Manipulation of gene expression in transgenic mice using ribozymes

P.J. L'HUILLIER AND J.L. VILOTTE[1]

Dairy Science Group, AgResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand.

ABSTRACT

This study describes the creation and analysis of transgenic mice carrying a ribozyme or RNA enzyme that specifically targets the bovine α-lactalbumin (α-lac) mRNA transcript. Using a MMTV-LTR promoter-based construct, mammary specific high-level expression of a bovine α-lac-targeted ribozyme was obtained in three out of twelve lines of transgenic mice created. When crossed with mice transgenic for the bovine α-lac gene (0.4 mg of α-lac.ml^{-1} of milk), expression of the ribozyme resulted in a reduction in the levels of the target mRNA to 50% of that observed in the non-ribozyme transgenic littermate controls. This activity was specific to the bovine α-lac transcript as no reduction in the levels of the endogenous murine α-lac mRNA was observed. These results demonstrate the feasibility of ribozyme-mediated down-regulation of highly-expressed transcripts in transgenic animals.

Keywords: milk composition; α-lactalbumin; mammary gland; transgenesis.

INTRODUCTION

Ribozymes are small RNA molecules capable of catalytic cleavage of RNA (Uhlenbeck, 1987; Symons, 1989). These molecules are composed of a substrate that possesses a three nucleotide recognition sequence (GUX), and an enzyme moiety which has a catalytic domain and flanking sequences complementary to the substrate. Such ribozymes can perform an enzymatic reaction, in which a target substrate is cleaved and the ribozyme itself is not altered during the reaction.

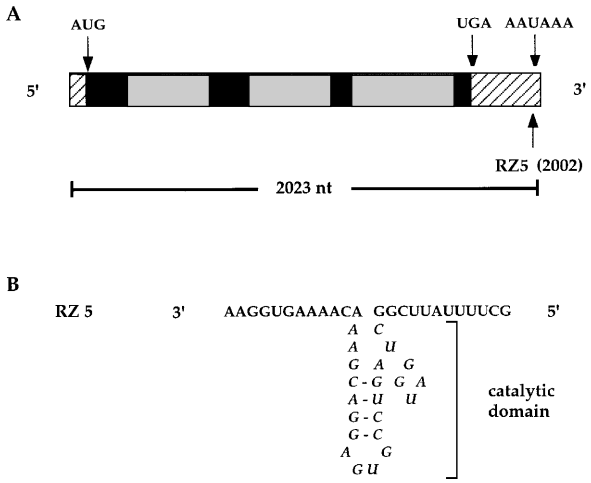
We have used a 'double' transgenic approach in mice to target a ribozyme against a highly expressed bovine milk protein transcript, α-lac (L'Huillier *et al.*, 1996). Since α-lac is involved in lactose synthesis, manipulation of its gene expression could lead to the development of animals with altered milk composition.

MATERIALS AND METHODS

Ribozyme construct and preparation of DNA for micro-injection

The ribozyme (RZ5) consists of the 24 nucleotide hammerhead catalytic domain (Haseloff and Gerlach, 1988) and two 12 nucleotide antisense sequences complementary to regions of the bovine α-lac transcript (Fig. 1 and L'Huillier *et al.*, 1992). It was inserted between the MMTV-LTR (mouse mammary tumor virus long terminal repeat) promoter and a SV40 early splice and polyadenylation signal to make the ribozyme transgene construct (Fig. 2). The recombinant plasmid was digested with *Hind*III and *Bam*HI, and the 2.4 kb fragment corresponding to the injected-construct was isolated.

FIGURE 1: Target site and ribozyme structure
(A) Structure of bovine α-lactalbumin (α-lac) pre-mRNA and target site of ribozyme 5 (RZ5). The α-lac pre-mRNA is composed of four exons (black segments, coding regions; hatched segments, 5' and 3' untranslated regions), and three introns (grey segments). Positions of the initiation and termination codons, and polyadenylation signal are shown. The number in brackets indicates the position of the U in the GUC target site. nt, nucleotides.
(B) Structure of RZ5. RZ5 is a hammerhead ribozyme that possesses two 12 nucleotide flanking regions (bold plain type) which are complementary to the bovine α-lac pre-mRNA. The catalytic domain of the hammerhead ribozyme, derived from the (+) strand of tobacco ringspot virus is shown in bold-italics.



Generation and analysis of Transgenic Mice

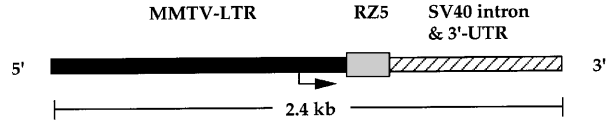
Micro-injection was performed on C57BL/6 x CBA F2 hybrid eggs and transgenic mice were generated and bred according to established procedures (Hogan *et al.*, 1986).

Tail biopsies of founder mice were taken 2-4 weeks after birth and genomic DNA was extracted and recovered. Southern analysis was performed with 10 µg of *Hae*III-

[1]Laboratoire de Génétique Biochimique et de Cytogénétique, INRA-CRJ, 78352 Jouy-en-Josas, cedex, France.

digested genomic DNA which was fractionated on a 1% agarose gel and transferred to Hybond N membrane. Hybridisation of membranes was carried out using a 1.3 kb probe (Fig. 2) complementary to 375 bp of the MMTV-LTR, and the ribozyme and SV40 sequences (data not shown).

FIGURE 2: Ribozyme transgene construct design.
Structure of DNA construct used for micro-injection. Construct possesses 1455 bp of the mouse mammary tumor virus long terminal repeat. Transcription initiation occurs approximately 268 bp upstream of the ribozyme insertion site as indicated by the arrow. Downstream of the ribozyme insertion site are 871 bp derived from SV40 (hatched segment). This sequence possesses the SV40 early splice region and small T antigen intron as well as the polyadenylation region. The polyadenylation site is at position 822 within this 3' untranslated sequence.



RNA extraction and Northern analysis

Total cellular RNA from 7-8 day lactating mammary glands was extracted using a modified version of the guanidinium thiocyanate-phenol method. RNA samples were fractionated on a 2% agarose/1.2M formaldehyde gel and transferred to Hybond N membrane. Northern hybridisation, and labelling of cDNA and oligonucleotide probes was carried out as previously described (L'Huillier *et al.*, 1996).

RESULTS AND DISCUSSION

Ribozyme construct, target site, and generation of transgenic mice.

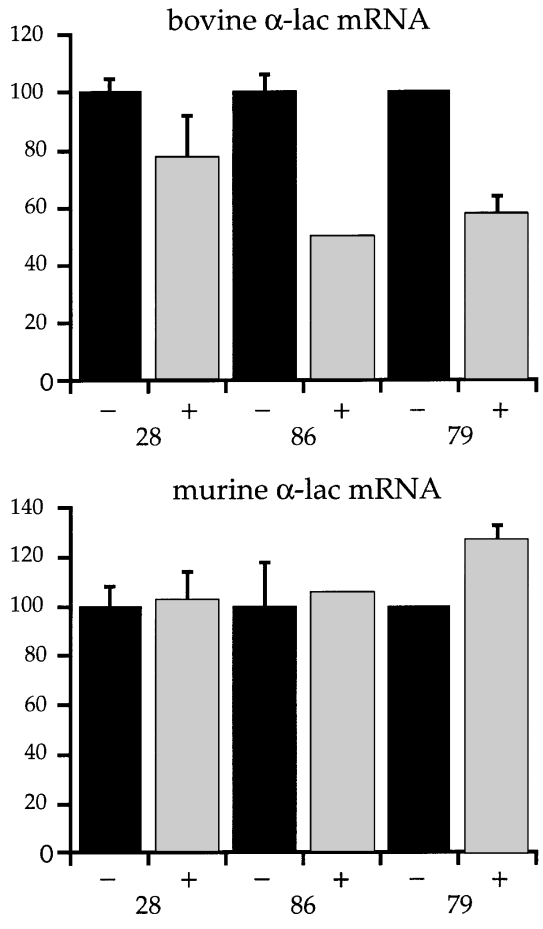
RZ5 targets a region of the 3' untranslated sequence of the bovine α-lac mRNA, near the polyadenylation signal that was previously found to be accessible to the ribozyme in C127I mouse mammary cells (L'Huillier *et al.*, 1992). The 12 nucleotide flanking sequences of this ribozyme are complementary to the bovine mRNA but have homology (16 of the 24 nucleotides are identical) with the murine α-lac sequence, and thus enable us to determine both the activity and specificity of this ribozyme.

By Southern analysis, the presence of the ribozyme transgene was identified in 12 founder mice. F1 mice, transgenic for the ribozyme were cross bred with a line of mice carrying a bovine α-lac transgene (line 53, previously described by Vilotte *et al.*, 1989). 'Double' transgenic mice were identified by Southern analysis. Unless otherwise stated, mice used in this analysis were heterozygous for the bovine α-lac transgene, and transgenic or non-transgenic (littermate controls) for the ribozyme transgene. Delivery of the ribozyme to transgenic mice did not apparently affect the health of the mice. In this study to date, only tissues from 7-8 day lactating mammary glands have been analysed. However given the observed high specificity of the ribozyme for bovine α-lac in the mammary gland, even if expressed in other tissues (Yom *et al.*, 1993) at low levels, RZ5 is unlikely to have any effect.

Expression of ribozyme specifically and efficiently reduces the level of bovine α-lac mRNA.

Expression of the ribozyme transgene in lines 79, 28 and 86 was correlated with a clear and highly-specific reduction in the level of bovine α-lac mRNA (Figure 3). In these lines, expression of the ribozyme transgene reduced the level of bovine α-lac mRNA to 58, 78 and 50% of that present in the non-RZ5 transgenic littermate controls. The specificity of the ribozyme-mediated reduction in bovine α-lac mRNA is demonstrated by the lack of effect on the level of the endogenous α-lac mRNA (Figure 3). The murine α-lac transcript possesses 16 of 24 nucleotides complementary to the antisense flanks of RZ5 plus the GUX cleavage site, and thus can be theoretically cleaved by the ribozyme. It is clear however from this study that in mice the action of the ribozyme is specific for the bovine sequence.

FIGURE 3: Quantitative analysis of ribozyme-mediated reduction of bovine α-lactalbumin mRNA in transgenic mice. Northern analysis autoradiographs (data not shown) were scanned using a Pharmacia LKB Imagemaster DTS scanning system. Bovine and murine α-lac mRNA values were corrected for the quantity of loading on the gel using the scan values obtained for 28S ribosomal RNA and are presented as arbitrary scan values. Values for ribozyme transgenic mice (+) from each line were normalised against their non-RZ5 transgenic littermate control (shown in black).



The ribozyme-mediated reduction in bovine α-lac in the milk closely paralleled that observed for the mRNA in mammary tissue. On average, the level of the bovine protein was reduced to 65, 76 and 51% of that observed for the non-RZ5 transgenic littermate controls for lines 79, 28 and 86 respectively (data not shown).

CONCLUSIONS

We have targeted ribozymes to the α-lac transcript with the objective of indirectly affecting lactose synthesis in the mammary gland. Using embryonic stem cells and gene targeting, Stinnakre *et al.,* (1994) recently created α-lac-deficient mice. A direct relationship between α-lac content and lactose synthesis was observed in these studies. Lactose, the predominant sugar in milk is also one of the major osmotic regulators of milk secretion (Morrissey, 1985); α-lac deficient mice are unable to feed their offspring as they produce a highly viscous milk that can not be removed from the gland (Stinnakre *et al.,* 1994). Furthermore, lactose accounts for the majority of milk intolerance in humans and intestinal lactase deficiencies affect >80% of mankind (Delmont, 1983). In this report, we have shown that delivery of a ribozyme can efficiently and specifically reduced bovine α-lac expression, and thus have demonstrated the feasibility of using a ribozyme approach for the modification of milk composition in mammals. At the present time, we are creating mice carrying a ribozyme that targets directly the murine α-lac transcript.

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