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DEVELOPMENT AND TESTING OF RETROVIRAL VECTORS EXPRESSING MULTIMERIC HAMMERHEAD RIBOZYMES TARGETED AGAINST ALL MAJOR CLADES OF HIV-1

Ali Ramezani, Xue Zhong Ma, Reza Nazari, and Sadhna Joshi

Department of Medical Genetics and Microbiology, Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 3E2, Canada

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1. ABSTRACT

Rz_{1-9} is a multimeric hammerhead ribozyme targeted against nine highly conserved sites within the *env* coding region of human immunodeficiency virus type-1 (HIV-1) RNA. This ribozyme was shown to inhibit HIV-1 replication (1, 2). However, Rz_{1-9} target sites are only conserved within the clade B of HIV-1. In this study, we have designed another multimeric ribozyme, Rz_{10-14}. This multimeric ribozyme is targeted against five sites that are highly conserved amongst all major clades of HIV-1. A third multimeric ribozyme, Rz_{1-14}, was obtained by combining both Rz_{1-9} and Rz_{10-14}. A mouse stem cell virus-based MGIN vector (3) was used to express these ribozymes in a human CD4+ T lymphoid cell line. Stable transductants expressed vector RNA containing ribozymes which were shown to be active. In HIV-1 challenge experiments, very little or no virus production could be detected in the pools of stable MT4 transductants expressing Rz_{1-14} for 60 days tested. Inhibition of virus replication was most prominent with Rz_{1-14}, followed by Rz_{1-9}, and then Rz_{10-14}. Thus, the combined multimeric ribozyme, Rz_{1-14}, is more effective than Rz_{1-9} or Rz_{10-14}. As Rz_{1-14} is targeted against all major clades of HIV-1, it will be further pursued for use in HIV-1 gene therapy.

2. INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by a lentivirus, HIV, which primarily infects the CD4+ T lymphocytes and monocytes/macrophages. Since the continuous proliferation and differentiation of a relatively small number of hematopoietic stem cells maintains all of the target cell types infected by HIV, introduction of a therapeutic gene(s) into hematopoietic stem cells is expected to confer long-term protection. Alternatively, delivering a therapeutic gene to the peripheral blood T lymphocytes may restore some degree of general immune functions. Both T lymphocytes and stem cells are therefore being considered for use in anti-HIV-1 gene therapy. Various anti-HIV-1 genes that are being considered for this purpose include interfering RNAs (*i.e.* antisense RNAs, sense RNAs, ribozymes) and interfering proteins (*i.e. trans*-dominants mutants, targeted nucleases) (4-7). However, most anti-HIV-1 genes, including several that are currently being evaluated in clinical trials, confer only partial inhibition of HIV-1 replication. Therefore, further development and testing of genes conferring complete protection is warranted. These genes must be designed to inhibit HIV-1 replication for a sustained period of time, while preventing the emergence of escape mutants of HIV-1 through mutations or recombinations. For the eventual reconstitution of a functional immune system, it is also crucial to focus on genes that would confer a positive survival advantage to the gene-modified cells, without perturbing their normal immune functions.

Hammerhead ribozymes are small catalytic RNAs that can be designed to specifically bind to and cleave the target RNA molecules *in trans* (6, 8, 9). Moloney murine leukemia virus (MoMuLV)-based vectors were used in a number of studies to express ribozymes

targeted against various sites within the HIV-1 RNA. A varying degree of inhibition of HIV-1 replication was reported using mostly monomeric, and in some cases dimeric ribozymes (6). A MoMuLV-based vector was designed in our laboratory to allow constitutive and Tat-inducible expression of a multimeric hammerhead ribozyme, Rz_{1-9}. This multimeric ribozyme is targeted against nine highly conserved sites within the *env* coding region of HIV-1 RNA (1, 2). Since these sites are only conserved in the clade B of HIV-1, Rz_{1-9} would not be effective in patients infected with other clades of HIV-1. Although the emergence of an HIV-1 escape mutant through point mutations is highly unlikely (as this would require HIV to mutate nine conserved sites within a single gene), a single recombination event between different clades of HIV-1 could confer resistance to all nine ribozymes in Rz_{1-9}. Thus, another multimeric ribozyme, Rz_{10-14}, was designed in this study. This multimeric ribozyme is targeted against five sites that are highly conserved amongst all major clades of HIV-1. A high degree of conservation of these target sites should make it very unlikely for an escape mutant of HIV-1 to emerge through mutations or recombinations. These two multimeric ribozymes were also combined to yield Rz_{1-14}. The mouse stem cell virus (MSCV)-based MGIN vector (3) was used to express the three multimeric hammerhead ribozymes. We report here, the design and construction of these multimeric ribozymes, the development of MGIN-based vectors expressing these ribozymes, the activity of multimeric ribozymes produced in the stable MT4 (a human CD4+ T lymphoid cell line) transductants, and the degree of inhibition of HIV-1 replication achieved.

3. MATERIALS AND METHODS

3.1. Construction of retroviral vectors

All ribozyme-encoding sequences were cloned in the MSCV-based MGIN vector (3), between the enhanced green fluorescence protein (*egfp*) gene and the internal ribosome entry site (IRES). All clones were identified and confirmed by restriction enzyme and PCR analyses. Before proceeding with the vector constructions, unique *Csp*451 and *Bgl*II sites were introduced 3' to the *egfp* gene. To this end, the EGFP-F primer (5'-GGA-TCC-GAA-TTC-GCC-AGC-ATG-GTG-AGC-AAG-GGC-GAG-GAG-3'; containing the *Eco*RI site) and the EGFP-R primer (5'-GCT-CGA-GGC-GGC-CGC-GGG-TGG-AGA-TCT-AGG-GCT-TTC-GAA-TTA-ATC-ATT-ACT-TGT-ACA-GCT-CGT-CCA-TGC-CGA-3'; containing the *Csp*451, *Bgl*II and *Not*I sites) were used to PCR amplify the *egfp* gene from the MGIN vector, as described earlier (10). The PCR product was then digested with *Eco*RI and *Not*I and cloned at the corresponding sites within the MGIN vector. The resulting vector was used in the subsequent cloning experiments.

MGIN-Rz_{1-9} vector construction: MoTiN-Rz_{1-9} contains a *Not*I site within the Rz_{1-9} region. This site was first inactivated as follows. MoTiN-Rz_{1-9} was digested with the *Not*I enzyme. The ends were then filled using Klenow, and the DNA was religated and transformed into *E. coli*. The modified MoTiN-Rz_{1-9} vector was then used as a template for PCR amplification of Rz_{1-9} using the Rz_{1-9}-F primer (5'-AGA-TCT-ATC-GAT-GGA-TCT-AAT-ATG-CCA-TAA-TAC-TG-3'; containing the *Cla*I site) and the Rz_{1-9}-R primer (5'-GTT-CGA-AAG-ATC-TCG-CGT-ACT-AGT-AGC-AAT-G-3'; containing the *Bgl*II site). The PCR product was then digested with *Cla*I and *Bgl*II, and cloned within the MGIN vector at the compatible *Csp*451 and *Bgl*II sites downstream of the *egfp* gene.

MGIN-Rz_{10-14} vector construction: HIV-1 target sites for the individual ribozymes within the Rz_{10-14} are as follows: the Rz_{10} target site, 5'-AUU-GGG-**GUC**$_{5254}$-UGC-AUA-CA-3'; the Rz_{11} target site, 5'-GGG-GCA-**GUA**$_{4976}$-GUA-AUA-CA-3'; the Rz_{12} target site, 5'-UUU-CGG-**GUU**$_{4904}$-UAU-UAC-AG-3'; the Rz_{13} target site, 5'-ACA-CCU-**GUC**$_{2498}$-AAC-AUA-AU-3'; and the Rz_{14} target site, 5'-ACU-CUG-**GUA**$_{586}$-ACU-AGA-GA-3'. The cleavage occurs after the **GUC/U/A** sequence, shown in bold. The nucleotide **C/U/A** within the HIV-1 RNA after which the cleavage occurs is numbered; the first nucleotide of the HIV-1 (NL4-3) provirus DNA denotes +1. All of these target sites were selected on the basis of 100% sequence conservation amongst all major clades of HIV-1 (11).

The primers used to construct Rz_{10-14} are as follows: Rz_{14}F, 5'-GCA-GAT-CTA-ATC-GCA-AGG-ATC-CGG-CCC-ATA-TGG-CCT-CTC-TAG-TCT-GAT-GAG-TCC-GTG-AGG-ACG-AAA-CCA-GAG-TCC-GCG-GAT-TAT-3'; Rz_{13}R, 5'-TAT-TAC-AGA-GGC-CTA-CAC-CTG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGA-ACA-TAA-TCC-GCG-GAC-TCT-G-3'; Rz_{11-12}R, 5'-CAT-ACA-ATG-CAT-GGG-TCA-GTT-TCG-TCC-TCA-CGG-ACT-CAT-CAG-GTA-ATA-CAT-CCG-GAT-TTC-GGG-TTT-CGT-CCT-CAC-GGA-CTC-ATC-AGT-ATT-ACA-GAG-GCC-TAC-A-3'; and Rz_{10}R, 5'-GGA-TAG-CGA-TCG-ATG-GCC-ATA-TGG-GCC-ATT-GGG-GTG-TCG-TCC-TCA-CGG-ACT-CAT-CAG-TGC-ATA-CAA-TGC-ATG-GGG-C-3'. The Rz_{14}F and Rz_{13}R primers were PCR amplified to yield a product, which was PCR amplified with the Rz_{11-12}R primer to yield another PCR product, which was then PCR amplified with the Rz_{10}R primer to yield the final 280 bp PCR product containing Rz_{10-14}. All PCRs were performed as described earlier (12). Starting from the 5' end, the order of various ribozymes in this multimeric hammerhead ribozyme is Rz_{14}, Rz_{13}, Rz_{12}, Rz_{11}, and Rz_{10}. For these ribozymes to meet their target sites within the HIV-1 RNA in a contiguous fashion, it would have been better to have them in a reverse order. Thus, at this stage, to partially reverse this order to Rz_{10}, Rz_{13}, Rz_{12}, Rz_{11}, and Rz_{14}, the final PCR product was PCR amplified using the Rz_{10}F primer (5'-ATC-GAT-AGA-TCT-GTA-TGC-ACT-GAT-GAG-TCC-GTG-AGG-ACG-AAA-CCC-CAA-TGG-GCC-CGC-GGA-TTA-TGT-TCT-G-3'; containing the *Bgl*II site, Rz_{10}, and a 17-nucleotide overlap) and the Rz_{14}R primer (5'-GGA-TCC-GCG-GCC-GCT-TCG-AAC-TCT-GGT-TTC-GTC-CTC-ACG-GAC-TCA-TCA-GAC-TAG-AGA-ATG-CAT-GGG-GCA-GTT-TC-3'; containing a 17-nucleotide overlap, Rz_{14}, and the *Not*I site). The resulting product was digested with *Bgl*II and *Not*I, and cloned at the same sites within the MGIN vector to yield MGIN-Rz_{10-14}.

MGIN-Rz_{1-14} vector construction: The *Eco*RI-*Bgl*II fragment of MGIN-Rz_{1-9} (containing the *egfp* gene

and Rz_{1-9}) was cloned into the *Eco*RI and *Bgl*II sites within the MGIN-Rz_{10-14} vector. In the resulting vector, the *egfp* gene in the MGIN-Rz_{10-14} was replaced with the *egfp* gene and Rz_{1-9}.

3.2. Transduction and selection of stable MT4 transductants

Amphotropic MGIN (as a control), MGIN-Rz_{1-9}, MGIN-Rz_{10-14}, and MGIN-Rz_{1-14} vector particles were produced (13) and were used to transduce the MT4 cells (14, 15), as described previously (13, 16). The pools of G418 resistant stable MT4 transductants were each selected for three weeks and were used in the subsequent experiments.

3.3. PCR analysis of genomic DNA from stable MT4 transductants

Genomic DNA was extracted from the individual pools of stable MT4 transductants expressing the multimeric ribozymes (17). PCRs were performed for 40 cycles (1 min at 95°C, 1 min at 56°C, and 1 min at 72°C each), as described previously (18), using MGIN-F (5'-CTC-TCG-GCA-TGG-ACG-AG-3') and MGIN-R (5'-ATG-CTG-GTC-AAG-AAG-AC-3') primers which bind to the vector sequences upstream and downstream of the multimeric ribozyme cloning site. Gap-5' (5'-TCT-ACT-GGC-GCT-GCC-AAG-3') and Gap-3' (5'-TCT-AGA-CGG-CAG-GTC-AGG-3') primers were used as a control to amplify the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

3.4. RT-PCR analysis of total RNA from stable MT4 transductants

Total RNA was extracted from stable MT4 transductants using acid guanidium thiocyanate-phenol-chloroform extraction (19). The RNA was then incubated with RQI RNase-free DNase (Promega Corp.; Madison, WI) for 15 min at 37°C to degrade any residual DNA. To ensure that the DNase treatment was complete, RNA samples were analyzed by PCR using the Gap-5'/Gap-3' primer pair. Semi-quantitative RT-PCRs were then performed as described previously (20). Reverse transcriptions were performed using the Neo-3' (5'-CTC-TTC-GTC-CAG-ATC-ATC-3') and Gap-3' primers. The cDNAs were then PCR amplified using the Neo-5' (5'-CAA-GAC-CGA-CCT-GTC-CGG-3') and ^{32}P-labelled Neo-3' primer pair and (as a control) the Gap-5' and ^{32}P-labelled Gap-3' primer pair; the 3' primers were 5'-end labeled (~1.3 x 10^9 cpm/mg) using [gamma ^{32}P] ATP (Amersham; 6000 Ci/mmol), as described previously (10). To ensure that a linear relationship exists between the input cDNA concentration and the intensity of the PCR products, the concentration of the 5' and the ^{32}P-labeled 3' primers was increased to 5 mg/l and the PCRs were performed for 25 cycles. Control PCRs were done to make sure that these conditions allow semi-quantitative amplification. To this end, the Neo-5' and the ^{32}P-labeled Neo-3' primers were used to PCR amplify 10 to 1,000,000 copies of MGIN vector DNA and the Gap-5' and the ^{32}P-labeled Gap-3' primers were used to PCR amplify 0.1 and 0.5 mg/l of cellular genomic DNA (20). The RT-PCR and PCR products were analyzed on a 2% agarose gel, followed by autoradiography.

3.5. *In vitro* cleavage activity of multimeric ribozymes amplified from pools of stable MT4 transductants

Total cellular RNA extracted from the MT4 transductants expressing Rz_{1-9}, Rz_{10-14}, or Rz_{1-14} was RT-PCR amplified, as described previously (18), to generate templates enabling T7-promoter driven transcription of multimeric ribozymes. Reverse transcription was performed using the MGIN-R primer. The cDNA was then used in a PCR using a T7-MGIN-F primer (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-CTC-TCG-GCA-TGG-ACG-AG-3'; containing the T7 promoter) and the MGIN-R primer. The PCR products were then transcribed *in vitro* using the T7 RNA polymerase (Life Technologies; Burlington, Canada) as described earlier (18). The reactions were stopped after 2 h by digesting the template DNA with 5 U of RQI RNase-free DNase for 10 min at 37°C. The RNA was extracted once with phenol and then ethanol precipitated.

The pNL4-3 DNA was PCR amplified to yield T7 promoter-containing templates, which were *in vitro* transcribed to generate alpha ^{32}P-labelled target RNAs as described previously (12). T7-Env-5' (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-GAA-GGA-GAA-ATA-TCA-GC-3') and Env-3' (5'-TCA-CTT-CTC-CAA-TTG-TCC-3') primers were used to yield a 1400 nt-long alpha ^{32}P-labelled T_{1-9} target RNA containing the Rz_{1-9} cleavage sites. T7-Pol-5' (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-CTC-TCG-GCA-TGG-ACG-AGG-GAC-GTG-ACA-TGG-GGG-G-3') and Vif-3' (5'-TTT-CTT-ATA-GCA-GAT-TC-3') primers were used to yield a 602 nt-long alpha ^{32}P-labelled T_{10-12} target RNA containing the Rz_{10}, Rz_{11}, and Rz_{12} cleavage sites. T7-Pro-5' (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-CAA-CTA-AAG-GAA-GCT-CTA-3') and Pro-3' (5'-TGC-GGC-CGC-AAT-TGG-AGG-3') primers were used to yield a 413 nt-long alpha ^{32}P-labelled T_{13} target RNA containing the Rz_{13} cleavage site. T7-leader-5' (5'-AAA-TTA-ATA-CGA-CTC-ACT-ATA-GGG-TCT-CTC-TGG-TTA-GA-3') and leader-3' (5'-CAA-GTC-CCT-GTT-CGG-GC-3') primers were used to yield a 200 nt-long alpha ^{32}P-labelled T_{14} target RNA containing the Rz_{14} cleavage site.

The resulting Rz_{1-9}, Rz_{10-14}, and Rz_{1-14} RNAs and the alpha ^{32}P-labelled T_{1-9}, T_{10-12}, T_{13}, and T_{14} target RNAs were used in the *trans* cleavage reactions as described previously (1, 18). Essentially, multimeric ribozymes and target RNAs were combined in a reaction mixture containing 40 mM Tris-Cl, pH 8.0, and 10 mM NaCl. The samples were heated to 65°C for 5 min, cooled to 37°C, and the cleavage reaction initiated by adding 20 mM $MgCl_2$. After incubation for 2 h at 37°C, the reaction was stopped by adding 5 mM EDTA. Samples containing the 1400 nt-long alpha ^{32}P-labelled T_{1-9} RNA and its cleavage products were loaded onto a 8 M urea-6% polyacrylamide gel and the electrophoresis performed for 1 h at 750 Volts. The alpha ^{32}P-labelled T_{10-12}, T_{13}, and T_{14} RNAs and their cleavage products were then loaded and the electrophoresis

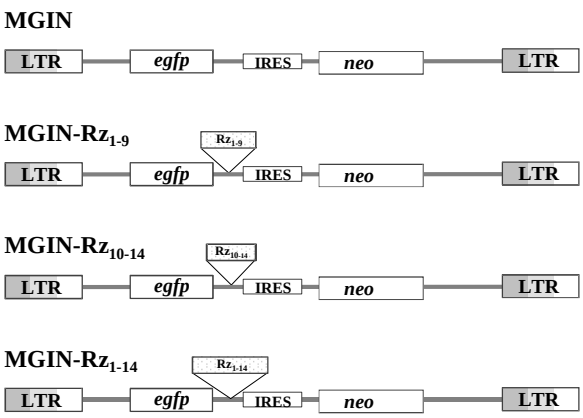

Figure 1. Schematic diagram of MGIN, MGIN-Rz_{1-9}, MGIN-Rz_{10-14}, and MGIN-Rz_{1-14} vectors. MGIN contains the *egfp* gene, an IRES element, and the *neo* gene. Multimeric ribozyme-coding sequences were cloned between the *egfp* gene and the IRES element. Only those sequences that are part of the retroviral vector are shown. LTR, long terminal repeat.

continued for another 45 min at 750 Volts. The gel was then exposed to an XAR-5 X-ray film (Kodak, Toronto, Canada).

3.6. HIV-1 susceptibility of stable MT4 transductants

Stable MT4 transductants were challenged with the HIV-1 strain NL4-3 (21). Challenge experiments were performed as described previously (16, 22), except that the challenge virus stock was produced as follows. HIV-1 was harvested every day (rather than every third day) by collecting the entire cell culture supernatant (rather than collecting half of the cell culture supernatant), and only the sample that contained the highest virus concentration (rather than all samples that contained virus) was aliquoted and used. The titer of this virus (infectious units/ml) was determined using U373-MAGI-$CXCR4_{CEM}$ cells (23).

The pools of actively dividing stable MT4 transductants (2×10^6 cells) were each inoculated with the HIV-1 strain NL4-3. HIV-1 inoculations were performed at a multiplicity of infection (m.o.i.) of 0.5 and 0.05 for 2 hours at 37°C, with gentle shaking (16, 22). Cells were washed twice with the RPMI 1640 medium, and resuspended in the same medium. Cells were then cultured at 37°C. Cells were routinely inspected for syncytia formation. The culture supernatants were collected every third day and replaced with the fresh medium. Experiments were terminated after 60 days. The amount of HIV-1 p24 antigen present in the frozen cell culture supernatants was then measured by an enzyme linked immunosorbent assay using a Kit (Abbott; Chicago, IL). For OD_{492} values >2.0, samples were diluted 10- to 5000-fold and retested. The OD_{492} values ranging between 0.1 to 1.9 were then multiplied with the dilution factor and converted to pg of p24 antigen per ml.

4. RESULTS

The MSCV-based MGIN vector was used to express the three multimeric hammerhead ribozymes, Rz_{1-9}, Rz_{10-14}, and Rz_{1-14}. These vectors were then tested in MT4 cells for the level of expression, ribozyme activity, and inhibition of HIV-1 replication.

4.1. MGIN-based retroviral vectors expressing multimeric ribozymes, Rz_{1-9}, Rz_{10-14}, or Rz_{1-14}

The multimeric ribozyme Rz_{1-9} is targeted against nine highly conserved sites within the *env* coding region of HIV-1 RNA (clade B) (1, 2). Rz_{10-14} was designed against one site within the *vif* coding region, two sites within the *pol* coding region, one site within the *pro* coding region, and one site within the 5' leader sequence of HIV-1 RNA. All five of these target sites were identified on the basis of sequence conservation amongst all major clades of HIV-1. Overlapping PCRs using synthetic oligodesoxynucleotides were performed to assemble Rz_{10-14}. The two multimeric hammerhead ribozymes were also combined to yield Rz_{1-14}.

The MGIN vector (3) used to express these ribozymes contains the *egfp* gene, an IRES element from the encephalomyocarditis virus, and the neomycin phosphotransferase (*neo*) gene (Figure 1). The MSCV 5' long terminal repeat (LTR) promoter-driven bicistronic transcript allows translation of both open reading frames. Genes encoding the three multimeric hammerhead ribozymes were cloned between the *egfp* gene and the IRES element.

4.2. Development of pools of stable MT4 transductants expressing multimeric ribozymes

Amphotropic MGIN, MGIN-Rz_{1-9}, MGIN-Rz_{10-14}, and MGIN-Rz_{1-14} vector particles were each used to transduce a human CD4+ T lymphoid (MT4) cell line (14, 15). Pools of G418 resistant stable MT4 transductants were then selected and tested without cloning.

The presence of DNA sequences encoding the multimeric ribozymes was confirmed by PCR analysis of genomic DNA isolated from various MT4 transductants. The MGIN-F/MGIN-R primer pair was used for this purpose. These primers were designed against sequences upstream (within the *egfp* gene) and downstream (within the IRES element) of the ribozyme-cloning site. PCR products obtained from untransduced (as a control) and transduced cells are shown in figure 2a. No PCR product was detected in the untransduced sample (figure 2a, lane 1). 217, 626, 497, and 906 bp products were detected in the MGIN, MGIN-Rz_{1-9}, MGIN-Rz_{10-14}, and MGIN-Rz_{1-14} vector-transduced samples, respectively (figure 2a, lanes 2-5). Genomic DNA from the untransduced and transduced cells was also analyzed using the Gap-5'/Gap-3' primer pair. As expected, a 122 bp product specific for the cellular GAPDH gene was detected in the untransduced and transduced samples (figure 2b, lanes 1-5).

Vector RNA expression from the 5' LTR promoter was confirmed by RT-PCR analysis of total cellular RNA from various MT4 transductants. Reverse transcriptions were performed using the Neo-3' and Gap-3' primers, followed by PCRs which were performed using the Neo-5'/^{32}P-labelled Neo-3' and Gap-5'/^{32}P-labelled Gap3' primer pairs. RT-PCRs were performed under semi-

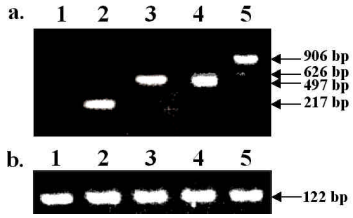

Figure 2. PCR analysis to determine the presence of vector DNA sequences in the transduced MT4 cells. Genomic DNA was extracted from the MT4 transductants and analyzed by PCR. **(a)** PCR products obtained from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-Rz_{1-9} (lane 3), MGIN-Rz_{10-14} (lane 4), and MGIN-Rz_{1-14} (lane 5) using the MGIN-F/MGIN-R primer pair. **(b)** PCR products obtained from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-Rz_{1-9} (lane 3), MGIN-Rz_{10-14} (lane 4), and MGIN-Rz_{1-14} (lane 5) using the Gap-5'/Gap-3' primer pair. PCR product sizes are indicated by an arrow.

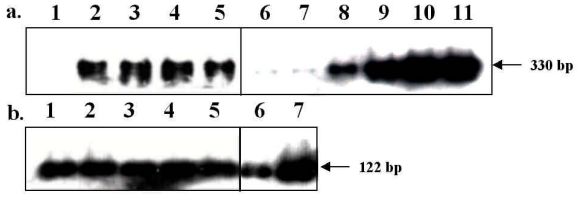

Figure 3. Semi-quantitative RT-PCR analysis of MGIN 5' LTR promoter-driven vector RNA and cellular GAPDH RNA produced in various MT4 transductants. Total RNA was extracted from untransduced and transduced MT4 cells, treated with DNase, and analyzed by semi-quantitative RT-PCR. **(a)** RNA was reverse transcribed using the Neo-3' primer, followed by amplification of *neo* sequences present within the vector RNA by semi-quantitative PCR using the Neo-5' primer and the ^{32}P-labelled Neo-3' primer. RT-PCR products were analyzed from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-Rz_{1-9} (lane 3), MGIN-Rz_{10-14} (lane 4), and MGIN-Rz_{1-14} (lane 5). Control PCRs were performed using 10 (lane 6), 100 (lane 7), 1000 (lane 8), 10,000 (lane 9), 100,000 (lane 10), and 1,000,000 (lane 11) copies of MGIN DNA to demonstrate a linear relationship between the intensity of the PCR product and the amount of cDNA used. **(b)** RT-PCR products obtained using the Gap-5' primer and the ^{32}P-labelled Gap-3' primer from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-Rz_{1-9} (lane 3), MGIN-Rz_{10-14} (lane 4), and MGIN-Rz_{1-14} (lane 5). This primer pair was used to amplify a 122 bp region within the endogenous *GAPDH* gene, which served as an internal control to assure that an equal amount of total RNA was analyzed in all cases. Control PCRs were performed using 100 (lane 6) and 500 (lane 7) ng of total cellular DNA. PCR product sizes are indicated by an arrow.

quantitative conditions allowing a linear relationship between the target RNA concentration and the RT-PCR product intensity (Figure 3a, lanes 6-11; figure 3b, lanes 6,7). A semi-quantitative RT-PCR using the Neo-5'/^{32}P-labelled Neo-3' primer pair resulted in the amplification ofa 330 bp product. RT-PCR products of similar intensities were obtained for all transduced samples (figure 3a, lanes 2-5), suggesting similar expression levels in all MT4 transductants. No RT-PCR product was obtained for the untransduced sample (figure 3a, lane 1). The Gap-5'/^{32}P-labelled Gap-3' primer pair was used as a control and yielded the expected 122 bp product in all untransduced and transduced samples (figure 3b, lanes 1-5). RNA samples were also analyzed by PCR using the Gap-5'/Gap-3' primer pair to ensure the lack of DNA contamination (results not shown).

4.3. *In vitro* cleavage activity of multimeric ribozymes produced from the stable MT4 transductants

To demonstrate the activity of the multimeric ribozymes produced in the transduced MT4 cells, Rz_{1-9}, Rz_{10-14}, and Rz_{1-14} were amplified from the cellular RNA by RT-PCR using a 5' primer, which contained the T7 promoter. The RT-PCR products were then transcribed *in vitro* to yield Rz_{1-9}, Rz_{10-14}, and Rz_{1-14}, which were used in *trans* cleavage reactions. The cleavage reactions were performed in the presence of alpha ^{32}P-labelled T_{1-9} RNA (containing the Rz_{1-9} target sites), T_{10-12} RNA (containing the Rz_{10}, Rz_{11}, and Rz_{12} target sites), T_{13} RNA (containing the Rz_{13} target site), or T_{14} RNA (containing the Rz_{14} target site). T_{1-9} RNA cleavage by Rz_{1-9} (Figure 4b, lane 1) and Rz_{1-14} (figure 4b, lane 5) yielded multiple products, confirming previous results (1, 2). The expected length of products resulting from T_{10-12}, T_{13}, and T_{14} target RNA cleavage by Rz_{10}, Rz_{11}, Rz_{12}, Rz_{13}, and Rz_{14} is indicated in figure 4a. T_{10-12} RNA cleavage by Rz_{10-14} (figure 4b, lane 4) and Rz_{1-14} (figure 4b, lane 8) yielded the 278, 152, 100, and 72 bp products. The 502, 450, 430, 350, and 172 bp partial cleavage products were also detected. This result indicates that the Rz_{10}, Rz_{11}, and Rz_{12}, present within Rz_{10-14} and Rz_{1-14}, are active. T_{13} RNA cleavage by Rz_{10-14} (figure 4b, lane 3) or Rz_{1-14} (figure 4b, lane 7) yield the 228 and 185 bp products, confirming the activity of Rz_{13} in both of these multimeric ribozymes. T_{14} RNA cleavage by Rz_{10-14} (figure 4b, lane 2) or Rz_{1-14} (figure 4b, lane 6) was expected to yield 129 and 71 bp products, which were not detected. This ribozyme was therefore inactive. These results demonstrate that except for Rz_{14} targeted against the 5' leader sequence, the ribozymes present within the three multimeric ribozymes produced in MT4 transductants are active.

Cleavage reactions were performed using 1400, 602, 413, and 200 nt-long target RNAs, which were subject to cleavage by the multimeric ribozymes containing nine, five, or fourteen monomeric ribozymes (figure 4b). Only the cleavage products of the expected sizes were detected, and no additional products were detected upon cleavage by Rz_{1-9}, Rz_{10-14} and Rz_{1-14}. Therefore, these results also demonstrate the target RNA specificity of these ribozymes.

4.4. HIV-1 susceptibility of pools of stable MT4 transductants expressing multimeric ribozymes

Stable MT4 transductants lacking or expressing Rz_{1-9}, Rz_{10-14}, or Rz_{1-14} were each challenged with HIV-1

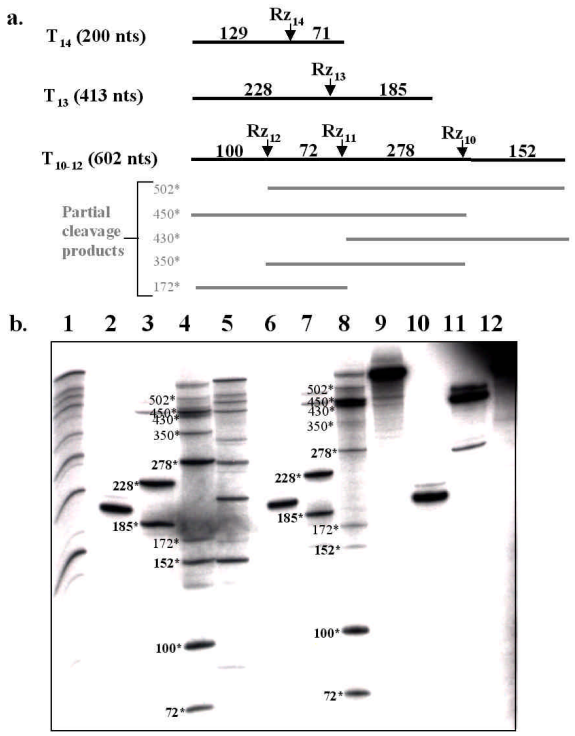

Figure 4. (a) Size of various target RNAs and of their 5' and 3' cleavage products. Partial cleavage products are shown with a thin line. **(b)** *In vitro* cleavage activity of multimeric hammerhead ribozymes amplified from the stable MT4 transductants. Rz_{1-9}, Rz_{10-14}, and Rz_{1-14} sequences were RT-PCR amplified from total RNA extracted from the MGIN-Rz_{1-9}, MGIN-Rz_{10-14}, and MGIN-Rz_{1-14} vector-transduced cells to generate T7 promoter-containing templates, which were then transcribed *in vitro*. Transcripts containing Rz_{1-9} (lane 1), Rz_{10-14} (lanes 2-4), and Rz_{1-14} (lanes 5-8) were used in trans cleavage reactions with the alpha ^{32}P-labeled T_{1-9} (lanes 1 and 5), T_{10-12} (lanes 4 and 8), T_{13} (lanes 3 and 7), and T_{14} (lanes 2 and 6) target RNAs. The cleavage products and the alpha ^{32}P-labeled T_{1-9} (lane 9), T_{10-12} (lane 12), T_{13} (lane 11), and T_{14} (lane 10) target RNAs were analyzed by 8-M urea-6% polyacrylamide gel electrophoresis, followed by autoradiography. Samples in lanes 1, 5, and 9 contained bigger size products and were, therefore, loaded 1 h before the other samples were loaded. Product sizes are indicated on the left. Partial T_{10-12} RNA cleavage products resulting from incomplete digestion by Rz_{10}, Rz_{11}, or Rz_{12} are also shown.

strain NL4-3 at a m.o.i. of 0.5 and 0.05. Virus production was measured by determining the amount of HIV-1 p24 antigen in the infected cell culture supernatants. In challenge experiments performed at a m.o.i. of 0.5, cells transduced with the MGIN vector (lacking any ribozyme) produced high amount of virus (Figure 5a). Cells expressing Rz_{10-14} or Rz_{1-9} produced lower amount of virus. Virus production from cells expressing Rz_{1-14} was significantly decreased and remained low for the 60 day period. At a m.o.i. of 0.05, significant inhibition of HIV-1 replication was observed with Rz_{1-14} followed by Rz_{1-9} (figure 5b). Thus, the combined ribozyme Rz_{1-14} confers the best inhibition, followed by Rz_{1-9}, and then Rz_{10-14}.

5. DISCUSSION

We have previously shown that a multimeric hammerhead ribozyme targeted against nine highly conserved sites within the *env* coding region of HIV-1 RNA confers excellent inhibition of HIV-1 replication (1). Since all nine of these sites are highly conserved within the clade B of HIV-1, it is unlikely that an escape mutant of HIV-1 could emerge through point mutations during reverse transcription. However, none of these sites is conserved amongst other HIV-1 clades. Thus, recombination in patients infected with multiple clades could easily result in an escape mutant of HIV-1 that would not be inhibited by any of the nine ribozymes present in Rz_{1-9}. Therefore, we designed another multimeric ribozyme, Rz_{10-14}. Rz_{10} is targeted against one site within the *vif* coding region. Rz_{11} and Rz_{12} are targeted against two sites within the *pol* coding region. Rz_{13} is targeted against one site within the *pro* coding region. And, Rz_{14} is targeted against one site within the 5' leader region of HIV-1 RNA. These sites were selected on the basis of 100% sequence conservation amongst all major clades of HIV-1. Such a high degree of sequence conservation across clades suggests that mutations in these target sites would be detrimental to HIV-1 replication. Thus, it is anticipated that in addition to conferring protection against all major clades of HIV-1, this ribozyme would greatly decrease the possibility of the emergence of a viable escape mutant of HIV-1 through point mutations or recombinations. The two multimeric ribozymes, Rz_{1-9} and Rz_{10-14}, were also combined to yield Rz_{1-14}. Rz_{1-14} was expected to confer an even greater degree of protection.

Previously, a MoMuLV-based MoTiN vector (2) was used to express Rz_{1-9} (1). In this vector, the multimeric ribozyme was expressed as part of transcripts produced from the 5' LTR promoter and an internal *tk*-TAR fusion promoter. The *tk*-TAR promoter was designed to allow constitutive gene expression in uninfected cells, and high-level Tat-inducible gene expression in the HIV-infected cells. Excellent inhibition of HIV-1 replication was observed in transduced MT4 cells (1). However, only partial inhibition of virus replication was observed in transduced peripheral blood T lymphocytes challenged with the laboratory or clinical isolates of HIV-1 (24).

Recent studies using the MoMuLV-based vectors have demonstrated that gene expression from the 5' LTR promoter is downregulated upon differentiation of transduced hematopoietic stem cells. Downregulation of gene expression seems to result from *de novo* methylation of the LTR and binding of negative regulatory transcription factors to the LTR and the primer-binding site (25). In the MSCV-based vectors, the LTR and the 5' untranslated sequences were modified to overcome these problems (26-28). These vectors were shown to maintain gene expression following differentiation of human hematopoietic stem cells (3, 26, 29, 30). Therefore, the multimeric hammerhead ribozymes were expressed using the MSCV-based MGIN vector (3). This vector contains the *neo* gene, the IRES

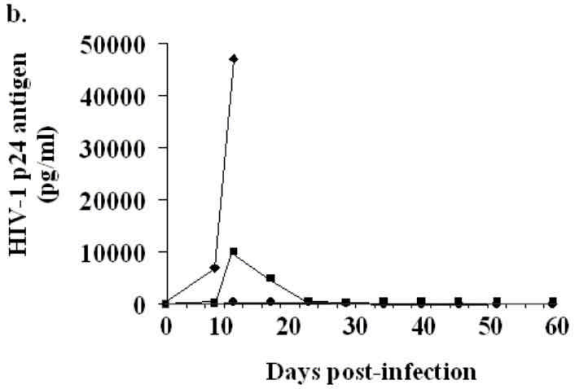

Figure 5. (a) HIV-1 susceptibility of MT4 cells transduced with MGIN (—◆—), MGIN-Rz_{1-9} (—■—), MGIN-Rz_{10-14} (—▲—), or MGIN-Rz_{1-14} (—●—). Transduced MT4 cells were infected with HIV-1 strain NL4-3 at a m.o.i. of 0.5, and the amount of HIV-1 p24 antigen released in the culture supernatants was measured at various time intervals. **(b)** MT4 cells transduced with MGIN (—◆—), MGIN-Rz_{1-9} (—■—), or MGIN-Rz_{1-14} (—●—) were infected with HIV-1 strain NL4-3 at a m.o.i. of 0.05. The amount of HIV-1 p24 antigen released in the culture supernatants was reported at various time intervals.

element, and the *egfp* gene. The MSCV 5' LTR promoter gives rise to a single transcript, which allows translation of both Neo and EGFP proteins. The three multimeric ribozymes were cloned downstream of the *egfp* gene, before the IRES elements.

Amphotropic vector particles were used to transduce a human CD4+ T lymphoid MT4 cell line. Pools of G418 resistant stable MT4 transductants were shown to contain ribozyme sequences by PCR analysis. Analysis of total cellular RNA under semi-quantitative RT-PCR conditions revealed similar level of interfering RNA production in all MT4 transductants. With the exception of Rz_{14} (targeted against the 5' leader sequence), all individual ribozymes within the three multimeric ribozymes tested were shown to be active. As the DNA sequences encoding the multimeric ribozymes were assembled by PCR, a mutation during PCR might have inactivated Rz_{14}. Production of multimeric ribozymes used during the cleavage reactions also involved an RT-PCR step. However, it is highly unlikely that a mutation during RT-PCR inactivated Rz_{14} in both Rz_{10-14} and Rz_{1-14}.

Our results also demonstrate that it is possible to use retroviral vectors to express up to fourteen different ribozymes. As each ribozyme contained the same 22 nt-long catalytic domain, a deletion during reverse-transcription could have eliminated some or all of the ribozymes. In the case of the MoMuLV-based double copy vector containing only a 16 nt-long duplication within the 3' LTR, inserted sequences were shown to be deleted by others (31) and us (Medina and Joshi, unpublished results).

Cells expressing the multimeric hammerhead ribozymes were viable and showed no sign of toxicity. As MoTiN-Rz_{1-9} was previously shown to confer excellent inhibition of HIV-1 replication, we were concerned that in the current study it will not be easy to determine which of the three multimeric hammerhead ribozymes confers the best protection. Thus, the HIV-1 used in these challenge experiments was produced by using a different procedure to ensure that it is highly infectious. Inhibition of virus replication was measured at a m.o.i of 0.05 and 0.5. In these experiments, Rz_{1-14} conferred the best protection, followed by Rz_{1-9}, and then Rz_{10-14}.

These results demonstrate the feasibility of a multimeric hammerhead ribozyme-based strategy to successfully inhibit HIV-1 replication using the MGIN vector. Experiments are now underway to test these vectors against challenge with all major clades of HIV-1. These vectors will also be tested for inhibition of HIV-1 replication in transduced peripheral blood T lymphocytes and in the progeny of transduced CD34+ stem cells. As these multimeric hammerhead ribozymes are targeted against all major clades of HIV-1, it is anticipated that they would provide benefit to HIV-positive patients worldwide.

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Abbreviations: AIDS: acquired immunodeficiency syndrome, *egfp:* enhanced green fluorescence protein, GAPDH, glyceraldehyde-3-phosphate dehydrogenase, HIV: human immunodeficiency virus, IRES: internal ribosome entry site, LTR: long terminal repeat, m.o.i.: multiplicity of infection, MoMuLV: Moloney murine leukemia virus, MSCV: mouse stem cell virus, *neo:* neomycin phosphotransferase

Key words: Gene therapy, HIV-1, Retroviral Vector, Multimeric Ribozymes

Send correspondence to: Dr Sadhna Joshi, Department of Medical Genetics and Microbiology, 150 College Street, #212, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 3E2, Canada. Tel: 416-978-2499, Fax:416-638-1459, E-mail: sadhnajoshi@yahoo.com