Expression analysis of the individual bovine β-, α_{s2}- and κ-casein genes in transgenic mice

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To identify *cis*-acting regulatory elements involved in the regulation of expression of the casein genes, the bovine β-, α_{s2}- and κ-casein genes were isolated from cosmid libraries and introduced into the murine germline. Bovine casein expression was analysed at the RNA and protein level. The bovine β-casein gene, including 16 kb of 5′- and 8 kb of 3′-flanking region, appeared to be expressed in all 12 transgenic mouse lines analysed. In 50% of these lines expression levels in milk exceeded 1 mg/ml. Three lines displayed expression levels comparable with or well above (20 mg/ml) the β-casein levels in bovine milk. Transgene expression was restricted to the mammary gland. Strong induction of expression occurred at parturition and thus resembled the bovine rather than the murine pattern. In spite of this high-level tissue-specific and developmentally regulated expression, β-casein expression levels were integration-site-dependent, suggesting that not all elements involved in regulation of expression were included in this β-casein clone. Neither the bovine α_{s2}- nor the κ-casein gene, including 8 kb and 5 kb of 5′- and 1.5 kb and 19 kb of 3′-flanking sequences respectively, were properly expressed in transgenic mice. However, they were transcribed in stably transfected mouse mammary epithelial cells. This indicates that regulatory elements required for high-level, mammary gland-specific expression are not present in the α_{s2}-and κ-casein clones used in this study and are probably located elsewhere in the casein gene locus.

INTRODUCTION

The four caseins constitute the major milk proteins in bovine milk. Together they account for 80% of the milk protein fraction. α_{s1}- and β-casein are the most abundant proteins at 10–12 mg/ml and 10 mg/ml respectively, α_{s2}-casein and κ-casein are present at lower levels, of 3.7 and 3.4 mg/ml [1]. The caseins are encoded by single-copy genes clustered in a region of about 200 kb on bovine chromosome 4 [2–4]. The genes encoding calcium-sensitive caseins (α_{s1}-, β- and α_{s2}) have evolved from a common ancestral gene [5,6]. All four casein genes are coordinately expressed in a tissue- and stage-specific fashion and the proximal 5′-flanking regions of the genes encoding calcium-sensitive caseins share common regulatory motifs [5,7]. However, the 5′ region of the κ-casein gene is organized differently from that of the other casein genes [8].

The promoters of the casein genes have been studied in tissue culture and, to some extent, in transgenic animals. Several regulatory motifs (transcription-factor binding sites, including hormone response elements) and *trans*-acting factors binding to them, have been identified, especially in the proximal promoter region of the β-casein gene [7,9–16]. Most *cis*-regulatory elements involved in mediating hormonal and extracellular matrix effects on β-casein gene expression were identified within the region from 1.7 kb upstream of the transcription initiation site to exon I [9,10,12,14,16–18]. Comparative expression analyses of β-casein wild-type and chimeric genes of rat [19,20], goat [21,22] and cow [23,24] in transgenic mice and rats [24] showed high variability in expression levels, while tissue- and stage-specificity of expression were retained. Similar expression characteristics have been obtained using transgenes based on regulatory elements derived from the α_{s1}-casein gene [23–26].

The only transgene based on κ-casein gene sequences (−552 bp to +64 bp) reported to date appeared to be non-functional in transgenic rats [24]. No expression studies with either the native α_{s2}-casein gene or a chimeric α_{s2}-casein transgene have been reported to date.

The *in vitro* analysis of milk protein gene expression in cell culture systems is seriously hampered by the inability of these cell models to completely mimic the entire repertoire of developmental signals essential for gene expression in the mammary gland [9,27–29]. Therefore, we have introduced the bovine α_{s2}-, κ- and β-casein genes into the murine germline with the specific aim of identifying elements involved in regulation of high-level mammary gland-specific expression. Such elements might be located anywhere in the casein gene locus. Moreover, interaction between these elements or between the proteins binding to them may be required for proper regulation of casein expression. By analysing the expression of intact bovine casein genes with relatively large flanking regions, rather than chimeric constructs containing only parts of a casein gene, the probability of identifying such elements is increased. In general, the level of expression of heterologous proteins in the milk of transgenic mice is affected by the site of integration, resulting in highly variable and copy-number-independent expression levels. Isolation of elements capable of overriding the effects of neighbouring sequences would contribute to improving mammary gland-specific expression systems, designed to express heterologous proteins in milk via transgenesis.

In the present paper, we describe the expression characteristics of the bovine β-, α_{s2}- and κ-casein genes in transgenic mice. Of these three genes only the β-casein gene, with 16 kb of 5′- and 8 kb of 3′-flanking sequence, was expressed at high levels in lactating mammary gland. Control elements capable of driving

Abbreviations used: WAP, whey acidic protein; CBB, Coomassie Brilliant Blue; LCR, locus control region.

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correctly regulated expression to the mammary gland appeared to be missing from the α_{s2}- and κ-casein genes used in this study.

MATERIALS AND METHODS

Isolation of cosmid clones containing the bovine casein genes

High-molecular-mass DNA was isolated from the placenta of a Jersey dairy cow and from white blood cells of a Holstein Friesian dairy bull as described by Little [30]. DNA was partially digested with limiting amounts of *Mbo*I and size-selected in the 40–50 kb range using pulsed-field gel electrophoresis (PFGE). The size-selected DNA was isolated from low-melting-point agarose gel (Seaplaque FMC BioProducts, Rockland, ME, U.S.A.) by agarase treatment [10 units of (100 μl) Sigma agarase of low-melting-temperature], ligated to a dephosphorylated *Bam*HI-digested pWE15 cosmid vector and transduced to *Escherichia coli* 1046, following the protocol described in the 'pWE15 cosmid vector instruction manual' (Stratagene, La Jolla, CA, U.S.A.). For the Jersey-DNA library, 2.6×10^5 colonies were plated on nitrocellulose (Schleicher & Schuell) and screened by colony hybridization following the manufacturer's instructions. For the Holstein Friesian-DNA library, 1×10^6 colonies were plated on Hybond-N$^+$ membrane (Amersham). Both bovine genomic cosmid libraries were screened using bovine β-, α_{s2}- and κ-casein cDNAs (pWV613, pWV610 and pWV619 respectively, kindly provided by Dr. M. Groenen, Agricultural University, Wageningen, The Netherlands). Several clones representing the β-, α_{s2}- and κ-casein genes were identified and isolated from frozen colony-filters as described by Heilig et al. [31].

The following three cosmid clones were used in this study (Figure 1): (1) cosmid 23A, containing the 9 kb β-casein gene with 16 kb of 5'- and 8 kb of 3'-flanking sequences; (2) cosmid 9A, containing the 19 kb α_{s2}-casein gene with 8 kb of 5'-flanking and 1.5 kb of 3'-flanking sequences; and (3) cosmid 30A, comprising the 13 kb κ-casein gene and 5 kb of 5'-flanking and 19 kb of 3'-flanking sequences. Clone 9A (α_{s2}) was isolated from the Jersey-DNA library, and the other two clones were from the Holstein Friesian-DNA library.

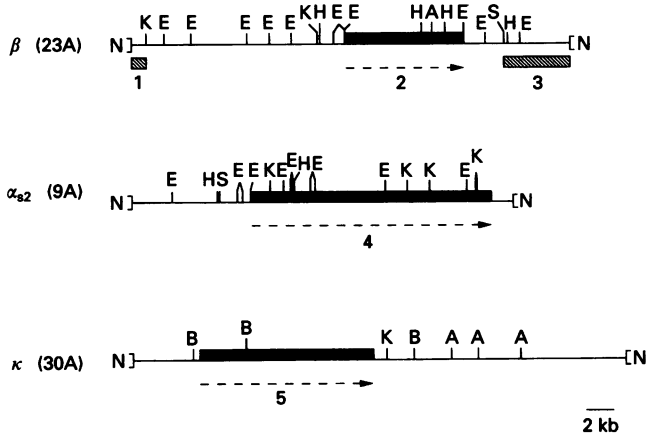

Figure 1 Casein gene restriction enzyme maps

Restriction maps of cosmid clones 23A (β-casein gene), 9A (α_{s2}) and 30A (κ). Abbreviations: A, *Apa*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sma*I. *Not*I (N) was used to release the genes from the vector (pWE15). Probes used in DNA and RNA analysis are indicated: (1) a 2 kb *Kpn*I–*Not*I fragment situated at the extreme 5'-end of 23A; (2) the 1.1 kb *Kpn*I insert from β-casein cDNA clone pWV613; (3) a 5 kb *Sma*I fragment detecting the 3'-end of 23A; (4) the 1 kb *Apa*I–*Eco*RI insert from α_{s2}-casein cDNA clone pWV610; and (5) the 850 bp *Kpn*I κ-casein cDNA insert from pWV619.

Generation of transgenic mice

The casein genes were released from the cosmid vector by *Not*I digestion and the 33 kb (β-casein), 28.5 kb (α_{s2}-casein) and 37 kb (κ-casein) DNA fragments were purified by 0.6% agarose gel electrophoresis and electro-elution. The DNA was microinjected into the most accessible pronucleus of fertilized mouse eggs (CBA/BrAxC57Bl/6) which were re-implanted in pseudo-pregnant females [32].

Southern blot analysis

Transgenic animals were identified by Southern blot analysis of *Eco*RI (for β-casein mice also *Hin*dIII + *Kpn*I)-digested genomic DNA extracted from tail biopsies. DNA fragments were separated on a 0.8% agarose gel and blotted to nylon membrane (Hybond-N, Amersham). To check integration and integrity of the three transgenes, blots were probed with (see Figure 1): (1) a 1.1 kb *Kpn*I–*Not*I fragment situated at the extreme 5'-end of 23A; (2) the 1.1 kb *Kpn*I insert of β-casein cDNA clone pWV613; (3) a 5 kb *Sma*I–*Not*I fragment located 3 kb downstream of the β-casein gene hybridizing to the 3'-end of 23A; (4) the 1 kb *Apa*I–*Eco*RI insert of α_{s2}-casein cDNA clone pWV610; and (5) the 850 bp *Kpn*I κ-casein cDNA insert of pWV619. Transgene copy numbers were estimated by comparison of the hybridization signal of dilutions of transgenic DNA to the signal of a known amount of bovine DNA using a Betagen Betascope 603 blot analyser (Waltham, MA, U.S.A.). All probes were labelled with [α-^{32}P]dCTP using random hexanucleotide primers [33]. Blots were hybridized, for 2 h, and washed as described by den Dunnen and van Ommen [34].

Northern blot analysis

Total RNA was extracted from mammary gland and eight other tissues (brain, heart, kidney, liver, tear gland, thymus, salivary gland and spleen) by the LiCl–urea method [35] or using RNAzol B (Tel-Test, Friendswood, TX, U.S.A.). To study transgene expression during mammary gland development RNA was isolated from mammary gland biopsies taken at different stages of gestation and early lactation. Total RNA (20 μg) was separated on 1% agarose/formaldehyde gels with circulation of running buffer [17.7% (w/v) paraformaldehyde, 14 mM sodium phosphate, pH 7.1] and transferred to Hybond-N membrane. RNA blots were probed with the appropriate bovine casein cDNA probes. Endogenous mouse milk protein gene expression was detected with a 200 bp *Eco*RI–*Pst*I murine β-casein cDNA fragment [36], a 900 bp murine γ-casein cDNA [37], a 404 bp PCR fragment (nucleotides 181–585) of murine κ-casein [38], and a 600 bp *Pst*I murine whey acidic protein (WAP) cDNA fragment [36]. To correct for RNA loading differences, blots were hybridized with a 1.5 kb human 28 S ribosomal probe. Hybridization and washing were carried out as described by Sambrook et al. [33]. Bovine casein transcript levels were quantified by comparison of the hybridization signal of transgene-derived mRNA to the signal of serially diluted total RNA isolated from lactating bovine mammary gland tissue. Hybridization signals were analysed on a Betagen Betascope 603 blot analyser (Waltham, MA, U.S.A.).

SDS/PAGE and Western blot analysis

Milk samples were diluted in milk dilution buffer composed of 10 mM EDTA, pH 7.4, 0.1% Tween-20 (J.T. Baker Chemicals

Table 1 Expression levels of bovine β-casein in mammary gland of 12 independent transgenic mouse lines

Female founder mice and transgenic progeny were analysed at the RNA and protein levels. The detection limit was at least 10^4-fold below the β-casein levels in lactating bovine mammary gland.

Line	Copy no.	RNA (day 8) (% of bovine)*	Protein range of highest expression level (mg/ml)†
1272	3	1–2	0.6–1.0
1605	11	5–10	1–10
1606	4	0.1	< 0.1
1607	2	0.1	< 0.1
1608	2	20–40	5–20
1609	1‡	0.5	< 0.1
1610	4	0.5–1	0.1–0.5
1611	2	1–4	2–4
1612	4	20	5–20
1613	4	0.5–1	0.5–2
1614	2	< 0.1	< 0.1
1615	2	< 0.1	< 0.1

* RNA expression levels in mammary gland tissue at mid-lactation in the first lactation are indicated as percentage of β-casein mRNA levels in a lactating cow.

† Protein range represents the highest expression levels detected in milk of individual mice from the same line. Of every line several lactating mice were analysed. For each mouse maximum expression levels were determined. The lowest and highest maximum levels of expression are presented in the Table as the range per line. In general, expression levels of the first lactation were 2- to 3-fold below those in later lactations. It should be noted that expression levels did not always reach a maximum at day 8.

‡ Line 1609 contains one copy of the transgene of which part of the 3′ flank is missing.

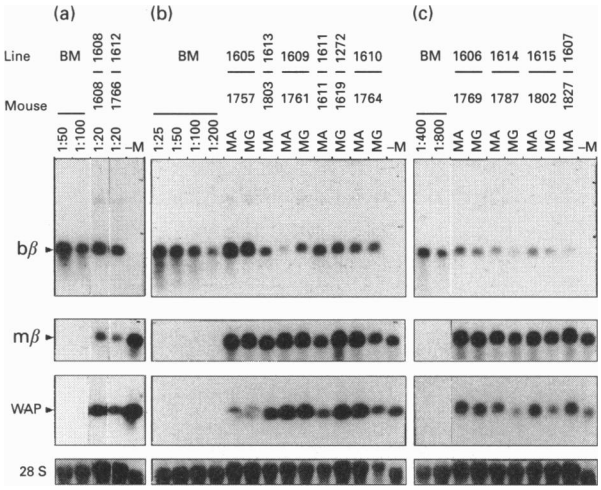

Figure 2 Transcription of the bovine β-casein gene in mouse mammary gland

Northern blot analysis of total RNA (20 μg/lane) of lactating mammary gland tissue from bovine β-casein transgenic mice. RNA was isolated from mammary gland biopsies (MA) taken at mid-lactation and, in most cases, mammary glands (MG) at late-lactation. Total RNA from a lactating bovine mammary gland (BM), diluted in non-transgenic mouse kidney total RNA as indicated, served as a standard for quantification of transgene-derived bovine β-casein transcript levels. Blots were hybridized to a bovine β-casein cDNA probe (bβ), murine β-casein cDNA probe (mβ), murine WAP and human 28 S ribosomal RNA probe. (**a**) Lactating mammary gland samples of line 1608 (biopsy of founder), third lactation (L3), day 8 (d8) 1:20 diluted; line 1612 (mouse 1766, L1, d11) 1:20 diluted; BM, dilutions 1:50, 1:100; —M, lactating non-transgenic mammary gland RNA. (**b**) Lactating mammary gland RNA samples of line 1605 (mouse 1757, L1, d8; L1, d18); line 1613 (mouse 1803, L1, d10); line 1609 (mouse 1761, L1, d9; L1, d17); line 1611 (biopsy of founder, L3, d9); line 1272 (mouse 1619, L1, d9); line 1610 (mouse 1764, L1, d10; L1, d18); BM, dilutions 1:25, 1:50, 1:100, 1:200; —M. The fuzzy signal for WAP in the 1605 samples is due to a blot artefact. (**c**) Lactating mammary gland RNA samples of line 1606 (mouse 1769, L1, d9; L1, d17); line 1614 (mouse 1787, L1, d9; L1, d16); line 1615 (mouse 1802, L1, d9; L1, d16); BM dilutions 1:400 and 1:800; —M.

Co.), 10 mM benzamidine hydrochloride (Janssen) and 0.1 mg/ml soy-bean trypsin inhibitor (type I-S, Sigma) in PBS. Samples (10 μl) of diluted transgenic mouse milk and diluted bovine milk, as a standard, were loaded under reducing conditions on to an SDS/12.5%-PAGE gel [33]. Western blotting was performed following the 'Mini-PROTEAN II dual slab cell' instruction manual (Bio-Rad, Richmond, CA, U.S.A.) using nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked overnight in PBS containing 3% BSA, 0.2% Tween-20, 0.1% sodium azide and 1% normal goat serum. As a first antibody a polyclonal rabbit anti-(bovine β-casein) antibody was used (kindly provided by Dr. C. Karatzas, McGill University, Montreal, Canada), diluted 1:5000 in 1% BSA/0.2% Tween-20/0.1% sodium azide in PBS, pH 7.4. Peroxidase-conjugated goat anti-(rabbit IgG), diluted 1:5000 in 1% BSA/0.2% Tween-20/0.1% sodium azide/1% normal goat serum in PBS, pH 7.4, was used as the second antibody. Chemiluminescence detection was performed with ECL reagents (Amersham) according to manufacturers' instructions. Bovine casein levels in murine milk were determined by comparison with samples of diluted pooled bovine milk run on the same gel. The average β-casein concentration in bovine milk was taken to be 10 mg/ml [1]. In addition, protein bands were visualized by staining of SDS/PAGE gels with Coomassie Brilliant Blue (CBB).

Culture and transfection of HC11 cells

HC11 cells [27] were cultured to 60% confluency in RPMI 1640 (Life-technologies) containing 10% (v/v) fetal-calf serum (Life-technologies), 5 μg/ml insulin (Sigma), 10 ng/ml epidermal growth factor (Life-technologies), 50 μg/ml gentamicin (Life-technologies) and 2 mM glutamine (Life-technologies). Transfection was carried out with 20 μg of bovine α_{s2}-casein cosmid clone 9A and κ-casein cosmid clones 30A or 39A. Aliquots (3 ml) of OPTI-MEM (Life-technologies) containing 0.1 mM spermine were mixed with 20 μg of cosmid DNA, then 15 μg of poly-L-lysine was added and incubated for 10 min at room temperature. Lipofectamine (50 μl) (Life-technologies) was added and left for 30 min at room temperature. Aliquots (1 ml per well) of this mixture were added and left on the HC11 cells for 24 h. Cells were cultured for 2 days under selective conditions (G 418: 400 μg/ml; Sigma). Surviving stably transfected clones were pooled (500 per pool) and expanded clones were grown without hormone treatment, i.e. under non-inducing conditions. Total RNA was isolated from these pools as described above.

RESULTS

Isolation of bovine casein genes and generation of transgenic mice

Several clones containing the β-, the α_{s2}- or the κ-casein genes were isolated from two independent bovine cosmid libraries together comprising 1.3×10^6 colonies. For the generation of transgenic mice, the following three cosmid clones were used (see Figure 1): (1) cosmid 23A, containing the 9 kb β-casein gene with 16 kb of 5′- and 8 kb of 3′-flanking sequences; (2) cosmid 9A, containing the 19 kb α_{s2}-casein gene with 8 kb of 5′- and 1.5 kb of 3′-flanking sequences; and (3) cosmid 30A, comprising the 13 kb κ-casein gene and 5 kb of 5′- and 19 kb of 3′-flanking sequences.

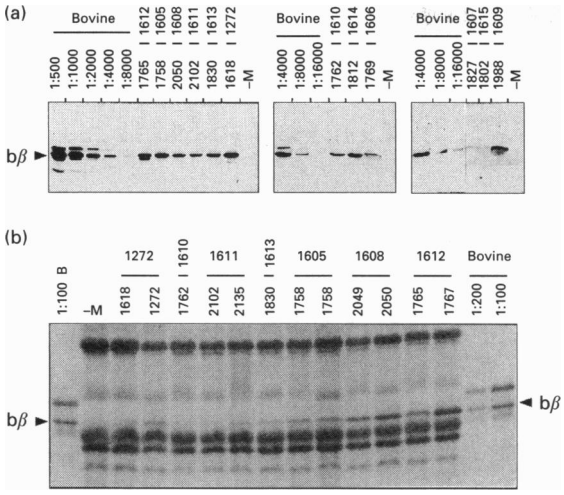

Figure 3 Expression of bovine β-casein in milk of transgenic mice

(**a**) Western blot analysis of milk samples from independent bovine β-casein mouse lines. Samples (10 μl) of diluted transgenic mouse milk were loaded under reducing conditions. Bovine β-casein was detected with a rabbit anti-(bovine β-casein) polyclonal antibody and a peroxide-conjugated goat anti-(rabbit IgG) polyclonal antibody as a second antibody. Casein bands were visualized by chemiluminesence. Diluted bovine milk was used as a reference. Panel I: diluted bovine milk (1:500, 1:1000, 1:2000, 1:4000, 1:8000); line 1612 [mouse 1765, second lactation (L2), day 10 (d10), diluted 1:4000]; line 1605 (mouse 1758, L2, d9, 1:2000); line 1608 (mouse 2050, L2, d13, 1:2000); line 1611 (mouse 2102, L1, d9, 1:400); line 1613 (mouse 1830, L2 ,d10, 1:400); line 1272 (mouse 1618, L2, d10, 1:400); —M, negative mouse milk 1:50. Panel II: diluted bovine milk (1:4000, 1:8000, 1:16000); line 1610 (mouse 1762, L2, d9, 1:400); line 1614 (mouse 1812, L2, d12, 1:160); line 1606 (mouse 1769, L1, d9, 1:20); —M, negative mouse milk 1:10. Panel III: diluted bovine milk (1:4000, 1:8000, 1:16000); line 1607 (mouse 1827, L1, d8, 1:10); line 1615 (mouse 1802, L1, d9, 1:10); line 1609 (mouse 1988, L2, d9, 1:10); —M, negative mouse milk 1:10. (**b**) Coomassie Brilliant Blue-stained SDS/PAGE gel with transgenic mouse milk samples of β-casein lines expressing at levels > 0.5 mg/ml. Diluted bovine milk was used as reference; 10 μl of 1:50 diluted transgenic and non-transgenic mouse milk were loaded under reducing conditions. Samples were derived from line 1272 (mouse 1618, L1, d9; mouse 1272, L2, d14); line 1610 (mouse 1762, L2, d9,); line 1611 (mouse 2101, L1, d9; mouse 2135, L2, d7); line 1613 (mouse 1830, L2, d10); line 1605 (mouse 1758, L3, d15 and L2, d9); line 1608 (mouse 2049, L2, d10; mouse 2050, L2, d13); line 1612 mouse 1765, L2, d9; (mouse 1767, L2, d5); —M, negative mouse milk 1:50; bovine milk diluted 1:100 and 1:200.

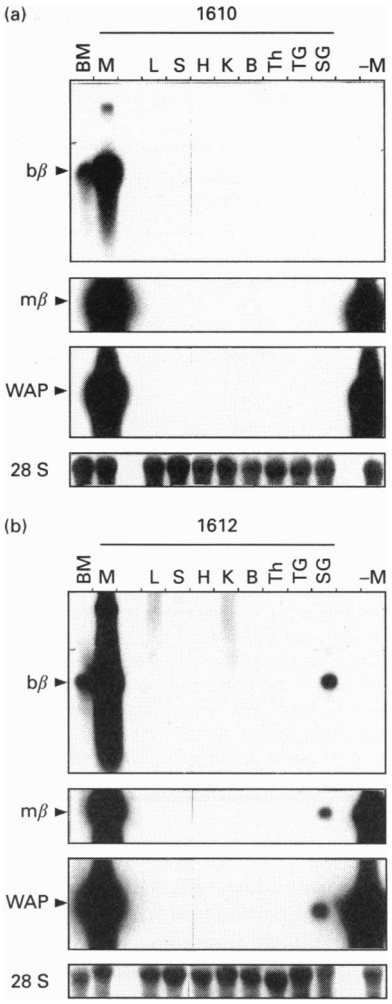

Figure 4 Tissue specificity of bovine β-casein expression

Northern blot analysis of total RNA (20 μg/lane) from tissues of two independent β-casein mouse lines, 1610 (**a**) and 1612 (**b**). Tissues analysed: BM, 1:2000 diluted lactating bovine mammary gland; M, lactating transgenic mouse mammary gland; L, liver; S, spleen; H, heart; B, brain; K, kidney; Th, thymus; TG, tear gland; SG, salivary gland; —M, non-transgenic mouse mammary gland. Blots were hybridized to bovine β-casein (bβ), murine β-casein (mβ), murine WAP cDNA probes and human 28 S RNA probe. Results were confirmed in an independent Northern blot with the same samples and with samples of non-transgenic tissues (results not shown).

Mice transgenic for the individual casein genes were generated via microinjection of the *Not*I fragments of β-casein cosmid 23A (33 kb), α_{s2}-casein cosmid 9A (28.5 kb), and κ-casein cosmid 30A (37 kb; for map see Figure 1). Transgene expression was analysed at the RNA and protein level. Tissue specificity and developmental regulation of transgene expression were analysed by Northern blotting.

Expression of bovine β-casein in transgenic mice

Twelve founder animals harbouring the 33 kb β-casein gene were generated and lines were established for each of these. Transgene integrity and copy number were analysed by Southern blotting (Table 1). All lines but one (line 1609) contained intact copies of the bovine β-casein gene with copy numbers varying from 2 to 11. Line 1609 contained only a single copy of the transgene, from which part of the 3′-flanking sequence was missing.

Transcription of the bovine β-casein gene in mouse mammary gland

Total RNA was isolated from mammary gland tissue of transgenic progeny at mid-lactation (days 6–10) and, in most cases, at late lactation (days 11–18). Bovine β-casein gene expression levels and transcript size were determined by Northern blotting.

RNA of the correct size was detected in mammary gland tissue of all lines (Figure 2). Transgene-derived transcript levels were quantified relative to RNA isolated from lactating bovine mammary gland tissue (Table 1). Roughly, the lines could be divided into two groups, those expressing at levels well below 0.5% of bovine mammary gland β-casein RNA levels (five lines), and a group of seven lines expressing from 1 to 40% of bovine levels.

In some cases the expression levels of bovine β-casein and endogenous mouse WAP had decreased by day 18, indicating that lactation was declining (Figure 2).

Bovine β-casein levels in the milk of transgenic mice

The bovine β-casein levels in the milk of transgenic mice were determined by Western blotting. Milk samples collected at several

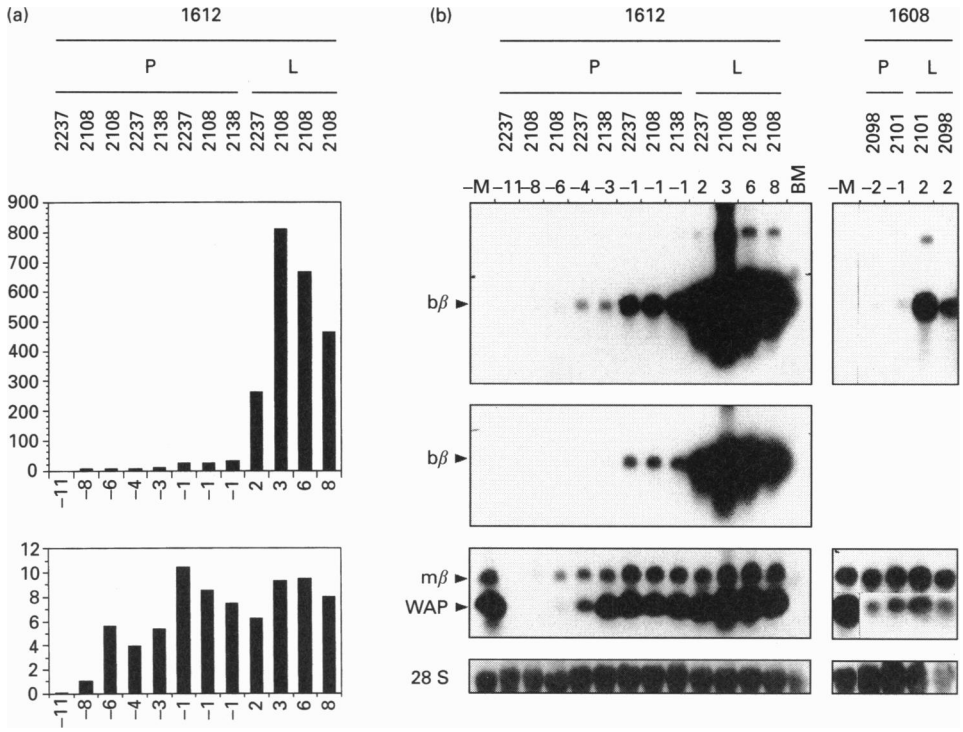

Figure 5 Stage-specific expression of bovine β-casein

Northern blot analysis of mammary gland RNA from β-casein transgenic mice of lines 1612 and 1608 at different points during the course of gestation (P) and lactation (L) and graphical representation of the results of line 1612. Line 1612: mouse 2237, biopsies taken at day −11, −4, −1 and 2; mouse 2108, day −8, −6, −1, 3, 6 and 8, and mouse 2138, day −3 and −1; line 1608: mouse 2098, day −2 and 2; mouse 2101, day −1 and 2. Blots were hybridized to a bovine β-casein cDNA probe (bβ), for which 1 and 4 day exposures are shown for line 1612 and a 2 day exposure for line 1608, a murine β-casein probe (mβ), a murine WAP and a human 28 S RNA probe. (**a**) Graphical representation of hybridization signals of the Northern blot containing samples of line 1612. Quantification was carried out using a beta-scan blot analyser. To determine the relative degree of induction, signal levels were divided by the amount of signal in the sample of day −8. Upper panel, bovine β-casein gene; lower panel, mouse β-casein gene. (**b**) −M, lactating non-transgenic mammary gland RNA, day 8 of lactation; −11, 11 days before parturition (2237−1612); −8 (2108−1612); −6, (2108−1612); −4, (2237−1612); −3, (2138−1612); −2, (2098−1608); −1, (2237−, 2108−, 2138−1612 and 2101−1608); 2, 3, 6, 8 days after delivery; BM, lactating bovine mammary gland RNA 1:2000 diluted in non-transgenic mouse kidney RNA. All lanes contain 20 μg of total RNA.

time points during lactation (mid-/late-) and different lactations (3–5 lactations/line), including samples corresponding to the RNA samples, were analysed. Correctly sized bovine β-casein was detected in the milk of all transgenic mouse lines (Figure 3). Maximum levels varied widely amongst lines, from less than 0.05 mg/ml to 20 mg/ml (Figure 3; Table 1). Six out of 12 lines (lines 1272, 1605, 1608, 1611, 1612 and 1613) expressed bovine β-casein in the mg/ml range (1–20 mg/ml). In these cases, a band migrating at the same position as β-casein in bovine milk was observed after SDS/PAGE and CBB-staining (Figure 3b). Three of these lines (1605, 1608 and 1612) expressed at levels similar to, or well above (2-fold), those found in bovine milk; the other lines (1272, 1611 and 1613) expressed from 1 to 4 mg/ml. Five lines exhibited levels below 0.1 mg/ml (1606, 1607, 1609, 1614 and 1615). The remaining transgenic mouse line (1610) expressed at intermediate levels (0.1–0.5 mg/ml). There was a positive correlation between the amounts of bovine β-casein mRNA and protein detected at mid-lactation (day 8; results not shown). Expression levels appeared to be independent of the number of integrated transgene copies.

Tissue-specificity of bovine β-casein expression

Tissue-specificity of bovine β-casein expression was determined in four transgenic animals [lines 1272, 1608, 1610 and 1612; results for lines 1610 (Figure 4a) and 1612 (Figure 4b) are shown]. Total RNA samples from nine different tissues of lactating transgenic mice, including the mammary gland, were analysed by Northern blotting (Figure 4). Expression of the bovine β-casein gene was detected exclusively in lactating mammary gland, with one exception. Ectopic expression was observed in the thymus of line 1612, at very low levels (< 0.1 % of bovine mammary gland levels; Figure 4b). The presence of transgene-derived transcripts in the salivary gland sample of line 1612 was most likely due to contamination by mammary gland tissue, as endogenous mouse milk protein gene transcripts (β-casein and WAP) were detected only in this sample and not in others.

Stage-specific expression of bovine β-casein

Induction of transgene expression during mammary gland development was studied in lines 1608 and 1612 (Figure 5). Total RNA isolated from mammary gland biopsies taken at different stages of mammary gland development and lactation were subjected to Northern blot analysis. Strong induction of bovine β-casein expression occurred in both lines upon parturition. Bovine β-casein transcript levels were at the threshold of detection at mid-pregnancy (days −8 and −6), these levels were only slightly higher (about 3-fold) at day −4, and from days −4 to −1 another 6-fold induction was observed. From day −1 to days 2 or 3 bovine β-casein expression increased dramatically (up to 50-fold; Figure 5). In contrast, expression of the mouse β-casein gene hardly increased upon parturition. Strong induction

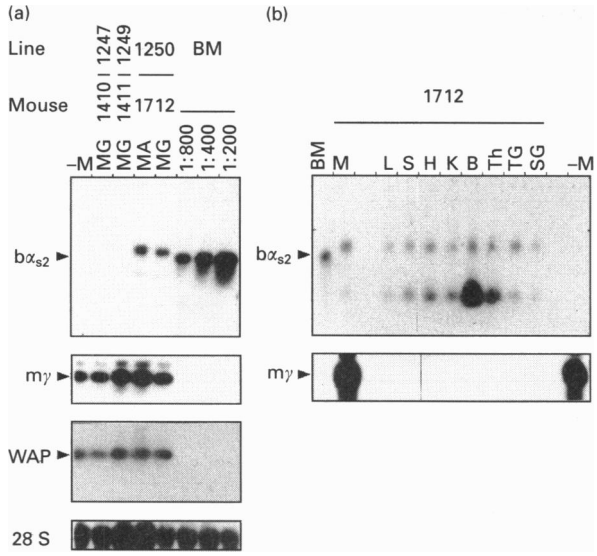

Figure 6 Northern blot analysis of bovine α_{s2}-casein transgenic mice

(**a**) Northern blot analysis of total RNA of mammary gland tissue of lactating α_{s2}-casein transgenic mice. Total RNA from lactating bovine mammary gland (BM) diluted in non-transgenic mouse kidney total RNA served as a standard for quantification of bovine α_{s2}-casein transcript levels. —M, lactating non-transgenic mammary gland RNA. Lactating mammary gland samples were derived from line 1247 (mouse 1410, L4, day 11); line 1249 (mouse 1411, L4, day 9) and line 1250 (mouse 1712 L1, day 10 and L1, day 17). The blot was hybridized to a bovine α_{s2}-casein (bα_{s2}), murine γ-casein (mγ; murine counterpart of bovine α_{s2}-casein), murine WAP cDNA probe and human 28 S probe. (**b**) Northern blot analysis of total RNA (20 μg/lane) from tissues of lactating α_{s2}-casein transgenic mouse 1712 of line 1250. Analysis was as described in the legend to Figure 5. The blot was hybridized to a bovine α_{s2}-casein cDNA probe (bα_{s2}) and a murine γ-casein probe (mγ; murine counterpart of bovine α_{s2}-casein).

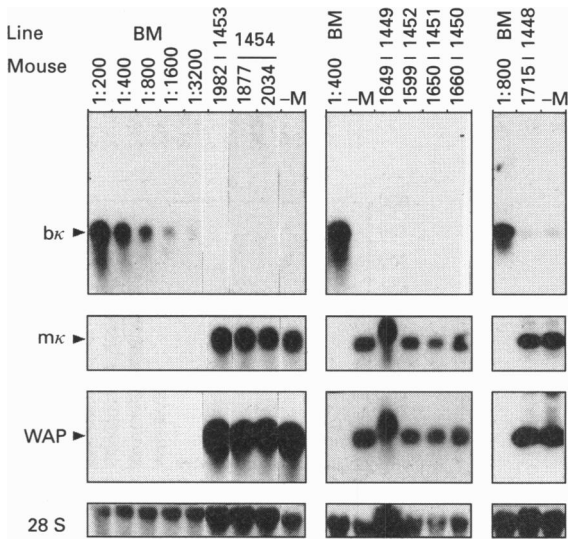

Figure 7 Northern blot analysis of κ-casein transgenic mice

Northern blot analysis of total RNA (20 μg/lane) from lactating mammary gland tissue of seven independent κ-casein transgenic mouse lines. — M and BM are as defined in previous Figures; lactating mammary gland samples of line 1453 [mouse 1982, first lactation (L1), day 6 (d6)]; line 1454 (mouse 1877, L1, d9 and 2034, L3, d8); line 1449 (mouse 1649, L1, d9); line 1452 (mouse 1599, L2, d10); line 1451 (mouse 1650, L1, d8); line 1450 (mouse 1660, L1, d9); and line 1448 (mouse 1715, L1, d9). Blots were hybridized to bovine κ-casein cDNA probe (bκ), murine κ-casein probe (mκ), murine WAP and human 28 S probe.

of murine β-casein gene expression occurred in mid-pregnancy and was followed by a steady increase in expression during further gestation and lactation (Figure 5). This developmental pattern of mouse β-casein expression has been described previously [21,26,39]. In the cow, casein RNA levels, which are low during gestation, also abruptly increase upon lactogenesis [40]. Therefore, our data indicate that the bovine β-casein transgene follows the bovine rather than the murine developmental expression pattern.

Analysis of bovine α_{s2}-casein transgenic mice

Five transgenic mice were generated carrying the 28.5 kb insert of bovine α_{s2}-casein clone 9A (8 kb of 5′- and 1.5 kb of 3′-flanking sequences). Three lines were established from these founders (1247, 1249 and 1250), all with transgene copy numbers higher than 10. The two other transgenic animals (842 and 1248) did not transmit the transgene.

RNA was isolated from mammary glands of lactating F_1 females and analysed by Northern blotting. Bovine α_{s2}-casein mRNA levels determined for these lines were either extremely low (line 1250, at least 1000-fold below bovine mammary gland levels), or not detectable (lines 1247 and 1249; Figure 6a). Transcripts detected in lactating mammary gland tissue of line 1250 were incorrectly sized; both larger and smaller transcripts were observed. Moreover, expression occurred in all tissues analysed, while the endogenous murine γ-casein transcript, the mouse equivalent of bovine α_{s2}-casein, was only present in the lactating mammary gland (Figure 6b). The ratio between the two transcripts was different in the various tissues; in brain the lower transcript was more abundant while in mammary gland the upper transcript was predominant. Apparently, not all regulatory elements required for correct expression are included in bovine α_{s2}-casein gene clone 9A.

Analysis of bovine κ-casein transgenic mice

Seven founder animals were generated harbouring the 37 kb insert of κ-casein clone 30A (5 kb of 5′- and 19 kb of 3′-flanking sequences), each of which yielded transgenic offspring. Transgene copy numbers varied between 2 and 10. All lines were analysed for κ-casein expression at the RNA level by Northern blot analysis (Figure 7). Bovine κ-casein transcripts could not be detected in any of these transgenic lines. This indicates that expression was either well below 0.03% of the bovine levels or completely absent. Similarly, no bovine κ-casein was detected in transgenic mouse milk by Western blotting, using a polyclonal rabbit antibody directed against bovine κ-casein (results not shown).

In vitro expression of bovine α_{s2}- and κ-casein genes

To investigate whether clones 9A and 30A contained intact, functional copies of the bovine α_{s2}- and κ-casein genes, these cosmid clones were each transfected into murine mammary epithelial cell line HC11 [27]. Total RNA was isolated from pools of stably transfected clones and analysed for the presence of the corresponding bovine transcripts. RNA derived from both genes was detected (Figure 8). This implies that the bovine casein gene clones used in this study contain an intact structural gene and functional promoter elements.

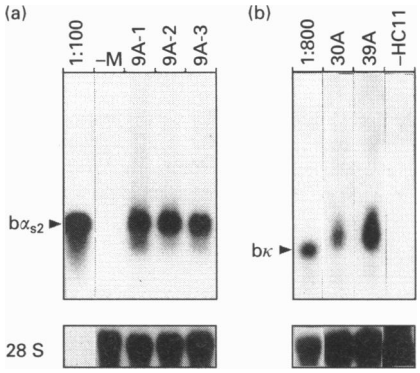

Figure 8 ***In vitro*** **expression of bovine α_{s2}- and κ-casein genes**

Northern blot analysis of total RNA from pools of HC11 clones stably transfected with bovine α_{s2}-casein cosmid clone 9A or bovine κ-casein cosmid clone 30A and 39A (κ-casein gene with 3 kb of 5′- and 21 kb of 3′-flanking sequences). —M as in previous Figures; (**a**) 1:100, 0.2 μg of total RNA isolated from lactating bovine mammary gland: 9A-1, 9A-2 and 9A-3, independent HC11 pools stably transfected with α_{s2}-casein cosmid clone 9A (20 μg/lane); (**b**) 1:800, 0.025 μg of lactating bovine mammary gland RNA mixed with non-transgenic kidney RNA to a total of 20 μg; 30A and 39A, HC11 pools stably transfected with bovine κ-casein cosmid clone 30A and 39A (≥ 20 μg/lane); —HC11, non-transfected HC11 cells. Blots were hybridized to the appropriate cDNA probes and human 28 S probe. The size difference between the κ-casein transcript in the bovine mammary gland control and in the stably transfected HC11 clones was caused by overloading and high salt concentrations of the HC11 samples.

DISCUSSION

As a first step towards identifying *cis*-acting elements involved in the coordinate regulation of the genes in the bovine casein locus, we isolated the individual β-, α_{s2}- and κ-casein genes and analysed their expression in transgenic mice. By using intact genes rather than hybrid constructs we aimed to augment the probability of including important regulatory elements.

To the best of our knowledge, this is the first report describing expression analysis in transgenic mice of the bovine α_{s2}- and κ-casein genes. Neither the bovine α_{s2}-casein gene with 8 kb of 5′- and 1.5 kb of 3′-flanking sequences nor the κ-casein gene flanked by 5 kb of 5′ and 19 kb of 3′ sequences, was transcribed at detectable levels in the mammary gland of transgenic mice. The promoter and structural gene of both transgenes were functional, as demonstrated by the detection of correctly sized bovine transcripts in stably transfected HC11 cells. Proximal 5′-flanking sequences of the bovine κ-casein gene (−552 to +64 bp) fused to a reporter gene (human growth hormone gene, hGH) appeared to be non-functional in transgenic rats [24]. Similarly, the goat κ-casein gene including 4.5 kb of 5′- and 3 kb of 3′-flanking sequences was expressed at extremely low levels (a few μg/ml) in transgenic mice, while a κ-casein mini-gene fused to goat β-casein gene 5′-flanking sequences was expressed at high levels (3 mg/ml; J.C. Mercier, personal communication).

These data suggest that sequences required for efficient expression of the bovine α_{s2}- and κ-casein genes, such as enhancers, are not closely linked to these genes and are situated elsewhere in the casein locus. Such elements might also play a role in the regulation of the other casein genes. Although the κ-casein gene is not evolutionarily related to the genes encoding the calcium-sensitive caseins it is physically and functionally linked to these genes, and all four genes are coordinately expressed. Combined with the data presented in this paper, this supports the notion that dominant *cis*-acting control elements might be involved in the expression of the entire casein gene locus [41], possibly in analogy to the locus control region (LCR) described for the β-globin gene cluster [42,43].

In contrast to the results obtained with the bovine α_{s2}- and κ-casein genes, the 33 kb bovine β-casein gene used in this study was functional in transgenic mice. Bovine β-casein expression was detected in all transgenic mouse lines. In 50% of these lines, relatively high levels of expression (> 1 mg/ml) were measured, while levels as high as 20 mg/ml were obtained in two of these, carrying two and four copies of the transgene. Apparently, regulatory elements capable of driving high-level tissue- and stage-specific expression, at levels similar to or even exeeding those observed in the cow, are included in this β-casein clone. However, they are not completely capable of overriding the effects of neighbouring murine sequences, since expression levels were highly variable and not related to copy number. This may indicate that *cis*-acting regulatory elements conferring high-level, position-independent expression of the β-casein gene are missing from the transgene. Alternatively, elements fully capable of

Table 2 Expression and structure of β-casein transgenes in mouse, rat and rabbit

Abbreviations: (g), genomic sequences; (c), cDNA sequences; –, no β-casein 3′ sequences in transgene; hLZ, human lysozyme; hIL-2, human interleukin-2.

Origin of regulatory sequences	5′ sequences (kb)	Nature of coding sequence	3′ sequences (kb)	Highest levels of expression	Lines expressing	Expressing at high levels (> 1 mg/ml)[1]	Transgenic species	Reference
Goat	4.2	β-casein (g)	5.3	50% of mβ-casein	7/7	4/7*	Mouse	[22]
Goat	3	β-casein (g)	6	24 mg/ml	9/9	6/9	Mouse	[21]
Rat	3.5	β-casein (g)	3	1% of mβ-casein	3/5	0/3*	Mouse	[19]
Bovine	16	β-casein (g)	8	20 mg/ml	12/12	6/12	Mouse	This paper
Bovine	−1.79 to +1.95 (exon 2)	hLZ (c)	Intron 8 to 3.5	RT-PCR†	1/8	0/1	Mouse	[23]
Bovine	−1.72 to +0.037 (exon 1)	hGH (g)	–	10.9 mg/ml	11/13	1/11	Rat	[24]
Rat	−2.3 to +0.490 (intron 1)	CAT	–	0.1% of mβ-casein	6/6	0/6[1]	Mouse	[20]
Rabbit	−2 to +0.051 (exon 1)	hIL-2 (c)	–	0.0004 mg/ml	4/4	1/4	Rabbit	[44]

* Expression > 5% of endogenous β-casein is taken as 'high level', assuming murine β-casein expression to be 20–30 mg/ml [21].
† Human lysozyme RNA could only be detected in one line by reverse-transcriptase PCR [23].

regulating bovine β-casein expression in their natural genomic environment may not be able to do so at every genomic position in mice.

The developmental expression pattern of the β-casein transgene followed that of the cow [40] rather than that of the mouse [39]. In the cow, casein mRNA levels are very low during gestation and increase abruptly at the onset of lactogenesis [40]. Induction of murine β-casein gene expression occurs in mid-pregnancy followed by a steady increase in expression during the second half of gestation and subsequent lactation [21,26,39]. The induction upon parturition is minor (1.5-fold). For a goat β-casein transgene [21] and transgenes containing bovine α_{s1}-casein regulatory sequences [23,26] developmental expression patterns similar to that of the bovine β-casein transgene have been observed in transgenic mice.

In transgenic mice expressing bovine β-casein, the endogenous mouse β-casein and WAP genes display the developmental profile as reported before [21,26,39]. Therefore, both the transgene and the murine β-casein gene have retained their cognate endogenous developmental expression pattern.

The proximal promoter regions (about 5 kb) of β-casein genes of rat, mouse and cow have been studied in tissue culture systems, resulting in the identification of regulatory motifs and *trans*-acting factors binding to them [9–16]. Most of these regulatory elements are situated within 1.7 kb upstream of the transcription start site. Wild-type β-casein genes of goat [21,22] and rat [19], and chimeric rat [20], rabbit [44] and bovine [23,24] β-casein genes, all including at least 1.7 kb of 5′-flanking region, have been introduced into the mouse, rabbit [44] and rat [24] germline (Table 2). High levels of expression were obtained with the goat β-casein gene in transgenic mice [21,22]. The goat gene was expressed in all transgenic mouse lines and about 50 % of the lines displayed expression levels in the mg/ml range (Table 2). Expression levels as high as 24 mg/ml [21] and up to 50 % of endogenous mouse β-casein transcript levels [22] were detected. These results are comparable with the findings reported here for the bovine β-casein gene.

The data summarized in Table 2 show that intact rat and various hybrid β-casein genes fail to be expressed at high levels in transgenic animals. This might, for a large part, be due to the fact that cDNA or bacterial rather than genomic sequences have been used in the latter cases [20,23,44]. The low expression levels of the intact rat β-casein gene might reflect differences between the rat and ruminant β-casein genes with respect to localization of regulatory elements in the flanking regions. Alternatively, the larger 3′-flanking region included in the ruminant β-casein transgenes (6 kb [21]; 5.3 kb [22]; 8 kb, this paper) as opposed to the 3 kb of 3′-flanking sequences included in the rat gene, might account for the higher and more consistent levels of expression.

In order to identify regions of the locus involved in casein gene regulation, introduction of the complete locus or large parts of it into the germline of transgenic mice would be desirable. This could be achieved by introducing Yeast Artificial Chromosomes (YAC), Bacterial Artificial Chromosomes (BAC) or P1 clones, containing the complete casein gene locus or parts of it, into the mouse genome by microinjection [45,46]. Alternatively, overlapping DNA fragments (e.g. cosmid clones) covering part of the casein locus could be co-injected, resulting in reconstitution of this region via extrachromosomal homologous recombination [47]. Once identified, the function of potential control elements could be studied directly by generating transgenic mice harbouring such sequences combined with the non-efficiently expressed α_{s2}- and κ-casein genes. Incorporation of casein locus-derived LCR-like elements into mammary gland-specific expression vectors would greatly improve the predictability and levels of heterologous protein expression in the milk of transgenic animals.

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