Expression and Bioactivity of Recombinant Human Lysozyme in the Milk of Transgenic Mice

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ABSTRACT

Human milk lysozyme is an important protein for innate immunity, but human breast milk is a fairly poor source for commercial production of this enzyme. Research on the expression of recombinant human lysozyme (rHlys) is therefore potentially valuable to the dairy industry. In this study, 2 different kinds of transgenic mice, PBC-hLY and PBC-sighLY, were generated and used as system models to express rHlys. Six lines of PBC-hLY transgenic mice with human lysozyme genomic DNA-based constructs were generated, and a maximum expression level of rHlys approaching 0.154 mg/mL was achieved. Antibacterial activity of the whey from PBC-hLY female transgenic mice was determined by a turbidimetric assay. Results showed that antibacterial activity of the whey was strongly enhanced, and confirmed that rHlys retained full activity. For rHlys to be secreted efficiently into the milk of transgenic mice, 5 lines of mice were also generated, in which the signal peptide DNA of bovine β -casein was substituted for that of lysozyme in PBC-hLY transgenic mice. Compared with PBC-hLY transgenic mice, both the expression levels of rHlys and the antibacterial activity of the whey were much higher in the PBC-sighLY transgenic mice. The concentration of rHlys in one of these mice amounted to 1.405 mg/mL—3 times higher than the level in human whey. The antibacterial activity of the whey was also 3 times higher than that of human whey. The rHlys from both PBC-hLY and PBC-sighLY transgenic mice had the same antibacterial activity as human milk lysozyme. The effect of the signal peptide and copy numbers of the transgene on expression of rHlys was also evaluated. This work will certainly permit a better understanding of how mammary gland bioreactor systems can be applied to produce rHlys in other mammals, such as cattle.

Key words: human lysozyme, whey, antibacterial activity, transgenic mice

INTRODUCTION

Milk provides the neonate not only with nutrients but also with a host of defense factors such as antibacterial, anti-inflammatory, and immunomodulatory agents (Goldman and Goldblum, 1995). Lysozyme is one of the most important antibacterial factors in milk, because it hydrolyzes the glycosidic β -(1-4) linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan polymer in the bacterial cell wall (Imoto et al., 1972). The lysozyme content and its physicochemical and enzymatic properties vary widely in the milk of different species. Human and equine milks are very rich in lysozyme (Chandan et al., 1964; Jauregui-Adell, 1975), whereas the milk of many other species contains low concentrations (Chandan et al., 1965; McKenzie and White, 1986; Elagamy et al., 1996). Among various species, the specific activities of lysozyme also differ because they possess different charges. Human milk lysozyme has about 3 times more lytic activity than that of egg white lysozyme because it possesses a greater positive charge than the latter (Parry et al., 1969). Bovine (Chandan et al., 1965) and horse (Bell et al., 1981) milk lysozymes possess even less positive charge than egg white lysozyme and also have less lytic activity. The specific activity of human milk lysozyme is about 10 times greater than that of bovine lysozyme. Therefore, human milk lysozyme plays an important role in host defenses. In human milk, the role of lysozyme in reducing microbial infections in the gastrointestinal tract of breast-fed infants has been extensively studied (Lönnerdal, 1985). Most gram-positive bacteria and a few gram-negative bacteria are damaged by human milk lysozyme, which helps to increase the levels of beneficial microorganisms in infants and strengthen their disease resistance. When lysozyme was added to infant formula, formula-fed infants showed reduced incidences of gastroenteritis and allergies and an increase in beneficial microflora in the gastrointestinal tract (Francis, 1980). The Food and Agriculture Organization/World Health Organization and many countries such as Australia, Belgium, Denmark, Finland, France, Germany, Italy, Japan, Spain, and the United Kingdom have acknowledged the nontoxicity of lysozyme and have approved its use in some foods and in pharmacological

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and therapeutic applications (Cunningham et al., 1991). However, because the source of human lysozyme is limited, research identifying recombinant sources of human lysozyme could be very beneficial. The first research on transgenic mice expressing human lysozyme was conducted by Maga et al. (1994). In their experiment, the cDNA sequence of human lysozyme was expressed using the α_{s1} -casein promoter. Recombinant human lysozyme (**rHlys**) mRNA was found in the mammary gland tissue of transgenic mice, and the milk of the transgenic mice exhibited antibacterial activity (Maga and Anderson, 1995). However, many studies have shown that the levels of gene expression obtained with genomic DNA-based constructs are generally higher than those obtained with cDNA-based constructs (Brinster et al., 1988; Palmiter et al., 1991), and other research has indicated that the signal peptide is associated with the secretion efficiency of recombinant proteins (Doud et al., 1993; Xiong et al., 2003). Therefore, as a first step toward the ultimate goal of improving the antibacterial properties of bovine milk through creating a mammary gland lysozyme bioreactor, 2 different kinds of transgenic mice containing human lysozyme genomic DNA-based constructs with different signal peptide DNA were respectively established as system models.

MATERIALS AND METHODS

Materials

Animals. Kunming white mice were purchased from the Beijing Laboratory Animal Center. The mice were kept under conditions of regulated temperature (22 to 25°C), humidity (40 to 50%), and illumination cycles (14 h light, 10 h dark) and were provided with food and water ad libitum.

Bacteria. TOP10 was used to clone pBC-hLY and pBC-sighLY expression, and *Micrococcus lysodeikticus* was used to check lysozyme activity.

Plasmids. The pBC1 expression vector and the pPCR-XL-TOPO vector were supplied by Invitrogen (Carlsbad, CA), and the bacterial artificial chromosome RP11-1143G9 was from Genentech Inc. (San Francisco, CA).

Molecular Biology Reagents. The following restriction enzymes were used: *NotI* and *SalI* (New England BioLabs Int., Herts, UK), *XhoI* (Promega, Madison, WI), *Csp45I* (TaKaRa Bio Inc., Otsu, Shiga, Japan), and *HindIII* (Huamei Biotechnology Co., Beijing, P. R. China). The following reagents were also used: T4 DNA ligase (New England BioLabs), proteinase K and RNase (Sigma, St. Louis, MO), chicken lysozyme and human lysozyme antibody (Huamei), and α -p32-dCTP (Yahui Co., Beijing, P. R. China).

Construction of the rHlys Expression Vectors.

Two rHlys expression vectors, pBC-hLY and pBC-sighLY, were constructed. For construction of the pBC-hLY expression vector, to gain the human lysozyme genomic DNA sequence, we first amplified the genomic region by PCR with the bacterial artificial chromosome RP11-1143G9 as the template. The forward primer was 5' CCC TCG AGA CTC TGA CCT AGC AGT CAA C 3' and the reverse primer was 5' CCG CTC GAG TCT GTT TCT ATC ATT TGG 3'. The reaction conditions were as follows: an initial denaturing step of 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 68°C for 5 min, followed by a final extension step of 68°C for 10 min, and 4°C for ∞ . The length of the resulting PCR product was 5,652 bp, incorporating the signal peptide DNA sequence and coding sequence (part of exon 1, intron 1, exon 2, intron 2, exon 3, intron 3, and part of exon 4) and a stop codon.

Second, the PCR product was cloned into the pPCR-XL-TOPO vector and was confirmed by sequencing as being the human lysozyme.

Third, the PCR product was cloned into the *XhoI* restriction site of the pBC1 expression vector (Invitrogen) using conventional molecular cloning techniques, and creation of pBC-hLY was confirmed by sequencing. The pBC-hLY expression vector contained the human lysozyme genomic DNA with its signal peptide DNA, the goat β -casein promoter, the 3' genomic sequence, exon 1, intron 1, part of exon 2 (before the initial codon), exon 7, intron 7, exon 8, intron 8, and exon 9 of goat β -casein and 2 copies of chicken β -globin insulator, as shown in Figure 1A.

The pBC-sighLY expression vector was then constructed with the aim of increasing the efficiency of secretion of rHlys in the milk of transgenic mice. The only difference from pBC-hLY was that the signal peptide DNA of bovine β -casein substituted for that of lysozyme (Figure 1B). We chose the β -casein signal peptide because β -casein is highly expressed in the mammary gland and is secreted efficiently into the milk. A second reason for choosing the β -casein signal peptide was that goat β -casein promoter was used to express rHlys in the pBC-sighLY mouse model, although the signal peptide sequences of the different milk proteins are quite similar. β -Casein is highly conserved between goats and cattle. Therefore, the mammary-specific goat β -casein promoter and the bovine β -casein signal peptide should be effective for directing transgene expression in the mammary gland.

First, the bovine β -casein signal peptide DNA sequence was synthesized (indicated by the boldfaced, underlined text) as follows: the forward sequence was 5' TCGAGATGA **AGGTCCTCAT CCTTGCCTGC** **CTGGTGGCTC TGGCCCTTGC** AAAGGTCTT 3',

Figure 1. Diagrammatic representation of (A) the pBC-hLY expression vector and (B) the pBC-sighLY expression vector. 2x globin insulator = 2 copies of chicken β -globin insulator; Pcasein = goat β -casein promoter; Casein E1-E2 = goat β -Casein exon1, intron1, part of exon 2 (before initial codon); hLY encoding region = human lysozyme encoding region; Casein E7-E8-E9 = goat β -Casein exon 7, intron 7, exon 8, intron 8, and exon 9; Casein 3genome DNA = goat β -casein 3' genomic region; sigpeptide DNA sequence = bovine β -casein signal peptide DNA sequence.

and the reverse sequence was 5' C GAAGACCTTT GCAAGGGCCA GAGCCACCAG GCAGGCAAGG ATGAGGA CCT TCATC 3'. The signal peptide DNA was then cloned into the site of pGEM-7zf between *XhoI* and *Csp45 I*, and this plasmid was named p7zf-sig.

Second, the human lysozyme genomic DNA was amplified by PCR. The forward primer was 5' ATT CGA AAG GTG TGA GTT GGC CAG AAC TCT G 3', and the reverse primer was 5' TAA GCT TCT CGA GAC CAT CCT GGC TAA CAC G 3'. The PCR conditions were: an initial denaturing step of 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 68°C for 5 min, followed by a final extension step

of 68°C for 14 min and 4°C for ∞ . The length of the PCR product was 5,255 bp, incorporating the coding sequence (part of exon 1, intron 1, exon 2, intron 2, exon 3, intron 3, and part of exon 4) and the stop codon but not the signal peptide DNA region. The PCR product was cloned into the pPCR-XL-TOPO vector and was confirmed by sequencing. It was then cloned into the site between the *HindIII* and *Csp45 I* sites of p7zf-sig, generating the p7zf-sighLY plasmid.

Third, the 5-kb DNA fragment containing the β -casein signal peptide DNA sequence and the human lysozyme DNA sequence was separated from p7zf-sighLY by digestion with *XhoI*. The insert was then cloned into the *XhoI* restriction site of the pBC1 expression vector, producing the pBC-sighLY expression vector, as confirmed by sequencing.

Generation of Transgenic Mice

After the pBC-hLY expression vector and pBC-sighLY expression vector plasmids digested by *NotI* and *SalI*, the 25-kb inserts were isolated and recovered by electroelution. To remove any contamination, the products were spot dialyzed against 40 mL of Tris-EDTA (10:0.1) for 30 min (VSWP02500 membrane; Millipore, Milford, MA). Purified DNA was diluted to 2 to 3 ng/ μ L in TE buffer (10:0.1) and microinjected into the pronuclei of fertilized Kunming White $\times$ Balb/C eggs. The injected eggs were transplanted into the oviduct of pseudo-pregnant mice.

PCR and Southern Blot Analysis

Chromosomal DNA was isolated from the tails of offspring. Transgenic mice were detected by PCR. The forward primer was 5' TTA TAC ACA CGG CTT TAC 3', and the reverse primer was 5' CAG CAT CAG CGA TGT TAT CT 3'. The reaction conditions were an initial denaturing step of 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 54°C for 40 s, and 72°C for 40 s, followed by a final extension step of 72°C for 7 min and 4°C for 1 h (30 cycles). The length of the PCR product obtained was 637 bp (spanning intron 2 and exon 3).

Ten micrograms of genomic DNA digested by *PstI* was used to detect transgenic mice. Nontransgenic mouse genomic DNA was used as a negative control and pBC-hLY vector DNA was used as a positive control. An α -p32-dCTP-labeled probe (from the former PCR product) was generated and used as the probe in a Southern blot. The positive hybridization signal was a 5-kb fragment.

Three separate positive controls of pBC-hLY or pBC-sighLY vectors containing different copies of the hLY gene were used to estimate the number of copies of the transgene in the transgenic mice by comparing the band

density of the positive control with that of the transgenic mice.

Western Blot Analysis

Milk from all viable female founder mice (i.e., I-1, I-3, I-4, II-2, II-4, and II-5) was collected at d 7 of lactation as previously described (Kim et al., 1999). To remove the fat fractions, milk samples were centrifuged at $3,000 \times g$ for 15 min. Liquids from the lower layer were then adjusted to pH 3.8 to 4.6 by 1 M HCl to eliminate the casein fraction. After electrophoresis on a 12.5% SDS-PAGE gel, the proteins were transferred to a nitrocellulose extra-blotting membrane (Sartorius, Goettingen, Germany). Polyclonal rabbit anti-Hly antibody (Bioscience International, Saco, ME) and horseradish peroxidase-conjugated goat antirabbit-IgG (Sino-American Co., Beijing, China) were used to detect rHlys.

Quantification of rHlys by Radioimmunoassay

For the radioimmunoassay, rHlys (Sigma) was radio-labeled with ^{125}I by the chloramine-T method (Liu et al., 2004). Competition binding was carried out at 4°C overnight in protein binding buffer (0.5% BSA, 0.1% NaN_3) using a polyclonal rabbit anti-hLY antibody. Separation of bound and free antibody was achieved by the polyethylene glycol-double antibody method, followed by centrifugation. The radioactivity was then measured using an automatic γ counter.

Assays for Lysozyme Activity

The cell wall of *M. lysodeikticus* is composed of peptidoglycan polymer, in which the glycosidic β -(1-4) bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine are easily degraded by lysozyme (Hemelt et al., 1979), so *M. lysodeikticus* is very sensitive to lysozyme. Therefore, the enzymatic activity of lysozyme is routinely determined by monitoring the reduction in turbidity of a suspension of *M. lysodeikticus* cells at 450 nm (Shugar, 1952; Nilsen et al., 1999; Huang et al., 2002). In this study, 90 μL of an optical density₄₅₀ = 1.0 *M. lysodeikticus* cell suspension was made in 1 mM of Tris-Cl buffer, pH 6.24. The cell suspension was equilibrated at room temperature and the reaction was initiated by adding 10 μL of samples containing chicken lysozyme with concentrations of 50, 75, 100, 125, 150, 175, and 200 U/mL. Recombinant human lysozyme activity was determined in the kinetic mode for 1 min at 450 nm. The activity of rHlys was calculated relative to a standard curve for native human lysozyme, and the rHlys activity of the whey was calculated according to the regression equation deduced from the standard curve.

Figure 2. Identification of (A) pBC-hLY and (B) pBC-sighLY transgenic mice by Southern blot. M = 1 kb DNA ladder; 10, 5, 1 in A represent 10, 5, 1 copies of pBC-hLY plasmid, respectively; 5, 3, 1 in B represent 5, 3, 1 copies of pBC-sighLY plasmid, respectively; ck = wild type; I-1, I-2, I-3, I-4, I-5, I-6 indicate no. 1, 2, 3, 4, 5, 6 pBC-hLY transgenic mouse, respectively; II-1, II-2, II-3, II-4, II-5 indicate no. 1, 2, 3, 4, 5 pBC-sighLY transgenic mouse, respectively.

RESULTS

Generation of Transgenic Mice

Transgenic mice were generated by standard microinjection (Hogan et al., 1980). For pBC-hLY, a total of 6 transgenic mice (4 females and 2 males) were identified from 83 mice by PCR and were further confirmed as containing the transgene construct by Southern blot analysis (Figure 2A). The efficiency of integration of the transgene was 7.2%. For pBC-sighLY, 5 transgenic mice (3 females and 2 males) were generated from 63 mice (Figure 2B). The efficiency of integration of the transgene was 7.9%.

To evaluate the copy number of the transgene, we performed a series of positive controls with different copy numbers in Southern blot. By comparing the band density of the transgenes from different transgenic mice with those of the positive controls, we estimated the copy number of the transgene in pBC-hLY and pBC-sighLY transgenic mice. We found that most of the transgenic mice had only a few copies (1 to 3), whereas some had high copy numbers (≥ 5), which is similar to the results by Liu et al. (2004).

All founder lines except the I-2 founder (which died before maturation) were back-crossed with Kunming

White mice to produce the F₁ generation. All offspring were screened by PCR. Detection of the transgene in F₁ mice indicated that the transgene could be transmitted from the founder transgenic mice to their offspring in a Mendelian manner (Table 1). The transmission rates in some lines, such as lines I-6, II-5, and II-3, were greater than 50%, which may have been due to multiple insertions. The transmission rate was predicted to be 75% if the transgene was inserted in 2 separate nonhomologous chromosomes, as could have been the case for lines I-6 (82.4%) and II-5 (76.9%). The 100% transmission rate in II-3 could have been due to the transgene being inserted into 2 homologous chromosomes, because the F₁ offspring of II-3 included male and female mice.

Detection and Quantification of rHlys

Recombinant human lysozyme was observed in the milk whey of most pBC-hLY and pBC-sighLY female transgenic mice by Western blot analysis (Figure 3A, 3B). The concentration of rHlys was then quantified by radioimmunoassay (Table 1). In pBC-hLY transgenic mice, the level of rHlys in the milk of founder I-1 was 0.154 mg/mL and that of founder I-3 was 0.062 mg/mL, whereas that of founder I-4 was too low to detect. In pBC-sighLY transgenic mice, the levels of rHlys in founders II-4 and II-5 transgenic mice were 1.405 and 0.959 mg/mL, respectively, whereas that of founder II-2 was negligible. The expression levels of rHlys for founders II-4 and II-5 pBC-sighLY transgenic mice were, respectively, 3 and 2 times greater than the concentration of lysozyme in human milk of 0.412 mg/mL. In general, the expression level of rHlys for pBC-sighLY female transgenic mice was much higher than in pBC-hLY transgenic mice.

Assays for rHlys Activity

To detect the activity of rHlys, a standard curve was established using human native lysozyme to lyse *M. lysodeiktitus*. One minute of lysis was plotted for a range of different lysozyme concentrations. One unit of lysozyme activity was defined as the change in unit absorbance per minute per milliliter of reaction mixture at 450 nm. Based on the standard curve, a regression equation was deduced as $y = 0.0005x - 0.002$, where y is the difference of optical density₄₅₀ and x is lysozyme activity (U/mL); $r = 0.9936$. Rearranging the formula gave $x = 2000 (y + 0.002)$. During preparation, the whey was diluted 3 times, so the formula for calculating lysozyme activity in the whey was as follows: $x = 2000 (y + 0.002) \times 3$.

We observed that antibacterial activity of the whey from pBC-hLY and pBC-sighLY female transgenic mice

Table 1. Comparison between pBC-hLY and pBC-sighLY transgenic mice¹

Founder line	pBC-hLY transgenic mice					pBC-sighLY transgenic mice					Human	
	I-1	I-2	I-3	I-4	I-5	I-6	II-1	II-2	II-3	II-4		II-5
Sex	♀	♀	♀	♀	♂	♂	♂	♀	♂	♀	♀	
Copy number	3	1	2	2	3	8	1	3	>30	>30	10	
Expression level of rHlys, mg/mL	0.154 ± 0.032	—	0.062 ± 0.013	0.002	—	—	—	0.006	—	1.405 ± 0.047	0.959 ± 0.039	0.412* ± 0.025
Antibacterial activity, U/mL	480.4 ± 61.08	—	301.6 ± 79.8	37.9 ± 41.4	—	—	—	52.3 ± 27.72	—	3,806.3 ± 462	2,737.5 ± 165	1,224.0 ± 478.2
Specific activity, U/mg	3,116.8	—	—	—	—	—	—	—	—	2,709.1	2,854.0	2,970.9
F1 mice	8	8	2	4	5	17	8	8	10	5	13	
Positive	3	—	2	2	1	14	3	4	10	1	10	
Inheritance rate, %	37.5	—	25	50	20	82.4	37.5	50	100	20	76.9	

¹The dash (—) indicates that data were not determined for the following mice: 1) Mouse I-2, which died of natural causes before checking; 2) mice I-5, I-6, II-1, and II-3, which were males; and c) mice I-3, I-4, and II-2, for which the rHlys expression levels were quite low. The asterisk (*) indicates human lysozyme.

Figure 3. Western blot analysis of the whey from (A) pBC-hLY and (B) pBC-sighLY female transgenic mice. ck = milk from wild type mouse; human = human milk; I-1, I-3, I-4 indicate milk from no. 1, 3, 4 pBC-hLY transgenic mouse, respectively; II-2, II-4, II-5 indicate milk from no. 2, 4, 5 pBC-sighLY mouse, respectively.

was strongly enhanced because of the expression of rHlys. The antibacterial activity of the whey from pBC-hLY female transgenic mice is shown in Table 1. The antibacterial activity of the I-1 transgenic mouse was 480.4 U/mL; of I-3 was 301.6 U/mL; and of I-4 was 37.9 U/mL, whereas that of the nontransgenic mouse and human were 25.9 and 1,224.0 U/mL, respectively. Those of the I-1 and I-3 transgenic mice were, respectively, 18 and 11 times greater than that of the nontransgenic mouse. The antibacterial activity of whey from mouse I-1 was 0.4 times greater than that in humans. Moreover, the antibacterial activity of whey from the pBC-sighLY transgenic mice increased more than that of the pBC-hLY mouse (Table 1). The antibacterial activity levels of the whey for the II-2, II-4, and II-5 transgenic mice were 52.3, 3806.3, and 2,737.5 U/mL, respectively, whereas those of the nontransgenic mouse and human were 25.9 and 1,224.0 U/mL, respectively. The antibacterial activity levels of II-4 and II-5 were 147 and 105 times as high as that in the nontransgenic mouse. Compared with the whey of humans, the antibacterial activity levels of the II-4 and II-5 transgenic mice were 3 and 2 times higher. The antibacterial activity levels of whey from the pBC-sighLY female transgenic mice were generally higher than those of pBC-hLY female transgenic mice, which was consistent with the increased expression levels of rHlys. These results showed that the expression of rHlys may have increased

the antibacterial activity of the whey in transgenic mice and confirmed that rHlys is biologically active.

The activity was also calculated in units per milligram (Table 1). The antibacterial activity and concentration of human lysozyme are 1,224.0 and 0.412 mg/mL, respectively, so the specific activity of human lysozyme should be 2,970.9 U/mg. The antibacterial activity in the whey of transgenic mice was mostly due to human lysozyme because mouse lysozyme is virtually inactive (Obita et al., 2003). Therefore, the specific activity of rHlys for the I-1 transgenic mouse was 3,116.8 U/mg. The specific activities of rHlys for the II-4 and II-5 transgenic mice were 2,709.1 and 2,854.0 U/mL, respectively. These results indicate that rHlys had the same specific activity as human lysozyme.

DISCUSSION

Human lysozyme is an important enzyme in breast milk because of its gentle but powerful role in preventing infections. Transgenically cloned farm animals would be a good source of milk containing highly active human lysozyme, which could be used to prevent bacterial and viral infections in weaned children. Milk containing rHlys could be used directly as food for humans, or indirectly as a source of purified human lysozyme for pharmacological and therapeutic applications. Before generating a transgenically cloned cow, we first generated the pBC-hLY and pBC-sighLY mouse models. In this study, we generated the model systems pBC-hLY and pBC-sighLY, 2 kinds of transgenic mice expressing rHlys in the mammary gland. Recombinant human lysozyme was highly expressed in the whey of both transgenic mice, and in 2 cases it even exceeded the lysozyme level in human milk. The lysozyme activity of the whey was markedly increased and exceeded that of human whey. These results suggest that it is feasible to produce milk containing fully active human lysozyme via a genetically engineered cow.

The transgenic mouse model expressing rHlys was first established by Maga et al. (1994). This model was a cDNA-based construct under the control of the α_{s1} -casein promoter (Maga et al., 1994; Maga and Anderson, 1995), in which the expression level of humans varied from 0.25 to 0.71 mg/mL and the specific activity of rHlys was about half that of human lysozyme. Strikingly, the specific activity levels of both pBC-hLY and pBC-sighLY were almost the same as that of human lysozyme. The specific activity of rHlys was similar to that of buffalo milk lysozyme (Parry et al., 1969), was 10 times that of bovine milk lysozyme (White et al., 1988), and was about 3 times that of egg white lysozyme, which is the main commercial lysozyme (Parry et al., 1969). The expression level of pBC-hLY was simi-

lar to that reported by Maga et al. (1994), but the expression level of pBC-sighLY was much higher. Although some results have shown that the levels of gene expression obtained with genomic DNA-based constructs are generally higher than those obtained with cDNA-based constructs (Brinster et al., 1988; Palmiter et al., 1991) and that the goat β -casein promoter can efficiently direct high expression of a foreign gene in the milk of transgenic mice (Roberts et al., 1992; Persuy et al., 1995), no advantage was found in pBC-hLY transgenic mice. Interestingly, the expression of rHlys in pBC-sighLY transgenic mice was remarkably enhanced. This may be for 2 reasons. First, the β -casein signal peptide may enhance rHlys expression. One possibility to explain this point is that the β -casein signal peptide is mammary specific. The other possibility is that the β -casein signal peptide has a favorable amino acid composition, which promotes secretion of rHlys. The idealized core regions of the different signal peptides contain different proportions of Leu and Ala residues, which effectively produce hydrophobicities above and below the threshold level required for efficient secretion (Izard et al., 1995). Doud (1993) observed that if the ratio of Leu and Ala is equal to 6:4, the signal peptide functions most efficiently. In our study, the human lysozyme signal peptide and the bovine β -casein signal peptide, respectively, were used in the pBC-hLY and pBC-sighLY transgenic mice. The ratio of Leu and Ala for the human lysozyme signal peptide was 5:1, whereas that of bovine β -casein was 5:4. The data indicate that the signal peptide of bovine β -casein was more efficient for secreting rHlys. Therefore, our results indicate that different signal peptides affected the expression efficiency of recombinant proteins. Second, the copy number of the transgene in the pBC-hLY and pBC-sighLY transgenic mice may correlate positively with the expression level of rHlys. Based on the data for pBC-hLY and pBC-sighLY (I-1, I-3, II-4, and II-5), a logarithmic regression analysis was performed between the transgene copy number and the lysozyme expression level, and R^2 was 0.9856, indicating a strong positive correlation between them. This result supported the view of Reichenstein et al. (2001) that the expression efficiency of a transgene depends on its integration copy numbers, and it is contrary to the conclusion of Thepot et al. (1995) that the copy number of the transgene does not affect its expression efficiency. In addition, we found that not every transgenic founder mouse in both types of transgenic mice expressed rHlys effectively. The chromosomal integration site may still affect the expression pattern of the transgene, although chicken β -globin insulator was used to prevent position-effect variegation in these 2 systems.

In conclusion, our results show that pBC-hLY and pBC-sighLY transgenic mice can efficiently express the fully active rHlys in the mammary gland, suggesting the feasibility of producing human lysozyme using transgenic farm animals. Compared with the pBC-hLY transgenic mice, the expression levels of rHlys in some lines of pBC-sighLY mice were much higher. This result indicates that the β -casein signal peptide or copy number of the transgene affects the expression level of rHlys in the mammary gland.

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