Abnormal Properties of Milk from Transgenic Mice Expressing Bovine β -Casein under Control of the Bovine α -Lactalbumin 5' Flanking Region

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ABSTRACT

Transgenic mice produced in this investigation contained the proximal promoter elements, the genomic coding sequence and the 3' flanking region of bovine β -casein and the 5' regulatory elements of bovine α -lactalbumin. Specifically, the gene construct included the TATAA box, CAAT box and polyadenylation signal of bovine β -casein in addition to the 5' regulatory elements of the bovine α -lactalbumin gene. Four lines of transgenic mice were generated; they expressed bovine β -casein in their milk at concentrations of up to 10 mg mL^{-1} . The milk from the transgenic mice tended to be very viscous and a proportion of these mice stopped lactating before the normal onset of involution. The time at which the abrupt involution occurred was directly correlated to the amount of β -casein produced. Mice secreting approximately 10 mg mL^{-1} of β -casein in their milk stopped lactating approximately 1–3 days after parturition while mice expressing $1\text{--}3 \text{ mg mL}^{-1}$ stopped lactating around days 10–18. The high expressing mice appeared to lactate normally on day 1 of lactation, but on day 3 the mammary gland morphology revealed alveoli and ducts which were very distended and contained milk components. However, the viscous milk solution could not be removed from the gland at this stage of lactation. There were also a number of alveoli that appeared to have gone through the process of involution by day 3 of lactation.

INTRODUCTION

Bovine α -lactalbumin (α -la) regulatory regions have been shown to drive the production of foreign proteins in the milk of transgenic mice in our laboratory as

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well as by others (Vilotte *et al.*, 1989; Bleck & Bremel, 1991; Stinnakre *et al.*, 1991; Soulier *et al.*, 1992). It appears from these studies that most of the cis-acting regulatory elements needed for maximal production of bovine α -la in the milk of transgenic mice are present in the 5' flanking region of the gene. To confirm that the bovine α -lactalbumin 5' flanking region was capable of driving high-level expression of a foreign protein in the milk of transgenic animals and to generate a gene construct that could be used to modify the characteristics and composition of bovine milk, transgenic mice were produced containing a bovine β -casein gene construct that was controlled by the bovine α -lactalbumin 5' flanking region.

Bovine β -casein was chosen for analysis due to its potential usefulness in the modification of physical properties of the milk. The addition of β -casein to cows' milk can increase the speed of curd formation and the cheese curd firmness (Richardson *et al.*, 1992). Thus, an increase in the production of β -casein will also increase the cheese yield of the milk. It has been hypothesized that the production of a modified β -casein in cows' milk can change the functional characteristics of the milk. The addition of a glycosylation site at amino acids 7 or 27 is postulated to increase the emulsifying, foaming and gelling characteristics of β -casein in the milk (Richardson *et al.*, 1992).

Transgenic animals containing a number of the casein genes have been generated, though transgenes controlled by the casein 5' flanking regions have been expressed at relatively low levels as compared to endogenous casein expression. The bovine α _{S1}-casein, rabbit β -casein and rat β -casein 5' flanking regions all have been used to drive the expression of transgenes in mice (Lee *et al.*, 1988; Buehler *et al.*, 1990; Meade *et al.*, 1990). Transgenic animals containing these casein constructs showed expression levels that were at most 1–10% of endogenous gene expression. From these data it was hypothesized that the casein gene cluster (Gupta *et al.*, 1982; Threadgill & Womack, 1990) is regulated by a major control element in addition to the basal promoter elements found in the 5' flanking region of each of the casein genes (Lee *et al.*, 1989). However, recently it was shown that a caprine β -casein gene construct could be expressed at high levels in the milk of transgenic mice (Persuy *et al.*, 1992). This gene construct was made up of 3.0 kb of 5' flanking, the entire genomic coding region, and 6.0 kb of 3' flanking region from the caprine β -casein gene. Milk from animals expressing this transgene contained caprine β -casein at concentrations up to 21 mg mL⁻¹ which is twice the level in normal caprine milk. These results indicate that the necessary cis-acting elements needed for maximal transgene expression may be present in the caprine β -casein gene construct.

Due to the discrepancies in the literature with respect to the ability of the casein 5' flanking regions to drive expression of genes in transgenic animals, we chose to test the ability of the bovine α -lactalbumin 5' flanking region to drive the production of bovine β -casein in the milk of transgenic mice and examine the characteristics of the mouse milk containing bovine β -casein.

MATERIALS AND METHODS

Isolation of the α -lactalbumin/ β -casein gene construct for microinjection

An α -lactalbumin/ β -casein gene construct (pA-lac/ β -casein) was developed in our laboratory that contained 2.0 kb of bovine α -lactalbumin 5' flanking region, the

entire 8.5 kb coding region of bovine β -casein and 150 bp of β -casein 3' flanking region (Fig. 1). This hybrid construct was restriction digested out of the plasmid clone (pA-lac 5'/ β -casein) using the restriction enzyme HhaI. This digestion was run on a 1.0% agarose gel and the band containing the 10.7 kb α -lactalbumin/ β -casein construct was purified from the gel using the Qiaex gel extraction kit (Qiagen, Chatsworth, CA). The resulting DNA was diluted in TE (10 mM Tris-HCl, pH = 7.4, 1 mM EDTA, pH = 8.0) for microinjection.

Production of transgenic mice

Mature C57B6 $\times$ DBA2J F1 (B6D2) female mice were superovulated using PMSG and hCG and mated with ICR or B6D2 males to yield fertilized embryos for pronuclear microinjection. The embryos were microinjected with the DNA construct and 40 normal-appearing two-cell embryos were then transferred to each pseudopregnant recipient (University of Wisconsin-Madison Biotechnology Center, Transgenic Mouse Facility). The resulting founder transgenic mice were then continually backcrossed into the ICR outbred strain of mice. The transgenic mice were maintained as heterozygotes for the transgene throughout the analysis.

Identification of transgenic mice

DNA was extracted from a portion of the mouse tail using the method described by Hogan *et al.* (1986). Polymerase chain reaction (PCR) was performed using 10 μ L 10 $\times$ PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl (pH = 8.8), 15 mM MgCl₂, 1% Triton X-100), 200 μ M each dNTP, 1.0 μ M each primer (Primer 1: 5' CTCTTCCTGGATGTAAGGCTT 3'; Primer 2: 5' CAAAGTAGAGGACAAGAAGT 3'; these primers amplify the junction

Fig. 1. Map of the α -Lac5'/ β -casein gene construct. The construct contains 2.0 kb of bovine α -lactalbumin 5' flanking region (Bleck & Bremel, 1993) and 8.5 kb bovine β -casein genomic clone (Bonsing *et al.*, 1988). The two clones were joined at position +92 bp from the α -lactalbumin transcription start point and position -91 bp from the β -casein transcription start point (tsp). The α -lactalbumin portion of the construct contains the 5' regulatory elements of the α -lactalbumin gene and the putative 'milk box' (Vilotte *et al.*, 1987) of the gene sequence. The β -casein portion of the construct includes the β -casein CAAT box, TATA box, transcription start point, nine exons, polyadenylation signal and 150 bp of 3' flanking region.

between the α -lactalbumin 5' flanking region and the β -casein gene), 1 unit Taq DNA polymerase and 1 μ g genomic DNA. Volume was adjusted to 100 μ L with double distilled sterile water and reaction was overlaid with light mineral oil. Samples were subjected to 30 cycles (94 °C 2 min, 50 °C 1.5 min, 72 °C 1.5 min). Products were run on a 1% agarose gel and stained with ethidium bromide. Transgenic mice were characterized by the presence of a PCR band corresponding to the junction between the α -lactalbumin 5' flanking region and β -casein gene.

Mouse milking

Mice were separated from their litters for 4 h and then anesthetized (0.01 mL g⁻¹ body weight intraperitoneal injection of 36% propylene glycol, 10.5% ethyl alcohol, 41.5% sterile water, and 12% sodium pentobarbital, 50 mg mL⁻¹). After being anesthetized the mice were injected intramuscularly with 0.3 international units of oxytocin and milked using a small vacuum milking machine.

Gel electrophoresis and western blotting

The mouse milk was examined on a 14% SDS-PAGE reducing gel (Laemmli, 1970) or on a 12.5% Urea-PAGE gel (Swainsgood, 1975). Gels were either stained with Coomassie Brilliant Blue R-250 or transferred onto Immobilon-P membranes (Millipore, Bedford, MA) for western blotting. Immunoblotting was carried out using a rabbit anti-bovine β -casein antibody (Dr Bruce Larson, Urbana, IL) and methods of Jiménez-Flores *et al.* (1989).

Histological analysis of mouse mammary glands

Mammary glands were removed from lactating mice and placed in 10% buffered formalin. The glands were then paraffin embedded, sectioned, affixed to microscope slides and stained with hematoxylin and eosin.

RESULTS

Generation of transgenic mice

Four founder transgenic mice containing the bovine α -lactalbumin/ β -casein gene construct were produced. This gene construct contained approximately 2.0 kb of bovine α -lactalbumin 5' flanking region (–1983 bp to +92 pb from the α -lactalbumin transcription start point) attached to a bovine β -casein genomic clone at position –91 bp from the β -casein transcription start point (Fig. 1). The α -Lac5'/ β -casein construct therefore contained the proximal promoter elements of β -casein and the 5' regulatory elements of α -lactalbumin. This 91 bp of β -casein 5' flanking region has been shown to be transcriptionally inactive in the mammary epithelial cell line CID-9 (Schmidhauser *et al.*, 1992). Gene constructs containing the 91 bp attached to the gene encoding chloramphenicol acetyltransferase (CAT) were unable to drive CAT expression in these cells.

One female founder mouse died before a transgenic line could be established,

however milk samples were obtained from this mouse for analysis. Transgenic lines have been produced from each of the three remaining founder mice and the transgenes exhibit normal Mendelian inheritance (~50% of offspring from a heterozygous transgenic mouse were transgenic).

Production of bovine β -casein in the milk of transgenic mice

Milk from transgenic mice was analysed using SDS-PAGE and western blotting of Urea-PAGE gels. One line of transgenic mice (UW α / β 41) containing the α -Lac5'/ β -casein gene construct produced bovine β -casein up to a concentration of approximately 10 mg mL⁻¹ in their milk (Fig. 2) and two other transgenic lines secreted detectable levels of bovine β -casein in their milk. The concentration of bovine β -casein in all three lines ranged from approximately 1 mg mL⁻¹ to 10 mg mL⁻¹ in the high producing UW α / β 41 line (Table 1). Concentrations of protein were estimated using Coomassie Blue stained gels and the Collage[™] image analysis software (FOTODYNE, New Berlin, WI). This is a crude estimate of bovine β -casein concentration. A more precise measurement could not be made due to the difficulty in getting a consistent milk sample from these animals and also because of the large number of bovine β -casein proteolytic and phosphorylation variants observed. A western blot of the transgenic mouse milk indicated a number of protein bands that reacted with the bovine β -casein antibody (Fig. 3). Since bovine β -casein is a known substrate of plasmin, it is possible that the smaller peptides are proteolytic products of the bovine β -casein. Also, bovine β -casein is known to have a number of phosphorylation variants that may show mobility differences in the urea-gel system.

Fig. 2. Coomassie Blue stained SDS-PAGE gel of bovine β -casein containing transgenic mouse milk. Lane 1: control cow milk. Lanes 2 and 3: line UW α - β 41 mouse milk (two dilutions). Lanes 4, 5 and 6: control mouse milk (samples from three different control mice). Lanes 7 and 8: line UW α / β 41 mouse milk (two dilutions). Arrows indicate location of bovine β -casein.

TABLE 1

Levels of Bovine β -Casein Expression in Mouse Milk and Observed Day of Premature Mammary Involution

<i>Transgenic line no.</i>	<i>Bovine β-casein expression in transgenic mouse milk (mg/mL)</i>	<i>Lactation length (days)</i>
UW α / β 34	1–3	12–20
UW α / β 41	8–12	1–3
UW α / β 59	Not detected	20
UW α / β 73	3–5	10–15

Characteristics of the transgenic mouse milk

The mouse milk containing bovine β -casein exhibited altered physical properties. The milk was much more viscous and more difficult to remove from the gland compared to control mouse milk. This difficulty in removing milk from the gland and the small quantities of milk that could be obtained made it difficult to produce consistent milk samples. For this reason, no accurate measurements of fat, protein and water were possible for milk of the mice producing the high levels of bovine β -casein. The mice producing low levels of the exogenous protein ($\sim 1 \text{ mg mL}^{-1}$) produced milk with fat, protein and water contents similar to control mice. Mice expressing bovine β -casein also underwent a premature involution of the mammary gland. Mice from the UW α / β 41 line which expressed bovine β -casein at concentrations of approximately 10 mg mL^{-1} stopped lactating on day 1–3 of lactation. Mice from the UW α / β 34 line which secreted bovine β -casein at concentrations of approximately $1\text{--}3 \text{ mg mL}^{-1}$ stopped lactating on day 12–18 of lactation, with some finishing a 20 day lactation. However, pups from UW α / β 34 mice that lactated for a normal length of time appeared much smaller

Fig. 3. Western blot of transgenic and control mouse milk. Samples were run on a 12.5% Urea-PAGE gel. Lane 1: partially purified bovine β -casein (Sigma Chemical Co., St Louis, MO). Lanes 2, 3 and 4: control mouse milk (samples from three different control mice). Lanes 5 and 6: transgenic mouse milk (UW α / β 41). Arrows indicate the relative size of endogenously produced bovine β -casein.

than control pups and some UW α / β 34 mice continued to lactate past the typical 20 days if the pups were unable to obtain dry feed and water on their own at day 20 of lactation. The founder transgenic mouse, UW α / β 73, also showed this same early involution on approximately day 12 of lactation (two lactations: approximate average involution day), bovine β -casein concentration in her milk was approximately 3–5 mg mL⁻¹. The premature involution was not observed in the UW α / β 59 line transgenic mice in which bovine β -casein could not be detected. The line UW α / β 34 and line UW α / β 41 mice that stopped lactating before the normal end of lactation went through a normal second pregnancy, started to lactate, but then again underwent the same premature involution. Pups from these transgenic mice were foster nursed to maintain the transgenic line.

Mammary gland morphology of transgenic mice producing bovine β -casein

On day 1 or day 3 after parturition, mammary glands from line UW α / β 41 transgenic mice and from control mice were removed, sectioned and stained to observe morphology. Mammary glands from control mice taken on day 1 of lactation showed normal, slightly distended alveolar structures and leftover milk components within the alveoli could be observed [Fig. 4(b)]. The mammary glands from a day 1 lactating UW α / β 41 mouse were much different as the alveolar structures in these glands were more compact and not as distended as the control alveoli [Fig. 4(a)]. Alveoli from the transgenic glands contained milk components that appeared to be much denser than the components within the control alveoli. Similar differences were observed in the glands on day 3 of lactation as the alveolar structures from the day 3 control gland were more distended and fuller of milk components than day 1 control alveoli [Fig. 5(b)]. However, the day 3 transgenic glands had alveolar structures that were smaller or the same size as those observed in the day 1 transgenic glands [Fig. 5(a)]. Milk components within these small alveoli again appeared very dense. Large ducts within the day 3 transgenic glands became very distended and filled with the dense milk components [Figs 5(a) and 6(a)] while the control large ducts were very irregular in shape and the milk components showed the same appearance as in the control alveoli [Fig. 6(b)]. The UW α / β 41 mice were milked before the glands were removed and no milk could be obtained, yet the glands appeared to be full of milk components after sectioning. In addition, glands obtained from transgenic mice on day 3 of lactation were approximately twice the size of control mice day 3 glands. Parts of the UW α / β 41 transgenic glands showed involution of the mammary alveoli already on day 1 of lactation [Fig. 7(a) and (b)]. The dense milk components remained visible within baskets formed by myoepithelial cells around the alveoli, but very few epithelial cells appeared to be present in this structure and just a small amount of epithelial cell staining occurred in this region of the gland.

DISCUSSION

Transgenic mice containing the α -Lac5/ β -casein gene construct were capable of producing a high concentration of bovine β -casein in their milk. This construct demonstrates the utility of combining genetic control regions of two milk protein

Fig. 4. Mouse mammary glands removed on day 1 of lactation. (a) Transgenic mammary gland section (UW α / β 41). (b) Control mammary gland section. Arrows indicate the alveolar lumen. Bar = 10 μ m.

Fig. 5. Mouse mammary glands removed on day 3 of lactation. (a) Transgenic mammary gland section (UW α /β41). (b) Control mammary gland section. Arrows indicate the alveolar lumen. Bar = 10 μ m.

Fig. 6. Large ducts from day 3 mouse mammary glands. (a) Transgenic mammary gland section (UW α / β 41). (b) Control mammary gland section. Arrows indicate the ductal lumen. Bar = 10 μ m.

Fig. 7. Transgenic mouse (UW α / β 41) mammary gland removed on day 1 of lactation. Transgenic mammary gland sections showing involution of the mammary alveoli on day 1 of lactation. Arrows point to regions of apparent mammary involution. Bar = 10 μ m.

genes to obtain high-level production of a transgene product. By linking mammary-specific enhancer regions of various milk protein genes it may be possible to obtain a very high production level of transgene products and yet maintain the mammary-specificity of the transgene. Similar experiments have been attempted using the rat β -casein promoter and the hormone response elements of mouse mammary tumor virus (Greenburg *et al.*, 1991). In those experiments, the hormone response elements increased transgene expression, but some of the mammary-specificity was lost. Enhancer elements from the milk protein genes may be able to maintain the mammary specificity and in addition increase expression of the gene construct in transgenic animals.

These data are the first report of a transgene product altering the physical properties of milk. This appears to be different from the *milchlos* phenotype that has been observed with the over-expression of whey acidic protein in the mammary gland of transgenic mice (Burdon *et al.*, 1991). In that study, the over-expression of whey acidic protein led to underdeveloped mammary glands that were not capable of synthesizing milk. Gland development in the bovine β -casein-expressing mice seemed normal, but the milk itself was altered. The modified milk characteristics then appeared to induce the premature involution of the mammary gland, and in addition the expression of the transgene increased the viscosity of mouse milk. The viscous milk was unable to be removed from the gland either by the pups or through the use of vacuum aspiration after an oxytocin injection. These properties of the milk and the mammary gland were very similar to results obtained when the α -lactalbumin gene was "knocked out" of mice (Stinnakre *et al.*, 1994). The build-up of milk components within the mammary gland may be causing the premature involution of the gland as it has been shown that the rate of mammary involution increases when milk is not removed from the gland (Larson, 1985). These additional milk components within the gland increase the alveolar pressure which leads to a stoppage of milk synthesis and a break up of the alveolar structure. Since the transgenic mice were able to go through a normal second pregnancy and start another lactation it appeared that no permanent damage was caused by the expression of bovine β -casein and the early involution of the gland. This indicated that the observed phenomenon was similar to a normal involution of the mouse mammary gland.

The altered physical properties of milk observed in this study were not reported to occur in transgenic mice expressing similar levels of caprine β -casein (Persuy *et al.*, 1992). Because the two β -caseins are more than 90% conserved at the amino acid level, these two sets of data may indicate that functional differences or processing variations of the β -casein molecule exist between the cow and the goat and that these differences are associated with the unique behavior of bovine β -casein in mouse milk. A second hypothesis explaining the difference between the bovine and the caprine data centers on the use of the bovine α -lactalbumin 5' flanking region to control β -casein expression. Typically, α -lactalbumin serves as a modulator of lactose production in the mammary gland. Lactose is the major osmotic component in milk and its production is associated with the movement of water into the lumen of the mammary gland. A reduction in the amount of α -lactalbumin produced may cause less lactose to be produced and less water movement into the gland, resulting in a more viscous milk. The α -lactalbumin 5' flanking region controlling the β -casein gene construct may be sequestering necessary transcription factors needed for the production of normal mouse α -lactalbumin and thus causing decreased production of mouse α -lactalbumin and

lactose. The possibility that a transcription factor may be rate limiting has been suggested by a number of authors (Hirst *et al.*, 1990; Wang & Miksicek, 1991; Colgan & Manley, 1992). Studies are underway to examine the functional differences between bovine β -casein-containing mouse milk and control mouse milk with regards to apparent viscosity, composition and the potential bovine β -casein variants observed in the mouse milk.

CONCLUSIONS

Transgenic mice producing a high level of bovine β -casein yielded abnormal milk and stopped lactating before the normal onset of involution. These mice may serve as a useful model to study the mechanisms of mammary involution as well as the functional changes induced by the presence of bovine β -casein in mouse milk. The bovine α -lactalbumin 5' flanking region proved to be a useful regulatory region for driving bovine β -casein production in the milk of transgenic mice. The α -Lac5'/ β -casein gene construct can be used to make mutations in the β -casein gene and then to test the effects of these changes on the physical characteristics of modified bovine β -casein in the milk of transgenic animals.

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