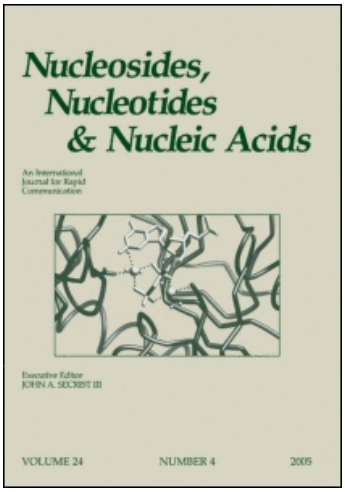

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Koizumi, Makoto , Ozawa, Yuji , Yagi, Ryuichi , Nishigaki, Takashi , Kaneko, Masakatsu , Oka, Shin-ichi , Kimura, Satoshi , Iwamoto, Aikichi , Komatsu, Yasuo and Ohtsuka, Eiko(1998) 'Design and Anti-HIV-1 Activity of Hammerhead and Hairpin Ribozymes Containing a Stable Loop', Nucleosides, Nucleotides and Nucleic Acids, 17: 1, 207 — 218
To link to this Article: DOI: 10.1080/07328319808005170
URL: http://dx.doi.org/10.1080/07328319808005170

NUCLEOSIDES & NUCLEOTIDES, 17(1-3), 207-218 (1998)

DESIGN AND ANTI-HIV-1 ACTIVITY OF HAMMERHEAD AND HAIRPIN RIBOZYMES CONTAINING A STABLE LOOP#

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ABSTRACT: Three ribozymes, a hairpin ribozyme (HR112) and two hammerhead ribozymes (RZ115 and RZ119) containing a 5'C(UUCG)G3' loop were designed to cleave the U5 region in the long terminal repeat (LTR) of HIV-1 RNA. The $t_{1/2}$ values of chemically synthesized substrates mediated by three ribozymes were measured. The transformed CEM cells possessing these three ribozyme-encoding genes were challenged with a HIV-1_{IIIB} strain, and two of these three ribozymes, HR112 and RZ119, were shown to possess strong anti-HIV-1 activity.

INTRODUCTION

Designed hammerhead ribozymes can act as endonucleases that cleave a targeted RNA with a UX*N (X=A, C or U; N=A, G, C or U; the cleavage site is indicated by an asterisk) sequence in high specificity.[1-4] Hairpin ribozymes have also been designed to cleave an RNA with a N*GUC sequence.[5,6] Some ribozymes have been designed to cleave RNAs that are related to diseases, such as AIDS and cancer.[7-9] Both hammerhead and hairpin ribozymes containing a stable 5'C(UUCG)G3' loop have been shown to have high cleavage

This paper is dedicated to the memory of the late Professor Tsujiaki Hata.

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activities.[10-12] A significant discovery was that an oligoribonucleotide derived from a mutated *ras* mRNA can be cleaved by a designed hammerhead ribozyme containing a stable C(UUCG)G loop.[10] The activity of these two types of ribozymes was compared in experiments that targeted β-amiloid peptide precursor mRNA or α-lactalbumin mRNA in the culture cells,[13, 14] and as a result it was found that these ribozymes had similar cleavage activity *in vitro* and *in vivo*. Furthermore, plasmid-vectors containing the *cis*-active sequences of each of the ribozymes, hammerhead, hairpin or hepatitis δ virus, were used for the comparison of these ribozyme activities.[15]

Some hammerhead ribozymes have been designed to cleave HIV mRNA as part of anti-HIV-1 gene therapy.[16-20] Recently, hairpin ribozymes were developed as the catalytic RNA for clinical gene therapy.[12, 21] The anti-HIV-1 activity of these ribozymes for the preclinical study was measured by the infection of HIV-1 virus in transformants with mouse retrovirus vectors or the plasmid vectors possessing ribozyme-encoding genes.

Herein we report on the design of two hammerhead ribozymes and a hairpin ribozyme with sequences targeted against the U5 region in the LTR of HIV-1 RNA, and the comparisons that could thus be made between the RNA cleavage activity and the anti-HIV-1 activity of these ribozymes.

MATERIALS AND METHODS

Synthesis of Oligonucleotides

Oligoribonucleotides were synthesized by the phosphoramidite method, using 5'-*O*-dimethoxytrityl-2'-*O*-*tert*-butyldimethylsilyl nucleosides 3'-*O*-phosphoramidite (Milligen), on Cyclone DNA/RNA synthesizer (Milligen). Deprotection and purification of synthetic oligoribonucleotides were performed with modifications to the procedures.[22, 23] In brief, the protected oligoribonucleotide was cleaved from the support (1 μmol scale) using c.NH_4OH-EtOH (3:1 v/v, 2 ml). The solution was heated at 55 °C for 16 hr. The reaction mixture was evaporated to dryness, then 1 M tetrabutylammonium fluoride in THF (1 ml, Aldrich) was added, and the solution was stirred for 18 hr. Triethylammonium acetate (0.1 M TEAA, 5 ml) was added, and the solution was chromatographed on a preparative C-18 reverse phase column (Waters) with a linear gradient of 10-40% CH_3CN in 50 mM triethylammonium bicarbonate. The fractions containing the 5'-dimethoxytritylated product were repeatedly evaporated with H_2O. Hydrochloric acid (0.01 N, pH 2.0) was added, the solution was stirred for 1 hr at room temperature, and then neutralized with 0.1 N NH_4OH. The solution was washed with AcOEt, and was evaporated. The deprotected oligoribonucleotide was

purified by reverse phase HPLC (column: Inertsil PREP-ODS, 10 x 250 mm., GL sciences, Japan) with a linear gradient of CH_3CN in 0.1 M TEAA (pH 7.0), and by anion-exchange HPLC (column: TSK gel DEAE-2SW, 4.6 x 250 mm., Tosoh) with a linear gradient of $HCOONH_4$ in 20% CH_3CN.

Oligodeoxyribonucleotides for the construction of plasmids with the ribozyme-encoding genes were synthesized using the phosphoramidite method.

RNA Cleavage Reaction *in vitro*

The substrate RNA (100 pmol) and the hammerhead ribozyme (5 pmol) were separately dissolved in 50 µl of 25 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), and 20 mM NaCl. In the case of the hairpin ribozyme, the substrate RNA (30 pmol) and HR112 (10 pmol) were separately dissolved in 250 µl of 12 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), and 4 mM spermidine 3HCl. The substrate and the ribozyme were separately heated at 70 °C for 5 min, and were then cooled at 0 °C for 2 min. To start the reaction, the substrate solution was mixed with the ribozyme solution, and then the reaction mixture was incubated at 37 °C. Aliquots (30 µl) of the reaction mixture were quenched by the addition of 30 µl of 50 mM EDTA (pH 8.0), and analyzed by reverse phase HPLC (column: Inertsil ODS-2, 6 x 150 mm., GL Sciences, Japan; solvent A: 5% CH_3CN, 0.1 M TEAA (pH 7.0); solvent B: 25% CH_3CN, 0.1 M TEAA (pH 7.0); B%: 5 - 40% (20 min); flow rate: 1 ml/min). In the case of the hairpin ribozyme, aliquots (125 µl) of the reaction mixture were quenched by the addition of 125 µl of 50 mM EDTA (pH 8.0), and analyzed by reverse phase HPLC. A chromatograph equipped with a data processor (Hitachi, model D-2000) was used to quantify the products of this cleavage reaction and thus cleavage rates could be determined.

Construction of plasmids

Plasmids pHR112neo, pRZ115neo and pRZ119neo were constructed by the ligation of two oligodeoxyribonucleotides with the ribozyme sequences into *Pst* I and *Kpn* I-digested pcDL-SRα296,[24] and followed by the ligation of *neo*^{r} gene that was derived from pMC1neo poly A (Stratagene). Plasmid pcDL-SRα296neo was also constructed by the ligation of *neo*^{r} gene into pcDL-SRα296.

Measurement of anti-HIV-1 activity by ribozymes

Each plasmid (pHR112neo, pRZ115neo, pRZ119neo or pcDL-SRα-296neo as a control) was transfected into CEM cells using the DEAE-Dextran method (the kit was purchased

from Pharmacia), and the transformed cells were selected in the medium containing G418. After infecting these transformed cells with a HIV-1_{IIIB} strain, the level of HIV-1 p24 antigen was determined by ELISA (RETRO-TEK™, Cellular Products Inc.).

RESULTS AND DISCUSSION

In vitro cleavage of HIV-1 LTR RNAs by ribozymes

Both the hammerhead and hairpin ribozymes containing a thermodynamically stable 5'C(UUCG)G3' loop[25] have been shown to have high cleavage activities.[10-12] We selected the HIV-1 LTR region as a target for the ribozymes (Fig. 1a), because these target sequences in this region were conserved in HIV-1 strains.[17,21] Two hammerhead ribozymes, RZ115 and RZ119 and a hairpin ribozyme, HR112 were designed as depicted in Fig. 1b-d. The cleavage sites of these three ribozymes have been located in 8 nucleotides in the 112 to 119 segment of the U5 region belonging to LTR as shown in Fig. 1a.

The cleavage reaction of the ribozymes was analyzed by reverse phase HPLC as shown in Fig. 2. The cleavage activity was measured by the quantification of the products of this cleavage reaction, and the cleavage site of the substrate (LTR 19; ^{106}GUG CCC GUC*UGU UGU GUG A^{124}, the cleavage site is represented by an asterisk) with RZ115 was determined by the co-injection of the reaction mixture and the chemically synthesized oligoribonucleotide (5' UGU UGU GUG A 3') as an authentic 3'-product. When the ribozyme activity in cleaving the two chemically synthesized substrates, LTR 19 and LTR 25 (^{103}UGU GUG CCC GUC*UGU UGU GUG ACU C^{127}; the nucleotide sequences longer than LTR 19 are underlined) were measured, the $t_{1/2}$ values of LTR 19 and LTR 25 mediated by RZ115 were lower than those of the RZ119 cases as shown in Table 1. The cleavage rates of LTR 19 by RZ115 and RZ119 were faster than those of LTR 25 by these ribozymes; this may be due to the secondary structures of the substrate with the longer nucleotides.[23] In the case of HR112 as a ribozyme, the $t_{1/2}$ value of LTR 19 was larger than that of LTR 25, which was three nucleotides longer at both 3'- and 5'-ends than LTR 19. These results were consistent with the data by Sekiguchi *et al.*[26] and Vinayak *et al.*[27] It may be shown that the complex between the longer substrate and the hairpin ribozyme easily forms the active bend conformation as shown in ref. 28.

Recently, Vinayak *et al.*[27] have reported on the assay of the ribozyme-substrate cleavage reaction using anion-exchange HPLC. In our system for the analysis of the cleavage reaction, reverse phase HPLC was used rather than anion exchange HPLC, the retention time of which might be influenced by the concentration of salt in the cleavage buffer. The utility of this HPLC does not always need to label the substrate RNA by the radioisotope or the fluorescence group, if a detectable amount of the substrate can be used.

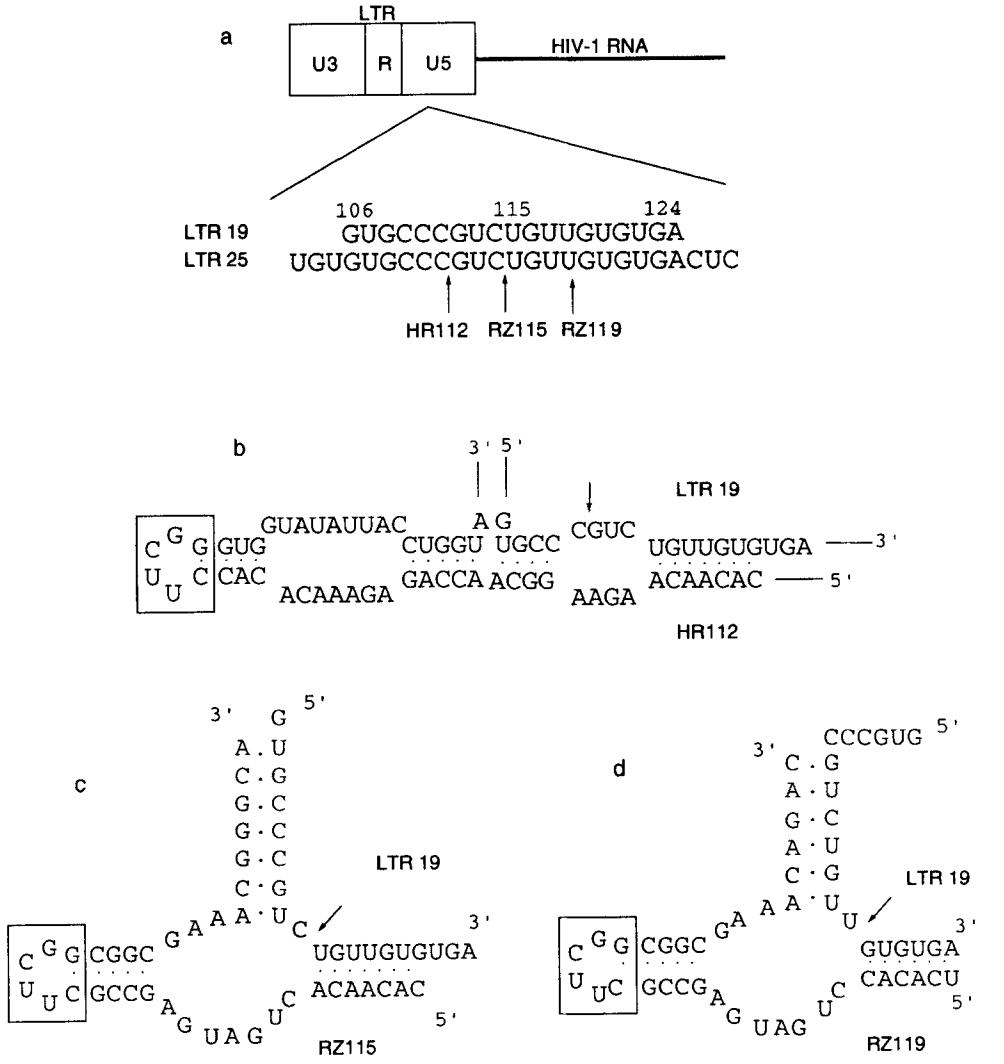

FIG. 1. (a) The location of the cleavage sites by ribozymes in HIV-1 RNA and the sequences of the substrates (LTR 19 and LTR 25) (b) Secondary structures of a hairpin ribozyme (HR112) and (c, d) two hammerhead ribozymes (RZ115 and RZ119), which contain a stable 5'C(UUCG)G3' loop, bearing the 19 mer (LTR 19) as a substrate. Numbering of the substrate is according to the reference by Muesing *et al.* [32] A stable 5'C(UUCG)G3' loop in the ribozyme is boxed.

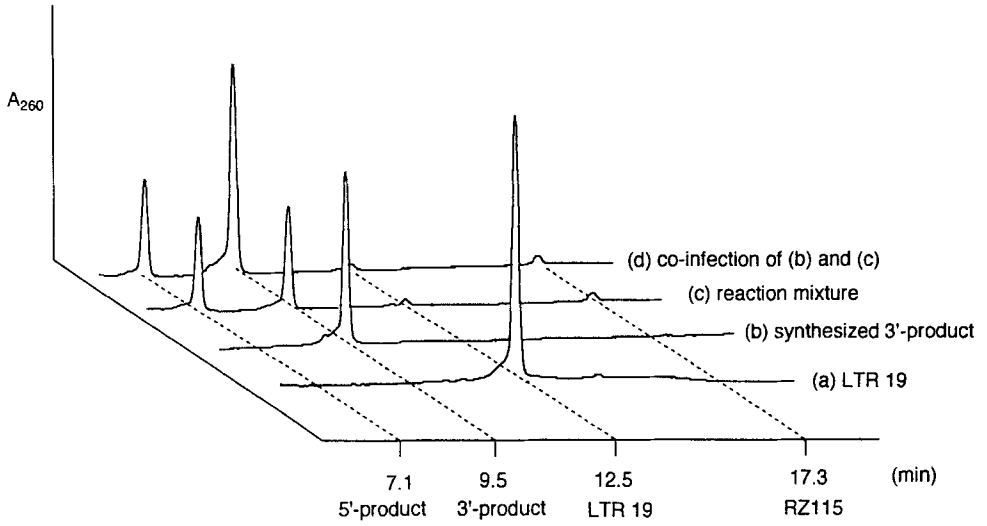

FIG. 2. Analysis of the cleavage reaction by reverse phase HPLC. The conditions of reverse phase HPLC were described in the Materials and Methods. LTR 19 (1.0 μM) was treated with RZ115 (0.05 μM) in the buffer described in the Materials and Methods for 1 hr at 37 °C. (a); LTR 19 (^{106}GUG CCC GUC*UGU UGU GUG A^{124}, the cleavage site is represented by an asterisk.), (b); chemically synthesized 3'-cleaved product (5' UGU UGU GUG A 3'), (c); the reaction mixture of LTR 19 and RZ115, (d); co-injection of (b) the 3'-cleaved product and (c) the reaction mixture.

TABLE 1. Half-lives ($t_{1/2}$) of synthetic substrates (LTR 19 and LTR 25) by ribozymes.

Ribozymes (type)	Half-lives of substrates (min)	
	$t_{1/2}$ of LTR 19	$t_{1/2}$ of LTR 25
HR112 (hairpin)*	140	64
RZ115 (hammerhead)**	9.1	19
RZ119 (hammerhead)**	38	110

* The substrate (0.06 μM) was treated with HR112 (0.02 μM) in the buffer described in the Materials and Methods at 37 °C.

** The substrate (1.0 μM) was treated with RZ115 or RZ119 (0.05 μM) in the buffer described in the Materials and Methods at 37 °C.

The HPLC system may be used in the automatic assay system of the cleavage reaction for the ribozymes.

Inhibition of expression of HIV p24 in ribozyme gene-transfected CEM cells

In order to investigate the anti-HIV-1 activity of these ribozymes, plasmids that expressed the ribozymes under the SRα promoter,[24] were designed as shown in Fig. 3. Plasmids pHR112neo, pRZ115neo and pRZ119neo were constructed to express ribozymes HR112, RZ115 and RZ119, respectively. When each plasmid, pHR112neo, pRZ115neo, pRZ119neo, or pcDL-SRα-296neo as a control, was transfected with CEM cells, stable transformants, pHR112neo/CEM, pRZ115neo/CEM, pRZ119neo/CEM, and pcDL-SRα-296neo/CEM, respectively, were obtained. These transformed cells were infected with HIV-1_{IIIB} strain, and then the p24 level was measured (Fig. 4). A reduction in the p24 level of two transformants, pHR112neo/CEM and pRZ119neo/CEM, was observed up to 12 days. However, inhibition of p24 by RZ115, which possesses high RNA cleavage activity, was weak.

The ribozymes, RZ115, RZ119, and HR112, all containing thermodynamically stable 5'C(UUCG)G3' loops, were designed and their ribozyme activity using two substrates, LTR 19 and LTR 25, was measured by reverse phase HPLC. These three ribozymes were proven to efficiently cleave LTR 19 and LTR 25 as shown in Table 1. Although cleavage sites for the ribozymes were located in 8 nucleotides of HIV-1 LTR (^{111}C*GUC*UGUU*G^{119}; the cleavage sites of these three ribozymes are indicated by asterisks), the anti-HIV-1 activities of these ribozymes were not all at the same level (Fig. 4). It was especially interesting that the anti-HIV-1 activity of RZ115 was weak even though the cleavage site of RZ115 was located in between the cleavage sites of two ribozymes, HR112 and RZ119, whose transcribed RNAs had high anti-HIV-1 activity. RZ115, with weak anti-HIV-1 activity, had the same cleavage site as active ribozymes which were reported by Dropulic *et al.*,[17] and Heidenreich and Eckstein.[29] We assumed that the weak anti-HIV-1 activity of RZ115 *in vivo*, in spite of having high substrate cleavage activity *in vitro*, might be due to the secondary structures of the transcribed RNA with ribozyme sequences. When secondary structures of RNA transcribed from the plasmids encoding the ribozymes were predicted using an RNA secondary structure fold program[30] (GENETYX ver. 8.0 Software Development Co., LTD. Japan) as shown in Fig. 5, the transcribed RNA with RZ115 formed a secondary structure different from that of the RNA transcribed with the other ribozymes, HR112 and RZ119, which efficiently reduced the

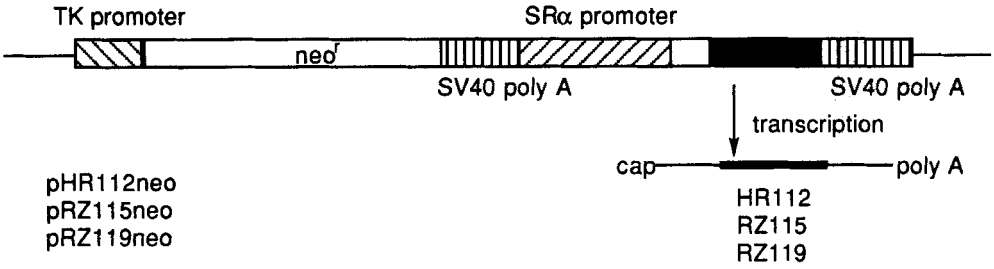

FIG. 3. Construction of plasmids containing the ribozyme-encoding gene (pHR112neo, pRZ115neo, and pRZ119neo).

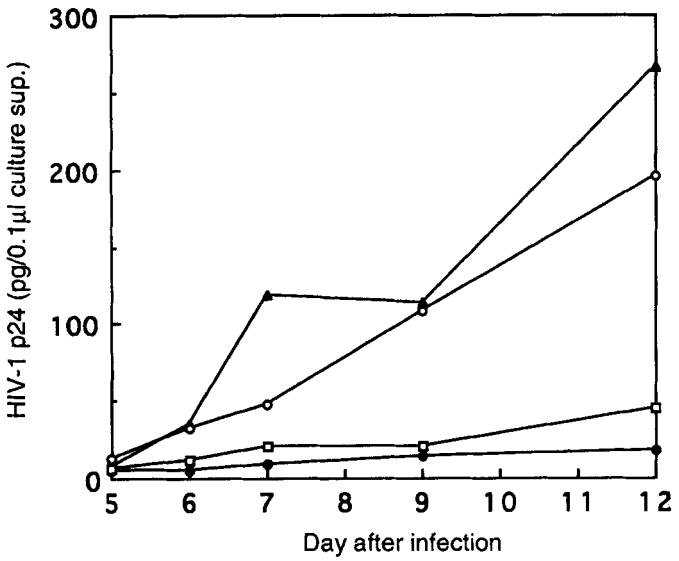

FIG. 4. Inhibition of expression of HIV p24 in ribozyme gene-transfected CEM cells. The p24 values on the culture supernatants of pcDL-SRα296neo/CEM, pRZ115neo/CEM, pHR112neo/CEM, and pRZ119neo/CEM, which were infected with a HIV_{IIIB} strain, are shown as solid triangles, open circles, open squares, and solid circles, respectively.

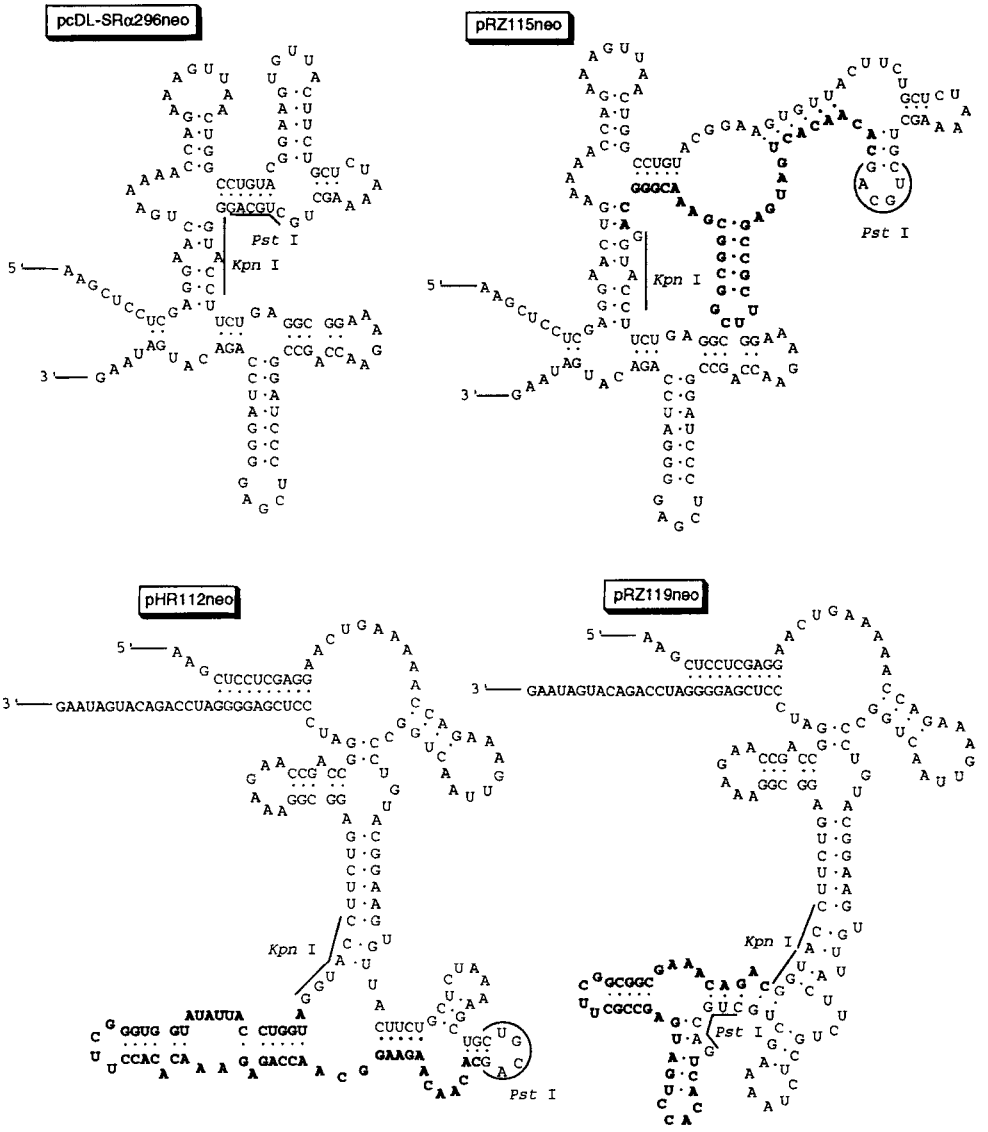

FIG. 5. Prediction of secondary structures of RNAs transcribed from plasmids pcDL-SRα296neo, pRZ115neo, pHR112neo, and pRZ119neo using GENETYX ver. 8.0. Bold letters indicate the ribozyme sequences.

HIV-1 p24 level. In the secondary structures of the transcribed RNA from pRZ115neo in Fig.5, it was shown that base-pairs formed between hybridized arms in the transcribed RNA with RZ115 and 5'-upstream regions of RZ115. However, secondary structures of 3'-upstream or 5'-upstream regions of this transcribed RNA were almost similar to that of the RNA transcribed from pcDL-SRα296neo. On the other hand, in the case of pRZ119neo and pHR112neo, secondary structures of their corresponding RNAs changed, compared to the RNA transcribed from pcDL-SRα296neo. Both 5'CUCCUCGAGG3' and its complimentary sequence formed new base-pairs, and a large internal loop consisting of 20 nucleotides appeared as shown in Fig. 5. The differences in the secondary structures of the ribozyme RNA may influence the resistance of the RNA to cellular nucleases and/or the accessibility of the RNA to the substrate. In these experiments, the importance of the nucleotide sequences derived from these vectors may be taken into account in designing the active ribozymes. Recently, the nucleocapsid protein of HIV-1 and the heterogeneous nuclear ribonucleoprotein A1 enhanced ribozyme catalysis.[31] These proteins may influence the ribozyme activity in our cellular assay system.

We found that the hammerhead ribozyme (RZ119) and the hairpin ribozyme (HR112) containing the 5'C(UUCG)G3' loop had strong anti-HIV-1 activity. When these ribozymes are incorporated into an expression vector, such as a retrovirus vector, they could possibly be used for AIDS gene therapy.

REFERENCES

1. Haseloff, J.; Gerlach, W.L. *Nature* **1988** *334*, 585-591.

2. Koizumi, M.; Iwai, S.; Ohtsuka, E. *FEBS Lett.* **1988a** *228*, 228-230.

3. Koizumi, M.; Iwai, S.; Ohtsuka, E. *FEBS Lett.* **1988b** *239*, 285-288.

4. Koizumi, M.; Hayase, Y.; Iwai, S.; Kamiya, H.; Inoue, H.; Ohtsuka, E. *Nucleic Acids Res.* **1989** *17*, 7059-7071.

5. Hampel, A; Triz, R.; Hick, M.; Cruz, P. *Nucleic Acids Res.* **1990** *18*, 299-304.

6. Joseph, S.; Berzal-Herranz, A.; Chowrira, B.M.; Butcher, S.E.; Burke, J.M. *Gene & Dev.* **1993** *7*, 130-138.

7. Scanlon, K.J.; Jiao, L.; Funato, T.; Wang, W.; Tone, T.; Rossi, J.J.; Kashini-Sabet, M. *Proc. Natl. Acad. Sci. U.S.A.* **1991** *88*, 10591-10592.

8. Sioud, M.; Natvig, J.B.; Forre, O. *J. Mol. Biol.* **1991** *223*, 831-835.

9. Koizumi, M., Kamiya, H.; Ohtsuka, E. *Gene* **1992** *117*, 179-184.

10. Koizumi, M., Kamiya, H.; Ohtsuka, E. *Biol. Pharm. Bull.* **1993** *16*, 879-883.

11. Komatsu, Y.; Kanzaki, I.; Koizumi, M.; Ohtsuka, E. *J. Mol. Biol.* **1995** *252*, 296-304.

12. Yu, M.; Poeschla, E.; Yamada, O.; Degraandis, P.; Leavitt, M.C.; Heusch, M.; Yees, J.K.; Wong-Staal, F.; Hampel, A. *Virology* **1995** *206*, 381-386.

13. L'Huillier, P.J.; Davis, S.R.; Bellamy, A.R. *EMBO J.* **1992** *11*, 4411-4418.

14. Denman, R.B.; Smedman, M.; Ju, W.; Rubenstein, R.; Potempska, A.; Miller, D.L. *Nucleic Acids Res.* **1994** *22*, 2375-2382.

15. Chowrira, B.M; Pavco, P.A; McSwiggen, J.A. *J. Biol. Chem.* **1994** *269*, 25856-25864.

16. Sarver, N.; Cantin, E.M.; Chang, P.S.; Zaia, J.A.; Ladne, P.A.; Stephens, D.A., Rossi, J.J. *Science* **1990** *247*, 1222-1225.

17. Dropulic, B,; Lin, N.H.; Martin, M.A.; Jeang, K.T. *J. Virol.* **1992** *66*, 1432-1441.

18. Chen, C. J.; Banerjea, A.C.; Harmison, G.G.; Haglund, K.; Schubert, M. *Nucleic Acids Res.* **1992** *20*, 4581-458.

19. Crisell, P; Thompson, S; James, W. *Nucleic Acids Res.* **1993** *21*, 5251-5255.

20. Ohkawa, J.; Yuyama, N.; Takebe, Y.; Nishikawa, S.; Taira, K. *Proc. Natl. Acad. Sci. U.S.A.* **1993** *90*, 11302-11306.

21. Ojwang, J.O.; Hampel, A.; Looney, D.J.; Wong-Staal, F.; Pappaport, J. *Proc. Natl. Acad. Sci. U.S.A.* **1992** *89*, 10802-10806.

22. Scaringe, S.A.; Francklyn, C.; Usman, N. *Nucleic Acids Res.* **1990** *18*, 5433-5441.

23. Koizumi, M.; Ohta, T.; Komatsu, Y.; Ohtsuka, E. *Nucleosides & Nucleotides* **1996** *15*, 505-517.

24. Takebe, Y.; Seiki, M.; Fujisawa, J.; Hoy, P.; Yokota, K.; Arai, K.; Yoshida, M.; Arai, N. *Mol. Cell. Biol.* **1988** *8*, 466-472.

25. Tuerk, C.; Gauss, P,; Thermes, C.; Groebe, D.R.; Gayle, M.; Guild, N.; Stormo, G.; Aubenton-Carafa, Y.; Uhlenbeck, O.C,; Tinoco Jr, I.; Brody, E.N.; Gold, L. *Proc. Natl. Acad. Sci. USA* **1988** *85*, 1364-1368.

26. Sekiguchi, A.; Koizumi, M.; Komatsu, Y.; Ohtsuka, E. *Nucleic Acids Res.* **1991** *24*, 6833-6838.

27. Vinayak, R.; Andrus, A.; Sinha, N.D.; Hampel, A. *Anal. Biochem.* **1995** *232*, 204-209.

28. Komatsu, Y.; Koizumi, M.; Nakamura, H.; Ohtsuka, E. *J. Am. Chem. Sci.* **1994** *116*, 3692-3696.

29. Heidenreich, O.; Eckstein, F. *J. Biol. Chem.* **1992** *267*, 1904-1909.

30. Zuker, M.; Stiegler, P. *Nucleic Acids Res.* **1981** *9*, 133-148.

31. Tsuchihashi, Z.; Khosla, M.; Herschlag, D. *Science* **1993** *262*, 99-102.

32. Muesing, M.A.; Smith, D.H.; Cabradilla, C.D.; Benton, C.V.; Lasky, L.A.; Capon, D.J. *Nature* **1985** *313*, 450-458.