

Development of transgenic mice harboring ovine beta lactoglobulin-calcitonin transgene

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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 gene construct was generated, consisting of 10.7 kbp of the ovine beta-lactoglobulin (oBLG) gene including its promoter and 3' flanking region with the calcitonin coding sequences inserted in-frame into the oBLG fifth exon. The gene construct was purified using CsCl gradient, released from vector, and gel-purified. It was microinjected into fertilized mouse oocytes. These oocytes were then transferred to pseudo-pregnant foster mice. The pups born from foster mice were genotyped using PCR, slot blot, and Southern blot techniques. Among 9 mice which showed positive PCR results, only 6 mice transmitted the transgene to the next generation. Therefore, 6 transgenic lines were established which stably transmitted their transgene to their progeny.

Keywords: transgenic mice, milk, calcitonin, beta-lactoglobulin

INTRODUCTION

Developing methods for the large-scale production of recombinant proteins is one of the most interesting challenges faced by biotechnologists. Recombinant proteins have been traditionally expressed in prokaryotes since the 1980s, and later in cultured eukaryotic cells. However, none of them was satisfactory for production of multi-subunit or highly-modifying proteins, which are too complex to be correctly expressed in these

immature expression systems. After making the first transgenic mice in the early 1980s, researchers increasingly used this approach to express different pharmaceuticals in the mammary glands of mice and various mammals, including rabbit, sheep, goat, and cow. Mammals' milk is both easily available and highly-produced, and 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 and 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. Thus, the expression of different proteins in the mammary gland is not only feasible, but also economic and scalable (Prunkard *et al.*, 1996 and Carver *et al.*, 1993). The mammary gland can be used as a bioreactor for the production, not otherwise feasible, of even complex proteins.

The milk contains several specific proteins, and their promoters are used to drive expression of foreign proteins in the milk. These proteins include caseins (α s1, α s2, β , and κ) which comprise about 80% of milk proteins and coagulate upon ripening, and whey proteins (beta-lactoglobulin, alpha-lactalbumin, whey acidic protein), which comprise the remaining 20%. Beta-lactoglobulin (BLG) is the most abundant whey protein in ruminant milk (Mercier and Vilotte, 1993). It has been suggested that BLG is involved in both fatty acid and retinol transport in milk (Flower *et al.*, 1996 and Perez and Calvo, 1995).

oBLG promoter has frequently been used to express foreign proteins in mammalian milk (Prunkard *et*

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al., 1996; John *et al.*, 1999; Kolb *et al.*, 2001 and McKee *et al.*, 1998). Although there is no BLG counterpart in rodents, it is well recognized by different mammary gland-specific transcription factors in transgenic mice and rabbits (Whitelaw *et al.*, 1992 and Hyttinen *et al.*, 1998). Using an oBLG promoter driven construct permits the expression of the desired proteins in farm animals, including sheep and goats as bioreactors, which are the preferred hosts for this promoter. Most reported transgenes used the BLG promoter, but in a few instances, the gene was used as a fusion partner as well.

Even though both cDNA and genomic sequences can be used under the control of a mammary gland-specific promoter, genomic sequences are usually expressed at levels several hundred times higher (mg/ml instead of ng- μ g/ml levels).

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 and 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 (McKee *et al.*, 1998).

The sCT although potent enough in humans, causes gastrointestinal problems and immunological hypersensitivity upon oral use, which limits its prescription as a widespread treatment. It has been shown earlier 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).

This study was initiated in order to develop a gene construct suitable for mouse transgenesis and produce transgenic mice harboring the desired gene.

MATERIALS AND METHODS

Gene construct: Three milliliters of fresh blood was collected from Moghan sheep, the most inbred sheep race in Iran, provided by the Iranian Research Center of Farm Animals. Sheep blood genomic DNA was extracted using a 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 (Fig. 1). Two PCR primer pairs were designed to amplify oBLG using the Expand Long Template

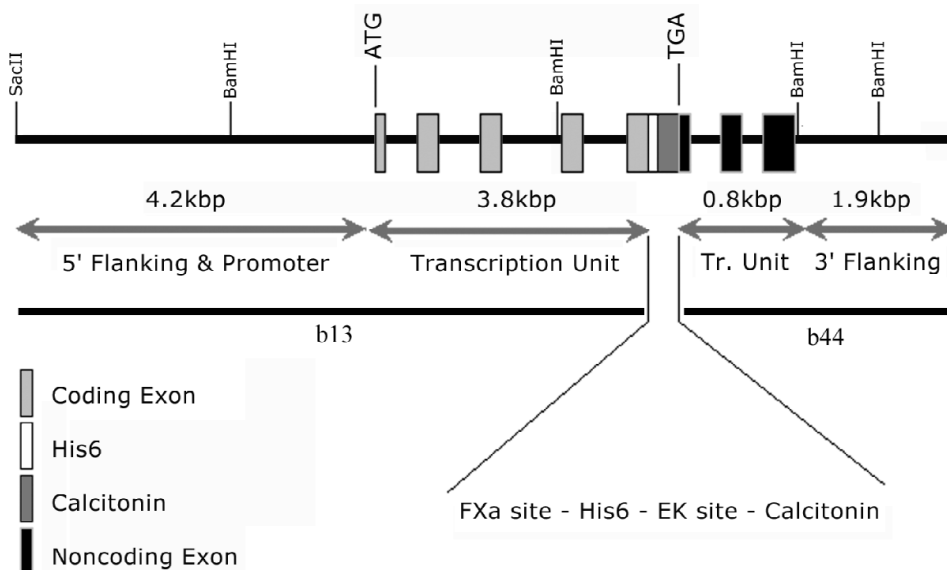


Figure 1. The final 10.9 kbp gene construct, microinjected into fertilized oocytes, consisting of entire oBLG gene and calcitonin gene construct inserted in-frame into the fifth exon of oBLG. Restriction sites used for transgene linearization and Southern blotting are shown too.

Table 1. Different primers used for making the gene construct.

Name	GenBank Reference	Sequence
b1f	X68105: 9-38	5'-GCAGGTCGCGGATCTCTGTGTCTGTTTTTC-3'
b3r	X12817: 4641-4613	5'-GGTTTGATATCAAGCCGGATGTGCATGGG-3'
b4f	X12817: 4608-4636	5'-CCCTATCGATGCACATCCGGCTTGCCCTTC-3'
b4r	X12817: 7343-7315	5'-GAAACTCGAGAGAAGCCTGCGCACCGAAC-3'
CTf	Synthetic, hybrid (97b)	5'-GATATCATCGAGGGCAGGCACCACCATCACCACCATGTGCGACGACGAC GATAAGTGCGGCAACCTGTCTACCTGTATGCTCGGCACATATACTCAGA-3'
CTr	Synthetic, hybrid (88b)	5'-ATCGATTACGCCCGGTGTGCCAGCGCCACGTCGGTTCCTAGGAT AGGTCTGCAGCTTGTGAGGAGTCTGAGTATATGTGCCGAGCATA-3'
496f	X12817: 7980-8001	5'-GCAGGTCGCGGATCTCTGTGTCTGTTTTTC-3'
768r	X12817: 8465-8444	5'-GGTTTGATATCAAGCCGGATGTGCATGGG-3'

PCR System (Roche, Germany): b1f and b3r for b13 fragment, and b4f and b4r for b44 fragment (Table 1, Fig. 1). The first pair amplified the 5' 8.0 kbp fragment of oBLG (b13), consisting of 4.2 kbp of the 5' flanking region and promoter in addition to 3.8 kbp of the transcription unit, and the second amplified the 3' 2.7 kbp fragment (b44), consisting of 0.8 kbp of the transcription unit in addition to 1.9 kbp of the 3' flanking region (Fig. 1). Both PCR products were cloned separately using TOPO XL Kit (Invitrogen, USA) to obtain topo-b13 and topo-b44, respectively.

Two 3' end complementary oligonucleotides of CTf (97b) and CTr (88b) were chemically synthesized, annealed and completed using Taq-Tgo DNA polymerase (Roche, Germany) mixture and dNTP using conditions similar to PCR to make the 165 bp hybrid CT fragment (Table 1). The completed CT fragment was subsequently cloned using TOPO XL Kit (topo-CT) and the structural integrity of the cloned fragment was confirmed by DNA sequencing.

The b44 fragment was removed from the topo vector by *NotI* and *Clal* double digestion and subcloned in *NotI-Clal* linearized topo-CT to get topo-CT-b44. Then topo-CT-b44 was released from the vector by *XhoI-EcoRV* double digestion and subcloned into *XhoI-EcoRV* linearized topo-b13 producing topo-b13-CT-b44 (now called b11ctv), containing the entire desired gene sequence. All junctions and some other parts of the gene construct were confirmed by DNA sequencing and compared with the reported GenBank sequence (X68105, X12817). Finally, b11ctv was purified by CsCl gradient ultracentrifugation to remove any broken plasmids and contaminants, digested using *SacII-XhoI* to obtain a linear 10.9 kbp b11ct fragment, and purified using 1% ultra-pure low-melting point agarose gel electrophoresis and Gel Extraction Kit (Qiagen, Germany). The purified fragment was dia-

lyzed against double-distilled water for 45 minutes using a Millipore 0.025 µm membrane, filtered on a 0.22 µm membrane, and diluted to 5 ng/µl in microinjection buffer {10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5}.

Generation of transgenic mice: F1 offspring resulting from crossing B6 and DBA/2 mice were used to obtain both fertilized oocytes for microinjection and foster mothers for embryo transfer. Recently fertilized oocytes (zygotes) were retrieved at 0.5 dpc (day post-coital) by sacrificing females showing vaginal plugs in the early morning during a normal oesterus cycle and cutting the oviduct in M2 medium (Sigma, USA). The zygotes were released from cumulus cells using sheep testis Hyaluronidase (Sigma, USA). They were then washed 3 times in M2 medium and incubated in M16 (Sigma, USA) at 37°C and 5% CO₂ concentration before microinjection.

For microinjection, the zygotes were transferred using a mouth pipette to M2 medium and covered with inert oil, and the transgene construct was microinjected into prominent male pronuclei using InjectMan (Eppendorf, Germany), causing them to enlarge by about twice their initial diameter. Under general anesthesia, 15-20 injected zygotes were unilaterally transferred to the oviduct of 7 female foster mice, which had mated earlier with vasectomized males. Foster mothers were then allowed to go to term under special environmental conditions controlling light, temperature and moisture.

Transgenic founders were determined as will be described, and they were back-crossed upon puberty to get the transgenic F1 mice. Upon puberty, transgenic F1 mice were back-crossed to obtain transgenic F2 mice.

Transgenesis evaluation

PCR: Two to three weeks after delivery of pups from the foster mice, 1 cm tail biopsies were performed. 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). Then 200 µl of 5M NaCl was added and centrifuged and its precipitated debris was discarded. DNA in supernatant was then precipitated with ethanol (99%). The pellet was further washed in ethanol (70%) and finally dissolved in 500 µl of TE buffer. Genomic DNAs were genotyped with PCR primers: 496f and 768r (Table 1), which amplify a 486 bp fragment (corresponding to transgene bases 7980 to 8465, deriving from both oBLG and CT) after 35 cycles of denaturation at 94°C, annealing at 59°C, and extension at 72°C.

DNA Slot Blotting: 1-2 µg of each DNA sample (the same used for PCR) 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 and Russell, 2001). Radioactivity was detected using Phosphoimager films (Fuji, Japan).

Southern Blotting: High-molecular weight DNA was extracted for Southern-blot analysis of samples using standard protocol (Sambrook and Russell, 2001). 10-12 µg of each sample was digested overnight with *Bam*HI (200U) 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 (Fuji, Japan).

RESULTS

Gene Construct: The fifth oBLG exon was selected for in-frame insertion of hybrid CT cDNA, just after the 149th codon (Fig. 1). A Histidine tag and appropriate restriction and peptidase sites were designed in CT gene construct. Hence, the final 10.9 kbp gene construct, which was microinjected into fertilized oocytes, b11CT, consisted of three main fragments, as shown in figure 1: (1) an 8.0 kbp fragment consisting of 4.2 kbp of the 5' flanking region and promoter in addition to 3.8 kbp of the transcription unit; (2) a 165 bp CT fragment; and (3) a 2.7 kbp distal fragment, consisting of 0.8 kbp of the distal transcription unit in addition to 1.9 kbp of the 3' flanking region.

Generation of Transgenic Mice: 18 mated mice were

sacrificed which yielded about 200 zygotes, each about 10-12 zygotes. After microinjection, 130 zygotes survived after 3hr incubation in M16 medium. They were divided into several groups of 15-20 zygotes, which were transferred to 7 foster mice. These mice produced 45 offsprings.

Mouse Genotyping: Foster mice delivered 45 pups, of them 41 survived after a week. Nine mice, 4 males and 5 females, found to be positive on PCR genotyping, which amplified a specific 486 bp fragment of the transgene, composed of both oBLG and synthetic CT (Fig. 2). The mice were further characterized using DNA slot blotting. This test confirmed that 4 mice were positive for the transgene (Fig. 3 and Table 2). Southern blotting further confirmed these transgenic mice (Fig. 3, Table 2). Results of DNA sequencing confirmed the integrity of different parts of the gene construct, particularly coding regions (not shown here).

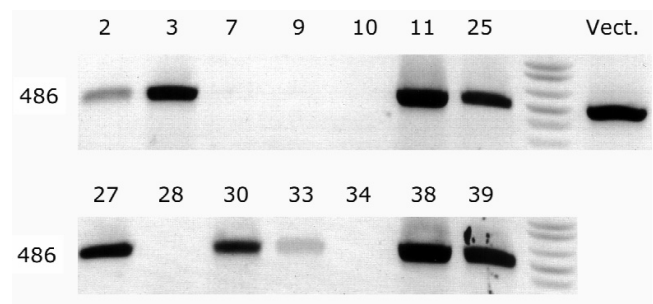


Figure 2. PCR genotyping of founder mice using the primer pair 496f-768r, which produces a 486 bp product in positive mice. Out of 9 positive mice obtained, 6 mice transmitted the transgene to progeny. "Vect" lane shows positive control.

Upon puberty, at 6-7 weeks, the positive mice were back-crossed to get the transgenic first generation (F1) mice. Six mice, 3 males and 3 females, resulted in pups with positive PCR tests (Fig. 2), hence regarded as transgenic founders (F0). As the main requirement for mouse transgenesis is transmission of the transgene to progeny, it can be concluded that 6 independent transgenic lines have been established (Fig. 4, Table 2).

At least one F1 of each line was tested for transgene transmission to F2. All F1 mice consistently transmitted their transgene to their progeny, F2 (results not shown).

Mice Health and Weight: All mice were in apparently good general health, with weights comparable to age-matched controls.

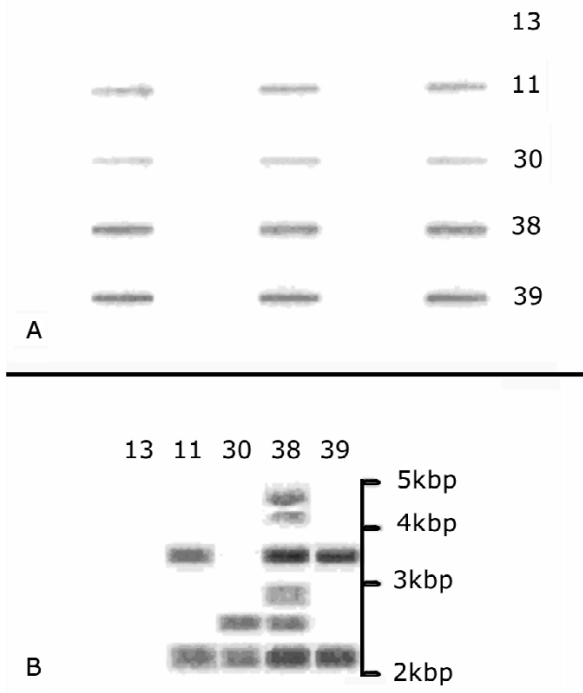


Figure 3. A. DNA slot blot of founders transgenic for b11ct, showing four transgenic founders. B. Southern blot analysis of founders transgenic for b11ct. The same mice positive in DNA slot blotting found to be positive in Southern blot too. The 2.2 kbp band is common in all lines, but the other probe 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 multiple integration events. The apparent length of the first transgene of a concatamer depends on how far the first genomic *Bam*HI site is located from transgene.

DISCUSSION AND CONCLUSION

In the present study, transgenic mice have been developed harboring a transgene consisting of hybrid human-salmon calcitonin and ovine beta-lactoglobulin (oBLG). Full length oBLG genomic sequences were used to benefit from enhancer-like effects of its introns.

Since microinjected DNA may integrate into genome after S phase of the zygote, the resultant mouse may become mosaic regarding the transgene. The severity of the mosaicism depends on the exact time of transgene integration regarding zygote division(s); For instance, if it integrates at 2-cell stage, at most 50% of resultant cells are transgenic, and if it integrates at 4-cell stage, no more than 25% of cells will harbor the transgene. This causes some apparent ambiguity during genotyping, because the cells harboring transgene may be too low to make a positive Southern or slot blotting, but still high enough to make a positive PCR genotyping test. This was the case at least for mice 2 and 3, which showed positive results on PCR, but failed to be positive by Southern and slot blotting.

If the mouse is mosaic and the germinal cells lack the transgene, it would not transmit the transgene to progeny eventhough it may show positive genotyping tests on somatic cells. In other words, if the cells making germinal cells harbor the transgene, the progeny will receive the transgene. This is the main factor for considering a mouse as transgenic founder. Accordingly, mice 2 and 3 are considered as transgenic founders, as they have resulted in pups with positive PCR even though they had not shown any positive blotting. Mouse 2 was so mosaic that just one out of its 9 pups were proved to be transgenic. However, mice 25, 27 and 33 could not be considered as transgenic founders, as none of their progeny showed any positive PCR results.

The transgene copy number is an additional factor affecting genotyping. Transgenic lines harboring lower transgene copy numbers result in weaker bands on DNA blotting. In order to assess the transgene copy number, the intensity of blot bands can be compared just in transgenic first generation (F1), as the founders (F0) may be mosaic for the transgene.

The mixture of 2.2 kbp and 2.5 kbp probes used for Southern blotting produced an apparently heterogeneous pattern, which can be explained using princi-

Table 2. Summary of transgenesis results.

Mouse No. (Male/Female)	2 (M)	3 (F)	11 (F)	25 (F)	27 (F)	30 (M)	33 (M)	38 (M)	39 (F)
PCR	+	+	+	+	+	+	+	+	+
DNA Slot Blotting	-	-	+	-	-	+	-	+	+
Southern Blotting	-	-	+	-	-	+	-	+	+
Positive First Generation PCR	1/9	7/12	6/12	0/8	0/8	7/12	0/11	4/8	3/10
Transgenic First Generation	+	+	+	-	-	+	-	+	+

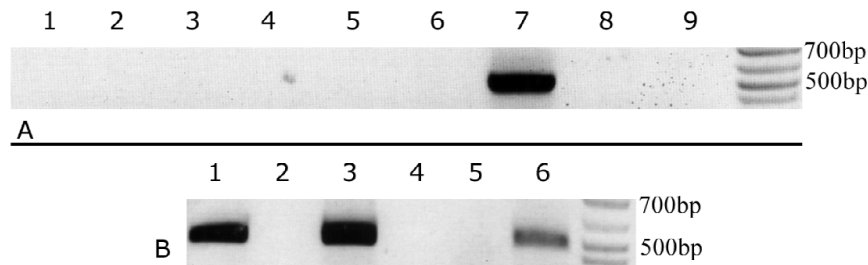


Figure 4. PCR genotyping of the transgenic first generation (F1) of the lines 2 and 11. Founder 2 transmitted the transgene to just one pup, an event incompatible with Mendelian inheritance and most likely due to mosaicism in the germinal cells. Founder 11 transmitted the transgene to half of its progeny, compatible with Mendelian inheritance. All founders except for founder 2 showed a similar pattern of transgene transmission.

ples of random integration. The linear gene construct injected into zygote tends to concatemerize end-to-head before random integration into genome. In addition, it tends to randomly integrate into just one point of genome, and so it is called “single integration” event, instead of “multiple integration”. In our experiment, the 2.2 kbp probe covering an internal region of the transgene showed the corresponding 2.2 kbp band on all transgenic lines tested. However, the flanking 2.5 kbp probe resulted in different bands on different lines; it made a 2.5 kbp band in lines 30 and 38, but an unexpected 3.4 kbp band in lines 11, 38 and 39. This is because the transgene concatemerizes before integration, and as neither the beginning nor the end of the transgene contain *Bam*HI site, the last *Bam*HI fragment (0.9 kbp) of each monomer joins to the first fragment of the next monomer (2.5 kbp), representing as a 3.4 kbp band in the lines with multiple transgene copies. The band length of the first fragment depends on how far the first local genomic *Bam*HI site is located.

Overall, 6 transgenic mice families were established successfully, harboring oBLG-CT transgene, and efficiently transmitting the transgene to their progeny. Further experiments are needed to find out tissue-specific expression of oBLG-CT in transgenic animals.

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