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In Vitro Activity of the Hairpin Ribozyme Derived from the Negative Strand of Arabis Mosaic Virus Satellite RNA[1]

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The negative strand of the satellite RNA of tobacco ringspot virus [(−)sTRSV] is a self-cleaving RNA, of which self-cleaving domain is called the hairpin ribozyme. The negative strand of the satellite RNA of arabis mosaic virus [(−)sArMV] has been suggested to have a hairpin ribozyme-like secondary structure, and we have previously shown that this hairpin domain of (−)sArMV has ribozyme activity. Here we report characterization of the cleavage reaction of the (−)sArMV hairpin ribozyme. Mutagenesis analyses in a *trans*-acting system revealed, surprisingly, that the wild-type ribozyme was less active than almost all the other mutant ribozymes tested. In a *cis*-acting system (self-cleaving reaction), however, the reaction of the RNA containing the wild-type sequence proceeds highly efficiently. This result suggests that the inefficient cleavage of the wild-type substrate in *trans*-acting system may be due to low efficiency at the substrate-binding step but not at the chemical cleavage step in the reaction. We also constructed a chimeric ribozyme between the catalytic hairpin domain from (−)sArMV and the substrate-binding site from (−)sTRSV. This chimeric ribozyme had the highest activity among the *trans*-acting hairpin ribozymes tested.

Key words: arabis mosaic virus, hairpin ribozyme, ribozyme, RNA enzyme, satellite RNA.

The hairpin ribozyme is the minimal self-cleaving domain of the negative strand of the satellite RNA of tobacco ringspot virus [(−)sTRSV] (*1-3*). The (−)sTRSV RNA autocatalytically cleaves itself at a specific site in the presence of Mg^{2+} to generate a 2′,3′ cyclic phosphate end and a 5′ hydroxyl end. A catalytic domain consisting of 50 nucleotides and a substrate domain of 14 nucleotides have been identified in the (−)sTRSV RNA sequence, and the catalytic domain interacts with the substrate as a true enzyme (*2*). Since the RNA having this ribozyme activity is thought to form a hairpin-loop structure, it has been named hairpin ribozyme (*3*). A model of the hairpin ribozyme derived from the (−)sTRSV is shown in Fig. 1A. At present, this secondary structure model is consistent with the results of many experiments including photocross linking, chemical modification and mutational analyses (*4-10*). The *in vitro* selection system has also revealed the sequence requirement for the catalysis (*11-13*). Several newly designed hairpin ribozymes have been found to correctly cleave various RNA sequences *in vitro* and *in vivo* (*14-17*). The tertiary interaction between two large internal loops has been reported to be important for the cleavage reaction (*18-21*).

It has been suggested that the negative strand of arabis mosaic virus satellite RNA [(−)sArMV] has a hairpin ribozyme-like secondary structure (Fig. 1B) (*22-25*), but for a long time the cleavage reaction of this RNA was not confirmed. In the previous paper (*26*), we reported that the hairpin region derived from (−)sArMV does indeed have hairpin ribozyme activity. In the present paper, we characterize this cleavage reaction and report that a chimeric ribozyme consisting of (−)sTRSV and (−)sArMV has very high cleavage activity. We also report the results of mutagenesis analyses for this ribozyme in *cis*- and *trans*-acting systems.

MATERIALS AND METHODS

Enzymes and Chemicals—T7 RNA polymerase was obtained from Toyobo (Osaka). Restriction endonucleases were from Toyobo, Takara Shuzo (Kyoto), or New England Biolabs (Beverly). Other enzymes and chemicals were purchased from commercial sources. DNA synthesis was done using an Applied Biosystems DNA synthesizer.

RNA Catalytic Reactions—All RNAs used in this study were prepared by *in vitro* transcription from appropriate synthetic DNAs or plasmids. The RNAs were labeled internally with [α-^{32}P]UTP (Amersham) during transcrip-

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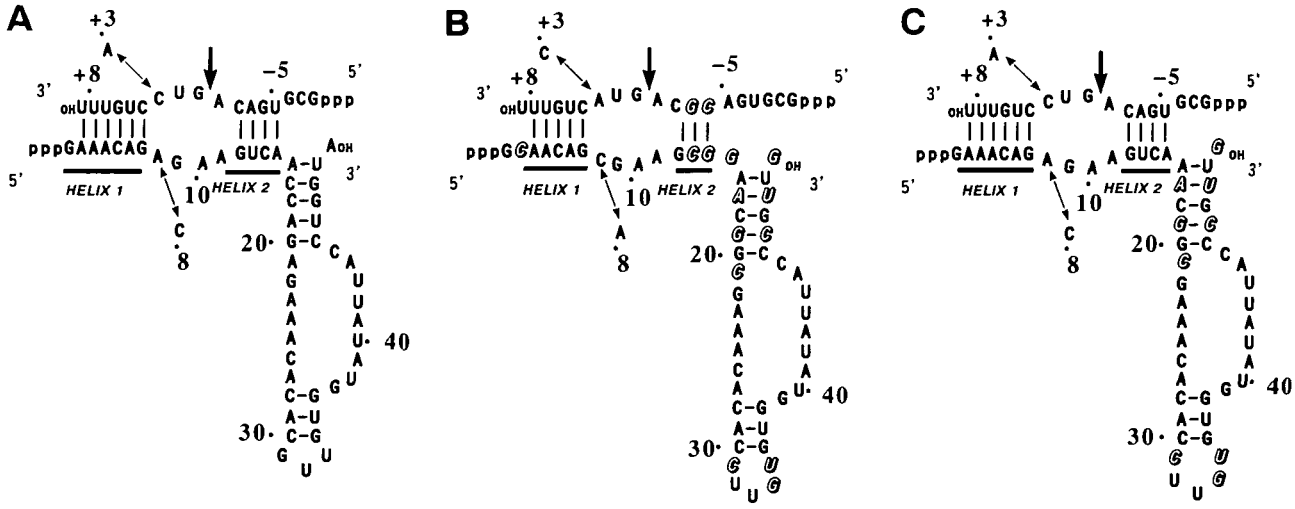

Fig. 1. **Secondary structure models of the hairpin ribozymes.** The *in vitro* transcripts used in this study are shown. A, (−)sTRSV; B, (−)sArMV; C, chimeric molecules between (−)sTRSV and (−)sArMV. Numbers with plus and minus signs, nucleotide numbers of the substrate. Numbers without signs, nucleotide numbers of the ribozyme. The thick arrow indicates the predicted site of cleavage. Thin arrows indicate the base substitutions in mutants. Different bases between A and B are indicated by outlined letters in B and C.

tion reaction as described (*14*). For the *trans*-acting hairpin ribozyme system, synthesis of substrate and ribozyme RNAs and RNA catalytic reactions were done as described previously (*2, 14, 25*). The standard reaction mixture containing 40 mM Tris-HCl (pH 7.6), 12 mM $MgCl_2$, 2 mM spermidine, 6 nM [^{32}P] substrate, and 15 nM ribozyme in a total volume of 20 μl was incubated at 37°C. To stop the reaction, aliquots were withdrawn from the mixture and put onto a dried urea-dye (Xylene Cyanol and Bromophenol Blue)-EDTA mixture to give final concentrations of 6.7 M urea, 0.04% dye, and 50 mM EDTA. Samples were analyzed by 20% polyacrylamide/8 M urea gel electrophoresis. The reaction products were measured by counting the radioactivity of the product bands on an autoradiogram of the gel by an Imaging Analyzer as described below.

Kinetic Analyses—Kinetic parameters of two ribozyme reactions were measured using a Lineweaver-Burk plot (Table I). The standard reaction mixture containing 1.5 nM ribozyme and 3.0, 4.5, 6.0, or 12.0 nM substrate RNA was incubated at 37°C. The products were analyzed as described (*14*).

Constructions of Ribozyme-Coding Plasmids—To obtain various types of *cis*-acting (self-cleaving) hairpin ribozymes (see Fig. 5), synthetic DNAs were cloned into a transcribable plasmid, pGEM-4Z (Promega). Four single-stranded DNAs, ACSA (5′-GACAAGCTTGACGCAGTAC-TGTTTCGGCAAGCAACAG-3′), ACSC (5′-GACAAGCT-TGACGCAGTCCTGTTTCGGCAAGCAACAG-3′), ACRT (5′-GACGAATTCAACGGGTAATATACCACACAAGGT-GTGTTTCGCCGTTCCGCTTCTCTGTTGCTTGCCGAA-ACAG-3′), and ACRG (5′-GACGAATTCAACGGGTAAT-ATACCACACAAGGTGTGTTTCGCCGTTCCGCTTCGC-TGTTGCTTGCCGAAACAG-3′) were synthesized. Since the 3′-terminal 19 nucleotides of ACSA or ACSC were hybridizable to the 3′-terminal 19 nucleotides of ACRT or ACRG, each pair of DNAs (ACSA-ACRT, ACSA-ACRG, ACSC-ACRT, or ACSC-ACRG) was annealed, and the long recessed 3′ ends were filled in with dNTPs and the Klenow fragment of DNA polymerase I. The resulting DNA (91 base pairs), containing *Hin*dIII and *Eco*RI recognition sites at the 5′ and 3′ terminal region, respectively, was digested with two enzymes and then cloned into the *Hin*dIII and *Eco*RI sites of the pGEM-4Z vector.

Transcription and Cis-Cleavage Reactions—The above described pGEM-4Z-based plasmids were digested with *Eco*RI. The *Eco*RI site is located at the end of the catalytic hairpin region of the plasmid (nucleotides 87–91 in Fig. 5). These linearized plasmids were transcribed with T7 RNA polymerase. The reaction mixture contained 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 26 units of ribonuclease inhibitor, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.05 mM [α-^{32}P]UTP (10 μCi in total), 0.05 μg of plasmid template and 25 units of T7 RNA polymerase in a total volume of 25 μl. The mixture was incubated for 1 h or 2.5 h at 37°C. The primary transcript is shown in Fig. 5. During the transcription reaction, the self-cleavage occurs concomitantly. The reaction products were precipitated in ethanol, dried and dissolved in a urea-dye-EDTA loading mixture to be electrophoresed in 10% polyacrylamide/8 M urea gels.

Nucleic Acid Manipulations and Analytical Methods—DNA manipulations, cloning techniques, and labeling and other analytical methods for RNA were performed essentially as described (*14*). Determination of cleavage sites was as described (*14*). Quantitative analyses of the cleavage reactions were performed by counting photo-stimulated luminescence of the product bands in a radiogram of the gel using a Bio-Image Analyzer BAS2000 (Fuji Film; Ref. *27*).

RESULTS AND DISCUSSION

The Hairpin Domain of the (−)sArMV Has Ribozyme Activity—In the previous communication (*26*), we reported that the hairpin domain of (−)sArMV has ribozyme activity. Here we describe the result in detail. Figure 1B shows the expected secondary structure of the ribozyme-

substrate complex derived from the (−)sArMV sequence. To examine whether this sequence has ribozyme activity, we have synthesized these RNAs and tested the ribozyme activity as described in "MATERIALS AND METHODS." Figure 2 shows the time courses of the reactions of hairpin ribozymes from (−)sTRSV and (−)sArMV. Although the ribozyme from (−)sArMV has very weak activity compared to the ribozyme from (−)sTRSV, the hairpin domain of the (−)sArMV has ribozyme activity. Almost 20% of the wild-type substrate was cleaved by the wild-type ribozyme in 1 h in the reaction mixture containing 15 nM ribozyme and 6 nM substrate (Fig. 2). Analyses of product bands confirmed that the cleavage occurred at the predicted location on the substrate (data not shown). Kinetic analyses showed that apparent K_m value of this ribozyme is similar to that of the (−)sTRSV but k_{cat} value is much smaller (one or two orders of magnitude) than that of the (−)sTRSV (Table I). Recently, De Young *et al.* also reported that the (−)sArMV hairpin domain has ribozyme activity (*28*).

The Wild-Type Ribozyme of the (−)sArMV Is Much Less Active than the Other Mutant Ribozymes Tested—In the (−)sArMV hairpin ribozyme, the nucleotides +3 and 8 are A and C, respectively (Fig. 1B), whereas (−)sTRSV has C+3 and A8 (Fig. 1A). It seems likely that compensatory mutations occurred during evolution of these satellite RNAs to conserve the unusual A-C (or C-A) pair at this set of positions. To investigate contribution of these nucleotides (+3 and 8) to ribozyme activity of (−)sArMV sequence, we have prepared such substrates (C+3 and A+3 substrates) and ribozymes (A8 and C8 ribozymes) (Fig. 1B) and tested their cleaving activities using all the combinations of A and C at this set of positions. Cleaving activity was revealed in every combination of the substrates and the ribozymes (Fig. 3). However, the wild-type combination (A+3-C8, closed circles in Fig. 3), surprisingly, was the most inefficient. The (−)sTRSV type of combination, C+3-A8, was the best combination for this cleavage (closed squares in Fig. 3). Table I shows the kinetic parameters of this reaction. The catalytic efficiency, k_{cat}/K_m, of the ribozyme reaction from (−)sArMV was much smaller than that of (−)sTRSV. When the nucleotides at A+3 and C8 were changed to C+3 and A8, the catalytic efficiency increased to more than twice of that of the wild type. This value is, however, still only 15% of that of the (−)sTRSV (Table I). In this condition, the ribozyme from (−)sArMV has very low activity. As described above, De Young *et al.* also presented data on the catalytic properties of (−)sArMV hairpin ribozyme (*28*). Their kinetic analyses showed that the k_{cat} value of the reaction with the wild-type substrate-ribozyme combination of (−)sArMV is similar to that of the (−)sTRSV ribozyme, although the K_m value is much higher. These results are in conflict with our data shown in Table I. As described above, we showed that the apparent K_m value of (−)sArMV ribozyme is similar to that of the (−)sTRSV but the k_{cat} value is much smaller. However, their catalytic efficiency (k_{cat}/K_m) ratio of (−)sArMV to (−)sTRSV is, interestingly, similar to our ratio. At present, we do not know why their kinetic data (*28*) are so different from ours. However, the difference may be explained by the differences in the terminal sequences between our RNAs and theirs. As shown in Fig. 1B, our substrate has a 5′-terminal sequence of pppGCGU-GACG-CA* (the asterisk denotes the cleavage site) but they used a substrate having pppGCGACGCA*. Also, the 5′-terminal sequences of the ribozymes are not the same. In our experience, an extensive conformational change may be caused by a single mutation in an RNA *in vitro* (*29*). It is possible that this small difference in sequence may cause a different mode of interaction between substrate and ribozyme and thus give different kinetic data.

TABLE I. **Kinetic parameters of (−)sArMV ribozymes.**

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min$^{-1}\cdot\mu$M^{-1})	k_{cat}/K_m (rel)	Ref.
(−)sTRSV (C+3, A8) (WT[a])	0.028	0.41	15	1.0	*30*
(−)sArMV (A+3, C8) (WT[a])	0.017	0.017	1.0	0.07	This work
(−)sArMV (C+3, A8)	0.025	0.054	2.2	0.15	This work

[a]WT, wild-type.

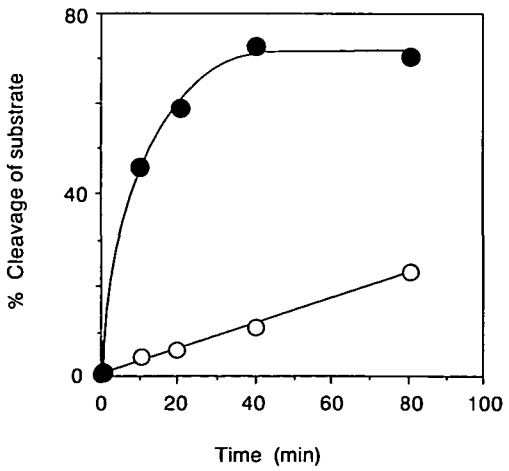

Fig. 2. **Time-course of *trans*-cleavage reactions.** The standard reaction mixtures containing wild-type substrate and wild-type ribozyme were incubated at 37°C and analyzed as described in the text. Filled circles, (−)sTRSV; open circles, (−)sArMV.

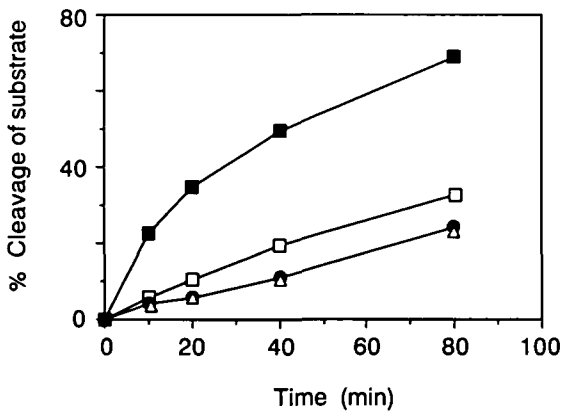

Fig. 3. **Time-course of *trans*-cleavage reactions of mutant (−)sArMV ribozymes.** The standard reaction mixtures containing mutant substrates and ribozymes were incubated at 37°C and analyzed as described in the text. Filled circles, A+3 substrate-C8 ribozyme (wild type); filled squares, C+3 substrate-A8 ribozyme; open squares, C+3 substrate-C8 ribozyme; open triangles, A+3 substrate-A8 ribozyme.

A Chimeric Ribozyme Consisting of Parts from (−)sTRSV and (−)sArMV Has Very High Activity—Why is the activity of the ribozyme from (−)sArMV so low? The hairpin ribozyme consists of two parts, the catalytic hairpin domain (the vertical domain in Fig. 1) and the substrate plus the substrate-binding site (the horizontal domain in Fig. 1). To investigate the ability of the hairpin (vertical) domain of (−)sArMV, we constructed a chimeric hairpin ribozyme between the (−)sTRSV and (−)sArMV sequences (Fig. 1C) and tested its cleaving activities. The substrate and the substrate-binding site of this chimeric molecule are derived from the (−)sTRSV sequence. The catalytic hairpin domain is from the (−)sArMV (Fig. 1C). Again, we prepared variants with respect to the positions at +3 and 8 to test the contribution of these nucleotides at this set of positions to the activity. In all of combinations of these chimeric molecules, the substrates were cleaved to higher extents than in the corresponding combinations of the (−)sArMV sequence (Fig. 4). In particular, the C+3-A8 (closed squares in Fig. 4) was the best combination for cleavage. More than 90% of substrate was cleaved in 20 min of incubation. This activity is higher than that of the wild type of (−)sTRSV (compare the closed squares in Fig. 4 with the closed circles in Fig. 2). Table II compares the reaction rates of these *trans*-reactions. The k_{app} value of chimera with the C+3-A8 is almost twice of that of (−)sTRSV (Table II). In this case, the A+3-C8 is also the poorest combination. These results indicate that the low activity of (−)sArMV shown in Fig. 2 is attributable not to the hairpin domain but to the substrate and the substrate-binding site. Moreover, we were able to construct a more active hairpin ribozyme than (−)sTRSV by adding the hairpin domain of (−)sArMV to the substrate-binding site of the (−)sTRSV.

A Cis-Acting System of the Hairpin Ribozyme from (−)sArMV—Although a chimeric ribozyme having the catalytic hairpin domain from (−)sArMV cleaved the substrate efficiently (Fig. 4), the wild-type combination of (−)sArMV, namely, A+3-C8, was still the poorest combination for cleavage. In the previous paper (*25*), we reported that the hairpin ribozyme derived from (−)sTRSV has different target-site specificities in *cis* and *trans* cleavages. We observed that the A+3-C8 pair is favorable for *cis*-cleavage, although this combination is unfavorable for *trans*-cleavage (*25*). To test whether the (−)sArMV ribozyme also has different target-site specificities in *cis* and *trans* cleavages, we constructed four types of self-cleaving RNAs having the (−)sArMV RNA sequence (Fig. 5). We observed that very efficient self-cleavage occurred when the nucleotides at position 22-42 (Fig. 5) were A-C, C-A, and A-A (Table III; the numbers, 22 and 42, correspond to those of Fig. 5). In this *cis*-acting system, the wild-type combination (A22-C42) had the highest activity among the mutants tested (Table III).

TABLE II. **Comparison of reaction rates of *trans*-reactions.** Apparent rate constants are presented. The nucleotides at +3 and 8 are shown at left. The data of (−)sTRSV are derived from Fujitani *et al.* (*25*).

N^{+3} / N^{8}	k_{app} (min^{-1})[a] (−)sTRSV	(−)sArMV	Chimera
C / A	0.063	0.025	0.127
A / C	0.031	0.004	0.009
C / C	0.067	0.005	0.043
A / A	0.029	0.004	0.030

[a]The apparent rate constant, k_{app}, is given by the slope of a plot of $\ln(S_0/S_t)$ *versus* time under the reaction conditions described in "MATERIALS AND METHODS," where S_0 equals the initial substrate concentration (100%) and S_t equals the substrate concentration (%) at a given time point.

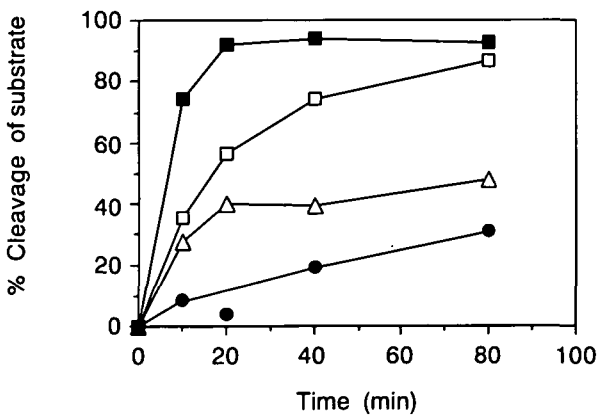

Fig. 4. **Time-course of *trans*-cleavage reactions of the chimeric ribozyme.** A chimeric ribozyme consisting of the substrate-binding site from (−)sTRSV and the vertical hairpin region from (−)sArMV was incubated with (−)sTRSV substrate at 37°C under the standard reaction conditions. Several mutants were also tested as described in text. Filled squares, C+3 substrate-A8 ribozyme; open squares, C+3 substrate-C8 ribozyme; open triangles, A+3 substrate-A8 ribozyme; filled circles, A+3 substrate-C8 ribozyme.

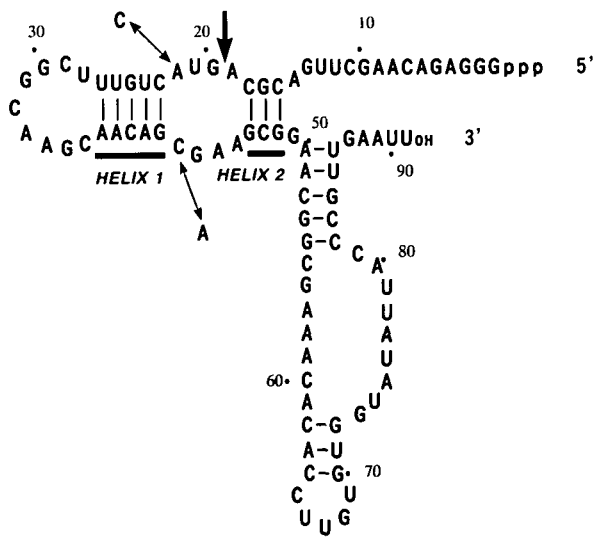

Fig. 5. **The secondary structure model of *cis*-acting (self-cleaving) hairpin ribozyme from (−)sArMV.** Numbers, nucleotide numbers of the primary transcript. The thick arrow indicates the predicted site of cleavage. Thin arrows indicate the base substitutions in mutants.

TABLE III. **Cleavage efficiency of the *cis*- and *trans*-acting ribozymes from (−)sTRSV and (−)sArMV.** Cleavage efficiency was determined as described in the text. The internal loop regions are shown. Changed nucleotides are in boldface. The data of (−)sTRSV were derived from Fujitani *et al.* (*25*).

Sequence in the internal loop	(−)sTRSV			(−)sArMV		
	Cis-cleavage (%) Incubation time		*Trans*-cleavage (%) Incubation time	*Cis*-cleavage (%) Incubation time		*Trans*-cleavage (%) Incubation time
	1 h	2.5 h	40 min	1 h	2.5 h	40 min
−C**UG**A− −A**GA**A−	98	98 (Wild-type)	73	88	99	50
−A**UG**A− −C**GA**A−	95	98	41	91	99 (Wild-type)	11
−C**UG**A− −C**GA**A−	45	80	76	14	36	20
−A**UG**A− −A**GA**A−	98	99	42	69	97	11

As presumed from the results of the *cis*-acting system from (−)sTRSV (*25*), we confirmed here that the hairpin ribozyme from (−)sArMV also has different target-site specificities in *cis* and *trans* cleavages. In the (−)sArMV ribozymes, the A+3 substrate (the wild-type) is the poorest substrate in the *trans*-acting system, whereas the wild-type sequence is the most efficient self-cleaving sequence (Table III). As described in the previous paper (*25*), the difference in the target-site specificities between *cis* and *trans* ribozyme systems may be explained by a difference in RNA folding specificities between *cis*- and *trans*-acting systems. In our *cis*-acting system, the secondary structure of the substrate and substrate-binding domain (the horizontal hairpin in Fig. 5) may easily form in the early phase of transcription. After the formation of this substrate part, the catalytic domain (the vertical hairpin domain in Fig. 5) may be generated by subsequent transcription. Then, the self-cleavage may take place smoothly. In *trans*-acting system described above, however, the substrate must bind to a pre-existing single-stranded substrate-binding site that should protrude from the vertical hairpin domain. The A+3 substrate is thought to be unfavorable for this substrate-binding step. From the results of the *trans*-acting system (Fig. 3), it is possible that the A+3 substrate might interact with the ribozyme to form non-productive complexes, whereas the C+3 substrate may interact with the ribozyme properly. The low activity of this A+3 substrate in the *trans*-acting system may be attributable to this substrate-binding step. At the chemical cleavage step, however, there may be no difference in cleavage efficiency between the A+3-C8 pair and the C+3-A8 pair.

In conclusion, although the A+3-C8 pair (Fig. 1B) was an unfavorable combination for the *trans*-cleavage reaction, this A-C pair in the natural (−)sArMV transcript may be effective for efficient self-cleavage *in vivo*, where a *cis*-acting system should operate. Finally, it should again be emphasized that we have incidentally obtained a very efficient chimeric hairpin ribozyme (Fig. 4). From this result, we expect to be able to create new ribozymes that are more active than the naturally occurring one.

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