J. Biochem. **127**, 531–536 (2000)

Functional Domain-Assembly in Hairpin Ribozymes[1]

Yasuo Komatsu,* Ikuyo Kanzaki,* Miho Shirai,* Izumi Kumagai,* Shigeko Yamashita,* and Eiko Ohtsuka*,†,[2]

**Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo 060-0812; and †Present Address: Health Sciences University of Hokkaido, Tobetsu 061-0293*

Received January 14, 2000; accepted January 26, 2000

Functional structures of hairpin ribozymes have been investigated by constructing various chemically modified molecules. Domain-exchange and linker insertion experiments were performed to find active conformations of the RNA enzyme showing cleavage activity. Our experiments and other evidence suggest that the active structure has a bent conformation, and that domain-interactions are essential for the cleavage activity.

Key words: bent structure, domain interaction, RNA cleavage, RNA enzyme, RNA synthesis.

Small catalytic RNAs, discovered in various types of viroid, virusoid, and virus satellite RNAs (*1*), are known to act as enzymes with RNA cleavage or ligation activities. The sequences required for the catalytic activities have been characterized and named hammerhead (*2*) or hairpin (*3*) depending on their secondary structures. Several ribozymes have been used to cleave target RNA in cultured cells (*4–7*). In particular, hairpin ribozymes have been used as effective gene therapeutic agents because of their high cleavage activity (*8*).

The hairpin ribozyme derived from the catalytic center of the negative strand of the satellite RNA of tobacco ring spot virus (sTRSV) is responsible for the cleavage and ligation reactions of the satellite RNA during replication (*9, 10*). This ribozyme contains fifty bases and can cleave RNA that binds to the ribozyme by base pairing (*2, 11*). The cleavage reaction occurs by an in-line mechanism, requires the presence of magnesium ions (*12*), and yields a 2′,3′-cyclic phosphate and 5′-hydroxyl group. The hairpin ribozyme contains four stem regions (helices 1–4) and two internal loops (loop A and loop B) in domains I and II (Fig. 1a) (*11, 13*). Essential bases required for the cleavage activity in the internal loops have been identified by an *in vitro* selection method (*13–15*). Point mutations (*16–18*), chemical modification (*19*), and UV cross-linking analyses (*20–22*) of the ribozyme have provided further information about the secondary structure and the required sequences. These experiments suggest that unusual base pairs, similar to the one found in the hammerhead ribozyme, exist in domain II. The 2-amino group of guanosine at the 3′-cleavage site is essential for the cleavage reaction (*18, 23*); this feature is unique, and is different from other small catalytic RNAs. Sequence requirements in loop A were investigated by mutational experiments, and base triplets following the 3′-cleavage site show diversity to some of extent (*24, 25*).

Although the reaction proceeds similarly to the hammerhead ribozyme, a phosphorothioate substitution in the cleavage site of the hairpin ribozyme does not affect the cleavage activity (*26*), in contrast to the hammerhead ribozyme (*12, 27*). It has been shown that cobalt hexamine, which is an exchange-inert metal complex, can also induce cleavage activity, as in the case of magnesium ions (*28–30*). These results indicate that the metal ion required for cleavage does not bind directly to the phosphate oxygens, but rather functions in outersphere binding to the ribozyme and as a structural factor for maintaining the active conformation of the ribozyme. It was also reported that hairpin ribozyme cleavage is affected by aminoglycoside antibiotics and polyamines (*31*).

The three dimensional structure of the hammerhead ribozyme has been demonstrated by X-ray analysis (*32, 33*). However, the ternary structure of the hairpin ribozyme has not been clarified. The structures of loop A (*34*) and loop B (*35*) have been analyzed independently by nuclear magnetic resonance. These data demonstrate the presence of non-canonical base pairs in both loops. Sheared G–A base pairs have been found to exist in both domains. A uracil next to the cleavage site on the 3′ side adopts a flexible conformation in loop A. In loop B, seven non-canonical base pairs are present.

Since the internal loop located apart from the cleavage site is also essential, a multi-folded structure may be the active conformation for cleavage activity. Linker insertion experiments suggested that the active conformation contains a bent form from (*36, 37*). Recently, domain docking has been supported by crosslinking (*38*) and fluorescence resonance energy transfer (FRET) experiments (*39–41*). In this review, various types of hairpin ribozymes, obtained during our efforts to investigate the topology of the domains, are described. We have proven that a bent conformation of the hairpin ribozyme is an active conformation.

J1 and J3 modification

We have investigated the structure–function relationship of the hairpin ribozyme by modifying the primary structure

[1] This work was supported by grants from The Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-11-706-3976, Fax: +81-11-706-4989, E-mail: ohtsuka@pharm.hokudai.ac.jp

Abbreviations: FRET, fluorescence resonance energy transfer; TriRz, a hairpin ribozyme with trimming activity; 5′FtriRz and 3′FTriRz, 5′- and 3′-side fragments processed from TriRz; TWJ, three way junction.

© 2000 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

at four junctions (J1, J2, J3, and J4) (Fig. 1b). First, we divided the hairpin ribozyme-substrate complex into three fragments by cleavage at the hairpin junction (J3). The ribozyme divided at J3 retains cleavage activity (*16*). Although division at the hairpin loop decreases the cleavage activity somewhat, the cleavage activity of the J3-divided hairpin ribozyme is rescued by extending helix 4 (*42*). In (–)sTobRV, junction 1 (J1) is covalently connected to 121 extra sequences. In order to probe the flexibility at J1, complexes were constructed by joining a substrate to the shortest strand of the three ribozyme fragments (Fig. 2b), using multiple units of 1,3-propandiol phosphate at the junction (*37*). Active conformations were obtained by increasing the number of non-nucleotidic linkers. Complexes without a linker, which are supposed to adopt an extended form as shown in Fig. 2a, did not show cleavage activity. Complexes with 1 or 2 linker units also showed no cleavage activity. Significant cleavage was observed in the molecule with three linker units, and the activity increased with linkers in molecules bearing 4 and 5 linkers. Complexes with 7, 10, and 13 linkers showed cleavage rates 3.6, 7.3, and 8.7-fold higher than that the complex with 5 linkers. Complexes with 10 and 13 propandiol linkers showed near maximal activities. These results suggest that extra space at J1 is required to form an active ribozyme structure, and a bent structure in the hairpin ribozymes caused by the extra space was proposed. We constructed a model of the bent complex by joining junctions with 13 linkers. The same result was obtained by Feldstein and Bruening (*36*). We further improved J1 by introducing a new helix (helix 5) to confirm the flexibility at J1 (Fig. 2c). An extra sequence was introduced at the 3′-end of the hairpin ribozyme to increase the binding to substrate RNA, as compared to the wild type hairpin ribozyme (*43*). A three way junction (TWJ) was formed between the newly designed ribozyme and the substrate RNA. The complex with a solid TWJ showed less RNA cleavage activity than the wild type hairpin ribozyme. However, the ribozyme with a TWJ comprising five unpaired bases or propandiol phosphate linkers had higher cleavage activity than the parent ribozyme without a TWJ. When a *cis*-cleavage system, in which the 5′-end of the substrate RNA is conjugated to the 3′-end of the ribozyme, was employed, the complex with the TWJ containing unpaired bases was also cleaved faster than the complex with the solid TWJ. This suggests that these differences in the cleavage activities are derived from the conformation, and this was proven by non-denaturing gel electrophoresis. The interaction between domains I and II might be poor in the complex with the solid TWJ, because the three helices at the junction are equally extended. On the other hand, a domain–domain interaction might occur in TWJ containing unpaired bases, since the structural stress originating in the TWJ of these complexes might be released by the unpaired adenine or the propandiol phos-

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

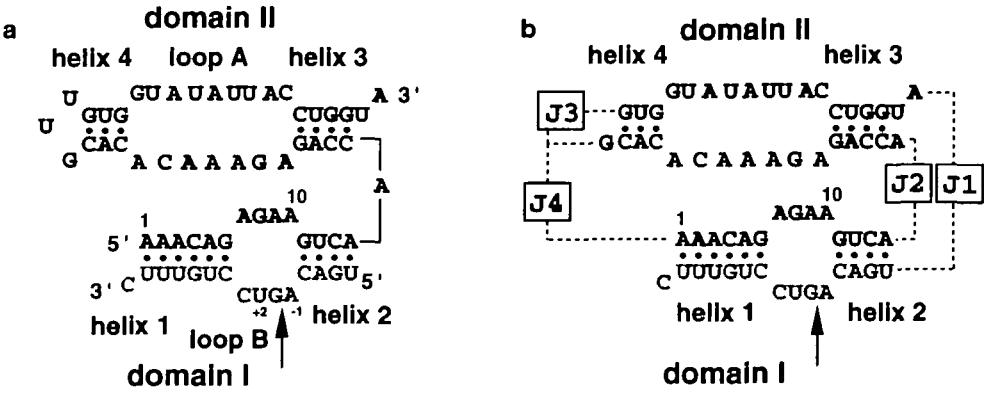
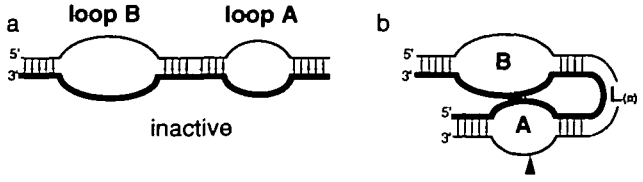


Fig. 1. **Secondary structures of hairpin ribozymes.** a) A wild type hairpin ribozyme (E50) and a substrate (S1). Domain I involves helix 1, helix 2, and an internal loop A; and domain II, helix 3, helix 4, loop B, and a hairpin loop (GUU). b) Modified hairpin ribozymes constructed for studies of structure-function relationships. The covalent bonds of J1 and J4 do not exist in the natural hairpin ribozyme, while bond J2 conjugates the two domains in the wild type ribozyme. J2 can be separated when J1 is connected. When domain I and domain II are connected by J4, the two domains are aligned in a reverse joined ribozyme. Cleavage occurs depending on the linker length at J1 and J4. Arrows indicate cleavage sites. Substrates are shown by gray letters.



L : -(CH₂CH₂CH₂PO₄)-	
n = 0~3	inactive
n =5,7,10,13	active

L : -(CH₂CH₂CH₂PO₄)-	kcat/Km (min⁻¹·μM⁻¹)	
n=0	less active	1.8
n=2	less active	1.9
n=5	active	7.2

Fig. 2. **Schematic drawing of modified hairpin ribozymes.** a) Extended form of the ribozyme to which J1 is covalently connected without a linker. b) Bent form of the ribozyme. J1 is joined through propandiol phosphate linkers. c) The hairpin ribozyme with a three-way junction (TWJ). Propandiol and adenosines were used as linkers (L). Arrows indicate cleavage sites. Enzyme parts and base pairs are shown by bold and thin lines, respectively.

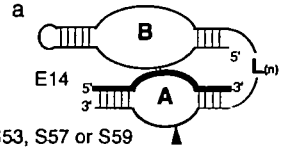
phate tract, and the flexibilities of the three helices might increase the domain–domain interaction. The difference in the activities between TWJs in the presence or absence of unpaired bases suggests that the flexibility of the TWJ is important for the cleavage. Burke and co-workers also demonstrated using FRET that a TWJ with mismatched bases at J1 allows domain docking (*41*). In the natural construct, a four-way junction is formed at J1, and, strikingly, gel mobility shift assay and FRET showed that the four way junction at J1 can achieved more domain docking than two or three-way junctions (*38*, *41*).

J2 modification

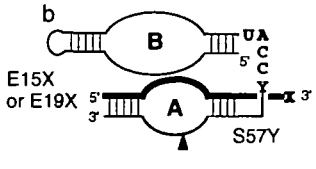
We divided the hinge region (A14–A15) of the hairpin ribozyme by joining J1 with tri- or penta-cytidylates (Fig. 3a) (*44*). In these constructs of the ribozyme, S53 without linker, S57 (n = 3) and S59 (n = 5) are substrates and the rest oligonucleotide, E14 is an enzyme. S59, S57 and S53 are inactive in the absence of E14, but S57 and S59 are cleaved at the same site as the wild type ribozyme in the presence of E14. S57 with three cytidine linkers shows an unexpectedly high cleavage activity. This result is interesting because it has been shown that the ribozyme joining at J1 with 3 propandiol linker units does not show high cleavage activity. The reason for this might be that the structural stress at the hinge is released by disconnecting the hinge. Division at the hinge might allow domain docking even though J1 is connected with a short linker. To investigate the structural properties of the hinge region in detail, E14 was substituted with 15-mer RNAs (E15X, X = A, U, or G) with an additional base X at the 3′ end (Fig. 3b). The extra base (X) was supposed to interact with one of the bases in the linker region, 5′ UA(C)n 3′, of S59 and S57. Although it was difficult to confirm the formation of the base pairs between the added A or U and the bases in the linker, S59 and S57 were cleaved in the presence of either E15A or E15U. However, E15G did not cause any cleavage. This inhibition of the cleavage reaction derives from base pairing between the 3′-end of E15X and the opposite base in the linker sequences of S57 or S59. To confirm whether or not these inhibitions derive from base pairing, the 3′-end of the linker was altered to either U or G (S57U (Y = U) and S57G (Y = G) in Fig. 3), and the cleavage of these substrates in the presence of E15X was analyzed. The S57U-E15G and S57G-E15G complexes were found to be active to the same extent as S57U-E14 or S57G-E14. However, the cleavage rates for both S57U-E15A and S57G-E15C were lowered to 43 and 10%, respectively. This means that the base pairing of the X in E15X with a base at the opposite side was responsible for the inhibition of the cleavage of the S57-E15G complex. The extent of inhibition for S57U-E15A was smaller than that for S57-E15G. This might have resulted from the difference in the stability of the G·C and A•U base pairs. The additional base at the 3′-end of E15X was further extended from one to five bases and a series of E19 induced the cleavage of both S57 and S59. This result indicates that the 3′-extra bases of these E19 series do not prevent the formation of the active bent conformation.

Hairpin ribozyme with replaced domains (J4 modification)

The results described above suggest that the active conformation of the hairpin ribozyme is a bent structure. If this tertiary structure is conserved, then the hairpin ribozymes would retain cleavage activity. We constructed a new type of hairpin ribozyme in which the two domains are separated and rejoined to yield ribozymes (Fig. 4a) (*44*). It had been shown that hairpin ribozymes can be divided at the hairpin loop (*16*, *42*). To replace domain II, E50 was divided between A15 and C16, and the 5′-end of E50 was linked to G30 in the hairpin loop using oligocytidylates (Cn, n = 0, 3, 6, 9, 12, and 18) as linkers. These newly rejoined enzyme strands, RC0–RC18, were hybridized with E19, and the cleavage reactions of S1 were analyzed. In 12 mM Mg^{2+}, RCn-19 complexes (n = 3, 6, 9, 12, and 18) gave cleavage products, while RC0-19, which has no linker, was found to be inactive. These ribozymes could cleave excess amounts of target RNA. As the linker length of RCn-19 is increased, the k_{cat}/K_m values for the rejoined ribozymes increases. The reason for the dependence of k_{cat}/K_m on the linker length is not clear, but the two domains might be in more favorable contact, due to the flexibility produced by the insertion of the cytidine linkers. However, the potential turnover of the rejoined ribozymes is lower than that of the parent ribozyme. Larger linkers are required for the cleavage activity of the rejoined ribozymes. It is thought that this result derives from the tertiary contact of the two domains. In the active bent conformation, there may be some distance be-



L : rC		
S53-E14	n=0	inactive
S57-E14	n=3	active
S59-E14	n=5	active



X	Y		k x 10^2 (min^{-1})
-A	C	active	2.7
-G	C	inactive	0.084
-C	C	active	3.3
-U	C	active	3.4
-C	G	inactive	0.12
-G	G	active	0.87
5′-UUUUU	3′C	active	3.6
-AAAAA	C	active	2.6
-GUUUU	C	inactive	0.065
-GGGUU	C	inactive	N.D.

Fig. 3. **The hairpin ribozymes divided at the hinge (J2) and rejoined at J1.** a) S53-E14, S57-E14, and S59-E14 complexes. J1 was connected by cytidine linkers. b) S57-E15X and S57-E19 complexes. E15X and E19 contain extra sequences at the 3′-end. Enzyme parts and base pairs are shown by bold and thin lines, respectively.

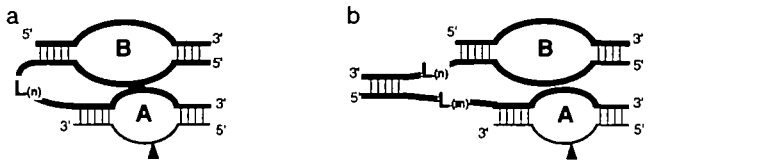
Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

tween helix 1 and helix 4 in the parent ribozyme.

Since this reversely joined ribozyme retains cleavage activity, the importance of the bent conformation for activity has been confirmed. Although completely separated domains of the hairpin ribozyme are active at high concentrations of ribozyme and magnesium ion (*45*), it is difficult for the completely separated domains to maintain the association form at low magnesium ion concentrations. Therefore, we made further constructions of hairpin ribozymes with separated domains, but containing complementary arms to allow the association of the two domains as shown in Fig. 4b (*46*). Linker nucleotides were inserted between the arms and domains to investigate the relative domain alignment. These ribozymes were active under the standard conditions (12 mM MgCl), depending on the length of the linker. Both L(m) and L(n) linkers are required for cleavage, and the L(m) linker in domain I influenced the cleavage activity more than L(n). These differences in the contributions of L(m) and L(n) to the cleavage activities derive from their conformations. When the complementary arms are covalently joined through a stable loop, these ribozymes show cleavage activities. However, the K_m values of the stem-loop ribozymes were found to be larger than that of the parent ribozyme, which can adopt both linear and bent conformations. Kinetic analyses of these modified hairpin ribozymes provide insight into a higher turnover of the hairpin ribozyme as compared to other small ribozymes. In these ribozymes, the bent conformation is thought to be enhanced, and may be suitable for physicochemical studies on the tertiary structure of the hairpin ribozyme.

Three-domain ribozyme

Based on the requirement of domain–domain interaction for ribozyme activity, we constructed a new type of hairpin ribozyme with three stem-loop domains in one molecule (Fig. 5a). In this ribozyme, another domain (domain I′) was connected to the 3′-end of domain II of the parent hairpin ribozyme, and the new ribozyme was trimmed at domain I′ during a run-off transcription reaction from the plasmid harboring the ribozyme gene with three domains. The trimming reaction appeared to proceed automatically as shown in Fig. 5b (*47*). The processed ribozyme was designed to



L : rC

nbozyme	kcat/Km (min^{-1}·μM^{-1})
RC0-19	—
RC3-19	0.025
RC6-19	0.092
RC9-19	0.32
RC12-19	0.35
RC18-19	0.89
E30-19	0.64

L : rA k x 10^2 (min^{-1})

	L1, m=			
L2, n=	0	2	4	6
0	0.048	0.11	0.48	0.79
2	0.059	0.53	5.0	9.5
4	0.088	0.95	6.2	9.5

Fig. 4. **Ribozymes joined in reverse.** a) J1 and J2 are separated and J4 is joined by cytidine linkers. The cleavage activities increase with the increase in linker length. b) Ribozymes with separated domains. The two domains have complementary anchor sequences to facilitate their association. Enzyme parts and base pairs are shown by bold and thin lines, respectively.

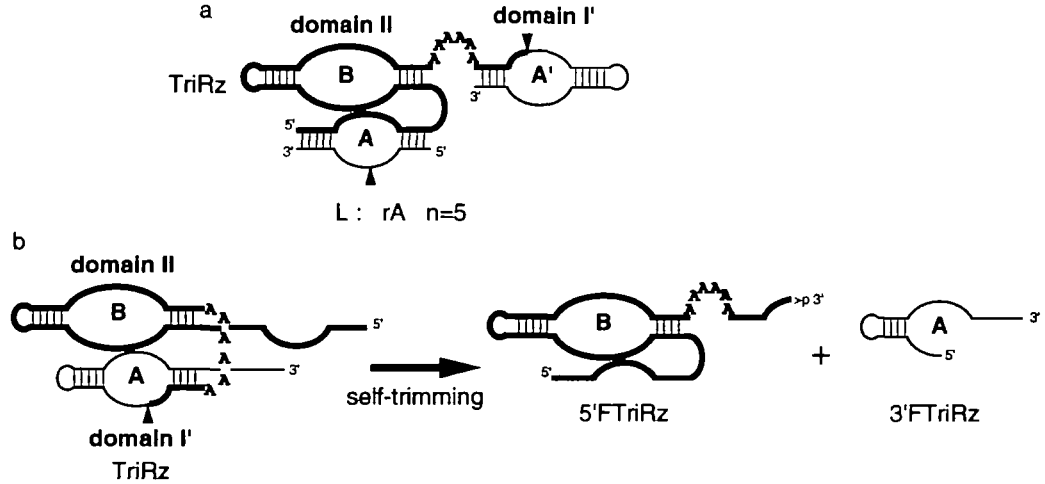


Fig. 5. **a) The hairpin ribozyme with three domains.** Domain I′ is connected to the 3′-end of domain II by adenosine linkers. The three domain ribozyme is autoprocessed at the cleavage site in domain I′ during transcription from the template DNA. **b) Scheme of the self-trimming reaction of the ribozyme.** The 5′-side fragment (5′FTriRz) cleaves the target RNA in *trans*. Enzyme parts and base pairs are shown by bold and thin lines, respectively.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

cleave an external substrate RNA derived from the mRNA of human inducible nitric oxide synthase, and was shown to cleave at the expected, unique site. Domain II of the ribozyme acts as the catalytic domain for both the *cis*- and *trans*-cleavage of RNA. Since this new ribozyme is able to process 3′-extra nucleotides autocatalytically without another ribozyme for trimming, this ribozyme is useful not only for proving the active conformation of RNA but also for *in vivo* applications. These ribozymes provide important models with which to improve our understanding of the role of the domain–domain interaction in the catalytic activity.

The cleavage activity of the three-domain hairpin ribozyme was further improved by increasing the number of linker bases connecting domain I′ with domain II from six to seven. The ribozyme was designed to release a *trans*-acting ribozyme that is able to bind target RNAs through three hybridizing arms (TWJ) (*48*).

We have assembled two domains of the hairpin ribozyme in various ways, and those constructs suggest that the two domains interact with each other in antiparallel fashion. Domain docking between domains I and II is supported by various experiments. However, it is still unclear what kinds of interactions promote the docking. Gait and co-workers proposed a model of domain docking that involves a zipper structure (*49*). The ribose zipper would be formed between the 2′-hydroxyl groups of residues A10–G11 of loop A and C25–A24 of loop B. We introduced 4-thio- (^{4S}U), 2-thio (^{2S}U), 4-*O*-methyluridine (^{4Me}U), and cytidine substitutions for U+2 in the substrate (*50*). A hairpin ribozyme cleaved the substrate RNA with either C+2, ^{4S}U+2, or ^{4Me}U+2 at about 14-, 6-, and 4-fold lower rates, respectively, than the natural substrate. In contrast, the substrate with a ^{2S}U+2 was cleaved with the same activity as the natural substrate. These results suggest that the O4 of U+2 is involved in hydrogen bonds at loop A, but the O2 of U+2 is not recognized by the active residues. Based on circular dichroism data in addition to these cleavage results, we propose the conformational change of U+2 from the ground state to the active molecule during the reaction. Domain docking is fast and not rate-limiting (*40*). Since this process is a key step in the cleavage and ligation reactions, the tertiary structure of the hairpin ribozyme should be determined for a detailed understanding of the reaction.

Conclusion

Modified hairpin ribozymes have been constructed chemically to investigate their active conformation, and a bend structure was found to be essential for cleavage activity. Based on these experiments, new ribozymes, which can be processed during transcription of the gene by self-cleavage, have been designed and used to cleave external RNA substrates. Tertiary interactions of internal domains of the ribozyme were investigated by alternating the primary structure or by introducing modified bases at essential positions. The results indicate that chemical synthesis is useful for investigating the structure–function relationships of active molecules.

REFERENCES

1. Symons, R.H. (1992) Small catalytic RNAs. *Annu. Rev. Biochem.* **61**, 641–671
2. Forster, A.C. and Symons, R.H. (1987) Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites. *Cell* **49**, 211–220
3. Hampel, A. and Triz, R. (1989) RNA catalytic properties the minimum (–)sTRSV sequence. *Biochemistry* **28**, 4929–4933
4. Sarver, N., Cantin, E.M., Chang, P.S., Zaia, J.A., Ladne, P.A., Stephens, D.A., and Rossi, J.J. (1990) Ribozymes as potential anti-HIV-1 therapeutic agents. *Science* **247**, 1222–1225
5. Koizumi, M., Kamiya, H., and Ohtsuka, E. (1992) Ribozymes designed to inhibit transformation of NIH3T3 cells by the activated c-Ha-ras gene. *Gene* **117**, 179–184
6. Christoffersen, R.E. and Marr, J.J. (1995) Ribozymes as human therapeutic agents. *J. Med. Chem.* 2023–2037
7. 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
8. Yu, M., Ojwang, J., Yamada, O., Hampel, A., Rapapport, J., Looney, D., and Wong-Staal, F. (1993) A hairpin ribozyme inhibits expression of diverse strains of human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* **90**, 6340–6344
9. Buzayan, J.M., Gerlach, W.L., and Bruening, G. (1986) Nonenzymatic cleavage and ligation of RNAs complementary to a plant virus satellite RNA. *Nature* **323**, 349–353
10. Haseloff, J. and Gerlach, W.L. (1989) Sequences required for self-catalyzed cleavage of the satellite RNA of tobacco ringspot virus. *Gene* **82**, 43–52
11. Hampel, A., Tritz, R., Hicks, M., and Cruz, P. (1990) 'Hairpin' catalytic RNA model: evidence for helices and sequence requirement for substrate RNA. *Nucleic Acids Res.* **18**, 299–304
12. van Tol, H., Buzayan, J.M., Feldstein, P.A., Eckstein, F., and Bruening, G. (1990) Two autolytic processing reactions of a satellite RNA proceed with inversion of configuration. *Nucleic Acids Res.* **18**, 1971–1975
13. Berzal-Herranz, A., Joseph, S., Chowrira, B.M., Butcher, S.E., and Burke, J.M. (1993) Essential nucleotide sequences and secondary structure elements of the hairpin ribozyme. *EMBO J.* **12**, 2567–2574
14. Berzal-Herranz, A., Joseph, S., and Burke, J.M. (1992) In vitro selection of active hairpin ribozymes by sequential RNA-catalyzed cleavage and ligation reactions. *Genes Dev.* **6**, 129–134
15. Joseph, S., Berzal-Herranz, A., Chowrira, B.M., Butcher, S., and Burke, J.M. (1993) Substrate selection rules for the hairpin ribozyme determined by in vitro selection, mutation, and analysis of mismatched substrates. *Genes Dev.* **7**, 130–138
16. Sekiguchi, A., Komatsu, Y., Koizumi, M., and Ohtsuka, E. (1991) Mutagenesis and self-ligation of the self-cleavage domain of the satellite RNA minus strand of tobacco ringspot virus and its binding to polyamines. *Nucleic Acids Res.* **19**, 6833–6838
17. Anderson, P., Monforte, J., Tritz, R., Nesbitt, S., Hearst, J., and Hampel, A. (1994) Mutagenesis of the hairpin ribozyme. *Nucleic Acids Res.* **22**, 1096–1100
18. Grasby, J.A., Mersmann, K., Singh, M., and Gait, M.J. (1995) Purine functional groups in essential residues of the hairpin ribozyme required for catalytic cleavage of RNA. *Biochemistry* **34**, 4068–4076
19. Butcher, S.E. and Burke, J.M. (1994) Structure-mapping of the hairpin ribozyme—Magnesium-dependent folding and evidence for tertiary interactions within the ribozyme-substrate complex. *J. Mol. Biol.* **244**, 52–63
20. Butcher, S.E. and Burke, J.M. (1994) A photo-cross-linkable tertiary structure motif found in functionally distinct RNA molecules is essential for catalytic function of the hairpin ribozyme. *Biochemistry* **33**, 992–999
21. dos Santos, D.V., Fourrey, J.L., and Favre, A. (1993) Flexibility of the bulge formed between a hairpin ribozyme and deoxy-substrate analogues. *Biochem. Biophys. Res. Commun.* **190**, 377–385
22. dos Santos, D.V., Vianna, A.L., Fourrey, J.L., and Favre, A. (1993) Folding of DNA substrate-hairpin ribozyme domains: use of deoxy 4-thiouridine as an intrinsic photolabel. *Nucleic*

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

Acids Res. **21**, 201–207
23. Chowrira, B.M., Berzal-Herranz, A., and Burke, J.M. (1991) Novel guanosine requirement for catalysis by the hairpin ribozyme. *Nature* **354**, 320–322
24. Shippy, R., Siwkowski, A., and Hampel, A. (1998) Mutational analysis of loops 1 and 5 of the hairpin ribozyme. *Biochemistry* **37**, 564–570
25. Perez-Ruiz, M., Barroso-DelJesus, A., and Berzal-Herranz, A. (1999) Specificity of the hairpin ribozyme. Sequence requirements surrounding the cleavage site. *J. Biol. Chem.* **274**, 29376–29380
26. Buzayan, J.M., Feldstein, P.A., Bruening, G., and Eckstein, F. (1988) RNA mediated formation of a phosphorothioate diester bond. *Biochem. Biophys. Res. Commun.* **156**, 340–347
27. Koizumi, M. and Ohtsuka, E. (1991) Effects of phosphorothioate and 2-amino groups in hammerhead ribozymes on cleavage rates and Mg^{2+} binding. *Biochemistry* **30**, 5145–5150
28. Hampel, A. and Cowan, J.A. (1997) A unique mechanism for RNA catalysis: the role of metal cofactors in hairpin ribozyme cleavage. *Chem. Biol.* **7**, 513–517
29. Nesbitt, S., Hegg, L.A., and Fedor, M.J. (1997) An unusual pH-independent and metal-ion-independent mechanism for hairpin ribozyme catalysis. *Chem. Biol.* **4**, 619–630
30. Young, K.J., Gill, F., and Grasby, J.A. (1997) Metal ions play a passive role in the hairpin ribozyme catalysed reaction. *Nucleic Acids Res.* **19**, 3760–3766
31. Earnshaw, D.J. and Gait, M.J. (1998) Hairpin ribozyme cleavage catalyzed by aminoglycoside antibiotics and the polyamine spermine in the absence of metal ions. *Nucleic Acids Res.* **26**, 5551–5561
32. Pley, H.W., Flaherty, K.M., and McKay, D.B. (1994) Three-dimensional structure of a hammerhead ribozyme. *Nature* **372**, 68–74
33. Scott, W.G., Finch, J.T., and Klug, A. (1995) The crystal structure of an all-RNA hammerhead ribozyme: a proposed mechanism for RNA catalytic cleavage. *Cell* **81**, 991–1002
34. Cai, Z.P. and Tinoco, I. (1996) Solution structure of loop A from the hairpin ribozyme from tobacco ringspot virus satellite. *Biochemistry* **35**, 6026–6036
35. Butcher, S.E., Allain, F.H., and Feigon, J. (1999) Solution structure of the loop B domain from the hairpin ribozyme. *Nat. Struct. Biol.* **6**, 212–216
36. Feldstein, P.A. and Bruening, G. (1993) Catalytically active geometry in the reversible circularization of 'mini-monomer' RNAs derived from the complementary strand of tobacco ringspot virus satellite RNA. *Nucleic Acids Res.* **21**, 1991–1998
37. Komatsu, Y., Koizumi, M., Nakamura, H., and Ohtsuka, E. (1994) Loop-size variation to probe a bent structure of a hairpin ribozyme. *J. Am. Chem. Soc.* **116**, 3692–3696
38. Pinard, R., Heckman, J.E., and Burke, J.M. (1999) Alignment of the two domains of the hairpin ribozyme-substrate complex defined by interdomain photoaffinity crosslinking. *J. Mol. Biol.* **287**, 239–251
39. Murchie, A.I.H., Thomson, J.B., Walter, F., and Lilley, D.M.J. (1998) Folding of the hairpin ribozyme in its natural conformation achieves close physical proximity of the loops. *Mol. Cell* **1**, 873–881
40. Walter, N.G., Hampel, K.J., Brown, K.M., and Burke, J.M. (1998) Tertiary structure formation in the hairpin ribozyme monitored by fluorescence resonance energy transfer. *EMBO J.* **17**, 2378–2391
41. Walter, N.G., Burke, J.M., and Millar, D.P. (1999) Stability of hairpin ribozyme tertiary structure is governed by the interdomain junction. *Nat. Struct. Biol.* **6**, 544–549
42. Chowrira, B.M. and Burke, J.M. (1992) Extensive phosphorothioate substitution yields highly active and nuclease-resistant hairpin ribozymes. *Nucleic Acids Res.* **20**, 2835–2840
43. Komatsu, Y., Shirai, M., Yamashita, S., and Ohtsuka, E. (1997) Construction of hairpin ribozymes with a three-way junction. *Bioorg. Med. Chem.* **5**, 1063–1069
44. Komatsu, Y., Kanzaki, I., Koizumi, M., and Ohtsuka, E. (1995) Modification of primary structures of hairpin ribozymes for probing active conformations. *J. Mol. Biol.* **252**, 296–304
45. Butcher, S.E., Heckman, J.E., and Burke, J.M. (1995) Reconstitution of hairpin ribozyme activity following separation of functional domains. *J. Biol. Chem.* **270**, 29648–29651
46. Komatsu, Y., Kanzaki, I., and Ohtsuka, E. (1996) Enhanced folding of hairpin ribozymes with replaced domains. *Biochemistry* **35**, 9815–9820
47. Komatsu, Y., Kanzaki, I., Shirai, M., and Ohtsuka, E. (1997) A new type of hairpin ribozyme consisting of three domains. *Biochemistry* **36**, 9935–9940
48. Komatsu, Y., Shirai, M., and Ohtsuka, E. (1998) Modification and application of self-trimming hairpin ribozymes to targeting a transcribed RNA in vitro. *J. Biochem.* **124**, 986–991
49. Earnshaw, D.J., Masquida, B., Muller, S., Sigurdsson, S.T., Eckstein, F., Westhof, E., and Gait, M.J. (1997) Inter-domain crosslinking and molecular modelling of the hairpin ribozyme. *J. Mol. Biol.* **274**, 197–212
50. Komatsu, Y., Kumagai, I., and Ohtsuka, E. (1999) Investigation of the recognition of an important uridine in an internal loop of a hairpin ribozyme prepared using post-synthetically modified oligonucleotides. *Nucleic Acids Res.* **27**, 4314–4323

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012