Genetic Modification of Bovine β-Casein and Its Expression in the Milk of Transgenic Mice

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Genomic vectors containing mutant bovine β-casein with putative glycosylation sites were constructed to study the functional properties of glycosylated β-casein and its possible effects in milk. The mutation was performed by PCR-based site-directed mutagenesis. The tripeptide sequence, **Asn-X-Ser,** was generated between Asn_{68} and Asn_{73} in mature β-casein. The resulting β-casein mutants were designated pCJB68 and pCJB6873. pCJB68 carries a substitution of $\mathbf{Ser_{70}}$ for $\mathbf{Leu_{70}}$ ($\underline{Asn}_{68}$-Ser_{69}-$\mathbf{Ser_{70}}$-Pro_{71}), and pCJB6873 carries a substitution of $\mathbf{Ser_{70}}$-$\mathbf{Ser_{71}}$ for $\mathbf{Leu_{70}}$-$\mathbf{Pro_{71}}$ ($\underline{Asn}_{68}$-Ser_{69}-$\mathbf{Ser_{70}}$-$\mathbf{Ser_{71}}$). The two mutated genomic constructs were placed under control of the bovine α-lactalbumin promoter, and lines of mice expressing the pCJB68 and pCJB6873 have been established. The milk from transgenic mice contained bovine β-casein at levels up to 2−3 mg/mL. N-Linked glycosylation of bovine β-casein in the pCJB6873 line was confirmed by peptide-*N*-glycosidase F treatment, but glycosylation of bovine β-casein did not occur in pCJB68 mice. In addition, mouse casein micelles containing glycosylated bovine β-casein showed the largest median diameter and rough outer surface, compared to normal mouse casein micelles and micelles from transgenic milk containing bovine β-casein.

Keywords: *Bovine β-casein; site-directed mutagenesis; transgenic*

INTRODUCTION

Milk proteins, besides being one of the main components in many dairy products, have been used widely in formulated foods, as nutritional supplements or functional ingredients (Morr, 1984). Bovine β-casein is calcium-sensitive, the second most abundant and the most hydrophobic of the caseins. The N-terminal region of bovine β-casein is hydrophilic due to a phosphoserine cluster, while its C-terminal region is very hydrophobic. This amphiphilic nature of β-casein has been correlated with some of its functional properties such as emulsifying, foaming, and gelling. However, some studies show that the functional properties of β-casein can be improved if the hydrophobic/hydrophilic contrast of the molecule is enhanced or if its solubility is improved (Courthaudon et al., 1989; Kato et al., 1992).

Efforts have been made to modify proteins to improve their functionalities using mainly chemical and enzymatic methods (Feeney and Whitaker, 1977; Richardson, 1977; Kinsella and Shetty, 1979; Chobert et al., 1988; Courthaudon et al., 1989; Cayot et al., 1991; Kato et al., 1992). In recent years, the availability of cDNA and genomic DNA encoding food proteins has opened the door for making genetic modifications, which are very specific compared to chemical and enzymatic ones. Consequently, genetic modifications at the molecular level now play an important role in understanding structure/function relationships of proteins. In addition, it becomes possible to engineer proteins with desired functional properties.

All of the major milk protein genes have been isolated and sequenced. The availability of milk protein genes has made genetic modification of milk proteins possible. Efforts have been made to enhance the functional properties of milk proteins (Lee et al., 1993; Simons et al., 1993) and to improve the nutritional quality (Oh and Richardson, 1991) of some milk proteins. For example, β-casein has been modified to resist chymosin cleavage (Simons et al., 1993), β-lactoglobulin has been altered to improve gelling properties (Lee et al., 1993), and methionine residues have been added to κ-casein to improve its amino acid balance (Oh and Richardson, 1991).

Transgenic mice have been used as an alternative model system for dairy farm animals to study the expression and regulation of milk protein genes and as a mechanism to predict possible effects in the milk of transgenic cattle (Simons et al., 1987; Vilotte et al., 1989; Yom et al., 1991; Plantenburg et al., 1991; Soulier et al., 1992; Bleck and Bremel, 1994). In this work transgenic mice were generated to study the functional properties of glycosylated β-casein and its possible effects in milk.

Bovine β-casein mutants were designed to increase the amphiphilic characteristics of bovine β-casein by placing glycosylation signals on its N-terminal region that lead to enzymatic glycosylation *in vivo*. That is, pCJB68 carries a substitution of $\mathbf{Ser_{70}}$ for $\mathbf{Leu_{70}}$, and pCJB6873 carries both the $\mathbf{Ser_{70}}$ for $\mathbf{Leu_{70}}$ substitution and a $\mathbf{Ser_{71}}$ for $\mathbf{Pro_{71}}$ substitution.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis using the polymerase chain reaction (PCR) was performed to alter specific nucleotides within a structural gene, the A^2 genetic variant of bovine β-casein (Jiménez-Flores et al., 1990). The mutagenic primers complementary to the *Apa*I site in exon 7 of β-casein (primer N68, 5′ CCC TTC CCT GGG CCC ATC

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CCT AAC AGC **TCC** CCA CAA AAC ATC CC 3′; primer N6873, 5′ CCT TTC CCT GGG CCC ATC CCT AAC AGC **TCC TCA** CAA AAC ATC CC 3′) and another primer in the opposite direction (5′ AGC CGG ATC CTC TTA GAC AAT AAT AGG G 3′) were used for PCR amplification. PCR was performed using Vent (exo-) DNA polymerase (New England BioLabs, Beverly, MA). Amplification was carried out as follows: 100 ng of template DNA, 10 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100 (pH 8.8, 25 °C), 20 μM of each dNTP, 50 μM of each primer, and 2.5 units of Vent (exo-) DNA polymerase in a final volume of 100 μL. These samples were overlaid with 100 μL of mineral oil (Sigma Chemical Co., St. Louis, MO) and subjected to one cycle each of 97 °C for 2 min, 55 °C for 1 min, and 72 °C for 1 min and then linked to 30 cycles of denaturation (97 °C, 1 min), annealing (55 °C, 1 min), and extension (72 °C, 1 min) using a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, PA). Additional extension (72 °C, 2 min) was done to ensure that the final extension step was complete. The PCR products were analyzed on an agarose gel containing 2% Nusieve, 1% SeaKem agarose (FMC, Rockland, ME), and 0.5 μg of ethidium bromide per milliliter in Tris−acetate buffer (pH 8.0; 40 mM Tris−acetate, 1 mM EDTA). Two mutant β-casein fragments (464 bp) generated by PCR amplification were subcloned into pCR II (Invitrogen, San Diego, CA) vector with single 3′ T-overhangs at the insertion site and conformed by DNA sequencing prior to cloning into β-casein cDNA plasmid.

Plasmid Construction. DNA manipulation was performed according to standard techniques (Sambrook et al., 1989) The pα-lac/β-CN genomic vector (Bleck et al., 1995) contains the α-lactalbumin promoter, which is mammary specific, and was used for the construction of mutant β-casein genomic vectors. The strategy for the construction of the expression vectors is described in Figure 1. The pα-lac/β-CN was digested with *Apa*I, *Stu*I, and *Avr*II to generate *Apa*I−*Stu*I fragments (6.8 kb). The resulting *Apa*I−*Stu*I fragments were then ligated with inserts harboring *Apa*I and *Stu*I sites from cDNA of mutant β-casein. The residual portion of pα-lac/β-CN, corresponding to 6.6 kb, was isolated by digestion with *Apa*I and *Pvu*I, followed by ligation of 7.1 and 6.6 kb fragments to complete β-casein mutant genomic vectors (13.7 kb). The resulting genomic vectors are designated pCJB68 and pCJB6873, respectively. These mutants were verified by enzyme digestion with *Alu*I, which cleaves at the modified sites, and DNA sequencing.

Isolation of α-Lactalbumin/Mutated β-Casein Gene Construct for Microinjection. The α-lactalbumin/mutated β-casein gene constructs containing 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 were digested out of the plasmid clone (pα-lac/β-CN) using the restriction enzyme *Hha*I. This digested DNA was separated on a 1.0% low melting point agarose gel (FMC) and the band containing the α-lactalbumin/β-casein construct was purified from the gel slice using the SpinBind DNA recovery system (FMC). The resulting DNA fragments were diluted in TE buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 7.5)] for microinjection.

Generation of Transgenic Mice. Mature C57BL/6J X SJL/J F1 (B6 SJLF1) female mice were superovulated using PMSG and hCG and mated with B6 SJLF1 males to yield fertilized embryos for pronuclear microinjection. The embryos were microinjected with the DNA construct, and 40 normal-appearing one-cell embryos were then transferred to pseudo-pregnant recipients (University of Illinois, Transgenic Animal Facility). The resulting founder transgenic mice were then continually backcrossed with the ICR outbred strain of mice. The transgenic mice were maintained as heterozyogotes for the transgene throughout the milk analysis.

Identification of Transgenic Mice. DNA was extracted from a portion of mouse tail using the method described by Hogan et al. (1986). Polymerase chain reaction (PCR) was performed using 10 mL of 10× PCR reaction buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.8), 15 mM $MgCl_2$, 1% Triton X-100], 200 mM of each dNTP, 1.0 mM of each primer (primer 1, 5′ CTCTTCCTGGATGTAAGGCTT 3′; primer 2, 5′ CAAAGTAGAGGACAAGAAGT 3′; primer 3, 5′ ATGTTTGTAACTCTCTCTGT 3′; primer 4, 5′ ATGCCAACA AAGAGACACT 3′; primers 1 and 2 amplify the junction between the α-lactalbumin 5′ flanking region and the β-casein gene construct, which are unique to transgenic mice, while primers 3 and 4 amplify the mouse α-lactalbumin 5′ flanking region), 1 unit of Taq DNA polymerase (Promega, Madison, WI), and 1 μg of genomic DNA. The final volume was adjusted to 100 μL with double-distilled sterile water, and the reaction was overlaid with mineral oil. Samples were subjected to 30 cycles (94 °C, 2 min; 50 °C, 2 min; 72 °C, 2min) in a DNA thermal cycler (Perkin-Elmer Cetus). PCR products were separated on a 3% agarose gel (2% Nusieve, 1% SeaKem) (FMC) and stained with ethidium bromide. Transgenic mice were identified by the presence of a PCR band corresponding to the junction of the α-lactalbumin and β-casein 5′ flanking regions.

Mouse Milking. Mice were separated from their litters for 4 h and then anesthetized [intraperitoneal injection of avertin (2.5% 2,2,2-tribromoethanol in 2.5% *tert*-amyl alcohol, Aldrich Chemical Co., Milwaukee, WI) at a dose of 0.018 mL/1 g of body weight). After being anesthetized, the mice were injected intramuscularly with 0.3 IU of oxytocin and milked using a small vacuum milking machine.

Gel Electrophoresis and Western Immunoblotting. Transgenic mouse milks were skimmed by centrifugation at 3000*g* for 15 min at 4 °C and then were filtered through an 0.8 μm membrane (Nalge Co., Rochester, NY). The skimmed mouse milk was separated on 12% SDS−PAGE reducing gels (Laemmli, 1970) and Western-blotted according to the method of Jiménez-Flores et al. (1990). The transferred PVDF membrane was incubated with a rabbit anti-bovine β-casein antibody (Dr. Bruce Larson, Urbana, IL) at a 1:3000 dilution, followed by incubation with a 1:5000 dilution of horseradish peroxidase-conjugated mouse anti-rabbit IgG. The results were visualized by means of a developing solution of 3,3′-diaminobenzidine (Sigma).

Deglycosylation and Dephosphorylation. Deglycosylation was performed according to the methods of Oxford GlycoSystem (Rosedale, NY). Skimmed transgenic mouse milks (300 μg) were denatured by heating at 100 °C for 2 min in 10 μL of buffer [phosphate/EDTA (pH 7.5−8) containing 0.5% SDS, 5% β-mercaptoethanol]. After cooling, 10% Triton X-100 was added at 5 times the concentration of the SDS prior to the addition of 2 units of peptide-*N*-glycosidase F (Oxford GlycoSystem) and incubated for 18 h at 25 °C. The treated samples were analyzed using SDS−PAGE and followed by Western immunoblot. For dephosphorylation of bovine β-casein, bovine β-casein was first partially purified using a Mono S HR 5/5 column (Pharmacia, Uppsala, Sweden), and the purified protein fraction was dephosphorylated using calf intestine phosphatase (CIP) (Boehringer Mannheim, Indianapolis, IN). The sample (500 ng) was resuspended in alkaline phosphatase buffer [20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM $MnCl_2$], and 0.1 unit of CIP was added. The reaction mixture was incubated at 37 °C for 30 min. For negative control, samples were treated without CIP and analyzed by urea−PAGE (Medrano et al., 1989).

Determination of Casein Micelle Size and Its Surface Conformation. Size of mouse casein micelles containing altered and normal bovine β-casein was determined using a particle size analyzer (Horiba LA-900; Horiba Instruments, Irvine, CA) by NSC Technologies Laboratory. Milk from two mice of each geneotype was combined for micelle analysis. The instrument test cell was rinsed with water and milk permeate, then more permeate was added, and the instrument was blanked. Skimmed mouse milk (not chilled) was added drop by drop to the permeate while the permeate was circulating through the instrument cuvette until the proper turbidity was obtained for measurement. The sample was mixed and circulated for 20 s before measurements were taken. Particle size analyses were performed in duplicate, and the results were calculated on a particle number and particle volume basis. The outer surface of casein micelles was analyzed using atomic force microscopy (NSC Technologies Laboratory, Mt. Prospect, IL).

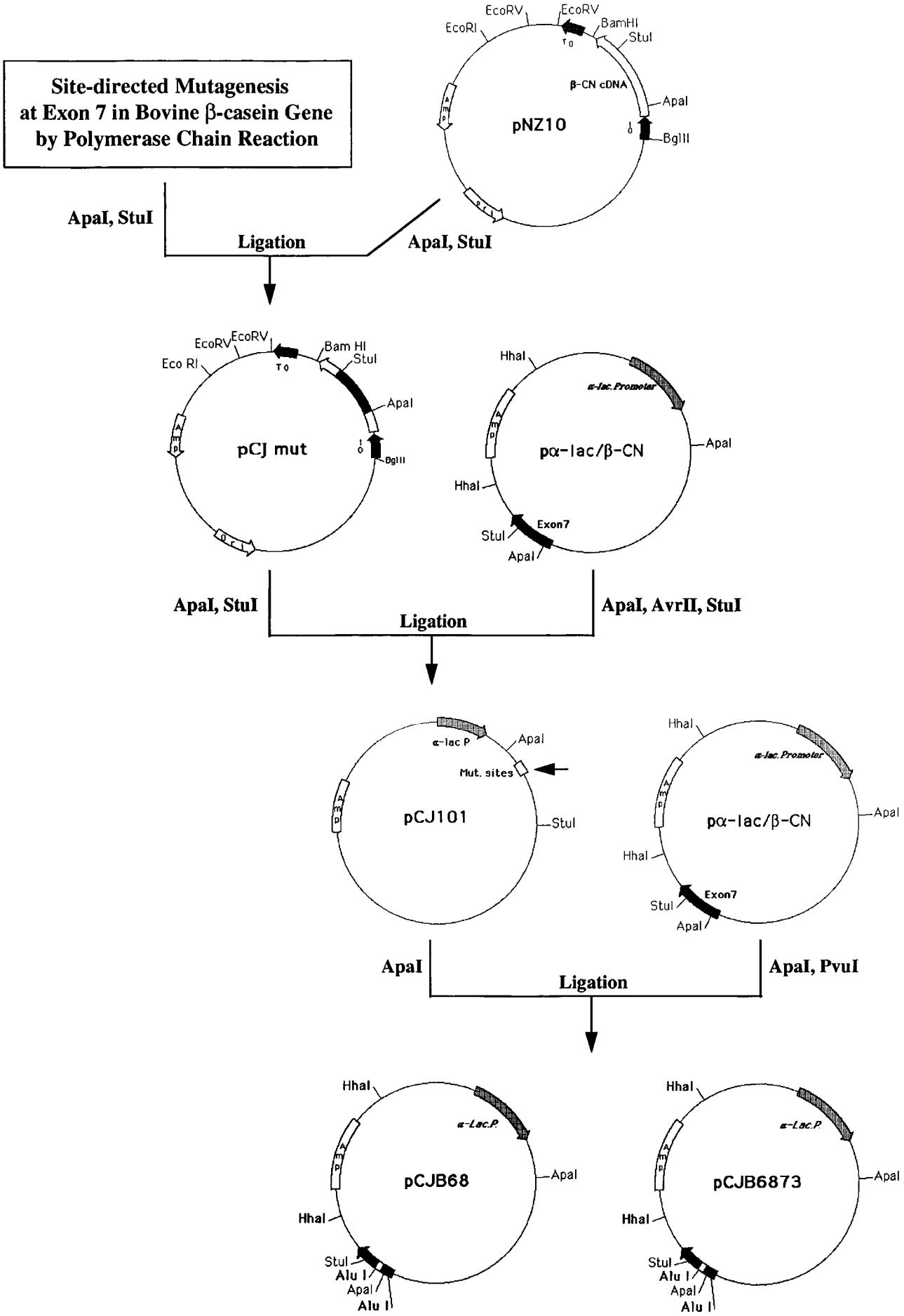

Figure 1. Construction of bovine β-casein genomic vectors carrying putative glycosylation sites.

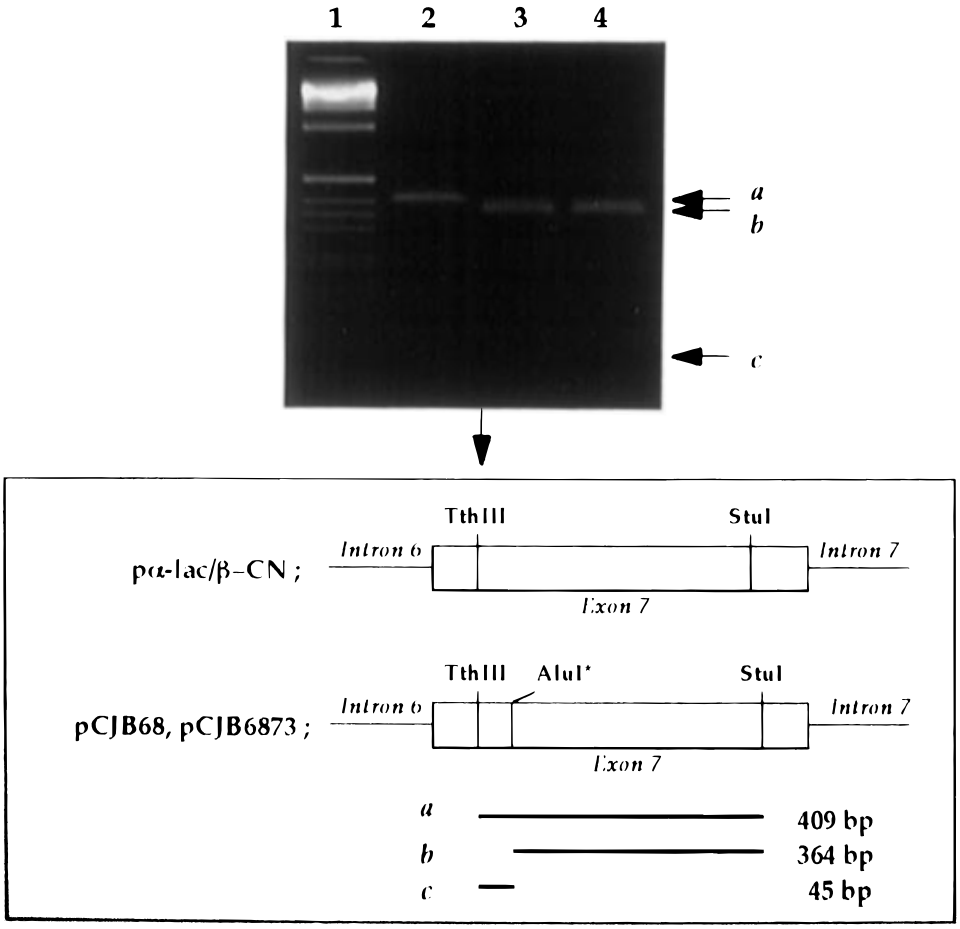

Figure 2. *Alu*I enzyme digestion of mutant bovine genomic vectors. Genomic expression vectors carrying putative glycosylation sites were digested with *Apa*I and *Stu*I, and then resulting *Apa*I/*Stu*I fragments (a portion of exon 7) were digested with *Alu*I for confirmation of mutated sites. *Alu*I* indicates new *Alu*I restriction enzyme site caused by mutation in pCJB68 and pCJB6873 constructs. Samples on gel are as follows: 1 kb DNA ladder (lane 1), pα-lac/*β*-CN carrying wild-type bovine *β*-casein gene (lane 2), pCJB6873 (lane 3), and pCJB68 (lane 4).

RESULTS

Plasmid Construction. The tripeptide sequence **Asn-X-Ser** was generated between Asn_{68} and Asn_{73} in mature *β*-casein by cloning two different mutant fragments into pα-lac/*β*-CN (Bleck et al., 1995) mammary expression vector using *Apa*I/*Stu*I restriction enzyme sites at exon 7. The resulting vectors were designated pCJB68 and pCJB6873, respectively. pCJB68 carries a substitution of Ser_{70} for Leu_{70}, and pCJB6873 carries both a substitution of Ser_{70} for Leu_{70} and the Ser_{71} for Pro_{71} substitution. The strategy for *β*-casein genomic vector construction is outlined in Figure 1. Mutant *β*-casein genomic genes are controlled by the bovine α-lactalbumin 5′ regulatory elements and promoter which can direct the synthesis of bovine *β*-casein to mammary gland (Bleck et al., 1994). The presence of mutated sites in genomic vectors was confirmed by *Alu*I enzyme digestion (Figure 2). The constructed genomic vectors were digested by *Hha*I enzyme to remove the unnecessary portion derived from bacterial plasmid and then isolated for microinjection (Figure 3).

Generation of Transgenic Mice. Two different genomic constructs were microinjected into pronuclei of fertilized one-cell mouse zygote (University of Illinois, Transgenic Animal Facility). Transgenic mice were identified using the polymerase chain reaction. Two founder transgenic mice were produced from the pCJB68 construct; one was designated α*β*68-14 and the other, α*β*68-30. In addition, one founder transgenic mouse was produced from the pCJB6873 construct and called α*β*6873-29. Transgenic mice were then continually backcrossed into the ICR outbred strain of mice.

Production of Mutant *β*-Casein in Transgenic Mice. After parturition, transgenic mouse milk was collected using a small vacuum machine from three transgenic mouse lines (α*β*68-30 and α*β*68-14, and α*β*6873-29 line). Skimmed mouse milks were subjected to 12% SDS−PAGE, followed by Western immunoblotting. As shown in Figure 4, bovine *β*-casein was not detectable in nontransgenic mouse milk (lane 2). α*β*68 lines (lane 4) showed the same *β*-casein band as a control bovine *β*-casein containing transgenic mouse milk UWα*β*34 line (Bleck et al., 1995), transgenic mouse milk containing wild-type bovine *β*-casein (lane 3), indicating that glycosylation did not occur. However, milk from the α*β*6873 line (lane 5) showed a higher molecular weight band than that of normal bovine *β*-casein, which indicates that the *β*-casein produced was in part glycosylated at Asn_{68}.

Total bovine *β*-casein produced by the α*β*6873 line was estimated using the Collage image analysis system (Fotodyne, New Berlin, WI) after Western blotting with bovine *β*-casein antibodies. Bovine *β*-casein concentration increased during the lactation period, and this is considered to be the effect of α-lactalbumin promoter according to a previous study (Bleck et al., 1994) using the same promoter. The estimated total bovine *β*-casein produced in milk of mice from the α*β*6873 line was about

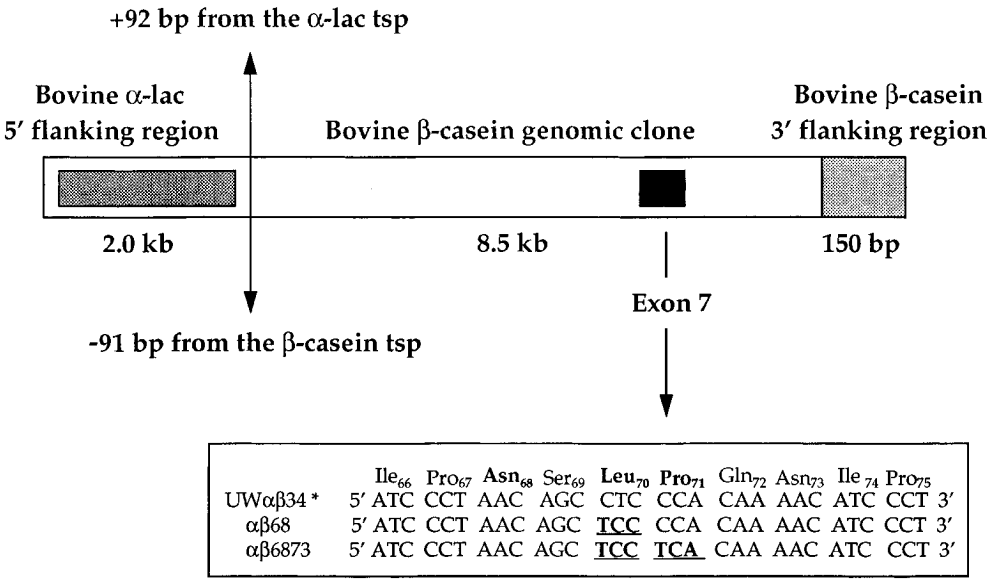

Figure 3. Microinjected bovine α-lactalbumin/β-casein gene construct. Two mutant genomic vectors (pCJB68 and pCJB6873) were digested with *Hha*I and then were purified for microinjection into mouse pronuclei. UWαβ34* indicates wild-type bovine β-casein.

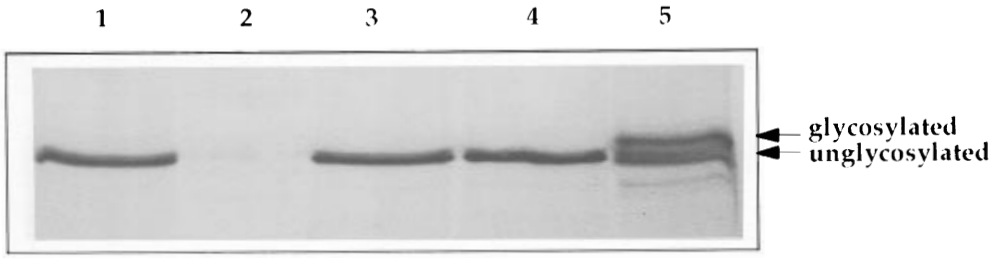

Figure 4. Western immunoblot of transgenic mouse milks. After 12% SDS−PAGE, proteins on the gel were transferred to PVDF membrane (Millipore), followed by immunostain with bovine β-casein antibodies. Lane 1, bovine β-casein control; lane 2, normal mouse milk; lanes 3−5, transgenic mouse milks [lane 3, UWαβ34 (wild-type bovine β-casein gene); lane 4, αβ68 (glycosylation site at an Asn_{68}); lane 5, αβ6873 (glycosylation site at Asn_{68} and a Ser_{71} substitution for Pro_{71})].

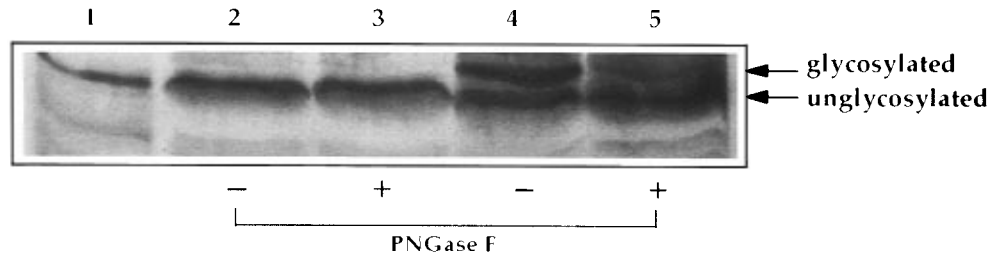

Figure 5. Western immunoblot of deglycosylated transgenic mouse milks. Samples were treated with peptide-*N*-glycosidase F (Oxford GlycoSystem), which cleaves only N-linked oligosaccharides. Lane 1, bovine β-casein standard; lanes 2 and 4, UWαβ34 and αβ6873; lanes 3 and 5, deglycosylated UWαβ34 and αβ6873.

3 mg/mL. The 3 mg/mL of total bovine β-casein was made up of 0.9 mg/mL unglycosylated β-casein and 2.1 mg/mL glycosylated β-casein. This would indicate that about 70% of the bovine β-casein produced was glycosylated. However, quantification of glyco-β-casein using β-casein antibody may result in underestimation because glycosylated β-casein has less affinity to bovine β-casein antibody than nonglycosylated β-casein (Storring, 1992).

Verification for N-Linked Glycosylation of Bovine β-Casein. To confirm that the sugar moiety had been attached to an Asn residue, skimmed transgenic mouse milks were treated with peptide-*N*-glycosidase F (PNGase F). Figure 5 shows deglycosylated bovine β-casein after PNGase F treatment, which was then detected by Western immunoblot. Bovine β-casein from control transgenic mouse milks (UWαβ34) remained unchanged (Figure 5, lanes 2 and 3), but the previously detected higher molecular weight band from αβ6873 was deglycosylated and shifted to control bovine β-casein size (lane 5). Results from the PNGase F treatment indicate that glycosylation of bovine β-casein is via N-glycosylation of the amino group of asparagine. However, we have yet to confirm if the sugar is attached specifically at Asn_{68}.

Physical Changes of Mouse Casein Micelle. Casein micelle size analysis was performed using a particle size analyzer. Casein micelle size was analyzed in duplicate, and the results were calculated on a particle number and volume basis (Figure 6). Median casein micelle size of control transgenic mouse milk containing wild-type bovine β-casein was similar to that

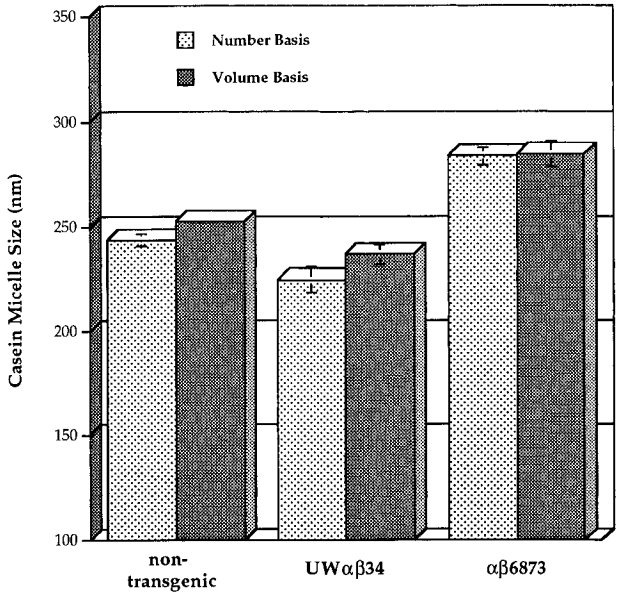

Figure 6. Median casein micelle size comparison in the milk of transgenic mice. Casein micelle size was analyzed on the basis of particle volume and number. Nontransgenic, normal mouse milk without bovine β-casein; UW$\alpha\beta$34, transgenic mouse milk with wild-type bovine β-casein; $\alpha\beta$6873, transgenic mouse milk with glycosylated bovine β-casein.

of nontransgenic mouse milk, whereas casein micelles containing glyco-β-casein showed a larger median micelle size.

To see any casein micelle conformational changes due to the addition of bovine β-casein or glyco-β-casein, transgenic mouse milk and nontransgenic mouse milk were scanned using atomic force microscopy. This technique is able to scan the surface of the micelle without causing any damage to the micelle structure due to sample preparation. According to the results from atomic force microscopy analysis (Figure 7), casein micelles from nontransgenic mouse milk and UW$\alpha\beta$34 milk have a smooth outer surface, whereas $\alpha\beta$6873 micelles have a rough outer surface. In addition, the overall shape of individual casein micelles among samples seemed to be spherical and there was no significant change of shape among the milks. The change of casein micelle surface was observed only in casein micelles containing glyco-β-casein.

DISCUSSION

Bovine β-casein expressing genomic vectors carrying putative glycosylation sites have been constructed to produce transgenic animals. The choice of mutation sites within the β-casein gene was based on its gene structure, hydrophobic and hydrophilic balance of mature β-casein, and location of Asn residues available. That is, bovine β-casein is known to have a hydrophilic region near its N terminus and a hydrophobic region at its C terminus (Swaisgood, 1992). This amphiphilic characteristic has been related with functionalities of bovine β-casein in a number of processes. Therefore, β-casein's mutation sites were designed to increase the hydrophilicity of the N-terminal region through N-linked glycosylation without disrupting the hydrophobicity of the C-terminal region.

After microinjection into mouse embryos, $\alpha\beta$68 and $\alpha\beta$6873 transgenic lines have been generated. According to Western blot analysis and deglycosylation using PNGase F, $\alpha\beta$68 and $\alpha\beta$6873 transgenic milks were

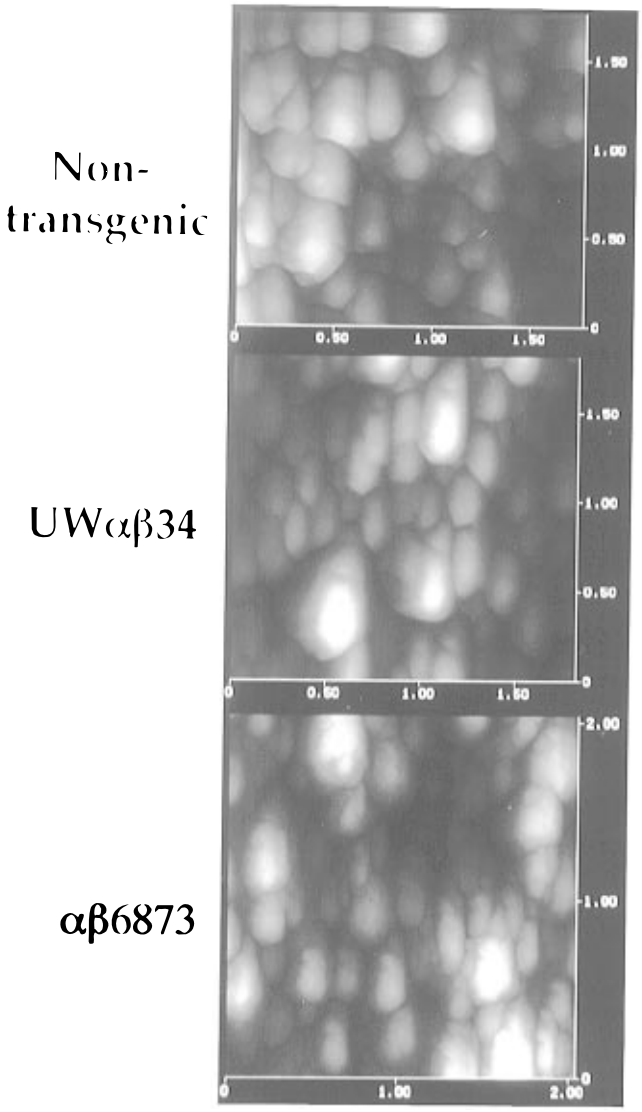

Figure 7. Comparison of casein micelle outer surface. Nontransgenic, normal mouse milk; UW$\alpha\beta$34, transgenic mouse milk containing wild-type bovine β-casein; $\alpha\beta$6873, transgenic mouse milk containing glycosylated bovine β-casein. Casein micelles were scanned by atomic force microscopy. (Picture scale unit: μm).

found to contain bovine β-casein. The mutant β-casein from $\alpha\beta$68 has not been glycosylated, whereas that from $\alpha\beta$6873 was N-glycosylated. One reason for the failure of glycosylation at Asn_{68} (-**Asn_{68}**-Ser_{69}-**Ser_{70}**-**Pro_{71}**-) might be the action of Pro_{71}; Roitsch et al. (1989) and Bause (1983) demonstrated that proline residues at the C terminus of the sequon inhibited N-glycosylation of Asn residues. In addition, $\alpha\beta$6873 carrying Ser_{71} in place of Pro_{71} (-**Asn_{68}**-Ser_{69}-Ser_{70}-**Ser_{71}**-Gln_{72}-Asn_{73}-) was N-glycosylated. This indicates the involvement of Pro_{71} in inhibition of N-glycosylation of Asn_{68}. However, besides inhibition by Pro, factors such as sensitivity of the glycosylation signal between Asn-X-Ser and Asn-X-Thr (Bause, 1984; Gavel et al., 1990; James et al., 1995) and accessibility of glycosylation enzymes to the Asn residue due to the local environment around glycosylation signals (Baenziger et al., 1974; Gosner et al., 1980; Ward et al., 1980) cannot be excluded as mechanisms preventing glycosylation.

The fact that only approximately 70% of the mutant β-casein was glycosylated in the $\alpha\beta$6873 line of mice can potentially be explained by a number of hypotheses.

First, the N-linked glycosylation machinery may be limiting in the mouse mammary gland, causing only a portion of the mutant β-casein to be glycosylated. A second possibility is that the mutant glycosylation site is not accessible in some molecules due to protein structure or because the β-casein has already been packaged in the normal micellar conformation, excluding any enzymes for glycosylation.

The same mutant bovine β-caseins have been produced using a yeast expression system (Choi et al., 1996) and showed consistent results with transgenic mice. As in the transgenic mice, glycosylation of Asn_{68} did not occur using the pCJB68 construct but did occur when the pCJB6873 construct was expressed in yeast.

Bovine β-casein (A^2 genetic variant) is a phosphoprotein that contains five phosphoseryl groups at its N-terminal region. Those phosphates are involved in formation of casein micelle structure by calcium phosphate bridges and are considered to be important for carrying mineral ions such as Ca^{2+} and Zn^{2+} (Swaisgood, 1992). According to urea−PAGE analysis after calf intestinal phosphatase treatment, glycosylated ($\alpha\beta$6873) β-caseins treated with CIP showed the same mobility as native bovine β-casein treated with CIP (data not shown). This indicates that bovine β-casein including glyco-β-casein produced in the milk of transgenic mice was phosphorylated to the same degree as endogenous bovine β-casein.

Particle size analysis showed that $\alpha\beta$6873 milk had larger median casein micelle size compared to nontransgenic mouse milk and control bovine β-casein containing mouse milk (UW$\alpha\beta$34). This could be due in part to the effect of glycosylated β-casein. That is, the hydrophilic group of sugar residues could increase water holding capacity, and steric hindrance or space occupied by sugar residues attached to the Asn residue may play an important role in increasing casein micelle size. In addition, the glycosylated β-casein may migrate to the casein micelle surface and change the normal surface structure dictated by κ-casein. We could not conclude whether glyco-β-casein in milk was associated with mouse casein micelles or remained as a monomer without association under normal conditions. However, on the basis of atomic force microscopic images of casein micelles, glyco-β-casein seemed to be associated with mouse casein micelles because the outer surface of casein micelles from milk containing glyco-β-casein was modified. As shown in Figure 7, casein micelles from $\alpha\beta$6873 had rough outer surfaces, whereas outer surfaces of nontransgenic and UW$\alpha\beta$34 micelles were smooth. Further studies will be required for localization and distribution of glyco-β-casein in casein micelle structure. In addition, the physical and chemical properties of purified glyco-β-casein produced by mice are being examined.

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