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Assessment of an anti-HIV-1 combination gene therapy strategy using the antisense RNA and multimeric hammerhead ribozymes

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1. ABSTRACT

A combination gene therapy strategy using an $AS_{Psi\text{-}gag}$ antisense RNA (targeted against the packaging signal and the *gag*-coding region) and a multimeric hammerhead ribozyme $Rz_{1\text{-}9}$ (targeted against nine sites within the *env*-coding region) or $Rz_{1\text{-}14}$ (targeted against 14 sites within the 5' leader and the *pro-*, *pol-*, *vif-* and *env*-coding regions) was assessed for inhibiting HIV-1 replication. A murine stem cell virus (MSCV)-based MGIN vector was used to express $Rz_{1\text{-}9}$, $Rz_{1\text{-}14}$, $AS_{Psi\text{-}gag}$, $Rz_{1\text{-}9}AS_{Psi\text{-}gag}$, or $Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ RNA in a CD4+ T lymphoid cell line. Stable transductants were shown to express similar levels of interfering RNA. HIV-1 replication was inhibited in cells expressing $Rz_{1\text{-}9}$ and $Rz_{1\text{-}14}$. Little inhibition of HIV-1 replication was observed in cells expressing $AS_{Psi\text{-}gag}$ RNA. Thus, the multimeric hammerhead ribozymes inhibit HIV-1 replication better than the antisense RNA. Inhibition of HIV-1 replication in cells expressing $Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ or $Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ RNA was worse than that obtained with the multimeric ribozymes alone. This result suggests that co-expression of antisense RNA decreases the anti-HIV potential of ribozymes. The multimeric ribozymes and the antisense RNA were designed to target different sites within the HIV-1 RNA. They are not expected to interact with each other. Neither are they expected to compete with each other for binding to the HIV-1 RNA. Instead, the antisense RNA binding to its (1553 nt-long) target site may have resulted in a decreased ribozyme turn over. Furthermore, since the antisense RNA/HIV-1 RNA hybrids are degraded by the cells, the co-expressed antisense RNA may have led to ribozyme degradation.

2. INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is caused by a lentivirus, human immunodeficiency virus type-1 (HIV-1), which infects the CD4+ T lymphocytes and monocytes/macrophages. Drug and gene therapy strategies are being developed to inhibit HIV-1 replication. The anti-HIV genes have been designed to express interfering RNAs or proteins (1-5). Of all the anti-HIV-1 genes tested to date in our laboratory, the multimeric hammerhead ribozymes rank at the top (6-8), followed by the antisense RNAs (9-11).

Antisense RNAs can be designed to prevent nuclear export, translation, and packaging of HIV-1 RNA as well as to inhibit progeny virus replication (3). Ribozymes are small catalytic RNAs that can be designed to specifically recognize and cleave the HIV-1 RNA in the nucleus, cytoplasm, and the progeny viruses such that either no virus is produced or the viruses produced are non-infectious (3).

The Moloney murine leukemia virus (MoMuLV)-based MoTN vector (12) was previously used to express two antisense RNAs. The $AS_{Psi\text{-}gag}$ RNA targeted against the packaging signal (Psi) and the *gag*-coding region of HIV-1 RNA (9) and the $AS_{LTR\text{-}GE}$ RNA targeted against the U3RU5 region and the *gag*- and *env*-coding regions of HIV-1 RNA (10). Both of these antisense RNAs were shown to confer excellent inhibition of HIV-1 replication for the 30-80 days for which they were tested (9,10). These antisense RNAs were also found to be packaged by the progeny viruses, which were further shown to have a markedly reduced infectivity (10). The antisense RNAs are very likely to have been copackaged with the HIV-1 RNA.

The MoMuLV-based MoTN vector (12) was previously modified in our laboratory to develop the MoTiN vector (13). This vector was designed to allow anti-HIV gene expression in a constitutive manner with further upregulation in the HIV-infected cells. It was used to express a number of monomeric hammerhead ribozymes targeted against various sites within the HIV-1 RNA (14,15). These ribozymes conferred varying degree of protection to a human CD4+ T lymphoid (MT4) cell line (14,15). However, none of them conferred complete protection, despite active ribozyme production and target site conservation (15). This vector was then used to express a multimeric hammerhead ribozyme ($Rz_{1\text{-}9}$) targeted against nine sites that are highly conserved within the *env*-coding region of HIV-1 RNA (clade B; 6,7). MoTiN-$Rz_{1\text{-}9}$ conferred excellent protection for the 60 days for which it was tested (7). The mouse stem cell virus (MSCV)-based MGIN vector (16) was then used to express $Rz_{1\text{-}9}$, $Rz_{10\text{-}14}$, (expressing five ribozymes that are targeted against the 5' leader region and the *pro*-, *pol*-, and *vif*-coding regions conserved in all major clades of HIV-1), and $Rz_{1\text{-}14}$ (containing $Rz_{1\text{-}9}$ and $Rz_{10\text{-}14}$) (8). $Rz_{1\text{-}14}$ was found to be even better than $Rz_{1\text{-}9}$ in inhibiting virus replication (8).

The MoTN-$AS_{Psi\text{-}gag}$ (9,11) and the MoTiN-$Rz_{1\text{-}9}$ (7) vectors were also shown to confer protection to the transduced peripheral blood T lymphocytes upon challenge with the clinical isolates of HIV-1 (10,17).

In this paper, we have assessed whether the antisense RNA and the multimeric hammerhead ribozymes can be co-expressed to develop a combination gene therapy strategy. The MGIN vector (16) was used in this study to express the genes encoding the multimeric hammerhead ribozymes ($Rz_{1\text{-}9}$ and $Rz_{1\text{-}14}$), the antisense RNA ($AS_{Psi\text{-}gag}$), or both the antisense RNA and the multimeric ribozymes ($Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ and $Rz_{1\text{-}14}AS_{Psi\text{-}gag}$).

3. MATERIALS AND METHODS

3.1. Construction of retroviral vectors

Genes encoding the multimeric hammerhead ribozymes and/or the antisense RNA were cloned in the MGIN vector (16) between the enhanced green fluorescence protein (*egfp*) gene and the internal ribosome entry site (IRES). Before proceeding with vector constructions, unique *Csp* 451 and *Bgl* II sites were introduced 3' to the *egfp* gene, as described previously (8). Briefly, a 5' primer (containing the *Eco* RI site) and a 3' primer (containing the *Csp* 451, *Bgl* II, and *Not* I sites) were used to PCR amplify the *egfp* gene. The PCR product was digested with the *Eco* RI and *Not* I enzymes and cloned at the corresponding sites within the MGIN vector. The modified MGIN vector was used in the subsequent cloning experiments.

The MGIN-$Rz_{1\text{-}9}$ and MGIN-$Rz_{1\text{-}14}$ vectors were constructed as described previously (8). For MGIN-$AS_{Psi\text{-}gag}$ vector construction, the 1553 nt-long $AS_{Psi\text{-}gag}$ sequences were amplified by PCR using the pNL4-3 (18) template DNA and the AS-F primer (5'-GTT-CGA-AAG-ATC-TGC-GGC-CGC-GGC-CGG-ATC-TTC-CCT-AAA-AAA-TTA-GCC-3'; containing the *Not* I site) and the AS-R primer (5'-GTG-ATC-AGG-GCC-CAA-GTA-GTG-TGT-GCC-CGT-CTG-3'), as described previously (8). Klenow (Amersham Pharmacia Biotech Inc., Quebec, Canada) was used to repair the ends of the PCR product, as described earlier (19). The 1591 bp product was then used in a blunt-end ligation into the MGIN vector at the *Bgl* II site, which was filled using Klenow. Orientation of $AS_{Psi\text{-}gag}$ gene in the MGIN-$AS_{Psi\text{-}gag}$ vector was confirmed by PCR and restriction enzyme analyses.

The MGIN-$Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ and MGIN-$Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ vectors were constructed as follows. The *Not* I fragment (containing the $AS_{Psi\text{-}gag}$ gene) of MGIN-$AS_{Psi\text{-}gag}$ was cloned at the same site downstream of the $Rz_{1\text{-}9}$ and $Rz_{1\text{-}14}$ genes in the MGIN-$Rz_{1\text{-}9}$ and MGIN-$Rz_{1\text{-}14}$ vectors. The correct clones, containing the MGIN-$Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ and MGIN-$Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ vectors, were screened by PCR and restriction enzyme analyses.

3.2. Transduction and selection of stable MT4 transductants

Amphotropic MGIN, MGIN-$Rz_{1\text{-}9}$, MGIN-$Rz_{1\text{-}14}$, MGIN-$AS_{Psi\text{-}gag}$, MGIN-$Rz_{1\text{-}9}AS_{Psi\text{-}gag}$, and MGIN-$Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ vector particles were produced and used to transduce the MT4 cells (20,21), as described previously

(22). The pools of $G418^R$ stable MT4 transductants were selected and then used in the subsequent experiments.

3.3. PCR analysis of genomic DNA from MT4 transductants

Genomic DNA was extracted from the stable MT4 transductants that either lacked (control) or expressed Rz_{1-9}, Rz_{1-14}, $AS_{Psi-gag}$, $Rz_{1-9}AS_{Psi-gag}$, or $Rz_{1-14}AS_{Psi-gag}$ (23). PCRs were performed, as described previously (8,15), using the MGIN-5' primer (5'-CTC-TCG-GCA-TGG-ACG-AG-3') and the MGIN-3' primer (5'-ATG-CTG-GTC-AAG-AAG-AC-3'), which bind to the vector sequences upstream and downstream of the *Rz_{1-9}*, *Rz_{1-14}*, and *$AS_{Psi-gag}$* genes. The Gap-5' primer (5'-TCT-ACT-GGC-GCT-GCC-AAG-3') and the Gap-3' primer (5'-TCT-AGA-CGG-CAG-GTC-AGG-3') were used to amplify a 122 bp region within the cellular glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The PCR products were analyzed by electrophoresis on a 1 or 1.5% agarose gel, as indicated.

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

Total RNA was extracted from the stable MT4 transductants (24) and treated with the RQI RNase-free DNase (Promega Corp.; Madison, WI), as described previously (8). The RNA samples were analyzed by PCR using the Gap-5'/Gap-3' primer pair to ensure that they are free of DNA contamination. The RNA samples were then analyzed by the semi-quantitative RT-PCR, as described previously (8,25). Reverse transcriptions were performed using the Neo-3' primer (5'-CTC-TTC-GTC-CAG-ATC-ATC-3') or the Gap-3' primer, followed by PCR using the Neo-5' primer (5'-CAA-GAC-CGA-CCT-GTC-CGG-3') and the ^{32}P-labelled Neo-3' primer or the Gap-5' primer and the ^{32}P-labelled Gap-3' primer; the 3' primers were 5'-end labeled (~1.3 x 10^9 cpm/mg) using [gamma ^{32}P] ATP (Amersham Canada Ltd.; Oakville, Canada; 6000 Ci/mmol), as described previously (19). PCRs were performed for 25 cycles using a higher concentration (5 mg/l) of the 5' and the ^{32}P-labeled 3' primers, as described previously (8). As a control, the Neo-5' primer and the ^{32}P-labeled Neo-3' primer were used to PCR amplify 100 to 100,000 copies of MGIN vector DNA and the Gap-5' primer and the ^{32}P-labeled Gap-3' primer were used to PCR amplify 0.1 and 0.5 mg/l of cellular genomic DNA (8,25). 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 the pools of MT4 transductants

Total cellular RNA extracted from the stable MT4 transductants expressing the Rz_{1-9} or Rz_{1-14} was RT-PCR amplified to generate a template enabling the T7-promoter-driven transcription of Rz_{1-9} and Rz_{1-14}. Reverse transcription was performed using the MGIN-3' primer, followed by PCR using the T7-MGIN-5' primer (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-CT-C-TCG-GCA-TGG-ACG-AG-3') and the MGIN-3' primer. The PCR products were then transcribed *in vitro* using the T7 RNA polymerase (Life Technologies; Burlington, Canada) for 2 h at 37°C, as described previously (8). The reaction was stopped by digesting the template DNA for 10 min with 5 U of RQI RNase-free DNase, followed by phenol extraction and ethanol precipitation.

A similar strategy was used to generate a 1400 nt-long alpha ^{32}P-labelled HIV-1 *env* target RNA, which contains the Rz_{1-9} and Rz_{1-14} target sites. To this end, the pNL4-3 plasmid was used in a PCR with the T7-Env-5' primer (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-GAA-GGA-GAA-ATA-TCA-GC-3') and the Env-3' primer (5'-TCA-CTT-CTC-CAA-TTG-TCC-3'). The PCR product was then transcribed *in vitro* using [alpha ^{32}P] UTP (Amersham Canada Ltd.; Oakville, Canada; 3000 Ci/mmol) and the T7 RNA polymerase for 2 h at 37°C, as described previously (8,15). The template DNA was digested for 10 min with 5 U of RQI RNase-free DNase, followed by phenol extraction and ethanol precipitation.

Rz_{1-9} or Rz_{1-14} and alpha ^{32}P-labelled *env* target RNA were used in a *trans* cleavage reaction as follows (7,8). The RNAs were combined in a reaction mixture containing 10 mM NaCl and 40 mM Tris-Cl, pH 8.0. The mixture was heated to 65°C for 5 min, cooled to 37°C, and the reaction was initiated by adding 20 mM $MgCl_2$. After 2 h incubation at 37°C, the reaction was stopped by adding 5 mM EDTA. The alpha ^{32}P-labelled target RNA and its cleavage products were analyzed by 8 M urea-6% polyacrylamide gel electrophoresis, followed by autoradiography.

3.6. HIV-1 susceptibility of MT4 transductants

To produce the HIV-1 NL4-3 challenge virus stock, HIV-1 was harvested by collecting the entire cell culture supernatant every day, and the sample containing the highest virus concentration was aliquoted and used (8). The titer of this virus (infectious units/ml) was determined using the U373-MAGI-$CXCR4_{CEM}$ cell line (26).

The pools of stable MT4 transductants (2 x 10^6 cells) were each inoculated with HIV-1 at a multiplicity of infection (m.o.i.) of 0.5, for 2 h at 37°C, as described previously (9,22). Cells were then washed twice with the medium, suspended in the same medium, and cultured at 37°C. The amount of HIV-1 p24 antigen released in the culture supernatants was then measured at different time intervals by enzyme linked immunosorbent assay using a kit (Abbott; Chicago, IL).

4. RESULTS

The MSCV-based MGIN vector (16) was used to express the antisense RNA ($AS_{Psi-gag}$), the multimeric hammerhead ribozymes (Rz_{1-9} and Rz_{1-14}), or the multimeric hammerhead ribozymes and the antisense RNA ($Rz_{1-9}AS_{Psi-gag}$ and $Rz_{1-14}AS_{Psi-gag}$). Amphotropic vector particles were used to transduce the human CD4+ T lymphoid (MT4) cell line (20,21). The stable MT4 transductants were then tested for the presence of vector DNA sequences, level of expression of interfering RNAs, ribozyme activity, and inhibition of HIV-1 replication.

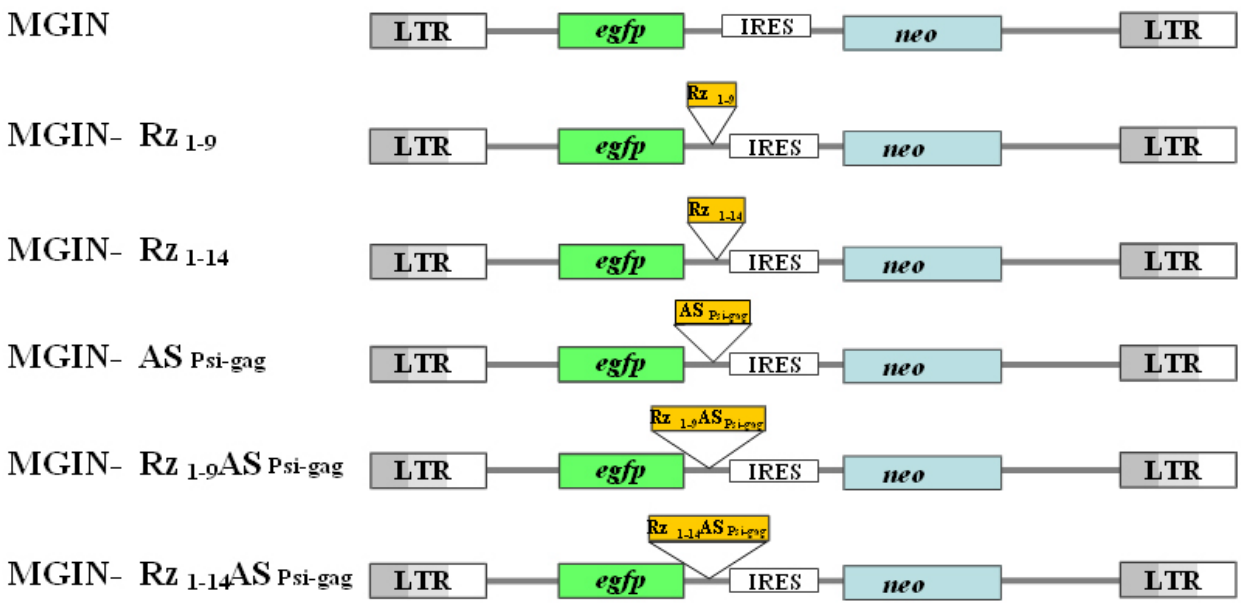

Figure 1. Schematic diagram of MGIN, MGIN-Rz_{1-9}, MGIN-Rz_{1-14}, MGIN-$AS_{Psi-gag}$, MGIN-$Rz_{1-9}AS_{Psi-gag}$, and MGIN-$Rz_{1-14}AS_{Psi-gag}$ vectors. Only those sequences that are part of the vector are shown.

4.1. MGIN-based vectors expressing the multimeric ribozymes, the antisense RNA, or the multimeric ribozymes and the antisense RNA

The MGIN vector contains the *egfp* gene, the IRES element, and the neomycin phosphotransferase (*neo)* gene; the *egfp* and the *neo* open reading frames are translated from a bicistronic mRNA transcribed from the 5' long terminal repeat (LTR) promoter (Figure 1) (16). Genes encoding the Rz_{1-9}, Rz_{1-14}, $AS_{Psi-gag}$, $Rz_{1-9}AS_{Psi-gag}$, or $Rz_{1-14}AS_{Psi-gag}$ RNA were cloned between the *egfp* gene and the IRES element. The resulting vectors were named MGIN-Rz_{1-9}, MGIN-Rz_{1-14}, MGIN-$AS_{Psi-gag}$, MGIN-$Rz_{1-9}AS_{Psi-gag}$, and MGIN-$Rz_{1-14}AS_{Psi-gag}$.

The $Rz_{1-9}AS_{Psi-gag}$ and $Rz_{1-14}AS_{Psi-gag}$ were designed to contain the multimeric ribozymes followed by the antisense RNA to enable ribozyme and antisense RNA hybridization to their target sites in a co-linear fashion (Figure 2).

4.2. Development of pools of stable MT4 transductants expressing the interfering RNAs

Amphotropic MGIN, MGIN-Rz_{1-9}, MGIN-Rz_{1-14}, MGIN-$AS_{Psi-gag}$, MGIN-$Rz_{1-9}AS_{Psi-gag}$, and MGIN-$Rz_{1-14}AS_{Psi-gag}$ vector particles were each used to transduce the MT4 cell line (20,21). Pools of $G418^R$ stable MT4 transductants were then selected and tested without cloning.

The presence of genes encoding the multimeric ribozymes and/or the antisense RNA was confirmed by PCR analysis of the genomic DNA isolated from untransduced and transduced MT4 cells. The MGIN-5'/MGIN-3' primer pair was used for this purpose. This primer pair was designed against sequences upstream (within the *egfp* gene) and downstream (within the IRES element) of the sites where the genes encoding the interfering RNAs were cloned. The expected size 217, 626, and 906 bp products were detected in the MGIN, MGIN-Rz_{1-9} and MGIN-Rz_{1-14} vector-transduced samples (Figure 3A, lanes 2-4), and 1617, 2026, and 2306 bp products were detected in the MGIN-$AS_{Psi-gag}$, MGIN-$Rz_{1-9}AS_{Psi-gag}$, and MGIN-$Rz_{1-14}AS_{Psi-gag}$ vector-transduced samples (Figure 3B, lanes 2-4). No PCR product was detected in the untransduced sample (Figure 3A, lane 1; Figure 3B, lane 1). As a control, the Gap-5'/Gap-3' primer pair was used to amplify a region within the cellular *GAPDH* gene. The expected 122 bp product was detected in all untransduced and transduced samples (Figure 3C, lanes 1-7).

Vector RNA expression from the 5' LTR promoter was confirmed by a semi-quantitative RT-PCR analysis of total cellular RNAs extracted from the untransduced and transduced MT4 cells. Under the conditions used, a linear relationship existed between the input RNA concentration and the RT-PCR product intensity (Figure 4A, lanes 8-11; Figure 4B, lanes 8,9). The semi-quantitative RT-PCR analysis using the Neo-5' primer and the ^{32}P-labelled Neo-3' primers resulted in the amplification of a 330 bp product, with a similar intensity for all RNA samples (Figure 4A, lanes 2-7). This result suggests that the level of expression of the 5' LTR promoter-driven transcript is similar in all MT4 transductants. No RT-PCR product was detected in the untransduced sample (Figure 4A, lane 1). The Gap-5' primer and the ^{32}P-labelled Gap-3' primers were used as a control. A 122 bp product specific for the cellular GAPDH RNA was detected upon semi-quantitative RT-PCR analysis of total cellular RNA from all untransduced and transduced MT4 samples (Figure 4B, lanes 1-7). Direct PCR analysis of RNA samples used in these RT-PCRs did

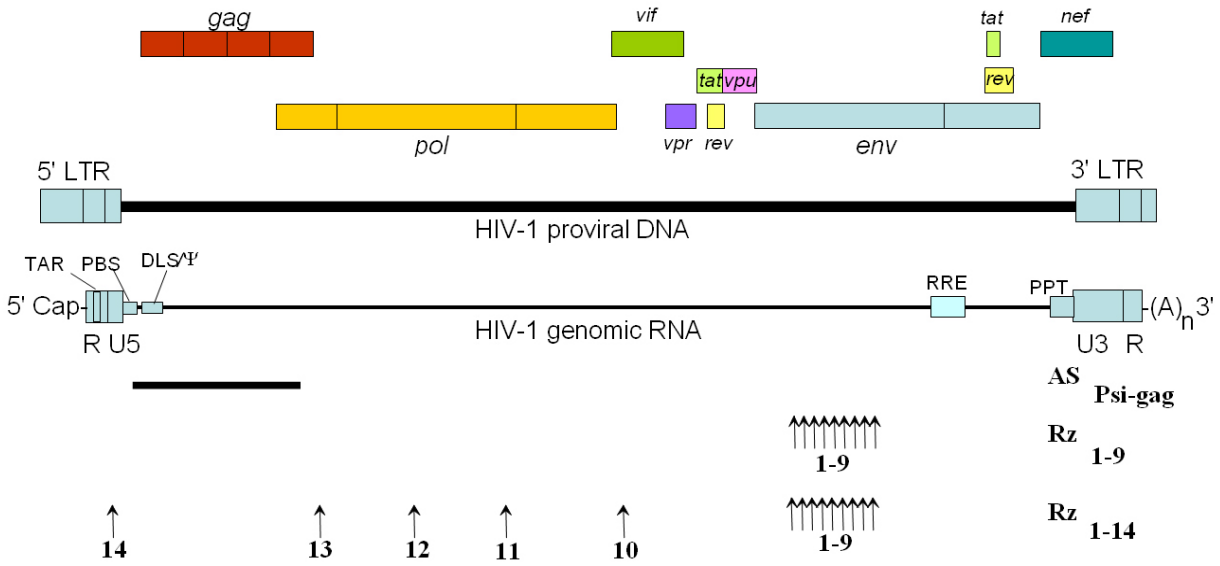

Figure 2. Genetic map of HIV-1 provirus DNA and genomic RNA. Shown below are the antisense RNA (horizontal line) and the multimeric hammerhead ribozyme (arrows) target sites within the HIV-1 RNA.

not yield any product, confirming the lack of DNA contamination in these samples (results not shown).

To demonstrate the activity of the multimeric hammerhead ribozymes produced in the transduced MT4 cells, Rz_{1-9} and Rz_{1-14} were amplified from the cellular RNA by RT-PCR using a T7 promoter-containing 5' primer, such that the RT-PCR product could be transcribed *in vitro*. Rz_{1-9} and Rz_{1-14} were then used in a *trans* cleavage reaction in the presence of an alpha ^{32}P-labelled 1400 nt-long HIV-1 *env* target RNA. This RNA contains the target sites for the nine ribozymes that are targeted against the *env*-coding region. These nine ribozymes are present in both multimeric ribozymes Rz_{1-9} and Rz_{1-14}. Therefore, similar cleavage products were obtained upon cleavage of this target RNA by Rz_{1-9} and Rz_{1-14} (Figure 5). This result indicates that the multimeric ribozymes, Rz_{1-9} and Rz_{1-14}, expressed in the transduced MT4 cells are active.

4.3. HIV-1 susceptibility of MT4 transductants expressing the interfering RNAs

The pools of stable MT4 transductants lacking or expressing the Rz_{1-9}, Rz_{1-14}, $AS_{Psi-gag}$, $Rz_{1-9}AS_{Psi-gag}$, or $Rz_{1-14}AS_{Psi-gag}$ RNA were each infected with the HIV-1 strain NL4-3 at an m.o.i. of 0.5. Progeny virus production was measured by determining the amount of HIV-1 p24 antigen present in the infected cell culture supernatants (Figure 6). The progeny virus production on day 6 post-infection is reported since only a few rounds of HIV-1 replication would have taken place by this time.

Cells transduced with the control MGIN vector (sample 1) produced a high amount of virus (6.9 ng p24/ml, Figure 6A). Compared to this, 96% or 100% inhibition of HIV-1 replication was observed in cells expressing the Rz_{1-9} (sample 2) or Rz_{1-14} (sample 3), whereas only 66% inhibition of HIV-1 replication was observed in cells expressing the $AS_{Psi-gag}$ RNA (sample 4). However, 76% or 96% inhibition of HIV-1 replication was obtained in cells expressing the $Rz_{1-9}AS_{Psi-gag}$ (sample 5) or $Rz_{1-14}AS_{Psi-gag}$ (sample 6). Similar results were obtained in two independent experiments (Figure 6A,B). As a negative control, cells expressing a multimeric hammerhead ribozyme that is not targeted against HIV-1 RNA were infected by HIV-1. No inhibition of virus replication was observed in these cells.

5. DISCUSSION

The MSCV-based MGIN vector was used to express multimeric hammerhead ribozymes, an antisense RNA, or the multimeric hammerhead ribozymes and the antisense RNA. The Rz_{1-9} is targeted against nine highly conserved sites within the *env*-coding region. It should therefore cleave the unspliced and singly spliced HIV-1 RNAs.[5,6] Rz_{1-14} is targeted against fourteen sites that are highly conserved within the 5' leader region and the *pro*-, *pol*-, *vif*-, and *env*-coding regions, and should therefore cleave the unspliced and singly spliced HIV-1 RNAs. One of the ribozymes in Rz_{1-14} is targeted against the HIV-1 5' leader sequence. However, since this ribozyme was previously found to be inactive *in vitro* (8), Rz_{1-14} is not likely to cleave the completely spliced HIV-1 RNAs. The $AS_{Psi-gag}$ RNA is targeted against the HIV-1 Psi signal and the *gag*-coding region of the unspliced HIV-1 RNA. The binding of this RNA to the unspliced HIV-1 RNA occurs over 1553 nts and to the singly and completely spliced RNAs over 196 nts. Antisense RNAs over 800 nts in length were previously reported to inhibit HIV-1 replication better than their smaller counterparts (9,11,27). Therefore, the $AS_{Psi-gag}$ RNA is expected to act primarily at the level of the unspliced RNA.

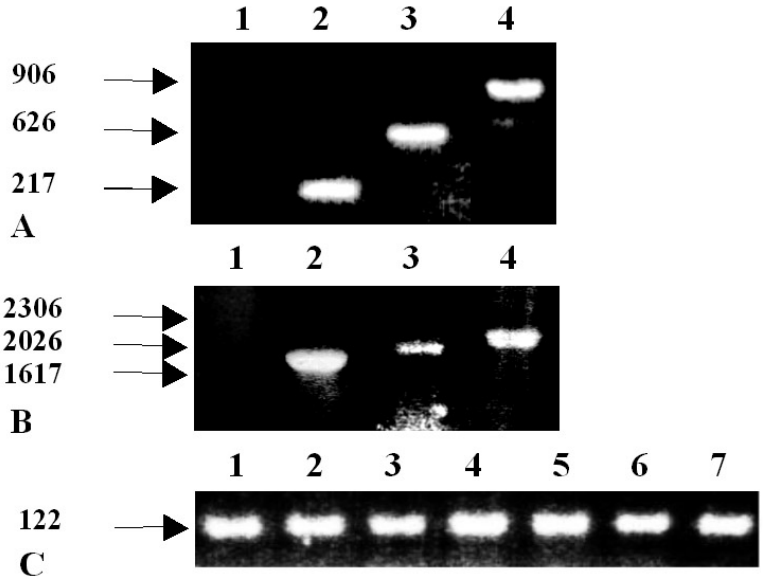

Figure 3. PCR analysis of genomic DNA to determine the presence of vector DNA sequences in the transduced MT4 cells. (A) DNA samples from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-$Rz_{1\text{-}9}$ (lane 3), and MGIN-$Rz_{1\text{-}14}$ (lane 4) were PCR-amplified using the MGIN-5'/MGIN-3' primer pair; the products were analyzed on a 1.5% agarose gel. (B) DNA samples from cells lacking (untransduced cells, lane 1) or expressing MGIN-$AS_{Psi\text{-}gag}$ (lane 2), MGIN-$Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ (lane 3), or MGIN-$Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ (lane 4) were PCR-amplified using the MGIN-5'/MGIN-3' primer pair; the products were analyzed on a 1% agarose gel. (C) DNA samples from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-$Rz_{1\text{-}9}$ (lane 3), MGIN-$Rz_{1\text{-}14}$ (lane 4), MGIN-$AS_{Psi\text{-}gag}$ (lane 5), MGIN-$Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ (lane 6), and MGIN-$Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ (lane 7) were PCR-amplified using the Gap-5'/Gap-3' primer pair. The products were analyzed on a 1.5% agarose gel. Arrows indicate the PCR product sizes.

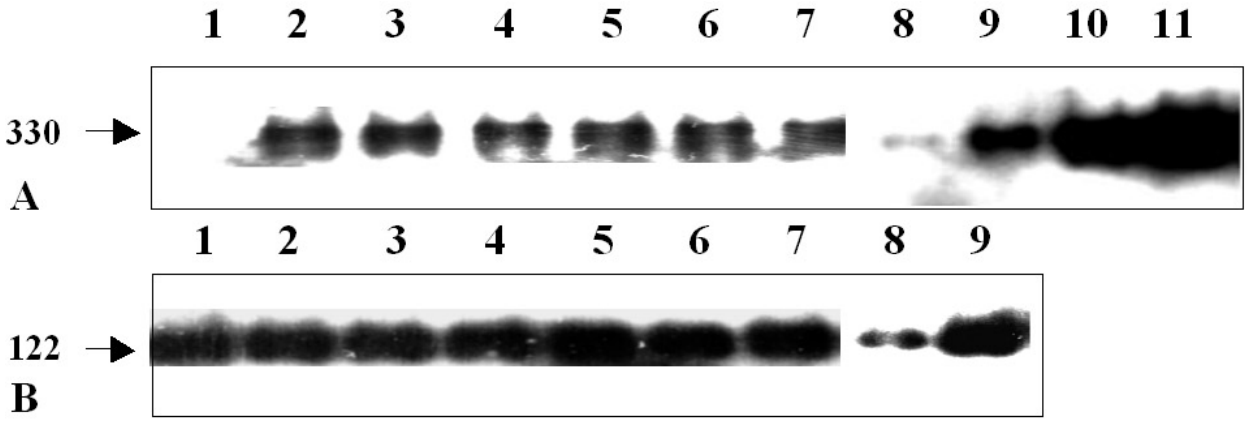

Figure 4. Semi-quantitative RT-PCR analysis of total RNA from the untransduced and transduced MT4 cells. (A) The RNA samples were reverse transcribed using the Neo-3' primer, followed by semi-quantitative PCR using the Neo-5' primer and the ^{32}P-labelled Neo-3' primer. RT-PCR products were analyzed from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-$Rz_{1\text{-}9}$ (lane 3), MGIN-$Rz_{1\text{-}14}$ (lane 4), MGIN-$AS_{Psi\text{-}gag}$ (lane 5), MGIN-$Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ (lane 6), and MGIN-$Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ (lane 7). Control PCRs were performed using 100 (lane 8), 1000 (lane 9), 10,000 (lane 10), and 100,000 (lane 11) copies of MGIN plasmid DNA (to assure a linear relationship between the intensity of the PCR products and the amount of cDNA used). The RT-PCR and PCR products were analyzed on a 2% agarose gel. (B) RT-PCR products obtained with the Gap-5' and the ^{32}P-labelled Gap-3' primers using the RNA from cells lacking (untransduced cells, lane 1) or expressing MGIN (lane 2), MGIN-$Rz_{1\text{-}9}$ (lane 3), MGIN-$Rz_{1\text{-}14}$ (lane 4), MGIN-$AS_{Psi\text{-}gag}$ (lane 5), MGIN-$Rz_{1\text{-}9}AS_{Psi\text{-}gag}$ (lane 6), and MGIN-$Rz_{1\text{-}14}AS_{Psi\text{-}gag}$ (lane 7). This primer pair was used to amplify a 122 bp region within the cellular GAPDH RNA, to monitor the amount of RNA used. Control PCRs were performed using 100 (lane 8) and 500 (lane 9) ng of total cellular DNA. The RT-PCR and PCR products were analyzed on a 2% agarose gel. Arrows indicate the product sizes.

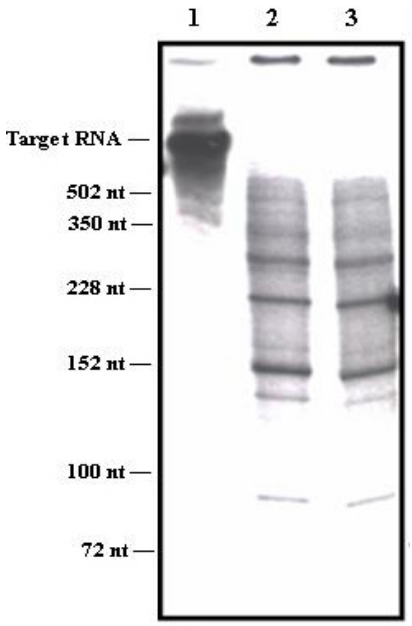

Figure 5. *In vitro* cleavage activity of multimeric ribozymes amplified from the stable MT4 transductants. Rz_{1-9} and Rz_{1-14} were RT-PCR amplified from the total RNA extracted from MGIN-Rz_{1-9} and MGIN-Rz_{1-14}-transduced cells to generate a T7 promoter-containing template DNA. This DNA was transcribed *in vitro* to obtain Rz_{1-9} and Rz_{1-14}, which were then used in a *trans* cleavage reaction with the alpha ^{32}P-labeled target RNA containing the HIV-1 *env* target sites. The alpha ^{32}P-labeled target RNA (lane 1) and its cleavage products (lanes 2,3) were analyzed by 8-M urea-6% polyacrylamide gel electrophoresis, followed by autoradiography. An arrow indicates the band corresponding to the uncleaved target RNA.

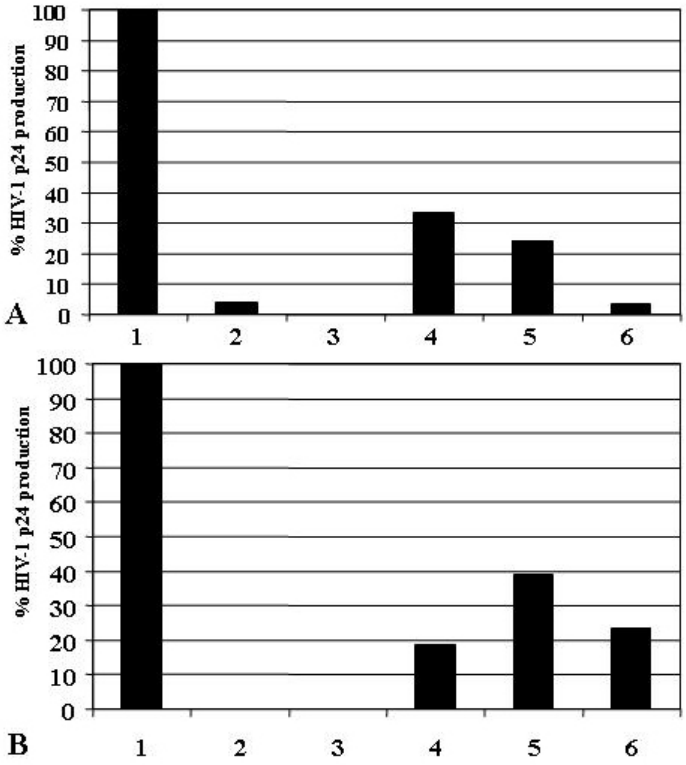

Figure 6. HIV-1 susceptibility of pools of MT4 cells stably transduced with MGIN (sample 1), MGIN-Rz_{1-9} (sample 2), MGIN-Rz_{1-14} (sample 3), MGIN-$AS_{Psi-gag}$ (sample 4), MGIN-$Rz_{1-9}AS_{Psi-gag}$ (sample 5), or MGIN-$Rz_{1-14}AS_{Psi-gag}$ (sample 6). The % HIV-1 p24 antigen produced in the culture supernatants is reported on day 6 post-infection for 2 independent experiments (A and B).

Amphotropic vector particles were used to transduce the human CD4+ T lymphoid MT4 cell line. The uninfected stable transductants were shown to contain the genes encoding the multimeric ribozymes and/or the antisense RNA (Figure 3), and to allow similar level of expression of all interfering RNAs (Figure 4). The multimeric ribozymes expressed in these cells were also shown to be active *in vitro* (Figure 5). Cells expressing the multimeric ribozymes and/or the antisense RNA were viable and showed no sign of toxicity (results not shown).

Stable MT4 transductants expressing various interfering RNAs were then challenged with HIV-1. Cells transduced with the control MGIN vector produced high amount of HIV-1. Significant inhibition of HIV-1 replication was observed in MT4 cells expressing Rz_{1-9} or Rz_{1-14}. Little inhibition of HIV-1 replication was observed in the cells expressing the $AS_{Psi-gag}$ RNA. In MT4 cells expressing the $Rz_{1-9}AS_{Psi-gag}$ or $Rz_{1-14}AS_{Psi-gag}$, virus replication was inhibited, but to a lesser degree than in the cells expressing the multimeric ribozymes alone (Figure 6).

These results indicate that the Rz_{1-9} and Rz_{1-14} inhibit HIV-1 replication better than the $AS_{Psi-gag}$ RNA. And, that the $AS_{Psi-gag}$ RNA co-expression with Rz_{1-9} or Rz_{1-14} decreases the antiviral potential of these ribozymes.

The multimeric hammerhead ribozymes and the antisense RNA are targeted against different regions of HIV-1 RNA. Therefore, although Rz_{1-9} and $AS_{Psi-gag}$ within the $Rz_{1-9}AS_{Psi-gag}$ RNA or Rz_{1-14} and $AS_{Psi-gag}$ within the $Rz_{1-14}AS_{Psi-gag}$ RNA might both interact with the same unspliced HIV-1 RNA, they are designed not to compete with each other for binding to a common HIV-1 target site. Rz_{1-9} and $AS_{Psi-gag}$ or Rz_{1-14} and $AS_{Psi-gag}$ are also not expected to interact with each other.

Similar length antisense RNAs (11,27) and Rz_{1-9} (7) were previously shown to inhibit the accumulation of HIV-1 RNA. Target site location within the HIV-1 RNA is not critical if the antisense RNA and the multimeric ribozymes act pre-splicing, since then the singly and completely spliced HIV-1 RNAs will not be generated. However, a post-splicing activity would be required for the spliced HIV-1 RNAs that escape degradation or cleavage by the antisense RNA or ribozymes. Although the $AS_{Psi-gag}$ RNA, Rz_{1-9}, and Rz_{1-14} all act at the level of unspliced HIV-1 RNA, the Rz_{1-9} and Rz_{1-14} have the added advantage of also acting at the level of singly spliced HIV-1 RNAs. This may be one of the reasons why these multimeric ribozymes inhibit better than the antisense RNA.

The $AS_{Psi-gag}$ would mainly target the unspliced HIV-1 RNA. Therefore, it would prevent the co-expressed Rz_{1-9} and Rz_{1-14} from acting at the level of unspliced (and not the singly spliced) HIV-1 RNA.

The antisense-HIV RNA hybrids are subject to RNase degradation. Therefore, the antisense RNA is consumed in the reaction, whereas the ribozymes are not – they only cleave the target RNA, which is further degraded by the cell. As a result, compared to the HIV-1-infected MT4 cells that express the Rz_{1-9} or Rz_{1-14}, ribozyme levels will be lower in the HIV-1 infected cells that express the $AS_{Psi-gag}$-Rz_{1-9} or $AS_{Psi-gag}$-Rz_{1-14}. This would further explain why the combination strategy was not better.

Our results indicate that the antisense RNA and ribozymes should not be co-expressed as a single transcription unit. Also, the multimeric ribozyme-based strategy seems to be better than the antisense RNA-based strategy. Therefore, the combination strategies must be carefully designed as the choice/design of the vector and the interfering RNAs present on a single or even a different transcript may interfere with each other's activity.

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Abbreviations: AIDS: acquired immunodeficiency syndrome, AS: antisense, 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, nts: nucleotides; Psi: packaging signal, Rz: ribozyme

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