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Different target-site specificities of the hairpin ribozyme in *cis* and *trans* cleavages

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The hairpin ribozyme cleaves a phosphodiester bond at the 5′ side of a 5′GUC3′ sequence of an RNA with high efficiency. An RNA having a 5′GUA3′ sequence instead of the GUC sequence is a poor substrate for this ribozyme Here, we show that this is indeed so in a *trans*-acting ribozyme system, but in a *cis*-acting ribozyme system this ribozyme cleaves the 5′ side of a GUA sequence as efficiently as the wild-type cleaves the GUC sequence. One base substitution in the ribozyme also affected the target-site specificity in the *cis*-acting system.

Catalytic RNA; Satellite RNA; Tobacco ringspot virus; Arabis mosaic virus; Chicory yellow mottle virus

1. INTRODUCTION

The negative strand of the satellite RNA of tobacco ringspot virus ((−)sTRSV) is a self-cleaving RNA [1,2]. 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 the 14 nucleotides has been identified in the (−)sTRSV RNA sequence [3–7], and the catalytic domain interacts with the substrate as a true enzyme [3]. Since the RNA forms a hairpin-loop structure it has been named hairpin ribozyme or hairpin catalytic RNA [7]. A model of the hairpin ribozyme derived from the (−)sTRSV is shown in Fig. 1: fig. 1A and B show models of the *trans* and the *cis* cleavage, respectively. Since, in the model of Fig. 1B, the 3′ end of the substrate is joined to the 5′ end of the ribozyme, this molecule is a self-cleaving RNA. The substrate domain interacts with the substrate binding site of the ribozyme through two helices (helix 1 and helix 2, Fig. 1) [7], and the cleavage occurs at a site in the internal loop shown by the thick arrow in Fig. 1. Based on some mutational experiments, it has been reported that the sequence requirement for the target-site of cleavage by the hairpin ribozyme is GUC [7]. Several hairpin ribozymes designed for such sites have been found to correctly cleave various RNA sequences in vitro and in vivo [8,9]. RNA containing a GUA sequence instead of a GUC has been reported to be a less efficient substrate [10,11]. From in vitro selection experiments, Joseph et al. [12] recently reported that the hairpin ribozyme requires the sequence 5′-NN(G/A)(U/C)N*G(A/U/C)(U/C)(G/U/C)NNNNN-3′ (the asterisk denotes the cleavage site; N = A/C/U/G) as a target-site for cleavage with high efficiency.

In the present paper, we report that the hairpin ribozyme can cleave the 5′ side of a GUA sequence with high efficiency in a *cis*-acting self-cleaving reaction, and that this ribozyme has different target-site specificities between *cis* and *trans* cleavages.

2. MATERIALS AND METHODS

2.1. *RNA catalytic reactions*

For the *trans*-acting hairpin ribozyme system, synthesis of substrate and ribozyme RNAs and RNA catalytic reactions were done as described by Hampel and Tritz [3].

2.2. *Constructions of ribozyme-coding plasmids*

To obtain various types of *cis*-acting (self-cleaving) hairpin ribozymes, the plasmid pNON 2 [8] was used as cloning vector. The pNON 2 encodes a modified hairpin ribozyme sequence that can be transcribed in vitro by SP6 RNA polymerase [8]. This plasmid has two specific restriction enzyme sites (*Hin*dIII and *Hpa*I), the former site at the 5′ end and the latter, at the 3′ end of the substrate binding site of the catalytic RNA sequence. The *Hin*dIII–*Hpa*I fragment of pNON2 was replaced by synthetic DNA of the desired sequence (corresponding to the sequence 10–46 in Fig. 1B).

2.3. *Transcription and cleavages reactions*

The pNON 2-based plasmids containing various mutant sequences were digested with *Eco*RI. The *Eco*RI site is located at the end of the catalytic hairpin region of the plasmid (the nucleotides 87–91 in Fig. 1B) [8]. These linearized plasmids were transcribed with SP6 RNA polymerase. The reaction mixture contained 40 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 U of RNasin, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, and 0.05 mM [α-^{32}P]UTP (10 μCi in total), 0.5 μg of plasmid template,

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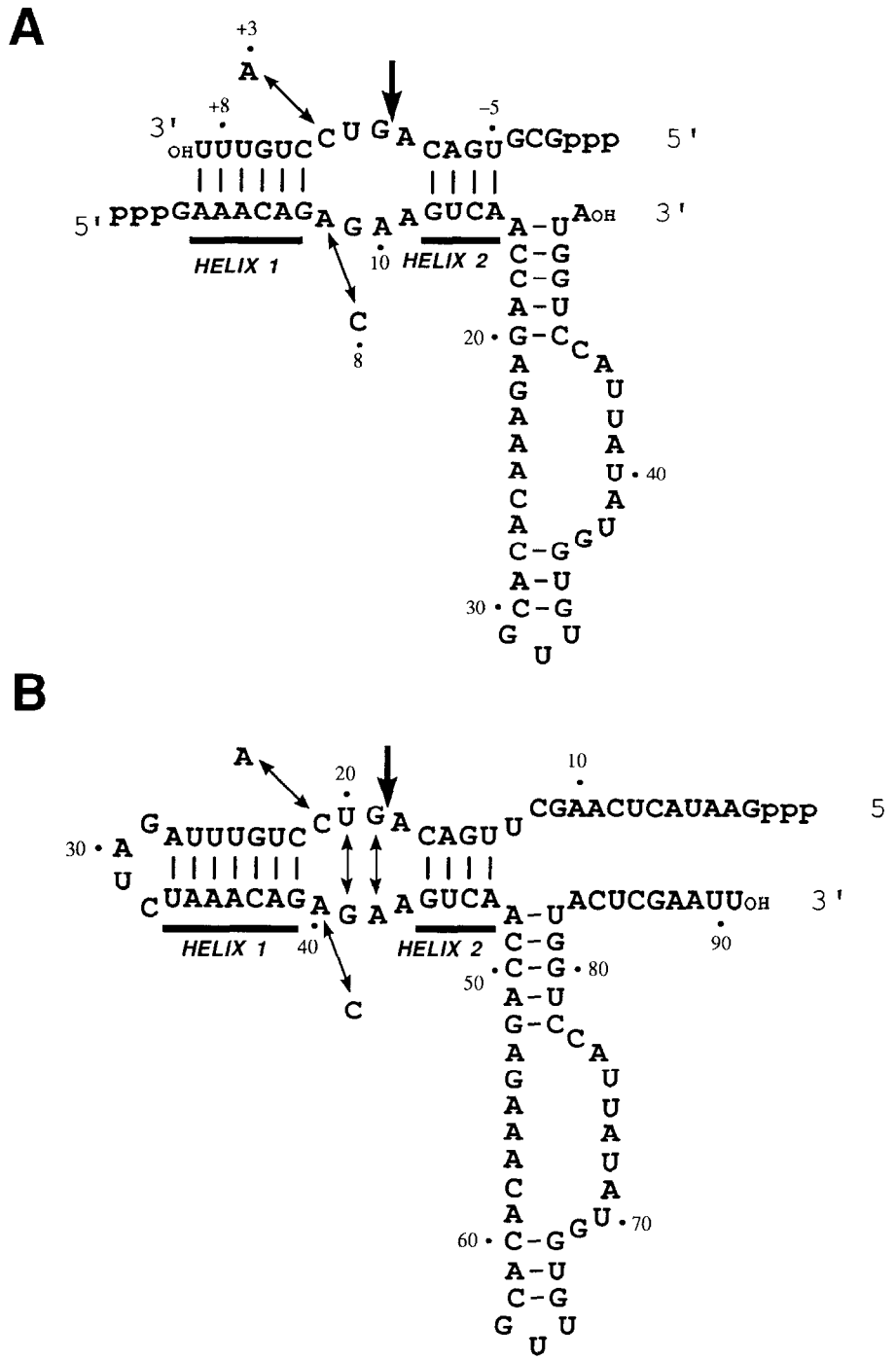

Fig. 1. The secondary structure models of the *trans*-acting hairpin ribozyme–substrate complex (A) and the *cis*-acting (self-cleaving) hairpin ribozyme (B) used in this study. The thick arrow indicates the predicted site of cleavage. Thin arrows indicate the base substitutions in mutants.

and 60 U of SP6 RNA polymerase in a total volume of 100 μl. The mixture was incubated for 1 h or 2.5 h at 37°C The primary transcript is shown in Fig. 1B. During the transcription reaction, the self-cleavage occurs concomitantly. The reaction products were precipitated in ethanol, dried, and dissolved in a urea/dye loading mixture to be electrophoresed in 20% polyacrylamide/8 M urea gels.

2.4. *DNA synthesis and analytical methods*

DNAs were synthesized using an Applied Biosystems DNA synthesizer. Quantitative analyses of the reactions were performed by counting photo-stimulated luminescence of the product bands in the autoradiogram of the gel using a Bio-Image Analyzer BAS2000 (Fuji Film Co.) [13]. Other analytical methods were as described previously [8].

3. RESULTS AND DISCUSSION

It has been suggested that the negative strands of

arabis mosaic virus satellite RNA ((−)sArMV) and chicory yellow mottle virus smaller satellite RNA ((−)sCYMV-S1) have possible hairpin ribozyme-like secondary structures [14–16]. However, the self-cleavage reactions have not been demonstrated for these RNAs. In these two RNAs, the nucleotides corresponding to positions +3 and 8 (Fig. 1A) are A and C, respectively, whereas (−)sTRSV has C + 3 and A8. 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. It has been reported that the substrate having a GUA sequence in the nucleotides corresponding to +1, +2 and +3 in Fig. 1A is a poor substrate for the hairpin ribozyme derived from (−)sTRSV in which the nucleotide corresponding to the +8 of Fig. 1A is A. Chowrira et al. reported that this substrate was cleaved with a catalytic efficiency 10-fold lower than the wild-type substrate [10]. Sekiguchi et al. also reported that this substrate was cleaved to an extent of only 9% after 15 h incubation with the wild-type ((−)sTRSV-type) hairpin ribozyme [11]. These results indicated that the C + 3–A8 pair was essential for cleavage with high efficiency in this system. Based on this phylogenetical data, we expected that the hairpin ribozyme would also cleave the 5′ side of a GUA sequence with high efficiency, when the ribozyme has a single base substitution from A8 to C (Fig. 1A).

To examine whether not only the C + 3–A8 pair but the phylogenetically conserved A + 3–C3 pair is also favorable to efficient cleavage, we have constructed such substrates (C + 3 and A + 3 substrates) and ribozymes (A8 and C8 ribozymes) (Fig. 1A) and tested their cleaving activities. Although we expected an efficient cleavage of A + 3 substrate by C8 ribozyme, as shown in Figs. 2 and 3, only the C + 3 substrate was

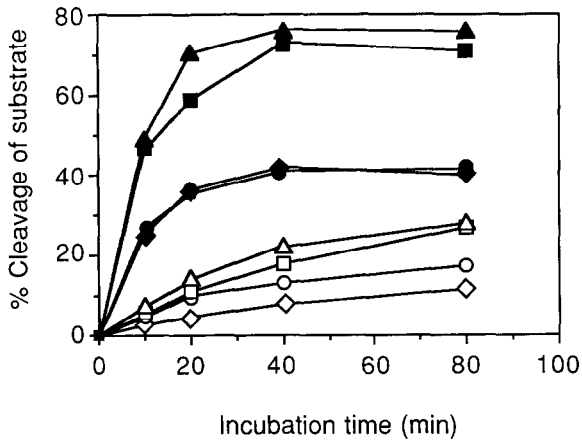

Fig. 2. Time-course of *trans*-cleavage reactions. The reaction mixture containing 40 mM Tris-HCl (pH 7.6), 12 mM $MgCl_2$, 2 mM spermidine, 6 nM [^{32}P]substrate, and 1.5 nM (open symbols) or 15 nM (filled symbols) ribozyme was incubated at 37°C and analyzed as described in the text. C + 3 substrate–C8 ribozyme, triangles; C + 3 substrate–A8 ribozyme, squares; A + 3 substrate–C8 ribozyme, circles; A + 3 substrate–A8 ribozyme, diamonds.

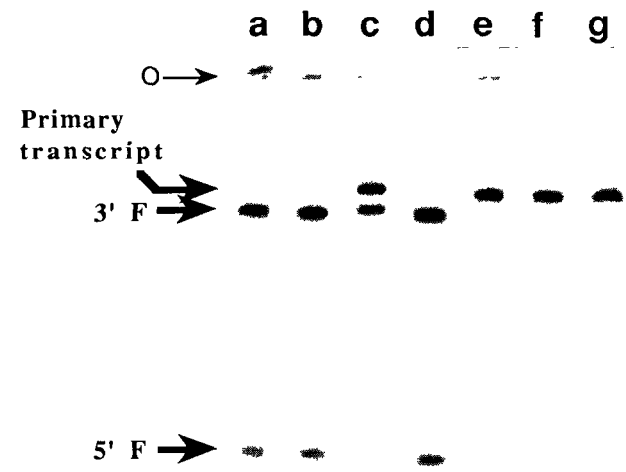

Fig. 3. Self-cleavage of the transcripts. Transcription mixtures were incubated for 1 h at 37°C and analyzed in 20% polyacrylamide/8 M urea gel. An autoradiogram is shown. The positions of primary transcripts, 3′- and 5′-fragments of self-cleavage reactions (3′F and 5′F) are indicated. O, origin of electrophoresis. Lane a, wild-type transcript (C21–A40); lane b, A21–C40 transcript, lane c, C21–C40 transcript; lane d, A21–A40 transcript, lane e, A19–G42 transcript; lane f, G20–U41 transcript; lane g, A19–G42 and G20–U41 transcripts.

cleaved with high efficiency. Irrespective of which nucleotide was at position 8 of the ribozyme the A + 3 substrate was a poor substrate: the A + 3 substrate was cleaved to almost 50% of the C + 3 substrate (Fig. 2). These results are essentially consistent with the data previously described [10,11].

The data described above were from a *trans*-acting ribozyme system. To test the specificity of the ribozyme in a *cis*-acting system, we have constructed four types of self-cleaving RNAs (Fig. 1B) and tested their self-cleaving abilities. As shown in Figs. 3 and 4, very efficient self-cleavage occurred when the nucleotides at position 21–40 (Fig. 1B) are A–C, C–A or A–A pairs. These three RNAs were cleaved to the extent of more than 95% during the transcription reactions over 1 h (Fig. 4). No significant difference was observed among the self-cleavage reactions of these three transcripts (Fig. 3, lanes a, b, and d). The RNA containing a C–C pair at these positions was also self-cleavable but only 45% of this transcript was cleaved after the 1 h incubation (Fig. 4c). Because the cleavage rate (k_{cat}) of the reaction of the wild-type substrate and the wild-type hairpin ribozyme has been reported to be 2.1 min^{-1} [3], the actual rate of the self-cleavage of the transcripts having a C–C pair at positions 21 and 40 (Fig. 1B and Fig. 4c) may be much lower than that of the wild-type transcript (Fig. 4a). These results indicate that the substrate domain having a GUA sequence at positions 19–21 is efficiently cleaved by both the A40 and C40 catalytic domains but that the GUC-containing substrate domain is efficiently cleaved only by the A40 catalytic

Lane	Sequence in the internal loop	Cis-cleavage (%) Incubation time 1 h	2.5 h	Trans-cleavage (%) Incubation time 40 min
a	-GUC C UGA CAG- / -CAG A GA A GUC- wild type	98	98	73
b	-GUC A UGA CAG- / -CAG C GA A GUC-	95	98	41
c	-GUC C UGA CAG- / -CAG C GA A GUC-	45	80	76
d	-GUC A UGA CAG- / -CAG A GA A GUC-	98	99	42
e	-GUC C UAA CAG- / -CAG A GG A GUC-	2	6	-
f	-GUC C GGA CAG- / -CAG A UA A GUC-	3	8	-
g	-GUC C GAA CAG- / -CAG A UG A GUC-	2	6	-

Fig. 4. Cleavage efficiency of the transcripts. Cleavage efficiency was determined as described in the text. Lanes a–g correspond to the lanes in Fig. 3. The internal loop regions of the transcripts are shown Changed nucleotides are in boldface. Hyphen, not determined

domain. These results from the *cis*-acting system are in conflict with the results from the *trans*-acting system described above.

The difference of the target-site specificities between the *cis* and *trans* cleavages might be explained by a difference in RNA-folding specificities between *cis*- and *trans*-acting systems. Berzal-Herranz et al. recently reported self-cleavage reactions of another self-cleaving construct of the hairpin ribozyme mutants [17]. The self-cleavage efficiency of their transcripts [17] seems to be much lower than that of ours (Fig. 3). Also, they reported that no difference in self-cleavage efficiency was observed between C21–A40 and C21 C40 constructs (the nucleotide numbers correspond to those of Fig. 1B) [17]. In contrast to our self-cleaving constructs (Fig. 1B), in their construct [17], the 5′ end of the substrate is joined to the 3′ end of the ribozyme. Therefore, the transcription of this construct starts from the 5′ end of the ribozyme domain, while our construct (Fig. 1B) is transcribed from the substrate domain. The difference in self-cleaving efficiency of their construct from ours may be due to a difference in the transcriptional order of the domains. This suggests that the presence of covalently bound substrate at the 5′ side of the ribozyme domain may facilitate the making of a productive conformation of a ribozyme or a ribozyme–substrate complex. Therefore, our data of *cis* cleavage (Fig. 4) are thought to be the efficiency only at the cleavage step of the reaction. The data of *trans* cleavage (Fig. 4) may represent the results of all steps of the reaction, including substrate binding and conformational change of the ribozyme or the ribozyme–substrate complex. In conclusion, the presented results suggest that the C21–C40 pair is unfavorable for the cleavage step of the reaction, compared with A–C, C–A or A–A pairs at this set of positions, but that this C–C pair may be effective in making a productive conformation of the ribozyme itself or the ribozyme–substrate complex.

Figs. 3 and 4 also show the effects of other exchanges between the top and the bottom bases in the internal loop on the self-cleaving activity of the transcripts. Although the base exchange between the positions 21 and 40 (Fig. 1B) did not cause any loss of activity, as described above, all other exchanges tested greatly reduced the efficiency of self-cleavage (Fig. 4).

In summary, although the A21–C40 pair (Fig. 1B) is an unfavorable combination for the *trans*-cleavage reaction, the phylogenetically conserved A21–C40 pair (Fig. 1B) in (−)sArMV and (−)sCYMV-S1 may be effective for efficient self-cleavage of the natural transcripts.

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