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Ribozyme cleaves *rex/tax* mRNA and inhibits bovine leukemia virus expression

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ABSTRACT **Bovine leukemia virus (BLV) encodes at least two regulatory proteins, Rex and Tax. Tax, the trans-activating protein, stimulates the long terminal repeat to promote viral transcription and may be involved in tumorigenesis. Rex is involved in the transition from early expression of regulatory proteins to later expression of viral structural proteins. We have targeted ribozymes against the mRNA encoding Rex and Tax. The ribozymes consist of the hammerhead catalytic motif flanked by antisense sequences that hybridize with the complementary *rex/tax* mRNA. To evaluate cleavage in a cell-free system, we transcribed portions of *rex/tax* mRNA and incubated them with synthetic RNA ribozymes. A ribozyme was identified that cleaves >80% of the target RNA. Synthetic DNA encoding this ribozyme was cloned into the expression vector pRc/RSV and transfected into BLV-infected bat lung cells. Intracellular cleavage of *rex/tax* mRNA was confirmed by reverse transcriptase PCR. In cells expressing the ribozyme, viral expression was markedly inhibited. Expression of the BLV core protein p24 was inhibited by 61%, and reverse transcriptase activity in supernatant was inhibited by 92%. Ribozyme inhibition of BLV expression suggests that cattle expressing these sequences may be able to control BLV replication.**

Bovine leukemia virus (BLV), a retrovirus structurally similar to human T-cell leukemia viruses I and II (HTLV-I and -II), causes persistent lymphocytosis and B-lymphocyte lymphoma in cattle and sheep (1). After initial infection, BLV expresses a doubly spliced transcript encoding the regulatory proteins Rex and Tax (2). Tax trans-activates the viral long terminal repeat and also cellular promoters, including c-*fos* and somatostatin (3). Cotransfection experiments have shown that Tax is necessary for viral expression *in vitro* (4). Rex regulates the transition from early expression of the doubly spliced transcript encoding regulatory proteins to the later expression of singly spliced or unspliced transcripts that express the *env* gene or the *gag* and *pol* genes (5). Recently, the 3′ region of HTLV-I and BLV has been shown to encode RNA with alternative splice patterns that may express other regulatory proteins (6–8).

Because of the critical role of regulatory proteins such as Tax and Rex in the HTLV/BLV group of viruses, we hypothesized that the *rex/tax* mRNA would be a rational target for ribozyme-mediated inhibition. The hammerhead motif, first identified in plant RNA pathogens (9, 10), cleaves the phosphodiester bond downstream of a GUC triplet (9, 10). By flanking the hammerhead motif with antisense sequences, Haseloff and Gerlach (11) demonstrated cleavage of specific target RNAs. In this study, we demonstrate a ribozyme that cleaves *rex/tax* mRNA and, when transfected into BLV-infected cells, markedly inhibits viral expression.

MATERIALS AND METHODS

Ribozymes. Ribozymes composed of the hammerhead motif (12) flanked by antisense sequences were targeted to cleave *rex/tax* mRNA at nt 7303 (ribozyme 2) (Fig. 1) or nt 7680 (ribozyme 3) [as designated by Sagata's numbering system (13)]. The sequence of ribozyme 2 is shown in Fig. 1, and that of ribozyme 3 is 5′-UUUCCGAGCUGAUGAGUCCGUGAGGACGAAACAGGAUGC-3′. Negative controls included a motif control, consisting of the hammerhead motif flanked by a random sequence 5′-CAGAGUGUCUGAUGAGUCCGUGAGGACGAAACAUUGGCA-3′, and an antisense control, consisting of the antisense sequences of ribozyme 2, but in which the hammerhead motif was replaced by a single base, U (5′-CAUCAUUGCAUAAAACCA-3′). RNA oligonucleotides were synthesized by National Biosciences (Plymouth, MN).

Substrate RNA. As described (14), plasmid pBT contains a cDNA copy of *tax*. A 203-base portion of *tax* RNA, which included the target of ribozyme 2, was generated by linearizing pBT at the *Sfu* I site (nt 7364) and transcribing with T3 RNA polymerase in the presence of [α-^{32}P]CTP (Fig. 2*A*). A 256-base RNA including the target of ribozyme 3 was generated by PCR amplification of pBT between nt 7578 and 7834. The 5′ primer also included the T3 RNA polymerase promoter sequence. The resulting PCR product was transcribed as described above (Fig. 2*A*).

Cell-free Cleavage Assay. Ribozyme and α-^{32}P-labeled RNA were incubated in 50 mM Tris·HCl (pH 7.6 or 8.0) and $MgCl_2$. Initially, Mg^{2+} concentrations from 2 to 15 mM were used; 12.5 mM was found to be optimal. The RNA substrate concentration was 0.75 μM and ribozyme concentrations varied from 0.375 to 15 μM. The ribozyme and RNA substrate in Tris were heated to 95°C for 2 min and cooled on ice; then $MgCl_2$ was added. Reactions were conducted at 37°C or 50°C for 1 hr, and the resulting RNA was subjected to electrophoresis in 8% polyacrylamide/7 M urea gels. Gels were fixed in 10% (vol/vol) methanol/10% (vol/vol) acetic acid/1% (vol/vol) glycerol and autoradiographed. The relative amounts of each RNA species were quantitated by densitometry followed by correction for cytosine content.

Ribozyme Expression Plasmids. Two expression plasmids were constructed: pRc/R2, which expresses ribozyme 2 RNA; and pRc/RMC, which expresses the motif control RNA, consisting of the hammerhead motif flanked by a random sequence. The parental vector was pRc/RSV (Invitrogen), which has a Rous sarcoma virus long terminal repeat promoter and a *Neo* gene under the control of the simian virus 40 early promoter. Two strands of DNA oligonucleotides were synthesized to encode ribozyme flanked by a 5′ *Hin*dIII site and a 3′ *Xba* I site. Each strand was 5′ phosphorylated using T4 polynucleotide kinase and the two strands were annealed at 65°C for 20 min, 45°C for 1 hr, and 22°C for 1 hr. After digesting concatamers with *Hin*dIII and *Xba* I, the

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Abbreviations: BLV, bovine leukemia virus; RT, reverse transcriptase; BLC, bat lung cell.

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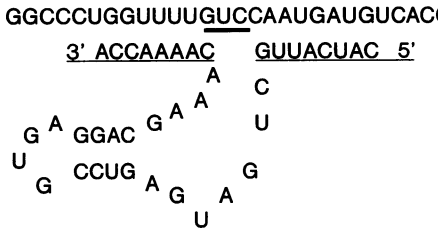

FIG. 1. Sequence of ribozyme 2 (below), hybridized to *rex/tax* mRNA (above). Underlined portions of ribozyme 2 are antisense sequences to target *rex/tax* mRNA. Shown here are bases 7289–7322 of *rex/tax* mRNA. The ribozyme is designed to cleave downstream of the underlined GUC (base 7303).

insert was ligated into the *Hin*dIII/*Xba* I sites of pRc/RSV and plasmid construction was confirmed by sequencing (data not shown).

Cells. BLV-infected bat lung cells (BLV-BLCs) (15) were used. BLV infection was confirmed by identification of p24, gp51, and Tax by immunoblot and by identification of intracellular p24 by immunochemistry (data not shown). Uninfected BLCs (Tb 1 Lu) were from the American Type Culture Collection.

Transfection of BLV-BLCs. BLV-BLCs were transfected by pRc/R2, pRc/RMC, or pRc/RSV, using calcium phosphate DNA precipitate formed in *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffer (pH 6.95) (16). Stably transfected, *neo*-expressing cell lines were selected by G418 for 25 days.

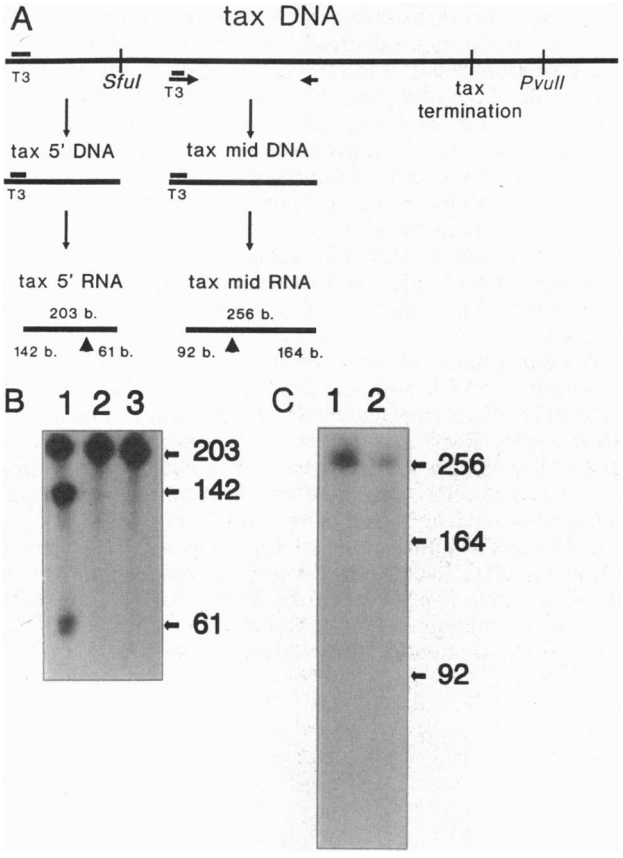

FIG. 2. Cell-free cleavage of RNA by ribozymes 2 and 3. (*A*) Schematic diagram of substrate RNA, as described in the text. Arrowheads designate predicted cleavage sites, and predicted fragment sizes are shown. (*B*) Cleavage by ribozyme 2. The 203-base *tax* 5' RNA was incubated with ribozyme 2 (lane 1), motif control (lane 2), or antisense control (lane 3). Size of each fragment is indicated. (*C*) Ribozyme 3 did not cleave substrate RNA. The 256-base *tax* mid-RNA was incubated with ribozyme 3 (lane 1) or motif control (lane 2).

PCR Analysis and Southern Blots. Transfection of cell lines by ribozyme-encoding DNA sequences was verified by PCR amplification of genomic DNA and Southern blotting. Primer 1 was composed of Rous sarcoma virus long terminal repeat bases 543–562 of the plasmid pRc/RSV (5'-TTAAGTGCCTAGCTCGATAC-3') and primer 2 was from bases 750–730 (5'-AGATGGCTGGCAACTAGAAG-3'). These primers were chosen to amplify either (*i*) a 208-base sequence, including the multiple cloning site, from the parental vector pRc/RSV, or (*ii*) a 159-base sequence, including the hammerhead motif, from pRc/R2 or pRc/RMC. PCR was conducted with AmpliTaq polymerase (Perkin–Elmer) in 50 mM KCl/10 mM Tris·HCl, pH 8.3/2.5 mM $MgCl_2$, using two amplifications, each consisting of 30 cycles of 95°C for 90 sec, 60°C for 90 sec, and 72°C for 90 sec, followed by electrophoresis in a 1.5% agarose gel and transfer to a positively charged nylon membrane (Boehringer Mannheim). Membranes were probed with a synthetic DNA oligonucleotide from the 24-base ribozyme hammerhead motif (Fig. 1) (2 pmol per ml of hybridization solution), which was 3' tailed with digoxigenin-11-dUTP/dATP and hybridized and washed according to the manufacturer's recommendations (Genius system; Boehringer Mannheim). Chemiluminescent signal was detected by anti-digoxigenin antibody conjugated to alkaline phosphatase and Lumi-Phos 530 substrate, according to the manufacturer's recommendations, followed by autoradiography.

Intracellular Cleavage Assay. Intracellular cleavage was evaluated by reverse transcriptase PCR (RT-PCR). Total RNA was extracted by acid guanidinium thiocyanate/phenol/chloroform (17). cDNA was synthesized with Moloney murine leukemia virus RT and random hexamer primers, according to the manufacturer's recommendations (GeneAmp RNA PCR kit; Perkin–Elmer). The *rex/tax* cDNA was PCR amplified by using one upstream primer (5'-AGGCGCTCTCCTGGCTACTG-3', from BLV nt 4758–4776) (13, 18) and two downstream primers [5'-GGCACCAGGCATCGATGGTG-3', from BLV nt 7333–7314 (13, 18), and 5'-CCCCAACCAACAACACTTGC-3', from BLV nt 7265–7246 (13)]. These primers span the second splice junction of *rex/tax* mRNA in order to specifically amplify the *rex/tax* transcript (18). Primer 7265–7246 is upstream of the ribozyme 2 cleavage site (nt 7303) and is chosen to amplify both cleaved and uncleaved *rex/tax* mRNA; primer 7333–7314 is downstream of the ribozyme cleavage site and should amplify only uncleaved *rex/tax* mRNA. PCR conditions were as described above, except for 2.0 mM $MgCl_2$ and a melting time of 75 sec. PCR product was subjected to electrophoresis in a 2.0% agarose gel and Southern blotted as described above. Blots were probed with the oligonucleotide 5'-GGCGTTTGCTGAAAGCCTTCAA-3', from BLV nt 4798–4820 (13), as described above, and autoradiographs were analyzed by densitometry (14).

Immunoblot Analysis. Cells (2.5×10^6) were lysed and sonicated (19), subjected to electrophoresis in 7.5–17.5% polyacrylamide gels, transferred to nitrocellulose, and reacted with Tax monoclonal antibody 5A5 (20), followed by reaction with peroxidase-labeled goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories). Reactivity was detected by a chemiluminescent assay, according to the manufacturer's recommendations (ECL; Amersham).

Immunochemistry to Detect p24 Expression. Monospecific polyclonal rabbit anti-BLV p24 antibody was used to detect intracellular p24 expression. A 1-kb p24-encoding *Sma* I fragment of BLV proviral clone pBLV913 was subcloned into pGEX2T and used to transform *Escherichia coli*. The p24–glutathione transferase fusion protein was bound to a glutathione-Sepharose column, and p24 was cleaved from glu-

tathione transferase with thrombin. Purity of isolated p24 was confirmed in silver-stained SDS/polyacrylamide gels. Rabbits were immunized with p24 using saponin as an adjuvant, and the specificity of the rabbit antibody was confirmed in immunoblot analysis. Preimmunization rabbit serum was used as a negative control.

Cells (10^5 cells per well) were grown overnight in 96-well plates, forming monolayers. The monolayers were fixed in 50% methanol/50% acetone/1.25% acetic acid, washed in phosphate-buffered saline (PBS), and blocked with 0.7% swine gelatin and 0.1% Tween 20 in PBS (pH 7.2). The wells were incubated with rabbit anti-p24 antibody, diluted 1:1000 in blocking solution at 37°C for 30 min; washed in PBS; incubated with a 1:200 dilution of biotinylated anti-rabbit IgG antibody (Vector Laboratories) at 37°C for 30 min; washed; and incubated with avidin DH and biotinylated alkaline phosphatase H (Vectastain ABC-AP kit; Vector Laboratories). The soluble substrate, 1.5 mg of *p*-nitrophenyl phosphate per ml in 100 mM $NaHCO_3$/10 mM $MgCl_2$, pH 9.5, was reacted at room temperature for 12–15 min in the dark, and the reaction was stopped with 0.2 M EDTA (pH 7.2). Optical density was quantitated by absorbance at 405 nm. Each reaction was performed in triplicate. At least four different BLV-infected cell lines transfected with the control plasmid pRc/RSV were analyzed in each experiment. The optical densities of BLV-infected cell lines transfected with pRc/R2, the ribozyme-encoding plasmid, were compared to the mean and 99% confidence interval of the cell lines transfected with control pRc/RSV; percentage inhibition was calculated. Uninfected BLCs were included as a negative control.

RT Assay. Supernatant from 3×10^6 BLV-BLCs transfected with pRc/R2 or pRc/RSV, or from uninfected BLCs was centrifuged at $1000 \times g$ for 15 min, filtered (0.45 μm), and centrifuged at $100,000 \times g$ for 30 min. The pellet was dissolved in 80 mM Tris·HCl, pH 8/150 mM KCl/0.02% Triton X-100/5 mM dithiothreitol/5 mM $MgCl_2$/0.3 mM reduced glutathione/0.5 mM EGTA, and incubated for 18 hr at 37°C with 20 μCi (1 Ci = 37 GBq) of [^{3}H]dTTP and 2.5 μg of poly(rA)/poly(dT)$_{12-18}$. Labeled DNA product was precipitated with trichloroacetic acid.

RESULTS

Cleavage of Target RNA. Incubation of ribozyme 2 with the 203-base substrate RNA from the 5′ end of *tax* RNA resulted in the predicted 142-base and 61-base cleavage products (Fig. 2*B*). Cleavage products were not seen when substrate RNA was incubated with motif control RNA or antisense RNA. Approximately 80% of the target RNA was cleaved when the ribozyme/target RNA ratio was >5:1 (Fig. 3). Ribozyme 3,

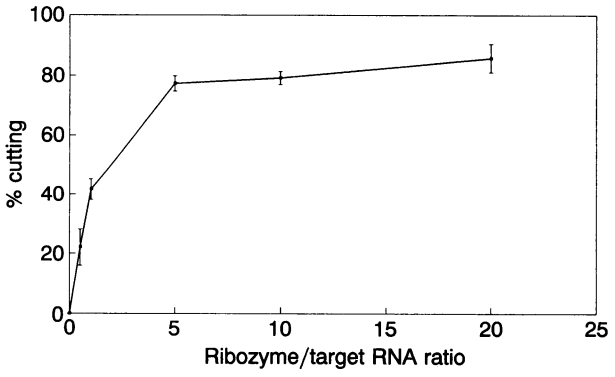

FIG. 3. Dose–response curve of cleavage by ribozyme 2. The 203-base substrate RNA (0.75 μM) was incubated with ribozyme 2 at concentrations ranging from 0.375 to 15 μM. The experiment was repeated three times; means ± SD are shown.

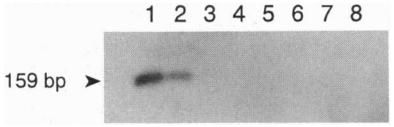

FIG. 4. Southern blot of PCR-amplified sequences from genomic DNA from BLV-infected cells transfected with ribozyme-encoding plasmid or control plasmids. The blot was probed with an oligonucleotide derived from the hammerhead motif. Lanes: 1, pRc/RMC (motif control plasmid); 2, pRc/R2 (ribozyme 2 plasmid); 3, pRc/RSV (parental plasmid); 4, untransfected cells; 5–8, PCR with no exogenous DNA.

directed at the middle of the *tax* RNA, failed to cleave its 256-base substrate RNA into the predicted 164- and 92-base products (Fig. 2*C*), even at a variety of Mg^{2+} conditions (12.5–22.5 mM) and at molar ratios of up to 20:1 ribozyme/target RNA.

PCR and Southern Blot Analysis of Transfected Cells. The presence of the hammerhead motif in cells transfected with the ribozyme-encoding plasmid pRc/R2 or with the motif-control plasmid pRc/RMC was demonstrated by PCR and Southern blotting (Fig. 4). In genomic DNA from both of these cell lines, PCR amplified a 159-bp fragment, which hybridized to an oligonucleotide derived from the hammerhead motif.

Intracellular Cleavage. Ribozymes hybridize with target mRNA due to flanking antisense sequences and then cleave. A ribozyme could inhibit gene expression by either an antisense effect or by cleavage of the target RNA. To verify that *rex/tax* cleavage occurred, we performed RT-PCR using one upstream primer and two downstream primers, one on either side of the ribozyme cleavage site. Uncleaved RNA is anticipated to produce both the short (133 bp) and the long (201 bp) PCR product; cleaved RNA is anticipated to produce only the short product. Fig. 5 demonstrates that RNA from the pRc/R2 cell line has more short product than long. When measured by densitometry, the ratio of short/long product was 3.75; ratios of BLV-infected cells without plasmid and with the control (pRc/RSV) plasmid were 1.0 and 0.82, respectively. This suggests that *rex/tax* mRNA was correctly cleaved.

Tax Expression. Immunoblot analysis using Tax monoclonal antibody 5A5 showed the expected 38-kDa band in lysate from BLV-BLCs transfected with the control plasmids pRc/RSV or pRc/RMC and in lysate from untransfected BLV-BLCs. The 38-kDa Tax band was not detected in lysate from BLV-infected cells transfected with the ribozyme-expressing plasmid pRc/R2 or in uninfected BLCs (Fig. 6).

p24 Expression. Intracellular expression of the core protein p24 was assayed in cell monolayers by immunochemistry. As shown in Table 1, p24 expression in the pRc/R2-transfected cell line was inhibited 64.7%, as compared to the mean of four cell lines transfected with the control plasmid pRc/RSV. This

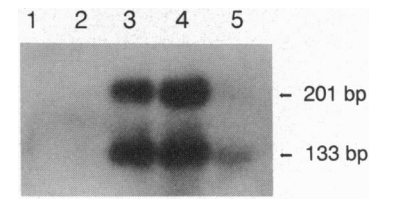

FIG. 5. Intracellular cleavage of *rex/tax* mRNA by ribozyme 2. RT-PCR was performed on RNA from uninfected BLCs (lane 2), BLV-BLCs (lane 3), BLV-BLCs transfected with pRc/RSV (lane 4), or BLV-BLCs transfected with pRc/R2 (lane 5). In lane 1, no RNA was added. Two downstream primers, positioned on either side of the anticipated ribozyme cleavage site, were used to amplify both 201- and 133-bp product from uncleaved RNA but only the 133-bp product from cleaved RNA.

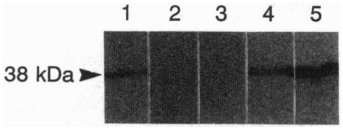

FIG. 6. Immunoblot of cell lysates reacted with Tax monoclonal antibody 5A5. Cells were transfected with pRc/RSV (parental plasmid) (lane 1), pRc/R2 (ribozyme 2 plasmid) (lane 2), or pRc/RMC (motif control plasmid) (lane 4). In lane 3, cells were uninfected with BLV; in lane 5, cells were BLV infected but not plasmid transfected.

experiment was repeated four times, with a mean percentage inhibition of 60.8% (range, 57.0–64.7%). In another experiment, p24 expression in pRc/RSV-transfected cell lines was compared with that in two pRc/RMC-transfected cell lines, which express RNA with the catalytic motif flanked by random sequences. In these lines, p24 expression was similar to that in the pRc/RSV control lines (data not shown).

RT Activity. RT activity was measured in virus pelleted from tissue culture supernatant. In supernatant from the cell line transfected with pRc/R2, RT activity was inhibited 86.1% in one assay and 98.6% in another assay, as compared with a cell line transfected with the control plasmid ($P < 0.05$ in each assay) (Table 2).

DISCUSSION

We demonstrated that transfection of BLV-infected cells by a ribozyme that is capable of cleaving *rex/tax* RNA is associated with marked inhibition of BLV expression. Ribozymes have been targeted to cleave a variety of sites of human immunodeficiency virus (HIV) (12, 21–24). Unlike Sarver *et al.* (12), who inhibited HIV expression with a ribozyme directed against *gag*, we targeted RNA-encoding regulatory genes in an effort to amplify the inhibitory effects. BLV transcribes at least three mRNAs, which, in addition to *rex/tax* mRNA, include unspliced 9.2-kb message expressing the *gag* and *pol* gene products, and singly spliced 5.1-kb message expressing the *env* gene products (25). Recently, alternative splice patterns have been shown to result in additional transcripts that may encode regulatory genes from the *env–rex* interregion (8). All of these transcripts contain a long 3′ untranslated region, which includes the sequences found in the *rex/tax* RNA, the target of ribozyme 2. In a variety of mRNAs, the 3′ untranslated region is important for extending mRNA persistence and avoiding RNase digestion (26). It is possible that ribozyme 2 not only cleaves *rex/tax* mRNA in its coding region, inhibiting translation, but also accelerates decay of other BLV transcripts.

Ribozyme 2 cleaved its target RNA in a cell-free assay and in BLV-infected cells. In the RT-PCR intracellular cleavage assay, the ratio of short PCR product to long product was markedly elevated, demonstrating intracellular cleavage. The ratio in this study, 3.75, compares closely with results of Dropulic *et al.* (21), who reported a ratio of 3.3 in a similar assay with a ribozyme directed against HIV. These figures are minimal estimates of cleavage, because of the possibility of accelerated decay of the cleaved fragments (26). The failure of ribozyme 3 to cleave emphasizes the importance of factors other than primary sequence in ribozyme action. For example, secondary structure such as a preexisting intramolecular RNA duplex may preclude access of a ribozyme to its target (27), or, if the ribozyme does hybridize with the target, alternative tertiary structures may form that are more favored than the hammerhead motif (27).

Table 2. Ribozyme inhibition of RT activity

Transfected plasmid	Corrected mean, cpm	SD, cpm	% inhibition
	Assay 1		
pRc/RSV (no ribozyme)	997,439*	224,382	
pRc/R2 (ribozyme)	138,268**	34,920	86.1
	Assay 2		
pRc/RSV (no ribozyme)	26,551†	12,920	
pRc/R2 (ribozyme)	365††	6,327	98.6

Each assay was performed at least three times. Data were compared by analysis of variance on log-transformed data followed by a *t* test on the corrected means. Different superscripts (* and **; † and ††) indicate significant differences ($P < 0.05$). Corrected mean RT activity was calculated by subtracting background cpm, as determined from uninfected cells. Background in assay 1 was 181,783 cpm (SD, 33,561) and in assay 2 it was 18,039 (SD, 7717).

Demonstration that this ribozyme or antisense (14, 28, 29) inhibits viral expression in persistently infected cells suggests possibilities for BLV control. BLV is a blood-borne disease primarily transmitted mechanically by vectors and fomites (30). Approximately 30% of infected cattle develop persistent lymphocytosis, a polyclonal expansion of B lymphocytes (31, 32). The development of persistent lymphocytosis, in which the absolute number of lymphocytes (33) and the percentage of infected lymphocytes are dramatically increased (34), markedly enhances the probability of transmission (35). The critical importance of persistent lymphocytosis in BLV transmission was shown by experiments in which <0.3 μl of blood from cattle with persistent lymphocytosis could transmit BLV, while >1 ml of blood was necessary to transmit from infected but nonlymphocytotic cattle (35). Moreover, vertical transmission from BLV-infected dams to their calves strongly correlates with persistent lymphocytosis (36).

In the present study, BLV expression was markedly inhibited but was not eliminated. Although the BLC line supports much higher levels of BLV replication than infected bovine B cells *in vivo*, it is quite likely that ribozyme inhibition of BLV expression in cattle will be less than complete. In cattle, the ability to transmit BLV varies (35, 37), and expression of antigen after *in vitro* culture has been shown to correlate with infectivity (38). The level of BLV expression in the animal also may correlate with the probability of development of persistent lymphocytosis (18, 39). If a ribozyme could inhibit viral expression enough to prevent persistent lymphocytosis, it may be that transmission within a herd would be markedly impaired.

Table 1. Ribozyme inhibition of p24 expression

Cell type	Transfected plasmid	Cell line designation	Absorbance	% inhibition
BLV-infected BLCs	pRc/RSV (no ribozyme)	RSV30-3-4	0.276 ± 0.009	—
		RSV30-1-1	0.244 ± 0.026	—
		RSV20-3-3	0.184 ± 0.009	—
		RSV20-1-2	0.245 ± 0.004	—
	pRc/R2 (ribozyme)	R230-2-1	0.084 ± 0.012	64.7
Uninfected BLCs	No plasmid	—	−0.004 ± 0.002	101.6

Absorbance is expressed as the mean of three wells treated with p24 antiserum minus the mean of three wells treated with preimmunization serum. Mean absorbance of the four control cell lines that were transfected with pRc/RSV was 0.237, and the 99% confidence interval was 0.125–0.350.

Moreover, persistent lymphocytosis is a strong risk factor for development of lymphoma. In 1–10% of the animals with persistent lymphocytosis, B-cell clones undergo neoplastic transformation, leading to leukemia or lymphoma (31), and cattle with persistent lymphocytosis are three times more likely to develop lymphoma than infected cattle without persistent lymphocytosis (31). By reducing BLV expression and persistent lymphocytosis, it may also be possible to significantly decrease the incidence of tumor development.

Whereas antisense and ribozyme nucleic acids are being actively investigated as therapeutic agents for control of human infectious diseases and neoplasias (40), it may be feasible to develop transgenic animals that are resistant to retroviral disease. Recently, transgenic mice were developed that express antisense RNA targeted to Moloney murine leukemia virus sequences and do not develop leukemia on challenge (41). Inhibition of BLV expression by ribozymes suggests the possibility of developing transgenic cattle that are resistant to BLV-induced persistent lymphocytosis and lymphoma.

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