

Cloned transgenic cattle produce milk with higher levels of β -casein and κ -casein

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To enhance milk composition and milk processing efficiency by increasing the casein concentration in milk, we have introduced additional copies of the genes encoding bovine β - and κ -casein (*CSN2* and *CSN3*, respectively) into female bovine fibroblasts. Nuclear transfer with four independent donor cell lines resulted in the production of 11 transgenic calves. The analysis of hormonally induced milk showed substantial expression and secretion of the transgene-derived caseins into milk. Nine cows, representing two high-expressing lines, produced milk with an 8–20% increase in β -casein, a twofold increase in κ -casein levels, and a markedly altered κ -casein to total casein ratio. These results show that it is feasible to substantially alter a major component of milk in high producing dairy cows by a transgenic approach and thus to improve the functional properties of dairy milk.

Today, milk represents an important food source consumed not only in its natural form, but also in a wide variety of processed products. Milk protein, 80% of which consists of casein, is one of the most valuable components of milk because of its nutritional value and processing properties. Therefore, casein is a prime target for the improvement of milk composition.

The casein fraction of bovine milk comprises four proteins (α_{s1} -, α_{s2} -, β -, and κ -casein), which exist naturally in a number of protein variants. They are aggregated into large colloidal micelles, the structure and stability of which govern many of the complex physicochemical properties of milk. Relatively small changes in casein ratios can affect the micelle structure and thus can have substantial effects on the functional properties of milk. Hence, higher casein concentration would not only increase a valuable milk component but could at the same time improve milk characteristics.

Of special importance is κ -casein, which is thought to coat the surface of the micelle¹. Increased κ -casein content has been linked to a reduction of the micelle size² and to improved heat stability and cheese making properties³. The interior of the micelle is composed of the highly phosphorylated α_{s1} -, α_{s2} -, and β -caseins that bind the otherwise insoluble calcium phosphate⁴. As one of the predominant milk proteins, β -casein is thus implicated in determining milk calcium levels. Moreover, increased β -casein content has been correlated with improved processing properties, including reduced rennet clotting time and increased whey expulsion³.

The production of the first transgenic livestock⁵ stimulated discussions about the application of a transgenic approach to improve the milk composition of dairy animals^{3,6}. Because of the low efficiencies in generating transgenic livestock by microinjection^{5,7,8}, the application of transgenic technologies in livestock animals has been largely restricted to the production of potentially highly valuable pharmaceutical proteins^{8–10}.

To date, the concept of altering milk composition to improve its nutritional or processing properties has been evaluated only in trans-

genic mice. Here, overexpression of the major milk proteins from livestock species has been achieved⁶. Overexpression of β -casein resulted in milk protein levels of more than 20 mg/ml for caprine¹¹ and bovine β -casein¹². In contrast, transgenes based on *CSN3* sequences were either nonfunctional^{12,13} or resulted in extremely low expression levels¹⁴. Only when these were constructed to contain *CSN2* regulatory sequences was production of more than 3 mg/ml κ -casein achieved^{14,15}. In these models, the transgenic β - and κ -casein contributed to the murine casein micelles^{14,16–18} and, in the case of κ -casein, resulted in smaller mouse casein micelles¹⁷.

Mouse studies, however, do not always accurately predict protein expression levels in ruminant species¹⁹. In addition, because of the intrinsic species differences in milk composition, protein concentration, and volume^{20,21}, mouse models do not provide great insight into the functional consequences of these alterations for dairy milk and its processed products. Recent developments in nuclear transfer (NT) technology offer a more efficient way to produce transgenic livestock animals^{22,23}, which will make it possible to evaluate the consequences of these concepts in dairy milk.

Here we report the production, by NT technology, of 11 transgenic cows carrying additional copies of *CSN2* and *CSN3*. Nine cows produced milk of a substantially altered composition with higher levels of β - and κ -casein and an increased ratio of κ -casein to total casein.

Results and discussion

Generation of expression constructs and isolation of transgenic lines. The genotype of the female bovine fetal fibroblast (BFF) cell line was determined to be *CSN2*^{A1/A2} *CSN3*^{A/A} by sequence and PCR²⁴ analysis. To permit discrimination between the endogenous and transgene-derived *CSN2* and *CSN3* alleles, we chose the rare naturally occurring variants *CSN2*^{A3} (ref. 4) and *CSN3*^B as transgenes. To ensure milk-specific expression of β -casein A3, we used a genomic *CSN2* fragment comprising 6.6 kb of 5' flanking sequence, the complete

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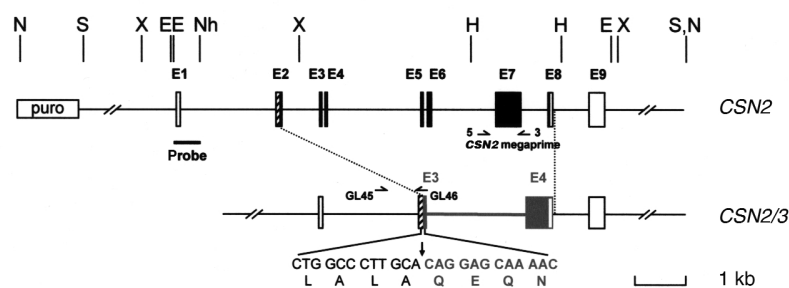


Figure 1. Schematic representation of the bovine *CSN2* and *CSN2/3* transgene constructs. The maps show *CSN2* sequences as a black line, nontranslated exons (E) as open boxes, sequences encoding the β -casein signal peptide as a hatched box and exons coding for the mature β -casein protein as black boxes. *CSN3* sequences are depicted in gray with solid and open boxes representing translated and nontranslated exonic sequences, respectively. The dotted lines indicate the position of the *CSN2* fragment replaced by *CSN3* sequences in the *CSN2/3* construct. The sequence at the fusion between exon 2, encoding the β -casein signal peptide (black) and exon 3, encoding the N-terminus of the mature κ -casein polypeptide (gray) is given below. The arrow indicates the predicted cleavage site of the β -casein signal peptide. The location of relevant PCR primers and hybridization probes are shown. Restriction enzyme sites: E, *EcoRI*; H, *HindIII*; N, *NotI*; Nh, *NheI*; S, *SalI*; X, *XbaI*. Puro, puromycin resistance marker.

8.5 kb coding region, and 2.6 kb of 3' flanking sequence²⁵ (Fig. 1, *CSN2*). The *CSN3* expression construct was generated as a *CSN2/3* fusion fragment that expressed a chimeric casein under the control of *CSN2* regulatory elements. It comprised the 15-amino-acid β -casein signal peptide and the complete κ -casein sequence²⁶ starting with the first amino acid of the mature κ -casein B protein (Fig. 1, *CSN2/3*).

When tested in transgenic mice, both constructs directed correct spatial and temporal expression, resulting in milk containing up to 20 mg/ml of bovine β -casein and 2 mg/ml of bovine κ -casein (unpublished results). This was consistent with results for similar constructs previously evaluated in transgenic mice^{12,14,15}.

The *CSN2*^{A3} and *CSN2/3*^B constructs were cotransfected into early-passage BFF cells and puromycin selection was used to derive stable cell clones. To account for the expected variability of expression resulting from the random nature of the transgene integration, we selected a total of four cell clones, designated TG2, TG3, TG5, and TG7, as donor cells for NT on the basis of normal chromosome numbers and medium-to-high copy numbers for both transgenes.

Generation of transgenic cattle by nuclear transfer. NT was used to reconstruct bovine embryos using the four transgenic cell lines and the parental, unmodified cell line BFF. The proportion of transferred embryos that developed into viable calves at weaning with transgenic donor cells was lower (11/126, 9%) than that achieved with BFF cells (7/35, 20%; Table 1). As in previous NT studies^{27–29}, approximately half of the cloned calves delivered at full term survived past weaning age (see Supplementary Note and Supplementary Fig. 1 online). After weaning, the 11 surviving transgenic calves (Table 1) continued to thrive; they are apparently healthy and currently between 20 and 26 months of age.

PCR analyses of DNA samples derived from the transgenic calves confirmed the presence of both transgenes in all animals (data not shown). Southern blot analysis specific for a 4.6 kb *CSN2/3* fragment and a 2.5 kb *CSN2* fragment demonstrated that in all four transgenic cattle lines both transgenes were integrated into the genome, with copy numbers per genome ranging from 4 to 84 for *CSN2/3* and 2 to 17 for *CSN2*^{A3} (Fig. 2). Cotransfected constructs normally co-integrate as large concatemeric structures into a single chromosomal locus³⁰. This was analyzed for line TG3. The *CSN2* and *CSN2/3* transgenes appear to be co-integrated, as genotypic analyses of biopsied TG3 embryos³¹ produced by *in vitro* reproductive technologies³²

indicate that both transgenes are inherited together (data not shown). The percentage of transgenic embryos produced from the hemizygous TG3 founder animals (51%, $n = 144$) is consistent only with a 50% mendelian ratio (95% confidence interval, 42–59%), which indicates the integration of both casein transgenes into a single chromosome.

Expression of transgene-derived casein variants in the milk of transgenic cattle. To evaluate the expression and secretion of the transgene-derived β - and κ -casein variants into milk, we induced into lactation transgenic and nontransgenic control cows (BFF animals and their maternal siblings CC-1 and CC-2). All animals responded well to the hormonal treatment, and small amounts of secretions that resembled colostrum were obtained from the first hand-milking. At the fourth milking, the milk yield ranged from 350 ml–1,000 ml.

To discriminate between the different casein variants, we analyzed the milks by two-dimensional gel electrophoresis. Both β - and κ -casein are post-translationally modified (β -casein is phosphorylated whereas κ -casein is glycosylated), which results in several isoforms. There was no obvious alteration in the ratio of

the different isoforms and so analyses focused on the main isoform for β - and κ -casein (Fig. 3). Milk samples from nine transgenic cows (TG2, TG3 1–8) revealed two additional prominent protein spots (arrows in Fig. 3, TG2 and TG3-2) when compared with nontransgenic control milk (Fig. 3, BFF), whereas the TG5 and TG7 animals showed only one additional protein.

One of the novel proteins (Fig. 3) migrated on two-dimensional gels to the same position as κ -casein B from a wild-type milk sample (Fig. 3, WT-B). This confirmed the spot as κ -casein B and also confirmed correct cleavage of the β -casein signal peptide. We were unable to obtain a sample of wild-type β -casein A3 milk. However, bovine β -casein A3, which was expressed from the same construct in transgenic mice (unpublished results), co-migrated with the novel β -casein protein in the TG2 and TG3 milk samples (Fig. 3), demonstrating its transgenic origin. Mass spectrometry (MS) analysis of the two κ -casein and three β -casein spots in the TG2 and TG3 samples positively identified the additional proteins as κ - and β -casein (data not shown). Taken together, these results show that all nine TG2 and TG3 animals expressed the transgene-derived β -casein A3 and κ -casein B variants in their milk.

We quantified the major protein isoforms on the two-dimensional gels to obtain an indication of the relative protein changes. The amount of transgene-derived κ -casein B was 126% of the endogenous κ -casein A in the TG2 and 179% of that in the TG3 samples. Interestingly, comparison of the κ -casein A spots in TG3 and wild-type samples revealed that there was 33% less κ -casein A in the transgenic samples ($P < 0.03$). In conjunction with the high κ -casein B levels, this resulted in an overall twofold increase of total κ -casein

Table 1. Summary of nuclear transfer results

Cell line	Total embryo development ^a	<i>In vivo</i> development to full term ^b	Viable calves at weaning ^b
TG2	90/129 (70%)	3/21 (14%)	1/21 (5%)
TG3	138/262 (53%)	13/46 (28%)	8/46 (17%)
TG5	52/71 (73%)	3/28 (11%)	1/28 (4%)
TG7	86/174 (49%)	5/31 (16%)	1/31 (3%)
BFF	90/165 (55%)	13/35 (37%)	7/35 (20%)

^aExpressed as proportions of reconstructed embryos placed into *in vitro* culture.

^bProportions of embryos transferred developing into calves.

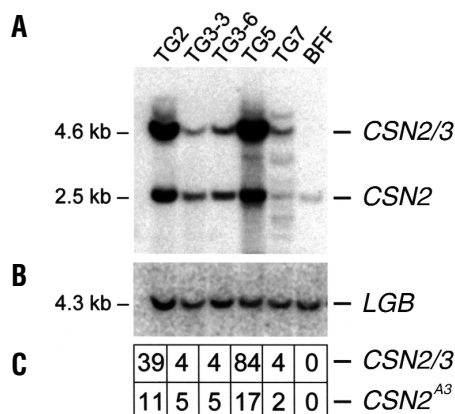


Figure 2. Southern blot analysis of transgenic cattle. Genomic DNA (5 µg) from transgenic cattle lines TG2, TG3, TG5, and TG7 and from a nontransgenic animal derived from cell line BFF was digested with *EcoRI-XbaI* and hybridized with a 500 bp *EcoRI-NheI* bovine *CSN2* fragment specific for both transgenes and the endogenous *CSN2* alleles (A) or with a 780 bp *MunI-StuI* bovine *LGB* promoter fragment (B). Resulting hybridization signals for *CSN2/3*, *CSN2*, and *LGB* are indicated and the sizes (kb) according to DNA standards given to the left. The deduced transgene copy numbers are summarized in (C).

for TG2 and TG3 ($P < 0.02$). The amount of the transgene-derived β -casein A3 spot was 26% and 66% of the endogenous β -casein A2 variant for TG2 and TG3 animals, respectively. Levels of β -casein A2 were not affected by the expression of the additional β -casein A3 variant; however, β -casein A1 levels were decreased by 48% in TG3 samples ($P < 0.01$). Taking this into account, the two-dimensional data are not sufficient to demonstrate statistically significant differences in total β -casein levels. The cow derived from cell line TG5 showed low κ -casein B expression (20% of endogenous κ -casein A) and did not produce enough β -casein A3 to be detected on the two-dimensional gels. κ -casein B was barely detectable in the TG7 milk sample (4% of κ -casein A), and β -casein A3 was not detected. The expression levels of the other major milk proteins α _{S1}-casein and β -lactoglobulin were unchanged in all transgenic lines.

A more accurate quantification of individual milk components, which includes all protein isoforms, was performed on the milk samples. Milk derived from the transgenic cows induced in July and December 2001 (Table 2) was analyzed for fat, lactose, and mineral content, all of which were within the normal range for bovine milk (not shown). Total milk protein was slightly increased in the highly expressing TG3 animals (13%, $P < 0.04$). Consistent with the results of the two-dimensional gels, total milk casein was increased by 17% in TG2 animals and 24–35% ($P < 0.001$) in TG3 animals (Table 2).

When further analyzed for β - and κ -casein content, milk samples from the one TG2 and eight TG3 animals were found to contain κ -casein levels of 8.4–14.1 mg/ml, approximately twice as high as in the nontransgenic BFF, CC-1, and CC-2 controls, which contained

5.0–5.8 mg/ml ($P < 0.001$, TG3 samples). The κ -casein concentration in these transgenic milk samples was also well above the normal range of 3–6 mg/ml observed in New Zealand milk samples⁴ (C. Prosser, personal communication). The lower expressing TG5 and TG7 animals had κ -casein levels within the normal range. Furthermore, the highly expressing TG2 and TG3 animals showed slightly higher β -casein levels (8% and 20%, respectively; $P < 0.026$, TG3 samples) than the nontransgenic controls. Casein expression from the additional gene copies in the transgenic animals resulted in a marked increase in the ratio of κ -casein to total casein (Table 2) in the TG2 and TG3 ($P < 0.019$) milk samples. In contrast, ratios of β -casein to total casein were not significantly different.

Although there is some compensation of expression between the β - and κ -casein variants, these results demonstrate that it is possible to increase total casein and total protein in cattle by increasing the gene dosage for β - and κ -casein. This is similar to the situation in sheep, where high level expression of α -antitrypsin does not affect the expression of other milk proteins¹⁹. In contrast, it appears that there is a physiological limit to the rate of protein synthesis and secretion in the murine mammary gland. Here, the expression of ovine β -lactoglobulin occurs at the expense of the endogenous mouse milk proteins^{33,34}.

When projected onto the production scale of the dairy industry, the increases observed in our study represent large changes that would translate into substantial economic gains³⁵. However, about four years would be required to introduce these transgenes into the dairy cattle population on a large scale. Once a highly expressing founder line has been identified, it is possible to expand the number of hemizygous animals within one year through advanced reproductive technologies, such as ovum pickup, *in vitro* embryo production³², and preimplantation genetic diagnostics³¹. The development of large production herds would, however, require the generation of homozygous transgenic bulls. Once this has been achieved, the

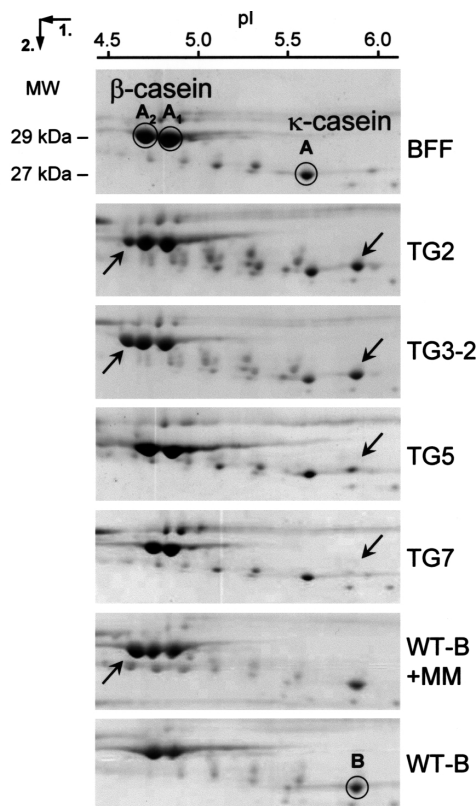


Figure 3. Expression of bovine β - and κ -casein in the milk of transgenic cows. Milk proteins (40 µg) were resolved by two-dimensional gel electrophoresis using isoelectric focusing in the first and size in the second dimension (as indicated) and visualized by Coomassie blue staining. Shown are representative gels for milk samples from the nontransgenic (BFF) animals, all four transgenic cattle lines (TG2, TG3, TG5, TG7), wild-type κ -casein B milk (WT-B) and WT-B milk spiked with mouse milk (40 µg of protein) from a transgenic mouse expressing bovine β -casein A3 (WT-B+MM). The positions of the major protein spots expressed from the endogenous *CSN2* and *CSN3* wild-type alleles in BFF (top panel), and *CSN3*^B in WT-B are indicated (bottom panel). Arrows denote the position of the main transgene derived spots for β -casein A3 (up) and κ -casein B (down). MW, molecular weight; pI, isoelectric point.

Table 2. Milk composition of transgenic and nontransgenic cows

Cow	Cell line	Age at induction, months	Protein ^b , %	Casein (CN) ^a , mg/ml	β-CN ^a , mg/ml	κ-CN ^a , mg/ml	β-CN:CN	κ-CN:CN
A Cows induced in July 2001								
TG2	TG2	8	5.6	44.5	15.8	11.2	0.36	0.26
TG3-1	TG3	7	6.9	54.9	19.9	10.7	0.36	0.20
TG3-2	TG3	7	5.2	42.0	17.6	11.6	0.42	0.29
TG3-3	TG3	8	5.9	47.5	20.9	12.0	0.44	0.26
TG3-4	TG3	7	5.9	47.2	18.4	10.1	0.39	0.22
TG3-5	TG3	7	5.3	43.0	14.1	8.4	0.33	0.20
Mean TG3			5.8	46.9	18.2	10.6	0.39	0.24
CC-1	NA	10	4.6	36.2	14.3	5.1	0.40	0.15
CC-2	NA	10	5.0	39.6	14.8	5.8	0.37	0.15
Mean			4.8	37.9	14.6	5.5	0.39	0.15
B Cows induced in December 2001								
TG3-6	TG3	9	4.5	33.8	14.0	10.7	0.41	0.32
TG3-7	TG3	9	6.4	34.8	17.8	13.0	0.51	0.37
TG3-8	TG3	9	4.5	33.8	17.0	14.1	0.50	0.42
TG5	TG5	9	3.8	25.6	12.2	5.7	0.48	0.22
TG7	TG7	7	4.5	25.3	13.9	5.6	0.55	0.22
Mean TG3			5.1	34.1	16.3	12.6	0.48	0.36
BFF-1	BFF	7	3.8	26.4	14.4	5.0	0.55	0.19
BFF-2	BFF	7	5.1	24.0	14.9	5.0	0.62	0.21
BFF-3	BFF	7	4.5	25.5	14.5	5.0	0.57	0.20
Mean			4.5	25.3	14.6	5.0	0.58	0.20

^aBased on skim milk. ^bBased on whole milk. Milk samples were collected after hormonal induction in July 2001 (A) and December 2001 (B). Milk from cows induced in July 2001 was analyzed for total protein and casein (CN) using infrared spectroscopy and milk from cows induced in December 2001 was analyzed by total combustion (protein) and HPLC (CN). β- and κ-casein concentrations were determined nephelometrically.

transgenic genotype can be rapidly propagated on a large scale at low cost through artificial insemination. This strategy relies on the stability of transgene inheritance and protein expression. Whereas high stability has been demonstrated for transgenic mice³⁶, failure to transmit some or all transgenes into the next generation has been observed for a number of transgenic sheep and pig founder lines¹⁹. These transgenic founders were, however, generated by microinjection and many of them were mosaic. Cloned transgenic cattle founders are not mosaic and thus instabilities are not expected, and none were identified by the embryo genotyping.

The highest expression of both β- and κ-casein was observed in TG3, which carries five and four copies of the respective transgenes (Fig. 2C). The expression levels were lower in TG2 and TG5, despite an increase in transgene copies, and were lowest in line TG7, with copy numbers similar to TG3. Thus, there is no correlation between the expression levels and the transgene copy numbers; this suggests locus-dependent expression of the transgenes similar to that observed for casein constructs in transgenic mouse studies^{11,12,14,15}.

Although all eight animals from line TG3 are cloned genomic copies of the transgenic cells used for NT, we have observed considerable variation of some milk protein values. This is not surprising given that milk production is greatly influenced by environmental factors. As a result, variations in milk composition are seen in milks produced at different times of the day³⁷. The availability of cloned animals should make it possible to determine the contribution of genetic and environmental factors in milk production.

These results were all based upon hormonally induced milk, which allowed an early analysis of the transgene expression in prepubertal animals. Although this is an artificial process, it has been shown that induced milk is representative of natural lactation milk for both wild-type³⁸ and transgenic cattle³⁹. It was further demon-

strated that the expression of recombinant human lactoferrin in the milk of transgenic cattle remained consistent throughout a 280 day lactation period³⁹. Thus, a stable expression of higher β-casein and κ-casein levels, with an alteration of the κ-casein to total casein ratio, can be expected for natural milk produced after calving. To obtain a more complete understanding of the milk produced by the transgenic cows, however, it will be necessary to analyze natural milk samples over an extended period of time.

Taken together, our data show that it is possible to substantially change milk composition in high-producing dairy cattle by introducing additional copies of casein genes. Moreover, the magnitude of the observed changes highlights the potential of transgenic technology to tailor milk composition in dairy cows. The milk produced by these transgenic cows will provide a useful model for studying the functional consequences of altered milk composition.

Experimental protocol

Animal studies. All animal studies were done in compliance with New Zealand laws and were approved by the Environmental Risk Management Authority (ERMA), New Zealand and the Ruakura Animal Ethics Committee.

DNA constructs. A 17.7 kb *SalI* CSN2 fragment (Fig. 1) was isolated from a λ-FixII bovine genomic library (Stratagene, La Jolla, CA). All exons were sequenced, which confirmed an intact reading frame encoding the β-casein variant A2. The CSN2 construct was changed

to the A3 variant by altering the His106 encoding triplet to the A3 specific codon for Gln106 (ref. 40) by megaprimer mutagenesis⁴¹ using the primers CSN2 megaprimer mutant (dTGGCTCTAAACAGAAAGAAATGC), CSN2 megaprimer 3 (dCTCGAATATCATACAACATCAGA), and CSN2 megaprimer 5 (dAAACAAACAAAATAAACCTCAGA). The amplified 950 bp fragment was subcloned as a 720 bp *PstI*-*StuI* fragment into the genomic CSN2 construct. For stable transfection of bovine cells, a puromycin selection marker⁴² driven by the phosphoglycerate kinase promoter was linked to the 5'-end of the CSN2 fragment.

A 1.7 kb CSN2 fragment from 100 bp upstream of a unique *NheI* site in intron 1 to the end of exon 2 was amplified by PCR with primers GL18 (dGCCGGTACCTCAGCCATAAAGGCAAGCA) and GL19 (dGGC GGGCC CAAGGGCCAGAGCCACCAG), introducing a new *ApaI*-site through the 3'-PCR primer GL19. PCR primers GL20 (dGCCGGGGCCACAGG AGCAAAACCAAGAACA) and GL30 (dGCGAAGCTTACCTGCGTTGTC TTCTTTGATGTC) with added *ApaI* and *HindIII* sites were used to amplify a 2.6 kb CSN3 fragment spanning parts of exon 3 and all of exon 4 from a bovine DNA sample previously genotyped as CSN3³⁸ (P. L'H., unpublished data). The CSN2 and CSN3 fragments were fused at the temporarily introduced *ApaI* site⁴³, generating a 4.3 kb CSN2/3³⁸ fragment. *NheI* and partial *HindIII* restriction digestion was used to insert the 4.2 kb *NheI*-*HindIII* CSN2/3 fusion fragment in place of the corresponding CSN2 sequence, from the *NheI* site within intron 1 to the *HindIII* site 3' of exon 8 in the genomic CSN2 construct.

Preparation, culture, and transfection of primary fibroblasts. A proven top Friesian bull and dairy cow of high genetic merit for milk production (including milk protein content) were mated to produce an elite female fetus that was recovered at day 60. The primary bovine fibroblast cell line BFF was isolated from this fetus by disaggregation of lung tissue. Passage 3 BFF cells were cotransfected with the CSN2⁴³ *NotI* fragment and the CSN2/3³⁸ *SalI* fragment using FuGENE 6 (Roche Diagnostics, Auckland, NZ) according to the manufacturer's guidelines. A cotransfection ratio of 1:10 was used to generate a high proportion of double transgenic cell clones and resulted in a range of copy numbers of the co-integrated transgenes (Fig. 2C). Puromycin

selection (2 µg/ml) was applied 24–48 h after transfection. Individual cell clones were isolated 7 d after transfection, cultured, and cryopreserved after a total of 19–29 d in culture. A proportion of cells from each cell clone was grown further and used to confirm correct chromosome numbers (M.G. Diagnostics, Dunedin, NZ) and to characterize transgene integration by PCR and Southern blot analyses.

Nuclear transfer. Cloned transgenic and control cows were produced following somatic cell nuclear transfer (NT) using methods essentially as described⁴⁴, except that the manipulation medium was based on HEPES-buffered TCM199 (Invitrogen, Auckland, NZ). Briefly, cytoplasts were prepared by enucleating *in vitro* matured bovine oocytes and were reconstructed with donor nuclei either in G0 or G1 of the cell cycle. G0 donor cells were induced into quiescence following serum deprivation for 5 d. G1 donors were obtained by fusing cells 1–3 h after observed mitosis. Artificial activation of the reconstructs occurred either simultaneously with fusion or after a delay of 3–9 h. Embryos were cultured *in vitro* for 7 d essentially as described⁴⁵, with the exception that the bovine serum albumin was obtained from ImmunoChemical Products Limited (Auckland, NZ). Individual blastocysts were transferred to synchronized recipient cows 7 d after estrus. Embryo and fetal survival were regularly monitored via ultrasonography and rectal palpation. Corticosteroids were administered near expected full term to aid fetal maturation and to induce parturition.

Transgene detection by nucleic acid analysis. DNA from cultured bovine cells was prepared according to standard protocols and DNA of calves was isolated from blood samples using a Nucleon BACC2 kit (Amersham Biosciences, Auckland, NZ). The presence of the transgenes was assayed by PCR with the CSN2-specific primer pair CSN2 megaprimer 3 and 5 and the primers GL45 (dTGACACCCCTGGTAATCACCAG) and GL46 (dTGGTGTCTGCTCCTGTGCAAG) specific for the CSN2/3 fragment. To identify the CSN2^{Δ3} transgene, CSN2 amplification products were digested with *Dde*I, which produced a variant specific restriction pattern.

Genomic DNA (5 µg) was digested with *Eco*RI and *Xba*I and analyzed by Southern blotting with the 500 bp *Eco*RI-*Nhe*I CSN2 hybridization probe (Fig. 1). Loading was normalized with a 780 bp *Mun*I-*Stu*I probe derived from the promoter of *LGB* (which encodes β-lactoglobulin). Transgene copy numbers were determined by signal quantification using a densitometer (GS-800; Bio-Rad, Auckland, NZ).

Milk analysis. Cows were hormonally induced into lactation³⁸ at 7–9 months of age. Due to differences in age, a group of six transgenic cows (TG2, TG3 1–5) was induced in July 2001 and a second group of five younger transgenic cows (TG3 6–8, TG5, TG7) in December 2001. Three 7-month old cows, generated by NT from the unmodified parental cell line BFF, were induced into lactation together with the second group in December. Because BFF animals of sufficient age were not available for the July induction, we induced control cows (CC-1, CC-2) that were maternal siblings of the BFF-derived animals.

The cows were hand milked every second day, and milk of the fourth milking was sampled for analysis.

Defatted milk samples containing 40 µg of milk protein as estimated by the method of Bradford⁴⁶ were subjected to two-dimensional gel electrophoresis using linear pH 4–7 immobilized pH gradients for the first dimension, and denaturing electrophoresis on 12.5% acrylamide gels for the second dimension. Protein spots were visualized by staining with colloidal Coomassie blue. Scanned images of multiple gels were analyzed, normalized, and the spots quantified using the PDQuest program (Bio-Rad).

For MS, in-gel trypsin (Promega, Madison, WI) digestion was performed on excised gel spots and followed by extraction of peptides. The κ-casein spots were analyzed by MALDI-TOF MS and peptide mass fingerprint matches were performed against public sequence databases using the ProFound program (Rockefeller University, New York, NY). The β-casein spots were analyzed by ion-trap MS. MS/MS data were matched to protein sequence databases using TurboSequest software (Thermo Finnigan, San Jose, CA).

Milk composition of whole milk samples from the July induction was determined by Fourier transform infra-red (FTIR) spectroscopy on a MilkoScan FT120 (Foss). Casein concentrations were converted to skim milk-based values. December samples were analyzed for casein (in skim milk) by high-performance liquid chromatography (HPLC) (Shimadzu LC10A, Kyoto, Japan) and for total nitrogen by combustion on a Carlo Erba NA 1,500 Analyser (CE Instruments, Milan, Italy); the latter values were converted to milk protein values using a factor of 6.38 g protein/g nitrogen. The concentration of β- and κ-casein in skim milk was determined with a nephelometric immunoassay⁴⁷ using bovine-specific anti-β-casein and anti-κ-casein IgY chicken antibodies.

Note: Supplementary information is available on the Nature Biotechnology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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