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Short Communication

Expression of a Bovine β-Casein/Human Lysozyme Fusion Gene in the Mammary Gland of Transgenic Mice

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Transgenic mice containing a bovine β-casein/human lysozyme fusion gene (pBZ) were generated in order to produce human lysozyme in their milk. The expression vector was a quadripartite fusion consisting of a 2 kb upstream DNA of the bovine β-casein gene, human lysozyme gene, intron II of the rabbit β-globin gene, and the polyadenylation/termination signals of SV40 DNA. Fertilized mouse zygotes were microinjected with pBZ, then transferred into the oviduct of foster mothers. Out of 20 mice born, 11 survived until post-weaning and three were identified as positive-transgenic by Southern blot analysis (one male and two females). The founder mice were mated to BCF1 mice to produce transgenic progeny. It was confirmed by RT-PCR and Northern blot analyses that the transgene was specifically expressed in the mammary gland of the founder mice. Furthermore, the artificial introns within the transgenic RNA was proven to be correctly spliced out as judged by RT-PCR analysis. These results indicated that transgenic mice generated in this study properly expressed the human lysozyme RNA in their mammary gland.

Keywords: Bovine β-casein, Human lysozyme, Mammary gland, Transgenic mice.

Introduction

Transgenic livestock producing human pharmaceuticals were developed in many species such as sheep (Clark *et al.*, 1989; Wright *et al.*, 1991; Carver *et al.*, 1993), goat (Denman *et al.*, 1991; Ebert *et al.*, 1991; 1994), rabbit (Buhler *et al.*, 1990), and swine (Swanson *et al.*, 1992). The gene of the desired protein is under the control of milk protein gene promoters which is either whey acidic protein (Gordon *et al.*, 1987; Bayna and Rosen, 1990), β-lactoglobulin (Simons and Land, 1987; Shani *et al.*, 1992), β-casein (Lee *et al.*, 1988; 1989), α-lactalbumin (Vilotte *et al.*, 1989), or αs1-casein gene (Meade *et al.*, 1990).

Human lysozyme, a lytic enzyme composed of 130 amino acids (15,000 Da) (Parry *et al.*, 1960), belongs to the chicken type class. It specifically catalyzes cleavage of the chemical bond between N-acetylglucosamine and N-acetylmuramic acid which are part of the cell wall structure of Gram–positive bacteria, and serves as a ubiquitous bacteriolytic enzyme present in external secretions, polymorphs, and macrophages (Jolles and Jolles, 1984). It contributes a bacteriostatic function in the gastrointestinal and respiratory tracts of breast-fed infants (Hanson and Winberg, 1972; McClelland *et al,* 1978), which is particularly important in the early neonatal period when the immune system is immature (Tomasi & Beinenstock, 1968). Amount of lysozyme in human milk is about 3000 times higher than in cow's milk which has on average only 0.13 μg/ml (Chandan *et al.*, 1968).

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Lysozyme has been considered with great interest because of its participation in the body defense mechanism, its effect on malignant growth, and its potential therapeutical applications (Osserman *et al.*, 1974). If transgenic animals including cattle can express human lysozyme protein efficiently in their milk, numerous possibilities are conceivable; increase in protein content of the milk, alteration in the ratio of milk proteins which may result in novel physical and functional properties (which include decreasing of rennet clotting time) (Jolles and Jolles, 1984), increasing of gel strength, cheese yield, and isoelectric coagulation of the micelles (Maga *et al.*, 1994), and novel milk with enhanced nutritional value. These considerations led us to consider the feasibility of an increase in the lysozyme content of bovine milk through the use of transgenic animals. However, the production of transgenic cattle is cost-expensive and must be refined. This study was carried out with mice to develop an animal model which can express human lysozyme in their mammary gland.

Materials and Methods

Construction of a human lysozyme expression vector Human lysozyme expression vector was constructed as follows. A 6.2 kb DNA fragment of the human lysozyme gene was prepared by polymerase chain reaction (PCR) from human chromosomal DNA. The primers used for the PCR are 5′-GTTGAATTCGGCCGCAAGGTCTTTGAAAGGTGTGA-3′ and 5′-CAGAAGCTTGGTATGAAGTTGC-3′ containing *Eag*I and *Hin*dIII sites at their 5′-ends. The PCR products were subcloned into the *Eag*I and *Hin*dIII sites of the pBluescriptSK vector (Stratagene, USA). The plasmid DNA was then digested with *Hin*dIII, gap-filled with Klenow enzyme, and digested with *Eag*I. The larger DNA fragment containing the human lysozyme gene was eluted from an agarose gel and ligated into the *Eag*I and gap-filled *Sfi*I site of the pBL1 vector (Kim *et al.*, 1994) to construct the lysozyme expression vector, pBZ (Fig. 1). The pBL1 vector is a human lactoferrin expression vector consisting of 1.8 kb 5′-flanking sequence, exon 1, signal sequence, and exons 8 and 9 of bovine β-casein gene, intron II of rabbit β-globin gene, human lactoferrin cDNA, and poly(A) signal of SV40 DNA.

Generation of transgenic mice Hybrid (C57BL × CBA) female mice were superovulated by intraperitoneal injection of 5 IU pregnant mare's serum gonadotropin (PMSG), followed by 5 IU human chorionic gonadotropin (hCG) 48 h later, and mated to hybrid males (Hogan *et al.*, 1986). On the following morning, females with vaginal plug were sacrificed by cervical dislocation and the fertilized zygotes were collected from the oviducts. After partial digestion of the pBZ plasmid DNA with *Sac*I and *Sal*I restriction enzymes, the fragment was separated on a 0.8% agarose gel, isolated from the gel using NA45 membrane (Schuleicher & Schuell, Germany), and purified by passing through Elutip-D column (Schuleicher & Schuell, Germany). Approximately 1–2 pl (4 ng/μl) of DNA solution was injected into the male pronuclei. Fifteen to twenty embryos were transferred into the oviduct of pseudopregnant recipients. For preparation of recipients, ICR females with spontaneous estrus were mated to vasectomized males. In the following morning, the plugged females were used. Pups born from DNA-injected embryos were weaned 4 weeks later and marked on the ear with dissecting scissors.

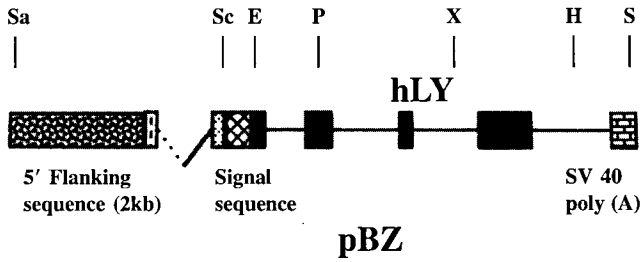

Fig. 1. Construction of a bovine β-casein/human lysozyme expression vector. Solid boxes and horizontal bars represent exons and introns of human lysozyme gene, respectively. Dashed line represents intron II region of the rabbit β-globin gene. 5′-flanking sequence and signal sequence of the bovine β-casein gene, and poly(A) signal of SV40 DNA are indicated. Restriction enzyme sites are abbreviated by Sa, *Sac*I; Sc, *Sac*II; E, *Eag*I; P, *Pst*I; X, *Xba*I; H, *Hin*dIII; S, *Sal*I.

Identification of transgenic mice Chromosomal DNAs from mouse tail tissues were isolated by the method of Hogan *et al.* (1986). The yield of DNA from each tail tissue was 50 to 100 μg. Transgenic mice were identified by Southern blot and PCR analysis. Ten μg of genomic DNA from founder mice or transgenic progeny was completely digested with *Xba*I and then separated on a 0.7% agarose gel. After transfer to a nylon membrane, the DNA was hybridized with a ^{32}P-labeled DNA probe for 24 h and then rinsed with 0.1× SSC/0.5% SDS washing solution. The DNA used for the probe was the 1.8 kb *Hpa*I/*Xba*I fragment of the pBZ vector.

Selection of the transgenic progeny was performed by amplification of transgene using PCR analysis (Saiki *et al.*, 1988). Transgene-specific DNA fragments were amplified from 1 μg of genomic DNA on a DNA thermal cycler for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The sequences of primers were 5′-CCGAAGCTTCTAGAGAATAAGATTG-3′ and 5′-CGCCTGCAGCTTTTTTCCAAAGTAGA-3′ which were complementary to the 5′-flanking sequence of bovine β-casein, and the expected size of amplified DNA fragment was about 800 bp.

Reverse transcriptase–polymerase chain reaction (RT–PCR) After sacrificing the mouse, total RNA was isolated from four organs (mammary gland, liver, spleen, and lung) by the acid-phenol method (Sambrook *et al.*, 1989). One μl of total RNA was reverse-transcribed in 30 μl for 2 h at 42°C using AMV reverse-transcriptase (Promega, USA). PCR was performed in a volume of 50 μl containing 2 μl of the first-strand cDNA. The primers used were: 5′-CCACCGCGGTATAGTGA GTCGTATTA-3′ and 5′-GGGATTCAGCTCCTCCTTCA-3′ which were complementary to the hybrid intron of pBZ, yielding a 120 bp fragment. After denaturation for 5 min at 94°C, 35 cycles of PCR (94°C for 1 min, 55°C for 1 min, and 72°C for 30 s) were performed.

Northern blot hybridization analysis RNA solution (30 μg of total RNA) dissolved in 0.5% SDS was denatured in the presence of 50% formamide, 2.2 M formaldehyde, 20 mM MOPS (3-N-morpholino propanesulfonic acid), 4 mM sodium acetate, and 0.5 mM EDTA. The RNA was separated by electrophoresis through a 1.0% agarose gel containing 2.2 M formaldehyde, and then transferred to a nylon membrane. Hybridization probe for detection of human lysozyme mRNA was 120 bp of RT-PCR product which was prepared as described above in the presence of [α-^{32}P]dCTP. Hybridization was performed in hybridization buffer (6× SSPE, pH 7.4, 5× Denhardt's solution, 0.5% SDS, 0.1 mg/ml denatured salmon sperm DNA, 50% deionized formamide) for 24 h at 42°C. After hybridization, the membrane was washed in a final stringency of 0.1× SSC/0.5% SDS for 20 min at 65° and exposed to an X-ray film at −70° for 2 days.

Results and Discussion

Construction of human lysozyme expression vector The human lysozyme expression vector, pBZ, was constructed in which human lysozyme (hLZ) genomic DNA was linked between the *Eag*I and *Sfi*I sites of the pBL1 expression vector (Fig. 1) (Kim *et al.*, 1994; 1995). pBL1 is a human lactoferrin expression vector and has been proven to properly express human lactoferrin in the mammary gland of transgenic mice. In pBZ, the human lactoferrin cDNA spanning the *Eag*I and *Sfi*I site was replaced with human lysozyme genomic DNA. We used genomic DNA because in many cases it expressed foreign proteins with higher levels than cDNA in transgenic animals. pBZ, therefore, was comprised by a 2 kb 5′-flanking sequence of the bovine β-casein gene, a hybrid intron, 5.5 kb human lysozyme DNA, and polyadenylation signal of SV40 DNA. To examine whether the lysozyme gene was correctly inserted into the pBL1 expression vector, the cloning sites were confirmed by DNA sequencing.

Generation and establishment of transgenic mice Totally, 105 zygotes were injected with the pBZ transgene and transferred into seven foster mothers. Out of 20 pups born, 11 survived until post-weaning. Tail DNA was isolated from founder and normal mice. Through Southern blot analysis, three were identified as transgenic. Founder mice were mated to normal F1 (CBA × C57BL) mice to pass the transgene through the progeny. Three transgenic founder mice raised their litter successfully. DNA samples were isolated from ears of progeny at the ages of 4 to 6 weeks and transgenicity was analyzed by PCR. One founder mouse (pBZ-2, female) transmitted the transgene at a high frequency (55.5%), but the others transmitted at lower frequencies (14.3%, pBZ-1, female), or did not transmit at all (pBZ-8, male). The pBZ-8 founder mouse was regarded as mosaic, which could not transmit the transgene through the germ-line.

Mammary gland-specific expression of the hLZ transgene Two lines of transgenic founder mice (pBZ-1; TG-1 and pBZ-2; TG-2) were sacrificed at day 10 of lactation and total RNA was isolated from various tissues including liver, spleen, lung, and mammary gland. To examine the tissue–specific expression and correct splicing of the hLZ transcripts, RT–PCR was performed (Fig. 2). The primers for PCR were designed to amplify a 120 bp DNA fragment which corresponds to the hybrid exon between the bovine β-casein and the rabbit β-globin genes in the pBZ vector. The pBZ vector DNA itself was also used as a template to represent the unspliced control transcript (lane P of Fig. 2). RT–PCR products (120 bp, lane Mg of Fig. 2) from the RNA of the transgenic mice was smaller than the product (420 bp, lane P of Fig. 2) from the pBZ vector by the size of the spliced-out intron segment. The 420 bp fragment from the mammary gland of TG-1, which did not appear in the case of TG-2, may have originated from mouse genomic DNA or from the unspliced RNA. These facts indicate that the transgene RNA was properly expressed in the mammary gland and underwent appropriate splicing at the artificial intron site.

In this study, 2.0 kb 5′-flanking sequence of the bovine β-casein gene was used as the promoter because its expression has been proven to be predominantly mammary-specific in our previous study (Kim *et al.*, 1994). It was reported that the expression level may be dependent on either the β-casein promoter or the extra introns put into the expression vector. This study confirmed that the expression cassette could be useful for the mammary gland-specific expression of foreign genes in transgenic mice.

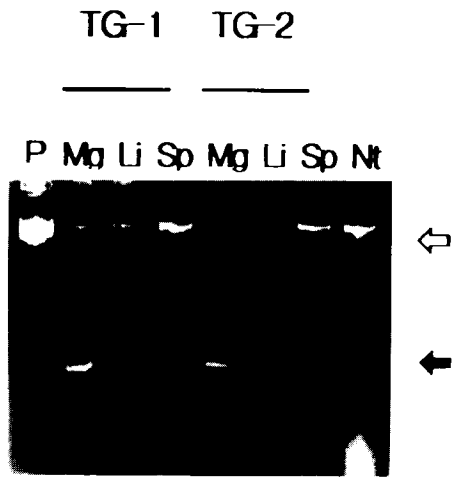

Fig. 2. RT-PCR analysis of total RNA from tissues of transgenic mice. RT-PCR was performed from total RNA of several tissues of transgenic mice. The tissues analyzed were mammary gland (Mg), liver (Li), and spleen (Sp). Mammary gland of non-DNA injected mouse (Nt) was also analyzed. Lane P is the PCR product from pBZ vector DNA. The opened arrow denotes the unspliced form; the closed arrow denotes the spliced from.

A Northern blot of total RNA from mouse tissues was probed with 120 bp RT–PCR products assigned to the transgene-specific hybrid exon located between the promoter and human lysozyme coding region in the pBZ expression vector. The human lysozyme DNA was not acceptable as a probe because it hybridized to both human and mouse lysozyme genes with equal intensity (data not shown). Human and mouse lysozyme showed considerably high homology (79%) when their amino acids sequences were compared. Transgene-specific RNA was expressed exclusively in the mammary gland (Fig. 3). Total RNA isolated from the mammary gland of a nontransgenic lactating female mouse did not show any human lysozyme expression (lane Nt of Fig. 3). Maga *et al.* (1994) reported that the 5′ promoter element of the bovine αs1-casein gene expressed the human lysozyme cDNA in the mammary gland of transgenic mice at the RNA level in a tissue-specific and developmentally-correct manner judging from Northern blot analysis. However, they were unable to detect lysozyme RNA when the promoter was replaced with that of the bovine β-casein gene. Only RT–PCR analysis was successful to detect human lysozyme RNA from one line out of eight transgenic lines. They postulated that the lower lysozyme expression level of bovine β-casein construct compared to the αs1-casein construct resulted from the absence of some, as yet unidentified, essential genetic elements. For the efforts to improve the expression level, we used human genomic DNA for the human lysozyme because it has been known that many genomic constructs expressed foreign genes more efficiently than cDNA constructs in transgenic mice (Gordon *et al.*, 1987; Brinster *et al.*, 1988; Pittius *et al.*, 1988; Simons *et al.*, 1988; Clark *et al.*, 1989; Tomasetto *et al.*, 1989; Palmiter *et al.*, 1991). Although it is yet unknown whether the improved expression of human lysozyme from pBZ vector is attributed by the genomic sequence, or by other factors of the pBZ construct such as the hybrid intron, the 2 kb 5′-flanking sequence of bovine β-casein certainly contains the elements necessary for mammary-specific expression.

To achieve a highly efficient, mammary-specific transgene construct, more detailed information on the actions of promoters and enhancers in eukaryotic gene expression has to be accumulated, and *cis*- and *trans*-acting elements must be defined. Through the study of the bovine β-casein/human lysozyme fusion gene construct, lysozyme was expressed at the mRNA level in the mammary gland of transgenic mice in a tissue-specific manner and the transgenic mice could be used as a model for studying the production of valuable human lysozyme.

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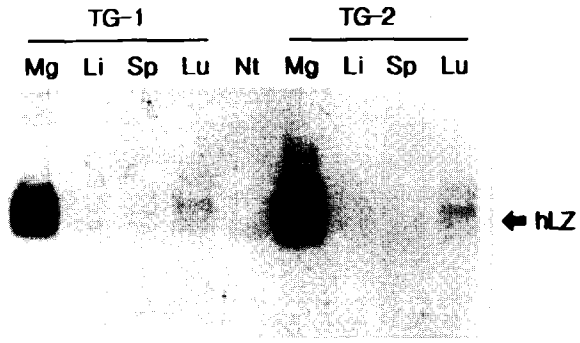

Fig. 3. Northern blot analysis of total RNA from tissues of transgenic mice. 30 μg of total RNA was hybridized with the transgene-specific DNA probe which spans exon 1 of the bovine β-casein gene and exon 3 of the rabbit β-globin gene in pBZ vector. Total RNAs were isolated from several organs of transgenic mice, TG-1 and TG-2. The organs are: Li, liver; Sp, spleen; Lu, lung; Mg, mammary gland of transgenic mice; Nt, nontransgenic mammary gland.

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