J. Biochem. 126, 320-325 (1999)

High-Level Expression of Human Lactoferrin in Milk of Transgenic Mice Using Genomic Lactoferrin Sequence[1]

Sun Jung Kim,* Bo Hwa Sohn,† Sangkyun Jeong,* Kyung-Won Pak,† Jung-Sun Park,† In-Young Park,† Tae-Hoon Lee,* Young-Hee Choi,† Chul-Sang Lee,† Yong-Mahn Han,† Dae-Yeul Yu,* and Kyung-Kwang Lee†,[2]

*Animal Molecular Physiology Research Unit and †Animal Developmental Biology Research Unit, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea

Received March 19, 1999; accepted May 25, 1999

In our previous study, transgenic mice were generated that expressed human lactoferrin (hLF) in milk using cDNA under control of the 2 kb bovine β-casein promoter. The expression level of the protein in milk of 7 mice ranged from 1 to 200 μg/ml; 1 to 34 μg/ml in 6 mice and 200 μg/ml in 1 mouse. With the aim of inducing higher expression of the protein, we constructed an expression cassette comprised of 10 kb of the bovine β-casein gene promoter and the hLF genomic sequence in place of the cDNA. The hLF genomic sequence of about 27 kb, spanning 23 kb of the entire coding region and 4 kb of the 3′-flanking sequence, was placed downstream the bovine β-casein promoter. In total, 8 transgenic mice were generated from 31 mice (transgenic rate of 25.8%) born from the embryos microinjected with the 40-kb hLF expression cassette. Mammary-specific expression of the transgene was addressed by performing Northern hybridization of the total RNAs from various tissues of transgenic mice. Immunoblot analysis showed that the recombinant protein expressed in milk has the same molecular weight as the native protein. The amount of the protein in milk of 5 mice ranged from 60 to 6,600 μg/ml when judged by ELISA analysis. Three mice expressed the protein at the level higher than 500 μg/ml. These data suggest that the genomic lactoferrin sequence represents a valuable element for the efficient expression of the protein in milk of transgenic animals.

Key words: bovine β-casein gene, human lactoferrin gene, mammary gland, milk, transgenic mice.

Transgenic animals are useful tools for the production of human proteins (*1*) as well as for the study of genes and their functions (*2*). Many useful human proteins such as growth hormone (*3*), protein C (*4*), α1-antitrypsin (*5*), serum albumin (*6*), and urokinase (*7*) were properly expressed in milk of transgenic animals. Milk gene promoters including α_{S1}-casein (*7*), β-casein (*3*), β-lactoglobulin (*5*), WAP (*4*), and α-lactalbumin gene (*8*) from various animal species were used in order to direct synthesis of the desired proteins to the mammary gland. Among these, sheep β-lactoglobulin, goat β-casein, and bovine α_{S1}-casein promoters are the most efficient at supporting good levels of heterologous protein expression (*1*). The bovine β-casein gene promoter adopted in this study also successfully directed synthesis of some foreign proteins such as human growth hormone (*3*) and lysozyme (*9*).

When cDNA encoding the protein was used it induced sufficient expression levels in some cases. For example, fusion genes comprised of mouse WAP promoter and human protein C cDNA (*10*), and bovine α_{S1}-casein promoter and human IGF-1 cDNA induced expression of the corresponding proteins at high levels in the order of g/liter (*11*). However, in many cases, the cDNA did not express the proteins at the desired level (*12, 13*). A few methods were developed to circumvent the lower expression. First, when a genomic sequence was adopted instead of the cDNA, it usually drove much higher level of the proteins. For example, genomic sequence of the human serum albumin gene expressed the protein at the level of 2.5 mg/ml, while almost no expression was detected when cDNA was used (*14*). Secondly, minigenes that have only a part of the introns also induced higher expression. For example, mice and sheep carrying minigenes composed of genomic sequences of human α1-antitrypsin behind the BLG promoter secreted high levels of the corresponding proteins, although almost no expression was detected in the mammary gland of transgenic mice carrying the cDNA of the gene (*15*). Thirdly, matrix attachment elements (MARs), locus control regions (LCRs), or YAC clones have been used to confer position-independent and high-level expression of

[1] This work was supported by research funds (HS2160 and NM1030) from the Ministry of Science and Technology of Korea.

[2] To whom correspondence should be addressed. Tel: +82-42-860-4424, Fax: +82-42-860-4608, E-mail: leekk@kribb4680.kribb.re.kr

Abbreviations: bLF, bovine lactoferrin; BLG, β-lactoglobulin; ELISA, enzyme-linked immunosorbent assay; IGF-1, insulin-like growth factor-1; hGH, human growth hormone; hLF, human lactoferrin; mLF, murine lactoferrin; RT-PCR, reverse transcription-polymerase chain reaction; UTR, untranslated region; WAP, whey acidic protein; YAC, yeast artificial chromosome.

proteins (*16-18*). These studies imply that there are some *cis*-acting sequences that are responsible for the higher expression of the proteins. The *cis*-acting elements could be enhancer elements where transcriptional factors bind and thereby produce the transcripts efficiently, or they may have essential elements that are needed for the stability of the transcribed message.

Human lactoferrin (hLF) is an 80-kDa iron-binding glycoprotein that is expressed in high concentration in milk and in lesser amount in the secondary or specific granules of neutrophils and in plasma (*19*). LF is classically considered to be related to the binding, transport, and storage of iron. It has a bacteriostatic activity through its strong ability to chelate the iron essential for microbial growth, and many other potential functions, such as regulation of myelopoiesis (*20*) and inflammatory-immune response (*21*), production of reactive oxygen metabolites (*22*), growth factor activity (*23*), DNA-binding activity (*24*), and potent RNase activity (*25*). It is also found in plaques of Alzheimer disease (*26*). These multi-functional properties of hLF invoke a need to produce the protein in large amounts for its potent nutritional or therapeutic application.

Transgenic mice that can express human lactoferrin in their milk were reported in studies by Platenburg *et al.* (*27*) and in our previous studies (*28, 29*). In both cases about 2.5 kb of cDNA for the hLF was used under control of either bovine α_{S1}-casein or bovine β-casein gene promoter and the expression level ranged from 0.1 to 36 and 1 to 200 μg/ml, respectively. This relatively poor performance of cDNA in directing the protein synthesis drove us to develop a more efficient expression system. In this study, we present a detailed analysis of transgenic mice harboring an hLF expression vector comprised of 10 kb of the bovine β-casein promoter and 30 kb of the hLF gene. The resulting transgenic mice expressed hLF specifically in the mammary gland with the highest expression level up to 6,600 μg/ml.

MATERIALS AND METHODS

Construction of the hLF Expression Vector—The genomic sequence of hLF gene used for the construction of the expression vector has been recently cloned from a cosmid library in our laboratory (*30*). The cosmid clone harboring the hLF gene was digested with *Sac*II and *Not*I, and the larger 27-kb fragment, which spans the entire hLF gene except for exon 1 and part of intron 1, was purified by electroelution. The missing 700 bp DNA fragment of the hLF gene was prepared separately by PCR amplification using primers 1 and 2 (Table I) from the human chromosomal DNA. Primer 1 has a *Bsr*I site at its 5′-end, which is compatible with the *Sac*II site. Following Kozak's rule (*31*), the primer was designed to have a purine residue (G) at the −3 nucleotide position from the translation initiation codon, ATG, when it was ligated into the promoter. The resulting PCR products were digested with *Bsr*I and *Not*I, and ligated to the *Sac*II site of the 10-kb 5′-flanking sequence of the bovine β-casein gene. Above two DNA fragments, hLF and bovine β-casein, were ligated together with the *Not*I digested pWE cosmid vector (Promega), *in vitro* packaged with packaging extract (Promega), and infected into *Escherichia coli* DH5α. Positive cosmid clones were screened by PCR analysis using primer sets 1, 2 and 3, 4 and restriction mapping, and the sequences of the junctions were confirmed by sequencing.

Generation of Transgenic Mice—The hLF expression cassette for microinjection was prepared by digestion of the recombinant cosmid DNA with *Not*I and purified by passing through a 0.6% agarose gel and an Elutip column (Schleischer & Schuell). The 40-kb DNA fragment was microinjected into the male pronuclei of the BCF1 (C57BL/6×CBA) mouse embryos, and the embryos were transferred into foster mothers following the methods described previously (*32*). The chromosomal DNAs from ear and/or nail tissues of the founder mice and their pups were isolated and analyzed for the determination of transgene copy number and transgenicity. Two sets of primers were used to screen transgenic animals: one set (intragenic primers, primers 3 and 4) can amplify a 400-bp DNA fragment of intron 3 of the hLF gene; the other set (intergenic primers, primers 5 and 6) was designed to amplify a 300-bp DNA fragment when the transgenes were integrated into the mouse genome in a concatemeric head-to-tail fashion. Copy numbers were determined by Southern blot hybridization of the chromosomal DNA with the 400-bp intron 3 of the hLF gene.

Northern Blot Analysis—Total RNA from various organs of transgenic or normal mice at day 10 of lactation was purified using Trizol (GibcoBRL) following the supplier's protocol. Total RNA (30 μg) was separated on 1.0% agarose-formaldehyde gels and transferred to Nylon membranes (Boehringer Mannheim). The blots were probed with a 35-mer oligonucleotde (primer 7 in Table I) whose sequence was complementary to the exon 17 of the hLF

TABLE I. **Oligonucleotides used in this study.**

Oligonucleotide	Corresponding DNA	Sequence[a]
1	hLF exon 1	GCACTGGCCATGAAACTTGTCTTCCT
2	hLF intron 1	CAGCGGCCGCATTTAGGTCACTAAT
3	hLF exon 3	TGGTTTCATATACGAGG
4	hLF exon 4	CATCCAGCGGTCCTGCG
5	hLF 3′-flanking	CTCTAGGTTATATTGCTG
6	bovine β-casein 5′-flanking	TGGCAACCCACTTCAGTA
7	hLF exon 17	GCCATCTTCTTCGGTTTTACTTCCTGAGGAATTCA

Sequences of oligonucleotides 1, 3, and 5 are deduced from the sense strand, and those of oligonucleotides 2, 4, 6, and 7 are complementary to the sense strand. Sequences of oligonucleotides 1, 2, 3, 4, and 7 are adopted from GenBank database accession no. X53961 and S52659, and those of oligonucleotides 5 and 6 are designed after determining the sequences of 3′-end of the hLF gene (*27*) and the 5′-end of the *Sac*I site of the bovine β-casein gene promoter (Fig. 1), respectively. [a]The initiation and termination codons of the hLF gene are underlined.

gene and was labelled with [γ-^{32}P]ATP. Hybridization was performed in Quick-Hybridization solution (Clontech) for 1 h at 42°C. The filters were then washed twice with 2×SSC/0.1% SDS for 20 min at 42°C and exposed to X-ray films (Fuji) for 12-24 h at −70°C.

ELISA and Western Blot Analysis—Milk was collected at day 10 of lactation. Briefly, mice were separated from their pups for 3 h and anaesthetized using 20 μl of diluted (250 μl of stock solution in 10 ml of 0.85% NaCl) Avertin (Sigma) per gram of body weight. They were then injected intraperitoneally with 0.3 IU of oxytocin (Sigma). Milk samples were collected by massaging the inguinal glands gently 30 min after the oxytocin treatment. Milk to be assayed was diluted with 2 volumes of phosphate-buffered saline and centrifuged at 4°C for 30 min at 14,000×g to separate the whey, casein, and fat fractions. For the Western blot analysis, the whey fractions diluted with PBS buffer were mixed with electrophoresis sample buffer (2% SDS, 10% glycerol, 0.08 M Tris-HCl, pH 6.8, 2 mM EDTA, 0.1 M dithiothreitol, and 0.01% bromophenol blue) and denatured at 95°C for 10 min before loading onto an 8% denaturing polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Boehringer Mannheim). Polyclonal rabbit anti-hLF antiserum (Sigma) was used to detect hLF by the immunoblot analysis. Bound antibody was detected by addition of alkaline phosphatase-conjugated goat anti-rabbit-IgG (Sigma). For ELISA analysis, milk samples were diluted 1:1,000-10,000 and the hLF expression levels were determined using an hLF ELISA kit (Calbiochem) following the supplier's protocol.

RESULTS AND DISCUSSION

Generation of Transgenic Mice—The hLF transgene used in this study is about 37 kb long, and the entire length is prepared for microinjection as a single DNA fragment. It is comprised of 10 kb of the bovine β-casein gene promoter and 27 kb of hLF gene. The promoter contains the proximal promoter as well as the 5′-untranslated region. The 27-kb hLF gene originated from a cosmid clone (*30*), and the expression cassette was constructed in a cosmid vector due to its large size. So far, the length of transgene used for the generation of transgenic animals has usually ranged from less than 10 to 20 kb. Only a few cases have been reported of transgenes of larger than 20 kb. When the length is too long for easy manipulation, overlapping fragments were prepared and microinjected together into the embryos (*33*). In another case, YAC (yeast artificial chromosome) clones of up to about 250 kb were directly microinjected into the embryos, and transgenic mice were born with a production rate of 21% (*34*). The transgenic efficiency from the hLF genomic sequence reached up to 25.8%, which is similar to that achieved with cDNA (25%). Therefore, we concluded that the length of the transgene does not affect the production rate of transgenic mice when they are produced by pro-nuclear injection, and the transgene can be easily prepared for microinjection if it has the proper restriction sites at its ends. Eight transgenic mice were obtained from 31 mice analyzed by PCR. Two primer sets, one intragenic (primers 3 and 4) and the other (primers 5 and 6) intergenic (Fig. 1), amplified fragments of 400 and 300 bp, respectively, from the chromosomal DNAs of transgenic animals. All transgenic mice that were positive for the intragenic primers were also revealed to be positive for the intergenic primers, implying that the transgene integrated into the mouse chromosome in at least two copies. Five of the mice were female and three were male. All except one line, #35 (Table II), successfully transmitted their transgene to their progeny.

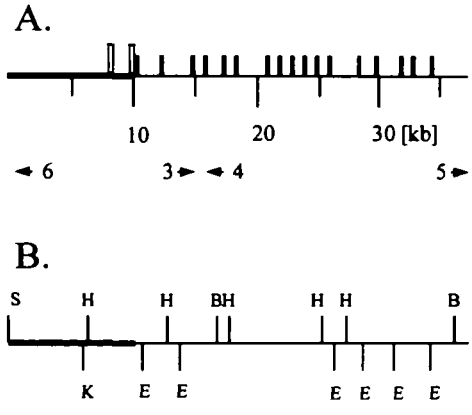

Fig. 1. **Schematic diagram of the hLF expression vector.** (A) A 37-kb hLF expression vector was constructed by combining 10 kb of the bovine β-casein promoter and 27 kb of the hLF gene, which were indicated by thick and thin horizontal lines, respectively. The promoter contains 8 kb of the 5′-flanking sequence, the untranslated exon 1 and 2 (vertical open boxes), and 2 kb of intron 1 of the bovine β-casein gene. The hLF gene contains all of the coding regions spanning from exon 1 to 17 (vertical black boxes) and 4 kb of the 3′-flanking sequences. Primers used for the screening of transgenic mice are denoted by arrows with the oligonucleotide number. (B) Restriction map of the expression vector. Restriction enzyme sites are abbreviated as: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sac*I.

Mammary Gland-Specific Expression of hLF—The mammary-specific expression of the transgene in the animal is a major concern when producing foreign proteins in milk of the animals. So far, many human proteins have been mammary-specifically expressed under the control of milk-specific promoters such as α_{s1}-casein, β-casein, α-lactalbumin, and β-lactoglobulin (*1*). However, in some cases the transgenes were also expressed in other tissues (*4, 6, 12*), even though this did not provoke any detrimental problems. Using the bovine β-casein gene promoter, lysozyme (*9*), hGH (*3*), and hLF (*28, 29*) were expressed in transgenic mice. In these cases, about 1.8 kb of the promoter was enough for the mammary-specific expression of the foreign genes. In our previous study, 2 kb of the bovine

TABLE II. **Profiles of the transgenic mice harboring the hLF transgene.**

Line	Sex of founder[a]	Copy number	Transmission[b] (%)	Expression level (μg/ml)
1	F	14	2/3 (67)	0
5	F	1	3/13 (25)	60
8	M	3	5/11 (45)	0
23	M	4	5/15 (33)	6,600
28	F	7	9/15 (60)	660
35	F	1	0/2 (0)	540
41	M	6	7/10 (70)	0
53	F	2	1/8 (13)	65

[a]The sex of animals is indicated by "F" for female and "M" for male. [b]The numbers are F1 transgenic animals per live births.

β-casein gene promoter directed the hLF expression to the mammary gland of transgenic mice (*29*). The 3′-side of the promoter ended in the middle of intron 1, 170 bp downstream of exon 1. In this study, 10 kb of the bovine β-casein promoter including the entire 2 kb of intron 1 and 5′-UTR of exon 2 was used.

Total RNAs of various organs from eight transgenic mice were analyzed by Northern hybridization. In five transgenic mice, the hLF was expressed in detectable amount, and in three mice it was not detected. Figure 2 shows the results for two transgenic mice. For the RNAs of the three mice that did not express the hLF RNA as judged by Northern blot analysis, RT-PCR was carried out and the result showed that the hLF message was synthesized. So, it is considered that the transgene is expressed in all transgenic mice, even though the expression level is variable due to the position effect (*35*). The transgene was expressed only in the mammary gland in all transgenic mice except the one that showed the highest expression level. In this mouse, the transgene was also expressed in lung, in much lower amount than in mammary gland. In general, we concluded that the transgene is expressed mammary-specifically by 10 kb of the bovine β-casein promoter. The expression of the correct-sized hLF RNA also implies that the recombinant transgene comprised of 19 exons was processed properly after transcription, like the native form, and could thereby synthesize the hLF protein in milk.

Expression of hLF Protein in Milk—The expression of the recombinant hLF protein in milk of transgenic mice was evaluated by immunoblot analysis after collecting the milk at day 10 of lactation. A polyclonal antibody raised against purified lactoferrin recognized a polypeptide that appeared only in the transgenic milk and that was approximately 80 kDa in size, like the purified hLF (Fig. 3). Among the milk samples, transgenic milk #23 showed the strongest color intensity. The bands corresponding to the recombinant hLF can also be seen on the Coomassie blue-stained SDS-PAGE gel (Fig. 3, lane Tg23), implying that the expression level of the protein in milk can reach up to several mg/ml.

The recombinant hLF expression level in milk of the transgenic mice was estimated by ELISA analysis after diluting the milk to 1:1,000-10,000 with PBS buffer. In five of eight milk samples from transgenic mice, hLF was detected and the expression level ranged from 60 to 6,600 μg/ml (Table II). The highest level, 6,600 μg/ml, represents an increase of more than 30-fold compared to that of cDNA, 200 μg/ml. When hLF cDNA was used, only one mouse expressed the protein at higher than 100 μg/ml and the others expressed it at 1 to 34 μg/ml. In the mice harboring the genomic sequence, three lines expressed the protein at the level of 540, 660, and 6,600 μg/ml, respectively. On average, the genomic sequence yielded expression levels higher by a factor of several tens than the cDNA.

When we generated transgenic mice harboring the 10-kb bovine β-casein gene/hLF cDNA expression cassette, the hLF expression level was not higher than 30 μg/ml (data not shown). Therefore, it could be considered that the genomic sequences of the hLF gene induced the elevated expression of hLF in the milk. In a few cases, it has been reported that in transgenic animals genomic sequences have shown higher expression of foreign proteins than cDNA. Minigenes with only partial introns also induced higher expression. However, in case of human erythropoietin (EPO), Korhonen *et al.* reported that higher expression was obtained using EPO cDNA than its genomic sequence (*36*). These results indicate that there might be elements in the intron sequences that can act as positive or negative elements, or elements related with the stability of RNA. However, it has not been shown whether there are

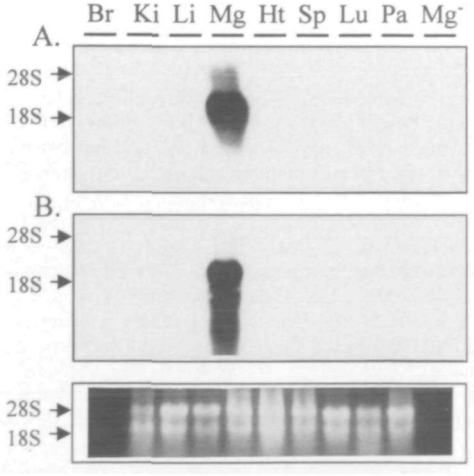

Fig. 2. **Northern blot analysis of the hLF transgene.** Total RNAs isolated from mouse tissues were size-fractionated on agarose gels, transferred to nylon membranes, and probed with an oligonucleotide (primer 7 in Table II) which can hybridize to exon 17 of the hLF gene. Tissues analyzed are brain (Br), kidney (Ki), liver (Li), mammary gland (Mg), heart (Ht), spleen (Sp), lung (Lu), pancreas (Pa), and mammary gland of a normal mouse (Mg^-). The RNAs are from transgenic mice, Tg#23 (A) and Tg#28 (B). Ethidium bromide staining of the gel for Tg#28 is shown at bottom to indicate loading.

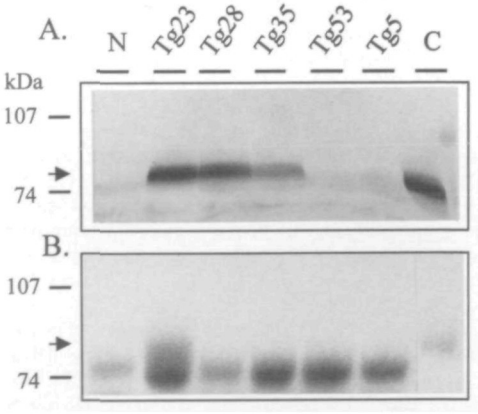

Fig. 3. **Immunoblot analysis of the hLF protein in milk of transgenic mice.** Whey fractions of the milk collected from the transgenic mice were loaded onto an 8% SDS-PAGE (B) and analyzed by immunoblotting (A). (A) A polyclonal anti-hLF antibody from rabbit serum and anti-IgG conjugated with alkaline phosphatase were used to detect the hLF. Lanes are: N, 1 μl of three-fold diluted normal whey; Tg23, Tg28, Tg35, Tg53, and Tg5, 1 μl of threefold diluted whey from each transgenic mouse line except for Tg23, in which 1 μl of twelve-fold diluted whey was loaded; C, 500 ng of purified hLF mixed with normal whey. (B) Coomassie brilliant blue staining of an SDS-PAGE gel. One microliter of the undiluted whey of transgenic mice and a normal mouse was loaded with the same loading order as in panel A. Lane C is 3 μg of purified hLF. The positions of the hLF are indicated as arrows and the band around 74 kDa is a major milk protein.

positive-acting elements in the introns of the hLF gene. Further studies with minigenes containing only part of the whole introns could help us to understand the positive-activity of introns of the hLF gene.

In conclusion, a hybrid transgene comprised of 10 kb of the bovine β-casein gene promoter and 27 kb of the hLF gene induced high levels of the recombinant protein in milk of transgenic mice with a mammary-specific pattern. Recently, we have generated transgenic cow harboring the hLF cDNA transgene (manuscript in preparation) and the genomic hLF expression vector could be used in place of the cDNA vector for the generation of large transgenic animals.

REFERENCES

1. Maga, E.A. and Murray, J.D. (1995) Mammary gland expression of transgenes and the potential for altering the properties of milk. *Bio/Technology* **13**, 1452-1457
2. Hatano, N., Eversole-Cire, P., Ferguson-Smith, A.C., Jones, P.A., Surani, M.A., and Sasaki, H. (1998) Enhancer-dependent, locus-wide regulation of the imprinted mouse insulin-like growth factor II gene. *J. Biochem.* **123**, 984-991
3. Ninomiya, T., Hirabayashi, M., Sagara, J., and Yuki, A. (1994) Functions of protein gene 5′-flanking regions on growth hormone gene. *Mol. Reprod. Dev.* **37**, 275-283
4. Wei, Y., Yarus, S., Greenberg, N.M., Whitsett, J., and Rosen, J.M. (1995) Production of human surfactant protein C in milk of transgenic mice. *Transgenic Res.* **4**, 232-240
5. Carver, A.S., Dalrymple, M.A., Wright, G., Cottom, D.S., Reeves, D.B., Gibdon, Y.H., Keenan, J.L., Barrass, J.D., Scott, A.R., Colman, A., and Garner, I. (1993) Transgenic livestock as bioreactors: stable expression of human α1-antitrypsin by a flock of sheep. *Bio/Technology* **11**, 1263-1270
6. Barash, I., Faerman, A., Ratovitsky, T., Puzis, R., Nathan, M., Hurwitz, D.R., and Shani, M. (1994) Ectopic expression of β-lactoglobulin/human serum albumin fusion genes in transgenic mice: hormonal regulation and in situ localization. *Transgenic Res.* **3**, 141-151
7. Meade, H., Gates, L., Lacy, E., and Lonberg, N. (1990) Bovine α_{s1}-casein gene sequences direct high level expression of active human urokinase in mouse milk. *Bio/Technology* **8**, 443-446
8. Soulier, S., Vilotte, J.L., Stinnakre, M.G., and Mercier, J.C. (1992) Expression analysis ruminant α-lactalbumin in transgenic mice: developmental regulation and general location of important *cis*-regulatory elements. *FEBS Lett.* **297**, 13-18
9. Lee, W.K., Kim, S.J., Hong, S., Lee, T.-H., Han, Y.-M., Yoo, O.J., Im, K.S., and Lee, K.-K. (1998) Expression of a bovine β-casein/human lysozyme fusion gene in the mammary gland of transgenic mice. *J. Biochem. Mol. Biol.* **31**, 413-417
10. Velander, W.H., Johnson, J.L., Page, R.L., Russel, C.G., Subramanian, A., Wilkins, T.D., Gwazdauskas, F.C., Pittius, C., and Drohan, W.N. (1992) High level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc. Natl. Acad. Sci. USA* **89**, 12003-12007
11. Brem, G., Hartl, P., Besenfelder, U., Wolf, E., Zinovieva, N., and Pfaller, R. (1994) Expression of synthetic cDNA sequences encoding human insulin-like growth factor. *Gene* **149**, 351-355
12. Hansson, L., Edlund, M., Edlund, A., Johansson, T., Marklund, S.L., Fromm, S., Stromqvist, M., and Tornell, J. (1994) Expression chracterization of biologically active human extracellular superoxide dismutase in milk of transgenic mice. *J. Biol. Chem.* **69**, 5358-5363
13. Velander, W.H., Page, R.L., Morcol, T., Russell, C.G., Canseco, R., Young, J.M., Drohan, W.N., Gwazdauskas, F.C., Wilkins, T.D., and Hohnson, J.L. (1992) Production of biologically active human protein C in the milk of transgenic mice. *Ann. N.Y. Acad. Sci.* **665**, 391-403
14. Shani, M., Barash, I., and Hurwitz, D.R. (1992) Expression of human serum albumin in the milk of transgenic mice. *Transgenic Res.* **1**, 195-208
15. Archibald, A.L., McClenaghan, M., Hornsey, V., Simons, J.P., and Clark, A.J. (1990) High-level expression of biologically active human α1-antitrypsin in the milk of transgenic mice. *Proc. Natl. Acad. Sci. USA* **87**, 5178-5182
16. Stief, A., Winter, D.M., Stratling, W.H., and Sippel, A.E. (1989) A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature* **341**, 343-345
17. Talbot, D., Collis, P., Antoniou, M., Vidal, M., Grosveld, F., and Greaves, D.R. (1989) A dominant control region from the human beta-globin locus conferring integration site-independent gene expression. *Nature* **338**, 352-355
18. Fujiwara, Y., Miwa, M., Takahashi, R., Hirabayashi, M., Suzuki, T., and Ueda, M. (1997) Position-independent and high level expression of human α-lactalbumin in the milk of transgenic rats carrying a 210-kb YAC DNA. *Mol. Reprod. Dev.* **47**, 157-163
19. Baggiolini, M., de Duve, C., Masson, P.L., and Heremans, J.F. (1970) Association of lactoferrin with specific granules in rabbit heterophil leukocytes. *J. Exp. Med.* **131**, 559-570
20. Yamada, Y., Amagasaki, T., Jacobsen, D.W., and Green, R. (1987) Lactoferrin binding by leukemic cell lines. *Blood* **70**, 264-270
21. Kulics, J. and Kijlstra, A. (1987) The effect of lactoferrin on complement mediated modulation of immune complex size. *Immunol. Lett.* **14**, 349-353
22. Klebanoff, S.J. and Waltersdorph, A.M. (1990) Peroxidant activity of transferrin and lactoferrin. *J. Exp. Med.* **172**, 1293-1303
23. Mazurier, J., Legrand, D., Lu, W.L., Montreuil, J., and Spik, G. (1989) Expression of human lactotransferrin receptors in phytohemagglutinin-stimulated human peripheral blood lymphocytes; isolation of the receptors by antiligand-affinity chromatography. *Eur. J. Biochem.* **179**, 481-487
24. He, J. and Furmansky, P. (1995) Sequence specificity and transcriptional activation in the binding of lactoferrin to DNA. *Nature* **373**, 721-724
25. Furmanski, P., Li, Z.-P., Fortuna, M.B., Swamy, Ch.V.B., and Das, M.R. (1989) Multiple molecular forms of human lactoferrin: identification of a class of lactoferrins that possess ribonuclease activity and lack iron-binding capacity. *J. Exp. Med.* **170**, 415-429
26. Kawamata, T., Tooyama, I., Yamada, T., Walker, D.G., and McGeer, P.L. (1993) Lactotransferrin immunocytochemistry in Alzheimer and normal human brain. *Am. J. Pathol.* **142**, 1574-1585
27. Platenberg, G.J., Kootwijk, E.P.A., Kooiman, P.M., Woloshuk, S.L., Nuijens, J.H., Krimpenfort, P.J.A., Pieper, F.R., de Boer, H.A., and Strijker, R. (1994) Expression of human lactoferrin in milk of transgenic mice. *Transgenic Res.* **3**, 99-108
28. Kim, S.J., Lee, K.-W., Yu, D.-Y., Han, Y.-M., Lee, C.S., Nam, M.-S., Moon, H.-B., and Lee, K.-K. (1997) Expression analysis of a bovine β-casein/human lactoferrin hybrid gene in transgenic mice. *J. Reprod. Dev.* **43**, 143-149
29. Kim, S.J., Cho, Y.-Y., Lee, K.-W., Yu, D.-Y., Lee, C.-S., Han, Y.-M., and Lee, K.-K. (1994) Expression of human lactoferrin in milk of transgenic mice using bovine β-casein/human lactoferrin cDNA fusion gene. *Mol. Cells* **4**, 355-360
30. Kim, S.J., Yu, D.-Y., Pak, K.-W., Jeong, S., Kim, S.-W., and Lee, K.-K. (1998) Structure of the human lactoferrin gene and its chromosomal localization. *Mol. Cells* **8**, 663-668
31. Kozak, M. (1987) An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**, 8125-8148
32. Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994) *Manupulating the Mouse Embryo: Laboratory Manual*, pp. 127-252, Cold Spring Harbor Laboratory Press, New York
33. Shimoda, K., Cai, X., Kuhara, T., and Maejima, K. (1991) Reconstruction of a large DNA fragment from coinjected small fragments by homologous recombination in fertilized mouse eggs. *Nucleic Acids Res.* **19**, 6654
34. Schedl, A., Montoliu, L., Kelsey, G., and Schutz, G. (1993) A yeast artificial chromosome covering the tyrosinase gene confers copy number-dependent expression in transgenic mice. *Nature*

362, 258-261
35. Hashido, K., Arai, Y., Kajihara, S., Joh, K., Yatsuki, H., Hori, K., Miyazaki, J., Yamamura, K., and Mukai, T. (1995) Copy-dependent and position-independent expression of rat aldolase A gene. *J. Biochem.* 118, 601-606
36. Korhonen, V.P., Tolvanen, M., Hyttinen, J.M., Uusi-Oukari, M., Sinervirta, R., Alhonen, L., Jauhiainen, M., Janne, O.A., and Janne, J. (1997) Expression of bovine β-lactoglobulin/human erythropoietin fusion protein in the milk of transgenic mice and rabbits. *Eur. J. Biochem.* 245, 482-489