Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6698–6703, June 1996
Applied Biological Sciences

Efficient and specific ribozyme-mediated reduction of bovine α-lactalbumin expression in double transgenic mice

PHIL J. L'HUILLIER*, SOLANGE SOULIER†, MARIE-GEORGES STINNAKRE†, LAURENCE LEPOURRY†, STEPHEN R. DAVIS*, JEAN-CLAUDE MERCIER†, AND JEAN-LUC VILOTTE†

†Laboratoire de Génétique Biochimique et de Cytogénétique, Institut National de la Recherche Agronomique, Centre de Recherches de Jouy-en-Josas, 78352 Jouy-en-Josas, cedex, France; and *Dairy Science Group, AgResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand

Communicated by George E. Seidel, Jr., Colorado State University, Fort Collins, CO, February 20, 1996 (received for review February 15, 1995)

ABSTRACT **Transgenic mice carrying a bovine α-lactalbumin (α-lac) specific ribozyme gene under the transcriptional control of the mouse mammary tumor virus long terminal repeat were generated and cross-bred with animals that highly express a bovine α-lac transgene (0.4 mg of α-lac·ml^{-1} of milk). The ribozyme contains the hammerhead catalytic domain, flanked by 12-nt sequences complementary to the 3′ untranslated region of bovine α-lac transcript. High-level expression of the ribozyme gene was detected by Northern blot analysis in the mammary gland of 7–8 day lactating transgenic mice, from 3 of 12 lines analyzed. Heterozygous expression of the ribozyme resulted in a reduction in the levels of the target mRNA to 78, 58, and 50% of that observed in the nonribozyme transgenic littermate controls for three independent lines. The ribozyme-mediated reduction in the levels of the bovine protein paralleled that observed for the mRNA, and was positively correlated with the level of expression of the ribozyme. In nonribozyme expressing transgenic mice, the level of bovine α-lac mRNA and protein was not affected. The specificity of this activity is demonstrated by the absence of a reduction in the levels of the endogenous murine α-lac mRNA or protein. These results demonstrate the feasibility of ribozyme-mediated down-regulation of highly-expressed transcripts in transgenic animals.**

Ribozymes are small RNA molecules capable of specific catalytic cleavage of RNA (1). In viroids and virusoids, this reaction is intramolecular (2). However, the hammerhead and hairpin ribozymes, respectively from the (+) and (−) strands of the satellite RNA of tobacco ringspot virus can act *in trans*. These molecules can be divided into a substrate that possesses a 3-nt recognition sequence (GUX), and an enzyme moiety that has a catalytic domain and flanking sequences complementary to the substrate (3). Such ribozymes can perform an enzymatic reaction in which a target substrate is cleaved, and the ribozyme itself is not altered during the reaction.

Ribozyme-mediated cleavage was first demonstrated *in vitro* by Uhlenbeck (1) and subsequently by Haseloff and Gerlach (3). Hammerhead and hairpin ribozymes have been successfully used in numerous studies to target mRNA in cell-free systems as well as mammalian cells, i.e., HIV and other viral RNAs (4–9), tumor necrosis factor α (10), c-*fos* (11), β-amyloid peptide precursor (12), acetyl-CoA carboxylase (13), BCR/ABL (14), MDR-1 (15), platelet-derived growth factor β (16), and growth factor pleiotrophin (17).

Recently, hammerhead ribozymes have been delivered to transgenic mice and *Drosophila*. Zhao and Pick (18) generated transgenic flies carrying a ribozyme against the *ftz* (*fushi tarazu*) transcript. The two developmental phases of *ftz* function were distinguished by timed induction of the ribozyme in the blastoderm. This activation resulted in disruption of the seven-stripe pattern and produced *ftz*-like pair-rule defective larvae. In transgenic mice, Efrat *et al.* (19) delivered a glucokinase transcript-targeted ribozyme, under the control of the insulin promoter to pancreatic β-cells. Expression of the ribozyme resulted in a reduction of glucokinase activity to 30% of control levels, but whereas insulin release in response to glucose from *in situ*-perfused pancreas was impaired, the plasma glucose and insulin levels remained normal. Similarly, expression in transgenic mice of a ribozyme targeting murine β_2-microglobulin mRNA resulted in up to 90% reduction in the target mRNA levels in specific tissues (20). In both studies, ribozymes were targeted to low-level mRNAs and despite the use of apparently strong promoters, detection of the ribozyme transcripts was only possible using reverse transcription (RT)-PCR techniques.

Here we report the development of transgenic mice carrying a ribozyme targeted against the milk protein bovine α-lactalbumin (α-lac) transcript. This protein induces the synthesis of lactose by interacting with the enzyme β1,4-galactosyltransferase (EC 2.4.1.38) and modifying its substrate specificity. Manipulation of α-lac gene expression should provide us with a better understanding of the roles of this protein and lactose in the lactation process and may lead to the development of milk with altered composition.

Ribozyme 5 (RZ5), which targets the 3′ untranslated region (UTR) of the bovine α-lac mRNA, has been shown previously to be highly active in the cytoplasm of transfected mouse mammary cells in culture (21). Mice carrying the ribozyme gene under the transcriptional-control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) were generated and subsequently cross-bred with animals that highly express a bovine α-lac transgene (line 53 in ref. 22). Expression of RZ5 in the mammary gland of transgenic mice was detected by Northern blot analysis and was positively correlated with a reduction of the target mRNA and protein of up to 50% of that observed in the non-RZ5 transgenic littermate controls. The specificity of this ribozyme activity is demonstrated by the absence of a reduction in the level of the endogenous murine α-lac mRNA or protein.

MATERIALS AND METHODS

Plasmid Constructs and Preparation of DNA for Microinjection. The ribozyme sequence, consisting of the 24-nt hammerhead catalytic domain (3), two 12-nt sequences complementary to regions of the 3′ UTR of bovine α-lac, and partial *Kpn*I and *Sal*I recognition sequences (5′-TCGACTTCCACTTTTGTTTCGTCCTCACCGACTCATCAGGCCGAATAAAAGCGGTAC-3′) was synthesized as an oligonucleotide using automated phosphoramidite chemistry and ligated into *Kpn*I/*Sal*I-digested Bluescript SK+ (21, 23). The

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: α-lac, α-lactalbumin; MMTV-LTR, mouse mammary tumor virus long terminal repeat; RZ5, ribozyme 5; UTR, untranslated region; SV40, simian virus 40.

full-length ribozyme insert was sub-cloned into *Sma*I/*Sal*I-digested pMSG plasmid (Pharmacia LKB). This inserted the ribozyme between the MMTV-LTR promoter and a SV40 early splice and polyadenylylation signal (Fig. 1). The sequences of the ribozyme constructs were confirmed by dideoxy sequencing using an Applied Biosystems model 373A automated sequencer. The recombinant plasmid was digested with *Hin*dIII and *Bam*HI, and the 2.4-kb fragment corresponding to the injected-construct was isolated by electrophoresis on a 1% agarose gel followed by electroelution and DEAE-cellulose chromatography purification (24).

Generation and Analysis of Transgenic Mice. Microinjection was performed on pronuclei of C57BL/6 × CBA F_2 hybrid eggs and transgenic mice were generated and bred according to established procedures (25).

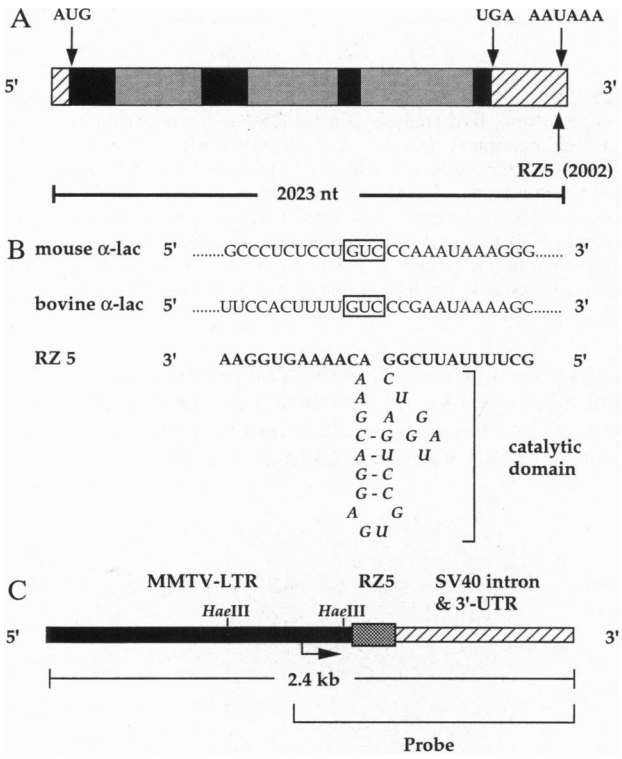

FIG. 1. Target site, ribozyme structure, and DNA construct design. (*A*) Structure of bovine α-lac pre-mRNA and target site of RZ5. The α-lac pre-mRNA is composed of four exons (solid segments, coding regions; hatched segments, 5′ and 3′ UTRs), and three introns (shaded segments; see ref. 22 for further details). Positions of the initiation and termination codons and polyadenylylation signal are shown. The number in parentheses indicates the position of the U in the GUC target site. (*B*) Target sequence for bovine and homologous sequence for murine α-lac mRNA and structure of RZ5. RZ5 is a hammerhead ribozyme (3) that possesses two 12-nt flanking regions (boldface type) that are complementary to the bovine α-lac pre-mRNA. Of the 24 nt in the bovine α-lac target region, 16 are identical in the murine α-lac sequence. The catalytic domain of the hammerhead ribozyme, derived from the (+) strand of tobacco ringspot virus (23) is shown in boldface italics. The tri-nucleotide GUC cleavage recognition sequence is boxed. (*C*) Structure of DNA construct used for micro-injection. Construct possesses 1455 bp of the MMTV-LTR. Transcription initiation occurs approximately 268 bp upstream of the ribozyme insertion site as indicated by the arrow. RZ5 was isolated from pTF29-RZ5 (21) by digestion with *Bam*HI and *Sal*I and cloned into *Sma*I and *Sal*I digested pMSG plasmid. 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 polyadenylylation region. The polyadenylylation site is at position 822 within this 3′ UTR sequence. The predicted transcript size is approximately 1150 nt [excluding the poly(A) tail]. Position of the probe used for Southern blot analysis is indicated (size = 1320 nt). The positions of two *Hae*III restriction enzyme sites in the MMTV-LTR are shown.

Tail biopsies of founder mice were taken 2–4 weeks after birth and genomic DNA was extracted and recovered as described (22). Southern blot analysis was performed with 10 μg of *Hae*III-digested genomic DNA, which was fractionated on a 1% agarose gel and transferred to Hybond N membrane. Hybridization of membranes was carried out as described (26, 27) using a 1.3-kb probe complementary to 375 bp of the MMTV-LTR, and the ribozyme and SV40 sequences (Fig. 1*C*).

RNA Extraction and Northern Blot Analysis. Total cellular RNA from 7- to 8-day lactating mammary glands was extracted using a modified version of the guanidinium-phenol method (28). Tissue (100–200 mg) was homogenized in a solution of 4 M guanidine thiocyanate and extracted once with water-saturated phenol:chloroform (10:2) followed by isopropanol precipitation. RNA samples were fractionated on a 2% agarose/1.2 M formaldehyde gel and transferred to Hybond N membrane. Northern blot hybridization was carried out as described (26, 27). Bovine and murine α-lac cDNA probes were labeled by standard random-priming techniques using Klenow DNA Polymerase I in the presence of [α-^{32}P]dCTP (23). Ribozyme and 28S oligonucleotide probes were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (23).

Milk Protein Analysis. Milk was collected from 7- to 8-day lactating mice as described (22). Milk samples were diluted 1:5 with distilled water and centrifuged at 1500 × *g* for 30 min. A fat-reduced fraction was collected and diluted with 1 vol of SDS/Laemmli loading buffer followed by fractionation on a 13% $NaDodSO_4$ polyacrylamide gel (29). Proteins were transferred to nitrocellulose by standard semi-dry electroblotting techniques. The membrane was blocked with 5% BSA in PBS overnight and incubated with a rabbit-anti bovine α-lac polyclonal antisera followed by peroxidase-labeled goat-anti rabbit IgG. Peroxidase activity was revealed using 4-chloro-1-naphthol.

RESULTS

Ribozyme and Target Site, Construct Design, and Generation of Transgenic Mice. RZ5 targets a region of the 3′ UTR of the bovine α-lac mRNA, near the polyadenylylation signal (nucleotide 2002 on the pre-mRNA, see Fig. 1*A*). The 12-nt flanking sequences of this ribozyme are complementary to the bovine mRNA but have homology also with the murine α-lac sequence; 16 of the 24 nt are identical (Fig. 1*B*). Both the murine and bovine sequences possess a GUC tri-nucleotide recognition site that is necessary for cleavage (24).

The MMTV-LTR has been used previously to direct high-level expression of the bovine α_{S1} casein cDNA to the mammary gland of transgenic mice (30). The ribozyme was inserted between the MMTV-LTR promoter and a 3′ UTR derived from SV40 (Fig. 1*C*). The possible structure of the ribozyme transcript was examined prior to its insertion in this vector using the MFOLD software package. This analysis suggested that the ribozyme would be placed close to the end of a long stem and that the catalytic domain should fold correctly. Furthermore, the majority of the antisense flanking nucleotides might be contained within unpaired regions (data not shown). This fragment was isolated and injected into pronuclei of C57BL/6 × CBA F_2 hybrid eggs. Southern blot analysis (Fig. 2) of genomic tail DNA identified the presence of the ribozyme transgene in 12 founder mice (11% of animals screened). Mice transgenic for the ribozyme were then cross-bred with a line of mice already carrying a bovine α-lac transgene (line 53, previously described in ref. 22). This line of mice has consistently secreted bovine α-lac at a high level in the milk (≈0.4 mg·ml^{-1} in heterozygous animals) for 12 generations (data not shown). The presence of this transgene does not influence the level of expression of the endogenous α-lac.

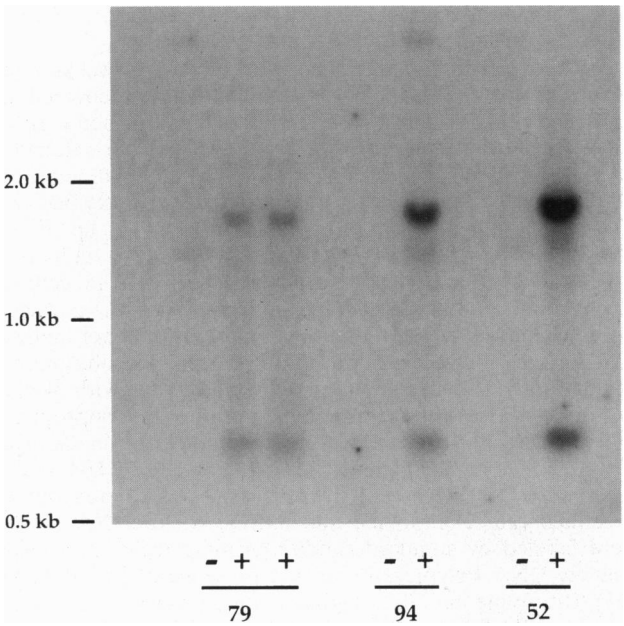

FIG. 2. Detection of transgenic mice carrying the RZ5 DNA construct by Southern blot analysis. DNA was isolated from tail biopsies, digested with *Hae*III, and fractionated on a 1% agarose gel prior to transfer to Hybond N membrane. The membrane was hybridized with a random-primed [α-^{32}P]dCTP-labeled DNA fragment that was complementary to the 3′ end of the DNA construct (see Fig. 1). The lower bands (≈750 bp) represent an internal fragment of the DNA construct released by *Hae*III digestion. The upper bands of approximately 1.65 kb indicate the presence of multiple copies arranged in tandem. Other hybridizing bands might represent junction fragments. Positions of DNA markers of known molecular weights are shown on the left. The presence or absence of the DNA construct is shown below the number for each line of mice by a + or −, respectively. Three independent lines of transgenic mice (lines 79, 94, and 52) are displayed.

"Double" transgenic mice were identified by Southern blot analysis (data not shown) and, unless otherwise stated, all mice analyzed were heterozygous for the bovine α-lac gene. The transmission of the ribozyme transgene was approximately 50%, and all transgenic mice appear not to suffer adverse effects due to the presence of this transgene.

Expression of Ribozyme Specifically and Efficiently Reduces the Level of Bovine α-lac mRNA. Heterozygous expression of the ribozyme transgene in lactating mammary glands was detected by Northern blot analysis in 3 of 12 independent lines of mice (Fig. 3). Fig. 3 shows a band of the expected molecular weight in RNA samples for mice positive for the ribozyme. It is possible that other lines express the RZ5 transgene, but at levels below the sensitivity of Northern blot analysis.

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 (Figs. 4 and 5). 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 ribozyme-mediated reduction in the steady-state levels of bovine α-lac mRNA was positively correlated with the level of expression of RZ5 (Figs. 3 and 4). No clear difference in the level of bovine α-lac mRNA between transgenic and non-RZ5 transgenic littermates was observed in "nonribozyme expressing" lines (data not shown). 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, no RZ5-expressing mice had less murine α-lac mRNA than their non-RZ5-expressing counterparts (Figs. 4 and 5). Dot-blot

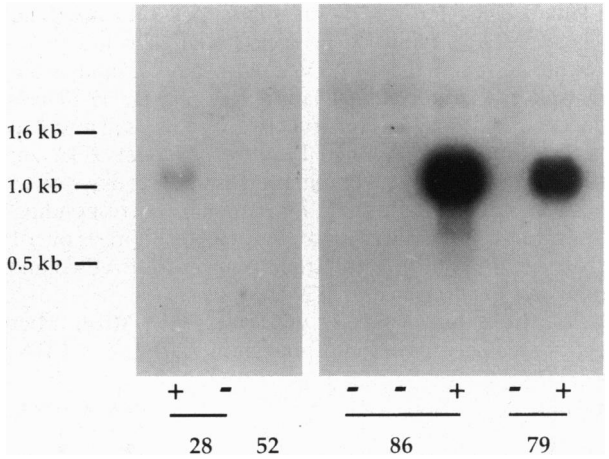

FIG. 3. Ribozyme expression in the mammary gland of transgenic mice. Northern blot analysis of total RNA extracted from 7- to 8-day lactating mammary gland tissue of transgenic (+) and non-RZ5 transgenic littermate control mice (−) for lines 28, 52 (+ mouse only), 86 (two − and one + mice), and 79. RNA extraction and Northern blot analysis were performed as described. Hybridization was performed with a [γ-^{32}P]ATP end-labeled ribozyme-specific oligonucleotide (the complementary sequence is shown in Fig. 1*B*). Four independent lines of mice transgenic for RZ5 are shown. Positions of DNA markers of known molecular mass are shown on the left.

analysis was used to confirm the ribozyme-mediated effects on both α-lac mRNAs and also further demonstrated the specificity of this activity as no effect on the levels of endogenous κ-casein mRNA was observed (data not shown).

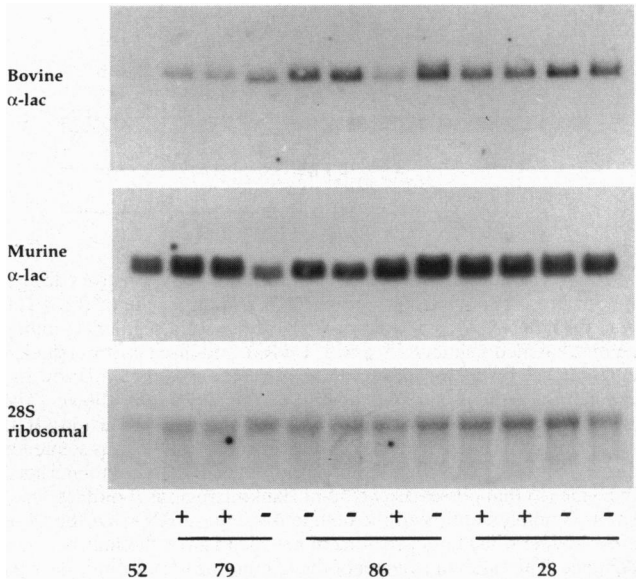

FIG. 4. Ribozyme-mediated reduction of bovine α-lac mRNA in transgenic mice. Northern blot analysis of total RNA extracted from 7- to 8-day lactating mammary gland tissue of transgenic (+) and non-RZ5 transgenic littermate control mice (−). RNA extraction and Northern blot analysis were performed as described. Hybridization was performed sequentially with a [α-^{32}P]dCTP random-primed bovine α-lac cDNA, a [γ-^{32}P]ATP end-labeled 28S ribosomal RNA-specific oligonucleotide (31), and a [α-^{32}P]dCTP random-primed murine α-lac cDNA. Before final hybridization with the murine α-lac cDNA probe, the membrane was stripped by boiling in 0.1× SSPE/0.1% SDS (23), and exposed to verify removal of the bovine α-lac probe. Specificity of the bovine α-lac probe is demonstrated by the absence of a signal in mouse 52, a control animal that does not possess bovine α-lac and RZ5 DNA. Three independent lines of mice are shown (79, 86, and 28).

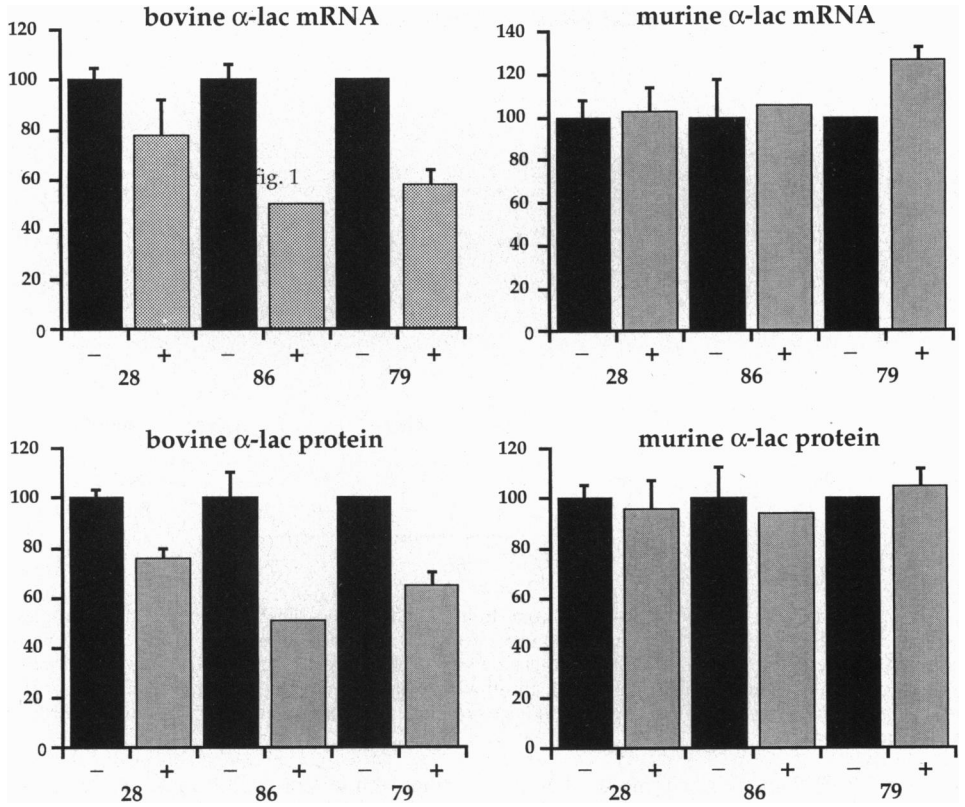

FIG. 5. Quantitative analysis of ribozyme-mediated reduction of bovine α-lac mRNA and protein in transgenic mice. The Northern blot analysis autoradiograph and Western blot analysis described in Figs. 4 and 6 were scanned using a Pharmacia LKB Imagemaster DTS scanning system. Calibration of the apparatus was performed according to the manufacturer's guidelines, and purified α-lac samples loaded on the protein gel were used as validation. In addition, to ensure that the bands scanned were below saturation on the film, multiple autoradiographs of different exposure times were examined. 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. Likewise, bovine and murine α-lac protein values were corrected for the quantity of loading on the gel using the scan values obtained for a high molecular weight protein (possibly albumin) that was present on the Western blot after overnight development and by Bradford estimation of total protein content of the milk samples (32). Values for ribozyme transgenic mice (+) from each line were normalized against their non-RZ5 transgenic littermate control (solid bars).

Ribozyme-Dependent Reduction in Bovine α-lac Content in Milk. To investigate whether the reduction achieved in bovine α-lac mRNA was paralleled by a reduction in the relevant α-lac content in the milk, samples were collected from 7-to 8-day lactating animals and examined by Western blot analysis (Fig. 6). 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 (Fig. 5). Neither the presence of the transgene in "nonexpressing" lines of mice (data not shown) or expression of the bovine α-lac-targeted ribozyme (lines 79, 28, and 86) affected the content of the endogenous α-lac protein in the milk (Fig. 5).

DISCUSSION

RZ5 targets a site immediately adjacent to the polyadenylylation signal in the 3′ UTR of the bovine α-lac transcript. We chose RZ5 because it efficiently reduced the levels of target mRNA and protein in C127I mouse mammary cells (21). These results suggested that this region in the 3′ UTR is accessible to a ribozyme molecule, as has been observed for the 3′ UTRs of certain transcripts with antisense molecules (33).

High-level expression of the ribozyme was detected by Northern blot analysis in 3 of 12 lines of mice analyzed. For MMTV-LTR-based constructs, this is a lower percentage of expressing lines than has been reported by others (30, 34–36). However, for constructs delivering a ribozyme to mice, the percentage of expressing lines appears to be low (19, 20). Possibly some of the RZ5 transgenic lines express the ribozyme gene but at levels below the sensitivity of Northern blot analysis, necessitating the use of RNase protection assay or RT-PCR for detection. However, given that RZ5 targets a highly expressed gene, low level ribozyme gene expression is unlikely to have any clear detectable effect on α-lac mRNA levels. Northern blot analysis was used to confirm the absence of a reduction in the level of bovine α-lac mRNA in apparently nonexpressing lines (data not shown).

To date, only low expression of ribozyme transgenes has been reported in animals, detected by RT-PCR analysis (19, 20). Problems with achieving high expression have also been observed in cells in culture (6, 9, 16) and probably relate to the apparent low stability of transcripts that contain ribozyme sequences (37, 38). Here we report high expression of a ribozyme transgene that was readily detected by Northern blot analysis using a ribozyme-specific oligonucleotide probe.

In ribozyme-expressing transgenic mice, Efrat *et al.* (19) observed a 70% reduction in the level of glucokinase activity, whereas Larsson *et al.* (20) observed 70, 22, and 81% reductions in β_2-microglobulin mRNA in three lines. In this study, the levels of bovine α-lac mRNA were reduced to 78, 58, and 50% of the levels in the non-RZ5 transgenic littermate controls for lines 28, 79, and 86, respectively. In contrast with glucokinase and β_2-microglobulin mRNA targets, the bovine α-lac transgene is highly expressed (22). The level of variation of bovine α-lac, within a line in non-RZ5 expressing mice, is

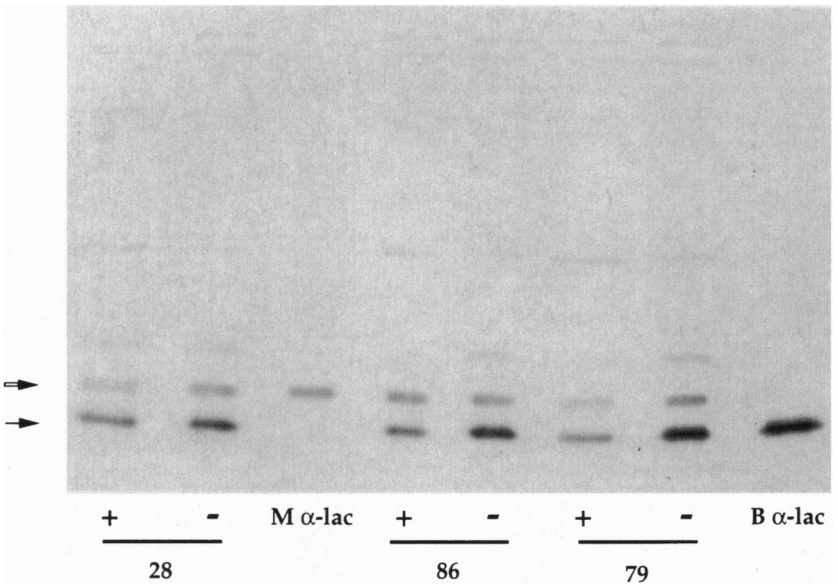

FIG. 6. Ribozyme-mediated reduction of bovine α-lac content in the milk from transgenic mice. Milk samples were collected from ribozyme-transgenic (+) and non-RZ5 transgenic littermate controls (−) 7–8 days after parturition. Proteins were fractionated on a 13% SDS/polyacrylamide gel and transferred to Hybond C nitrocellulose by semi-dry electro-transfer. Equal volumes of milk for each sample were loaded on the gel. α-lac was detected using a polyclonal rabbit anti-bovine α-lac antiserum. This antiserum also weakly crossreacts with the endogenous murine α-lac. Open arrow indicates murine α-lac; solid arrow indicates bovine α-lac. Pure protein samples of 100 ng of bovine α-lac and 180 ng of murine α-lac are shown.

substantially lower (12.4 ± 2.7%) than the ribozyme-mediated reduction observed in lines 79 and 86 (unpublished observation and Fig. 5).

This ribozyme-mediated reduction in the level of bovine α-lac mRNA was positively correlated with ribozyme expression (Figs. 3 and 4). Furthermore, the ribozyme action was highly-specific as neither the presence of the transgene in nonexpressing mice or expression of the ribozyme gene effected the endogenous murine α-lac mRNA or protein levels. The level of variation in murine α-lac within a line is however higher (16.8 ± 6.2%) than that observed for the bovine α-lac transgene. The levels of murine α-lac mRNA were particularly low for two non-RZ5 transgenic mice (Fig. 4). The murine α-lac transcript possesses 16 of 24 nt complementary to the antisense flanks of RZ5. Of these nucleotides, 3 reside immediately 5′ of the cleavage site and 7 of the 8 nt immediately 3′ to the GUC have homology with the RZ5 sequence. Recently Tabler *et al.* (39) demonstrated that an asymmetrical hammerhead ribozyme with only 3 nt in helix 1 inhibited HIV replication with equal efficacy as the symmetric control molecule. In addition, the murine α-lac transcript possesses the GUC tri-nucleotide recognition sequence that is necessary for cleavage. However, in *in vitro* studies, RZ5 does not cleave this target (data not shown). Similarly, Bennett and Cullimore (40) observed a high degree of specificity in cleavage with closely related glutamine synthetase transcripts. The extent of mismatch that can be tolerated by antisense sequences *in vivo* is not well defined. Using antisense oligonucleotides targeted to the human collagen transcript, Laptev *et al.* (41) observed a reduction of the murine protein with oligonucleotides that were complementary to only 10 of the 22 nt in the murine sequence. It is clear from this study however that in mice the action of the ribozyme was specific for the bovine sequence and the level of the murine transcript was not affected.

We are unable to determine the extent of antisense action compared with catalytic cleavage for this ribozyme as no mice are available with a catalytically-inactive or mutant ribozyme. Equally however, due to the effects of site of integration on expression level of a transgene, it would be difficult to compare two ribozyme molecules without analyzing many independent lines of mice for each transgene. Perhaps more appropriate in mice is the use of "double" transgenics that possess two possible target molecules, one of which cannot be cleaved by the ribozyme but has complementarity with the antisense flanks of the ribozyme, such as the bovine and murine α-lac mRNAs. In a previous study with RZ5 about 25% of the down-regulation of target RNA levels was attributed to antisense action in C127I mouse mammary cells (21).

Delivery of the ribozyme to transgenic mice, under the transcriptional control of the MMTV-LTR, did not apparently affect the health of the mice in either nonexpressing or expressing animals. In previous studies, low expression of transgenes under the control of this LTR has been observed in several tissues other than the mammary gland (30, 34, 35). In this study to date, only tissues from 7- to 8-day lactating mammary glands have been analyzed. However, given the observed high specificity of the ribozyme for bovine α-lac in the mammary gland, even if expressed in other tissues at low levels, RZ5 is unlikely to have any effect.

We have targeted ribozymes to the α-lac transcript with the objective of indirectly affecting the regulation of lactose synthesis in the mammary gland. Lactose, the predominant sugar in milk is also one of the major osmotic regulators of milk secretion (42). Hayssen and Blackburn (43) have suggested that the origin of lactation was related to the molecular evolution of α-lac from lysozyme. Lactose accounts for the majority of milk intolerance in humans and intestinal lactase deficiencies affect >80% of mankind (44). Using embryonic stem cells and gene targeting, Stinnakre *et al.* (45) recently created α-lac-deficient mice. They observed a direct relationship between α-lac content and lactose synthesis. α-lac deficient mice were unable to feed their offspring as they produced a highly viscous milk that could not be secreted. However, in the heterozygous phenotype, α-lac was decreased by 40% and lactose by 10–20% (45). This resulted in a significantly more concentrated milk. In this report, we have shown that delivery of a ribozyme can efficiently and specifically reduce bovine α-lac expression. We thus have demonstrated the feasibility of using a ribozyme approach for the modification of milk composition in mammals. Further toward this goal, we are in

the process of creating mice carrying a ribozyme that targets directly the murine α-lac transcript to study more closely the relationship between α-lac concentration and the lactose and water content of milk.

We thank Jacques Buri and Pascal Laurent for synthesis and purification of oligonucleotides and Dick Wilkins for photos. W. L. Hurley supplied a full-length cDNA clone of bovine α-lac. This work was supported by Ministere de la Recherche et de la Technologie (France), Institut National de la Recherche Agronomique (France), and AgResearch (New Zealand).

1. Uhlenbeck, O. C. (1987) *Nature (London)* **328,** 596–600.
2. Symons, R. H. (1989) *Trends Biochem. Sci.* **14,** 445–450.
3. Haseloff, J. & Gerlach, W. L. (1988) *Nature (London)* **334,** 585–591.
4. Crisell, P., Thompson, S. & James, W. (1993) *Nucleic Acids Res.* **21,** 5251–5255.
5. Yu, M., Ojwang, J., Yamada, O., Hampel, A., Rapapport, J., Looney, D. & Wong-Staal, F. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6340–6344.
6. Homann, M., Tabler, M., Tzortzakaki, S. & Sczakiel, G. (1994) *Nucleic Acids Res.* **22,** 3951–3957.
7. Sun, L.-Q., Warrilow, D., Witherington, C., Macpherson, J. & Symonds, G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9715–9719.
8. Tang, X.-B., Hobom, G. & Luo, D. (1994) *J. Med. Virol.* **42,** 385–395.
9. Cantor, G. H., McElwain, T. F., Birkebak, T. A. & Palmer, G. H. (1994) *Proc. Natl. Acad. Sci. USA* **90,** 10932–10936.
10. Sioud, M. (1994) *J. Mol. Biol.* **242,** 619–629.
11. Scanlon, K. J., Ishida, H. & Kashani-Sabet, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11123–11127.
12. Denman, R. B., Smedman, M., Ju, W., Rubenstein, R., Potempska, A. & Miller, D. L. (1994) *Nucleic Acids Res.* **22,** 2375–2382.
13. Ha, J. & Kim, K.-H. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9951–9955.
14. Lange, W., Cantin, E. M., Finke, J. & Dolken, G. (1993) *Leukemia* **7,** 1786–1794.
15. Kiehntopf, M., Brach, M. A., Licht, T., Petschauler, S., Karawajew, L., Kirschning, C. & Herrmann, F. (1994) *EMBO J.* **13,** 4645–4652.
16. Dorai, T., Kobayashi, H., Holland, J. F. & Ohnuma, T. (1994) *Mol. Pharmacol.* **46,** 437–444.
17. Czubayko, F., Riegel, A. T. & Wellstein, A. (1994) *J. Biol. Chem.* **269,** 21358–21363.
18. Zhao, J. J. & Pick, L. (1993) *Nature (London)* **365,** 448–451.
19. Efrat, S., Leiser, M., Wu, Y.-J., Fusco-DeMane, D., Emran, O. A., Surana, M., Jetton, T. L., Magnuson, M. A., Weir, G. & Fleischer, N. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2051–2055.
20. Larsson, S., Hotchkiss, G., Andang, M., Nyholm, T., Inzunza, J., Jansson, I. & Ahrlund-Richter, L. (1994) *Nucleic Acids Res.* **22,** 2242–2248.
21. L'Huillier, P. J., Davis, S. R. & Bellamy, A. R. (1992) *EMBO J.* **11,** 4411–4418.
22. Vilotte, J.-L., Soulier, S., Stinnakre, M.-G. Massoud, M. & Mercier, J.-C. (1989) *Eur. J. Biochem.* **186,** 43–48.
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
24. Symons, R. H. (1991) *Crit. Rev. Plant Sci.* **10,** 189–234.
25. Hogan, B., Costantani, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab. Press, Plainview, NY).
26. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.
27. Westneat, D. F., Noon, W. A., Reeve, H. K. & Aquadro, C. F. (1988) *Nucleic Acids Res.* **16,** 4161.
28. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
29. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
30. Yom, H.-C., Bremel, R. D. & First, N. L. (1993) *Anim. Biotechnol.* **4,** 89–107.
31. Barbu, V. & Dautry, F. (1989) *Nucleic Acids Res.* **17,** 7115.
32. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
33. Strickland, S., Huarte, J., Belin, D., Vassalli, A., Rickles, R. J. & Vassalli J.-D. (1988) *Science* **241,** 680–684.
34. Stewart, T. A., Pattengale, P. K. & Leder, P. (1984) *Cell* **38,** 627–637.
35. Matsui, Y., Halter, S. A., Holt, J. T., Hogan, B. L. M. & Coffey, R. J. (1990) *Cell* **61,** 1147–1155.
36. Ross, S. R., Hsu, C.-L. L., Choi, Y., Mok, E. & Dudley, J. P. (1990) *Mol. Cell. Biol.* **10,** 5822–5829.
37. Cameron, F. H. & Jennings, P. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9139–9143.
38. Bertrand, E., Pictet, R. & Grange, T. (1994) *Nucleic Acids Res.* **22,** 293–300.
39. Tabler, M., Homann, M., Tzortzakaki, S. & Sczakiel, G. (1995) *J. Cell. Biochem.*, Suppl. **19A,** 217.
40. Bennett, M. J. & Cullimore, J. V. (1992) *Nucleic Acids Res.* **20,** 831–837.
41. Laptev, A. V., Lu, Z., Colige, A. & Prockop, D. J. (1994) *Biochemistry* **33,** 11033–11039.
42. Morrissey, P. A. (1985) in *Developments in Dairy Chemistry,* ed. Fox, P. F. (Elsevier, New York), Vol. 3, pp. 1–34.
43. Hayssen, V. & Blackburn, D. G. (1985) *Evolution* **39,** 1147–1149.
44. Delmont, J. (1983) *Milk Intolerances and Rejection* (Karger, Nice, France).
45. Stinnakre, M.-G., Vilotte, J.-L., Soulier, S. & Mercier, J.-C. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6544–6548.