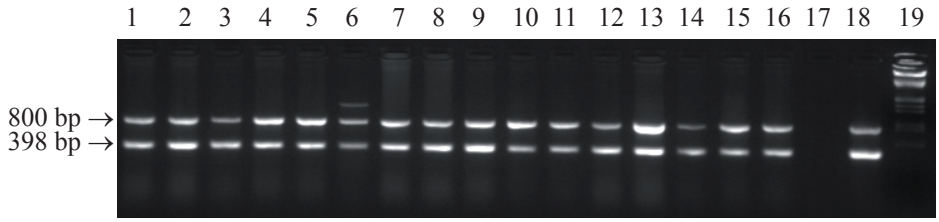

Photo 2. Analysis of the presence of pbLGIFN-GFPBsd transgene in transfected ear skin fibroblasts. PCR products were fractionated in 1.2% agarose gel. Screening for the transgene was performed by PCR encompassing 398 bp and 800 bp DNA fragments. Lanes 1-6 – ear skin fibroblast line 6350; lanes 7-12 – ear skin fibroblast line 6349; lanes 13-16 – ear skin fibroblast line 6335; lane 17 – negative control (without DNA); lane 18 – positive control (vector with transgene); lane 19 – size marker (λ DNA/*Hind*III, *EcoR*I). PCR observation was confirmed by sequencing.

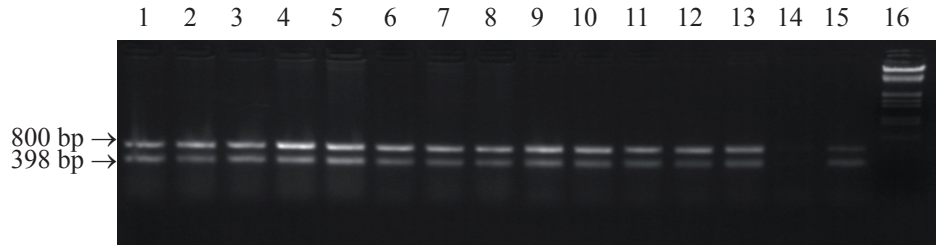

Photo 3. Analysis of the presence of pTracer-EF/Bsd A control vector in transfected ear skin fibroblasts. PCR products were fractionated in 1.2% agarose gel. Screening for the transgene was performed by PCR encompassing 250 bp and 393 bp DNA fragments. Lanes 1-5 – ear skin fibroblast line 6350; lanes 6-9 – ear skin fibroblast line 6349; lanes 10-13 – ear skin fibroblast line 6335; lane 14 – negative control (without DNA); lane 15 – positive control (vector pTracer-EF/Bsd A); lane 16 – size marker (λ DNA/*Hind*III, *EcoR*I). PCR observation was confirmed by sequencing.

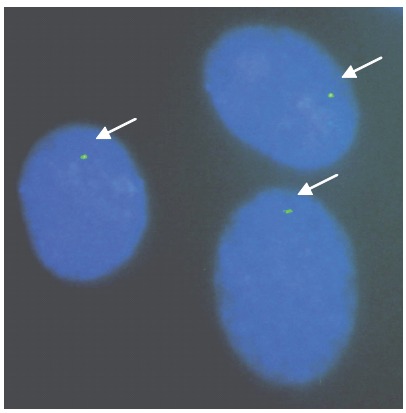

Photo 4. FISH results on interphase nuclei from heifer's ear skin fibroblasts transfected with pbLGIFN-GFPBsd gene construct. One hybridization signal (arrow) indicates the presence of the transgene integrated into the genome.

All Bsd- and GFP-positive cell lines were identified by PCR analysis and confirmed by fluorescence *in situ* hybridization (FISH).

Using PCR, transgenes were detected in all cell lines that survived selection and were also GFP-positive (Tab. 1). The screening results are presented in Photos 2 and 3. FISH was conducted on interphase nuclei to show the presence of the transgene in all transgenic cell lines (Photo 4). For mapping the transgene in a specific region of a genome, FISH should be accomplished on the metaphase chromosomes. In this study it was impossible to obtain chromosome slides at that stage. Cells after transfection and following 8 days of blasticidin selection showed very low frequency of cell division. It is known that long-term culture *in vitro* and the selection time can lead to reduction in the proliferative activity and to cytological aging of cells [Watanabe *et al*. 2005]. In spite of this, the FISH signal on interphase nuclei confirmed the presence of the integrated transgene in the ear skin fibroblasts of heifers no. 6335, 6349 and 6350.

Summarizing, the GFP-positive and bsd-positive transgenic cell lines have been obtained from three Polish Red heifers as confirmed by molecular analysis (PCR) and cytogenetic evaluation (FISH).

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Uzyskanie transgenicznych linii fibroblastów bydła z integracją genu ludzkiego interferonu alfa

S t e s z c z e n i e

Badania prowadzono w celu uzyskania linii transgenicznych fibroblastów bydlęcych z zamiarem wykorzystania ich w klonowaniu bydła. Wykorzystano konstrukcję genową pbLGIFN-GFPBsd zawierającą gen kodujący interferon alfa człowieka (*IFNα*) pod kontrolą promotora β-laktoglobuliny (bLG) oraz gen kodujący białko świecące na zielono (*gfp*) połączony z genem kodującym odporność na blastycydynę (*bsd*) pod kontrolą wczesnego promotora cytomegalowirusa (CMV). Konstrukcję genową wprowadzono metodą lipofekcji do fibroblastów izolowanych z uszu jałówek rasy cp. Uzyskane linie komórkowe okazały się GFP- i Bsd-pozytywne. Analiza molekularna (PCR) i cytogenetyczna (FISH) potwierdziła uzyskanie linii transgenicznych fibroblastów z integracją ludzkiego genu interferonu alfa.