Alterations of the Physical Characteristics of Milk from Transgenic Mice Producing Bovine κ-Casein

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ABSTRACT

κ-Casein is the protein fraction of milk that allows formation of micelles and determines micelle size and function, thus affecting many of the physical characteristics of milk. Several lines of transgenic mice were generated bearing the B allele of the bovine κ-CN gene under the control of the regulatory sequences of the caprine β-CN gene that specifically directed expression of bovine κ-CN to the lactating mammary tissue of these mice. High expression of bovine κ-CN protein was observed in the lines studied; the total level of protein in milk was not significantly affected. A high degree of conservation in the amino acids involved in the predicted three-dimensional structure exists between murine and bovine κ-CN. Milk from transgenic lines expressing high bovine κ-CN had a significantly smaller micelle size than did control milk. Therefore, bovine κ-CN appears to have effectively participated in assembly of murine casein micelles. There was no effect on the time of rennet coagulation, but the association was significant between the milk of transgenic lines and the production of a stronger curd in rennet-induced gels. We conclude that bovine κ-CN is an appropriate candidate for transgenic technology that would increase the ratio of κ-CN to the calcium-sensitive caseins, therefore affecting the physical properties of the colloidal casein suspension.

(**Key words**: transgenic mice, bovine κ-casein, micelle size, gel strength)

INTRODUCTION

Several reviews (2, 6, 20, 21, 29, 31) have been published over the last decade that discuss the potential application of transgenic technology for the modification of milk protein. One potentially useful alteration to bovine milk is an increase in κ-CN content, which affects thermal stability and gelation properties (20, 21).

The major proteins in milk, the caseins, are aggregated into large micelles containing calcium and phosphate in colloidal suspension in milk. Assembly of the caseins into micelles allows milk to have high protein concentration and low viscosity. The structure and physical stability of the micelles govern many of the complex properties of milk and its industrial uses. A number of models for micelle structure have been proposed [for review, see (12, 14)]. In the most accepted model, the micelles are composed of calcium-sensitive caseins (α_{s1}-, α_{s2}-, and β-CN in bovine milk), which associate with one another, form the interior of the micelles, and interact with the calcium and κ-CN that are predominant on the surface of the micelle (40). The protein that allows the formation of micelles and determines micelle size and function is κ-CN, which differs from other caseins in that it is soluble over a wide range of calcium ion concentrations and has a hydrophilic carboxy-terminal region. κ-Casein is cleaved by chymosin (or rennet) during the manufacture of cheese to give a milk gel of aggregated micelles, which then shrinks to exude whey.

The presence of additional unmodified κ-CN may reduce micelle size (8, 42), thereby increasing the thermal stability of CN aggregates in milk and reducing the danger of coagulation and gelation in various milk products during the sterilization process of fluid milk (13). Smaller, more homogeneous micelle size could also have other effects on cheese making. Bovine κ-CN has two common genetic variants, κ-CN A and κ-CN B. The B allele of κ-CN results in an elevated concentration of κ-CN in bovine milk (44) that is consistent with the κ-CN genotype effect (BB is larger than AB, which is larger than AA) on the proportion of κCN in the total casein fraction of milk (27, 33). The milk of cows with κ-CN A genotype has a greater proportion of large micelles, and milk from cows genotyped κ-CN B has a smaller, more homogeneous distribution of micelle size (30). The

Received August 28, 1995.
Accepted January 17, 1996.
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smaller micelle diameter results in a larger micelle surface area, allowing the formation of a firmer and more consistent curd. This dense coagulum retains a greater proportion of solids in the curd, resulting in higher cheese yield (25, 37, 38).

To date, our knowledge of the effects of κ-CN has been based on comparisons of milk from cows with κ-CN A and B genotypes or on the addition of κ-CN to milk. This paper describes the changes produced in the functionality of milk from transgenic mice that express a bovine κ-CN genetic variant B transgene (15). The transgenic protein, when produced in the mouse milk, has the same molecular mass and immunoactivity with polyclonal antibodies as does the endogenous bovine κ-CN (15). Hence, these transgenic lines provide an appropriate in vivo model for studying the effect of overexpression of κ-CN on micelle size, rennet clotting time, and gel strength.

MATERIALS AND METHODS

Transgenic Mice

Three transgenic lines of mice (BC-31, BC-67, and BC-7) containing bovine κ-CN cDNA under the control of the caprine β-CN 5′ promoter elements and 3′ flanking regions were used in this study (15). Transgene copy number and expression levels for these lines are listed in Table 1. Mice were housed in a facility for animal care approved by the American Association for the Accreditation of Laboratory Animal Care. Mice were kept in a room maintained at 21 ± 1°C, under a light regimen of 14 h/d, and with free access to water and feed (Purina Mouse Chow 5015; Purina Inc., St. Louis, MO).

Mouse Milking, Preliminary Processing, and Total Protein Measurement

Transgenic and nontransgenic female mice in first lactation, and with 9 or 10 pups, were milked at d 5, 10, and 15 of lactation. Before milking, females were separated from their pups for at least 3 h and injected intraperitoneally with 0.2 IU of oxytocin (Sigma Chemical Co., St. Louis, MO). Collection of milk was carried out as previously described (24). A 1- to 1.5-ml milk sample was obtained from each mouse. When possible, milk was collected from all 10 glands. Fresh milk samples were used in all milk functionality assays.

Skim milk was prepared by centrifugation (14,000 rpm) at 4°C for 5 min in an Eppendorf® microfuge (Brinkmann Instruments Inc., Westbury, NY) and then placed on ice for 5 min. Protein content of milk from 5 control mice and 5 transgenic mice per line at d 5, 10, and 15 of the first lactation was determined by a colorimetric assay based on the Bradford dye-binding procedure (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA). Bovine casein was utilized to generate the standard curves used in the protein quantification.

TABLE 1. Bovine κ-CN expression and micelle size in the milk of transgenic mice at d 10 of lactation.

Mouse line	Bovine κ-CN in milk[1]			Micelle diameter			Range in mean micelle diameters[2]
	— (mg/ml) —		(no.)[3]	— (nm) —		(no.)[3]	(nm)
	$\bar{X}$	SD		$\bar{X}$	SD		
Control	. . .	. . .	. . .	259[a]	9	9	250–274
BC-7							
Hemizygous	0.9	1.4	5	245[ab]	22	5	207–263
Homozygous	1.7	0.8	5	242[ab]	27	5	198–269
BC-31							
Hemizygous	2.6	1.0	5	227[bc]	15	8	202–242
Homozygous	3.8	2.1	5	200[d]	13	8	176–217
BC-67							
Hemizygous	2.9	0.9	5	222[c]	11	8	208–237
Homozygous	3.7	2.1	5	191[d]	26	8	141–211

[a,b,c,d]Means with no common superscript letter differed (ANOVA and Duncan multiple range test; $P < 0.05$).

[1]From Gutiérrez et al. (15).

[2]Minimum and maximum values.

[3]Number of animals analyzed for each line.

Micelle Size Analysis

Micelle sizes were measured in fresh skim milk samples from lactating mice at d 10 in eight separate replicates using a particle size analyzer with a single modular light-scattering system (Microtrac UPA; Leeds and Northrup, North Wales, PA). For each milk sample, casein micelle size was calculated as the mean diameter (nanometers) of the area distribution of the particles in solution obtained from two 180-s scans and was compared with the micelle size of control, nontransgenic mice. Mean diameter = $\Sigma V_i / \Sigma(V_i/d_i)$, where V = volume percentage in channel size, and d = channel diameter (width of path of light).

Rennet Clotting Time

One milliliter of fresh whole milk was gently mixed with 50 μl of chymosin [1:20 (vol/vol) dilution (double-strength) Chymax™; Dairy Ingredients Division, Pfizer Inc., Milwaukee, WI)/milk], placed in a 35°C shaking incubator, and checked for formation of curd at 5-min intervals. The rennet clotting time was the time that had passed until a solid gel formed in the bottom of the test tube without any remaining liquid phase. Six mice per line (3 homozygous and 3 hemizygous) were analyzed at d 10 of lactation. Duplicate measurements were made on each mouse.

Gel Strength Analysis

A controlled stress rheometer (Carri-Med CSR Model CSL 100, version 5.3 software; T.A. Instruments, New Castle, DE) was used to evaluate the overall firmness of casein gels induced by rennet. To obtain sufficient fresh milk for studies on gel strength, milks from 2 mice of the same genotype and at the same stage of lactation were pooled. Unrefrigerated whole milk (1.5 g) with added rennet (115 μl; double-strength Chymax™; 1:13, vol/vol) was placed on the rheometer under a 4-cm diameter plate. The temperature was increased and held at 32°C for 90 min for formation of the gel and then decreased and held at 20°C for 30 min before measurements were taken (35). Dynamic oscillatory measurements were carried out (over the range of 100 to 1000 dyne·cm at a frequency of 1 Hz). The $|G^*|$ (a measure of the overall firmness of the gel) and tan δ (the tangent of the phase shift, a measure of the degree of viscoelasticity and the type of bonds present) were measured (1). Three sets of measurements were taken for each gel that was formed. Milks from 6 control and 8 transgenic mice (4 homozygous and 4 hemizygous) from each transgenic line were analyzed.

Statistical Analysis

The particle size and total protein measurements were analyzed by ANOVA. Duncan's multiple range tests were used to evaluate the differences between means. Mean differences of rennet clotting time and gel strength between control and transgenic mice were analyzed using independent Student's *t* test.

RESULTS AND DISCUSSION

Homology Between Bovine and Murine κ-CN

Although the structure, properties, and behavior of nonbovine caseins and casein micelles were the same as bovine micelles in the 19 species studied [review, (36)], no in vivo studies have established the possible interaction among κ-CN from different species. To predict whether the bovine κ-CN expressed in transgenic mice could participate in murine micelle assembly and influence properties of the milk protein system, we examined the degree of conservation of amino acid sequences between murine and bovine κ-CN. The amino acid sequence of caseins is generally not highly conserved across species (19), but the κ-CN are the most conserved of the caseins (19). Although a number of mutations and small insertion and deletion events have occurred, the overall structure of the molecule, such as the primary structure around the protease-sensitive peptide bond at amino acids 105–106, has been conserved (28), as well as the three-dimensional structure (23).

Alignment of the amino acid sequences of mouse and bovine κ-CN (Figure 1) revealed that the majority of the amino acids that were essential for prediction of the "horse and rider" three-dimensional structure are conserved between the two proteins (23). Three Pro residues located in an important hydrophobic region are conserved in mouse (Pro-27, Pro-47, and Pro-57). Kumosinski et al. (23) argued for an important biological function of these residues: they might serve as a universal signature for κ-CN and its role in micelle interactions. The region where chymosin acts on the casein micelles by hydrolyzing the Phe-Met or Phe-Leu peptide bond (residues 105–106) is located in a conserved Pro-rich region of the molecule. Moreover, Ser-104 and Ala-107 are conserved. Glycosylation and phosphorylation sites of κ-CN at Thr-133 and Ser-149 are conserved in the mouse. Amino acid residues 35 to 68 represent a

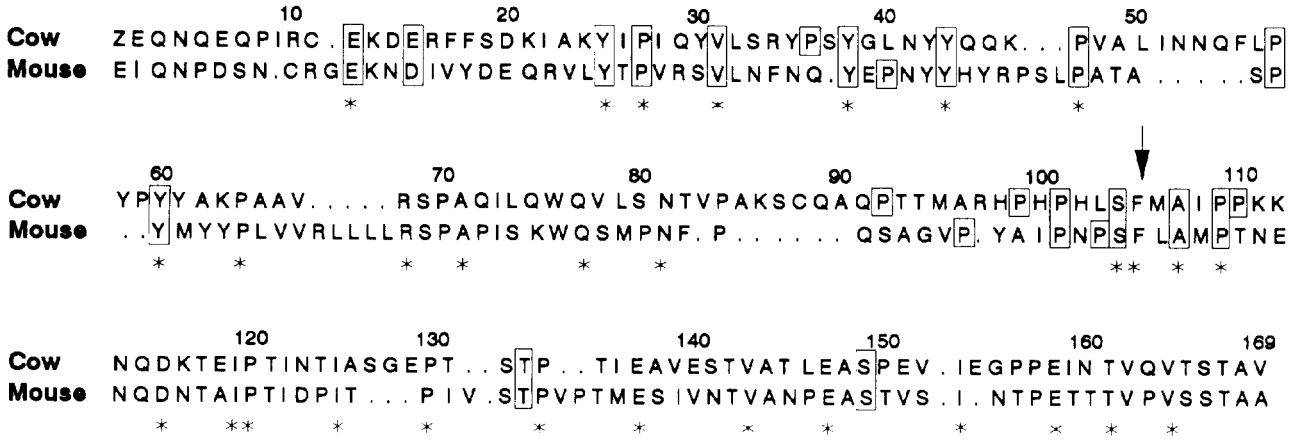

Figure 1. Alignment of the sequence of bovine κ-CN B (15) and murine κ-CN (43). Standard one-letter symbols for amino acids are used. Alignments follow those of Kumosinski et al. (23). The numbers above the sequences are those of the bovine protein. Asterisk indicates identical residues conserved in cow, ewe, goat, human, rat, and mouse; □ = essential residues for the three-dimensional molecular model described by Kumosinski et al. (23); and ↓ = the chymosin-sensitive bond.

hydrophobic region with almost no charge in both bovine and murine κ-CN. This region is of significance in the formation of the "legged" secondary structure of the κ-CN molecule, a site for interaction with hydrophobic domain of other caseins or κ-CN molecules (23). Moreover, Tyr-25, Tyr-38, Tyr-43, and Tyr-60 are invariant between murine and bovine κ-CN, as is Val-31; of 9 Tyr residues in bovine κ-CN, 7 are located between Tyr-35 and Tyr-68, and, of 10 Tyr residues in murine κ-CN, 7 are located in this area. Sulfhydryl groups in residues Cys-10 or Cys-11 also appear to be conserved. In relation to physical and chemical studies, a positive charge is conserved at residue 49 or 50, which, in the formation of κ-CN aggregates, is brought into proximity with the conserved Glu-12 residue and Glu-15 or Asp-15, allowing for dimer aggregates of κ-CN. Based on this structural comparison, bovine κ-CN could plausibly participate in the assembly of murine casein micelles. The results of our study indicated that this suggestion was in fact true.

Expression of Bovine κ-CN and Total Milk Proteins During Lactation of Transgenic Mice

The production of bovine κ-CN in the milk of transgenic mice was variable within a lactation, between mice of the same line and stage of lactation, and between transgenic lines. Concentrations of bovine κ-CN protein in transgenic murine milk paralleled the pattern of expression of endogenous murine caseins during lactation, increasing throughout lactation (15). At d 5 of lactation, the mean for hemizygous mice of the three lines ranged from 0.47 to 0.65 mg/ml of κ-CN, peaking at d 10 of lactation (0.9 to 2.9 mg/ml) and then decreasing by d 15 (0.57 to 0.93 mg/ml). The concentration of total proteins in the milk from the transgenic lines did not show extensive variation among d 5, 10, and 15 of lactation (Figure 2), although, at d 10 of lactation, line BC-7 had 19% more protein than did lines BC-31 and BC-67 ($P < 0.05$; one-way ANOVA). Even though the production of bovine κ-CN in the milk of some individual homozygous mice from lines BC-31 and BC-67 was 6.7 and 7.3 mg/ml, respectively, in general total milk protein concentration from transgenic mice did not increase significantly. Total milk protein concentration for transgenic mice remained within the typical range for milk from nontransgenic mice, suggesting that the increase in κ-CN might be accompanied by a decrease in the production of other milk proteins. Our results are in agreement with those of Wilde et al. (46), suggesting the existence of a physiological factor common to the mammary gland that limits the rate of milk protein synthesis and secretion.

Relationship Between Expression of Bovine κ-CN and Casein Micelle Size

Because κ-CN is on the surface of micelles, increasing the κ-CN content of milk should result in decreased micelle size, as the ratio of surface area to volume is increased. The mean sizes of the casein

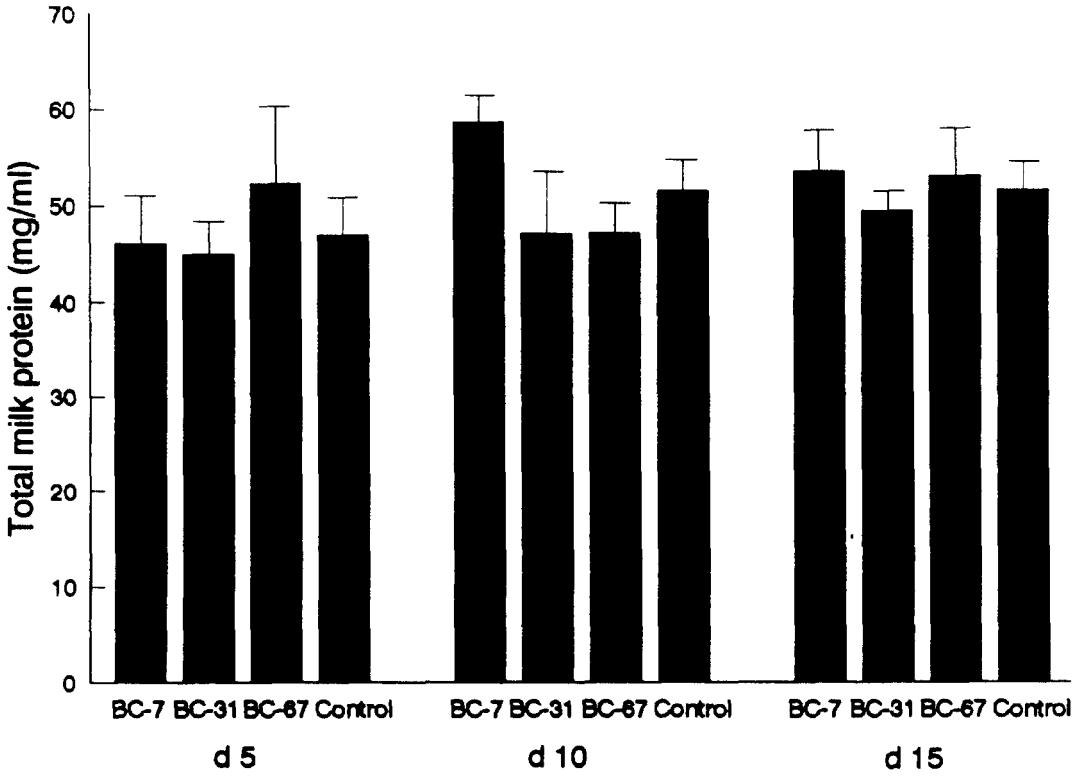

Figure 2. Mean concentration (± SD) of total protein in milk of the three bovine κ-CN transgenic lines BC-7, BC-31, and BC-67 and the control line of mice at d 5, 10, and 15 of lactation.

micelles in the milk of transgenic lines BC-31 and BC-67 were smaller ($P < 0.05$) than those for control mouse milk (Table 1). Moreover, the micelles in milk from homozygous mice were smaller than those from hemizygous mice ($P < 0.05$). Micelles from both hemizygous or homozygous line BC-7 mice tended to be smaller than micelles in milk from control mice. The failure to obtain significantly smaller micelles in milk from line BC-7 may be attributed to the lower bovine κ-CN expression in this line.

The distribution of casein micelle sizes was generally unimodal (Figure 3); however, approximately 20% of the milk samples had apparent bimodal distributions (Figure 3D). Similar distributions have been described for bovine milk but not conclusively explained (18), suggesting that this distribution could have been due to an intrinsic property of casein micelle formation. The variability in the micelle diameter for different mice from the same line (Figure 3D) could be attributed to the variation among transgenic mice of the same line and lactation stage in the expression of bovine κ-CN in their milk (15).

Transgenic mice that were deficient in κ-CN also had casein micelles that were smaller than micelles from nontransgenic mice (22). The reduction in micellar diameter was consistent with that expected if the volumes contributed to the micelles by the caseins were in proportion to their respective concentrations (22). Those results and ours suggested a high insensitivity of casein micelle assembly to major changes in composition. In agreement with our results, an inverse relationship between κ-CN content and micelle size has been reported (10, 26, 39); the smaller micelles in milk had a higher ratio of κ-CN to the calcium-sensitive caseins (9).

Rennet Clotting Time of Transgenic Milk

To test whether the smaller micelles could affect the renneting properties of milk, we analyzed the rennet-induced coagulation of milk. We found no differences between the rennet clotting time for milk from transgenic and nontransgenic mice (range 115 to 125 min). Rennet clotting times of milk have been suggested to be highly dependent on micellar size (11). Our results are in accord with those of Dalgleish et al. (7), who suggested that the degree of aggregation did not affect access of the enzyme to the

chymosin-sensitive bond in κ-CN, and with the results of Chaplin and Green (4), who reported that skim milk and milk with dissociated micelles showed equivalent proteolysis by chymosin.

Bovine κ-CN Expression and Gel Strength

During the first step in cheese making, rennet-induced coagulation of milk by hydrolysis of κ-CN forms a weak viscoelastic gel or curd. Curd firmness at cutting is a primary factor that influences cheese yield (3). The effect of milk constituents on gel structure can be probed with nondestructive viscoelastic measurements in the linear viscoelastic range. These rheological experiments provide information on the firmness of a gel by studying the relationship between force and deformation as a function of time [for review, see (5)]. The overall firmness of the gel formed with transgenic mouse milk, or average $|G^*|$ value, from lines BC-31 and BC-67 was increased over those formed from nontransgenic mouse milk (Table 2; $P < 0.001$ for both homozygous and hemizygous mice). Furthermore, gels produced from the milk of homozygous mice of lines BC-31 and BC-67 were firmer than those produced from the milk of hemizygous animals from those lines ($P < 0.001$; Table 2 and Figure 4). The difference between milks from hemizygous and homozygous mice reflected the increased amount of bovine κ-CN produced in the milk of the homozygous mice.

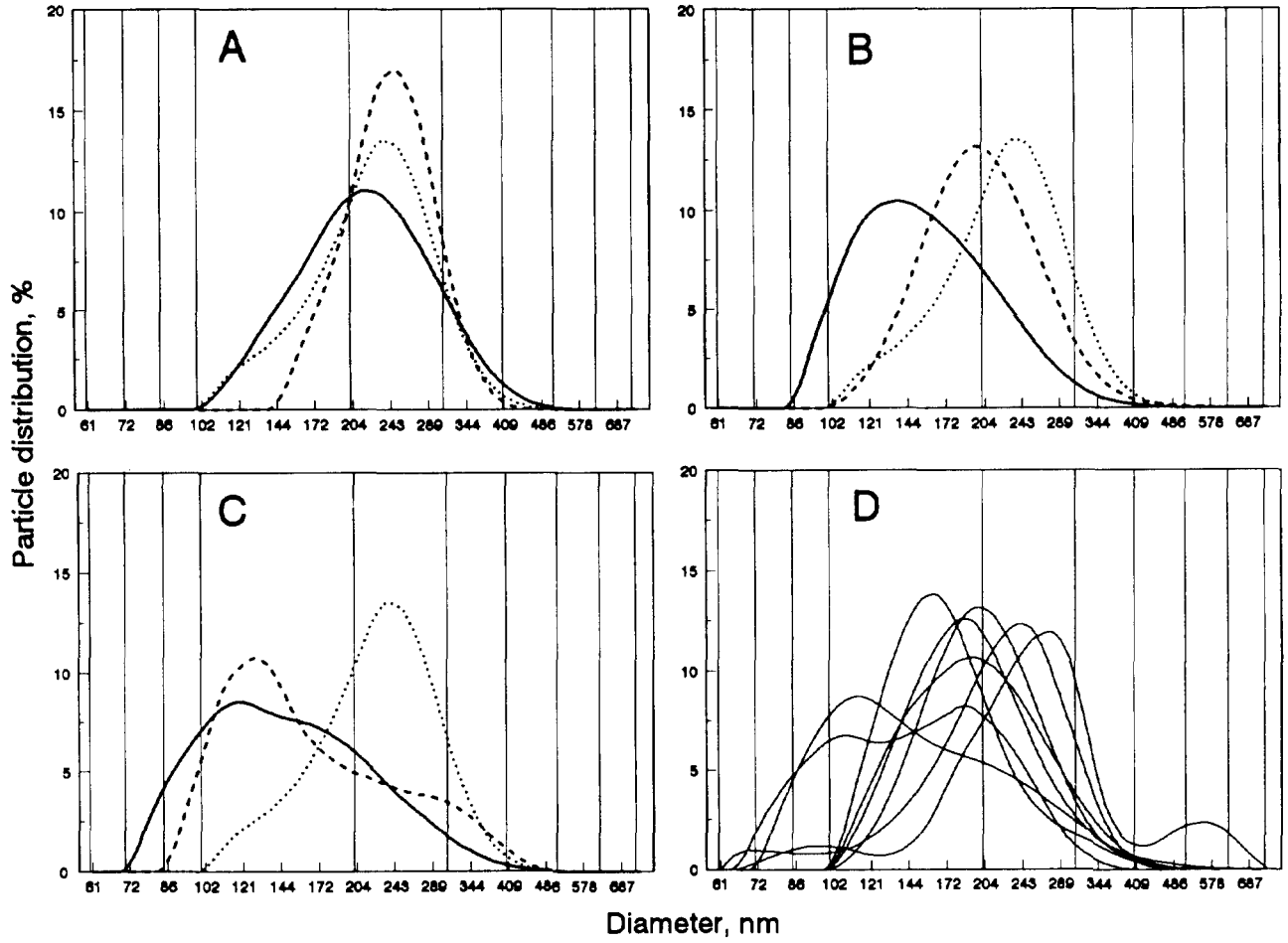

Figure 3. Example of mean diameter distribution of casein micelles in one replicate of three lines of transgenic mice. Casein micelle size of control nontransgenic mice (. . .) was compared with both hemizygous (- - -) and homozygous (—) mice of lines BC-7 (A), BC-31 (B), and BC-67 (C). Diameter distribution of casein micelles from hemizygous mice of transgenic line BC-31 in eight replicates was performed on different days (D).

TABLE 2. Gel firmness (| G* |) and degree of viscoelasticity (tan δ) of rennet-induced milk gels in two transgenic mouse lines with high expression of bovine κ-CN and in control mice.

Mouse line	|G*|			tan δ		
	(dyne/cm²)			(×10⁻³)		
	$\bar{X}$	SD	(no.)[1,2]	$\bar{X}$	SD	(no.)[1,2]
Control	2644	969	6	247	19	6
BC-31						
Hemizygous	5607***	2056	4	262	4	4
Homozygous	9144***	2923	4	279**	7	4
BC-67						
Hemizygous	5796***	1249	4	262	4	4
Homozygous	8047***	674	4	269	7	4

[1]Number of animals analyzed for each line.

[2]Milks from two mice (d 10 of lactation) were pooled, and the combined sample was run three times.

**Different from the control by Student's t test ($P < 0.01$).

***Different from the control by Student's t test ($P < 0.001$).

Halim and Shoemaker (16) observed that the addition of κ-CN to skim milk produced a stronger curd. They suggested that greater amounts of κ-CN allowed more bonds to form by facilitating formation of disulfide bridges through oxidation of sulfhydryl groups and thiodisulfide exchange. Intermolecular disulfide bonding has been shown to allow the multimerization of bovine κ-CN in the cow (34). Bovine *p*-κ-CN retains two cysteine groups after rennet cleavage (45), but mouse κ-CN retains only one, which could account for the large differences observed in our transgenic mice, because these sulfhydryl groups might facilitate intermolecular bonds and play an important role during gel formation (17).

Shoemaker et al. (41) found that gel strength clearly differed between the rennet-induced milk gels from κ-CN A cows and those from κ-CN B cows. Bovine milk containing the κ-CN B variant has greater amounts of casein present [for review, see (32)] and a smaller, more homogeneous distribution of micelle size (30). Shoemaker et al. (41) suggested that the stronger rennet gel produced by the κ-CN B variant was due to the greater amounts of casein and κ-CN, allowing for the formation of a greater number of

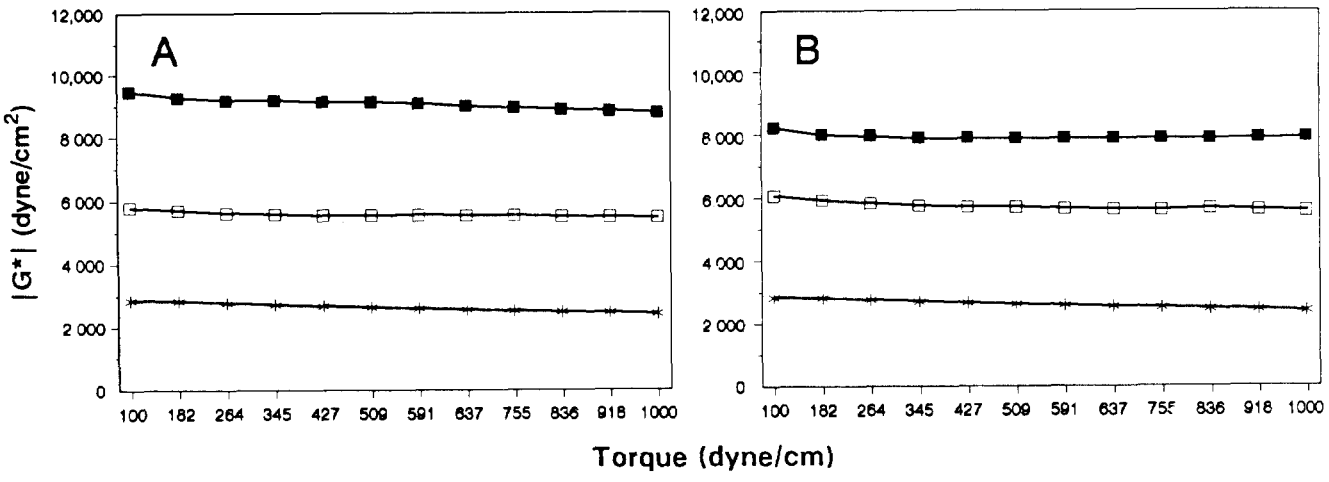

Figure 4. Overall gel strength of milk from bovine κ-CN transgenic or control lines of mice. Representative gel firmness (| G* |) values for 1 torque sweep of rennet gels made from the milk of homozygous (■) and hemizygous (▲) mice of line BC-31 versus control (●) (A) and homozygous (■) and hemizygous (▲) mice of line BC-67 versus control (●) (B).

casein micelles. We have not determined the number of casein micelles that were present in the milk of bovine κ-CN transgenic mice, but our gel strength results (Table 2 and Figure 4) support this hypothesis.

The tan δ values (Table 2), or measures of the degree of viscoelasticity over the torque range, were not different between the transgenic and control lines, except for homozygous line BC-31, which was not different from the rest of the transgenic lines but was significantly higher than the control. This result suggests that the expression of bovine κ-CN does not affect the type of interactions or bonds being formed in the milk gels.

Transgenes to Alter the Milk Protein System

Maga et al. (24) produced transgenic mice to assess the effect of the secretion of human lysozyme on the milk protein system. The expression of human lysozyme in the milk of transgenic mice resulted in significantly decreased rennet clotting time, increased gel strength, and a smaller, but not significantly smaller, micellar size. These effects might be attributed to the positive charge of lysozyme at physiological pH. The presence of extra positive charges might have led to the titration of the negatively charged caseins, resulting in a shorter coagulation time and increased curd strength. In this study, the presence of extra κ-CN resulted in a significantly increased curd strength and a smaller micelle size, but not a reduced rennet clotting time. The main function of κ-CN is in micelle size and function, and extra κ-CN acted to change these properties. The lack of effect on rennet clotting time was probably because cleavage of κ-CN still occurred at the same rate, but, with human lysozyme, although cleavage is the same, the rate of coagulation of caseins is increased because of the presence of extra positive charges.

The in vivo expression of both the bovine κ-CN and of human lysozyme transgenes demonstrated that the physical and functional properties of the milk protein system could be altered by transgenic technology without affecting the total protein content of the milk or the health of the mice or of the pups suckling on transgenic dams. Even though some of the effects caused by the two transgenes were the same, the precise mechanism by which each gene affects the overall properties discussed might be different.

CONCLUSIONS

The results of our in vivo transgenic studies showed that the production of additional κ-CN could affect the functional properties of transgenic mouse milk. Increasing the κ-CN content of bovine milk by transgenic technology should not only affect the cheese manufacturing process but also enhance the thermal stability of milk and increase cheese yield.

ACKNOWLEDGMENTS

This research was supported by a grant from the California Dairy Foods Research Center and the California Milk Advisory Board. Alfonso Gutiérrez-Adán was supported by a postdoctoral fellowship from the Ministerio de Educacion y Ciencia of Spain.

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