

DESIGN AND CLONING OF A HAMMERHEAD RIBOZYME TARGETED TO HIV-1 *VIF* MRNA

Katherine L. Anderson and William H. Jackson*

*Department of Biology and Geology, University of South Carolina Aiken, Aiken, SC 29801
billj@usca.edu

ABSTRACT

Human Immunodeficiency Virus (HIV), the etiological agent of the Acquired Immune Deficiency Syndrome (AIDS) poses a worldwide threat to public health. The ability of HIV to adapt to current antiviral drug therapy places some importance on other means to inhibit viral replication. Ribozymes may offer a promising avenue of research in the battle against HIV. Hammerhead ribozymes are RNA molecules that act as enzymes, catalyzing the substrate-specific cleavage of target RNAs. Ribozyme targeting of HIV RNAs essential to replication have been shown to reduce or inhibit viral replication. Among the genes most commonly targeted are *tat* and *rev*, which regulate viral transcription and post-transcriptional modification, respectively. In this paper we describe the design, synthesis, and cloning of a hammerhead ribozyme targeted to HIV-1 *vif* (Virion Infectivity Factor). This gene encodes a protein that enhances viral infectivity by blocking a host antiviral defense pathway, which relies on the host protein APOBEC3G. *Vif*'s ability to inhibit this cellular defense mechanism makes it a potentially advantageous target for ribozyme-mediated down-regulation of HIV.

INTRODUCTION

The identification of the Human Immunodeficiency Virus as the causative agent of AIDS in 1983 was accompanied by a tremendous research effort directed at limiting or preventing the spread of this virus into susceptible populations. While these measures have temporarily succeeded in reducing the number of HIV infected individuals who progress to AIDS, they are not curative. To this end, the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated in late 2003 that about 40 million people worldwide were infected with HIV, and in that year alone, approximately 5 million new HIV infections and 3 million AIDS-related deaths occurred (NIAID, 2004).

The rapid adaptation of HIV-1 to antiviral drugs (Freeman and Herron, 2001) gives some urgency to investigating other means to inhibit viral replication. One such area of investigation involves the use of catalytic RNAs, or ribozymes, to target and cleave viral mRNAs. Ribozymes are RNA molecules that act as enzymes, catalyzing the substrate-specific cleavage of substrate RNAs (Cech, 1989). Ribozyme targeting of HIV mRNAs could potentially inhibit viral replication by reducing HIV gene expression and limiting assembly of viral progeny. One class of catalytic RNA was found to exist naturally in the satellite RNA of tobacco ringspot virus (sTobRV) (Prody et al., 1986). Forster and Symons (1987)

identified characteristic conserved sequences of the ribozyme and noted the secondary hammerhead-shaped structure. Haseloff and Gerlach (1988) defined the minimal ribozyme sequence necessary for cleavage and developed a model for ribozyme design. Cleavage of substrate RNAs by hammerhead ribozymes depends on the availability of target sites that were initially defined by Haseloff and Gerlach (1988) and later broadened by Perriman et al. (1992). These sites can be defined as any XUX', where X is any ribonucleotide and X' is an A, C, or U (Perriman et al., 1992). Ribozyme-mediated cleavage occurs immediately 3' of the X' nucleotide (Perriman et al., 1992). Several investigations have studied the use of hammerhead ribozymes to inhibit specific viral functions. Studies published by Zhou et al. (1994) and Jackson et al. (1998) confirmed the ability for hammerhead ribozymes to target HIV-1 RNA and inhibit subsequent replication. Favored HIV targets have been *tat* and *rev*, which encode regulatory proteins responsible for regulating transcription from the viral promoter and post-transcriptional splicing of transcripts, respectively. Other HIV targets may also prove to be valuable targets for viral inhibition. One such target is HIV-1 *vif* (Virion Infectivity Factor), which encodes a protein that neutralizes an inhibitory host defense mechanism mediated by apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) (Navarro and Landau, 2004). APOBEC3G is a cellular cytidine deaminase that is encapsulated into assembling virions in the absence of *vif* and is inhibitory during the next round of viral replication (Navarro and Landau, 2004). *Vif* neutralizes APOBEC3G by reducing its translation and by rapid degradation of the native protein (Navarro and Landau, 2004).

Vif's ability to inhibit the host cell's retrovirus defense mechanism makes it a potentially advantageous target for ribozyme binding and associated decreased expression of HIV. This paper describes the design, synthesis, and cloning of a catalytic and noncatalytic (Δ) control hammerhead ribozyme targeted to HIV-1 *vif* mRNA.

METHODS AND MATERIALS

Ribozyme Design and Synthesis. The HIV-1 NL43 mRNA sequence (accession number M19921) was analyzed for potential ribozyme target sites (Perriman et al., 1992; Haseloff and Gerlach, 1988). One such target, a pGUU was located at nucleotide 5154. An anti-*vif* ribozyme, Vif5154, consisting of the hammerhead ribozyme catalytic core and 12-nucleotide complementary flanking regions was designed to this sequence (Figure 1). An identical non-catalytic control ribozyme, Vif5154 Δ , was designed by changing the adenine located at position 21 in the catalytic core to a guanine (Zhou et al., 1994). Each ribozyme was converted to DNA and used to design forward and reverse primers for PCR. The two ribozyme DNA templates (5'-GTG ATG TCT ATA ACT GAT GAG TCC GTG AGG ACG (A/G)AA CCA GTC CTT AGC-3') and associated PCR primers (forward, 5'-GTG ATG TCT ATA ACT GAT GAG TC-3' and reverse, 5'-GCT AAG GAC TGG TT(T/C) CGT C-3') were synthesized (Integrated DNA Technologies, Inc., Coralville, IA) and used to generate dsDNA ribozymes.

Amplification of Vif5154 and Vif5154 Δ Ribozymes. To generate dsDNA ribozymes suitable for cloning, the synthesized Vif5154 and Vif5154 Δ ribozyme DNAs were used as templates for the polymerase chain reaction (PCR). Briefly, 200pMol ribozyme template, 1000pMol of the appropriate forward and reverse primers were added to 1x thermostable buffer, 4mM dNTPs, 100mMol MgSO₄, and 2.5 units of Vent DNA polymerase (New England Biolabs, Beverly, MA). PCR was conducted under the following conditions: dissociation at

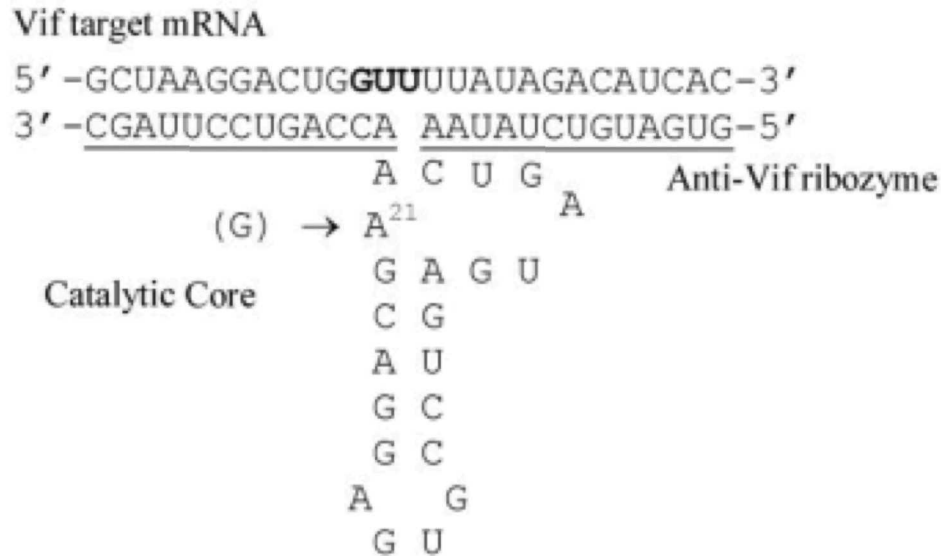


Figure 1. Design of anti-Vif ribozymes. Two hammerhead ribozymes targeted to a pGUU target in HIV-1 NL43 Vif mRNA were designed based on the Haseloff and Gerlach model (1988). Each ribozyme consisted of a catalytic core and complementary flanking regions (underlined) that provide substrate specificity. Catalytic activity was abolished by substituting a guanine at position 21 in the catalytic core (Zhou, 1994).

94°C for 30 seconds, primer annealing at 52.5°C (Vif5154) or 52.8°C (Vif5154Δ) for 30 seconds, and extension at 72°C for 60 seconds. This process was repeated for 30 cycles. Amplified DNA was analyzed by gel electrophoresis for the presence of a 48 base pair fragment indicative of both ribozymes (data not shown).

Ligation and Transformation of Vif5154 and Vif5154Δ Ribozymes. Each amplified ribozyme was cloned into the shuttle vector pPCR-Script Amp Sk(+) (Stratagene, La Jolla, CA) by blunt-end ligation according to the manufacturer's recommendation. Briefly, each ligation reaction was carried out in 50μl using 10ng pPCR-Script, 0.5mM rATP, 5U *Srf*I, 4U T4 DNA ligase, 2μl PCR product, and 1x reaction buffer. Ligation reactions were incubated at room temperature for 1 hour, followed immediately by transformation of XL10 Gold Ultracompetent *E. coli* cells (Stratagene, La Jolla, CA).

XL10 Gold cells were thawed on ice, and 40μl were aliquoted into sterile, prechilled 17x150mm tubes along with 1.6μl of β-mercaptoethanol. Two microliters of the respective ligation reaction were added to each tube, and the cells were incubated on ice for 30 minutes then heat-pulsed in a 42°C water bath for 30 seconds. The cells were removed from the bath and incubated on ice for two minutes. Four hundred microliters of preheated (42°C) SOC broth were added to each tube and the cells were incubated for 1 hour at 37°C and 225 rpm. Fifty microliters of cells were plated onto Luria-Bertani (LB)/Amp (50μg/ml) along with 10μl of 50mg/ml X-gal and 10μl of 10mM IPTG. Each plate was incubated overnight at 37°C. Four white colonies from the Vif5154 plate and five white colonies from the Vif5154Δ plate were picked for analysis. Each colony was used to inoculate 2ml of LB/Amp (50μg/ml) broth and incubated overnight at 37°C and 225 rpm.

Clone Analysis of Vif5154 and Vif5154Δ Ribozymes. Plasmid DNA was extracted from transformed *E. coli* using a miniprep procedure (Sambrook et al., 1989). One milliliter of each cultures was centrifuged for 4 minutes, the medium was aspirated, and the pellets were resuspended in 100μl of ice cold Solution I (50mM glucose, 25mM Tris Cl [pH 8.0], 10mM EDTA [pH 8.0]). Two hundred microliters of freshly prepared Solution II (10N NaOH [20μl], 10% SDS [10μl], 970μl dH₂O) were added and mixed by inversion. The lysates were placed on ice and 150μl of ice cold Solution III (5 M potassium acetate [60ml], glacial acetic acid [11.5ml], dH₂O [28.5 ml]) were added and mixed by gentle inversion. The lysate was allowed to precipitate on ice for 5 minutes, and centrifuged for 5 minutes. The supernatant was transferred to a new microcentrifuge tube, and plasmid DNA was precipitated with 2 volumes of ethanol for 2 minutes. The supernatant was decanted after a 10-minute centrifugation. Remaining pellets were washed with an additional volume of ethanol and the dried pellets were redissolved in 25μl of dH₂O.

Presence and Orientation Analysis of Cloned Ribozymes. To determine ribozyme presence within plasmid DNA, PCR was conducted using M13 primers. Briefly, each reaction was carried out in 50μl using 100pMol each of M13 forward and reverse primers, 2μl miniprep DNA, and 1.25U of 2x Taq (Takara, Madison, WI). PCR was carried out as described with annealing temperatures of either 55°C for potential catalytic ribozyme clones or 54°C for potential noncatalytic ribozyme clones. PCR fragments were analyzed by gel electrophoresis for the presence of a 274 base pair fragment indicative of both ribozymes.

Orientation of the ribozymes with respect to the T7 promoter of pPCR-Script was determined by gel electrophoresis of PCR reactions using M13 primers, ribozyme primers, and Taq (Takara, Madison, WI). Each reaction was carried out in 50μl using 100pMol of a premix containing M13 forward primer and either ribozyme forward or reverse primer, 2μl miniprep clone DNA, and 1.25U 2x Taq (Takara, Madison, WI). PCR was conducted as previously mentioned, with annealing temperatures of 54°C for pVif5154 clones and 55°C for pVif5154Δ clones. PCR fragments were analyzed by gel electrophoresis for the presence of a 176 base pair fragment for both ribozymes. Positive clones was sent to the Medical College of Georgia's Core Facilities for direct sequencing to confirm cloning (Medical College of Georgia, Augusta, Georgia).

RESULTS

Design and cloning of anti-*vif* ribozymes. A pGUU at nucleotide 5154 of HIV-1 *vif* mRNA was identified as a potential site for ribozyme cleavage (Perriman et al, 1992). A hammerhead ribozyme was designed to target this mRNA based on the model of Haseloff and Gerlach (1988). Additionally, a control, non-catalytic ribozyme was designed by substituting a guanine for an adenine residue at position 21 of the catalytic core (Zhou et al, 1994). Each ribozyme sequence was converted to DNA and used to design forward and reverse primers for PCR. The Vif5154 catalytic and Vif5154Δ noncatalytic ribozyme DNAs were synthesized along with the appropriate PCR primers. Each ribozyme DNA was amplified and visualized by gel electrophoresis prior to cloning into pPCR-Script. Gel analyses revealed PCR fragments of approximately 48bp for both Vif5154 and Vif5154Δ ribozymes (data not shown), indicating successful ribozyme amplification. Amplified ribozymes were cloned into pPCR-Script by blunt-end ligation and transformed into XL10 Gold Ultracompetent cells.

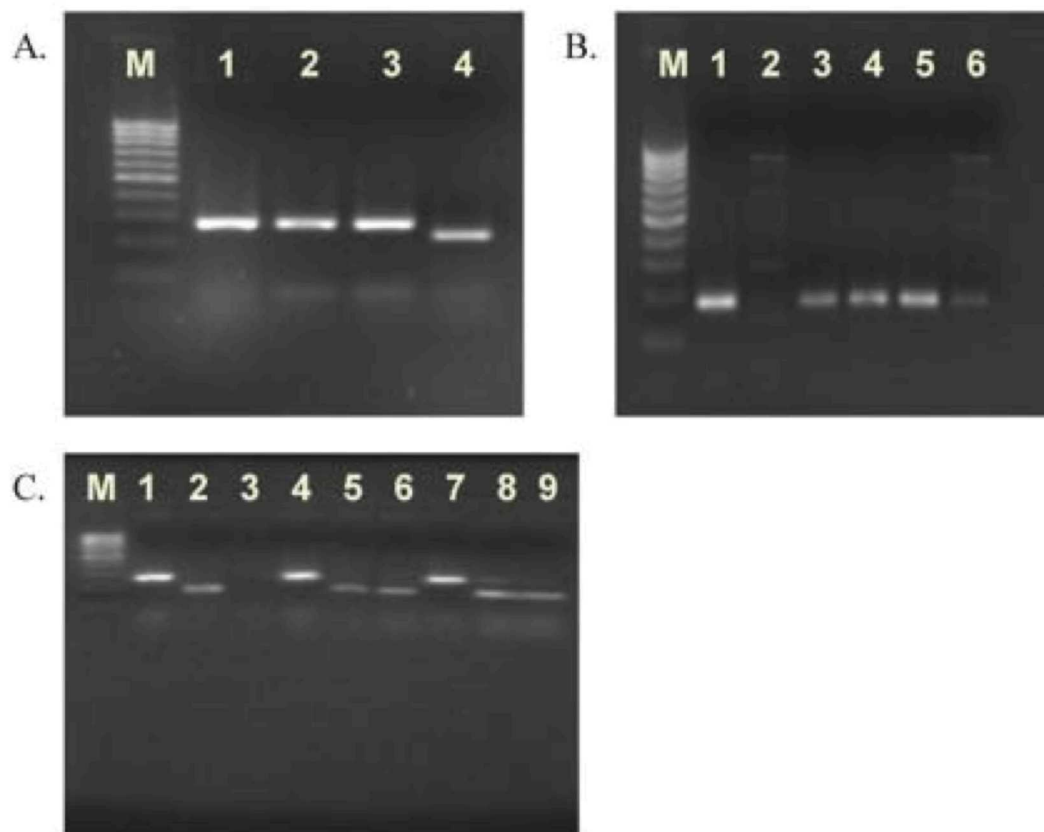


Figure 2. Analysis of ribozyme presence and orientation. Plasmid DNAs were screened for ribozyme presence and orientation by PCR using plasmid- and ribozyme-specific primers. Presence was confirmed by the amplification of a 274bp fragment: panel A, lanes 1-3 for Vif5154 and panel C, lanes 1, 4, and 7 for Vif5154Δ ribozymes. Orientation was determined by the amplification of a 176bp fragment in one of two paired reactions using a plasmid-specific primer in combination with each ribozyme-specific primer. The presence of a 176bp fragment in panel B, lane 1 along with the absence of a band in lane 2 indicated Vif5154 ribozyme orientation inverted with respect to the plasmid T7 promoter. Similar results were observed in panel C, lane 2 which along with the absence of a band in lane 3 indicated Vif5154Δ ribozyme orientation was also inverted. The remaining reactions gave no clear indication of orientation and were discarded. All agarose gels were run in 1XTAE.

Analysis of ribozyme presence and orientation. Miniprep DNA was analyzed for ribozyme presence and orientation by PCR using plasmid-specific and ribozyme-specific primers. Three of four clones analyzed for Vif5154 ribozyme resulted in amplification of a 274bp fragment (Figure 2A) as determined using the plasmid map. In addition, three of five clones analyzed for Vif5154Δ ribozyme resulted in amplification of a 274bp fragment (Figure 2C). Each of these six clones was further analyzed by PCR for ribozyme orientation with respect to the pPCR-Script T7 promoter. Two reactions were carried out for each clone using a combination of M13 forward/ribozyme forward or M13 forward/ribozyme reverse primers, each of which could result in a 176bp fragment. However, in this analysis successful PCR is solely determined by the ribozyme orientation such that only one positive reaction should occur for any clone. The orientation analysis clearly revealed that one Vif5154 ribozyme clone and one Vif5154Δ ribozyme clone contained the cloned sequences in an

orientation inverted with respect to the T7 promoter (Figure 2B and 2C). These clones were subsequently sent for direct sequencing (Medical College of Georgia Genomics Core Facility, Augusta, GA). Sequencing results for Vif5154: GCT AAG GAC TGG TTT CGT CCT CAC GGA CTC ATC AGT TAT AGA CAT CAC; and Vif5154Δ: GCT AA G GAC TGG TTC CGT CCT CAC GGA CTC ATC AG T TAT AGA CAT CAC verified that each was inverted with respect to the T7 promoter.

DISCUSSION

Hammerhead ribozymes are catalytic RNA molecules that cleave in a substrate-specific manner (Forster and Symons, 1987). Targeting of HIV mRNAs by ribozymes could potentially inhibit viral replication by reducing HIV gene expression and limiting viral progeny assembly (Zhou, 1994, Jackson et al, 1998). Although *tat* and *rev* have been favored HIV targets due to their functions of transcription regulation and post-transcriptional splicing, *vif*'s ability to inhibit the host cell's retrovirus defense mechanism also makes it a potentially advantageous target for ribozyme binding and associated decreased expression of HIV.

To test this hypothesis, a pGUU cleavage site at nucleotide 5154 of HIV-1 NL43 *Vif* mRNA was identified and a catalytic hammerhead ribozyme was designed. A non-catalytic control ribozyme was also designed by substituting a guanine for the adenine residue at position 21 of the catalytic core (Zhou et al, 1994). Each ribozyme was cloned into the shuttle vector, pPCR-Script and successful cloning was verified by direct sequencing. These

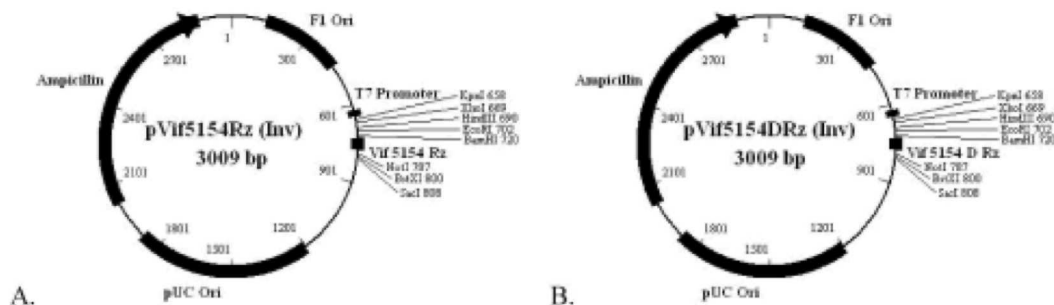


Figure 3. Plasmid maps of anti-*vif* ribozymes. Direct sequencing results were used to design plasmid maps showing the pPCR-Script Amp SK(+) shuttle vector containing cloned ribozymes, pVif5154 ribozyme (A.) and pVif5154Δ ribozyme (B.). Each ribozyme is cloned into the Multiple Cloning Site (MCS) of the pPCR-Script, and is inverted with respect to the T7 promoter.

plasmids, pVif5154Rz and pVif5154ΔRz (Figure 3) complement two previously cloned ribozymes that target HIV-1 *vif* mRNA at nucleotides 5113 and 5127. The cleavage activity of these anti-*vif* ribozymes will be determined in an *in vitro* cleavage assay. Individual ribozyme kinetics will be calculated and used to determine those ribozymes best suited for further testing in tissue culture models of HIV-1 replication.

ACKNOWLEDGMENTS

This work was funded by a grant from the National Institutes of Health (NIH AREA Grant 1 R15 GM66689-01).

REFERENCES

- Cech, T.R. 1989. Ribozymes: Tools for Sequence Specific Cleavage of RNA. Editorial Comments 16(2):1-5.
- Forster, A.C. and R.H. Symons. 1987. Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell* 49:211-220.
- Forster, A.C. and R.H. Symons. 1987. Self-cleavage of virusoid RNA is performed by the proposed 55-nucleotide active site. *Cell* 50:9-16.
- Freeman, S. and J.C. Herron. 2001. *Evolutionary Analysis*, 2nd ed. Prentice Hall: New York, NY.
- Haseloff, J. and W.L. Gerlach. 1988. Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* 334:585-591.
- Jackson, W.H., Jr., Moscoso, H., Nechtman, J.F., Galileo, D.S., Garver, F.A., Lanclos, K.D. 1998. Inhibition of HIV-1 replication by an anti-tat hammerhead ribozyme. *Biochemical and Biophysical Research Communications* 245:81-84.
- National Institute of Allergy and Infectious Diseases. 2004. How HIV causes AIDS. World Wide Web. Available from: <https://www.niaid.nih.gov/factsheets/howhiv.htm>. via the INTERNET. Accessed 10 April 2005.
- Navarro, F. and N.R. Landau. 2004. Recent insights into HIV-1 *Vif*. *Current Opinions in Immunology* 16:477-482.
- Perriman, R., Delves, A., Gerlach, W.L. 1992. Extended target-site specificity for a hammerhead ribozyme. *Gene* 113:157-163.
- Prody, G.A., Bakos, J.T., Buzayan, J.M., Schneider, I.R., Bruening, G. 1986. Autolytic processing of dimeric plant virus satellite RNA. *Science* 231:1577-1580.
- Sambrook, J., Fritsch, E., Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Springs Harbor Laboratory Press: Plainview, New York.
- Zhou, C., Bahner, I.C., Larson, G.P., Zaia, J.A., Rossi, J.J., Kohn, D.B. 1994. Inhibition of HIV-1 in human T-lymphocytes by retrovirally transduced anti-tat and rev hammerhead ribozymes. *Gene* 149:33-39.