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Expression of human lysozyme mRNA in the mammary gland of transgenic mice

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Owing to its inherent antimicrobial effect and positive charge, the expression of human lysozyme in bovine milk could be beneficial by altering the overall microbial level and the functional and physical properties of the milk. We have used transgenic mice as model systems to evaluate the expression of human lysozyme containing fusion gene constructs in the mammary gland. Expression of human lysozyme was targeted to the mammary gland by using the 5′ promoter elements of either the bovine β (line B mice) or α_{s1} (line H mice) casein genes coupled to the cDNA for human lysozyme. Expression of human lysozyme mRNA was not found in mammary tissue from any of line B mice. Tissues were analysed from six lines of H mice and two, H6 and H5, were found to express human lysozyme mRNA in the mammary gland at 42% and 116%, respectively, of the levels of the endogenous mouse whey acidic protein gene. At peak lactation, female mice homozygous for the H5 and H6 transgene have approximately twice the amount of mRNA encoding human lysozyme as hemizygous animals. Expression levels of human lysozyme mRNA in the mammary gland at time points representing late pregnancy, early, peak and late lactation corresponded to the profile of casein gene expression. Human lysozyme mRNA expression was not observed in transgenic males, virgin females or in the kidney, liver, spleen or brain of lactating females. A very low level of expression of human lysozyme mRNA was observed in the salivary gland of line H5.

Keywords: human lysozyme; transgenic; mammary gland

Introduction

Much of the work carried out with transgenes directed to the mammary gland of animals has been focused on producing foreign proteins in the mammary gland of livestock with the intent of recovering the protein of interest from the milk (Clark *et al.*, 1987; Jimenez-Flores and Richardson, 1988; Bremel *et al.*, 1989; Wilmut *et al.*, 1991). The desired protein is put under control of a milk protein gene promoter in order to direct synthesis to the mammary gland. Transgenic mice have been generated with a number of mammary gland-specific genes, including sheep β-lactoglobulin (Simons *et al.*, 1987), rat β-casein (Lee *et al.*, 1988), and bovine α-lactalbumin (Vilotte *et al.*, 1989), in order to test the ability of the promoter elements of these genes to direct synthesis of proteins by the mammary gland of the mouse. In addition, promoter and 3′ elements from the mouse whey acidic protein gene (Gorden *et al.*, 1987), bovine α_{s1}-casein gene (Meade *et al.*, 1990), and sheep β-lactoglobulin gene (Shani *et al.*, 1992) have been used to express fusion gene constructs in the mouse mammary gland. Fusion genes encoding human α_1-antitrypsin and human tissue plasminogen activator have been expressed successfully in the milk of transgenic sheep (Wright *et al.*, 1991) and goats (Ebert *et al.*, 1991). Here, a murine model is being developed to determine if the addition of human lysozyme to the bovine milk protein system in order to directly alter the composition will produce a milk with novel properties.

Lysozyme specifically hydrolyses the glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine found in the peptidoglycan component of bacterial cell

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walls (Phillips, 1966). Lysozymes serve as a defence mechanism against bacterial infection or digestion of intestinal bacteria, are the primary product of macrophages and are found in mammalian secretions such as tears, saliva and milk. Lysozyme is found in human milk at levels 3000 times greater than in bovine milk (Chandan *et al.*, 1968). Lysozyme is considered to play a part in passive immunity, acts as a natural defence mechanism against bacteria, virus, parasites and fungi in human milk (Chang, 1990), and is inhibitory together with lactoferrin when present at the levels observed in human milk (Carlsson *et al.*, 1989).

There are two potential advantages of genetically adding human lysozyme to bovine milk. Firstly, because of its antimicrobial activity, lysozyme could help to reduce the number of bacteria in the milk and udder if present at sufficiently high levels. Lysozyme has been shown to inhibit the growth of a strain of *Listeria* in several different foods (Hughey *et al.*, 1989). At levels between 10 and 200 mg per litre of medium, lysozyme was effective against bacteria involved in food-borne disease and food spoilage (Hughey and Johnson, 1987). Additionally, it has been shown that human milk is more effective at stopping the growth of *Bacillus pertussis* than is bovine milk (Redhead *et al.*, 1990). At 100 mg per litre of milk, lysozyme was effective at inhibiting the growth of non-acid-forming bacteria (Panfil-Kuncewicz and Kisza, 1976). However, other bacteria in milk have been shown to be more resistant to lysozyme than bacteria in media alone (Grinde, 1989).

Secondly, lysozyme is positively charged at physiological pH (Jolles and Jolles, 1984). This could allow lysozyme to interact with the negatively charged caseins of milk to give the milk novel physical and functional properties. For instance, rennet clotting time could be decreased, gel strength and cheese yield could be increased and isoelectric coagulation of the micelles could occur at a higher pH if lysozyme was associated with the caseins by charge-charge interactions. The addition of lysozyme has been reported to increase the aggregation of micelles and lower the rennet clotting time by absorption to the micelle (Green and Marshall, 1977).

In this paper, we report the expression of human lysozyme mRNA in the mammary gland of transgenic mice as a means to evaluate the feasibility of genetically engineering an animal to produce increased levels of lysozyme in its milk at a sufficiently high level to affect the physical and functional properties of the milk.

Materials and methods

DNA constructs

Expression of human lysozyme (HLZ) was targeted to the mammary gland using the 5′ and 3′ regulatory regions of either the bovine β- or α_{s1}-casein genes coupled to the HLZ cDNA. The final constructs were derived from the partial human lysozyme cDNA clone HL-1421 (Castanon *et al.*, 1988). Bovine β-casein regulatory elements were obtained from genomic clones pH 9.3, containing 9.0 kb of 5′-flanking DNA and the coding regions (through exon 7), and pH 4.5 consisting of the rest of the β-casein genomic DNA including nontranslated exon 9 and 3.5 kb of 3′ flanking DNA (Gorodestsky *et al.*, 1988). Bovine α_{s1}-casein regulatory elements were from clone pCAS 1288 which contains 20 kb of 5′-flanking α_{s1} DNA, including exon 1 and part of the non-translated region of exon 2 hooked to the COOH terminal coding portion of the gene and 2 kb of 3′ flanking DNA by a unique *Xho* I restriction site (Meade *et al.*, 1990).

Construct B-HLZ. The clone pH 4.5 was cut with *Hin d*III and the entire 4.5 kb fragment containing the 3′ portion of the β-casein gene was inserted into the *Hin d*III linearized clone HL-1421 at the 3′ end of the 540 base pair HLZ cDNA. Correct orientation was verified by restriction digest. The resulting clone, H3′, was missing the first 12 base pairs at the 5′ end of the cDNA, including the start codon and the first three amino acids of the HLZ signal sequence. The missing coding region and a *Xho* I site upstream of the ATG codon were incorporated at the 5′ end of the clone by polymerase chain reaction (PCR) with a primer (ML1) containing the missing sequence. The 5.0 kb fragment containing the complete human lysozyme cDNA with a *Xho* I site on its 5′ end and the 3′ β-casein sequences at its 3′ end was amplified by PCR as described previously (Maga and Richardson, 1991). The resulting fragment was inserted into a TA PCR 1000 vector (Invitrogen, San Diego, CA, USA) to give H3′xTA.

The 5′ end of the β-casein gene (including promoter elements) was isolated from the 9.0 kb clone pH 9.3 by PCR. The direct pUC primer (Operon) was used at the 5′ end in order to obtain as much of the 5′ promoter sequences as possible from clone pH 9.3. For the 3′ end, a primer was designed that ended in the non-translated region of exon 2 and also incorporated a *Xho* I site at its 3′ end. Reaction conditions were the same as previously reported (Maga and Richardson, 1991) but with extension times of 4, 6 and 10 min. The resulting fragment contained 3.7 kb of β-casein 5′ DNA, including exon 1, intron 1 and the non-translated portion of exon 2, followed by a *Xho* I site. This fragment was cloned into another TA PCR 1000 vector to give the clone B5′xTA.

The clone H3′xTA was cut with *Apa* I and *Xho* I and the 5.0 kb fragment was then inserted into B5′xTA, which had been linearized with *Apa* I and *Xho* I. The resulting clone, B-HLZ, was used in subsequent experiments. It contained 3.7 kb of 5′ β-casein DNA joined to the complete HLZ cDNA at a *Xho* I site at the junction of the end of the noncoding region in β-casein exon 2 and the ATG initiation codon for HLZ, followed by 4.5 kb of 3′

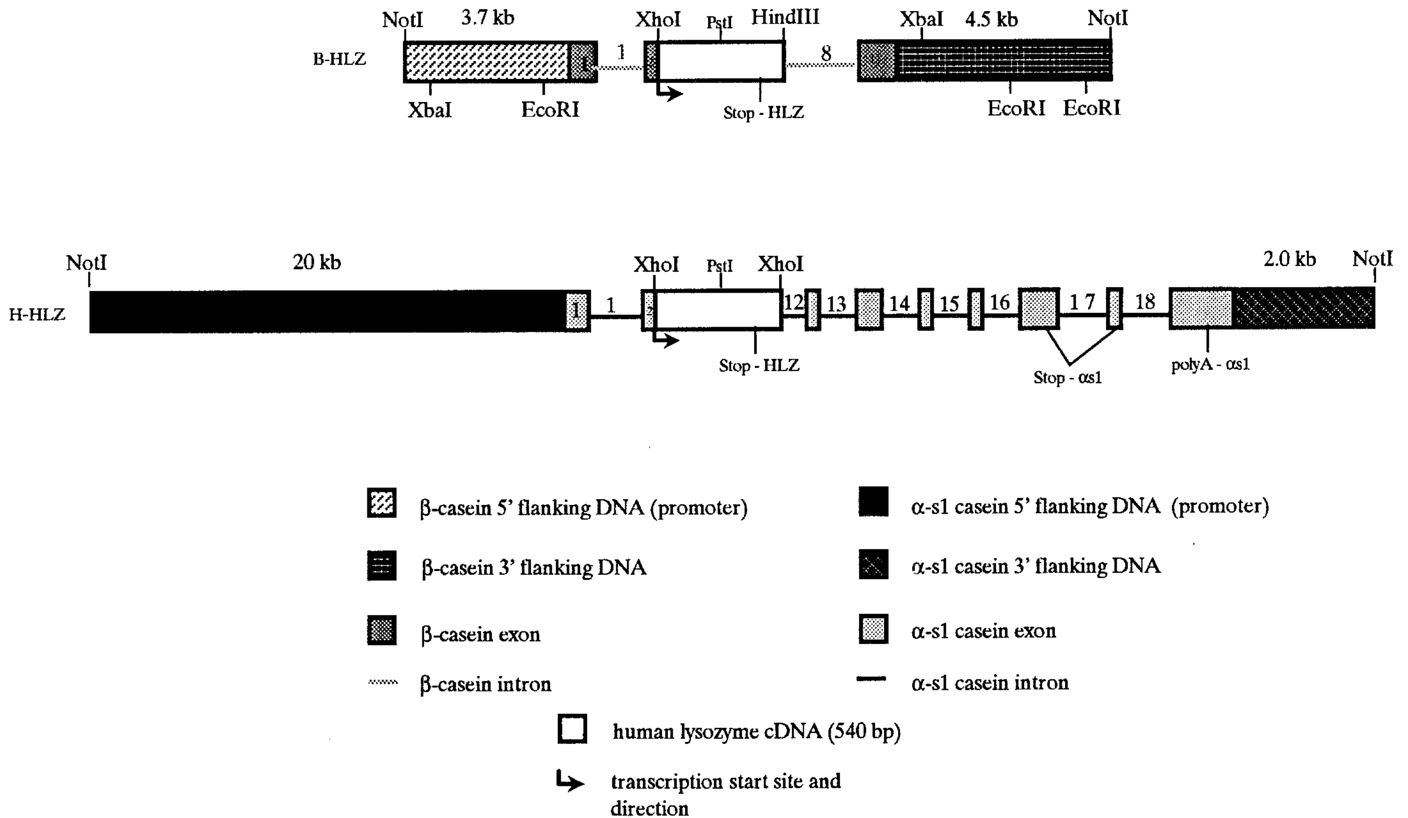

Fig. 1. Schematic representation of bovine casein-HLZ expression vectors.

flanking β-casein DNA including non-translated exon 9 (Fig. 1). The casein-lysozyme junctions and entire cDNA were sequenced using standard dideoxy DNA sequencing techniques.

Construct H-HLZ

PCR was used to incorporate *Xho* I sites at both ends of the HLZ cDNA. Primers specific for the ends of the HLZ cDNA (ML1 and ML2) were used in a PCR consisting of one cycle of 90°C, 2 min; 55°C, 1 min; 72°C, 1 min and 29 cycles of 92°C, 1 min; 55°C, 1 min; 72°C, 1 min. The remainder of the PCR conditions were as described previously. The 540 bp PCR fragment was cloned into a TA PCR 1000 vector, cut with *Xho* I and inserted into pCAS 1288 at the unique *Xho* I site. This gave the final construct H-HLZ, which consisted of 20 kb of 5′ flanking α_{s1} DNA including exon 1 and six of the 12 bp of the non-translated portion of exon 2, the complete HLZ cDNA followed by the COOH-terminal coding region of the α_{s1} gene including introns 12–18 and coding exons 13–17, non-coding exons 18 and 19, which contains the α_{s1} polyA site followed by 2 kb of 3′ α_{s1} flanking DNA (Fig. 1). Correct orientation of the human lysozyme insert and the lysozyme-casein junctions were verified by sequencing.

Generation of transgenic mice

For microinjection, each construct was removed from its vector by restriction digests, separated on a low melting agarose gel, purified on an Elutip column (Schleicher and Schuell, Keene, NH USA), and diluted to a final concentration of 2.5 ng μl^{-1} in a 10 mM Tris and 0.25 mM EDTA pH 7.5 solution. The DNA solution was microinjected into the male pronucleus of JU (Eklund and Bradford, 1976) mouse embryos, which were then transferred to pseudopregnant recipient JU females. Transgenic animals were identified by DNA extraction from tail or toe clips of pups as described by Evans *et al.* (1990) with the exceptions of using 1 μl 50 mg ml^{-1} Proteinase K and incubation for 2 h at 55°C, followed by 10 min at 95°C. The presence of the transgene was assayed using PCR of each extraction with primers specific for the ends of the HLZ cDNA, followed by detection on a 1% agarose gel. PCR reaction conditions were those used to incorporate *Xho* I sites onto the cDNA. PCR-positive founder animals were confirmed, and orientation and possible rearrangements of the transgene were detected by Southern blotting. Twenty μg of tail DNA were digested with *Xho* I and run on a 0.75% agarose gel and transferred to a nitrocellulose membrane. The membrane was baked at 80°C

for 2 h then prehybridized in a 6× SSC, 0.5% SDS, 5× Denhardt's and 100 μg ml^{-1} salmon sperm DNA solution at 68°C for 2–3 h. The HLZ cDNA was labelled with ^{32}P by random priming and was allowed to hybridize with the membrane overnight at 68°C. The membrane was then washed with 2× SSC, 0.1% SDS at room temperature for 15 min followed by one wash of 0.1× SSC, 0.5% SDS at 68°C for 2 h. The membrane was exposed to x-ray film for 3 to 5 days at −70°C. Copy number was determined by densitometer scanning of the autoradiographs with respect to a known amount of HLZ plasmid.

RNA analysis

Total RNA was isolated from the mammary gland, kidney, spleen, liver, brain and sub-lingual and sub-mandibular salivary gland of day 10 lactating transgenic and control mice by an acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Saachi, 1987). For initial analysis of expression, mammary gland tissue from two day 10 lactating transgenic females was analysed per line. Thirty μg of total RNA was run on a 1.2% MOPS formaldehyde gel and transferred to a nitrocellulose membrane for northern analysis. The probe and reaction conditions were the same as used for Southern blotting except washings consisting of one wash of 1× SSC, 0.1% SDS for 20 min at room temperature followed by one or two washes of 0.2× SSC, 0.1% SDS at 68°C for 20 min.

Mature hemizygous transgenic F_2 and F_3 females from expressing lines H5 and H6 were bred at 8 to 15 weeks of age and mammary glands were taken at different time points throughout gestation and lactation and tested for expression and quantity of HLZ message. A total of three mice were analysed per time point per line. The time points used corresponded to late gestation (day −2 with respect to birth), early lactation (day 4), peak lactation (day 10), and late lactation (day 17). Mature virgin transgenic females and mature transgenic males were also tested for the presence of the HLZ message. Negative controls were non-transgenic siblings of the positive animals used for analysis.

Autoradiographs from each time point were scanned with a densitometer to obtain an average absorbance value relating to the amount of signal present. All levels of message were quantified with respect to the amount of message present at peak lactation (day 10). In addition, the day 10 blot was stripped and reprobed with a 560 bp fragment of the mouse whey acidic protein (WAP) cDNA (gift of R. McKnight) and scanned by densitometry to determine the amount of lysozyme mRNA present relative to the amount of WAP mRNA. The average absorbances for each probe were divided and then averaged in order to account for loading differences.

Transgenic animals from each line were bred to homozygosity and test mated to JU mice. An animal producing 15 or more transgenic offspring was considered to be homozygous for the transgene. Total RNA was isolated from the mammary gland of day 10 lactating homozygous females and quantitated by densitometry as above.

Results

The DNA constructs used to promote expression of human lysozyme in the mammary gland consisted of the genetic regulatory elements of either the bovine β- or α_{s1}-casein gene and the complete cDNA for human lysozyme including its signal sequence (Fig. 1).

Ten lines of transgenic mice were generated with the construct B-HLZ (Table 1). Copy number of the transgene ranged from 1 to 12. Eight of the 10 primary transgenic mice transmitted the transgene to their offspring and hemizygotes from the F_1 generation were used for expression analysis. Two mice from each of the eight lines were analysed by northern blotting with 30 μg of total RNA isolated from the mammary gland. Expression of the HLZ cDNA could not be demonstrated in any of the eight lines by northern analysis. One line did show expression at very low levels, detectable only by RT-PCR (data not shown), and was not considered for further analysis.

Six lines of transgenic mice were generated with construct H-HLZ (Table 1). All lines transmitted the transgene to their offspring; however, Southern analysis showed one line had a rearrangement at the 3′ end and was not used for further study. Two of the remaining five lines (H5 and H6) showed expression at the mRNA level as detected by northern blotting (Fig. 2). Both lines showed a single band of approximately 1100 nucleotides corresponding to a HLZ-α_{s1} fusion mRNA, just under the 18S ribosomal band. Table 1 shows the relative levels of expression of the H-HLZ transgene in lines H5 and H6.

Table 1. Characteristics of human lysozyme transgenic mouse lines

Characteristic	*B-HLZ*	*H-HLZ*
Primary transgenics	10	6
Transmission	8/10	6/6
Rearrangements	0	1/6
Copy number range	1–12	1–138
Expression	1[a]/8	2/5
Quantity of HLZ mRNA as compared to WAP	ND	42%[b] 116%[c]

[a]mRNA detectable only by RT-PCR.
ND = Not determined.
[b]Line H6.
[c]Line H5.

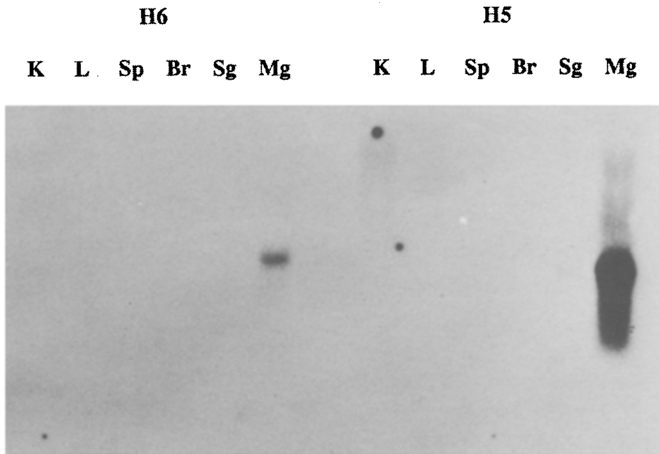

Fig. 2. Tissue specificity of H-HLZ transgene expression. Northern blot analysis of total RNA isolated from day 10 lactating transgenic females of lines H5 and H6. Blots were probed with ^{32}P HLZ cDNA. K, kidney; L, liver; Sp, spleen; B, brain; Sg, salivary gland; Mg, mammary gland.

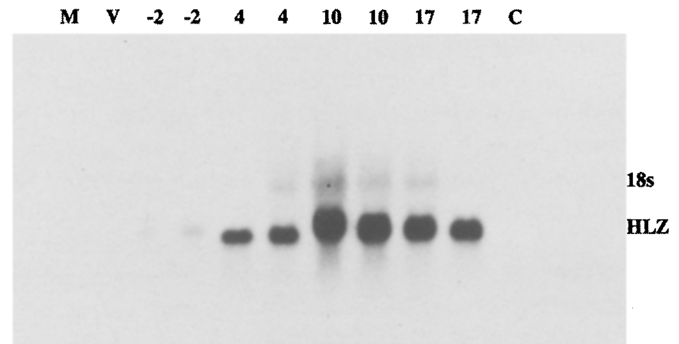

Fig. 3. Summary of the developmental regulation of HLZ expression in the mammary gland of line H5 H-HLZ transgenic mice. Northern blot analysis of two of the three animals analysed per time point with ^{32}P HLZ cDNA as probe. M, transgenic male; V, transgenic virgin female; C, nontransgenic day 10 lactating female; −2, 2 days before parturition; 4, day 4; 10, day 10; 17, day 17 of lactation.

At day 10 of lactation, line H6 expressed the HLZ mRNA at 42% and line H5 at 116% of the mRNA level of the endogenous mouse WAP gene.

Proper tissue specificity of transgene expression was observed in both lines of H-HLZ mice. Twenty μg of total RNA was used for northern blotting. There was no detectable expression of HLZ mRNA in the RNA isolated from any tissue tested other than mammary gland, with the exception of the salivary gland in line H5 (Fig. 2). It was also noted that the HLZ cDNA probe used could not detect any endogenous mouse lysozyme message in any of the tissues tested, including the mammary gland.

Both lines H5 and H6 had displayed proper developmental regulation of HLZ expression. Line H5 showed detectable expression of the transgene 2 days before birth, increasing amounts through day 4 to day 10 of lactation and then decreasing amounts of mRNA by day 17 (Figs 3–4). Line H6 demonstrated a similar developmental pattern except no HLZ transcript could be detected at day −2 (data not shown). No expression was seen in mature transgenic males or in virgin transgenic females of both lines (Fig. 3).

At peak lactation, homozygous females from both lines H5 and H6 were found to have twice as much HLZ mRNA as hemizygous females. Average densitometer absorbance values adjusted for loading differences were 0.601 and 0.343 for homozygous animals from lines H5 and H6 respectively, as compared to 0.308 and 0.130 for hemizygous animals from lines H5 and H6 respectively.

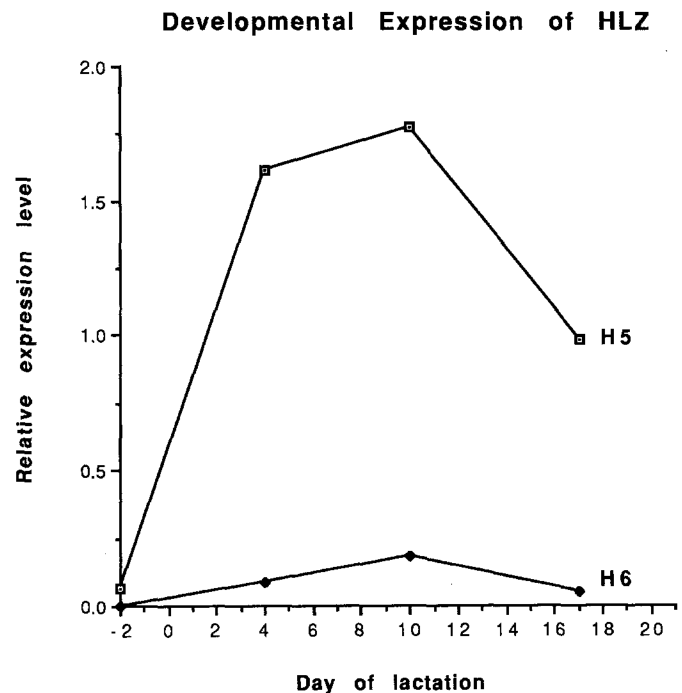

Fig. 4. HLZ expression during lactation. Relative level of HLZ mRNA as compared to HLZ mRNA at day 10 of lactation. Expression values are the averages of the absorbance values obtained by densitometer scanning of northern blots of three individual mice per time point.

Discussion

Two different DNA constructs were made in order to promote the expression of human lysozyme in the mammary gland of transgenic mice. Both fusion gene constructs contained the complete HLZ cDNA and either the bovine β- or α_{s1}-casein 5′ and 3′ regulatory regions. Eight of 10 lines of transgenic mice carrying the B-HLZ construct transmitted the transgene but did not express

human lysozyme in the mammary gland at levels detectable by northern blotting. The B-HLZ construct contained only 3.7 kb of β-casein promoter region. Lee *et al.* (1988) reported poor expression of the intact rat β-casein gene in transgenic mice. Therefore, it seems likely that the lack of expression with this construct is the result of the absence of some, as yet unidentified, essential genetic elements at the 5′ end of the gene.

The sequences used in the H-HLZ construct have been shown to express the genomic gene for human urokinase at a high level in the mammary gland of transgenic mice (Meade *et al.*, 1990). The presence of three transcripts in the mammary gland of the mice generated were attributed to incomplete splicing or human urokinase-α_{s1} fusion transcripts due to the α_{s1} coding exons present in the construct. Here, using the same α_{s1} sequences, only one transcript with a size of approximately 1100 nucleotides was present in the mammary gland of transgenic mice. This corresponds to a HLZ-α_{s1} fusion mRNA with approximately 700 nucleotides being contributed by the α_{s1} 3′ coding sequences present in construct H-HLZ. The HLZ sequences contain a stop codon and should be translated correctly. The HLZ cDNA did not cross-hybridize with any endogenous mRNA transcripts in any of the tissues analysed. The HLZ transcript was not found in the kidney, spleen, liver or brain of day 10 lactating females, which agrees with the observation on the tissue specificity of the α_{s1}-urokinase transgene (Meade *et al.*, 1990). However, a small amount of transcript was detected in the salivary gland of line H5 day 10 lactating transgenic females. While Meade *et al.* (1990) did not test salivary gland for the presence of transcript, Whitelaw *et al.* (1991) reported the presence of a transcript directed by the sheep β-lactoglobulin promoter in the salivary gland of transgenic mice. Expression of mammary gland-specific genes in the salivary gland could be due to the fact that the mammary and salivary glands share a common developmental pathway. Both are secretory, and are derived from the interaction between ectodermal epithelium and mesodermal mesenchyme during development. These results suggest that the α_{s1} promoter contains regulatory elements in common with salivary gland-specific genes. Human lysozyme transcript was not found in transgenic males or in transgenic virgin females, again showing the tissue specificity of the transgene.

The transgene was under proper developmental control of the α_{s1} promoter before and throughout lactation, as expression profiles of HLZ message corresponded with those of the caseins (Harris *et al.*, 1991). No message was seen at day −2 in line H6, owing to the overall lower level of expression in this line.

Homozygous females from both lines produced approximately twice the amount of HLZ mRNA at peak lactation as hemizygous animals. This would be expected for alleles with codominant expression and demonstrates that neither insertion site is in an imprinted region of chromatin.

From these transgenic studies, it can be concluded that the bovine α_{s1}-casein-human lysozyme DNA construct can successfully promote expression of human lysozyme at the mRNA level in the mammary gland of transgenic mice in a tissue-specific and developmentally correct fashion. Levels of human lysozyme mRNA expression in line H5 were comparable to the levels of endogenous WAP expression. Preliminary western analysis using a polyclonal antibody to human lysozyme indicate that HLZ protein is being made in the mammary gland of day 10 lactating females from both lines. The milk from these animals will be useful in evaluating the functional and physical effects of *in vivo*-produced lysozyme in milk.

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