J. Biochem. **132**, 149–155 (2002)

Cleavage of an Inaccessible Site by the Maxizyme with Two Independent Binding Arms: An Alternative Approach to the Recruitment of RNA Helicases

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Received April 10, 2002; accepted May 9, 2002

To overcome obstacles to target site selection, we recently created a novel hybrid ribozyme that could access any chosen site by the recruitment of intracellular RNA helicases [Warashina *et al.* (2001) *Proc. Natl. Acad. Sci. USA* 98, 5572–5577; Kawasaki *et al.* (2002) *Nat. Biotech.* 20, 376–380]. We also demonstrated previously that pol III–driven maxizymes with two substrate-binding arms that were directed against two different sites within a target mRNA formed very active heterodimers *in vivo* [Kuwabara, *et al.* (2000) *Trends Biotechnol.* 18, 462–468; Tanabe *et al.* (2001) *Nature* 406, 473–474]. Despite the complicated dimerization process, all the maxizymes that we tested in cultured cells had greater catalytic activity than the parental ribozymes. To investigate the action of maxizymes in cells, we designed a specific maxizyme with two substrate-binding arms that was directed against endogenously expressed LTR-luciferase chimeric mRNA, where LTR refers to the long terminal repeat of HIV-1. One substrate-binding arm of the maxizyme was designed to bind to a site within HIV-1 TAR RNA that is known to form a stable stem structure that normally prevents binding of a ribozyme. The other substrate-binding arm was directed against a relatively accessible site within the luciferase gene. As expected, the conventional ribozyme failed to cleave the TAR region *in vivo* because of the latter's stable secondary structure. However, to our surprise, the maxizyme cleaved the TAR region within the stem with high efficiency *in vivo*. The enhanced cleavage *in vivo* by the maxizyme might have resulted from an entropically favorable, intramolecular, second binding process that occurred during the breathing of the stem structure of the target mRNA. Importantly, our data suggest that this maxizyme technology might be used as an alternative approach to the recruitment of RNA helicases in cleaving sites previously found to be inaccessible.

Key words: HIV-1 TAR, maxizymes, ribozymes, substrate-binding.

Hammerhead ribozymes are small and versatile catalytic RNAs that can cleave target RNAs at specific sites (Fig. 1A) (*1–15*). In studies directed towards the development of therapeutic applications and potentially useful tools *in vivo*, various ribozymes have been designed and tested *in vivo* (*16–32*). During attempts to shorten a hammerhead ribozyme, we developed the maxizyme, which functions as a dimer (*5, 33–43*). Kinetic and NMR analyses indicated that the shortened ribozyme was essentially inactive as a monomer but exhibited strong catalytic activity as a dimer (Fig. 1B) (*5, 34*). We also designed a heterodimer composed of two different monomers, designated maxizyme left (MzL) and maxizyme right (MzR) (Fig. 1C) (*5*). Such a heterodimer has two substrate-binding arms and two catalytic cores and the substrate is cleaved only when MzL and MzR form a dimer. The heterodimeric maxizyme simultaneously cleaved HIV-1 tat mRNA at two independent sites both *in vitro* and in cultured cells (*33, 37*). The catalytic activity of heterodimeric maxizymes depends on a complex dimerization process, and inactive homodimers are formed in addition to the complex of heterodimers with the target (Fig. 1C) (*37–39*). Because of the mixed populations of dimeric forms generated *in vitro*, the cleavage activities of heterodimeric maxizymes examined previously were lower than those of parental hammerhead ribozymes and of the homodimeric maxizyme (*35–43*). Nonetheless, the intracellular activities of heterodimeric maxizymes were as high as those of homodimeric maxizymes and higher than those of parental ribozymes that targeted the two independent cleavage sites.

Successful gene inactivation by ribozymes *in vivo* depends strongly on the design of the expression vector. The design can determine both the level of expression and the half-life of the expressed ribozyme (*25*). In previous studies we found that pol III–mediated expression of ribozymes as tRNA fusions resulted in highly expressed stable ribozymes (*27–43*). However, even these improved ribozymes were

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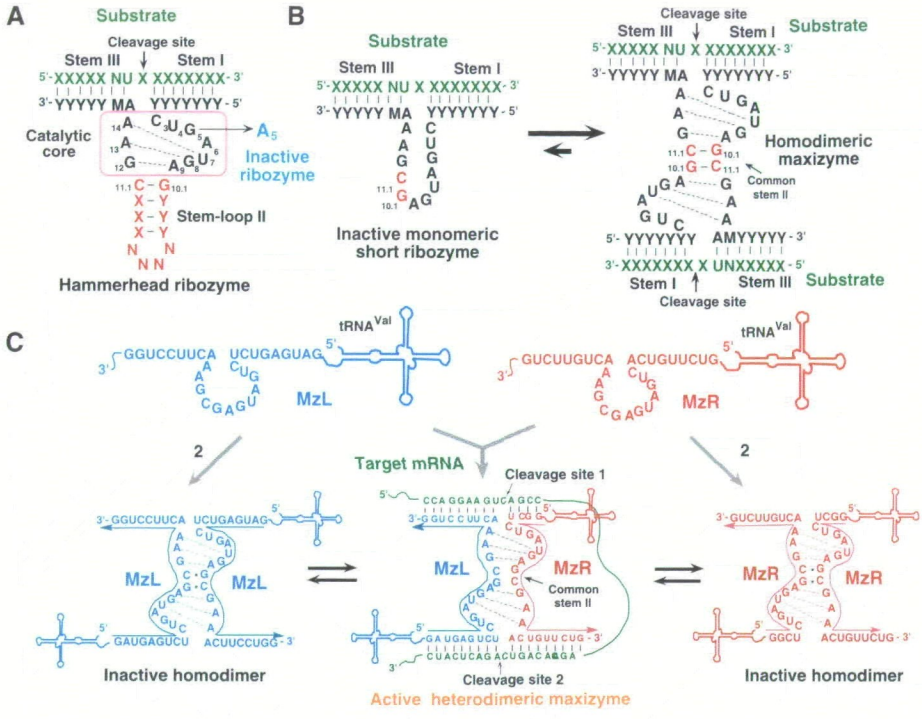

Fig. 1. **Secondary structures of ribozyme and maxizyme.** (A) Secondary structures of the wild-type hammerhead ribozyme. (B) Secondary structures of the inactive monomeric short ribozyme (left) that is capable of forming a homodimeric maxizyme (right). (C) Secondary structures of the heterodimeric maxizyme that can cleave an mRNA at two sites simultaneously. The active heterodimeric and inactive homodimeric forms of dimeric maxizymes under the control of a human $tRNA^{Val}$-promoter are shown at the bottom.

sometimes ineffective, probably because the ribozyme was unable to locate its target. One potential explanation for this ineffectiveness is that the rate-limiting step *in vivo* for the cleavage of phosphodiester bonds is the annealing/association of the ribozyme with its target site (*28*). To overcome the problem of accessibility, we reasoned that it would be useful to design a ribozyme that could recruit a protein that could, in turn, relieve any interfering secondary structure, thereby making any site accessible to the ribozyme. To accomplish this, we linked a ribozyme to an RNA helicase(s), proteins demonstrated to have nonspecific RNA binding, sliding and unwinding activities (*27, 44–47*). The introduction of an RNA motif, the constitutive transport element (CTE), that appears to interact with RNA helicases *in vitro* and *in vivo* (*48–54*) led to cleavage *in vivo* at any chosen target site regardless of the local RNA secondary or tertiary structure (*27*).

In this study, we examine the features of the maxizyme in greater detail. Our present analysis indicates that, once a maxizyme has been anchored at a more accessible site, its activity *in vivo* can be improved significantly by permitting cleavage of sites previously found to be inaccessible. We suggest that the enhanced cleavage *in vivo* by the maxizyme resulted from an entropically favorable, intramolecular, second binding process that occurred during the breathing of the stem structure of the target mRNA. Importantly, the improved efficacy as a result of anchoring by one of the binding arms of the maxizyme makes it even more suitable for a wide range of applications in the post-genome era.

MATERIALS AND METHODS

Kinetic Analysis—Kinetic measurements of reactions catalyzed by $tRNA^{Val}$-driven ribozymes (tRNA-Rz) and $tRNA^{Val}$-driven maxizymes (tRNA-Mz) were performed with 2 nM 5′-^{32}P–labeled LTR-Luc RNA as substrate. Reaction rates were measured, in 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0), under single-turnover conditions with high concentrations of tRNA-Mz and tRNA-Rz (5 μM) at 37°C. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and product with a Bio-Image Analyzer (BA2000; Fuji Film, Tokyo).

Cell Culture and Luciferase Assay—Methods for cell culture were described previously (*25, 37, 55*). Luciferase activity was monitored basically as described elsewhere (*37*). LTR-Luc HeLa cells were plated at 80% confluence in twelve-well plates and incubated at 37°C in a CO_2 incubator. The cells were washed twice with phosphate-buffered saline (PBS) before (co-)transfection. Four μg of each ribozyme-expression plasmid and 150 ng of Tat-expressing plasmid (pCD-SRα/tat) (*55, 56*) were mixed with 4 μl of Lipofectin reagent (Gibco-BRL, Rockville, MD) in 400 μl of serum-reduced medium (OPTI-MEM I; Gibco-BRL) and incubated for 30 min at room temperature, as described in the user's bulletin from Gibco-BRL. Then the mixture was gently added to cells. After 12 h, the medium was replaced by growth medium (DMEM) supplemented with 10% fetal calf serum and cells were cultured for another 24 h. Luciferase activity was measured with a PicaGene kit (Toyoinki, Tokyo) as described elsewhere (*25, 37, 55*). To normalize the efficiency of transfection by reference to β-galactosidase activity, cells were co-transfected with 200 ng of the pSV-β-galactosidase control vector (Promega, Madison, WI) and the chemiluminescent signal due to the activity of β-galactosidase was determined with a luminescent b-galactosidase Genetic Reporter System (Clontech, Palo Alto, CA), as described elsewhere (*55*).

RESULTS

Design of Maxizyme with Two Independent Binding Arms—Heterodimeric maxizymes have two substrate-binding arms and can bind to two different sites on a target mRNA simultaneously (*29–32*). We wondered what would happen when the apparent accessibility of the respective binding sites differed significantly. We postulated that, even if one of the maxizyme-binding sites were hidden within the stable stem of an mRNA, the maxizyme might still be able to access and cleave the site provided that the other site were accessible to the maxizyme. We reasoned that, if the maxizyme can bind to a substrate with one accessible site, the binding to the second but less accessible site should become an intramolecular interaction, which should be entropically favored. To analyze the effect of such double binding of a maxizyme to its substrate on intracellular cleavage activity, we designed a maxizyme that was specifically targeted against endogenously expressed chimeric LTR-luciferase mRNA (where LTR is the long terminal repeat of HIV-1). Figure 2A shows the secondary structure of the target mRNA (as predicted by the MulFold program). One substrate-binding region of the maxizyme was targeted to an apparently inaccessible site within HIV-1 TAR RNA. This site is known to be located within a tight stem structure that prevents a normal ribozyme from binding (*27*). The other substrate-binding region was directed against a site that is considered to be relatively accessible within the luciferase gene (Fig. 2A). Using such a maxizyme with binding arms directed against two sites with different accessibilities, we hoped to gain some insight into

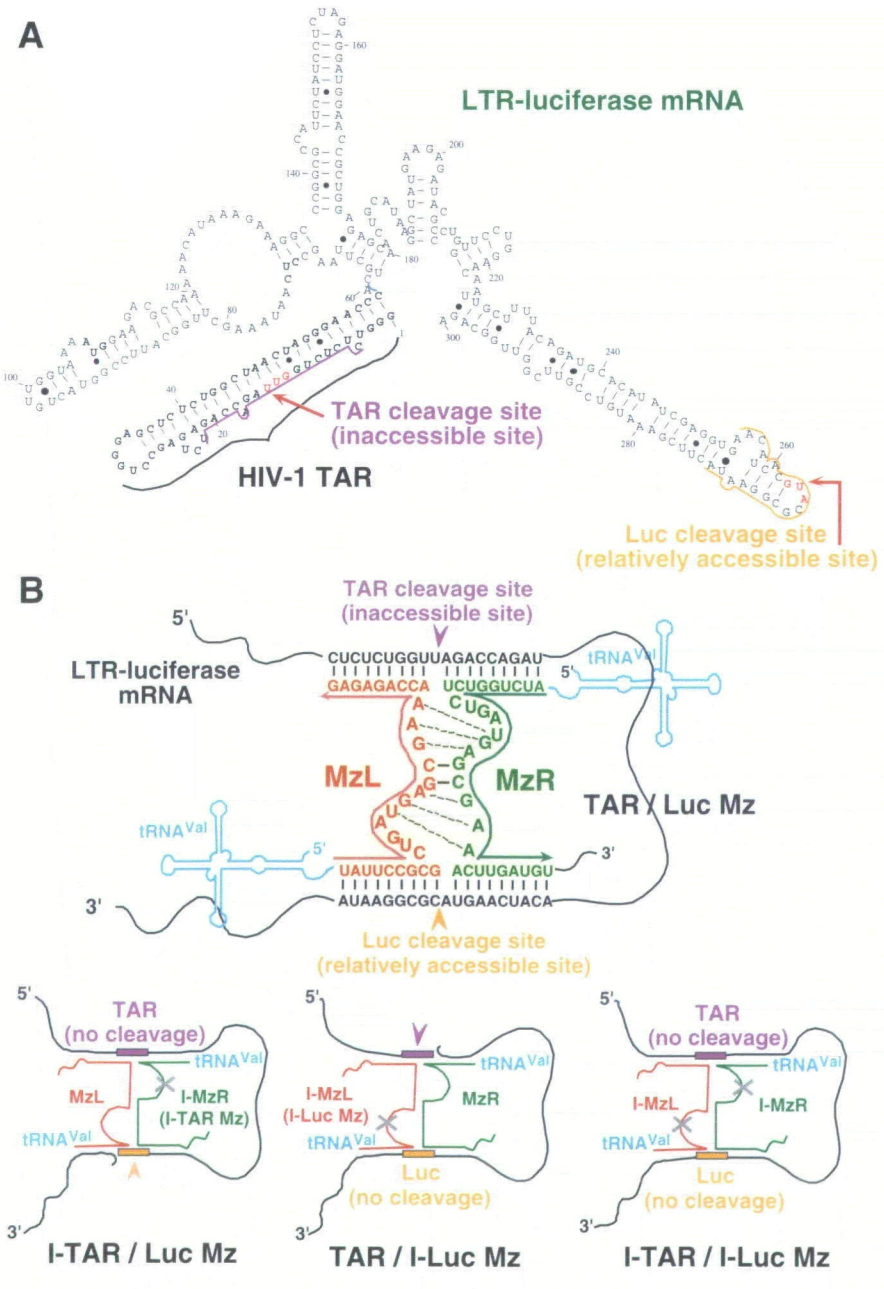

Fig. 2. **Design of heterodimeric maxizymes directed against chimeric LTR-Luc mRNA.** (A) Predicted secondary structure, based on calculations with the MulFold program (Biocomputing Office, Biology Department, Indiana University, IN, USA), of the target LTR-Luc mRNA. (B) Various combinations of maxizymes for targeting the TAR cleavage site and/or the Luc cleavage site in LTR-Luc mRNA. X indicates inactivation by a point mutation (G5 to A5) within respective catalytic cores. I indicates an inactivated monomer. Thus, I-MzR indicates inactivated MzR.

the molecular mechanism of action of the maxizyme *in vivo*.

To ensure efficient formation of dimers in cells, we expressed maxizymes under the control of the promoter of a human gene for $tRNA^{Val}$ which is recognized by RNA polymerase III (*25*) The sequences of the monomers of the maxizyme were inserted downstream of a modified gene for $tRNA^{Val}$, and the expression cassettes of the monomer units were subcloned into one vector (TAR/Luc Mz) as described elsewhere (*34, 35, 37*). For comparisons of activity, we prepared plasmids that encoded wild-type ribozymes directed against the two individual sites (TAR Rz and Luc Rz). We also prepared plasmids that encoded inactive maxizymes with a point mutation (G5 to A5 in Fig. 1A) within the catalytic core specific for each cleavage site (TAR/I-Luc Mz and I-TAR/Luc Mz; Fig. 2B, bottom).

Characterization of Cleavage Activity In Vitro and In Vivo—We first examined the cleavage activities and the accessibilities of maxizymes and ribozymes and the extent to which they were able to interact with or gain access to the substrate RNA *in vitro*. The cleavage activities of the various maxizymes and ribozymes *in vitro* are shown in Table I. The time courses of reactions are plotted in Fig. 3. As we had anticipated, the tRNA-ribozyme directed against the accessible Luc cleavage site (Luc Rz) was two orders of magnitude more active than the tRNA-ribozyme directed against the inaccessible TAR cleavage site (TAR Rz). Apparently, access to the target had determined the cleavage activity of each ribozyme. In the reactions with maxizymes, as observed previously, in the absence of facilitators such as cetyltrimethylammonium bromide (CTAB) (*38, 39*), the majority of all maxizymes were basically trapped in their inactive forms, which included homodimers, at least under the reaction conditions that we used *in vitro*.

It should be emphasized here that our recent studies demonstrated that no relationships were found between the activities of catalytic RNAs in cultured cells and the kinetic parameters of their respective chemical cleavage reactions *in vitro*, indicating that in no case was chemical cleavage the rate-limiting step *in vivo* (*28*). Therefore, in order to gain insight into the molecular mechanism of action of the maxizyme *in vivo*, analysis must be carried out *in vivo*.

Accordingly, we next evaluated the intracellular activities of the ribozymes and maxizymes in HeLa cells that expressed LTR-Luc (Fig. 4). The target chimeric gene was stably expressed and consisted of the long terminal repeat (LTR) of HIV-1 and a gene for luciferase. The expression of this reporter is Tat-dependent and, therefore, an expression plasmid for Tat was co-transfected and transiently expressed together with each ribozyme-expression vector (Fig. 4A) (*55, 56*). As anticipated, Luc Rz reduced the expression of the reporter (lane 3; Fig. 4B) while TAR Rz had no effect on the activity of the luciferase reporter (lane 2).

In contrast to the results *in vitro* (Fig. 1, Table I), when maxizymes were expressed in HeLa cells, the maxizyme that had been designed to cleave both the TAR and the Luc cleavage sites (TAR/Luc Mz) had higher activity than the corresponding ribozymes (lane 7; Fig. 4B). The maxizymes, whose active dimeric form is in equilibrium with many inactive forms (*34–41*), must have been converted preferentially to the active form by endogenous intracellular facilitators, since only the active heterodimeric maxizyme is stabilized when its two binding arms anneal to the target regions (Fig. 1C, bottom) (*37*). The individual maxizyme monomers (MzL and MzR, lanes 5 and 6) by themselves were ineffective. In addition, the completely inactive maxizyme (I-TAR/I-Luc Mz) with double point-mutations (G5 to A5) within the two catalytic cores was also ineffective (lane 10). These results clearly indicated that, in cells, the active maxizyme was a heterodimer and, furthermore, that it was cleavage by the maxizyme, not an antisense effect, that was responsible for the specific suppression of expression of LTR-Luc mRNA.

Both half-inactive maxizymes, in which just one catalytic core had a point mutation (G5 to A5), namely, TAR/I-Luc

TABLE I. **Kinetic parameters of cleavage of the LTR-Luc RNA substrate.**

	$K_{obs} \times 10^{-2}$ (min^{-1})
Cleavage at the Luc cleavage site	
TAR/Luc Mz	0.060
I-TAR/Luc Mz	0.076
Luc Rz	2.4
Cleavage at the TAR cleavage site	
TAR/Luc Mz	0.032
TAR/I-Luc Mz	0.037
TAR Rz	0.028

Rate constants were measured in 50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ under enzyme-saturating conditions (5 μM ribozyme or maxizyme and less than 2 nM substrate) at 37°C.

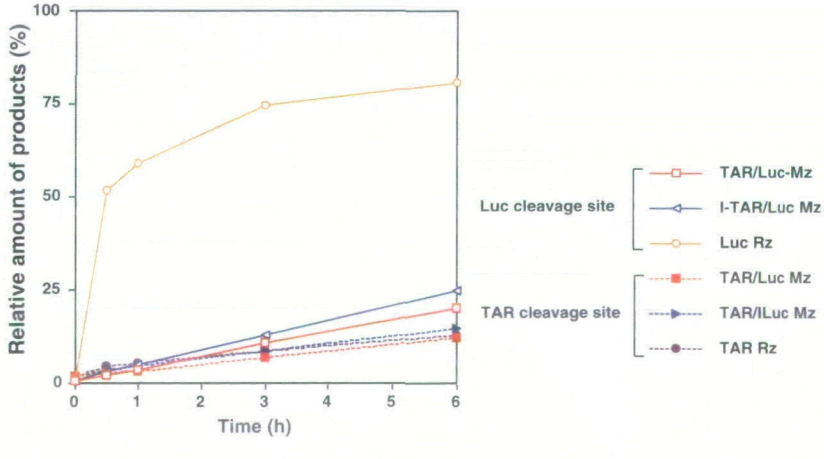

Fig. 3. **Time courses of reactions catalyzed by $tRNA^{Val}$-ribozymes and maxizymes *in vitro*.** Reactions were carried out in 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0) under single-turnover conditions with high concentrations of tRNA-Mz and tRNA-Rz (5 μM) at 37°C. Reaction conditions and the rate constants obtained are summarized in Table I.

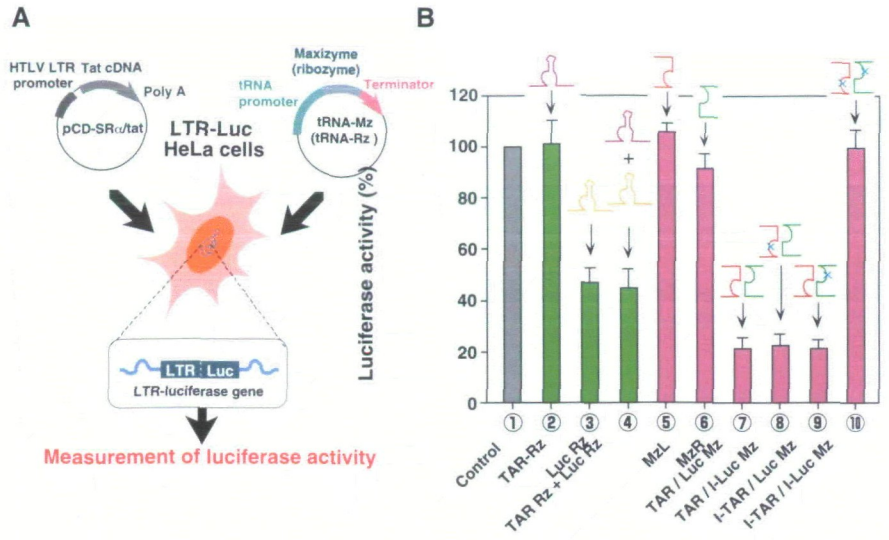

Fig. 4. **Intracellular activities of tRNA^{Val}-enzymes in HeLa cells that stably expressed LTR-Luc.** (A) Assay system for measurement of the activities of tRNA^{Val}-enzymes in LTR-Luc HeLa cells. (B) The effects of tRNA^{Val}-enzymes on the Tat-mediated transcription of the chimeric LTR-Luc gene. The results shown are the averages of results from three sets of experiments. Luciferase activity was normalized by reference to the efficiency of transfection, which was determined by monitoring the activity of β-galactosidase encoded by a co-transfected gene.

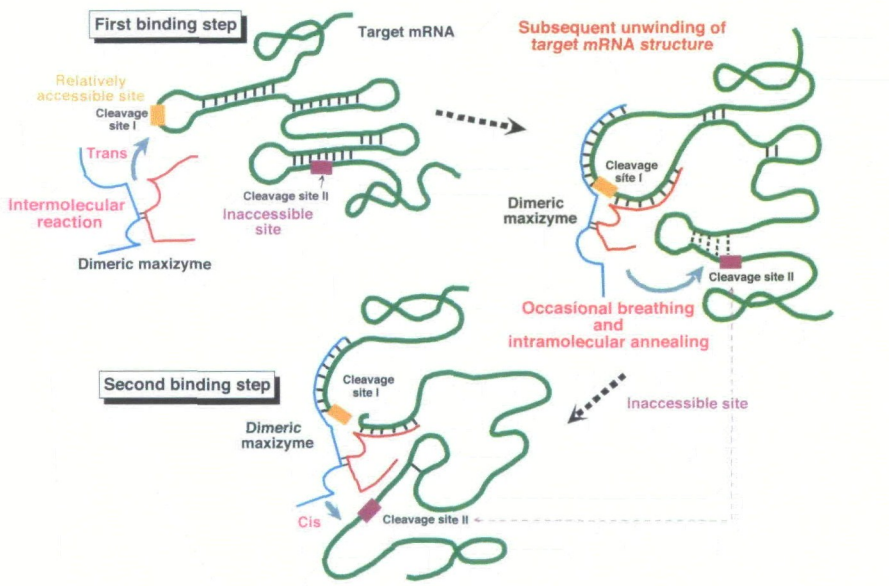

Fig. 5. **Schematic representation of the advantages of maxizymes.** The considerable effects that might enhance the activity of the activity of maxizymes designed to cleave a target mRNA at two sites *in vivo via* two sequential bindings to the substrate.

Mz and I-TAR/Luc Mz (Fig. 2B, bottom), cleaved the substrate with remarkable efficiency (lanes 8 and 9). In fact, the activity of the half-inactive I-TAR/Luc Mz maxizyme was higher (80% reduction in luciferase activity) than that of the wild-type ribozyme, Luc Rz (50% reduction). The activity was actually equal to that of the normal maxizyme (lane 7), indicating the potential advantage of any maxizyme with an additional binding arm.

In general, the rate-limiting step *in vivo* of a reaction mediated by a catalytic RNA, such as a ribozyme, has been considered to be the substrate-binding step (*28, 57–62*). Our present analysis further strengthens this hypothesis, since the efficacy of our maxizymes *in vivo* apparently reflected enhanced accessibility to the substrate. It is also noteworthy that TAR/I-Luc Mz, which was designed to cleave only the inaccessible TAR cleavage site (lane 8), suppressed the expression of the LTR-luciferase gene to the same extent as the normal heterodimeric maxizyme. The binding arm of the maxizyme has to gain access to the TAR region prior to cleavage of the target mRNA. Once one binding arm of the maxizyme has bound to the more accessible Luc cleavage site (TAR/I-Luc Mz cannot cleave the Luc cleavage site but can bind to this site with the same affinity as the normal maxizyme), binding of the other arm to the TAR cleavage site becomes an entropically favorable intramolecular interaction that can occur during the breathing of mRNA, which is most probably the result of the action of some endogenous RNA helicase(s) (*27, 44–47*). Even within the tight structure of the TAR region, the stem structure can be disrupted by interactions with cellular factors and the maxizyme, having already bound *via* the Luc cleavage site, should be able to bind efficiently to the disrupted region. The additional binding arm appears to allow the maxizyme to attack efficiently even at an otherwise inaccessible target site.

DISCUSSION

Under conditions where the rate-limiting step is the substrate-binding step, once the maxizyme with two binding arms has bound to the more accessible target site *via* one of its binding arms, the second binding step at the second site becomes an intramolecular interaction (Fig. 5). This intramolecular annealing process is much more favorable entropically than the intermolecular annealing process that must occur when independent standard ribozymes are involved. Figure 5 summarizes the considerable effects that might enhance the activity of maxizymes *in vivo* specifically *via* the two sequential bindings to the target mRNA. As a result of the advantage of the intramolecular interactions in the second binding step, as described above, the maxizyme can gain access to a structured region in the target RNA by increasing the chance of an encounter, once the structured region has been disrupted by cellular factors (yellow panel in Fig. 5). Structural changes in the target RNA caused by the initial binding of the maxizyme might also trigger the second binding/cleavage reaction. The enhancement of the intracellular activity of the present maxizymes is reflected by the results of several earlier successful studies *in vivo* (*34–41*). Maxizymes, which can easily be designed (*40*), should be useful as tools in molecular biology, with potential utility *in vivo* and in a clinical setting.

Our present studies suggest that, once a maxizyme has been anchored at a more accessible site, its activity *in vivo* can be improved significantly. Our present maxizyme technology should complement the hybrid ribozyme technology that uses endogenous RNA helicases (*27, 63–65*). It may be possible to devise a universal anchor, for example, an anchor that binds to the poly(A) tails of mRNAs to create universally active maxizymes *in vivo*. Such an anchoring strategy might not be limited to maxizymes exclusively but might also apply to parental ribozymes. Such possibilities are currently being investigated in our laboratory.

REFERENCES

1. Uhlenbeck, O.C. (1987) A small catalytic oligoribonucleotide. *Nature* **328**, 596–600
2. Haseloff, J. and Gerlach, W.L. (1988) Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* **334**, 585–591
3. Symons, R.H. (1989) Self-cleavage of RNA in the replication of small pathogens of plants and animals. *Trends Biochem. Sci.* **14**, 445–450
4. Sawata, S., Komiyama, M., and Taira, K. (1995) Kinetic evidence based on solvent isotope effects for the nonexistence of a proton-transfer process in reactions catalyzed by a hammerhead ribozyme: implication to the double-metal-ion mechanism of catalysis. *J. Am. Chem. Soc.* **117**, 2357–2358
5. Amontov, S.V. and Taira, K. (1996) Hammerhead minizymes with high cleavage activity: A dimeric structure as the active conformation of minizymes. *J. Am. Chem. Soc.* **118**, 1624–1628
6. Zhou, D.-M., Usman, N., Wincott, F.E., Matulic-Adamic, J., Orita, M., Zhang. L.-H., Komiyama, M., Kumar, P.K.R., and Taira, K. (1996) Evidence for the rate-limiting departure of the 5′-oxygen in nonenzymatic and hammerhead ribozyme-catalyzed reactions. *J. Am. Chem. Soc.* **118**, 5862–5866
7. Zhou, D.-M., Kumar, P.K.R., Zhang. L.-H., and Taira, K. (1996) Ribozyme mechanism revisited: evidence against direct coodination of a Mg^{2+} ion with the pro-R oxygen of the scissile phosphate in the transition state of a hammerhead ribozyme-catalyzed reaction. *J. Am. Chem. Soc.* **118**, 8969–8970
8. Birikh, K.R., Heaton, P.A., and Eckstein, F. (1997) The structure, function and application of the hammerhead ribozyme. *Eur. J. Biochem.* **245**, 1–16
9. Kuimelis, R.G., and McLaughlin, L.W. (1998) Mechanism of ribozyme-mediated cleavage. *Chem. Rev.* **98**, 1027–1044
10. Zhou, D.-M. and Taira, K. (1998) The hydrolysis of RNA: from theoretical calculations to the hammerhead ribozyme-mediated cleavage of RNA. *Chem. Rev.* **98**, 991–1026
11. Lilley, D.M.J. (1999) Structure, folding and catalysis of the small nucleolytic ribozymes. *Current Opinion Struct. Biol.* **9**, 330–338
12. Wang, S., Karbstein, K.; Peracchi, A., Beigelman, L., and Herschlag, D. (1999) Identification of the hammerhead ribozyme metal ion binding site responsible for rescue of the deleterious effect of a cleavage site phosphorothioate. *Biochemistry* **38**, 14363–14378
13. Yoshinari, K. and Taira, K. (2000) A further investigation and reappraisal of the thio effect in the cleavage reaction catalyzed by a hammerhead ribozyme. *Nucleic Acids Res.* **28**, 1730–1742
14. Warashina, M., Takagi, Y., Stec, W.J., and Taira, K. (2000) Differences among mechanisms of ribozyme-catalyzed reactions. *Curr. Opin. Biotech.* **11**, 354–362
15. Takagi, Y., Warashina, M., Stec, W.J., Yoshinara, K., and Taira, K. (2001) Recent advances in the elucidation of the mechanisms of action of ribozymes. *Nucleic Acids Res.* **29**, 1815–1834
16. Rossi, J.J. and Sarver, N. (1990) RNA enzymes (ribozymes) as antiviral therapeutic agents. *Trends Biotechnol.* **8**, 179–183
17. Sarver, N., Cantin, E.M., Chang, P.S., Zaida, J.A., Ladne, P.A., Stephenes, D.A., and Rossi, J.J. (1990) Ribozymes as potential anti-HIV-1 therapeutic agents. *Science* **247**, 1222–1225
18. Erickson, R.P. and Izant, J. (eds.) (1992) *Gene Regulation: Biology of Antisense RNA and DNA*, Raven Press, New York, NY
19. Murray, J.A.H. (ed.) (1992) *Antisense RNA and DNA*, Wiley-Liss, New York, NY
20. Rossi, J.J. (1995) Controlled, targeted, intracellular expression of ribozymes: progress and problems. *Trends Biotechnol.* **13**, 301–306
21. Eckstein, F. and Lilley, D.M.J. (eds.) (1996) *Nucleic Acids and Molecular Biology*, in Catalytic RNA, 10, Springer-Verlag, Berlin
22. Turner, P.C. (ed.) (1997) *Methods in Molecular Biology*, in Ribozyme Protocols, 74, Humana Press, Totowa, NJ
23. Kawasaki, H., Ecker, R., Yao, T.-P., Taira, K., Chiu, R., Livingston, D.M., and Yokoyama, K.K. (1998) Distinct roles of the coactivators p300 and CBP in retinoic-acid-induced F9-cell differentiation. *Nature* **393**, 284–289
24. Scanlon, K.J. (ed.) (1998) *Therapeutic Applications of Ribozymes: Methods*, in Molecular Medicine, vol. 11, Humana Press, NJ
25. Koseki, S., Tanabe, T., Tani, K., Asano, S., Shioda, T., Nagai, Y., Shimada, T., Ohkawa, J., and Taira, K. (1999) Factors governing the activity in vivo of ribozymes transcribed by RNA polymerase III. *J. Virol.* **73**, 1868–1877
26. Krupp, G. and Gaur, R.K. (eds.) (2000) *Ribozyme: Biochemistry and Biotechnology*, Eaton Publishing, Natick, MA
27. Warashina, M., Kuwabara, T., Kato, Y., Sano, M., and Taira, K. (2001) RNA-protein hybrid ribozymes that efficiently cleave any mRNA independently of the structure of the target RNA. *Proc. Natl. Acad. Sci. USA* **98**, 5572–5577
28. Kato, Y., Kuwabara, T., Warashina, M., Toda, H., and Taira, K. (2001) Relationships between the activities *in vitro* and *in vivo* of various kinds of ribozyme and their intracellular localization in mammalian cells. *J. Biol. Chem.* **276**, 15378–15385
29. Kurata, H., Miyagishi, M., Kuwabara, T., Warashina, M., and Taira, K. (2000) *J. Biochem. Mol. Biol.* **33**, 359–365
30. Kuwabara, T., Warashina, M., and Taira, K. (2000) Allosterically controllable ribozymes with biosensor functions. *Curr. Opin. Chem. Biol.* **4**, 669–677
31. Kuwabara, T., Warashina, M., and Taira, K. (2000) Allosterically controllable maxizymes cleave mRNA with high efficiency and specificity. *Trends Biotechnol.* **18**, 462–468
32. Warashina, M., Kuwabara, T., and Taira, K. (2000) Working at the cutting edge: the creation of allosteric ribozymes. *Structure*

8, 207–212
33. Kuwabara, T., Warashina, M., Ohkawa, J., and Taira, K. (1996) Characterization of several kinds of dimer minizyme: simultaneous cleavage at two sites in HIV-1 tat mRNA by dimer minizymes. *Nucleic Acids Res.* **24**, 2302–2310
34. Kuwabara, T., Warashina, M., Orita, M., Koseki, S., Ohkawa, J., and Taira, K. (1998) Formation of a catalytically active dimer by tRNAVal-driven short ribozymes. *Nature Biotechnol.* **6**, 961–965
35. Kuwabara, T., Warashina, M., Tanabe, T., Tani, K., Asano, S., and Taira, K. (1998) A novel allosterically trans-activated ribozyme, the maxizyme, with exceptional specificity *in vitro* and *in vivo*. *Mol. Cell* **2**, 617–627
36. Hamada, M., Kuwabara, T., Warashina, M., Nakayama, A., and Taira, K. (1999) Specificity of novel allosterically *trans*- and *cis*-activated connected maxizymes that are designed to suppress *BCR-ABL* expression. *FEBS Lett.* **461**, 77–85
37. Kuwabara, T., Warashina, M., Nakayama, A., Ohkawa, J., and Taira, K. (1999) tRNAVal-heterodimeric maxizymes with high potential as geneinactivating agents: simultaneous cleavage at two sites in HIV-1 Tat mRNA in cultured cells. *Proc. Natl. Acad. Sci. USA* **96**, 1886–1891
38. Nakayama, A., Kuwabara, T., Warashina, M., and Taira, K. (1999) CTAB-mediated enrichment for active forms of novel dimeric maxizymes. *FEBS Lett.* **448**, 67–74
39. Nakayama, A., Warashina, M., Kuwabara, T., and Taira, K. (2000) Effects of cetyltrimethylammonium bromide on reactions catalyzed by maxizymes, a novel class of metalloenzymes. *J. Inorg. Biochem.* **78**, 69–77
40. Tanabe, T., Takata, I., Kuwabara, T., Warashina, M., Kawasaki, H., Tani, K., Ohta, S., Asano, S., and Taira, K. (2000) Maxizymes, novel allosterically controllable ribozymes, can be designed to cleave various substrates. *Biomacromolecule* **1**, 108–117
41. Tanabe, T., Kuwabara, T., Warashina, M., Tani, K., Taira, K., and Asano, S. (2000) Oncogene inactivation in a mouse model. *Nature* **406**, 473–474
42. Zhou, J.-M., Nakamatsu, Y., Kuwabara, K., Warashina, M., Tanaka, Y., Yoshinari, K., and Taira, K. (2000) Chemical and enzymatic probing of effector-mediated changes in the conformation of a maxizyme. *J. Inorg. Chem.* **78**, 261–268
43. Kuwabara, T., Hamada, M., Warashina, M., and Taira, K. (2001) Allosterically controlled single-chained maxizymes with extremely high and specific activity. *Biomacromol* **2**, 788–799
44. Lee, C.-G. and Hurwitz, J. (1993) Human RNA helicase A is homologous to the maleless protein of Drosophila. *J. Biol. Chem.* **268**, 16822–16830
45. Wagner, J.D.O., Jankowsky, E., Company, M., Pyle, A.M., and Abelson, J.N. (1998) The DEAH-box protein PRP22 is an ATPase that mediates ATP-dependent mRNA release from the spliceosome and unwinds RNA duplexes. *EMBO J.* **17**, 2926–2937
46. de la Cruz, J., Kressler, D., and Linder, P. (1999) Unwinding RNA in *Saccharomyces cerevisiae*: DEAD-box proteins and related families. *Trends Biochem. Sci.* **24**, 192–198
47. Jankowsky, E., Gross, C.H., Shuman, S., and Pyle, A.M. (2000) The DExH protein NPH-II is a processive and directional motor for unwinding RNA. *Nature* **403**, 447–451
48. Tang, H., Gaietta, G. M., Fischer, W. H., Ellisman, M. H., and Wong-Staal, F. (1997) A cellular cofactor for the constitutive transport element of type D retrovirus. *Science* **276**, 1412–1415
49. Grüter, P., Tabernero, C., von Kobbe, C., Schmitt, C., Saavedra, C., Bachi, A.,Wilm, M., Felber, B.K., and Izaurralde, E. (1998) TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus. *Mol. Cell* **1**, 649–659
50. Braun, I.C., Rohrbach, E., Schmitt, C., and Izaurralde, E. (1999) TAP binds to the constitutive transport element (CTE) through a novel RNA-binding motif that is sufficient to promote CTE-dependent RNA export from the nucleus. *EMBO J.* **18**, 1953–1965
51. Hodge, C.A., Colot, H.V., Stafford, P., and Cole, C.N. (1999) Rat8p/Dbp5p is a shuttling transport factor that interacts with Rat7p/Nup159p and Gle1p and suppresses the mRNA export defect of xpo1-1 cells. *EMBO J.* **18**, 5778–5788
52. Kang, Y. and Cullen, B.R. (1999) The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences. *Genes Dev.* **13**, 1126–1139
53. Li, J., Tang, H., Mullen, T.M., Westberg, C., Reddy, T.R., Rose, D.W., and Wong-Staal, F. (1999) A role for RNA helicase A in post-transcriptional regulation of HIV type 1. *Proc. Natl. Acad. Sci. USA* **96**, 709–714
54. Schmitt, C., von Kobbe, C., Bachi, A., Pante, N., Rodrigues, J.P., Boscheron, C., Rigaut, G., Wilm, M., Seraphin, B., Carmo-Fonseca, M., and Izaurralde, E. (1999) Dbp5, a DEAD-box protein required for mRNA export, is recruited to the cytoplasmic fibrils of nuclear pore complex *via* a conserved interaction with CAN/Nup159p. *EMBO J.* **18**, 4332–4347
55. Koseki, S., Ohkawa, J., Yamamoto, R., Takebe, Y., and Taira, K. (1998) A simple assay system for examination of the inhibitory potential *in vivo* of decoy RNAs, ribozymes and other drugs by measuring the Tat-mediated transcription of a fusion gene composed of the long terminal repeat of HIV-1 and a gene for luciferase. *J. Controlled Release* **53**, 159-173
56. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) SR alpha promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8**, 466–472
57. Pontius, B.W. and Berg, P. (1991) Rapid renaturation of complementary DNA strands mediated by cationic detergents: a role for high-probability binding domains in enhancing the kinetics of molecular assembly processes. *Proc. Natl. Acad. Sci. USA* **88**, 8237–8241
58. Ellis, J. and Rogers, J. (1993) Design and specificity of hammerhead ribozymes against calretinin mRNA. *Nucleic Acids Res.* **21**, 5171–5178
59. Crisell, P., Thompson, S., and James, W. (1993) Inhibition of HIV-1 replication by ribozymes that show poor activity *in vitro*. *Nucleic Acids Res.* **21**, 5251–5255
60. Sullenger, B.A. and Cech, T.R. (1993) Tethering ribozymes to a retroviral packaging signal for destruction of viral RNA. *Science* **262**, 1566–1569
61. Kronenwett, R., Haas, R., and Sczakiel, G. (1996) Kinetic selectivity of complementary nucleic acids: *bcr-abl*-directed antisense RNA and ribozymes. *J. Mol. Biol.* **259**, 632–644
62. Sioud, M. and Jesperson, L. (1996) Enhancement of hammerhead ribozyme catalysis by glyceraldehyde-3-phosphate dehydrogenase. *J. Mol. Biol.* **257**, 775–789
63. Kawasaki, H., Onuki, R., Suyama, E., and Taira, K. (2002) Identification of genes that function in the TNF-α-mediated apoptotic pathway using randomized hybrid ribozyme libraries. *Nat. Biotech.* **20**, 376–380
64. Kawasaki, H. and Taira, K. (2002) Identification of genes by hybrid ribozymes that couple cleavage activity with the unwinding activity of an endogeneous RNA helicase. *EMBO Report* **3**, 443–450
65. Akashi, H., Kawasaki, H., Kim, W.J., Akaike, T., Taira, K., and Maruyama, A. (2002) Enhancement in the cleavage activity of a hammerhead ribozyme by cationic comb-type polymers and an RNA helicases *in vitro*. *J. Biochem.* **131**, 687–692