Transgenic Research (2005) 14:719–727
DOI 10.1007/s11248-005-7217-x

Development of transgenic mice expressing calcitonin as a beta-lactoglobulin fusion protein in mammary gland

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Received 27 August 2004; accepted 11 May 2005

Key words: beta-lactoglobulin, calcitonin, fusion protein, milk, recombinant protein, transgenic mice

Abstract

Expression of foreign proteins in mammalian milk is becoming a widespread strategy for high-level production of recombinant pharmaceuticals, especially those with the most complex post-translational modifications. A milk-specific ovine beta-lactoglobulin (oBLG) promoter was used to drive expression of recombinant calcitonin in mouse milk. A gene construct was generated, consisting of 10.7 kbp of the oBLG gene including its promoter and 3′ flanking region with the calcitonin coding sequences inserted in-frame into the oBLG fifth exon. After microinjection, six founder mice transmitted the transgene to their progeny. RT-PCR confirmed mammary-gland specific expression of recombinant mRNA in most transgenic mice and Western blot analysis confirmed expression of chimeric protein. Calcitonin can thus be expressed under the oBLG promoter and regulatory elements in a mammary-gland specific manner.

Introduction

One of the challenges created by the biotechnology revolution is developing methods for the large-scale production of purified proteins. Recent developments indicate that expression of different proteins in the mammary gland is not only feasible, but also economic and scalable (Carver et al., 1993; Prunkard et al., 1996). Proteins expressed in the mammary gland show extensive similarities to their native forms. These similarities include secondary, tertiary, and even quaternary structures (John et al., 1999; Kolb et al., 2001) and post-translational modifications including glycosylation, hydroxylation, carboxylation and amidation (McKee et al., 1998). These characteristics are often essential for their biological activity, which in most instances are to be used as pharmaceuticals.

There are several milk-specific genes, all of which have been tested for their capacity to drive mammary gland-specific expression of foreign proteins. Caseins, which contribute to about 80% of milk protein, coagulate upon ripening, while whey proteins, accounting for the remaining 20%, remain soluble.

Beta-lactoglobulin (BLG), the most abundant whey protein in ruminant milk (Mercier and Vilotte, 1993), exists as a dimer. It is a member of the superfamily of hydrophobic molecule transporters or lipocalins (Flower, 1996). The ovine BLG (oBLG) subunit is a polypeptide of 162 residues. It has been suggested that BLG is involved in both fatty acid and retinol transport in milk (Perez & Calvo, 1995; Flower, 1996).

oBLG promoter has frequently been used to express foreign proteins in mammalian milk (Prunkard et al., 1996; McKee et al., 1998; John

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et al., 1999; Kolb et al., 2001). Most reported transgenes used the BLG promoter, but in a few instances, the gene was used as a fusion partner as well.

Genomic sequences are usually expressed at levels several hundred times higher than cDNA sequences. However, it is not yet clear if the addition of intronic sequences derived from a fusion partner can fully compensate for the lack of intronic sequences in a cDNA. In one study, human erythropoietin (hEPO) cDNA which was inserted in-frame into the fifth exon of a bovine BLG gene construct, resulted in relatively high level expression of the bBLG-hEPO fusion protein (Korhonen et al., 1997).

It was shown that the proximal 4.3 kbp fragment of oBLG contains both the elements essential for high-level mammary gland-specific expression, and those which sequester transgene expression from integration site-effects (Whitelaw et al., 1992). The 1.9 kbp of 3′ flanking fragment contains matrix attachment region (MAR) (Whitelaw et al., 2000), which play an important role in preventing localized genomic effects. Intronic sequences also have profound effects on transgene expression (Webster et al., 1997).

Calcitonin is a 32-amino acid single-chain circulating polypeptide secreted from thyroid parafollicular C cells of mammals and is involved in skeletal homeostasis (Bittar & Bittar, 1997). Although the detailed functional significance of calcitonin remains an intriguing question, there is a growing consensus that calcitonin protects the skeleton during periods of calcium stress, namely growth, pregnancy and lactation. In addition, its importance in clinical pharmacology has been established beyond any doubt (Bittar & Bittar, 1997). Although human calcitonin (hCT) shows just a weak pharmacologic potency in osteoblastic calcium disposition in humans, its salmon counterpart (sCT) is among the most pharmacologically potent osteogenic forms of the hormone and has found widespread clinical use in osteoporosis (e.g. post-menopausal osteoporosis), Paget's disease, and hypercalcemic shock. There are common characteristics in calcitonin structure among different species, including an N-terminal lariat due to a disulfide bond between Cys1 and Cys7, and a C-terminal amide group resulting from an extending Gly residue. The C-terminal amide group is particularly essential for biological activity of calcitonin (McKee et al., 1998).

sCT although potent enough in humans, causes gastrointestinal problems and immunological hypersensitivity upon oral use, which limit its prescription as a widespread treatment. It has been shown that a hybrid calcitonin protein consisting of sCT C-terminal 16 amino acids and hCT N-terminal 16 amino acids has sCT potency without causing gastrointestinal and immunologic problems in humans (Maier, 1976). In addition, it has been shown that the rabbit mammary gland has PAM (peptidyl-glycine α-amidating mono-oxygenase) activity, which converts a calcitonin C-terminal Gly-extension to an amide group, which is essential for biological potency of the hormone (McKee et al., 1998). One may hope that a similar enzyme activity is present in the mouse mammary gland.

Materials and methods

Gene construct

Fresh blood was collected from Iranian Moghan sheep, the most inbred sheep race in Iran and its genomic DNA was extracted using Genomic DNA Extraction Kit (Promega, USA) to obtain high molecular weight DNA for the long-PCR isolation of the oBLG gene and flanking regions.

The fifth oBLG exon was selected for in-frame insertion of hybrid CT cDNA, just after the 149th codon (Figure 1). Two PCR primer pairs were designed to amplify oBLG using the Expand Long Template PCR System (Roche, Germany): b1f–b3r and b4f–b4r (Table 1). The first pair amplified 4.2 kbp of the 5′ flanking region and promoter in addition to 3.8 kbp of the transcription unit, and the second amplified remaining 0.8 kbp of the transcription unit in addition to 1.9 kbp of the 3′ flanking region. Both PCR products were cloned separately using TOPO XL Kit (Invitrogen, USA) to obtain topo-b13 and topo-b44, respectively.

The following sequences were built into the CT construct: *Eco*RV–Factor Xa–His6–Enterokinase–CT–*Cla*I. Enterokinase site permits release of calcitonin polypeptide before final purification. 16 N-terminal codons of CT were selected from human calcitonin gene, while 17 C-terminal codons from salmon one. Accordingly, two 3′ end complementary oligonucleotides, CTf (97b) and CTr (88b) (Table 1), were chemically synthesized

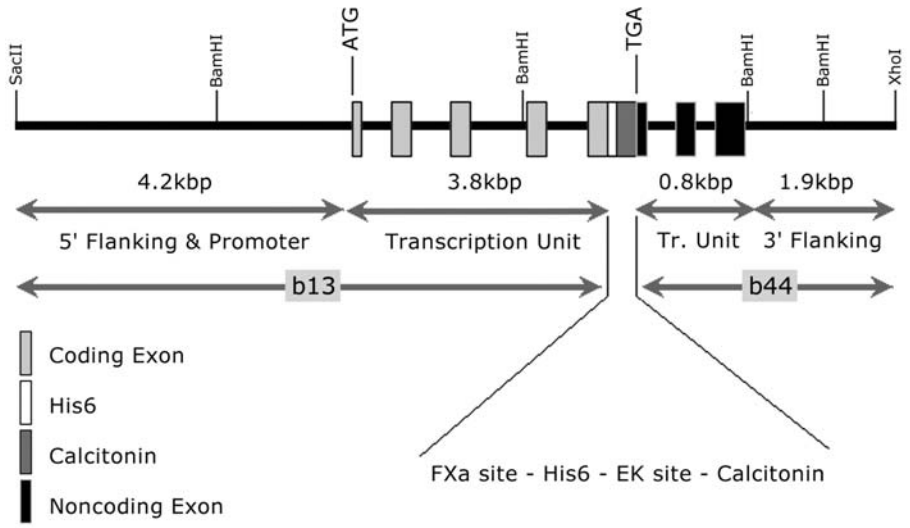

Figure 1. The final 10.9 kbp gene construct, consisting of entire oBLG gene and calcitonin gene construct inserted in-frame into oBLG fifth exon. This construct results in a fusion protein of 149 amino acids of oBLG and 51 amino acids of calcitonin construct. Restriction sites used for gene construct linearization and Southern blotting have been shown too.

to get the hybrid CT fragment. In a PCR-like reaction, these oligonucleotides were annealed and polymerized into the 165 bp CT (and appendices) fragment. The CT fragment was subsequently cloned using Topo XL Kit (topo-CT) and its structure was confirmed using DNA sequencing.

The b44 fragment was removed from the TOPO vector by *Not*I–*Cla*I double digestion and subcloned in *Not*I–*Cla*I linearized topo-CT to get topo-CT-b44. Then CT-b44 was released from the vector by *Xho*I–*Eco*RV double digestion and subcloned into *Xho*I–*Eco*RV linearized topo-b13 producing topo-b13–CT-b44 (now called b11ctv), containing the entire desired gene sequence. All junctions were confirmed by DNA sequencing and compared with the reported GenBank sequence (Table 1). Finally, b11ctv was purified by CsCl gradient ultracentrifugation, digested using *Sac*II–*Xho*I, and resultant linear 10.9 kbp b11ct fragment was purified by agarose gel electrophoresis using a Gel Extraction Kit (QIAgen). The eluted fragment was filtered on a 0.22 μm membrane and diluted to 5 ng/μl in microinjection buffer.

Generation of transgenic mice

F1 offspring resulting from crossing B6 and DBA/2 mice were used to obtain both zygotes for microinjection and foster mothers. About 200 zygotes were retrieved from ovulating mice, which after microinjection into male pronuclei, were transferred to the oviduct of 7 foster mice. Foster mothers were then allowed to go to term. Transgenic founders were determined as will be described, and they were back-crossed upon puberty to get the F1 transgenic mice.

Transgenesis evaluation

PCR

About 1-cm-long tail biopsies were performed on the 3-weeks-old resultant pups. DNA was extracted by overnight incubation at 55°C in 600 μl of TNES-PrK solution (Tris 50 mM, NaCl 0.4 M, EDTA 0.1 M, SDS 0.5%, Proteinase K 0.6 mg/ml), followed by addition of 200 μl 5 M NaCl to centrifuge out precipitated debris, precipitation of DNA from the supernatant with 99% ethanol, washing the pellet in 70% ethanol, and resuspending the pellet in 500 μl TE. Genomic DNAs were genotyped with PCR primers: 496f and 768r (Table 1), which amplify the transgene bases 7980 to 8465, deriving from both oBLG and CT.

DNA slot blotting

About 1–2 μg of each DNA sample was analyzed by slot blot with a radio-labeled 165 bp CT fragment as probe, corresponding to bases 8020 to 8184 by standard protocols (Sambrook & Russell, 2001).

Table 1. Different primers used for making the gene construct and assessment of tissue expression

Name	GenBank reference	Sequence
b1f	X68105: 9–38	GCAGGTCCGCGGATCTCTGTGTCTGTTTTC
b3r (551r)	X12817: 4641–4613	GGTTTGATATCAAGCCGGATGTGCATGGG
b4f	X12817: 4608–4636	CCCTATCGATGCACATCCGGCTTGCCTTC
b4r	X12817: 7343–7315	GAAACTCGAGAGAAGCCTGCGCACCGAAC
CTf	Synthetic, hybrid	GATATCATCGAGGGCAGGCACCACCATCACCACCAT GTCGACGACGACGATAAGTGCGGCAACCTGTCTACCTG TATGCTCGGCACATATACTCAGA
CTr	Synthetic, hybrid	ATCGATTCAGCCCGGTGTGCCAGCGCCCACGTCG GTTCTAGGATAGGTCTGCAGCTTGTGAGGAGTCT GAGTATATGTGCCGAGCATA
496f	X12817: 4585–4606	GAAATTCGACAAAGCCCTCAAG
768r	X12817: 4892–4871	GGCTCACCTAGACGTGGCACTG
42f	X12817: 843–862	TGAAGTGCCTCCTGCTTGCC
964r	X12817: 5461–5438	AGGTTTATGCCTTTATTGGTGAAG
542f	Synthetic, hybrid	GATATCATCGAGGGCAGGCACCACCATC
706r	Synthetic, hybrid	ATCGATTCATTAGCCAGGTGTTCCGCTTC

Southern blotting

High-molecular weight DNA was extracted using standard protocol (Sambrook & Russell, 2001). About 10–12 μg of each sample was digested overnight with *Bam*HI (200 U) and after electrophoresis on a 0.8% agarose gel, transferred to nylon membrane and probed with 2 radio-labeled *Bam*HI fragments of b11ct, corresponding to bases 1–2493 and 6930–9121. Radioactivity was detected using Phosphoimager films.

RT-PCR

Besides founder 38, which died and its tissues were stored at −80°C, several transgenic mice from different generations were killed and about 50–100 mg of the different tissues were freshly used for RNA extraction using Trizol (Invitrogen, USA) according to the manufacturer's protocol. cDNA was made using 1 μg of total RNA with random hexamers and MMLV Reverse Transcriptase (Promega, USA) according to the manufacturer's protocol, and submitted to PCR with various primer pairs including 496f, 768r, 551r, 42f, 964r, 542f, and 706r (Table 1, Figure 3). Female mice were killed while lactating, and male mice were killed after puberty. These primers amplify different fragments of mRNA, from base 42 down to 964. RT-PCR on RNA extracted from extra-mammary tissues (including ovary, uterus, prostate, salivary glands, lung, brain, liver, heart, and kidney) using primers 42f–768r was done to assess tissue-specificity of transgene expression.

Milk collection and clarification

Lactating mice were separated from their pups for 6 h on days 10, 11 or 12 of lactation. Mice were anesthetized using intra-peritoneal injection of a mixture of Rampun (Bayer, Germany) and Imalgine 1000 (Merial, France), followed by the intra-peritoneal injection of oxytocin (6.25 U/kg) and 50–600 μl of milk was collected from the mammary glands. After addition of 3–4 volumes of distilled water to the milk samples, they were centrifuged at 14,000 *g* for 5 min and the middle aqueous phases were stored in a separate tube at −20°C.

Western blotting

Milk samples were electrophoresed on 12% SDS-PAGE using standard protocol (Sambrook & Russell, 2001), and transferred to PVDF membrane in Tris–Glycine buffer containing 40% ethanol using 100 V for 2 h. The PVDF membrane was blocked in PBS containing 1% Tween 20 and 5% low-fat dry milk, washed in PBS containing 1% Tween 20, incubated in PBS containing 1% Tween 20 and 100 mU/ml mouse anti-His6-peroxidase (Roche, Germany) overnight at 4°C, and washed again in PBS containing 1% Tween 20. Finally,

bound antibodies were detected using ECL reagents (Sigma Aldrich), exposed for 15–30 s to autoradiography films (Kodak, Germany), prior to developing.

Enterokinase digestion of clarified milk

The clarified milk from different transgenic founder and F1 mice was digested using Enterokinase (Roche, Germany), one of the specific peptidases whose recognition site had been designed in gene construct. 2 µl of Enterokinase, 1 µl of ddH_2O, and 2 µl of 2× buffer (Tris 0.1 M, Urea 6 M) was added to 2 µl of diluted milk sample, and the mixture was incubated 18 h at room temperature. The digested sample was then assessed along with raw milk sample and molecular weight marker in a Western blotting using anti-His6 antibody.

Results

Gene construct

The final 10.9 kbp gene construct, b11CT, consisted of three main fragments (Figure 1): (1) an 8.0 kbp fragment including the oBLG promoter, regulatory sequences, and proximal part of the transcription unit; (2) a 165 bp CT fragment; and (3) a 2.7 kbp distal fragment of the oBLG transcription unit and downstream sequences. CT sequences are expressed as an oBLG fusion protein. In addition to a hybrid human-salmon calcitonin cDNA, they encode a Histidine tag and specific peptidase recognition sites.

Mouse genotyping

Of 41 surviving pups born, 6 transmitted the transgene to their progeny (Figure 2a). These independent transgenic founders carried various copy numbers (Southern blot and DNA slot assays; Figure 2b, c). On Southern blot, the 2.2 kbp probe covering the internal region of the transgene showed the corresponding 2.2 kbp band. However, the flanking 2.5 kbp probe showed the corresponding 2.5 kbp band in lines 30 and 38, and a 3.4 kbp band in lines 11, 38 and 39, both specific for the transgene. At least one F1 of each line was tested for transgene transmission to F2.

RT-PCR

Different fragments of mammary gland transgene mRNA were amplified using different primer pairs (Figure 3). As shown in Figure 3, the fragments expected from different regions of the mRNA coding region were amplified, thus confirming the integrity and correct size of the entire coding region, from the signal peptide down to the polyadenylation signal.

RT-PCR on RNA extracted from various tissues of most founders showed high-level expression of transgene in mammary glands, moderate expression in uterus, very low expression in brain, heart, ovary, prostate, and salivary glands, and undetectable expression in lung, kidney, and liver (Figure 4). Expression pattern was more or less the same for founder, F1 and F2 mice. However, in founder 38, transgene expression in lung, brain, and heart was comparable to mammary gland expression.

Mice health and weight

All mice were in apparently good general health, with weights comparable to age-matched controls.

Western blotting

Western blot analysis of different lines using anti-His6 antibody showed that transgene had been expressed in founder mice 3 and 11, and F1 mice 30-4, 38-5, and 39-6 (Figure 5).

Enterokinase assessment of expressed protien

The clarified milk as described in Materials and Methods from different transgenic founder and F1 mice were digested using Enterokinase, one of the specific peptidases whose recognition site had been designed in transgenic construct. The digested sample was then assessed along with raw milk sample and molecular weight marker in a Western blotting using anti-His6 antibody. This resulted in two bands of about 26 and 27 kDa on Western blotting, compared to 31 and 32 kDa bands of raw milk sample (Figure 6). As the Enterokinase cuts the fusion protein just before the calcitonin sequence, the apparent 5 kDa difference in molecular weight is attributed to calcitonin, which lacks any His6 to be visualized on Western blotting. If the transgene was expressed correctly, the fusion

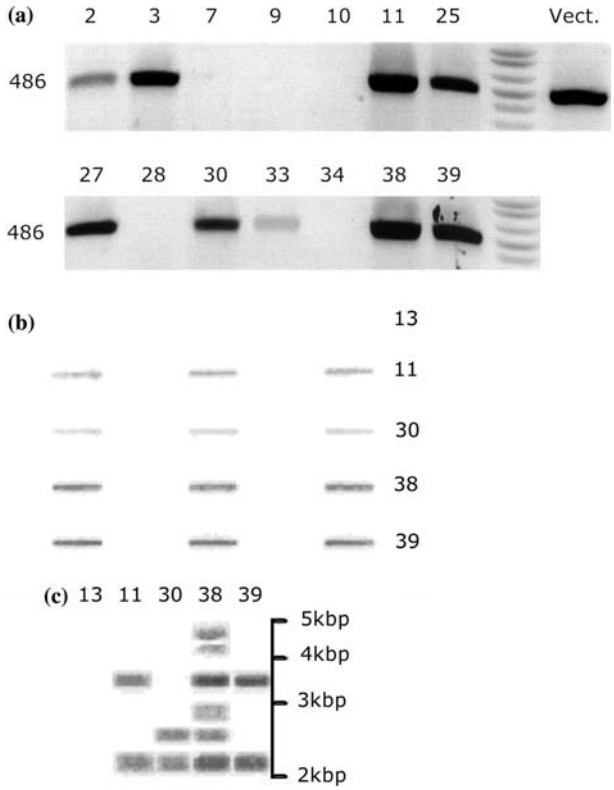

Figure 2. (a) PCR genotyping of founder mice using the primer pair 496f–768r, which produces a 486 bp product in positive mice. ('Vect': positive control.) six mice transmitted the transgene to progeny. (b) DNA slot blot of four transgenic founders. (c) Southern blot analysis of transgenic founders, confirming results of DNA slot blotting. The 2.2 kbp band is common in all lines, but the other probe which detects transgene proximal 2.5 kbp produced different bands; (a) 2.5 kbp band representing probe itself, a 3.4 kbp band representing a 0.9 + 2.5 kbp concatamerized fragment, and nonspecific bands in line 38 most likely due to different integration sites.

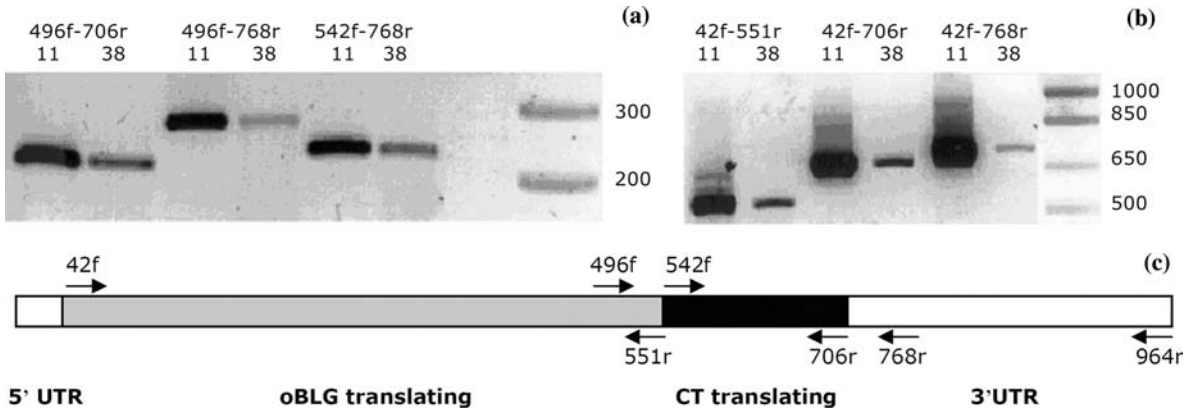

Figure 3. RT-PCR on mammary gland total RNA of founders 11 and 38 using three different primer pairs amplified expected products. (a) The primer pairs 496f–706r, 496f–768r, and 542f–768r produced products corresponding to 211, 273, and 227 bp, respectively. (b) RT-PCR using the primer pairs 42f–551r, 42f–706r, and 42f–768r produced relevant fragments of 510, 665, and 727 bp, respectively. (c) Primer map, depicting position of different primers on the 966 bp transgene mRNA.

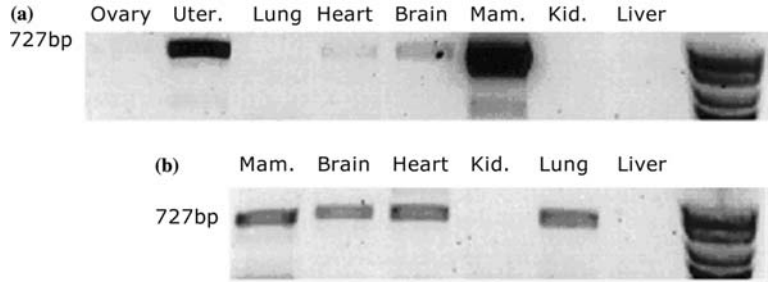

Figure 4. (a) RT-PCR on RNA extracted from different tissues of founder 11 using primers 42f and 768r. This transgenic mouse shows reasonably high tissue-specificity, with high level expression in mammary gland, moderate expression in uterus, and minimal expression in brain and heart. Most founders showed a similar pattern. (b) Ectopic expression of transgene in founder 38, showing moderate to high-level expression in brain, heart, and lung.

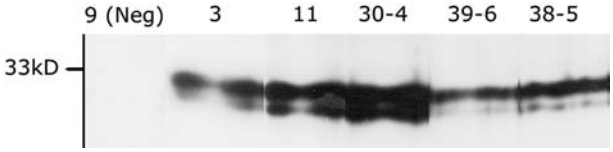

Figure 5. Western blot analysis of 2 μl of milk obtained from five different transgenic mice, showing two distinct bands of about 31 and 32 kDa. These distinct bands may be due to differences in glycosylation status of expressed fusion protein. Mouse 9 is negative control, mice 3 and 11 are transgenic founders, and 30–4, 39–6 and 38–5 are transgenic F1 mice.

protein should have a molecular weight of about 22.5 kDa, with 3.5 kDa for calcitonin. Western blot analysis of the raw and Enterokinase-digested milk samples showed that the fusion protein and calcitonin appear heavier than expected, 8–9 kDa for fusion protein and about 1.5 kDa for calcitonin.

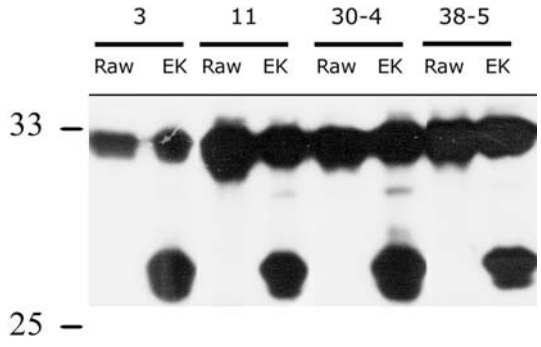

Figure 6. Western blotting of the Enterokinase-digeseted clarified milk using anti-His6 antibody. Enterokinase (EK) treatment has made two distinct bands of about 5 kDa less in size compared to raw milk bands. As the Enterokinase cuts the fusion protein just before the calcitonin sequence, it should have released a calcitonin fragment of about 5 kDa, not visualized using anti-His6 antibody, as expected.

Discussion

We have shown expression of a fusion protein, expressed under the control of the oBLG promoter, consisting of ovine β-lactoglobulin (oBLG) and calcitonin sequences in the milk of transgenic mice. Full length oBLG genomic sequences were used to benefit from enhancer-like effects of its introns, and from the oBLG protein as a fusion partner for calcitonin, which by itself may be too small to ensure its stability in the milk environment. This strategy can be used for expression of highly active proteins. Combining the desired protein with a fusion partner, one may produce a larger and less active protein, less likely to leak to the circulation, or that would be less active if leaking.

The transgenic lines lacked any apparent systemic signs of hypercalcitoninemia, although a limited transcriptional leakage was detected by RT-PCR in several lines. This suggests that no apparent translational leakage took place.

We showed that a transgene containing the entire oBLG transcription unit and proximal 4.2 kbp and distal 1.9 kbp fragments of oBLG in addition to calcitonin cDNA is expressed mainly in the mammary glands of most transgenic mice.

In addition to high level expression in the mammary gland, the transgene was expressed at a moderate level in the uterus, but little or no expression was found in other tissues including brain, heart, ovary, lung, liver, and kidney. Low level ectopic expression in the uterus driven oBLG promoter which has also been observed in an earlier study (Korhonen et al., 1997) seems to be a general phenomenon, involving other milk specific regulatory sequences (Archibald et al., 1990; Barash et al., 1994). This effect may be due to the more or less similar endocrine environments of these tissues, disruption of the integrity of oBLG sequences by the inserted calcitonin cDNA (Barash et al., 1994), and or higher sensitivity of detection afforded by RT-PCR vs. Northern blotting, which presumably allowed us to detect physiologically low levels of ectopic expression.

All transgene mRNA fragments were amplified correctly by RT-PCR, verifying the correct structure of the chimeric mRNA. As the distal transcription unit and 3′ flanking sequences are kept intact, changes in oBLG RNA splicing are minimized, and this may help to improve transgene mRNA stability.

If the transgene was expressed correctly, the fusion protein should have a molecular weight of about 22.5 kDa, with 3.5 kDa for calcitonin. According to Western blot analysis of the raw and Enterokinase-digested milk samples, the fusion protein and calcitonin appear systematically heavier than expected size. According to glycosylation prediction analysis using NetOGlyc and NetNGlyc tools at Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services), these are most likely due to post-translational modification of the fusion protein, since there are several highly potential glycosylation sites throughout the fusion protein, particularly an N-linked glycosylation on 3rd amino acid residue (Asn) of calcitonin sequence.

In fact, there are some reports supporting above hypothesis of post-translational modification of calcitonin. In first article (Flanders et al., 1986) in which a rat medullary thyroid carcinoma cell line (CA-77) was investigated to study calcitonin biosynthesis, calcitonin was expressed as a 5.5 kDa peptide. In second report using a transplantable rat medullary thyroid carcinoma (Gkonos et al., 1989), glycosylated calcitonin constituted 2.3% of the total extracted immunoreactive calcitonin. The structure of this peptide differed from nonglycosylated calcitonin only by the oligosaccharide modification of Asn3.

In contrast to analysis of transgene expression at the RNA level, which is fairly straight-forward, analysis of the protein level and particularly protein characterization and activity is more complex, because of possible post-translational modifications or polymerizations, and will thus require further investigation.

Overall, calcitonin can be expressed under the control of the oBLG promoter and regulatory elements in a mammary-gland specific pattern. In addition, it seems that this fusion partner strategy might also prevent transgene translational leakage.

Acknowledgements

We would like to thank Max Radjkumar, Franck Paput, and David Mercurio for support with the animal work. We are especially grateful to Dr. Francois Cuzin of INSRM U636, and Dr. Kenneth B. Marcu of SUNY, Stony Brook, for their critical reading of the manuscript. This work was supported by funds from the Pasteur Institute of Iran and INSERM U636.

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