The Construction and Expression of Lysine-Rich Gene in the Mammary Gland of Transgenic Mice

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Lysine is the limiting amino acid in cereal grains, which represent a major source of human food and animal feed worldwide, and is considered the most important of the essential amino acids. In this study, β -casein, α S2-casein, and lactotransferrin cDNA clone fragments encoding lysine-rich peptides were fused together to generate a lysine-rich (*LR*) gene and the mammary gland-specific expression vector pBC1-*LR-NEO*^r was constructed. Transgenic mice were generated by pronuclear microinjection of the linearized expression vectors harboring the *LR* transgene. The transgenic mice and their offspring were examined using multiplex polymerase chain reaction (PCR), Southern blotting, reverse transcriptase-PCR, *in situ* hybridization, and Western blotting techniques. Our results showed that the *LR* gene was successfully integrated into the mouse genome and was transmitted stably. The specific *LR* gene expression was restricted to the mammary gland, active alveoli of the transgenic female mice during lactation. The lysine level of the two transgenic lines was significantly higher than that of non-transgenic controls ($p < 0.05$). In addition, the growth performance of transgenic pups was enhanced by directly feeding them the *LR* protein-enriched transgenic milk. Our results demonstrated that lysine-rich gene was successfully constructed and expressed in mammary gland of transgenic mice. This study will provide a better understanding of how mammary gland expression systems that increase the lysine content of milk can be applied to other mammals, such as cows.

Introduction

THE INABILITY OF HUMANS and many farm animals to synthesize certain amino acids has triggered tremendous interest in increasing the levels of these so-called essential amino acids in the human diet. Among the essential amino acids, lysine has received the most attention because it is the most limiting amino acid in cereal crops, which represent a major source of human food and animal feed worldwide. Lacking lysine leads to fatigue, nausea, dizziness, loss of appetite, agitation, bloodshot eyes, slow growth, anemia, and reproductive disorders. Therefore, enriching essential amino acids in the human diet, especially lysine, has both economical and humanitarian benefits. This can be achieved using transgenic technology with genes encoding stable proteins that are rich in lysine or modifying lysine biosynthesis (Frizzi *et al.*, 2008). To date, several recombinant genes encoding lysine-rich proteins have been expressed in transgenic plants (Guenoune *et al.*, 2003; Bicar *et al.*, 2008). Recently, a high-lysine expressing maize line (LY038) (Dizigan

et al., 2007) has been approved for commercial use in the livestock feed industry in a number of countries.

Milk represents an important food source that is consumed not only in its natural form but also in a wide variety of processed products (Brophy *et al.*, 2003). Because milk proteins are a natural component of animal diets and have excellent nutritional parameters, they have been proposed as alternative proteins that can be expressed in plants to improve their nutritional content (Chong *et al.*, 1997; Yang *et al.*, 2002). Therefore, milk that contains a high level of lysine could be used directly as food for humans or indirectly as a source of protein for animals, thus complementing the deficiency found in cereal crops. Transgenic farm animals that secrete recombinant proteins into their milk are attractive systems for the production of valuable proteins. Although approximately 100 recombinant proteins have been expressed in mouse milk using transgenic technology (Soler *et al.*, 2006), a transgenic mouse that expresses a high level of lysine in its milk through its mammary glands has not been reported.

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In this study, we successfully generated transgenic mice that expressed lysine-rich proteins in their milk, which will help to determine the feasibility of producing lysine-enriched milk in transgenic cows. To our knowledge, this is the first time to produce transgenic mice expressing a lysine-rich protein in their milk. This work lays the foundation for producing transgenic cows that express high levels of lysine in their milk through their mammary glands.

Materials and Methods

Reagents and animals

All reagents were purchased from Sigma unless otherwise noted. Kunming (KM) mice and ICR mice were purchased from the Center of Experimental Animals of Jilin University and Changchun Hi-Tech Laboratory Animal Research Center, respectively, and were housed under controlled light cycle (14 h light/10 h dark) conditions. Animal treatments complied with a protocol approved by the Jilin University Institutional Animal Care and Use Committee.

Construction of the lysine-rich gene and the mammary gland-specific expression vector

For better eukaryotic expression efficiency in mammary glands' epithelium, the naturally occurring lysine-rich peptide sequences of milk proteins casein beta (aa 111–129, bp 391–447 in exon 7 of GenBank: NM_181008.2), casein alpha-s2 (aa 151–215, bp 507–701 in exon 2 of GenBank: NM_174528.2), and lactotransferrin (aa 282–310, bp 984–1043 in exon 8 of GenBank: NM_180998.2) were selected to form lysine-rich protein as 3' alpha s2-casein, lactotransferrin and beta-casein 5' (Fig. 1B). The three peptides have no homology with any nocuous protein by BLAST. Then, the lysine-rich (LR) cDNA sequence was fused with a bovine beta-casein signaling peptide on its 5'-end. The recombinant LR gene was artificially synthesized and insert it into the vector pBluescript II SK (+) (Shanghai Sangon Biological Engineering and Technology Service Co. Ltd). The length of the LR gene is 376 bp and includes a signal peptide sequence (bp 9–53), an LR gene coding sequence (sequences bp 54–248, bp 249–308, and bp 309–362 is derived from casein alpha-s2, casein beta, and lactotransferrin, respectively.), two stop codon (TAATAG), two *XhoI* restriction site (CTCGAG), and a pair of protective bases (CG and CG) (Fig. 1C). The LR protein is 14.02 kDa (cutting away signal peptide); 25.24% (26/103) of the protein is lysine. It contains 118 amino acids, including a 15-amino-acid bovine β -casein signal peptide sequence (aa 1–15), casein alpha-s2 peptide sequence (aa 16–80, lysine content is 21.54%), casein beta peptide sequence (aa 81–100, lysine content is 26.31%), and lactotransferrin peptide sequence (aa 101–118, lysine content is 30%) (Fig. 1C).

The pBC1 vector (Invitrogen) was designed to facilitate the expression of recombinant proteins in the milk of transgenic animals. The pBC1 vector contained a 5' genomic fragment of goat β -casein gene, which served as a promoter (Persuy *et al.*, 1992; Roberts *et al.*, 1992), a 3' β -casein genomic fragment, including exons, introns, as well as polyadenylation sequences for the efficient transcription termination of mRNA. The LR gene was inserted into the pBC1 vector using the *XhoI* restriction site between exon 2 and exon 7 of the

β -casein sequence (Fig. 1A), and the generation of the plasmid pBC1-LR was confirmed by DNA sequencing. For the stable transfection, a *Neo^r* selection marker (the neomycin resistance gene) driven by the phosphoglycerate kinase promoter was inserted into the *NotI* restriction site of the pBC1 expression vector, and the neomycin resistance cassette was flanked by two *Loxp* cassettes to allow the removal of the selectable marker gene. The creation of pBC1-LR-*Neo^r* was purified and subsequently digested with *SalI* restriction enzymes to remove the prokaryotic sequences. The isolated fragment (Fig. 1A) was purified and used to produce transgenic mice by pronuclear microinjection.

Generation of transgenic mice

Six-week-old female ICR mice were superovulated by intraperitoneal injection of 10 IU pregnant mare's serum gonadotropin (PMSG), followed by intraperitoneal injection of 10 IU human chorionic gonadotropin (hCG) at intervals of 48 h.

To obtain zygotes, ICR mice were mated with KM (Kunming) male mice in a 1:1 ratio after hCG injection and females with vaginal plugs were used for zygote preparation. Plugged females were sacrificed at day 0.5 and the zygotes were collected and denuded from the cumulus cells by treatment with 0.2% hyaluronidase. For pronuclear microinjection, the mammary gland-specific expression cassette of the pBC1-LR-*Neo^r* expression vector was excised by digesting with *SalI*. The purified transgene was diluted in TE buffer (2 to 3 ng/mL) and was microinjected, under inverted microscope (Nikon), into the male pronucleus of fertilized eggs that were isolated from superovulated female mice. Injected zygotes were cultured overnight in KSOM with 5% CO₂ at 37°C. Embryos at the two-cell stage of development that survived microinjection were transferred into the oviducts of 0.5-dpc pseudopregnant female ICR mice.

Transgene detection by multiplex polymerase chain reaction and Southern blotting

Genomic DNA was isolated from the tail tissue of mice. The multiplex polymerase chain reaction (PCR) procedures were performed in a final volume of 25 μ L with the following reagent: 10 $\times$ PCR Buffer (25 mM MgCl₂; TaKaRa) 2.5 μ L, dNTP (2.5 mM; TaKaRa) 2 μ L, *Ex Taq* DNA polymerase (5 U/ μ L; TaKaRa) 0.125 μ L, genomic DNA template (200 ng/ μ L) 0.5 μ L, and 17.875 μ L ddH₂O. According to different amplification efficiencies, the concentration of each prime pair (4 pairs, 10 pM) was accurately calibrated: the LR gene 0.4 μ L, the *Neo^r* gene 0.15 μ L, the β -casein promoter 0.9 μ L, and β -actin 0.4 μ L. The PCR parameters were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 s and 54°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 7 min. The primers used were as follows:

LR 187: Upstream, 5'-AATCAGCCAGCGTTACCAGA-3',
Downstream, 5'-AGCCATAGCCTCCTTCACTT-3';

Neo^r gene: Upstream, 5'-TTCCTTGCGCAGCTGTGCTC-3',
Downstream, 5'-CATTCGCCGCCAAGCTCTTC-3';

β -actin: Upstream, 5'-GATATCGCTTGCGCTGGTCGTC-3',
Downstream, 5'-ACGCAGCTCATTGTAGAAGGTGRRG-3';

β -casein promoter: Upstream, 5'-GGAAATGTTGAATGGG AAGGATA-3',

FIG. 1. Structure of the expression vector pBC1-LR-NEO', LR gene, and LR protein. **(A)** Structure of the expression vector pBC1-LR-NEO'. The 2×β-globin insulator: two copies of chicken β-globin insulator; β-casein promoter: goat β-casein promoter; E1, E2, E7, E8, and E9: β-casein exon sequences; IVS1, IVS7, and IVS8: goat β-casein intron sequences; 3' genomic fragment: goat β-casein 3' genomic fragment; LR: LR coding region, flanked by exon 2 and exon 7 of β-casein sequences; pGK-NEO': the neomycin resistance gene cassette; *SalI* restriction sites were used to linearize the pBC1-LR-NEO' construct to remove the prokaryotic sequences. **(B)** Structure of the LR coding region. Casein beta sequences (aa 111–129, bp 391–447 of GenBank: NM_181008.2), casein alpha-s2 sequences (aa 151–215, bp 507–701 of GenBank: NM_174528.2), milk lactotransferrin sequences (aa 282–310, bp 984–1043 of GenBank: NM_180998.2) were fused together to form the lysine-rich (LR) gene, as 5' alpha2-casein, lactotransferrin and beta-casein 3'. Then, the LR gene sequence was fused with a bovine beta-casein signaling peptide on its 5' end. The LR gene includes a signal peptide sequence, an LR gene coding sequence, stop codon (TAATAG), two *XhoI* restriction site (CTCGAG) and a pair of protective base (GC and CG). **(C)** Annotated sequence of the fusion LR cDNAs and protein. The positions of the *XhoI* restriction sites are underlined. TAATAG is the stop codon. LR protein aa 1–15 is bovine beta-casein signaling peptide corresponding to LR cDNA sequences bp 9–53. LR sequences bp 54–248, aa 16–80 is derived from casein alpha-s2 (GenBank: NM_174528.2) sequence, which is corresponding to bp 507–701, aa 151–215. Sequences bp 249–308, aa 81–100 and sequences bp 309–362, aa 101–118 of LR are corresponding to aa 111–129, bp 391–447 of casein beta (GenBank: NM_181008.2) and aa 282–310, bp 984–1043 of lactotransferrin (GenBank: NM_180998.2).

Downstream, 5'-AGAAGAGAGAAAGAGAAAGGGGT-3';

Genomic DNA (20 μg) from the tail tissues of transgenic and nontransgenic mice were digested using *EcoRI*, and the digestion products were electrophoresed on a 0.8% agarose gel using TAE buffer and were transferred onto Hybond-N membranes (Invitrogen). Southern blotting analysis was performed using a 277-bp LR-specific hybridization probe that was labeled with digoxigenin (DIG) using a PCR DIG Probe Synthesis kit (Roche Applied Sciences). The PCR parameters were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s and 58°C for 30 s, 72°C for 1 min, followed by a final extension step of 72°C for 7 min. The sequences of the hybridization probe primers were as follows:

Upstream, 5'-GAAGACCGTTGACATGGAAT-3'

Downstream, 5'-AGCCATAGCCTCCTTCACTT-3'

Hybridization and immunological detection were carried out according to the instructions provided with the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Applied Sciences). The positive hybridization signal was a 3-kb fragment. Genomic DNA from nontransgenic mice was used as a negative control. The pBluescript II SK(+)-LR plasmid DNA (3.4 kb) was used as a positive control. The

copy number of the transgene was quantified using Quantity One software (Bio-Rad).

Analysis of LR expression using reverse transcriptase-PCR

Three lactating females (in lactation day 5) and three males of each transgenic line were sacrificed and tissue samples were collected from mammary gland, muscle, heart, liver, lung, spleen, kidney, and ovary/testis to determine LR mRNA expression. Total RNA was extracted using TriReagent (TaKaRa). To generate the cDNA template for PCR, RNA pellets were resuspended in DEPC-water and were treated with 10 units of DNase I (Promega) to remove DNA contamination, after which cDNA was synthesized using an oligo-dT primer and the SuperScript reverse transcriptase according to the manufacturer's instructions (TaKaRa). Reverse transcriptase-PCR (RT-PCR) primers were designed based on the LR coding sequences. The primers amplified the LR coding region and the length of the PCR product obtained was 376 bp. *GAPDH* was used as a control. The primers used were as follows:

LR gene: Upstream, 5'- GCGAGCTCATGAAGTTCTTCAT CTTTAC -3', Downstream, 5'- GCCTCGAGCTATTATTAG GGAAGGGCATTTTC -3'; *GAPDH*: Upstream, 5'- ACGTGCC GCCTGGAGAAACC -3', Downstream, 5'- GGCCATGAGG TCCACCACCTG -3'.

Analysis of LR distribution in mammary gland by in situ hybridization

The amplification of *LR* fragments (277 bp) was performed using *LR*-specific primers. The amplified *LR* fragments (277 bp) were recovered from the 0.8% agarose gel and were cloned into the pGEM-T plasmid (pGEM-T Vector System 1; Promega). The orientation of the *LR* fragment in pGEM-T plasmid was determined by PCR using a combination of T7-, SP6-, and *LR*-specific primers. The identity of the cloned *LR* fragment was verified by sequencing. To prepare templates for labeling, the *LR*-containing plasmid was amplified using primers for T7 and S6. DIG-labeled antisense and sense cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche Diagnostics GmbH). The sequences of the hybridization probe primers used were as follows:

Upstream, 5'- GAAGACCGTTGACATGGAAT -3'

Downstream, 5'- AGCCATAGCCTCCTTCACTT -3'

Frozen sections of mammary gland tissues from a lactating female on lactation day 5 were mounted on 3-aminopropyltriethoxy-silane-coated slides and were fixed in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS). The sections were washed twice with PBS, incubated in 1% Triton-100 for 20 min, and washed three times in PBS. After pre-hybridization in a solution containing 50% formamide and 5×SSC (0.15 M sodium chloride and 0.015 M sodium citrate) at room temperature for 15 min, the sections were hybridized at 55°C for 16 h in hybridization buffer (5×SSC, 50% formamide, 0.02% bovine serum albumin [BSA], 250 mg/mL yeast tRNA, 10% dextran sulfate, and 1 mg/mL denatured DIG-labeled antisense or sense RNA probe for *LR*). After hybridization, the sections were washed sequentially in 50% formamide/5×SSC at 55°C for 15 min, 50% formamide/2×SSC at 55°C for 30 min, 50% formamide/0.2×SSC at 55°C twice for 30 min each, and 0.2×SSC at room temperature for 5 min. After nonspecific binding was blocked using a 1% blocking reagent (Roche) for 1 h, the sections were incubated in alkaline phosphatase-conjugated sheep anti-DIG antibody (1:5000; Roche) in 1% blocking reagent overnight at 4°C. The signal was visualized using 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitroblue tetrazolium in a buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂. Endogenous alkaline phosphatase activity was inhibited using 2 mM levamisole. The sections were counterstained with 1% methyl green in 0.12 M glacial acetic acid and 0.08 M sodium acetate for 30 min. The positive signal was a dark brown color.

Collection of mouse milk

Milking of the transgenic and nontransgenic female mice was performed beginning on day 5 after the birth of the pups until day 19. In general, mice may be milked once every other day; pups must remain healthy to maintain the mother's lactation cycle. Mice were separated from their litters for at least 3 h before the planned milking time to allow milk to accumulate. Just before milking the mother was stimulated

for milk ejection by a hypodermic injection of 5 IU oxytocin. The hormone takes effect within 1 to 5 min after injection. A modified human breast pump was used to collect mouse milk as previously described (DiTullio *et al.*, 1992).

Analysis of the expression of the LR protein by Western blotting

The *LR* protein expressed in 293T cells by using the transfection of pcDNA3.1-*LR* vector served as a positive control. The *LR* gene was cloned into the *Xho*I restriction site of the expression vector pcDNA3.1 to generate expression vector pcDNA3.1-*LR*. 293T cells were transfected with pcDNA3.1-*LR* vectors using Lipofectamine 2000 Reagent (Invitrogen). The transfectants were incubated at 37°C for 48 h and lysed with extraction buffer followed by Western blotting analysis.

Milk samples were diluted (1:20) with distilled water and 4×protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Loading Buffer (TaKaRa), followed by centrifugation (10,000 g for 10 min at 4°C) to remove the fat layer and insoluble precipitates. Heat-denatured samples were separated by Tricine-SDS-PAGE (Schagger, 2006) and were stained with Coomassie blue.

In Western blotting analysis, the milk samples from non-transgenic mice were used as negative controls, the *LR*-transfected 293T whole-cell lysates were used as positive controls and the mouse β -casein served as the internal control. The diluted milk samples were centrifuged at 3,000 g for 5 min at 4°C and separated by 12% Glycine-SDS-PAGE. Gels were transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen) using transfer buffer (15% methanol, 200 mM glycine, and 25 mM Tris) at 10 V for 10 min for *LR* and 10 V for 20 min for β -casein using a BioRad semi-dry blotting system. Membranes were blocked using 5% skim milk in PBS with 0.1% Tween 20 (PBS-T) for 2 h with gentle agitation. Blots were probed overnight with a primary antibody at 4°C with gentle agitation. The mouse anti-*LR* monoclonal antibody (antigen epitope: YLKTVYQHQQ, designed and produced by Abmart Company) and mouse anti- β -casein monoclonal antibody (H-4, sc-166530; Santa Cruz Biotechnology, Inc.) was used as the primary antibody and diluted 1:500 in PBS-T containing 1% BSA. After several washes, blots were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG second antibody diluted to 1:5000. The protein bands were visualized using enhanced chemiluminescence detection reagents. For rennet coagulation (Ausar *et al.*, 2001) milk samples were incubated 15 min at 37°C with 0.01 U of rennet/mL. Then, samples were centrifuged at 5,000 g for 10 min to separate the precipitate (casein) and the supernatants (whey). The milk sample from the same transgenic mouse was used as a whole milk control. 12% SDS-PAGE and Western blotting analysis were performed to detect if the *LR* protein secreted in the whey or a fraction of it is associated with the casein micelles.

Determination of lysine content in transgenic milk

Different generations (F_0 , F_1 , and F_2) of the 0, 4, and 10 transgenic lines were selected for the analysis of lysine content in the first lactation period. Four nontransgenic mice from generations F_1 and F_2 were used as controls. Milk samples were collected from mice every other day beginning on day 5 during their natural lactation period. Amino acid

quantitation was performed using a Hitachi L-8800 Amino Acid Analyzer by Ginseng and Antler Products Center of the Ministry of Agriculture PRC. Levels of lysine were analyzed with software SPSS 17.0. All experimental data are expressed as the mean value $\pm$ standard deviation (SD). A difference between two means was considered to be statistically significant based on $p < 0.05$ (*).

Pup weight gains measurement and statistical analysis

A total 92 pups from transgenic line 10 and 25 pups from wild-type offspring were used in this study. Mothers were 8–10 weeks old and weighed 24–26 g (first lactation). To ensure accuracy of the study, the litter size was adjusted to 8–10 pups for each mother on postnatal day 3 while maintaining as close to a 1:1 sex ratio as possible for each group. Individual pups in each litter were weighed daily for the first 3 weeks at 9:00 a.m. from third day after birth. Weight data were analyzed with software SPSS 17.0. All experimental data were expressed as the mean value $\pm$ standard deviation (SD). A difference between two means was considered statistically significant when $p < 0.05$.

Results

Generation of transgenic mice that express the LR gene

The mammary gland-specific expression vector pBC1-LR-NEO^r containing the LR coding region and the neomycin selection marker (Neo^r) was used (Fig. 1A). The linearized expression vector containing the transgene was purified and microinjected into 823 zygotes. Surviving zygotes (712) were then transferred to 25 recipients. The recipients delivered 19 pups, among which three transgenic founder (0, 4, 10) animals were identified by multiplex PCR analysis (Fig. 2A) and were confirmed by Southern blotting analysis (Fig. 2B). The results show that the LR transgene was integrated into the genome of the three transgenic mice. The efficiency of integration of the transgene was 15.8%. Of the three transgenic founders, two were female and one was male. These founders were backcrossed with wild-type mice to produce the F₁ generation and were screened using multiplex PCR. The three mice (0, 4, and 10) transmitted the LR transgene to their offspring at ratios of 37.50% (3/8), 46.15% (6/13), and 48.27% (14/29), suggesting that the transgene was inherited in a Mendelian fashion (Table 1).

Southern blotting analysis

The integrity of the transgene and its copy number was determined by Southern blot analysis using *Eco*RI digestion, which made a double cut in pBC1-LR-NEO^r, resulting in the

FIG. 2. The selection of transgenic founders. **(A)** Multiplex polymerase chain reaction (PCR) for the selection of transgenic founders. M: DNA ladder 2000; 1: LR 187 (187 bp); 2: β -actin (342 bp); 3: NEO^r gene (438 bp); 4: β -casein promoter (899 bp); 5,6,7: transgenic founders 0, 4, 10; 8: nontransgenic mouse; 9: positive control of plasmid pBC1-LR-NEO^r; 10: blank control. **(B)** Southern blotting analysis of LR transgenic mice. Genomic DNA (20 μ g per lane) was isolated from mouse-tail tissue and was digested with *Eco*RI. The bands were hybridized to a 277-bp specific probe that was complementary to a fragment of the LR gene. Lanes 5, 25, and 50: positive controls (pBluescript II SK(+)-LR, 3 Kb) containing 5, 25, and 50 copies, respectively. N: genomic DNA from a nontransgenic mouse; 1, 2, 3: genomic DNA from three transgenic founders 10, 4, and 0.

generation of a 3.4-kb fragment. To evaluate the copy number of the transgene, a series of positive controls with different copy numbers was examined using a Southern blotting. Comparison of the band density of the transgenes from different transgenic mice with those of the positive controls allowed the estimation of the copy number of the transgenes in LR transgenic mice. Southern blotting analysis revealed that the transgene founders carried different numbers of copies of the LR gene in their genomes (2, 5, and 20 copies were found in the 0, 4, and 10 founder mice, respectively) (Fig. 2B).

Expression of LR mRNA in the mammary gland of transgenic mice

The expression of the LR mRNA was detected by RT-PCR. The LR mRNA was restricted to the mammary gland in female transgenic mice and ectopic expression was not

TABLE 1. LYSINE LEVEL IN THE MILK OF TRANSGENIC MICE

Transgenic lines (sex)	Transgene copy no.	Transmission rate (%) ^a	Lysine level in milk (g/100 g) ^b	Generation (no. of mice) ^c
0 (♂)	2	37.50% (3/8)	0.718 $\pm$ 0.018	F ₁ (n = 2), F ₂ (n = 3)
4 (♀)	5	46.15% (6/13)	0.822 $\pm$ 0.035	F ₀ (n = 1), F ₁ (n = 1), F ₂ (n = 2)
10 (♀)	20	48.27% (14/29)	0.966 $\pm$ 0.062	F ₀ (n = 1), F ₁ (n = 2), F ₂ (n = 2)

^aTransmission rate (%) = the number of positive F₁ transgenic pups/total number of transgenic F₁ offspring.

^bLysine concentrations were determined using the Hitachi L-8800 Amino Acid Analyzer.

^cLR expression and lysine concentrations in the milk were measured in different generations (F₀, F₁, and F₂) in the first lactation period.

detected (Fig. 3A). Direct sequencing of the RT-PCR products confirmed 100% homology with the *LR* gene that we designed. However, in the male mice, a low level of ectopic expression was detected in the testes of transgenic line 4 (Fig. 3B), but ectopic expression was not detected in transgenic line 0 and 10 (Fig. 3C).

LR mRNA expression in lactation of the mammary gland

Whole mount analyses showed that the mammary epithelium of transgenic animals (Fig. 4B, C) isolated at the lactation day 5 and day 19 exhibited distended alveolar lumina that were filled with milk containing fat droplets, and the alveolar walls were thin with barely discernable nuclei, which was similar in extent to that observed in control glands (Fig. 4A).

In situ hybridization was performed in the mammary gland to examine the tissue and cellular distribution of *LR* mRNA. The mammary gland of a wild-type mouse was used as a negative control. There were no detectable hybridization messages in the negative control (Fig. 4A). When a DIG-labeled antisense probe for the *LR* mRNA was used in transgenic mouse, we found that the *LR* mRNA was mainly localized in active alveoli (Fig. 4B, C). There was no difference in the morphology of lactating mammary glands between transgenic and wild-type mice. *In situ* hybridization experiments have shown that *LR* mRNA is expressed predominantly in the active or mature alveoli. Thus, it is likely that the *LR* protein is correctly targeted, it undergoes normal secretion in mammary gland epithelial cells, and it is not toxic to the mammary gland.

High-level expression of LR protein in the milk of transgenic mice

To determine the production of the recombinant *LR* protein and to compare the secretion efficiency of the *LR* protein among the different transgenic mouse lines, milk was col-

lected from three transgenic lines of female progenies of the F_1 generation during the first lactation. These mice were chosen for further examination of the expression of the transgene. Milk of nontransgenic mice served as a negative control, the vector pcDNA3.1-*LR*-transfected 293T cell lysates served as a positive control. Proteins from the negative control, the positive control, and transgenic mice milk were separated by 12% SDS-PAGE. A band with a molecular weight of ~14.02 kDa for transgenic lines 10 and 4 indicated the high-level expression of the *LR* protein in their milk (Fig. 5A). Western blotting analysis was carried out to detect the *LR* protein. A 14.02-kDa protein band appeared only in the milk collected from the transgenic mice and had the similar size with positive control using an *LR*-specific monoclonal antibody. No such band was detected in the milk collected from control mice (Fig. 5B, N). Protein bands generated from transgenic lines 10, 4, and 0 of the F_1 generation had different intensities (Fig. 5B, lanes 1–3). The results suggest that the female progeny of two transgenic lines (10 and 4) produced a significant amount of *LR* protein in their milk, whereas the expression level in the other transgenic line (0) was low (Fig. 5B, lane 3). Western blotting analysis revealed that the expression of the *LR* protein varied among the three transgenic lines. In addition, there was no significant decline in *LR* expression among F_0 , F_1 , and F_2 generations of transgenic mice (Fig. 5C). To determine if the *LR* protein secreted in the whey or a fraction of casein micelles, the transgenic mouse milk caseins were precipitated by rennet coagulation. The proteins that appeared in the precipitate and the supernatant were analyzed by 12% SDS-PAGE to show the protein composition in both fractions (Fig. 5D). These results indicate that caseins are the major component in the precipitate (Fig. 5D, lane 3), whereas the whey proteins remain soluble (Fig. 5D, lane 2). The results of Western blotting showed that there was no significant difference in band intensity between supernatants (Fig. 5E, lane 2) and whole milk control (Fig. 5E,

FIG. 3. Expression profile of *LR*-specific mRNA detected by reverse transcriptase-PCR in mammary glands and diverse tissues. (A) Transgenic mice from line 10. (B) Transgenic mice from line 4. (C) Transgenic mice from line 0. Female transgenic mice in lactation day 5. 1: heart; 2: liver; 3: spleen; 4: lung; 5: kidney; 6: muscle; 7: ovary; 8: mammary gland; 9: nontransgenic control (mammary gland); 10: positive control; 11: negative control (water). Male transgenic mice. 1: heart; 2: liver; 3: spleen; 4: lung; 5: kidney; 6: muscle; 7: testis; 8: nontransgenic control (testis); 9: positive control; 10: negative control (water).

FIG. 4. *In situ* hybridization of the *LR* gene in the mammary gland tissue from normal mice (A) and transgenic mice in the lactating stage day 5 (B) and day 19 (C). No hybridization messages were observed in the nontransgenic control (A). *LR* mRNA was mainly localized in active alveoli (B, C). ▼ indicates positive messages in mammary gland alveoli. Scale bar represents 20 μ m.

lane 1), whereas the precipitate (Fig. 5E, lane 3) had no band. These observations indicated that the LR protein secretes in the whey. To express LR protein as a positive control, 293T cells were transfected with the pcDNA3.1-LR vector. A 14.02-kDa protein band was specifically detected by Western blotting analysis (Fig. 5F).

Determination of the lysine content in transgenic milk

To determine if the expression of the LR protein in milk from the transgenic mice led to changes in its lysine content, a complete amino acid analysis was performed by Amino Acid Analyzer on milk collected from the three transgenic lines in different generations (as shown in Table 1). Milk harvested from nontransgenic mice during the lactating stage was used as a negative control ($n=4$; the mean lysine level is 0.704 ± 0.023 g/100 g). Statistically significant changes in the lysine levels of the milk from lines 10 ($n=5$; the mean lysine level is 0.966 ± 0.062 g/100 g) and 4 ($n=5$; the mean lysine level is 0.822 ± 0.035 g/100 g) were observed (Fig. 6A). However, no such significant changes were observed in the milk from line 0 ($n=5$; the mean lysine level is 0.7181 ± 0.018 g/100 g). The level of lysine was significantly higher in transgenic lines 10 and 4 than in the negative controls ($p < 0.05$). Of these, transgenic mice 10–45 (F_2 generation in the first lactation period from line 10) have the highest lysine level, up to 1.057 g/100 g (Fig. 6C), increased by 50.1%, compared with nontransgenic control (Fig. 6B).

Growth performance of transgenic pups monitored by daily weight gains

The effect of the lysine-rich milk on the preweaning growth performance of transgenic mouse pups during the nursing stage was measured by monitoring their daily weight gain. The weights of total 92 pups from transgenic line 10 including two generations (F_2 and F_3) were measured. After identified by PCR analysis, mice were divided into two groups: group 1 (56 transgenic mice) and group 2 (36 nontransgenic mice), all of them suckled transgenic milk. The 25 pups from wild-type offspring (nontransgenic mice) suckled normal milk (group 3) were used as a control to evaluate growth performance. Individual pups in each litter were weighed daily during the nursing period. The results show that the growth rate of pups from the transgenic line 10 (group 1) and the negative mice from the

transgenic line 10 (group 2) increased significantly by 13.2% and 11.1% ($p < 0.05$), respectively, compared with the growth rate of the normal pups (Fig. 7). There were no significant differences observed between group 1 and group 2. The results suggest that this increase in growth performance was due to the lysine-enriched milk and not the transgenic mice themselves.

Discussion

Improvement of milk production has been performed for centuries and achieved by genetic selection and by optimizing lactating female alimentation. Modifying milk composition (e.g., increase the levels of essential amino acids) may also be obtained to some degree by using the same techniques. However, the major impact of genetic selection so far concerns the amount of milk rather than its composition. Nutrition accounts for perhaps 50% of the variation in milk protein content. Several recent studies already exist on the potential to modify milk protein concentrations and yields nutritionally (DePeters and Cant, 1992; Xu *et al.*, 1998; Vyas and Erdman, 2009; Wang *et al.*, 2010; Appuhamy *et al.*, 2011). These findings suggest that it is possible to increase the total levels of essential amino acids by improving efficiency of milk protein synthesis using proper feeding strategies, but there is little ability to alter single amino acid content and its percentages, for example, lysine. Modification of milk composition may be carried out for different reasons by transgenic technology. It may be useful to remove allergenic proteins and to reduce lactose concentration (Jost *et al.*, 1999; Vilotte, 2002). Some alleles of milk protein genes may be added to improve curd quality or to increase total milk nutritional value (Brophy *et al.*, 2003; Yang *et al.*, 2008). Milk may also be a carrier to provide consumers with anti-pathogen molecules (Soler *et al.*, 2005) or with high-quality lipids (Kao *et al.*, 2006). Milk is also a possible source of pharmaceutical proteins. All these domains are currently being investigated. In this study, we report the expression and characterization of LR proteins in the milk of transgenic mice using a goat β -casein gene promoter to express the LR protein in the mammary glands of lactating animals and to increase the level of lysine in their milk. This study is the first step toward the ultimate goal of improving the nutritional value of bovine milk through the expression of lysine-rich proteins in the milk.

FIG. 5. Analysis of LR protein secretion in the milk of transgenic mice. **(A)** Analysis by Tricine-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the LR protein in transgenic milk, stained with Coomassie blue. PC (positive control): LR-transfected 293T whole-cell lysates; N (negative control): milk protein harvested from nontransgenic mice during the lactating stage; lanes 1–3: milk proteins from different lines of transgenic mice, 10–16, 4–15, and 0–17, respectively; M: protein molecular marker. **(B)** Western blotting analysis of LR protein using a mouse anti-LR monoclonal antibody. PC (positive control): LR-transfected 293T whole-cell lysates; N (negative control): milk protein harvested from nontransgenic mice during the lactating stage; lanes 1–3: milk proteins from different lines of transgenic mice, 10–16, 4–15, and 0–17, respectively; M: protein molecular marker; β -casein in mouse milk served as a loading control. **(C)** F_0 , F_1 , and F_2 generations of transgenic mice from line 10; β -casein served as a loading control. **(D)** Analysis by Tricine-SDS-PAGE of the proteins that appear in the precipitate and the supernatant of rennet coagulated transgenic milk (10–45, F_2 generation in the first lactation period from line 10) and its control (the equal mount whole transgenic milk); M: protein molecular marker; N: nontransgenic mouse milk control; 1: transgenic whole milk control; 2: the supernatant (whey fraction); 3: the precipitate (casein fraction). **(E)** Western blotting analysis to detect LR protein in the supernatant (whey) and precipitate (casein). M: protein molecular marker; N: nontransgenic mouse milk control; 1: transgenic whole milk control; 2: the supernatant (whey fraction); 3: the precipitate (casein fraction); β -casein in mouse milk served as a loading control, LR-transfected 293T whole-cell lysates served as a positive control. **(F)** LR protein expressed in 293T cells transfected with pcDNA3.1-LR. The LR protein was expressed in the transfected 293T cells. The nontransfected 293T cells served as negative controls. β -actin served as a loading control.

We have generated transgenic mice expressing lysine-rich protein in their milk using mammary gland-specific expression vector pBC1. First, transgene is under the control of the goat β -casein promoter (Roberts *et al.*, 1992; Persuy *et al.*, 1995), which is derived from a milk-specific gene, and was included in the construct to direct the efficient expression of the LR transgene in the mouse mammary gland. Other studies have shown that the goat β -casein promoter can efficiently direct the high expression of a foreign gene in the milk of transgenic animals, such as human serum albumin (up to 40 g/L) in bovine milk (Echelard *et al.*, 2009), lysozyme

(up to 1.45 g/L) in transgenic mice (Yu *et al.*, 2006), and lactoferrin (2.5 g/L and 3.4 g/L) in two transgenic cows (Yang *et al.*, 2008). The levels of recombinant protein expression reported in those studies were high, suggesting that the goat β -casein gene promoter is a successful expression system. Second, naturally occurring codons of milk proteins were used. The genetic code is redundant in most amino acids using multiple codons. In many organisms, codon usage is biased toward particular codons (Shah and Gilchrist, 2011). Moreover, genes are selectively expressed in several tissues: such genes exhibit characteristic codon usage that, in

FIG. 6. Amino acid analysis of different transgenic mice lines. (A) Levels of lysine in the milk of different transgenic mice lines (0, $n=5$; 4, $n=4$; 10, $n=5$) and nontransgenic mice (N, $n=4$). Lysine concentrations in the milk were measured for different generations (F_0 , F_1 , and F_2) in the first lactation period. The lysine content in the 2 transgenic lines (lines 10 and 4) was significantly higher ($p<0.05$) than that of the nontransgenic controls, $*p<0.05$. (B) L-8800 AAA system manager report: levels of lysine in the milk from nontransgenic mouse (lysine level was 0.667 g/100 g); (C). L-8800 AAA system manager report: levels of lysine in the milk from 10–45 (F_2 generation in the first lactation period from line 10; lysine level was 1.057 g/100 g). ▼ indicates peak quantitation of lysine.

many cases, distinguishes the genes expressed in one tissue from those expressed in another. An implication of this result is that major codons are allocated for the group of genes with high expression levels to allow efficient translation of the mRNA, and that genes with low expression levels use minor codons to avoid competition with highly expressed genes. All above predict that codon-usage bias should be higher in genes expressed at high levels (Bulmer, 1991; Akashi, 1996; Eyre-Walker, 1996). In this study, cow milk predominant proteins casein alpha-s2, casein beta, and lactotransferrin were found for the selection of the lysine-rich peptides whose codons usage is biased by bovine mammary gland to obtain a high expression level of the LR protein in milk. The corresponding cDNA sequences of the lysine-rich peptides were fused together to form the LR cDNA to construct transgene expression vector. Finally, the bovine β -casein signal peptide DNA sequence was used in our study to enhance the expression of the LR gene. Different signal peptides affect the expression efficiency of recombinant proteins in different expression systems (Doud *et al.*, 1993; Izard *et al.*, 1995). The β -casein signal peptide was chosen because it is mammary specific and β -casein is highly conserved between goats and cattle. Yu *et al.* (2006) made a similar observation in a transgenic mouse model that utilizes the plasmids pBC-sighLY (includes the bovine β -casein signal peptide) and pBC-hLY (no bovine β -casein signal peptide) where the expression of rHLY was remarkably enhanced in pBC-sighLY transgenic mice.

Transgenic animals show differences in transgene copy numbers (Nakanishi *et al.*, 2002; Yang *et al.*, 2008). The copy number of the transgene in the transgenic mice may affect the expression level of the LR gene (Reichenstein *et al.*, 2001; Chandler *et al.*, 2007; Kong *et al.*, 2009). In random transfections, the transgene usually integrates into the host genome in a tandem fashion (Kong *et al.*, 2009). High-copy-number tandem integration is thought to lead to transgene silencing. After examining the LR expression levels in the different lines of transgenic mice that contained various numbers of

FIG. 7. Effects of LR protein in the milk of transgenic mice on body weight gain during the lactation period. Growth curve of pups during the lactation period among three different groups: group 1, positive transgenic mice from transgenic line 10 ($n=56$, transgenic mice suckling transgenic milk); group 2, negative siblings of group 1 ($n=36$, nontransgenic mice suckling transgenic milk); group 3, wild-type mice ($n=25$, suckling normal milk). The asterisks represent significant differences ($*p<0.05$). B. Enhanced growth performance of preweaning mice from group 1 and group 2 (mice suckling transgenic milk) compared with group 3 (the nontransgenic suckling normal milk). Values are expressed in relative growth performance taking the group 3 as 100%. The asterisks represent significant differences ($*p<0.05$).

copies of the *LR* transgene, we found that high-copy-number groups of transgenic lines (e.g., transgenic lines 10) continued to show high expression levels, whereas the expression levels in the middle-copy-number and low-copy-number groups of transgenic mice were relatively low (Table 1). This position-independent, copy number-dependent expression may be caused by the use of the commercial expression vector pBC1, which was intentionally designed to avoid position effect of transgene by inducing two tandem copies of the chicken β -globin insulator. This well-characterized insulator contains silencers that prevent cross talk between genes of neighboring loci, chromatin openers to give the transcription machinery free access to transgenes and enhancers (Recillas-Targa *et al.*, 2002). In addition to their ability to guard against position effects, insulators appear to have other favorable properties applicable to transgenesis. One possibility, though not well explored, is that insulators may contribute to the integration event to make this process more efficient. Insulators may also stabilize the transgene avoiding deletions or recombination. This may have been a relevant factor in a transgenic study observed by O'Malley (Wang *et al.*, 1997), where no founder mice were obtained for an inducible transgene in the absence of insulators. In contrast, when the same transgene was shielded with two copies of β -globin insulator, four founder mice were obtained, with three of them showing high and constant levels of expression. In our study, the three founder transgenic mouse lines appeared to stably inherit the transgene through three generations and were used in a breeding program to follow the inheritance and expression of the transgene from generation to generation (Table 1). Western blotting (Fig. 5C) revealed that there was no significant decline in *LR* protein expression among F_0 , F_1 , and F_2 generations of transgenic mice.

The expression of the *LR* protein that was detected in the milk varied among the three different transgenic lines according to the results of Western blotting and lysine analysis. A variation in the levels of transgene expression among different generations has been observed in several transgenic studies (Yu *et al.*, 2006; Wu *et al.*, 2007; Shi *et al.*, 2009) and has been postulated to be partly due to a variation in the chromosomal location where transgenes are integrated into the genome and to differences in copy number (Ye and Signer, 1996). It is possible that in some lines, the transgene integrated near hypermethylated chromosomal regions, which can result in reduced transgene expression (Zhang *et al.*, 2009). This implies that insulators may still be subject to some position effects. Although chicken β -globin insulator can function to reduce some position effects after transgene integration, these sequences fail to exert complete dominance (Walters *et al.*, 1999; Potts *et al.*, 2000).

Our results indicate that there was no ectopic expression of the *LR* gene in the heart, liver, spleen, lung, kidney, muscle, or ovary in lactating female transgenic mice. Transgene expression was largely confined to the mammary gland of lactating transgenic mice. However, a small amount of *LR* mRNA was detected in the testis of transgenic males (from line 4). It is possible that this occurs because the testis is the male gonad, which responds to at least some of the hormones that stimulate milk protein synthesis in the mammary gland (Hatakeyama *et al.*, 1988). Therefore, the expression of milk protein genes in the male gonad may reflect the pres-

ence of similar *trans*-acting factors in this tissue and the mammary gland (Whitelaw *et al.*, 1992).

The objective of this work was to increase the level of lysine in milk by expressing an *LR* protein in the mammary gland of transgenic mice. Two transgenic lines, 10 and 4, displayed consistently higher lysine levels in their milk (0.785–1.057 g/100 g) when amino acid composition analysis was carried out. The increase of lysine in transgenic lines 10 and 4 ranged from 11.5% to 50.1%, compared with nontransgenic control. These results suggest that the expression of *LR* proteins in milk is a potentially useful strategy to improve milk quality.

Several studies have shown that lysine promotes the growth of neonatal animals. Our transgenic mouse model demonstrates that the growth rate of the pups was enhanced by directly feeding them the transgenic milk containing a high level of lysine (Fig. 7). Furthermore, enhanced growth performance was observed in nontransgenic mice suckling lysine-rich milk.

In conclusion, we have successfully produced transgenic mice capable of expressing and secreting recombinant lysine-rich proteins in the whey of their milk, and confirmed the relatively stable inheritance and efficient expression of the *LR* gene in the three transgenic generations. In addition, the transgenic mouse milk with a high-level lysine content is beneficial to the growth of mice pups as suggested by a feeding experiment using *LR* milk from transgenic mice. Finally, we demonstrate the feasibility of improving the lysine content of milk by the introduction of a foreign protein, thus providing a valuable way to enrich the human diet in essential amino acids, especially in lysine.

Acknowledgments

This work was supported by grants from National Transgenic Animal Program (No.2009ZX08007-004B), National Basic Research Program (No. 2009CB941001), and National Natural Science Foundation (No. 31072027) in China.

Disclosure Statement

No competing financial interests exist.

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Received for publication December 27, 2011; received in revised form April 2, 2012; accepted April 9, 2012.