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Ribozyme-dependent inactivation of *lac*Z mRNA in *E. coli*: a feasibility study to set up a rapid *in vivo* system for screening HIV-1 RNA-specific ribozymes

Research Article

Maria Fe C. Medina and Sadhna Joshi

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

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Correspondence: Sadhna Joshi, Department of Medical Genetics and Microbiology, Faculty of Medicine, University of Toronto, 150 College St. # 212, Toronto, Ontario M5S 3E2, Canada. Tel: (416)-978-2499; Fax: (416)-638-1459;
E-mail: sadhna.joshi.sukhwal@utoronto.ca

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Summary

Ribozymes are potentially useful tools with widespread applications in gene therapy of several diseases. In order to assess the *in vivo* cleavage efficiency of human immunodeficiency virus (HIV)-1 RNA-specific ribozymes, a bacterial indicator cell system could be developed in which the degree of inhibition of β-galactosidase activity would correlate with ribozyme activity. The suitability of this indicator cell system was assessed using a ribozyme targeted against the *env* coding region within the HIV-1 RNA. To this end, a pGEM4Z-based plasmid was engineered wherein oligodeoxynucleotides containing a hammerhead ribozyme and its target site were cloned in frame within the *lac*Z coding region that encodes for the α fragment of β-galactosidase. Extra nucleotides were included in the insert to ensure that the *lac*Z open reading frame was not interrupted due to a frameshift or nonsense mutation. In *E. coli* indicator cells harbouring this plasmid, ribozyme-mediated cleavage of the target site provided *in cis* and the subsequent loss of β-galactosidase activity should correlate with ribozyme activity. However, frameshift mutations were observed upon sequence analysis of plasmid DNA isolated from the selected light blue to white colonies. Because these mutations affected the production of the β-galactosidase α fragment, a direct correlation between β-galactosidase and ribozyme activities could not be established *in vivo*. Thus, in clones which demonstrated visibly lower β-galactosidase activities than the control, the effect of the frameshift mutations on *lac*Z mRNA translation can not be discounted. In clones expressing ribozymes but displaying dark blue colour, it is possible that *lac*Z mRNAs were cleaved but that the β-galactosidase substrates used were sensitive enough to allow detection of proteins translated from residual *lac*Z mRNA transcripts. The use of alternative β-galactosidase substrates with less sensitivity may enable the use of the proposed indicator cell system.

I. Introduction

Hammerhead ribozymes are small, catalytic RNA molecules first identified in the avocado sunblotch viroid as well as in the satellite RNAs of lucerne transient streak and tobacco ringspot viruses (reviewed by Vaish, 1998). The hammerhead ribozyme catalytic and substrate binding domains have been well characterized (Haseloff and Gerlach, 1988; Uhlenbeck, 1987). Hammerhead ribozymes may be targeted against any given RNA (reviewed by Birikh et al, 1997) provided that the ribozyme catalytic domain is flanked by antisense sequences to allow ribozyme binding to the target RNA. The cleavage site within the target RNA must be immediately preceded by NUH (Ruffner et al, 1990), with N being any nucleotide and H being any nucleotide except G. Cleavage results in a 5' product with a 5' hydroxyl group and a 3' product with a 2', 3' cyclic phosphate.

AIDS is caused by HIV, a retrovirus with an RNA genome. During its life cycle, HIV produces numerous mRNAs which are all potential targets for designing ribozymes (reviewed by Joshi and Joshi, 1996). Monomeric hammerhead ribozymes have been developed and tested against several sites within the HIV-1 RNA (reviewed by

Macpherson et al, 1999); however, virus breakthrough was eventually observed in each case (reviewed by Ramezani and Joshi, 1999).

Ribozymes with increased catalytic activity have been selected *via in vitro* selection/evolution (reviewed by Pan 1997). However, the *in vivo* cleavage activity of these ribozymes may be less than what is anticipated from results *in vitro*. The *in vitro* cleavage activity of HIV-1 RNA-specific ribozymes has been shown not to correlate with their *in vivo* cleavage activity in human cell lines (Koseki et al, 1999; Crisell et al 1993; Ramezani and Joshi, 1996; Ventura et al, 1994; Domi et al, 1996; Kuwabara et al, 1999). A ribozyme targeted against the HIV-1 5' leader sequence, although active *in vitro*, was less active upon testing in HeLa and H9 cells (Koseki et al, 1999). A ribozyme against the first coding exon of the HIV-1 *tat* which possessed short flanking sequences performed better *in vitro* than ribozymes with longer flanking sequences (Crisell et al, 1993). However, upon testing in Jurkat cells, the opposite was the case. Similarly, a ribozyme targeted against the HIV-1 *env* coding region cleaved poorly *in vitro*, but demonstrated the highest inhibition against viral replication in the MT4 cell line (Ramezani and Joshi, 1996). On the other hand, ribozymes targeted against the HIV-1 R region (Ventura et al, 1994) or 5' leader sequence (Domi et al, 1996) were catalytically inactive *in vitro* but were found to be active in a cellular environment. A dimeric maxizyme possessing a 2-bp common stem loop II demonstrated weak activity *in vitro* against the HIV-1 *tat* coding region, but in transiently transfected HeLa cells expressing a chimeric HIV-1 LTR and luciferase gene, luciferase activity was inhibited by up to 90% (Kuwabara et al, 1999). Thus, selection of ribozymes on the basis of their *in vitro* activity alone may eliminate molecules with increased therapeutic potential *in vivo*. *In vivo* systems are therefore required for screening ribozymes with increased/altered catalytic activities. The development of such screening systems should greatly accelerate ribozyme applications, for example in gene therapy.

Ribozymes have been shown to be active in bacterial cells. A ribozyme targeted against the *A2* coding region of RNA coliphage SP was tested in *E. coli*. Cells expressing this ribozyme produced less progeny phage than those expressing the inactive ribozyme (Inokuchi et al, 1994). Ribozyme cleavage of HIV-1 RNA target sites have also been demonstrated in bacterial cells. RNA containing the *IN* coding region of HIV-1 and a ribozyme targeted against it were expressed under control of the T7 promoter in bacteria producing T7 RNA polymerase (Sioud and Drlica, 1991). Upon induction, integrase mRNA could not be detected by analyzing RNA extracted from bacteria expressing the active ribozyme. However, it was present when an inactive ribozyme was expressed. Induction of target RNA synthesis prior to ribozyme induction led to the detection of one of the cleavage products. The amount of integrase protein produced *in vivo* was also shown to be decreased by Western blot analysis. Ribozymes targeted against the *RT* and *pro* coding regions within the HIV-1 RNA were also tested in *E. coli* expressing an RNA containing HIV-1 *pro* and *RT* coding regions (Ramezani et al, 1997). *Trans* cleavage of HIV-1 RNA was demonstrated by semi-quantitative RT-PCR and HIV-1 RT activity assay. However, although ribozyme activity against HIV-1 RNA could be demonstrated in both of these studies (Sioud and Drlica, 1991; Ramezani et al, 1997), the assays used were rather time consuming, and thus would not allow the fastest possible screening of ribozyme activity *in vivo*.

We were interested in designing an *E. coli* based indicator cell system for rapid initial screening of active ribozymes without performing extensive biochemical characterizations. In the proposed bacterial indicator cell system (**Figs. 1, 2**), a ribozyme and its target site were cloned in frame within the *lac*Z open reading frame (ORF) present in the plasmid pGEM4Z, which gives rise to the α fragment of β-galactosidase. Accordingly, the *lac*Z transcript would contain the ribozyme and its target site *in cis*. Ribozyme-mediated cleavage of the target RNA would prevent its translation and thus production of the α fragment of β-galactosidase. Complementation between the α fragment and the ω fragment (expressed in certain *E. coli* strains) of β-galactosidase would not occur. In the presence of a chromogenic substrate such as 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), β-galactosidase would catalyze the formation of 5-bromo-4-chloro-indigo, a blue-coloured product. If the enzyme is absent, the substrate would not break down and remain colourless. Thus, an effective ribozyme should lead to the formation of white, in contrast to blue, colonies on agar plates containing X-gal and isopropylthio-β-D-galactoside (IPTG), an inducer of the *lac* operon.

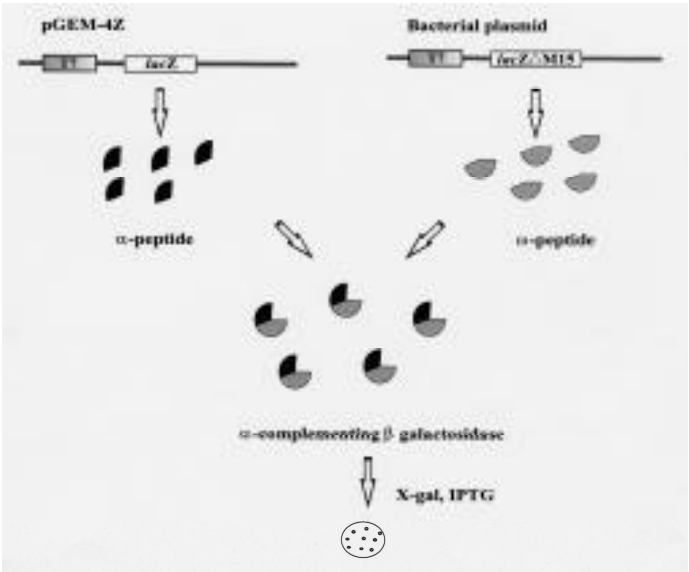

FIG. 1A. Indicator cell system for monitoring ribozyme cleavage activity *in vivo*. α-complementation between the α-peptide produced from plasmids containing the N-terminal portion of the *lac*Z gene and bacteria which express the ω-peptide leads to formation of blue colonies in agar plates with X-gal and IPTG.

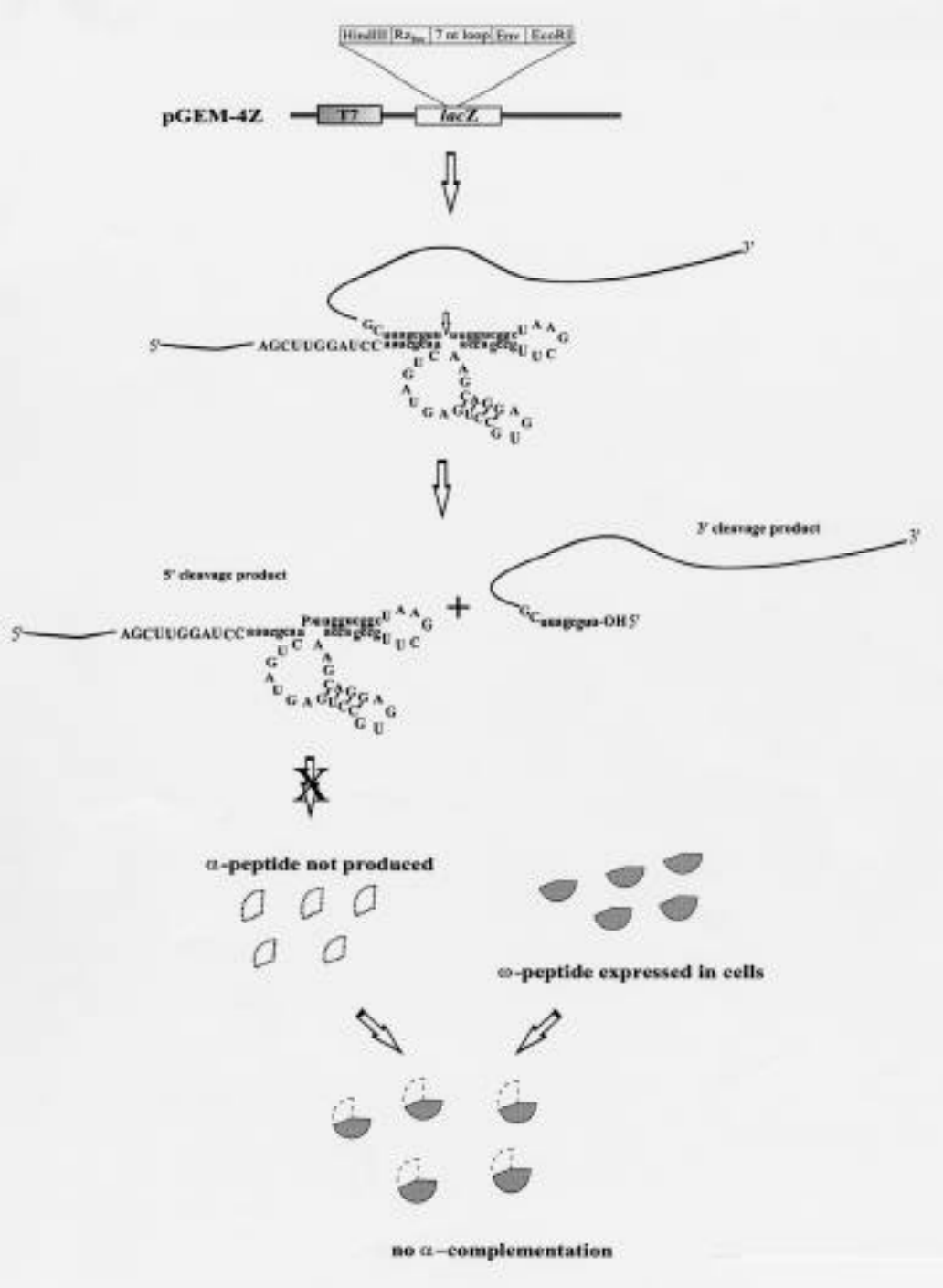

Fig 1 (Cont.) (1B) An oligonucleotide was designed which contained the ribozyme and its target sequence downstream. A 7-nt loop was placed between the ribozyme and its target sequence to allow folding and consequent hybridization of the two sequences. This loop (UUCGAAU) was designed so that it closely resembles a naturally occurring loop such as the tRNA anticodon loop (U/CUNNNG/AN; 31). *Hin*d III and *Eco*R I sites were added on either side of the oligonucleotides to allow cloning between the *Hin*d III-*Eco*R I sites of the plasmid pGEM4Z located at the *lac*Z gene. The oligonucleotide was thus *Hin*d III-Rz_{Env}-loop-Env-*Eco*R I. Additional nucleotides were added such that insertion by itself of the oligonucleotide would not affect the reading frame of the *lac*Z gene present in pGEM4Z. Upon *in vitro* transcription, the ribozyme cleaves its target site, thereby inactivating the *lac*Z mRNA. Because the α-peptide is not produced, α-complementation does not occur, which leads to formation of white colonies in agar plates with X-gal and IPTG. Ribozyme catalytic domain and 7-nt loop are shown in large case. Ribozyme flanking sequences and the target sequences to which they bind are shown in small case. ⇩ denotes cleavage site.

A yeast splicing protein was found to interact *in vivo* with a ribozyme and block its intracellular activity (Castanotto et al, 1998), whereas the nucleocapsid protein of HIV-1 (Tsuchihashi et al, 1993; Bertrand and Rossi, 1994; Herschlag et al, 1994; Moelling et al, 1994; Muller et al, 1994; Mahieu et al, 1995; Hertel et al, 1996), the heterogeneous nuclear ribonucleoprotein A1 (Bertrand and Rossi, 1994; Herschlag et al, 1994) and glyceraldehyde-3-phosphate dehydrogenase (Sioud and Jespersen, 1996) were found to enhance ribozyme activity. Since hammerhead ribozymes are found in plant pathogens (viroid and satellite RNAs of viruses), plant proteins may also be found which could enhance ribozyme cleavage. Lack of complete cleavage both *in vitro* and *in vivo* in bacterial and mammalian cells may reflect the absence of proteins which enhance ribozyme activity. Thus, aside from the assessment of ribozyme cleavage activity *in vivo*, a bacterial system may also be used for cloning protein co-factors which could affect ribozyme activity *in vivo*.

II. Results

A. Bacterial indicator system for identification of ribozymes capable of *in vivo* cleavage

E. coli DH5α cells contain a portion of the *lac*Z gene which encodes for the ω fragment of β-galactosidase. Transformation of these cells with plasmids expressing the α fragment of β-galactosidase leads to complementation between the α and ω fragments and the consequent assembly of an active enzyme, whose activity can be detected by chromogenic substrates (**FIG. 1*A***). A ribozyme (Rz_{Env}) was therefore designed to cleave the *lac*Z mRNA coding for the α fragment of β-galactosidase. This was achieved by cloning Rz_{Env} and the *env* target sequence in frame within the *lac*Z gene of plasmid pGEM4Z. Upon transcription of this modified *lac*Z gene, *lac*Z mRNA would be produced which contains Rz_{Env} and its *env* target sequence. If this mRNA remains intact, then the ribozyme must have been incapable of *in vivo* cleavage. This should lead to the formation of blue colonies on agar plates containing X-gal and IPTG. In contrast, if the conditions *in vivo* are suitable for cleavage, then the ribozyme should hybridize to its target located downstream and cleave it, effectively cutting the *lac*Z mRNA into two. Bacteria harbouring ribozymes capable of *in vivo* cleavage would not produce the α fragment of β-galactosidase and, as a result, would give rise to white colonies on plates containing X-gal and IPTG (**FIG. 1*B***). The colour of the colonies should thus correlate with *in vivo* cleavage of the ribozyme target site present in the *lac*Z mRNA. A ribozyme's ability to cleave *in vivo* may therefore be easily and quickly assessed by monitoring the colour of the colonies which result after transformation in *E. coli* cells.

B. Ribozyme cloning, *in vivo* screening and characterization

Oligonucleotides containing the ribozyme and its target sequence were synthesized. A 7-nt loop was placed between the ribozyme and its target sequence to allow folding and consequent hybridization of the two sequences. This loop (UUCGAAU) was designed to closely resemble a naturally occurring loop, such as the tRNA anticodon loop (U/CUNNNG/AN; Stryer, 1988). Additional nucleotides were added so that insertion by itself of the oligonucleotide would not affect the reading frame of the *lac*Z gene present in pGEM4Z. After cloning, ligated plasmids were used to transform *E.coli* cells. Cells were then plated on X-gal/IPTG plates. Twenty-four colonies which ranged in colour from white to light blue were screened by restriction enzyme analysis and quickly assayed for β-galactosidase activity. Clones #4, #18 and #21 demonstrated correct restriction enzyme patterns and lower β-galactosidase activities compared to cells expressing the plasmid pGEM4Z. Colonies #4 and #21 were light blue, while #18 was white on LB agar plates containing X-gal and IPTG. β-galactosidase activities of extracts from all three colonies were consistently lower, compared to extracts from cells expressing pGEM4Z (**Table 1**).

Table 1. β-galactosidase activity of pGEM4Z clones*

clone #	colour of colony	β-gal activity*
4	light blue	1.42
18	white	9.60
21	light blue	<0
pGEM4Z	dark blue	23.14

*The values listed are the average of two experiments.
Unit of β-galactosidase activity = $1000 \times [A_{420} - (1.75 \times A_{550})] / (t \times 0.1 \times A_{600})$, where t = time in minutes
N.A., not applicable

Upon sequencing, all three clones were found to contain mutations (**FIG. 3**). Clone #4 contained an insertion (G) in the ribozyme flanking sequence. Clone #18 contained a substitution (G → T) in stem loop II of the ribozyme catalytic domain and a deletion (C) in the ribozyme flanking sequence. Clone #21 contained an insertion (C) in the 7-nt loop connecting the ribozyme and the target sequence. Three additional clones that were picked and sequenced also contained mutations (data not shown). In a second set of experiment, twenty-four colonies picked from the ligation using the partially overlapping oligonucleotides were also characterized. Three colonies from this set were sequenced. Instead of single point mutations, tracts of mutated sequences were observed (data not shown). These could have resulted from mis-alignment of the partial overlap during the extension reaction performed prior to cloning.

C. Cis and *trans* cleavage activity *in vitro* of a cloned ribozyme

Of the three clones selected, clone #21 contained a mutation in the loop region between the ribozyme and the

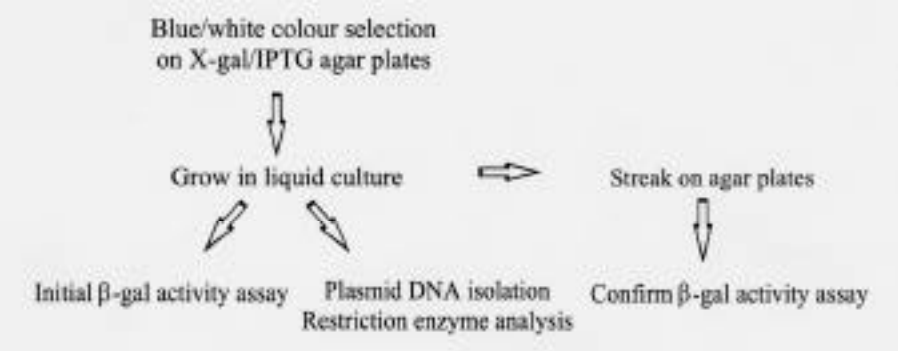

FIG. 2. Overview of the selection procedure for colonies with reduced β-galactosidase activity. Selection of clones able to cleave *in vivo* was mediated by the chromogenic substrate X-gal which was added to agar plates. Lighter coloured clones expressing ribozymes were picked and grown in liquid culture, and used for an initial assay. Plasmid DNA isolated was subjected to restriction enzyme analysis. The cultures were re-streaked on agar plates. Isolated colonies were used in a β-galactosidase assay to confirm lack of *lac*Z mRNA expression.

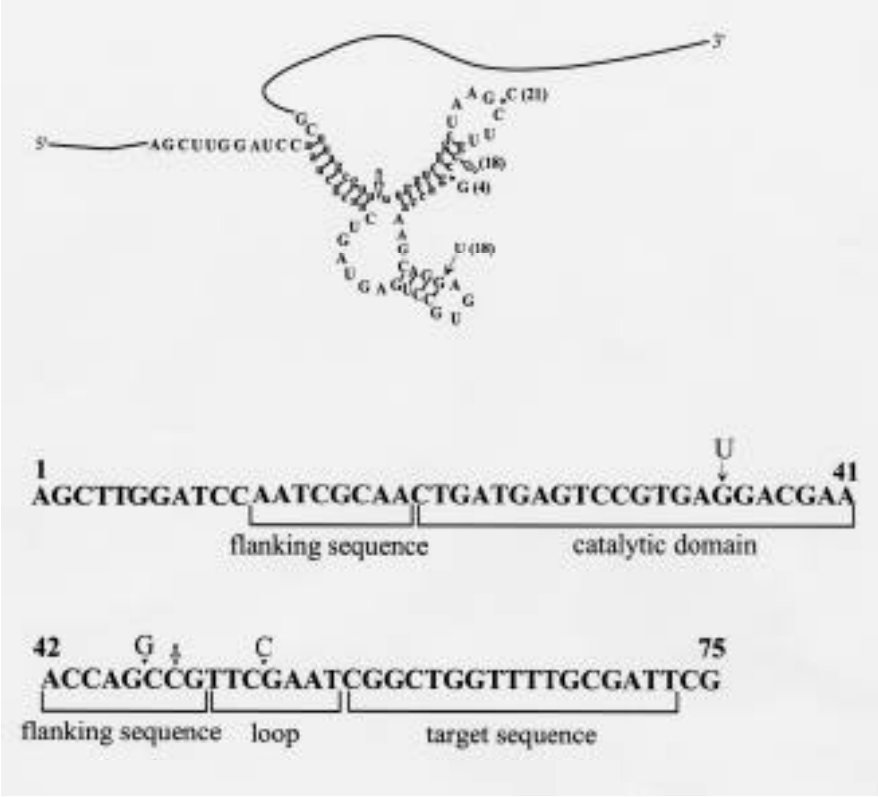

FIG. 3. Sequences of the Rz_{Env} clones. Mutations in the sequences of clones 4, 18 and 21 are indicated as → for substitution, ▲ for insertion and ⇨ for deletion. The numbers in parentheses correspond to the clone # in which the mutation was found. Ribozyme catalytic domain and 7-nt loop are shown in large case. Ribozyme flanking sequences and the target sequences to which they bind are shown in small case. ⇩ denotes cleavage site. The sequence of the 75-nt insert is shown at the bottom. Locations of the different mutations within the flanking sequences, ribozyme catalytic domain and the loop region are indicated by arrows.

target site. This mutation was not expected to affect ribozyme cleavage *per se*. The ribozyme and target site from pGEM-Rz_{Env}-Env #21 were PCR amplified and the PCR products transcribed *in vitro*. The PCR product was then used in an *in vitro* transcription and cleavage reaction (**FIGS. 4*A*, 4*B***). *Cis* cleavage occurred during the *in vitro* transcription reaction itself. This demonstrates that the ribozyme cloned in pGEM-Rz_{Env}-Env #21 was functional *in vitro*.

Relative occurrence of *cis* and *trans* cleavage *in vitro* of RNA containing Rz_{Env}-Env sequences was determined as follows. The RNA containing the Rz_{Env} target site was transcribed separately and added to the *in vitro* transcription mixture of pGEM-Rz_{Env}-Env #21, and the *cis* and *trans* cleavage products were analyzed by PAGE (**FIG. 4*C***). *Trans* cleavage did not occur for up to 2 h incubation. Thus, only *cis* cleavage occurred under the conditions used for *in vitro* transcription.

To determine whether the ribozyme possesses *trans* cleavage ability, ribozyme (without the *cis* target site) was PCR amplified from clone #21 and the PCR product transcribed *in vitro*. This RNA was then used in an *in vitro trans* cleavage reaction using a target RNA which was PCR amplified and transcribed separately. The ribozyme was able to cleave the target RNA *in trans* (**FIG. 5**). Thus, lack of *trans* cleavage in the presence of a *cis* target site (**FIG. 4*C***) is due to the higher efficiency of *cis* cleavage.

III. Discussion

Although *in vitro* selection techniques may allow the identification of ribozymes with improved catalytic activity, the *in vivo* performance of these ribozymes may not correlate with their activity *in vivo*. *In vivo* ribozyme activity may be rapidly assessed using a bacterial indicator system, provided that a strategy is designed which allows correlation of *in vivo* ribozyme activity with a bacterial phenotype.

We attempted to test activity of the enzyme β-galactosidase produced by l*ac*Z mRNA to monitor ribozyme activity *in vivo* (**FIG. 1**). Sequences encoding Rz_{Env} and its target site were cloned *in cis* within the N-terminal region of the *lac*Z gene in pGEM-4Z. Bacterial cells were then transformed with pGEM-Rz_{Env}-Env plasmids. Upon transcription, Rz_{Env} should have bound to and cleaved its target site, thereby inactivating the *lac*Z transcript coding for the α fragment of β-galactosidase. Absence of the α fragment should have prevented formation of a functional enzyme *via* complementation. White colonies likely to contain active Rz_{Env} were identified on agar plates containing X-gal and IPTG.

Ribozyme's ability to cleave *in cis* was demonstrated during *in vitro* transcription (**FIGS. 4*A* and *B***). Upon addition of a target RNA containing Rz_{Env} target site, only the products corresponding to *cis* cleavage were detected (**FIG. 4*C***). However, this does not rule out the ability of Rz_{Env} to cleave *in trans*. The ribozyme was indeed able to cleave the target RNA under *trans* cleavage conditions (**FIG. 5**). Thus, *cis* cleavage occurs with higher efficiency than *trans* cleavage. The use of a *cis* cleaving ribozyme is therefore a logical choice in establishing a bacterial indicator cell system.

pGEM-Rz_{Env}-Env plasmid designed to contain the ribozyme and its target sequence was used to transform *E. coli* cells. Colonies which had reduced β-galactosidase activity based on their colour on LB agar plates containing X-gal and IPTG were identified. Lack of β-galactosidase activity within the bacterial cell extracts was confirmed by performing an assay using ONPG as a substrate (**Table 1**). Plasmid DNA from the clones was isolated and analyzed by restriction enzyme analysis. However, sequencing results revealed that mutations were present in the insert (**FIG. 3**).

The mutations present in the clones may have caused formation of white colonies by disruption of the *lac*Z ORF. In addition, Rz_{Env} could have cleaved its target site *in vivo* which could have further decreased the number of *lac*Z mRNAs available for translation of the α fragment of β-galactosidase. Therefore, the observed reduction in β-galactosidase activity in these clones could be due to an additive effect between the mutations and ribozyme activity. However, because clones containing both an active ribozyme and a frameshift mutation were the only ones which reduced β-galactosidase activity to a detectable level, only these clones were selected. Clones containing the correct ribozyme and target sequence may have been missed, as these may have appeared blue on agar plates with X-gal/IPTG and therefore not selected for further analysis. As seen during the *in vitro* transcription and cleavage reaction using pGEM-Rz_{Env}-Env #21, some of the RNA may have remained uncleaved in *E. coli*, which could then be used in translation.

Using a similar blue/white colour selection, Chuah & Galibert (1989) could successfully demonstrate the activity of a *cis* cleaving ribozyme but not of a *trans* cleaving ribozyme. In this study, a ribozyme targeted to *lac*Z mRNA was cloned within the *lac*Z coding region of plasmid $M_{13}mp_8$ to allow co-expression of the ribozyme and its target site within the same RNA molecule *in vivo*. Upon transcription, the ribozyme was expected to cleave the *lac*Z mRNA *in cis*. Out of 18 white plaques tested, 15 contained the correct ribozyme sequence, while 3 were due to cloning of aberrant sequences leading to the loss of the ORF. When the ribozyme was designed and expressed to *trans* cleave the *lac*Z RNA encoding ω fragment of β-galactosidase transcribed in *E. coli* from the episome, all of the isolated white plaques were due to the presence of incorrect sequences (Chuah & Galibert, 1989).

In our study, all of the isolated white colonies were due to mutations. The discrepancy between our results and those by Chuah and Galibert (1989) could be due to a number of reasons. The ribozyme that we designed cleaved the 5' end of the *lac*Z mRNA coding for the α fragment. This may have been less effective in reducing the amount of protein produced than if the target chosen was further downstream as is the case in Chuah and Galibert's study (1989). The ribozyme used in our study may have been less active than the ribozyme used by Chuah and Galibert (1989). However, Rz_{Env} was shown to cleave the *lac*Z mRNA ***in vitro*** **(FIG. 4*A*, 4*B*)**; the majority of the RNA was cleaved *in cis*, suggesting that the design of the construct was appropriate. Since the ribozyme was in very close proximity to its target site, it is also unlikely that the ribozyme was bound to sequences other than its downstream target, forming an inactive complex.

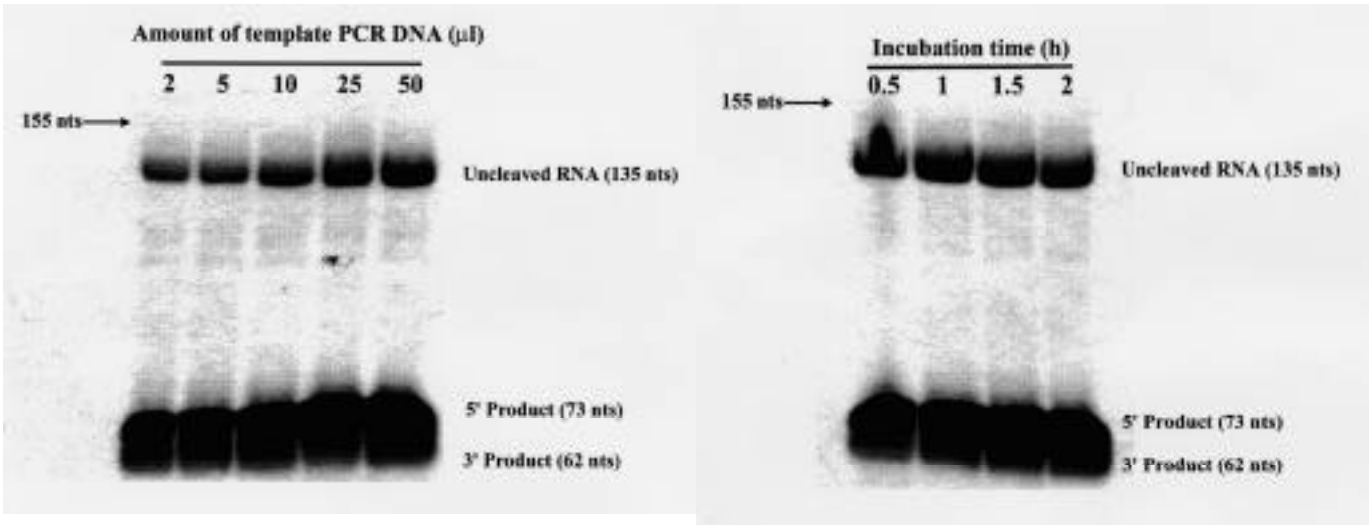

FIG. 4A,B. Cis and trans cleavage activity of Rz_{Env}-Env. **(4A, Left):** Increasing amounts of PCR DNA (2, 5, 10, 25 or 50 µl) were used for in vitro transcription for 2 h. **(4B, Right):** PCR DNA (30 µl) was used for in vitro transcription and incubated at increasing time intervals (0.5, 1, 1.5 or 2 h). The full-length transcript (135 nts) along with the 5' (73 nts) and 3' (62 nts) cis cleavage products were detected.

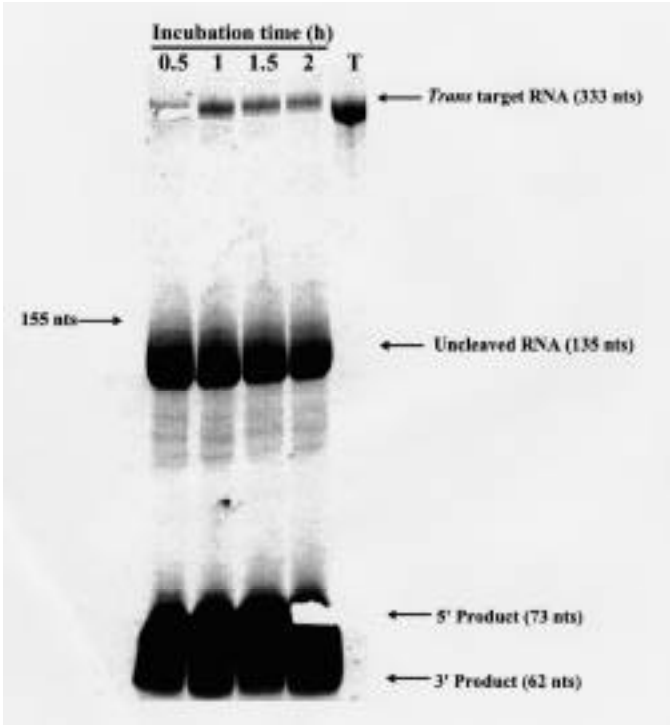

Fig. 4C: Same as B, except that RNA (333 nts) containing the ribozyme target site was added to each transcription mixture. Products (170 nts and 163 nts) which would result from *trans* cleavage were not detected. Only the *cis* cleavage products (73 nts and 62 nts) and the full-length transcript (135 nts) were detected. T, target RNA alone. The uncleaved target RNA in lane "0.5" and 5' cleavage product in lane "2" have been excised from the gel and used for subsequent experiments.

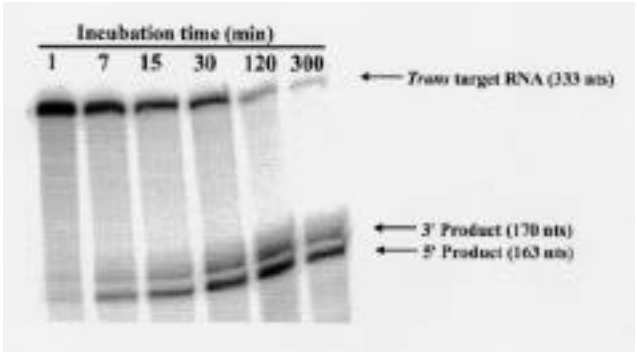

FIG. 5. *Trans* cleavage activity of pGEM-Rz_{Env}. Rz_{Env} and [α-^{32}P]-labelled target RNA were used in a *trans* cleavage reaction. Aliquots were taken at the indicated time intervals and analyzed by 8 M - 8% polyacrylamide gel electrophoresis followed by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA).

Another possibility is that the white plaques obtained by Chuah and Galibert (1989) may have been due to mutations which occurred elsewhere in the cloning vector and not in the insert. Also, the substrate (X-gal) concentration we have used in our system (800 µg X-gal/plate) was higher than the amount used by Chuah and Galibert (4 µg X-gal/plate). Thus, it is conceivable that our system is too sensitive, allowing small amounts of β-galactosidase to produce a detectable blue-coloured product.

Ribozymes tested against HIV-1 *pro* (Ramezani et al, 1997), *RT* (Ramezani et al, 1997) and *IN* (Sioud and Drlica, 1991) coding regions were shown to be active in *E. coli*. However, in these studies ribozyme activities were demonstrated by assays that relied on the presence of cleaved RNA and their translation products. On the other hand, the system we and Chuah and Galibert (1989) have utilized detected the presence of uncleaved products. Thus, although the majority of the *lac*Z RNA may have been cleaved *in vivo*, protein translated from the remaining uncleaved transcripts catalyzed the breakdown of the substrate to a blue coloured product, which could still be detected by the assays used. As such, the blue/white colour selection may not accurately report the *in vivo* cleavage activity of a ribozyme, since colonies containing mutations were the only ones that could be isolated in our study. For successful development of a ribozyme screening system, the amount of substrate used may have to be titrated for each ribozyme. However, this may be time-consuming. Alternatively, substrates may have to be used which have a higher cut-off limit of detection, requiring a higher amount of β-galactosidase before a colour change is observed. Thus, only those cells which are producing high amounts of β-galactosidase may turn blue.

IV. Materials and methods

A. Oligonucleotide design and cloning of Rz_{Env}-Env into pGEM4Z

Cloning of sequences encoding Rz_{Env} (Medina and Joshi, 1999) and its HIV-1 *env* target site was performed using two sets of oligonucleotides. The first set consisted of partially overlapping oligonucleotides (53-54-nt) which were first extended *in vitro* and then cloned. The second set consisted of two complementary oligonucleotides that contained ribozyme and target sequences flanked by restriction sites, which were cloned directly into the plasmid pGEM4Z. Both ligations yielded >100 colonies upon transformation into *E. coli*.

Rz_{Env} was designed to cleave after a highly conserved GUU (nt 665 to nt 667) sequence within the *env* coding region of HIV-1 HXB2 RNA (Myers et al, 1995). Partially overlapping oligonucleotides (5'-CCC-CCC-*AAG-CTT-GGA-TCC*-aat-cgc-aa**C-TGA-TGA-GTC-CGT-GAG-GAC-GAA**-acc-agc-3' and 5'-GGG-*GAA-TTC*-C**aa-tcg-caa-aac-cag-ccg**-*att-cga-a*cg-gct-ggt-**TTC-GTC-CTC-AC**-3') were synthesized using the Expediteä Nucleic Acid Synthesis System (Millipore; Etobicoke, Canada). Before cloning, these oligonucleotides were extended to full-length complementary oligonucleotides for 1 h at 37°C in a 40 µl reaction containing 50 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 8µM of each dNTP, and 5 units of Klenow (Life Technologies; Burlington, Canada). The fill-in products were ethanol-precipitated and resuspended in water and digested with *Hin*d III and *Eco*R I. In a second set of experiment, complementary oligonucleotides with 5' overhangs to allow cloning (5'-AGC-TT*G-GAT-CC*a-atc-gca-a**CT-GAT-GAG-TCC-GTG-AGG-ACG-AA**a-cca-gcc-g*tt-cga-at***c-ggc-tgg-ttt-tgc-gat-t**CG-3' and 5'-AAT-TCG-**aat-cgc-aaa-acc-agc-cg***a-ttc-gaa*-cgg-ctg-gt**T-TCG-TCC-TCA-CGG-ACT-CAT-CAG**-ttg-cga-tt*G-GAT-CC*A-3') were synthesized. Ribozyme catalytic domain is in uppercase bold, 8-nt flanking sequences complementary to either side of the cleavage site are in lowercase, target sequence is in lowercase bold, loop sequence is in lowercase italics, restriction enzyme sites are in uppercase italics, and 5' overhangs are underlined. The full-length oligonucleotides (75 nts) are of comparable length to the *Hin*d III-*Eco*R I fragment (54 nts) being removed from pGEM4Z.

Plasmid pGEM4Z (Promega Corp.; Madison, USA) was transformed into *E. coli* strain DH5α, isolated by a miniprep procedure and digested with *Hin*d III and *Eco*R I. The DNA band corresponding to the *Eco*R I-*Hin*d III fragment was eluted using the Geneclean kit (BIO 101; Vista, USA) following 1% agarose gel electrophoresis. Full-length oligonucleotides were then cloned as described in (Sambrook et al, 1989) at the *Hin*d III and *Eco*R I sites within the *lac*Z gene of pGEM4Z. Ligation reactions (10 µl) containing 50 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000, double stranded insert (300-5000 ng), vector (10 ng) and 1 unit T4 DNA ligase (Life Technologies; Burlington, Canada) were performed at 23°C for 1h. DH5α competent cells were transformed with the ligation mix and plated on Luria-Bertani (LB) agar plates containing ampicillin (50 µg/ml), X-gal (800 µg) and IPTG (0.4 µmol). A positive transformation control consisting of pGEM4Z DNA yielded over 300 colonies, a negative transformation control without DNA yielded no colonies, and the ligation mixtures each yielded ~50 colonies. Colonies which ranged in size and colour from white to light shades of blue were picked and screened by *Csp*45 I, *Dra* I, *Sma* I, *Bam*H I, *Eco*R I and *Hin*d III restriction enzyme analyses. DNA sequencing was performed using the T7 Sequencing Kit (Pharmacia Biotech Inc.; Baie d'Urfé, Canada) using instructions provided by the supplier.

B. β-galactosidase activity of individual pGEM-Rz_{Env}-Env clones

Individual colonies were picked and grown overnight in LB medium containing ampicillin (50 µg/ml). The next day, β-galactosidase activity was assayed using cultures at an optical density at wavelength of 600 (OD_{600}) equivalent to 1.00. The cultures were incubated for another 4 h after adding IPTG (0.1 µmol) and X-gal (200 µg). Cells were pelleted by spinning for 3 min at 8000*g*. OD_{550} and OD_{420} of the supernatants were measured to quickly assess β-galactosidase activity using culture medium containing IPTG and X-gal as a blank.

For clones selected for further characterization, β-galactosidase activity was assayed using *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) as substrate (30) and LB cultures at the logarithmic growth phase with OD_{600} values between 0.28-0.70. After cooling on ice for 20 minutes, cell cultures (100 µl) were mixed with 50 µl 0.1% SDS, 100 µl chloroform and 900 µl Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), vortexed for 10 seconds, and incubated at 28°C for 5 minutes. ONPG (4

mg/ml, 200 µl) was added to each tube, and the incubation continued for 80 to 220 min at 37°C. Reactions were stopped by adding 500 µl of 1M Na_2CO_3. OD_{550} and OD_{420} were then measured.

C. Cis and *trans* cleavage activity of Rz_{Env}-Env

To detect *cis* cleavage activity, ribozyme and HIV-1 *env* target site were PCR amplified from pGEM-Rz_{Env}-Env #21 using a forward primer (5-CGA-AAT-TAA-TAC-GAC-TCA-CTA-TA-3') which binds to the T7 promoter and a reverse primer (5'-GTA-AAA-CGA-CGG-CCA-GT-3') which binds downstream of the ribozyme target site. PCRs were performed for 30 cycles (1 min, 95°C; 1 min, 56°C; 1 min, 72°C each). PCR DNA (2-50 µl) containing the T7 promoter sequence was transcribed *in vitro* at 37°C in a reaction mixture (100 µl) containing 40 mM Tris-Cl (pH 8.0), 25 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM DTT, 1 mM of each NTP, and 200 units of T7 RNA polymerase (Life Technologies; Burlington, Canada). The reaction was stopped after 0.5-2 h by digesting the template DNA with 5 units of RQI RNase-free DNase (Promega Corp.; Madison, USA) for 10 min. *Cis* cleavage at the HIV-1 *env* target site by Rz_{Env} occurred under the condition used for transcription, without further incubation or addition of reagents. To compare *cis* and *trans* cleavage activities, the *env* target sequence was PCR amplified from the plasmid pHEnv using a primer (5'-ATA-TCA-TAT-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CGA-GTG-CAG-AAA-GAA-TAT-GC-3') which binds upstream of the ribozyme target site and contains the T7 promoter sequence and a primer (5'-GTC-CGT-GAA-ATT-GAC-AG-3') which binds downstream of the ribozyme target site. PCR DNA was transcribed *in vitro* for 2 h at 37°C as described above. After phenol extraction and ethanol precipitation, the target RNA was resuspended in water and then added to the *in vitro* transcription mixture. The cleavage products were analyzed by 8 M urea-8 % polyacrylamide gel electrophoresis (PAGE) followed by methylene blue staining (Sambrook et al, 1989).

D. Trans cleavage activity of Rz_{Env}

Target RNA was transcribed in the presence of [α-^{32}P] UTP (3000 Ci/mmol; Amersham Canada Ltd.; Oakville, Canada). Rz_{Env} was transcribed from PCR DNA which was amplified from pGEM-Rz_{Env}-Env #21 using a forward primer (5-CGA-AAT-TAA-TAC-GAC-TCA-CTA-TA-3') which binds to the T7 promoter and a reverse primer (5'-ATA-TAT-ATC-GAT-AAA-AAA-CGG-CTG-GTT-TCG-TCC-TC-3') which binds near the 3' end of the ribozyme. It was then used in a *trans* cleavage reaction with [α-^{32}P]-labelled target RNA. Essentially, Rz_{Env} and target RNA were combined in a reaction mixture containing 40 mM Tris-Cl (pH 8.0) and 10 mM NaCl. The sample was heated to 65°C for 5 min, cooled to 37°C, and the reaction initiated by adding 20 mM $MgCl_2$. Aliquots were taken after 7, 15, 30, 120, 300, 600 and 900 min incubation at 37°C, and the reaction stopped by addition of loading buffer containing 5 mM EDTA. Cleavage products were analyzed by 8 M urea-8 % PAGE followed by exposure to a phosphor screen and scanning by Storm phosphorimager (Molecular Dynamics; Sunnyvale, USA).

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