Expression of bovine β-lactoglobulin in the milk of transgenic mice

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The use of transgenic animals to manipulate milk composition has considerable potential, both for the production of biomedical proteins and for the direct manipulation of milk composition for the improvement of dairy animals and their products (for reviews, see Wall *et al.* 1992; Yom & Bremel, 1993). Promoters from a number of milk protein genes from a variety of species have been tested for their ability to direct the expression of foreign proteins to the mammary gland (for review, see Maga & Murray, 1995).

β-Lactoglobulin (β-lg) is the major whey protein produced in ruminant milk and is part of the normal milk composition of most mammals except humans and rodents (Pervaiz & Brew, 1985). It is expressed at high levels in the mammary gland and is developmentally regulated. Transgenic mice have been produced using the complete ovine (Simons *et al.* 1987; Shani *et al.* 1992) and caprine (Ibañez *et al.* 1997) β-lg genes. In general, high levels of expression were obtained with the ovine β-lg gene, and expression was also seen in a position-independent manner (Whitelaw *et al.* 1992). Lower levels of expression were reported using the caprine β-lg gene. Here we report the production of transgenic mice using the bovine β-lg gene. We describe high expression, position-dependent, and copy number-related expression of bovine β-lg protein in the milk of six lines of transgenic mice.

MATERIALS AND METHODS

The DNA construct was prepared from a genomic clone of the bovine β-lg gene (Alexander *et al.* 1993) by restriction enzyme digest with *Eag*I and *Sph*I (Fig. 1). The transgene consisted of the entire genomic coding region for bovine β-lg as well as 1·2 kb 5′ and 1 kb 3′ of bovine β-lg flanking regions. The DNA construct was purified using a NaCl gradient (Fink, 1991), the gradient fractionated and the fractions containing the construct desalted using NAP-25 columns (Pharmacia Biotech, S-751 28 Uppsala, Sweden).

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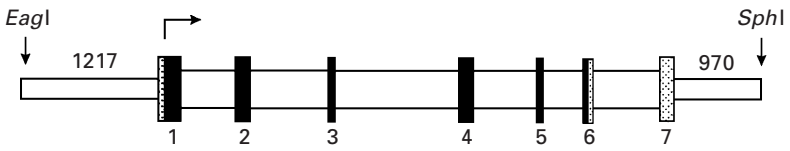

Fig. 1. Structure of the bovine β-lactoglobulin transgene (7 kb). Filled boxes represent bovine β-lactoglobulin translated exons. Hatched boxes represent non-translated exons. Narrow boxes represent 5′ and 3′ flanking regions of bovine β-lactoglobulin DNA.

Transgenic mice were generated by microinjection of construct DNA (10 ng/μl) into pronuclear-stage mouse embryos obtained from CBA × C57B1/6 F1 female mice. Microinjected embryos were transferred into pseudopregnant recipient mice. Transgenic mice were identified by Southern analysis of DNA extracted from tail biopsies. Blots were probed with a digoxigenin–dUTP (DIG/Genius™ System, Boehringer Mannheim, Indianapolis, IN 46250, USA) 340 bp polymerase chain reaction fragment (described below) of bovine β-lg DNA. Transgene copy number was determined by using slot blots of tail biopsies from two animals of the F1 generation from all lines except line 20, where DNA from the founder animal was used. The blots were probed with the same probe used for Southern analysis and copy number determined by comparison of the band intensity of the bovine β-lg gene (obtained from bovine DNA) with that of the β-lg transgene on a densitometer (Ultroscan XL; LKB, S-161 26 Bromma, Sweden). Tissue specificity of transgene expression was analysed by dot blot analysis. Total RNA (5 μg) was extracted from mammary gland, liver, kidney, spleen and salivary gland of two F1 transgenic and non-transgenic control females at peak lactation as previously described (Maga *et al.* 1994). The blots were probed with the same probe used for Southern anlaysis.

DNA from progeny of founder mice was extracted from tail or toe clips of pups as described by Gutiérrez-Adán *et al.* (1996), and the presence or absence of the transgene was detected by polymerase chain reaction analysis. The primers used were specific for the amplification of a 340 bp fragment between exon 4 and intron 4 of the β-lg DNA (Van Eenennaam & Medrano, 1991). This polymerase chain reaction product was also used as a probe for Southern and slot blot analyses.

Milk samples were collected on day 10 of lactation from transgenic and non-transgenic control mice. The mother was separated from her pups for at least 3 h and injected intraperitoneally with 0·2 i.u. oxytocin (Sigma, St Louis, MO 63178, USA) to stimulate milk ejection. The milk was collected under reduced pressure by a mechanical suction apparatus into 1·5 ml Eppendorf tubes (Gutiérrez-Adán *et al.* 1996). Milking was continued until there was no further milk yield from any nipple, $\sim$ 1–1·5 ml milk being collected from each mouse and stored at -70 °C until analysis.

Bovine β-lg protein expression in mouse milk was detected by SDS-PAGE (Basch *et al.* 1985). Samples were centrifuged at 16000 ***g*** and 4 °C for 2 min to remove fat. The skim milk samples were then diluted 1 : 10 with distilled H_2O and added to an equivalent volume of SDS loading buffer. Samples were resolved on a standard SDS-PAGE gel and the proteins electrophoretically transferred on to a nitrocellulose membrane. Bovine β-lg was detected by using a 1 : 7500 dilution of rabbit anti-bovine β-lg antibody (Nordic Immunological Laboratories, Capistrano Beach, CA 92624, USA) and a 1 : 15000 dilution of goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (EC 1.11.1.7; BioRad, Hercules, CA 94547, USA). The signal

Table 1. *Characteristics of bovine β-lactoglobulin transgenic mouse lines*

Line	Copy no.	Estimated bovine β-lactoglobulin in milk, mg/ml†
BLA-9	5	0·74 ± 0·4
BLA-10	2	1·75 ± 1·0
BLA-11	10	—
BLA-16	5	1·29 ± 1·0
BLA-20	8	ND
BLA-22	12	—
BLA-26	1–2	3·37 ± 1·1
BLA-27	6	0·95 ± 0·4
BLA-29	1–2	2·6 ± 1·2

—, Not detected; ND, not determined.
† Values are means ± SD.

was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL 60005, USA), which can detect nanogram amounts of protein.

Quantification of bovine β-lg in mouse milk was carried out by densitometer scanning of Western blots in triplicate using a digital densitometer with the Collage@Software (Fotodyne, Hartland, WI 53220, USA). Each membrane contained seven mouse milk samples and two controls with different concentrations of bovine milk. After the Western blots had been developed, the images were digitized and a quantitative integration of the intensity and area for each band was performed. A linear standard curve was constructed from the controls (assuming 3 mg bovine β-lg/ml in cows' milk) and used to assign the bovine β-lg present in each of the mouse milk samples.

RESULTS

Nine lines of transgenic mice carrying a 7 kb bovine β-lg gene were generated, with the transgene copy number varying between 1 and 10 (Table 1). In eight of the nine lines, the transgene was stably integrated and transmitted as a Mendelian locus. In line BLA-20, no transmission was observed owing to the infertility of the founder male.

Expression of bovine β-lg at the protein level was detected by Western blotting in six of the eight transgenic lines studied (Fig. 2). Cross reaction with the rabbit anti-bovine β-lg antibody was not observed in milk from non-transgenic control mice. Bovine β-lg protein levels in the milk of hemizygous mice at peak lactation ranged from 0·74 to 3·37 mg/ml (Table 1). The expression of bovine β-lg in mouse milk did not appear to affect the physiology of the mammary gland, and the growth of the pups was observed to be normal (results not shown). Simple unweighted regression analysis showed a significant ($P < 0{\cdot}001$) inverse relationship between transgene expression level and copy number with low copy number having the highest levels of expression and the highest copy number having no expression (Fig. 3). Furthermore, dot blot analysis of five tissues (mammary gland, liver, spleen, kidney and salivary gland) from two animals of lines BLA-10, BLA-16 and BLA-26 showed that the expression of the transgene was mammary gland specific (results not shown).

DISCUSSION

Transgenic mice carrying the complete bovine β-lg gene transcription unit together with 1·2 kb and 1 kb respectively of the 5′ and 3′ flanking regions are capable of expressing high levels (0·75–3·4 mg/ml) of bovine β-lg in their milk. It has

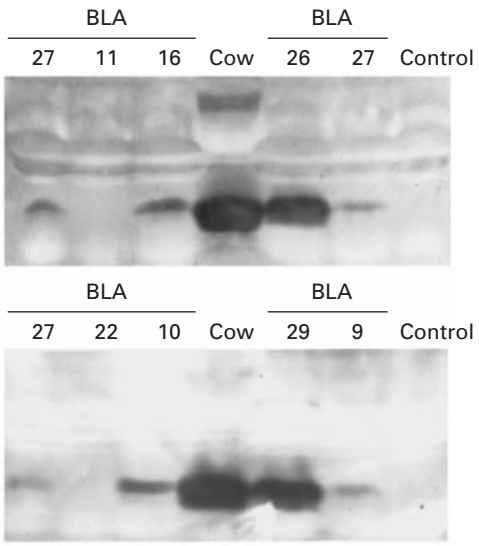

Fig. 2. Detection of bovine β-lactoglobulin in the milk of transgenic mice. Milk from three hemizygous mice from each transgenic line was collected on day 10 of lactation and analysed by Western blotting. Equal amounts of milk equivalent to 1·5 μl milk from transgenic expressing lines (BLA-27, BLA-16, BLA-26, BLA-10, BLA-29, BLA-9), non-expressing lines (BLA-11 and BLA-22), control non-transgenic mouse milk (Control) and skim bovine milk (Cow) were resolved on an SDS-PAGE gel (150 g/l), transferred on to a nitrocellulose membrane and probed with a polyclonal rabbit anti-bovine β-lactoglobulin antibody.

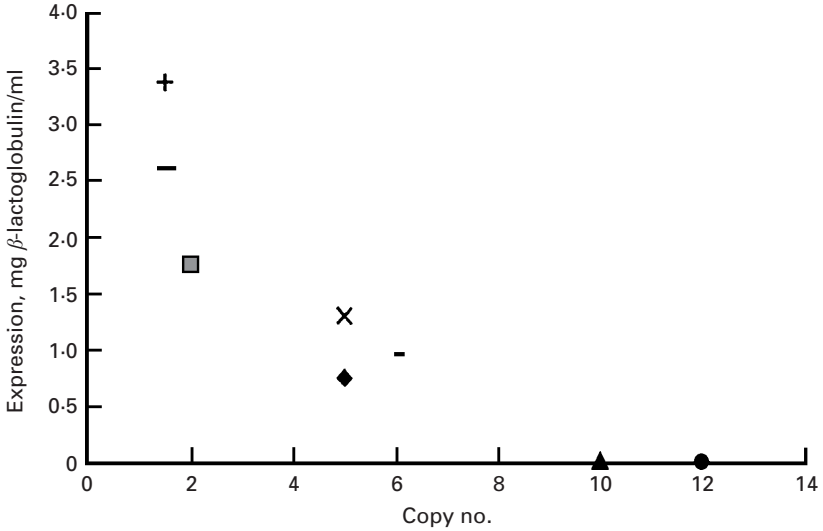

Fig. 3. Copy number-dependent expression of bovine β-lactoglobulin protein in transgenic mouse lines: ◆, BLA-9; ■, BLA-10; ▲, BLA-11; ×, BLA-16; ●, BLA-22; +, BLA-26; ‾, BLA-27; ‾, BLA-29. Mean bovine β-lactoglobulin protein levels in the milk of transgenic mice were determined by Western blotting. Copy number was determined from F1 mice by Southern blotting.

also been reported that a bovine β-lg transgene containing 2·8 kb of 5′ and 1·9 kb of 3′ flanking regions is specifically expressed in the mammary gland at levels of 1–2 mg/ml (Hyttinen *et al.* 1998). While high levels of expression (3–23 and 1–8·5 mg/ml) of ovine β-lg transgenes in mice have been reported by Simons *et al.*

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(1987) and Shani *et al.* (1992) respectively, lower levels of expression (10 μg/ml–0·5 mg/ml) were reported using the caprine β-lg gene (Ibañez *et al.* 1997). The use of bovine β-lg sequences resulted in protein expression at levels between those observed with the ovine and caprine sequences, but tending towards that of the ovine gene. The bovine and ovine transgenes contained less 5′ flanking DNA than did the caprine transgene (1·2 or 2·8 and 3 or 4 kb *v.* 6·1 kb respectively). Increasing the length of the 5′ flanking region of ovine β-lg constructs also had a negative effect on expression of the transgenes (Shani *et al.* 1992). The lower expression reported using the caprine β-lg construct could be due to the differences in the lengths of the 5′ and 3′ flanking regions, and indicates the possible presence of inhibitory *cis*-acting elements in the longer (6·1 kb) promoter used.

Moreover, ovine β-lg transgenes with 4·3 kb of 5′ flanking sequences are expressed at high levels in a position-independent manner in transgenic mice (Simons *et al.* 1987; Whitelaw *et al.* 1992; Clark *et al.* 1994). Here, the shortening of the 5′ flanking region to 1·2 kb in our bovine β-lg transgene does not confer position-independent expression. Clark *et al.* (1994) proposed that the position-independent expression observed using the ovine β-lg transgene requires the presence of *cis*-acting regulatory elements located downstream of the gene. Such elements appear not to be present in the 1·2 kb of 5′ and 1 kb of 3′ flanking sequences used in our bovine β-lg transgene.

Protein expression of the bovine β-lg transgene follows an inverse relationship with transgene copy number as low copy numbers express at a higher level than the high copy numbers and the non-expressing lines have the highest copy numbers of all lines. It has been reported that the number of transgene copies within an array can exert a repressive influence on expression, with several mouse studies indicating a decrease in the level of expression per copy as copy number increases (Davis & MacDonald, 1988; Gourdon *et al.* 1994; Dorer, 1997; Garrick *et al.* 1998). A model has been proposed in which homologous pairing between monomers within the array induces heterochromatinization at the transgene locus (Garrick *et al.* 1998). However, the molecular mechanism for repeat-induced silencing of multimeric arrays has yet to be fully elucidated. Repeat-induced silencing may often be responsible for poor transgene expression when transgene constructs do not contain genetic elements that function to insulate individual monomers and prevent silencing. This could be the case for the short 5′ and 3′ flanking sequences that were used in our bovine β-lg construct. The inverse relationship between transgene copy number and expression has not been seen with the ovine β-lg transgene, perhaps because the long 5′ and/or 3′ flanking sequences used contain a locus control element that confers position-independent expression, 'rescue of expression', and positively related copy number-dependent expression (Whitelaw *et al.* 1992; Clark *et al.* 1994). However, an ovine β-lg construct comprising 406 bp of the 5′ flanking sequences showed a positive relationship between copy number and level of expression (Whitelaw *et al.* 1992). Clark *et al.* (1994) hypothesized that the position-independent expression of the ovine gene requires elements that may be located within as well as downstream of the gene.

The endogenous β-lg gene produces 3 g β-lg/l in the milk of lactating dairy cattle. Substantial amounts of bovine β-lg, equivalent to endogenous levels in cows' milk, were produced from a single transgenic locus (line BLA-26) by gene insertion. Our results indicate that this transgene is mammary gland-specific. This makes the bovine β-lg construct described here a useful addition to the list of mammary-specific promoters that can be used to determine the basis for the specific expression of genes in the mammary gland, and for the production of heterologous proteins in the milk of transgenic animals.

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