



Detection of recombinant human lactoferrin and lysozyme produced in a bitransgenic cow

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ABSTRACT

Lactoferrin and lysozyme are 2 glycoproteins with great antimicrobial activity, being part of the nonspecific defensive system of human milk, though their use in commercial products is difficult because human milk is a limited source. Therefore, many investigations have been carried out to produce those proteins in biological systems, such as bacteria, yeasts, or plants. Mammals seem to be more suitable as expression systems for human proteins, however, especially for those that are glycosylated. In the present study, we developed a bicistronic commercial vector containing a goat β -casein promoter and an internal ribosome entry site fragment between the human lactoferrin and human lysozyme genes to allow the introduction of both genes into bovine adult fibroblasts in a single transfection. Embryos were obtained by somatic cell nuclear transfer, and, after 6 transferences to recipients, 3 pregnancies and 1 viable bitransgenic calf were obtained. The presence of the vector was confirmed by fluorescent in situ hybridization of skin cells. At 13 mo of life and after artificial induction of lactation, both recombinant proteins were found in the colostrum and milk of the bitransgenic calf. Human lactoferrin concentration in the colostrum was 0.0098 mg/mL and that in milk was 0.011 mg/mL; human lysozyme concentration in the colostrum was 0.0022 mg/mL and that in milk was 0.0024 mg/mL. The molar concentration of both human proteins revealed no differences in protein production of the internal ribosome entry site upstream and downstream protein. The enzymatic activity of lysozyme in the transgenic milk was comparable to that of human milk, being 6 and 10 times higher than that of bovine lysozyme pres-

ent in milk. This work represents an important step to obtain multiple proteins or enhance single protein production by using animal pharming and fewer regulatory and antibiotic-resistant foreign sequences, allowing the design of humanized milk with added biological value for newborn nutrition and development. Transgenic animals can offer a unique opportunity to the dairy industry, providing starting materials suitable to develop specific products with high added value.

Key words: bitransgenic cow, human lactoferrin, ELISA, human lysozyme

INTRODUCTION

Breast milk provides not only nutrients to a newborn, but also bioactive components for the development of tissues and physiological functions (Pereira, 2014). In some cases breastfeeding must be substituted with formula milk that is mainly derived from bovine milk; however, several studies have shown that breastfed babies have fewer infections than those fed infant formula (O'Connor and Unger, 2013). Bovine milk has a higher content of total protein than human milk, but some of the proteins of the human secretion are not present, or are at lower levels, in bovine milk. Among those at lower levels, lactoferrin and lysozyme are proteins that provide strong defense to newborn due to their antimicrobial activity against a broad spectrum of bacteria, viruses, yeasts, fungi, and parasites (Farnaud and Evans, 2003; Bayarri et al., 2014). Lactoferrin is the major antimicrobial protein in the milk of most mammals; it is a glycoprotein of approximately 80 kDa that belongs to the family of iron-binding proteins called transferrins (González-Chávez et al., 2009). Lactoferrin is synthesized by the epithelial cells of the external mucous membranes, present also in some bodily fluids, such as tears and saliva, although the highest levels are detected in milk secretions (Farnaud and Evans, 2003). However, the amount of lactoferrin is quite different between human and bovine milk; in human colostrum

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the concentration is about 6 mg/mL, whereas in bovine colostrum it is around 1 mg/mL (Sánchez et al., 1988; Montagne et al., 2001). In both species, the concentration of lactoferrin decreases dramatically in the first days of lactation, remaining around 2 and 0.1 mg/mL in human and bovine mature milks, respectively (Sánchez et al., 1988; Montagne et al., 2001).

Lysozyme is an enzyme of about 14 kDa consisting of a polypeptide chain of 130 AA. It is widely distributed in body fluids such as tears, saliva, blood, and other secretions, playing an important role in the nonspecific defenses of the individual (Zhou et al., 2014). Lysozyme is a hydrolase enzyme able to damage the bacterial cells by breaking the β -(1-4)-glycosidic bond between *N*-acetylglucosamine and *N*-acetylmuramic acid of the peptidoglycan chain present in the cellular bacterial wall (Niyonsaba and Ogawa, 2005). In human milk the lysozyme concentration is about 0.37 and 0.24 mg/mL in colostrum and mature milk, respectively (Montagne et al., 2001), whereas in bovine milk its concentration is practically undetectable, about 10 ng/mL (Król et al., 2010).

Transgenic animals can be used for several purposes: they have been used as animal models of human disease to find new treatments (Duverger et al., 1996; Carter, 2004), as sources of organs for xenotransplantation (Houdebine, 2000; Niemann, 2001), for the study of gene expression, promoter regulation, and codifying sequences (Montoliu, 2002; Giraldo et al., 2003), and as "biofactories" to produce human therapeutic proteins for pharmaceutical or nutraceutical purposes. The composition of an animal product, such as milk or meat, can be altered to make a functional food (Brem et al., 1994; Houdebine, 1994; Limonta et al., 1995; Hwang et al., 2004) with enhanced functionality due to mammalian post-translational modification of recombinant proteins. Considering that the availability of human milk-derived natural proteins, such as human lactoferrin and human lysozyme, is limited, alternative ways of production for these biopharmaceutical products are being extensively researched (Maga et al., 2006; Zhang et al., 2008; Goldman et al., 2012; Meng et al., 2013).

Multitransgenic animals have been obtained by using reproductive and molecular biotechnologies (Webster et al., 2005; Deng et al., 2011). The method to obtain multitransgenic animals for medical and veterinary purposes should be simple and efficient so that it allows for simultaneously modifying certain traits or enhancing the production of a certain protein by the inclusion of multiple copies of the same gene in the vector construction. Different strategies, such as mating transgenic pigs obtained for the production of single recombinant proteins, have been used to achieve this in pigs (Zhou et al., 2005). However, in addition to the low efficiency

of the principal techniques to obtain transgenic animals (pronuclear microinjection, viral vectors, sperm-mediated gene transfer, nuclear transfer), these protocols include time-consuming and expensive breeding programs (Niemann and Kues, 2003). One of the most questionable aspects of the use of more than one vector for obtaining multitransgenic animals is the presence of different markers that are constitutively expressed and multiple viral or bacterial sequences for each transgenic process. Moreover, as we found in previous experiments, repeated transfection rounds of a single cell line render the line unviable for nuclear transfer procedures; cells grew in a different way, adopting globose shape, and we could not obtain embryos after the cloning procedure. Moreover, the use of heterologous promoters sometimes generates promoter interference; that is, transcription from one promoter suppresses transcription from another (Shearwin et al., 2005; Blazeck and Alper, 2013).

Internal ribosome entry sites (**IRES**) have been proposed to express 2 distinct coding sequences under the control of only 1 promoter, which is of great interest to molecular and cellular biologists. Unlike that observed in most eukaryotic mRNA, in which ribosomes scan from the 5' end until the initiation codon is reached, in mRNA of picornaviruses (Jackson et al., 1990), such as encephalomyocarditis virus, ribosomes are able to begin translation at IRES. These IRES elements can be removed from their viral setting and linked to unrelated genes to produce polycistronic RNA (Gurtu et al., 1996). We hypothesized that IRES sequences inserted in a vector with a tissue-specific promoter may allow the expression of 2 human proteins from only 1 mRNA (bicistronic expression) in the cattle mammary gland, avoiding the successive transfections and multiple antibiotic insertions into donor cells before nuclear transfer. Thus, the objective of our work was to assess the feasibility of introducing, in a single transfection, a bicistronic vector containing an IRES fragment with 2 human genes (lactoferrin and lysozyme) in a bovine cell culture, and to obtain embryos by somatic cell nuclear transfer (**SCNT**), pregnancies, and the birth of double transgenic individuals able to express the human proteins in the mammary gland.

MATERIALS AND METHODS

Generation of Transgenic Cow

Ethics Statement. Because the expected births were genetically modified organisms, the facilities used for this experiment were designed to achieve resolution number 443/12 of the Ministerio de Agricultura, Ganadería y Pesca de la Nación (Argentina). This work was carried out after its approval by the National Commis-

sion of Agricultural Biotechnology (CONABIA, Buenos Aires, Argentina), presentation # S01:0325784/2011.

All procedures performed with animals were in accordance with the ethical standards of the Instituto Nacional de Tecnología Agropecuaria (Balcarce, Argentina) and Universidad Nacional de San Martín (San Martín, Argentina), where the studies were conducted. The procedures for animal care and methods of sacrifice were approved by the CICUAE (for Internal Commission for Experimental Animal Care, in Spanish) of the Veterinary Council (National Universities, Buenos Aires, Argentina), resolution 011/2011. The health of all bovine individuals used or obtained in this work was controlled by veterinarians.

The raw human milk samples used for lactoferrin isolation were donated by the human milk bank of Zaragoza (Spain). The study was approved by the Ethical Committee for Clinical Research of the Government of Aragón (Zaragoza, Spain) and informed consent was obtained from all donors.

Chemicals and Reagents. Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Construction of Expression Vectors. A 1,960-bp cDNA (GenBank accession number NM_002343) obtained from American Type Culture Collection encompassing the entire coding region of human lactoferrin was used. The gene was amplified from the original vector by PCR and cloned in the pGEM-T easy vector (Promega, Madison, WI). Primers contained the recognition sequence for *Xho*I (New England Biolabs, Ipswich, MA) in their extremes.

Human lysozyme cDNA was obtained by reverse-transcription PCR using as a template mRNA of an in vitro macrophage culture obtained from peripheral blood. The 438-bp fragment corresponding to the complete coding sequence of the gene was cloned in the pGEM-T vector, but primers had different restriction sequences, specifically *Msc*I (forward) and *Eag*I (reverse; New England Biolabs).

The pIRES2-EGFP vector (Clontech Inc., Mountain View, CA) was used to construct the mammary gland expression vector. The cytomegalovirus promoter was replaced by the goat β -CN obtained from the pBC-1 milk expression system (Invitrogen, Carlsbad, CA), by PCR with Platinum TAQ DNApol (Gibco, Thermo Fisher Scientific, Waltham, MA), and a primer containing restriction sequences *Asel* (forward) and *Nhe*I (reverse; New England Biolabs). The Kozak sequence was added to each oligonucleotide used to amplify the human genes to achieve better ribosomal recognition and their correct expression (Figure 1). The presence and correct orientation of the vector were verified by PCR and automatic sequencing, respectively. The vec-

tor was kept in *Escherichia coli* Top Ten (Invitrogen) until use.

Bovine Primary Cell Line Transfection. A primary cell line was obtained from an ear biopsy of a 5-yr-old Jersey cow. The sample was processed as stated by Baldassarre et al. (2004).

For transfection, the culture medium from 70% confluent cells was replaced with Opti-MEM medium (Gibco). Resistant single colonies were isolated and expanded in the presence of 25 μ g/mL of neomycin and then cryopreserved in liquid nitrogen. Cell lines were analyzed as previously described by Keefer et al. (2001) using fluorescent in situ hybridization (FISH) to confirm the presence of the entire vector and to determine the integration sites. Bovine transgenic cells (0.5 to 1.3×10^9) were plated onto 24- or 96-well plates and cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 15% fetal bovine serum (FBS) until they reached 100% confluence. After this, cells were cultured at 38°C, 5% CO₂ in air in Dulbecco's modified Eagle medium with 0.5% FBS, 20 mg/mL gentamicin, and 25 μ g/mL neomycin for 4 d until the day of nuclear transfer.

Embryo Production by SCNT

Oocyte Maturation. Ovaries were collected at a local slaughterhouse and transported to the laboratory in sterile 0.9% NaCl solution at 20°C within 3 h. Cumulus-oocyte complexes (COC) were recovered by aspiration of 2 to 10 mm follicles using a 21-gauge needle attached to a vacuum pump; only COC with more than 3 layers of compact and unexpanded cumulus cells were selected and cultured, as described in Mucci et al. (2006). Between 40 and 50 COC were incubated in 400 μ L of maturation medium at 38.5°C in 5% CO₂ with maximum humidity. The maturation medium consisted of M199 (Gibco) supplemented with 100 μ M cysteamine, and 0.1 IU of recombinant human FSH (Gonal F75, EMD Serono, Rockland, MA) and 10% FBS (Gibco).

Nuclear Transfer. All procedures were performed as described by Keefer et al. (2002) and Baldassarre et al. (2004), with minor modifications. After 18 h of maturation, the cumulus cells were removed by vortexing for 1 to 2 min in HEPES synthetic oviductal fluid (H-SOF; 20 mM HEPES, 5 mM NaHCO₃, 107.7 mM NaCl, 7.1 mM KCl, 1.2 mM KH₂PO₄, 1.5 mM MgSO₄, 7.3 mM sodium pyruvate, 0.2 mM L-glutamine, 1.8 mM citrate, 1.8 mM CaCl₂·2H₂O, 5.4 mM sodium lactate, and 1% BSA) containing 1 mg/mL hyaluronidase. Denuded oocytes that presented polar bodies were washed and returned to the maturation medium in the incubator. The enucleation process was initiated within 30

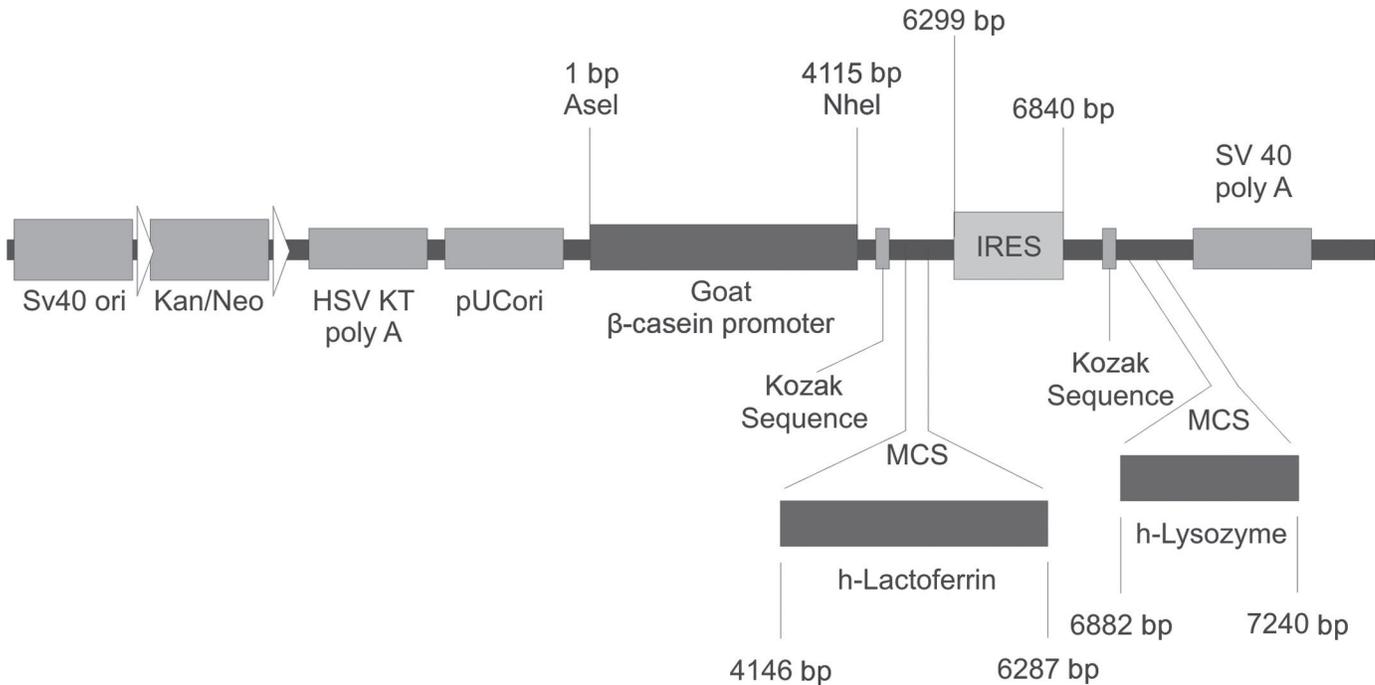


Figure 1. Schematic presentation of the vector used to transfect NMuMG and Jersey cow cells. The pIRES2-EGFP vector (Clontech Inc., Mountain View, CA) was used as backbone. Cytomegalovirus promoter was replaced by the goat β -CN promoter obtained from the pBC-1 milk expression system (Invitrogen, Carlsbad, CA). Kozak sequences were added to each cDNA (human lactoferrin and lysozyme). SV40 = simian virus 40 origin of replication; Kan/Neo = kanamycin/neomycin; HSV KY Poly A = polyadenylation signal herpes simplex virus thymidin kinase gene; pUCori = pUC vector origin of polyadenation signal of simian virus 40; IRES = international ribosome entry site.

min of oocyte denuding using an inverted microscope (Nikon Eclipse TE-300, Nikon, Tokyo, Japan) and micromanipulators (NT 88 V3, Nikon).

Oocytes were incubated for 10 min in H-SOF supplemented with 10 μ g of Hoechst 33342 per milliliter at 38°C, placed into manipulation drops (H-SOF supplemented with 1% FBS) covered with mineral oil, and finally enucleated after a brief exposure to UV light (Nikon Filter Set 01) to determine the location of DNA. Donor cells were collected from the culture plate by trypsinization using 0.05% trypsin-EDTA, washed twice, and finally resuspended in H-SOF. Cells were picked up with the transfer needle and slipped into the perivitelline space of enucleated oocytes. Cell-cytoplasmic couplets were fused immediately after cell transfer using a 0.5-mm gap fusion chamber (BTX, San Diego, CA) overlaid with sorbitol fusion medium (0.25 M sorbitol, 0.5 mM magnesium acetate, 0.1% BSA) with a 25- μ s pulse of 2.4 kV/cm (BTX Electrocell Manipulator 630, Harvard Apparatus, Holliston, MA). The postfusion culture medium consisted of H-SOF plus 5% FBS with 5 mg/mL of cytochalasin B. After 1 h, fused couplets were activated with 5 mM ionomycin in H-SOF for 4 min, and then 4 h of culture in 2 mM 6-dimethylaminopurine at 38.5°C in 5% CO₂ in air with maximum humidity. After this, presumptive embryos were rinsed

in H-SOF and placed into 4-well plates with culture medium (SOF citrate) under mineral oil. Embryos were cultured for 7 d at 38.5°C in 5% CO₂, 5% O₂, 90% N₂ with maximum humidity (Sanyo MCO 175M, Moriguchi, Japan) until they were transferred to recipients.

Embryo Transfer and Pregnancy Detection. Three-year-old multiparous Aberdeen Angus cows were used as embryo recipients. Estrus was synchronized by the intramuscular application of 2 doses of 2 mL of PGF_{2 α} separated by 11 d. Embryos were transferred 7 d after heat was detected. Pregnancies were confirmed by ultrasonography scanning using a transrectal 5-MHz linear array probe (Aloka 500, Hitachi-Aloka, Tokyo, Japan) 23 d after embryo transfer. Pregnant recipients were monthly evaluated by rectal palpation and ultrasonography scanning.

Birth and Neonatal Care

A neonatology unit was equipped to prevent or treat the most common pathologies associated with the birth of clones obtained by SCNT. Ten days before the estimated day of birth, the fetal position and viability were examined by rectal palpation. On the same day, birth was induced by intramuscular administration of 30 mg of dexamethasone and 25 mg of PGF_{2 α} . In cases

in which no signs of parturition were observed, 36 h after induction obstetric maneuvers were performed to evaluate the fetal position and response to stimuli. When necessary, a cesarean section was performed in a standing position through a left flank approach for the recovery of fetuses. Immediately after delivery the calf was transferred to the neonatology unit to assess its physiological status and receive the first veterinary care.

Genotyping of Cloned Animals

To corroborate that the born animal shared the genotype of the transfected cells, a genotyping test for bovines was performed (StockMarks for Cattle Bovine Genotyping Kit, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). The test was based on the detection of 11 microsatellites and performed in an automatic genetic analyzer (ABI Prism 3730, Applied Biosystems). Markers were authorized by the International Society of Animal Genetics and the results processed with Data Collection and Gene Mapper v0.4 software (Applied Biosystems).

FISH

To determine the integration site and the number of insertions of the vector, FISH was performed using the expression vector as probe. Briefly, 10 μ g of the vector was digested with DNase I, and a nick translation reaction with *Escherichia coli* polymerase I using deoxycytidine-5'-triphosphate (dCTP)-Rhodamine-labeled nucleotides was performed.

Skin samples were obtained from the presumptive transgenic calf and processed as previously described. Once obtained, cells were cultured in the presence of 50 μ g/mL of colchicine (Colcemid, Gibco) for 4 h, resuspended, and washed with hypotonic solution (0.05 M KCl in water). Mounting was performed by directly dropping cells onto a slide before fixation with Carnoy solution (3:1 methanol:acetic acid). The insert was visualized at 400 \times in an Eclipse 2000 microscope (Nikon) by using rhodamine and DAPI (4',6-diamidino-2-phenylindole; DNA staining) filters to contrast with the red positive potential signals of the hybridized probe.

Induction of Artificial Lactation

The calf that was born was kept in a pen under controlled growing conditions, which included a complete sanitary plan and feeding schedule until lactation. To obtain milk from the calf before its physiological lactation, an artificial induction was performed. The 1-yr-old calf was treated following the protocol sug-

gested by a field veterinary. Treatment consists of a daily intramuscular dose of 20 mg of estradiol cypionate (Konig, Buenos Aires, Argentina) from d 1 to 14. On d 1, a progesterone injection of 125 mg (Syntex, Buenos Aires, Argentina) and the introduction of an intravaginal device of 1 g of progestogen (Syntex) was performed. The device was replaced by a new one on d 4 and removed on d 7, when another progesterone injection was given (5 mL). On d 15 and 16, 25 μ g of PGF_{2 α} (Syntex) was administered, followed by 20 mg of dexamethasone at d 18, 19, and 20 of treatment. On d 21, 3 mL of Orastine (Intervet, Buenos Aires, Argentina) was administered and manual milking started. Milking was performed for 57 d twice a day (morning and evening) and then ceased. Samples were fractioned and frozen at -20 and -80°C . Colostrum from second day and milk from d 35 were used for protein analysis.

Lyophilization of Colostrum and Milk Samples

Fluid raw colostrum and milk samples (200 mL) were placed in the lyophilizer VirTis Benchtop 6K (VirTis, SP Scientific, Warminster, PA) in 50-mL tubes (Falcon, Carlsbad, CA). The drying process was conducted for 12 h using a vacuum lyophilizer and cooling to -5°C . Freeze-dried colostrum and milk were stored in a polyethylene bag with zipper until use.

Detection and Quantification of Human Proteins in Milk

Specific Antibodies Against Human Lactoferrin. Lactoferrin was purified from human milk by cation exchange chromatography using an SP-Sepharose column, and polyclonal antiserum against human lactoferrin (**hLF**) was obtained in rabbits as described previously using the purified protein (Conesa et al., 2008). To eliminate the antibodies that gave cross-reactivity with bovine lactoferrin (**bLF**) from the anti-hLF antiserum, the polyclonal antiserum was passed through an immunoaffinity column with insolubilized bLF (donated by Tatua Nutritionals Company, Morrinsville, New Zealand). To eliminate cross-reactivity with bLF, the antiserum anti-hLF was added to the gel with insolubilized bLF. The mixture was incubated for 1 h with gentle agitation at room temperature and afterward mounted into a column. The antibodies that did not react with bLF eluted with 25 mM phosphate potassium buffer, 0.5 M NaCl, pH 7.4, and were stored for further isolation of specific antibodies against hLF. An immunoaffinity column (similar to the one for bLF) with insolubilized hLF was used to obtain specific antibodies against hLF. The more strongly retained

antibodies were eluted with the buffer described above added with 10% of dioxane.

Immunodotting Technique. The elimination of cross-reactivity of the antiserum anti-hLF with bLF and the presence of hLF in bitransgenic milk and colostrum were verified by immunodotting. The samples (1 μ L) were applied onto a nitrocellulose membrane with a pore size of 0.45 μ m and left to dry. The membrane was then saturated with 5% ovalbumin (wt/vol) in 2 mM potassium phosphate, 8 mM sodium phosphate, 3 mM KCl, 0.14 M NaCl, pH 7.4 (PBS) by incubation for 1 h at room temperature. After 5 washes with PBS, the membrane was incubated with antiserum monospecific against hLF, diluted 1:50 in PBS with 3% ovalbumin for 1 h. The membrane was washed again 5 times with PBS and incubated with goat anti-rabbit IgG antibodies conjugated with peroxidase and diluted 1:1,000 in PBS with 3% ovalbumin. Finally, after 5 washes, the membrane was revealed with a solution of peroxidase substrate prepared as follows: 3 mg of 4-chloro-1-naphthol, 1 mL of methanol, 4 mL of 10 mM potassium phosphate, 0.15 M NaCl (pH 7.4), and 5 μ L of H₂O₂. The reaction was stopped by washing the membrane with distilled water. At the point of sample application, a greyish coloration was observed when a positive reaction with the antibodies occurred.

Western Blot. Colostrum and milk samples from the transgenic heifer, human (positive control), and cow (negative control, raw milk obtained from pooled dairy farm cows) were tested for the presence of hLF and human lysozyme. The samples (100 μ L) were mixed with cracking buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol]. The samples were resolved in SDS-PAGE, 12% polyacrylamide gel (acrylamide/bisacrylamide, 19:1), and separated at 200 mA for 2 h in running buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS). Proteins were transferred to a nitrocellulose membrane (Millipore, Billerica, MA) by using a Mini subcell chamber (Bio-Rad, Hercules, CA) set up at 25 mA for 2 h. The membrane was washed and blocked for 1 h with Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.6 and 150 mM NaCl) with 5% cold fish gelatin and 2% normal horse serum. The membranes were incubated with commercial monoclonal antibodies for human lactoferrin (Abcam, Cambridge, UK) or human lysozyme (Abcam), both generated in rabbit. After several washings with buffer, a goat anti-rabbit antibody marked IRD 800CW IgG (Li-Cor, Lincoln, NE) was used as secondary antibody diluted 1:20,000 in TBS 3% cold fish gelatin solution for 1 h. Washings with TBS 0.1% Tween 20 were done and signal detection performed by an Odyssey Quantitative Fluorescence Imaging System (Li-Cor).

Development of an ELISA to Quantify hLF.

This technique was developed to quantify the concentration of hLF in bitransgenic milk samples that were provided in powder (obtained by conventional lyophilization of the whole secretion) and reconstituted with distilled water to the original volume. The wells of Maxisorp strips (provided by Nunc, Thermo Fisher Scientific) were coated with 100 μ L per well of anti-hLF antibodies at 5 μ g/mL, dissolved in 0.05 M sodium carbonate buffer, pH 9.6, and kept overnight at 4°C. The wells were washed 5 times with PBS with 0.05% Tween 20 in an automatic washer. Then, wells were incubated with 300 μ L of 3% ovalbumin in PBS for 2 h at 37°C. Afterward, they were washed 5 times and incubated with 100 μ L of hLF standards (0, 10, 25, 50, 100, 250, 500, and 1,000 ng/mL) and bitransgenic milk samples for 1 h at 37°C. After 5 washings, the wells were incubated with 100 μ L of anti-hLF antibodies conjugated with horseradish peroxidase according to Catty and Raykundalia (1989; dilution 1:5,000 in PBS with 1% ovalbumin). Finally, the wells were washed 5 times and incubated with 100 μ L of peroxidase substrate consisting of 3,3',5,5'-tetramethylbenzidine (Zeulab, Zaragoza, Spain) for 30 min at room temperature. The reaction was stopped by adding 50 μ L of 2 M sulfuric acid, and the absorbance at 450 nm was measured in a plate reader Multiskan MS (Labsystem, Helsinki, Finland). The ELISA was validated as described in the Supplemental Material (<https://doi.org/10.3168/jds.2016-11173>).

Isolation of Lysozyme from Human Milk.

Human lysozyme was isolated from the fractions corresponding to the excluded volume obtained in the isolation of hLF by cation exchange chromatography. Those fractions were pooled, concentrated 10 times by UF and subjected to Sephadex G-50 (ϕ 1 \times 65.5 cm) chromatography. The elution was carried out with PBS containing 1 M NaCl at a flow rate of 1 mL/min and collecting 2-mL fractions. The purity of the protein was assessed by SDS-PAGE in Phast System equipment (GE Healthcare Life Sciences, Pittsburgh, PA).

Competitive ELISA to Quantify Human Lysozyme.

The content of lysozyme in bitransgenic milk and colostrum was determined by the Lysozyme Human ELISA kit of Abcam. The 96-well plate was supplied coated with specific lysozyme antibodies. The standards (3, 1.5, 0.750, 0.375, 0.188, 0.094, and 0.047 μ g/mL) or samples were added in a volume of 25 μ L per well and immediately 25 μ L of biotinylated lysozyme was added on top. The plate was incubated for 2 h at room temperature and, afterward, the wells were washed 5 times with washing buffer and incubated with 50 μ L of streptavidin-peroxidase conjugate for 30 min. The wells were washed 5 times and the chromogen

substrate was added per well and incubated 10 min or until the optimal color developed. Finally, 50 μ L of stop solution was added per well and the absorbance was determined at 450 nm.

Determination of Enzymatic Activity of Lysozyme in Milk Secretions. The activity of lysozyme was determined by a turbidimetric method, measuring the decrease in absorbance at 450 nm of a fresh suspension of *Micrococcus lysodeikticus* versus time. For this purpose, 8 mg of this bacterium was suspended in 25 mL of 100 mM potassium phosphate buffer, pH 6.2. A volume of 950 μ L of the bacterial suspension was placed in a glass cuvette, adding 50 μ L of the sample in which lysozyme activity had to be determined. The decrease in absorbance was measured continuously at 450 nm. The enzymatic activity was determined from the slope of the curve obtained by representing the absorbance versus time.

Detection of Casein and Whey Proteins

Casein and milk serum proteins were detected by reverse-phase HPLC (ISO, 2004). The area of each peak in the chromatogram obtained was relativized to total casein and concentration for each casein expressed as a percentage.

RESULTS

Embryo Production and Transfer

Two SCNT procedures were performed using the bitransgenic cell lines. In the first, we obtained single Day-7 embryos from 27 reconstructions (62% fusion rate). The embryo was transferred to a recipient and resulted in a 30-d pregnancy. The recipient was sent to a local slaughterhouse to recover the fetus for studies. Transgenesis was confirmed by FISH of the primary cell culture from the fetus (data not shown).

The second nuclear transfer procedure was performed with the same transgenic cell batch, from which we obtained 8 Day-7 transferable embryos from 54 reconstructions after SCNT (69% fusion rate). Six embryos were transferred to previously synchronized recipients, 4 single transfers with embryos rated 1 to 2 (according to International Embryo Technology Society ratings; www.iets.org) and 2 double transfers with embryo quality 3. Two cows were detected pregnant (1 single and 1 double) on d 30 (pregnancy rate: 33% per transferred recipient, 37.5% per transferred embryo).

Birth and Neonatal Care

Thirty hours after birth induction, fetal viability of the double pregnancy was compromised and, after

a cesarean section, 2 dead Jersey female fetuses were recovered. No evident pathologies were found during fetal necropsy. Each fetus weighed 21 and 16 kg, respectively. Placentas presented fewer than 30 placentomes each, the larger ones 15 cm in diameter. Tissue samples were sent to the pathology laboratory (INTA, Balcarce, Argentina), resulting in mild pericarditis and interstitial pneumonia in one fetus and hemorrhage in the central nervous system in the other fetus. One placenta presented mild lymphocytic placentitis.

The remaining pregnant cow gave birth by a cesarean section to a female Jersey calf that weighed 45 kg. The calf received 25 mg of doxapram (Viviram, Holliday, Argentina) and an antimicrobial rational preventive therapy immediately after birth. The calf was kept in the neonatal unit until d 80, receiving specific therapies in case of need. On d 81, the calf started a normal life following normal feeding and health care protocols.

Genotyping and FISH

Genotyping results from the born calf, cell line, and Jersey cow from which the tissue sample was taken (founder) shared the same microsatellite markers (data not shown). To verify the presence of the vector (pIRES/LF/lyso) in the calf genome and to determine the number of insertions and the target chromosomes, a FISH was performed. A clear sign of the vector was found as a single copy in chromosome 7 (Figure 2).



Figure 2. Fluorescent microscopic image (400 \times) of the vector insertion (arrow). The probe was labeled with Rhodamine (Sigma-Aldrich, St. Louis, MO), and DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) was used to stain DNA.

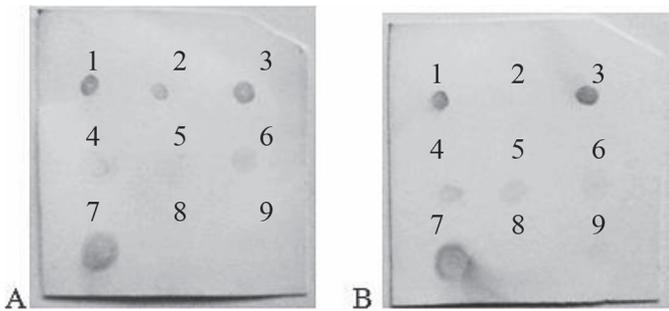


Figure 3. Immunodotting to test the specificity of antibodies against human lactoferrin (hLF) in which the nitrocellulose membranes were incubated with a polyclonal antiserum against hLF diluted 1:50 (A) and with specific antibodies anti-hLF after elimination of cross-reactivity with bovine lactoferrin (bLF) diluted 1:50 (B). Samples: 1 = hLF (1 mg/mL); 2 = bLF (1 mg/mL); 3 = hLF + bLF (0.5 mg/L + 0.5 mg/L); 4 = whey of bitransgenic colostrum; 5 = whey of bitransgenic milk; 6 = BSA (1 mg/mL); 7 = human milk; 8 = human α -LA; 9 = bovine milk.

Lactation

The treatment to induce lactation started when the calf was 13 mo old. The calf started producing 400 mL of milk per day and reached 6 L per day on d 60, when milking stopped. The teats were then sealed with a commercial product (Bovigam R, Bayer, Buenos Aires, Argentina).

Specific Antibodies Anti-Human Lactoferrin and Immunodotting

Immunodotting results are shown in Figure 3. No cross-reactivity with bLF was detected, and the presence of hLF in bitransgenic milk and colostrum was confirmed by the positive reaction of specific antibodies with both samples. A positive reaction in the position corresponding to human milk was also observed, whereas we observed no reaction in the position corresponding to bovine milk.

Western Blot for the Detection of hLF and Human Lysozyme

We found a clear positive sign of the presence of both human proteins in the bitransgenic cow milk using monoclonal antibodies against hLF (Figure 4) and human lysozyme (Figure 5) in the milk and colostrum

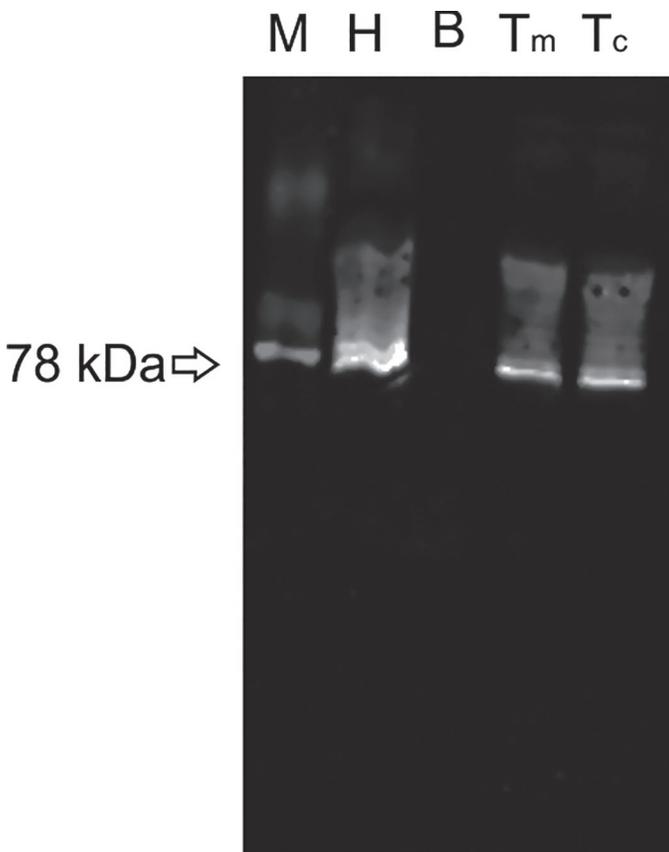


Figure 4. Expression of human lactoferrin (hLF) in the bitransgenic cow's mammary gland. Western blot was performed and samples were detected with specific antibodies for hLF. M = molecular marker; H = human milk; B = pooled milk from dairy cows; Tm = milk from a bitransgenic cow (10 d after milking started); Tc = colostrum from bitransgenic cow (1 d after milking started).

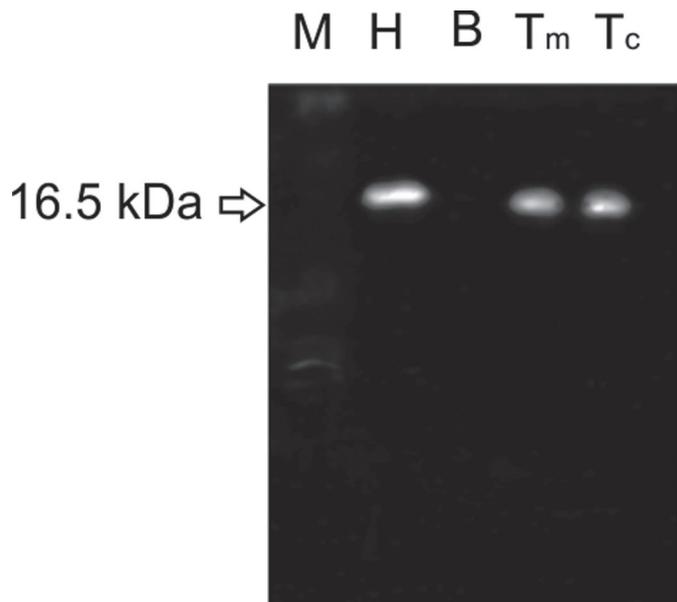


Figure 5. Expression of human lysozyme (hLZ) in bitransgenic cow. Western blot was performed and samples were detected with specific antibodies for hLZ. M = molecular marker; H = human milk; B = pooled milk from dairy cows; Tm = milk from bitransgenic cow (10 d after milking started); Tc = colostrum from bitransgenic cow (1 d after milking started).

of the bitransgenic cow, which coincided with the expected molecular weights. The positive control (human milk) revealed a band according to the protein molecular weight, whereas the negative control (cow milk) gave no sign.

Quantification of hLF and Human Lysozyme

Human protein concentrations in the calf colostrum and milk were assessed by ELISA. Human lactoferrin concentration was 0.0098 mg/mL in cow colostrum and 0.011 mg/mL in milk, whereas human lysozyme concentration was 0.0022 mg/mL in cow colostrum and 0.0024 mg/mL in milk. Considering the molecular weight of each protein (80 and 16.5 kDa for hLF and human lysozyme, respectively), the transformation to molar concentration shows that hLF is present in cow milk at 139.74 nM (8.31×10^{13} molecules/mL) and that human lysozyme is present at 145.45 nM (8.75×10^{13} molecules/mL).

Enzymatic Activity of Lysozyme

The enzymatic activity of lysozyme was determined by the turbidimetric method and the results are shown in Table 1. The sample with the highest activity against *M. lysodeikticus* was the recombinant human lysozyme from rice (donated by Ventrria Bioscience, Sacramento, CA), which was used as reference, followed by lysozyme isolated from human whey. It is necessary to note that enzymatic activity was measured in pure isolated lysozymes from recombinant rice and human whey, which were used as references, whereas the values of activity for the other samples were measured directly in milk or colostrum secretions without isolating previously the enzymes. With regard to bitransgenic milk samples, the highest enzymatic activity was measured in colostrum.

Casein and Whey Protein Concentration

Total casein concentration, determined by HPLC in the milk of the bitransgenic cow, was 41.5 g/L, whereas that in colostrum was 72 g/L. Among total casein, α -CN represented 51.2 (21.25 g/L) and 64.8% (46.65 g/L) and β -CN represented 48.8 (20.25 g/L) and 35.2% (25.35 g/L), respectively; κ -CN was found in trace amounts. The α -LA concentration was 2.4 g/L in milk and 1.8 g/L in colostrum, whereas β -LG concentration was 6.4 and 8.4 g/L, respectively.

DISCUSSION

The mammary gland is one of the readily available animal bioreactors, and by the use of recombinant

protein technologies it can be directed to produce pharmaceuticals and add beneficial molecules for human health to the milk. Expression vector construction for transgenic expression of recombinant proteins still needs to be optimized (Houdebine, 2009). One strategy for optimization includes using elements such as insulators and matrix-attached regions, but the expression level of proteins is still often low. For these reasons, construction of the targeting vector is one of the most important goals for mammary gland bioreactors, but currently available techniques are laborious, time-consuming, and inefficient (Yu et al., 2012). One approach to enhance protein production is to introduce several copies of the same gene under the effect of a single promoter, by using bi- or polycistronic vectors. We obtained the first bicistronic cow expressing hLF and human lysozyme by using a modified IRES-containing vector. The principal use of IRES-containing plasmids was the simultaneous expression of 2 independent cistrons for the co-transfection of a marker gene with the gene of interest. Vectors containing resistance genes (e.g., to neomycin, hygromycin), in addition to the gene of interest, have been constructed (Gurtu et al., 1996; Rees et al., 1996; Veelken et al., 1996; Hobbs et al., 1998) and demonstrated to be useful in establishing stable cell lines expressing a gene of interest. We found that the 2 transgenic proteins obtained from the bitransgenic cow had a similar molar concentration in colostrum and milk. However, Mizuguchi et al. (2000) reported the differential expression of a second gene while using IRES co-expression in several cultured cell lines and in mouse liver *in vivo*. The expression of the IRES-dependent second gene ranged from 6 to 100% (in most cases between 20 and 50%) compared with the first gene's expression. Analysis of transgenic animals resulting from microinjection of IRES constructs showed that expression levels of both genes vary and are generally lower than those of the same genes transcribed individually (Jankowsky et al., 2001); this is likely because polycistronic mRNA are unusual for higher eukaryotic cells and mechanisms preventing their normal function

Table 1. Enzymatic activity of lysozyme in samples from different origin, estimated by the decrease in absorbance at 450 nm (Δ Abs 450) of a *Micrococcus lysodeikticus* suspension¹

Sample	Enzymatic activity (Δ Abs 450/min)
Rice recombinant human lysozyme (1 mg/mL)	230
Human milk lysozyme (1 mg/mL)	126.2
Bitransgenic colostrum	47.2
Bitransgenic milk	29.3
Bovine milk	4.7
Human milk	24.1

¹The values represent the mean of values corresponding to 3 independent experiments.

are triggered. Similarly, transgenic animals containing more than 2 genes have been obtained by using sperm-mediated gene transfer (Webster et al., 2005), resulting in 7 triple transgenic, 7 double transgenic, and 4 single transgenic piglets for fluorescent proteins. Deng et al. (2011) used a 2A peptide approach and obtained 11 multitransgenic piglets, 7 of which co-expressed 4 fluorescent proteins at equivalently high levels and 4 of which showed slight discrepancies in protein expression. These results were achieved with vectors constructed with no more than 2 genes under the control of a promoter. When 4 genes were driven by a single cytosine-adenine-guanine (CAG) promoter it was not efficient enough to mediate the co-expression of the 4 genes in the primary fetal fibroblast cell line and the embryos obtained. The expression of the last 2 genes was much weaker than that of the first 2 genes in the polycistronic cassette, suggesting that the cleavage efficiencies at the downstream 2A sites were lower than those at the upstream 2A sites. In our experiment, only 2 proteins were included in the vector; thus, the functionality of the vector for more than 2 genes should be assessed in the future.

Different promoters, including α_{S1} -CN, β -CN, β -LG, α -LA, and hybrid promoters, which coincide with the proteins that account for approximately 90% of all proteins in bovine milk (Ikonen et al., 1999), have been used for the specific expression of recombinant proteins in milk (Yuan et al., 2014). We used a β -CN gene promoter, which is one of the most efficient and most commonly used in the mammary glands of mice, goats, and cows (for review, see Maksimenko et al., 2013). Because the length of the β -CN promoter can be optimized for efficient recombinant protein expression from 950 bp (Ahn et al., 1995) to 10 kb (Sohn et al., 2003), we used a 4,112-kb promoter. Casein promoters possess binding sites for transcription factors, including activators of transcription, enhancer binding proteins, and glucocorticoid receptors (Raught et al., 1995; Buser et al., 2007), and expression of milk protein genes are regulated by interactions between particular hormone-activated transcription factors during mammary gland development. Western blot results demonstrated that the recombinant proteins were expressed in milk and colostrum, both of the expected size. A novel approach of using a hybrid promoter or enhancer has been reported to increase specific expression of hLF in the mouse mammary gland (Cheng et al., 2012), although inherent regulatory sequences of different species and the differences of the protein genes are the main subjects to be taken into account to optimize transgenic protein production (Yuan et al., 2014).

After artificial induction, milk production was similar to that observed by Jewell (2002), who found more

than 5 kg/d of milk yield in 80% of induced Jersey heifers. The concentration of total protein in the bi-transgenic calf milk was high (47.3 g/L), whereas Jewell (2002) found an average concentration of 38 g/L, higher than that observed in noninduced Jersey cows (33 g/L). Similarly, Sawyer et al. (1986) reported that percentages of protein and fat were higher in milk from lactation-induced heifers than in milk from heifers after normal calving for the first 14 d. In the present study, increased protein concentration did not alter the proportion of 80% caseins to 20% whey proteins. An increase in β -CN (almost 50% of the caseins) was observed in milk, whereas the other major casein (i.e., α -CN) remained at normal concentrations (21–25 g/L). Likewise, whey proteins were increased, and the expected α -LA + β -LG concentration of 8.8 g/L was obtained in the milk of the transgenic calf.

In bovine milk, lactoferrin is in its highest levels in the first 2 d of lactation and then decreases very markedly (Sánchez et al., 1988); however, the milk secretions obtained in our study do not seem to have the same evolution. It has to be considered that the lactation of the bitransgenic calf was hormone-induced; therefore, the pattern of protein secretion might be altered respect to a natural lactation, as described in previous studies (Jewell, 2002). Similar concentrations of both lactoferrin and lysozyme have been obtained in colostrum compared with milk, perhaps due to the use of the β -CN promoter, a protein that has been found in similar amounts or slightly reduced in colostrum compared with milk (Ontsouka et al., 2003), marking no effect of the lactation period on the casein family promoters.

The concentration of hLF obtained in the present study was similar to that found by Neville and Zhang (2000), who reported 0.01 to 0.03 mg/mL during bovine lactation. The first recombinant human lactoferrin (**rhLF**) bull was produced by the Pharming Group NV (Leiden, the Netherlands) in 1990 (Krimpenfort et al., 1991) by microinjection, and the hLF gene was under the control of the bovine α_{S1} -CN promoter. van Berkel et al. (2002) obtained 0.8 mg/mL of lactoferrin in a transgenic cow in 2002 using the same technique and promoter; those authors also obtained 3 male transgenic individuals, whose F1 daughters produced between 0.3 and 2.3 mg/mL of lactoferrin. The rhLF of the transgenic cows and natural hLF show similar structural and functional properties *in vitro* (Thomassen et al., 2005) and have been reported as structurally and functionally similar, as the homology of hLF and bLF at the protein level is 69% (Nuijens et al., 2009). Hyvönen et al. (2006) obtained a basic mean concentration of rhLF in the milk of transgenic cows of 2.9 mg/mL during early lactation, and a concentration

of bLF of 0.07 mg/mL. These cows were produced by multiple ovulation and embryo transfer using semen from a transgenic bull obtained by pronuclear injection. Yang et al. (2008) obtained 2 transgenic calves by using microinjection of bacterial artificial chromosome DNA containing the entire genomic sequence of hLF. Two copies were inserted in one calf and one in the other. Analysis by RIA further demonstrated that rhLF was highly expressed in the transgenic milk, at concentrations of 2.560 and 3.460 mg/mL. Recombinant hLF was also obtained from transgenic goats, for which the levels of hLF achieved were of 0.765 mg/mL (Zhang et al., 2008), and transgenic rabbits, with levels of 0.103 mg/mL (Li et al., 2006). However, in transgenic mice, hLF levels obtained were low, between 0.001 and 0.036 mg/mL (Platenburg et al., 1994). Regarding recombinant human lysozyme, Yang et al. (2011) reported that the expression level of this protein in milk was between 0.013 and 0.02596 mg/mL, as measured by RIA by using a bovine β -CN promoter and different kinds of fetal cells. Recombinant human lysozyme has also been expressed in other mammals, such as goats (Maga et al., 2006), obtaining higher levels than in our study (0.270 mg/mL). Although the level of human lysozyme is low in the bitransgenic milk and colostrum of our study, its enzymatic activity is comparable to that of human milk. Furthermore, the lysozyme activity found for the bitransgenic milk would correspond mainly to that of human recombinant lysozyme, as we found very low levels of lysozyme activity at the same conditions and to previous reports (Benkerroum, 2008).

Low levels of transgenic proteins could be explained by the insertion of the foreign DNA into a gene-poor region, characterized by frequent DNA breaks (Goldman et al., 2004). The chromatin in these regions typically exert a negative influence on the expression of the transgene integrated nearby. Several regulatory elements are used to protect the transgene expression, which in our case was excluded from the fragments of the pBC1 vector used for the construction to achieve a smaller vector size. For the same reason, we used cDNA instead of the complete DNA sequence of the human genes. The use of introned or partly introned genes is preferable due to the presence of regulatory sequences, enhanced stability of the primary transcripts, and increased transgene expression efficiency (Whitelaw et al., 1991; Hennighausen, 1992). Bacterial artificial chromosome generally contain all the regulatory elements necessary for gene expression (Giraldo and Montoliu, 2001), probably diminishing the position effect caused by the chromosomal insertion site of an exogenous gene.

Although most milk parameters were not analyzed in our work, Zhang et al. (2012) demonstrated that the parameters measured in milk from transgenic cloned

cattle (almost 70 proteins) were all within the normal range compared with control milk. The authors (Zhang et al., 2012) used 3 different transgenic cell lines to obtain cows expressing approximately 0.01 mg/mL of lysozyme, 3.5 mg/mL of lactoferrin, and 1.5 mg/mL of α -LA.

We suggest that using the IRES approach and DNA editing techniques (such as CRISPR, clustered regularly interspaced short palindromic repeats) for targeted recombination could enhance protein production, by using native promoters and active genome zones and maintaining the physiological expression patterns for the transgenic proteins. In summary, a bitransgenic cow expressing 2 human proteins in the mammary gland was produced by using an IRES-containing vector and the SCNT technique. This study demonstrates that transgenic animals producing more than one protein of interest in bovine milk can be efficiently obtained by using a single round of transfections and less antibiotic-resistance markers, allowing the use of dairy herds as a source of high-quality nutraceutical or pharmaceutical proteins.

CONCLUSIONS

The presence of hLF and human lysozyme has been detected in the milk of the first bitransgenic cow in the world expressing 2 proteins of interest. This achievement is very important as a key step in the process of designing humanized milk with added biological value for newborn nutrition and development. The fact that the 2 proteins expressed in the bitransgenic milk have antibacterial activity and constitute the nonspecific defensive system of milk is of great relevance. Although the levels of hLF and human lysozyme in the bitransgenic milk and colostrum are low, the success in the generation of this bitransgenic cow is very promising because it could provide great benefits to human nutrition and health in the near future.

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