

Expression of Human Serum Albumin in Milk of Transgenic Mice Using Goat β -casein/Human Serum Albumin Fusion Gene*

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ABSTRACT : The gene encoding human serum albumin (HSA) was cloned from human liver cDNA library by PCR. The HSA cDNA in size of 2,176 bp, including 1,830 bp of open reading frame, was cloned into the plasmid carried with the 5' flanking sequence of goat β -casein gene (-4,044 to +2,025 bp) to get a tissue specific expression vector in mammary gland named pGB562/HSA (12.5 kb). A 9.6 kb DNA fragment in which the sequence is in order of goat β -casein gene regulatory sequence, HSA cDNA and SV40 polyadenylation signals was isolated from the pGB562/HSA by *SacI* and *DraIII* cutting, and used to microinject into the pronuclei of mouse fertilized eggs to produce transgenic mice. Three transgenic mice (2 female and 1 male) were identified by PCR and dot Southern blot analysis. The copy numbers of integrated transgene were more than 10 copies in line #21 and #26 as well as over 50 copies in line #31 of transgenic mice. HSA protein collected from the milk of lactating transgenic mice was confirmed by immuno-detection of Western and slot blot. The concentrations of HSA in the milk were from 0.05 to 0.4 mg/ml. An obvious antigen and antibody conjugate could be observed in immunohistochemical stain of mammary gland tissue from lactating day 11 of HSA transgenic mice. The transmission of transgene and its expression was recognized according to the results of RT-PCR and sequences analyses of their progeny. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 6 : 743-749)

Key Words : Human Serum Albumin, β -Casein Gene, Transgenic Mouse, Gene Expression

INTRODUCTION

Expression of major milk-encoding genes and derived hybrid constructs has been investigated in transgenic animals, such as mice, rabbit, swine, and ruminates. These genes included β -lactoglobulin (Shani et al., 1992), whey acidic protein (Bischoff et al., 1992), α -lactalbumin (Bleck and Bremel, 1994), α s1-casein (Maga et al., 1995) and β -casein (Gutierrez et al., 1996). The transgenic technique provided a powerful system for studying the control of gene expression in the mammary gland and determining the consequences of transgene expression on the development of this organ. Casein genes are tissue-specifically expressed in mammary glands during lactation, in which mammary epithelial cells lining the alveoli synthesize and secrete numerous components of milk by active transcription and

translation of those genes (Faerman et al., 1995). β -casein is the most abundant protein among caseins, with an average content of 37% in cattle milk and over 50% in sheep milk (Provot et al., 1995). Human serum albumin (HSA) is a globular, non-glycosylate protein (MW 65,000) synthesized by the liver. HSA is the most abundant serum protein. It is involved in the transport of molecules and its presence is required for sustaining normal bloodstream osmolarity as well as blood pressure (Peter, 1996). The HSA is an important protein and high quantity needed in pharmaceuticals (Wall et al., 1997), but with high risk from human blood contamination such as HIV or prion. In the present study, we report a high stage- and mammary tissue specific expression of HSA transgene investigated in 3 lines of transgenic mice. In the mice and their offspring, the HSA expression in milk was detectable. The transgenic mice model of produce recombinant protein may be show the way to apply in domesticated livestock.

MATERIALS AND METHODS

Construction of recombinant vectors

Goat β -casein 5' regulatory region was cloned and tested to identify its expression capability. Briefly, a 6.2 kb fragment from pGB5-1 (Huang and Lin, 1996) was cloned into the promoterless GFP expression vector pEGFP-1 (Clontech, Palo Alto, CA, USA). The new clone named pGB562/GFP, and encompassed the sequences from -4,044 to +2,123 bp of the β -casein gene 5' flanking region, exon 1 and 2 including intron 1 to drive the GFP open reading

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Table 1. List of primer pairs used in this study

Primer sets	Sequence	Length of PCR product	Purpose
HALB5	5'-TCT CTT CTG TCA ACC CCA CG-3'	2,176 bp	Amplify HSA cDNA fragment with full length of coding region.
HALB3	5'-GCC CAC AGA AAC TAG AAA TCC-3'		
GB562-F	5'-ACT CTC AAA ATT CTC CAA GCC-3'		
GB562-R	5'-TCC CAT CCA ACC ATC TCA TC-3'	491 bp	Detect the transgene GB562/HSA integrated in the genome of transgenic mice. The partial promoter region of GB562 is amplified.
GB562/HSA-F	5'-CTG TAA CTT CTC TTC TGT CTT TCC-3'	671 bp	Transgene detection. The partial promoter/HSA junction is amplified.
GB562/HSA-R	5'-CAT CTC GAC GAA ACA CAC C-3'		
HSA5d	5'-TAA TGC TGAAAC ATT CAC CT-3'	1,260 bp	Transgene detection. The partial 3' fragment region of transgene is amplified.
3polyA	5'-CAC GAA CTC CAG CAG GAC CAT G-3'		
HSA5-2	5'-TCT CTT CTG TCA ACC CCA CGC CTT TGG CAC-3'	2,133 bp	Detect the HSA mRNA expressed in the mammary gland of transgenic mice by RT-PCR.
HSA3-2	5'-TAA GAG AGAACA CTG GAA CTT CCA CAG AAC C-3'		

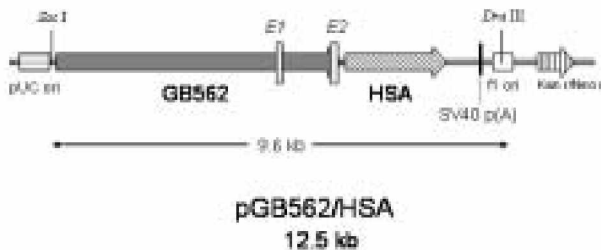


Figure 1. Restriction map of pGB562/HSA recombinant plasmid. The *SacI/DraIII*-restricted fragment in size of 9.6 kb, which contains GB562, HSA cDNA, and SV40 polyadenylation signal (SV40 p(A)), was cut and collected for the use to produce transgenic mice by microinjected into mouse fertilized eggs. *E1*, *E2*, Exon 1 and 2 respectively; pUC ori, fl ori, origin of replication in pUC and fl, respectively; Kan *r*/Neo *r*, kanamycin and neomycin resistance genes.

frame. *In vitro* and *ex vivo* green fluorescent protein expression in alveolar mammary epithelial cells and mammary glands had been confirmed that GB562 present great capability to promote GFP expression (Wu et al., 2003).

The gene fragment encoding HSA protein was cloned from human liver cDNA bank (Human liver Quick-Clone cDNA, Clontech, Co.). According the GenBank of NCBI (accession number: L00132, L00133, J00078, V00495), we designed primers to amplify 2,176 bp HSA cDNA fragment (HALB5: HALB3) (Table 1) and constructed the DNA fragment into the plasmid pCR2.1 designed as pCR2.1/HSA. The cDNA contained the complete HSA coding sequences as well as 33 bp of 5' untranslated and 313 bp of 3' untranslated sequences (NCBI accession number: AF542069). The pCR2.1/HSA was digested with *ApaI/BamHI*, the HSA cDNA fragment was inserted into the same sites of pGB562/GFP to generate a clone designated pGB562/HSA (12.5 kb) (Figure 1).

Generation and identification of transgenic animals

The 9.6 kb fragment GB562/HSA prepared for microinjection including goat β -casein gene promoter, HSA cDNA and SV40 polyadenylation signals was released from pGB562/HSA by *SacI* and *DraIII* digestion (Figure 1). This 9.6 kb DNA fragment diluted to final concentration of 2.5 ng/ μ l in a 10 mM Tris and 0.25 mM EDTA pH 7.5 solution. The 2 μ l of DNA solution was microinjected into pronucleus of *in vitro* fertilized FVB/NCr1 mouse eggs (Wu et al., 2003) which were then re-implanted into pseudopregnant females CB6 F1 (BALB/c \times C57BL/6) mice (Huang, 1988; Hogan et al., 1994; Nakao et al., 1997) to generate the transgenic mice. All animals were maintained, handled and treated following NRC (1996) guidelines.

Three primer sets were designed to identify the GB562/HSA transgenic mice. The targeting sequences of these 3 primer pairs are the promoter region of GB562 (GB562-F: GB562-R), the joining regions between GB562 and HSA (GB562/HSA-F: GB562/HSA-R) as well as between HSA and 3' fragment region (HSA5d; 3polyA), respectively (Table 1).

Transgene copy numbers were estimated by comparison of hybridization signal of dilutions of transgenic DNA to the signal of a known amount of GB562/HSA DNA fragment by the method of DIG-labeled dot blot (Rijnkels et al., 1995). A 671 bp DIG-labeled probe, which crosses the β -casein gene promoter and HSA cDNA junction, was prepared with PCR synthesis (primer set: GB562/HSA-F: GB562/HSA-R) (Table 1). The transgene detection of DNA hybridization was followed by the manufacturer's manual (DIG DNA Labeling and Detection Kit, Roche Applied Science, Mannheim, Germany).

Collection and fraction of milk

Milk was collected from nursing transgenic mice after 10 to 12 days parturition. Milk samples were diluted 1:5 in distilled water containing 2 mM phenylmethylsulfonyl

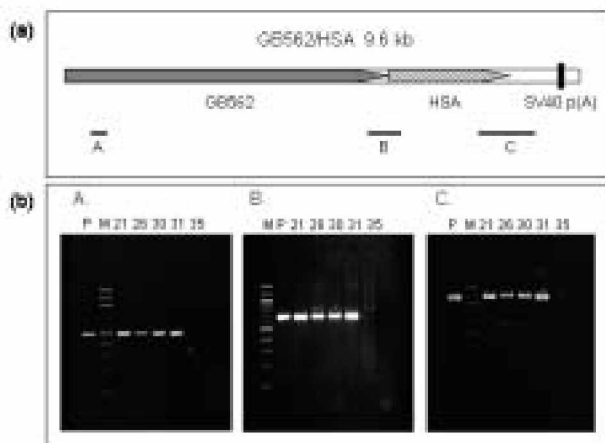


Figure 2. PCR analysis of transgenic mice tail genomic DNA. (a) a diagram of GB562/HSA and the primer sets for used in analyzing the transgene integrated into the genome of transgenic mice. A: GB562-F; GB562-R, product length 491 bp. B: GB562/HSA-F; GB562/HSA-R, product length 671 bp. C: HSA5d; 3polyA, product length 1,260 bp. (b) The results of PCR analysis. #21, #26, #30, #31 and #35 were the founder mice from microinjected mice eggs, respectively. P, positive control DNA of pGB562/HSA; M, 100 bp DNA ladder.

fluoride (PMSF) and Aprotinin (Sigma, St Louis, MO, USA) (Shani et al., 1992). The milk was defatted by centrifugation at 12,000 g for 5 min to collect milk whey and stored at -70°C for further Western blot assay.

Western blot analysis

Milk whey proteins were analyzed for the presence of HSA in transgenic mice. Milk whey samples were fractionated on 8% SDS-polyacrylamide gels. Proteins were either stained with Coomassie Brilliant Blue or transferred onto nitrocellulose membrane (Hybond-c extra, Amersham Inc., UK). HSA was detected using a mouse anti-HSA monoclonal antibody (HSA1/25.1.3, Cedarlane Laboratories Limited, Canada), visualized by chemiluminescence (Western-Star, Tropix Inc., USA) and exposed to Kodak X-AR film. The levels of HSA secreted into the milk were determined by slot-blot assay compared to HSA standards (Sigma) (Shani et al., 1992). The 1, 5 and 10 μ l of diluted milk whey were loaded in slot blot for evaluating the HSA product of transgenic mice.

Immunohistochemistry analysis

The tissues of mammary glands of lactating HSA transgenic and control female mice (day 11) were employed for the immunohistochemistry analysis carrying out on formaldehyde-fixed, paraffin-embedded sections. The monoclonal antibody of mouse anti-human albumin (1:50 dilution, HSA1/25.1.3, Cedarlane Laboratories Limited) was used to detect expression and

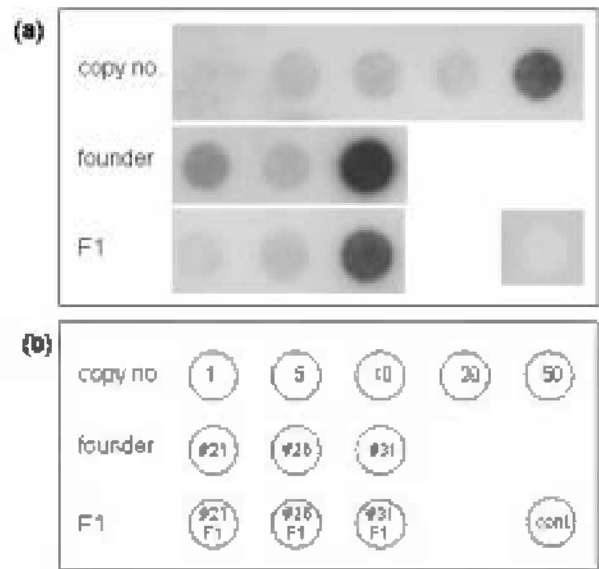


Figure 3. Copy number determination of transgene integrated into the transgenic mice and progeny by DIG-Southern dot blot. Row 1 presents the control DNA representing different copy numbers from 1, 5, 10, 20 and 50, respectively. Row 2 shows the detected result of transgenic founder mice #21, #26 and #31 and row 3 shows the result of F1 progeny of transgenic mice #21, #26, #31 and non-transgenic mice (cont.). (b) indicates the arrangement of blot (a).

distribution of HSA transgene on mammary tissue. The avidin biotinylated enzyme complex immunostain (ABC immunostain, Vector[®] M.O.M.[™] kit, Vector Laboratories, Burlingame, CA, USA) was used by following the method of Liang et al. (2000). Sections were counterstained with hematoxylin then examined using a Nikon E600 microscope.

RT-PCR analysis

Total RNA from mammary gland and tissue (liver) of lactating transgenic and nontransgenic mice was isolated by acid guanidium-isothiocyanate method (RNesay mini kits, Qiagen Inc., Germany). The primer pair used for RT-PCR was HSA5-2 and HSA3-2 (Table 1), which yield a 2,133 bp fragment. Reverse transcription and PCR amplification was performed using total RNA as the template in one step method followed by the manufacture's protocol (OneStep RT-PCR kit, Qiagen Inc., Germany).

Sequence analysis

To compare multiple sequence alignment from HSA founder mice and their F1, F2 generations of related sequences, we used PileUp program (SeqWeb 2.0, Accelrys, Inc.) to analysis the transgene transmission in heredity. Genomic DNA of transgenic mice and pGB562/HSA plasmid DNA were sequencing both two directions by used

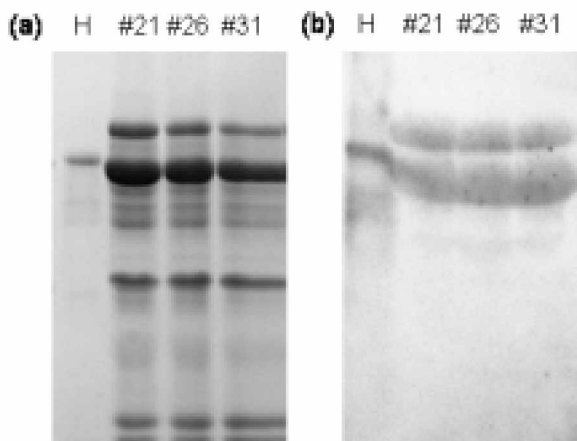


Figure 4. Western blot analysis of HSA in the milk of transgenic mice. (a) Coomassie blue stained 8% SDS-polyacrylamide gels fraction and (b) anti-HSA monoclonal antibody immunoblot analysis. H, standard HSA protein; #21, #26, #31, indicate samples milk whey from transgenic mice #21, #26 (F1 progeny) and #31, respectively.

primer GB562/HSA-F and GB562/HSA-R according to the method described by Lin et al. (2002) (Table 1).

RESULTS

Recombinant transgenic expression vector

The expression vector of pGB562/HSA was constructed as illustrated in Figure 1. The HSA cDNA fragment was cloned into goat β -casein promoter expression vector to designate pGB562/HSA. The HSA cDNA fragment (2,176 bp) included 1,830 bp of complete sequence of open reading frame. The regulatory fragment GB562 (from -4,044 to +2,123 bp) contains 6.2 kb of 5'-regulatory region, exon 1, 2 and intron 1 of goat β -casein gene. The sequences have been identified to have several regulatory elements (Roberts et al., 1992; Ebert et al., 1994; Kim et al., 1994; Cerdan et al., 1998), which could drive the coding gene to express. *In vitro* and *ex vivo* expression of GFP derived by the GB562 promoter in alveolar mammary epithelial cells and mammary glands had been confirmed by our prior studies (Wu et al., 2003). The expression vector pGB562/HSA also carries a SV40 polyadenylation signal following the 3' terminus for stabilizing the transcript and a kanamycin/neomycin resistance gene for selection procedures.

Generation of transgenic mice

Reimplantation of 134 microinjected eggs from total of 224 one-cell eggs yielded 34 pups, 4 of them carrying the HSA transgene as deduced from both concordant PCR and dot blot analysis of genomic DNA (Figure 2 and 3). Breeding of transgenic mice demonstrated that #30 (male)

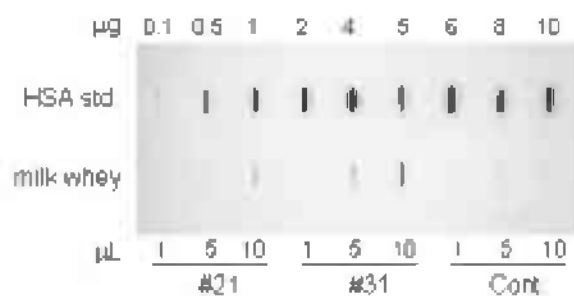


Figure 5. Immuno-detection of HSA in the milk of transgenic mice. Upper row was blotted with standard HSA proteins (HSA std.), concentration of HSA were from 0.1 to 10 g. Diluted (1:5) milk whey (1, 5 and 10 L) from transgenic mice #21 and #31 were blotted at down row. Immuno-slot blot analysis was reacted with anti-HSA monoclonal antibody. Cont., non-transgenic mouse milk whey.

founder was a mosaic and did not pass its transgene onto its offspring. The other lines (#21, #26 and #31) did pass their transgenes to subsequent generations. Transgene segregation data of founders #21 (female), #26 (male) and #31 (female) were compatible with the Mendelian transmission of a single locus of multiple integrated transgene copies. Moreover, levels of HSA expression in the milk from these offspring were similar to levels expression by their parent founders. The copy numbers of each line were calculated by densitometry of the transgenic mice genomic DNA Southern signals relative to the multiple copies of GB562/HSA gene. The result showed the copy numbers in the 3 lines are high copy numbers intergraded transgenic mice. There were 20, 10 and over 50 copy numbers of GB562/HSA gene detected in the line #21, #26 and #31, respectively (Figure 3).

Expression of HSA gene in the milk of transgenic mice

Milk samples from 3 of the HSA expressing lines were subjected to SDS-PAGE and immunoblot analysis (Figure 4). Mouse milk contains a significant amount of endogenous mouse serum albumin which slightly comigrates with human serum albumin in SDS-PAGE gel, but an immunoreactive band comigrating with expressed HSA (65 kDa) was detected in the milk of all transgene positive lines. The HSA expression level in transgenic mice milk was measured by Western blot analysis (Figure 5). The results showed that there were about 0.05, 0.25, 0.4 mg/ml of HSA detected in the line #21, #26 and #31, respectively.

Expression of HSA RNA in different tissues of transgenic mice

RT-PCR analysis showed that the transcription of the HSA transgene was restricted to the mammary gland (Figure 6). The sizes of the HSA mRNA as well as the splicing pattern were similar in transgenic mice mammary

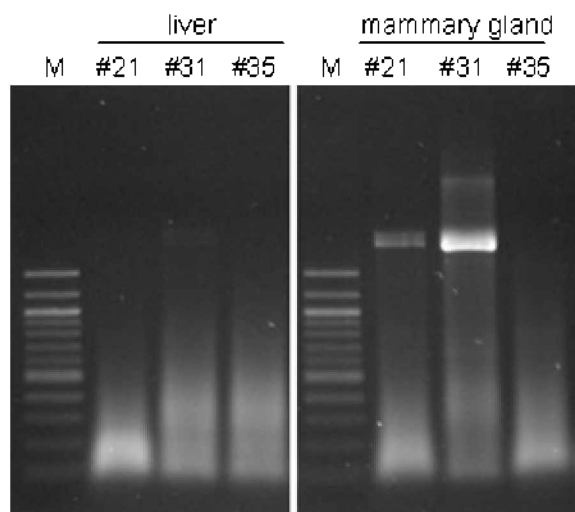


Figure 6. RT-PCR analysis of HSA transgene expression in the liver and mammary gland of transgenic female mice. Primer HSA5-2 and HSA3-2 were used in this study for HSA cDNA amplification. M, 100 bp DNA ladder; #21 and #31, transgenic mice; #35, non-transgenic mouse.

gland but not found in liver. The transgene specific mRNA levels in lactating mouse mammary gland correlated with the HSA protein concentration in the milk of the transgenic mice (Figures 5 and 6). The RT-PCR result showed #31 reproduced more PCR product than #21, the same tendency showed the HSA protein concentration in milk of #31 was higher than that of #21.

Immunohistochemistry analysis of mammary glands

The tissue specific expression of the transgene GB562/HSA was confirmed by RT-PCR. The result of immunohistochemistry analysis from mammary glands gave a strong evidence of HSA expression in the milk of lumen (red staining), which circled with luminal epithelial cell at out layer (Figure 7).

Transgene transmission between generations

From the sequences analyses of founder mice and their F1, F2 generations, the sequences between 400 bp of each mice were confirmed to be totally the same (Figure 8). The results showed transgene can transmit to their offspring by nature mating according Mendelian's rule.

DISCUSSION

We successfully established three transgenic mouse lines that expressed the exogenously-introduced HSA in their mammary glands. The HSA expression level in transgenic mice milk was from 0.05 to 0.4 mg/ml. The copy numbers of insert HSA were more than 10 copies in line #21 and #26 and over 50 copies in line #31.

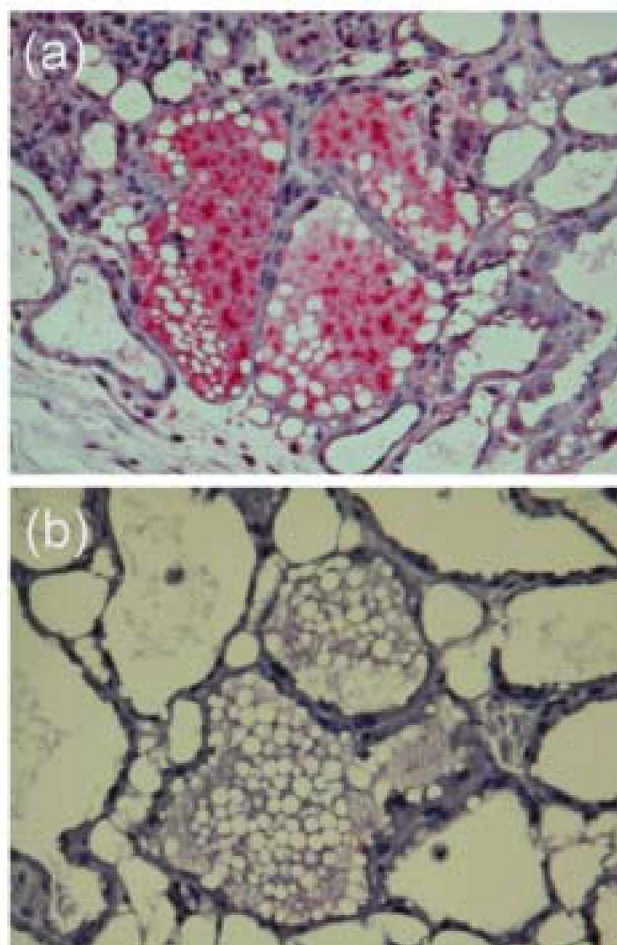


Figure 7. Immunohistochemical staining of mammary gland tissues from (a) lactating (day 11) HSA transgenic mice and (b) control mice. Mammary gland tissues were formalin fixed, paraffin embedded and immunohistochemistry stained by ABC method. The monoclonal antibody of mouse anti-human albumin was used to detect expression and distribution of HSA transgene on mammary tissue (red staining).

β -casein is the most abundant protein in goat and cow milks. Accordingly, it is suggested that β -casein gene has a powerful promoter and is suitable for use in driving the transgene expressed in the mammary glands of transgenic animals (Roberts et al., 1992; Ebert et al., 1994). In the former studies we described the results that the 6.2 kb regulatory sequence of goat β -casein gene GB562 is sufficient and effective for directing exogenous gene (GFP) expression in the NMuMG cells and mammary gland fragments (Wu et al., 2003). The 6.2 kb fragment GB562 contains 5' regulatory sequences, exon 1, 2 and intron 1 of goat β -casein gene. Exon 2 encodes a 15-aa secretion signal and the first two residues of the mature protein product (Roberts et al., 1992). Introns are essential for gene expression, but their rule is complex, especially the first intron of genes contains binding sites for transcription factors. This feature contributes to the maintenance of an

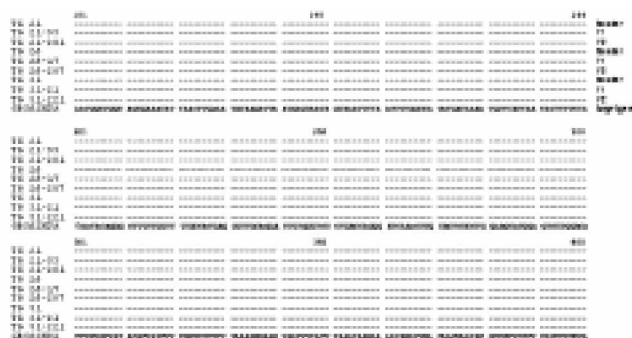


Figure 8. Sequence comparison among HSA transgenic founder mice and their F1 as well as F2 progeny. The conserved sequences were present in “-”. TG21-53 and TG21-201 were the F1 and F2 generation of transgenic line #21; TG26-17 and TG26-207 were the F1 and F2 generation of line #26; TG31-21 and TG31-221 were the F1 and F2 generation of line #31.

open and active form of chromatin around the cap. Alternatively, sequences for transcription factor binding may be added within the intron (Petitclerc et al., 1995). Kang et al. (1998) indicated that prolactin-inducible enhancer activity localized in the intron 1 of bovine β -casein gene. In addition, several elements in the intron 1 of bovine β -casein gene cooperatively interact not only with each other but also with its promoter for hormonal induction.

The HSA cDNA was inserted into the expression vector pGB562/GFP. The 2,176 bp HSA cDNA fragment included 1,830 bp of complete amino acid translation sequences. The promoter GB562 would activate the HSA cDNA to express its protein and terminated at stop codon.

The expression levels in the various founder animals are variable between and among species and have ranged from 0.025 g/ml to 35 mg/ml (Ebert et al., 1994). These orders of magnitude differences may be attributed to the level of effectiveness of the promoter itself, the post-transcriptional processing of the mRNA, the site of integration of transgene within the chromatin, or an incompatibility of the protein product itself within the mammary gland (Ebert et al., 1994). From the results of gene copy number, protein expression and RT-PCR, we found there is a positive correlation between integrate gene number and gene expression. Another evidence of HSA gene expression was the result of immunohistochemical staining of mammary gland. The HSA can be detected at alveoli contained secreted material (milk), which was secreted by luminal epithelial cell. The sequence analysis from founder, F1 and F2 genome also confirmed the HSA transgene can be transmitted to their offspring.

We have demonstrated mammary gland specific expression of human serum albumin in transgenic mice. The 6.2 kb of goat β -casein 5' regulatory region may serve to identify mammary gland specific elements involved in

regulatory of heterologous genes expression. From the results of HSA transgenic mice, we assume the regulatory elements derived from goat β -casein gene can be employed to target the expression of heterologous protein to the lactating mammary gland of others transgenic animal.

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