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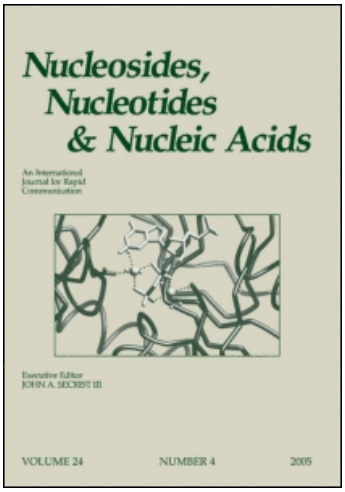

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Addition of an Extra Substrate Binding Site and Partial Destabilization of Stem Structures in HDV Ribozyme Give Rise to High Sequence-Specificity for its Target RNA

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ADDITION OF AN EXTRA SUBSTRATE BINDING SITE AND PARTIAL DESTABILIZATION OF STEM STRUCTURES IN HDV RIBOZYME GIVE RISE TO HIGH SEQUENCE-SPECIFICITY FOR ITS TARGET RNA

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□ *Because the substrate binding site (P1) of HDV ribozyme consists of only seven nucleotides, cleavage of undesired RNA is likely to occur when applied for a specific long RNA target such as mRNA. To overcome this problem, we designed modified* trans*-acting HDV ribozymes with an extra substrate-binding site (P5) in addition to the original binding site (P1). By inserting an additional seven base-pair stem (P5 stem) into the J1/2 single-stranded region of the ribozyme core system and partial destabilization of the P2 or P4 stem, we succeeded in preparation of new HDV ribozymes that can cleave the target RNA depending on the formation of P5 stem. Moreover, the ribozyme with a six-nucleotide P1 site was able to distinguish the substrate RNA with a complete match from that with a single mismatch in the P1 region. These results suggest that the HDV ribozyme system is useful for the application* in vivo.

Keywords HDV ribozyme; RNA cleavage; Substrate recognition

INTRODUCTION

Ribozymes are RNA molecules that can catalyze specific chemical reactions.[1–4] The ribozymes that cleave other RNA molecules in a sequence-specific manner can be used as molecular tools for inhibiting the expression of genes, which is related to genetic diseases or elucidating unknown gene function.[5] For these applications, the flexibility of cleavage site selection, high sequence fidelity, and high activity for the target RNA cleavage are very important. For such purposes, hammerhead ribozymes[6] are frequently used. Hairpin and hepatitis delta virus (HDV) ribozymes[7–9] are also used. These ribozymes catalyze an RNA cleavage reaction in the presence of divalent cations. However, hammerhead and hairpin ribozymes require most

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This article is dedicated to Professor Eiko Ohtsuka on the occasion of her 70th birthday.
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effective GUC sequence at the cleavage site, restricting the selection of the cleavage site in the target RNA.[10,11] Moreover, these ribozymes allow several mismatch base-pairs in the recognition of target RNA sequence.[12] This problem is due to the limitation of catalytic core itself of the ribozymes and thermodynamics of RNA–RNA hybridization between the ribozyme and its substrate RNA.

Human hepatitis delta virus (HDV) is a satellite virus that specifically infects cells already infected with hepatitis B virus. The HDV genome consists of a circular, single-stranded RNA of about 1700 nucleotides (nt) in length.[13] The genome replicates through the rolling-circle mechanism, and self-cleavage reactions transforming polymeric forms of genomic and antigenomic RNAs into active monomer unit forms are involved in this process. Minimum catalytic domains for the cleavage activity are obtained from both the genomic and antigenomic HDV RNAs. A domain of about 85 nt is necessary for the self-cleavage activity of both HDV RNAs.[14,15] From secondary and tertiary structural analyses, it is assumed that both HDV ribozymes have a very similar folding structure, double-pseudoknot structure, consisting of four double-stranded stems (P1 to P4), two hairpin loops (L2 and L4), and three internal loop structures (J1/2, J2/4 and J4/1).[16,17]

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Trans-acting HDV ribozymes are generated by deleting J1/2 single-stranded region, and available for the genetic applications like other ribozymes.[18,19] There are two advantages of HDV ribozymes to use for the application. First, since HDV ribozymes originally work in human cells, high enzymatic activity may be expected also in the case of medical usage. Secondly, the sequence required for target RNA is YGN_6 (Y: pyrimidine, N: any base),[20] which is easier to find in the target RNA than those of hammerhead- or hairpin-type ribozymes. Moreover, one mismatch base-pairing in the center of P1 stem abolishes cleavage activity of the *trans*-acting HDV ribozymes,[21] indicating that HDV ribozymes require a high sequence fidelity for the target RNA. However, the substrate recognition by seven base-pairs in P1 is not sufficient for complete discrimination between desired substrate and false substrate. In order to overcome this problem, we have tried to prepare novel HDV ribozymes with high sequence fidelity for the target RNAs by modification of the antigenomic HDV ribozyme.[22] In this article, we report design and characteristics of new HDV ribozyme with an additional substrate-binding site and further modifications to make the ribozyme high sequence-specific.

MATERIALS AND METHODS

RNA Preparation

Each enzyme part and substrate part of the ribozyme systems used in this study were prepared by *in vitro* transcription with T7 RNA polymerase using

fully double-stranded DNA promoter-templates as previously described[23,24] with some modification. The transcription reaction was carried out at 37°C for 30 min. The reaction mixture contained 2 μM promoter-template DNA, 0.1 mg/ml T7 RNA polymerase, 7.5 mM each NTP, 20 mM GMP, 35 mM $MgCl_2$, 160 mM Tris-HCl (pH 8.1), 5 mM DTT, 2 mM spermidine and 0.01% Triton X-100 (v/v). The transcripts were purified by denaturing (7 M Urea) polyacrylamide gel electrophoresis and extracted by crush and soak method using 0.3 M sodium acetate buffer (pH 7.0). The extracted RNAs were isolated by two rounds of ethanol precipitation and dissolved in distilled RNase/DNase-free water (Nippon Gene).

Ribozyme Activity Assay

Cleavage reactions were carried out under the single-turnover conditions. The substrates were saturated with a large excess of the ribozyme prior to the reaction so that the cleavage reaction can be assumed to be a pseudo-first-order reaction.[18,25] Two picomoles enzyme part (final concentration: 0.1 μM) and 1,000 cpm 5′-end-labeled substrate RNA (final concentration: <1 nM) were mixed in 18 μl standard reaction buffer (50 mM Tris-HCl, pH 7.0). The mixture was annealed by heating at 70°C for 5 min and gradually cooling to room temperature. Following preincubation at 37°C for 10 min, the cleavage reaction was started by addition of $MgCl_2$ (final concentration: 10 mM). Aliquots of the reaction mixture (0.5 μl) were removed after 0, 1, 3, 5, 10, and 40 min and quenched by addition of 5 μl of 50 mM EDTA, 9 M urea, 0.1% bromophenol blue. The cleavage products (6 nt) were separated from uncleaved substrate RNA by 20% polyacrylamide gel electrophoresis and detected by autoradiography (Imaging Plate and FLA200, FUJIFILM). The cleavage rate constants (k_{obs}) were obtained by fitting the data to the equation: $Y_t = EP(1 - e^{-kobst})$, where Y_t is the ratio of cleavage at time t and EP is the end point of cleavage reaction.

RNase Structural Mapping

The 5′-end-labeled longer part of RzP5ΔL4 (RzP5ΔL4-43, 20,000 cpm) was mixed with the other enzyme part and the substrate RNA (1 μM each) in the standard reaction buffer containing 5 μg yeast tRNAs (Sigma). The mixture was heat-denatured and then cooled gradually. After 30 min, 0.05 U of RNase T1 (Ambion) or 0.005 U of RNase V1 (Ambion), and 10 mM $MgCl_2$ were added and the mixture was incubated at 37°C for 5 min. The reaction was terminated by addition of an equal volume of 9 M urea loading buffer, and the products were electrophoresed on a 10% polyacrylamide gel and visualized by autoradiography.

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RESULTS AND DISCUSSION

Design and Characterization of Novel *trans*-Acting HDV Ribozymes

The *trans*-acting HDV ribozymes have been usually generated by breaking the J1/2 single-stranded region between P1 and P2 (Figure 1A). We introduced an additional stem structure (P5) into the middle of this J1/2 region, dividing the ribozyme into a substrate part and an enzyme part. In this way, the self-cleaving HDV ribozyme can be converted to a *trans*-acting one, which contains two substrate RNA-binding sites, P1 and P5, in a single ribozyme molecule (Figure 1A).

The RNA sequence of this novel *trans*-acting HDV ribozyme is based on that of the antigenomic HDV ribozyme (Figure 1B). The extra P5 stem contains seven base pairs just as the P1 stem. The P5 sequences were chosen so that they do not bind to the other parts of the enzyme, and the substrate component by secondary-structure calculation with a computer. In the enzyme part of this ribozyme system (RzP5), P4 stem of the natural HDV ribozyme is truncated and closed with the tetranucleotide loop (UUCG).[26] In the substrate part (S24), five nucleotides are added to the 5′-end of the minimum eight-nucleotide substrate (S8) for convenient detection of the cleavage product in acrylamide gel electrophoresis, and seven (for P5) plus four nucleotides (as a linker) are also added to the 3′-end of the minimum substrate. We prepared a substrate RNA, S24(P5-), where the 3′-terminal 7-nt sequence of S24 is replaced with a reverted complementary sequence, 5′UUCCGCG3′ (Figure 1B). Thus S24(P5-) can form only a P1 stem with

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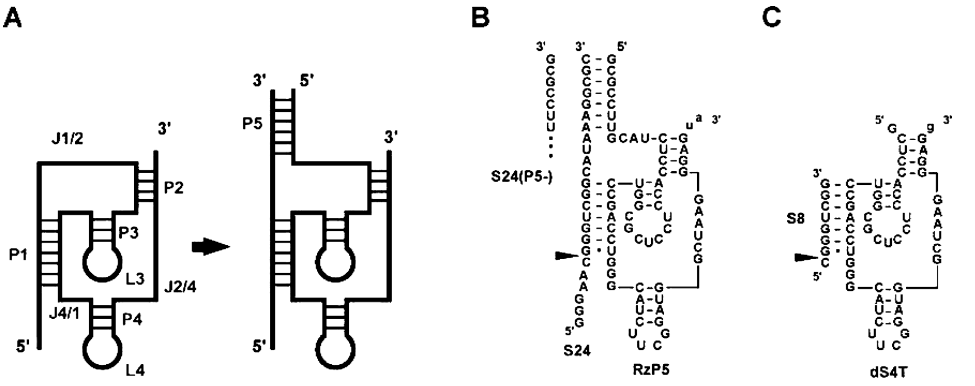

FIGURE 1 Design and sequences for novel *trans*-acting HDV ribozymes. (A) Schematic representation of secondary structures of *cis*-acting and novel *trans*-acting HDV ribozymes. Each stem is referred to as P1 to P4 along the RNA chain 5′- to 3′-direction, and an additional stem inserted into J1/2 single stranded region is referred to as P5. (B) and (C) show sequences and secondary structures of RzP5 and ds4T complexed with the corresponding substrate RNAs (S24 and S8). Cleavage site of the substrate RNA is indicated by an arrowhead. Small characters at the 3′-end of enzyme part RNAs represent extra sequences for *in vitro* transcription.

RzP5. We also prepared the conventional *trans*-acting HDV ribozyme system (dS4T and S8) based on dSIV described previously as a reference system (Figure 1C).[18] Using these RNA oligomers, we performed detailed characterization of cleavage activity of RzP5.

Each RNA molecule was transcribed by T7 RNA polymerase *in vitro* from chemically synthesized complementary DNA template and purified as described in Materials and Methods. For carrying out the cleavage reactions under the single-turnover conditions, 0.1 μM ribozyme and 5′-end-labeled substrate RNA (<1 nM) were used. After preincubation at 37°C, the cleavage reaction was started by addition of Mg^{2+} solution. Time course of the cleavage reaction was monitored by the appearance of the 5′-fragment, and then the observed rate constant (k_{obs}) and reaction endpoint (EP) were calculated as described in Materials and Methods. As a start, we compared the cleavage activities of conventional *trans*-acting dS4T and our newly designed RzP5 ribozymes against S24 and S8 substrates.

dS4T exhibited similar cleavage rates and end points against both substrates: S24 ($k_{obs} = 0.35$ min^{-1}, EP = 75%); S8 ($k_{obs} = 0.31$ min^{-1}, EP = 68%) (Figure 2A). Therefore, it turned out that the extra sequence at 3′-portion of S24 does not interfere with the cleavage activity of the core ribozyme but has rather enhancing effect. On the other hand, RzP5 exhibited cleavage activity against S24 ($k_{obs} = 1.2$ min^{-1}, EP = 44%), definitely higher than those against S24(P5-) ($k_{obs} = 0.75$ min^{-1}, EP = 33%) and S8 ($k_{obs} = 0.50$ min^{-1}, EP = 34%), though the extent of overall cleavage is rather low (Figure 2B). The slightly lower EP for S24(P5-) than that for S8 may be due to possible dimerization of the substrate. These results indicate that S24, which can form

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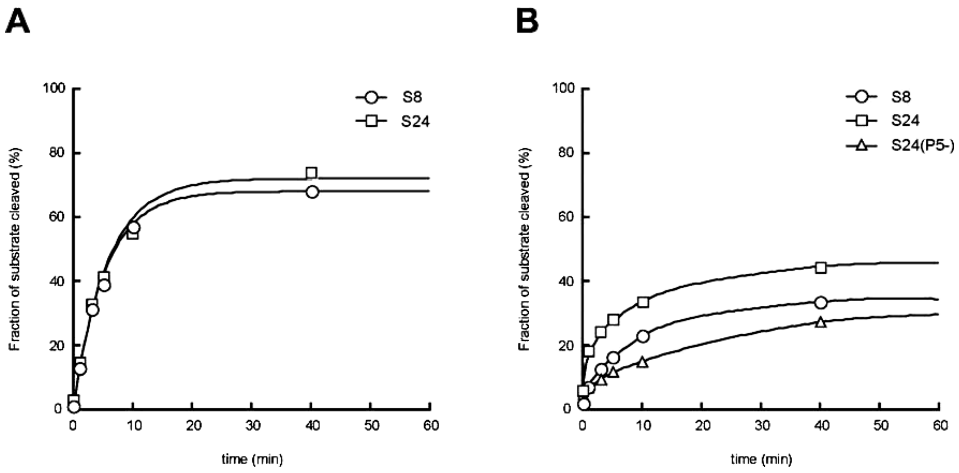

FIGURE 2 Kinetics of cleavage reactions catalyzed by dS4T (A) and RzP5 (B). Graphical representation of time course for cleavage reaction for each substrate RNA, S8 (circles), S24 (squares) and S24(P5-) (triangles). The reaction was carried out under the single-turnover conditions and aliquots were removed after 0, 1, 3, 5, 10, and 40 min.

P5 stem as well as P1 stem, is preferentially cleaved by RzP5 with respect to S24(P5-) and S8, which can form only P1 stem with the ribozyme. The selectivity and cleavage yield of RzP5, however, are not satisfactory for practical use. Therefore, we then tried to improve these points by modification of this system.

Destabilization of a Stem Structure Generates P5-Dependent Cleavage Activity for RzP5

One possible way to make the desired P5-dependent ribozyme, which is inactive when bound by a substrate that can form only P1 stem but is active when bound by a substrate that can form both P1 and P5 stems, is to destabilize the ribozyme core structure, which can be stabilized only by formation of both P1 and P5 stems with a substrate. For these purpose, we prepared some modified RzP5 ribozymes, which have point mutations in P2: RzP5(G58C) and RzP5(A59C) or in P4: RzP5(A48C) and RzP5(U49C) (Figure 3A), and examined whether these mutant ribozymes exhibit P5-dependent cleavage activity or not.

Two modified ribozymes, which have mutation in P2, RzP5(G58C), and RzP5(A59C), efficiently cleaved S24, though the cleavage rate is relatively low, but showed very little activity against S24(P5-), which cannot form P5 stem with the ribozymes (Figure 3B). The cleavage activity of RzP5(A59C) seems to be completely P5-dependent.

On the other hand, two modified ribozymes, which have a mutation in P4, RzP5(A48C) and RzP5(U49C), efficiently cleaved S24 showing almost the same EPs (68 and 65%, respectively) but showed quite different activity

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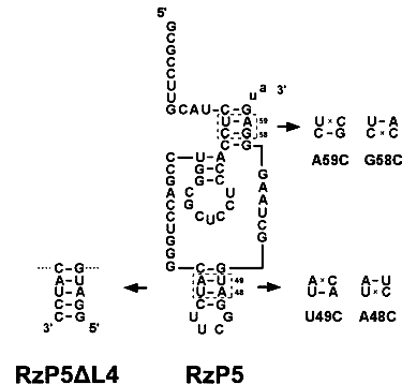

B

Ribozyme	S24 k_{obs} (min^{-1})	S24 EP (%)	S24(P5-) k_{obs} (min^{-1})	S24(P5-) EP (%)
Mutation in P2				
RzP5(A59C)	0.22	58	N.D.	N.D.
RzP5(G58C)	0.22	45	0.19	2.7
Mutation in P4				
RzP5(U49C)	0.19	65	0.11	6.0
RzP5(A48C)	0.14	68	0.05	26
L4 deletion				
RzP5ΔL4	0.14	44	N.D.	N.D.

FIGURE 3 The effects of single point mutations in P2 or P4 stem and deletion of L4 loop. (A) Sequences and secondary structures of mutant RzP5 and RzP5ΔL4. (B) The kinetic data for the cleavage reactions of S24 and S24(P5-) catalyzed by various mutant ribozymes under the single-turnover conditions. N.D.: not detectable.

against S24(P5-). RzP5(U49C) showed activity much more dependent on P5 stem formation than that of RzP5(A48C) (EP: 6 and 26%, respectively).

Another way to destabilize the P4 stem of RzP5 is assumed to be deletion of the closing tetranucleotide loop (L4). We prepared RzP5ΔL4, in which L4 loop is replaced with one G:C-pair, and examined its activity (Figures 3A and B). RzP5ΔL4 efficiently cleaved S24 with the same rate (k_{obs} = 0.14 min^{-1}) as that of RzP5(A48C) but with lower EP (44%). However, RzP5ΔL4 did not cleave S24(P5-) at all like RzP5(G58C) and RzP5(A59C). These results demonstrate that by introducing a single point mutation in P2 region or deleting L4 loop of RzP5, the ribozyme activity of RzP5 becomes dependent on the formation of P5 stem.

Sequence Specificity of P1 and P5

Because it is important for RNA-based gene inactivation system to cleave only one specific target RNA *in vivo*, we investigated the specificity of the RzP5ΔL4 system against various substrate RNAs with one or two base substitution mutation (Figures 4A and 4C). Using RzP5ΔL4 as an enzyme, the ef-

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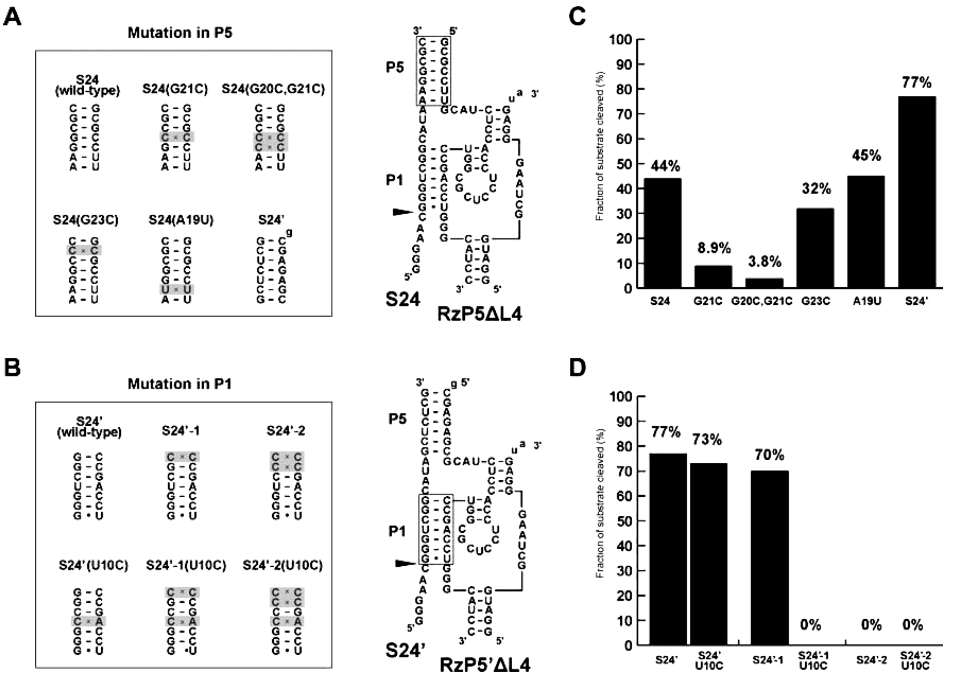

FIGURE 4 Sequence specificity for the substrates with mismatch mutations in P1 and P5 sites in the RzP5ΔL4 and RzP5′ΔL4 systems. (A) Substrate RNA sequences with mutation(s) in P5 sites and putative P5 stem structures in the S24-RzP5ΔL4 system. (C) Substrate RNA sequences with mutation(s) in P1 sites and putative P1 stem structures in the S24′-RzP5′ΔL4 system. (B) and (D) Graphs showing the cleavage efficiency in terms of the reaction end point (EP) for each substrate in the S24-RzP5ΔL4 system (B) and S24′-RzP5′ΔL4 system (D). Mutation sites are highlighted in gray boxes (A and C). An arrowhead indicates the cleavage site of the substrate RNA.

fects of positions of mismatch mutation in P5 or P1 of the substrate RNA were examined by comparing the estimated end points of the cleavage reaction. The substrate RNAs with the mutation in the center of P5, S24(G21C), and S24(G20C, G21C), showed markedly reduced EPs of 8.9 and 3.8%, respectively, with respect to that (44%) for the wild-type substrate (S24) (Figure 4C). On the other hand, the substrate RNAs with mutation in the peripheral part of P5, S24(G23C), and S24(A19U), showed EPs (32 and 45%, respectively) comparable to that of S24. These results indicate that RzP5ΔL4 has high sequence-specificity for the substrate if the expected mismatches are in the central region of P5. To confirm the sequence flexibility of P5 in the present system, a substrate (S24′), in which the 3′-terminal sequence (−AAGGCGC) in the P5 region of S24 was replaced by a different sequence (−GCUCUCG) containing the same GC content, and the corresponding ribozyme (RzP5′ΔL4) were prepared and the cleavage reaction in the new system was examined (the enzyme part is shown in Figure 4). RzP5′ΔL4 cleaved S24′ with higher EP (77%) than that (44%) observed for the RzP5ΔL4-S24 system (Figure 4C). This result suggests that the cleavage efficiency is greatly affected by the sequence of P5.

The conventional *trans*-acting HDV ribozymes prepared by disrupting the J1/2 single-stranded region like dS4T can efficiently distinguish the substrate RNAs with a complete match for P1 from that with a one-base mismatch in the center of P1.[21] We performed experiments using RzP5′ΔL4 and substrates, which contain one to three mismatch-bases, and assessed the sequence specificity for P1 (Figure 4C). At first, we compared the cleavage activity against S24′ and S24′(U10C), which has a substitution mutation (U10 to C) in the center of P1 region (Figure 4C). Both of the substrate RNAs were cleaved with similar high EPs, 77 and 73%, respectively, indicating that RzP5′ΔL4 is not able to discriminate the substrate RNAs with one base difference in P1. Next, we prepared substrate RNAs, which have further mutation at the end of P1 region and examined their activity. S24′-1(U10C), which contains double mutations at the center and the terminus of P1 region, was not cleaved at all, while S24′-1, which contains a single mutation in the terminus of P1 region, was cleaved with EP (70%) similar to that of S24′. Moreover, S24′-2, which has two-base substitution at the terminal region of P1, as well as S24′-2(U10C), was not cleaved at all. These results suggest that reduction of the number of base-pairs in the P1 stem from 7 to 6 by design enables RzP5′ΔL4 to efficiently discriminate the substrate RNAs with one-base difference at the P1 site.

In the conventional *trans*-acting antigenomic ribozyme like dS4T, disruption of one base-pair at the 3′-end of P1 site in the substrate RNA causes about tenfold reduction in the cleavage activity.[21] It is assumed that the base pairing at the center of P1 stem play an important role for active-site formation from mutational analysis of *trans*-acting antigenomic HDV ribozymes.[27] In contrast to those results, RzP5′ΔL4, which can form a 7-base-paired P1 stem,

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efficiently cleaved substrates with one-base substitution not only at the end but also at the center of the P1 site. In this system, formation of the extra P5 stem may enhance the thermal stability of the ribozyme-substrate complex and maintain the complex in an active state despite of one base-pair loss in the P1 stem. The system of S24′-1 plus RzP5′ΔL4 can be assumed as a system with a 6-base-pair P1 stem. In this system, the substrate containing one-base mismatch in the P1 site (S24′-1(U10C)) was not cleaved at all, suggesting that the thermal stability of the substrate-ribozyme complex is reduced to the level for the complex without an extra P5 stem.

Formation of P5 Stem Stabilizes P2 Stem Structure

We have been able to prepare new ribozymes such as RzP5(A59C) and RzP5ΔL4, which cleave the substrates in a P5-dependent manner, by destabilization of P2 or P4 in the RzP5 system. To elucidate the mechanism of this P5-dependent activity, we examined the solution structure of S24′-RzP5′ΔL4 and S24′(P5-)-RzP5′ΔL4 complexes by RNase footprinting experiments using structure- and sequence-dependent RNases (RNase V1, specific for RNA in helical and stacked regions; RNase T1, specific for G located in a single-stranded region) and compared the results. Only the long strand of RzP5′ΔL4 (RzP5′ΔL4-43) was 5′-end labeled and mixed with the unlabeled short strand of RzP5′ΔL4 (RzP5′ΔL4-16) and each substrate RNA in the reaction buffer containing Mg^{2+}. The mixtures were then subjected to limited digestion for 5 min by the enzymes under the conditions for cleavage reaction (Figure 5).

In the case of RNase T1 digestion (Figure 5, lane 3), strong cleavage was observed at G8 and G24 of RzP5′ΔL4-43 in the S24′-ribozyme complex, suggesting that these two G residues are located in a single-stranded region, but cleavage at G4 and G6, which are involved in formation of the putative P5 stem in the complex, was extremely weak. On the other hand, in the case of RNase V1 digestion, strong cleavage was observed in the region from G2 to G6 of RzP5′ΔL4-43, which are located in P5 site, in the S24′-ribozyme complex (Figure 5, lane 4), while only weak cleavage was observed at these residues in the S24′(P5-)-ribozyme complex (Figure 5, lane 5). These results are consistent with that a stable P5 stem is formed in the S24′-ribozyme complex in the reaction solution but not in the S24′(P5-)-ribozyme complex. The weak cleavage observed for the latter complex could be due to possible weak interaction between P5 sequences of S24′(P5-) (3′-CGAGAGC 5′) and RzP5′ΔL4-43 (5′-CGAGAGC-3′).

The two complexes show difference in the cleavage pattern also in the other regions, around P1, P1.1, and P2. S24′-ribozyme complex shows strong cleavage in the P2 region, whereas S24′(P5-)-ribozyme complex shows only weak cleavage in the same region. These results suggest that P2 stem is stabilized depending on the P5 stem formation in S24′-ribozyme complex. It is

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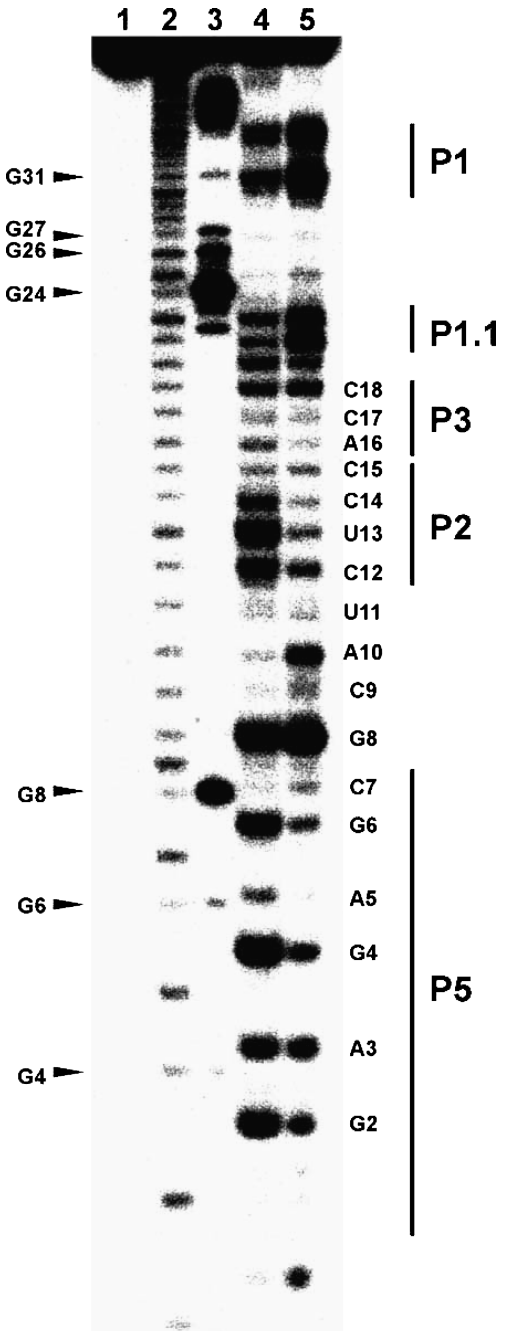

FIGURE 5 Secondary structure analysis and comparison of the effect of formation of P5 stem. A mixture of equal concentrations of 5′-^{32}P-labeled long strand of RzP5′ΔL4 (RzP5′ΔL4-43), the short part of the enzyme and each substrate RNAs was subjected to partial digestion with sequence- or structure-dependent nucleases, then to electrophoresis on a 10% polyacrylamide sequencing gel. Lane 1: RzP5′ΔL4-43 as a control; lane 2: alkaline hydrolysis products of RzP5′ΔL4-43 as markers; lane 3: digestion products of S24′-RzP5′ΔL4 complex by RNase T1; lane 4: digestion products of S24′-RzP5′ΔL4 complex by RNase V1; lane 5: digestion products of S24′(P5-)-RzP5′ΔL4 complex by RNase V1.

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known that high stability of P2 stem is necessary for efficient cleavage activity of HDV ribozyme.[28,29] The P5-dependent cleavage activity of RzP5 ribozyme with P2 mutation shown in Figure 3 may be induced by stabilization of the P2 stem, which is destabilized by introduction of base-pair mismatch, by P5 stem formation.

The significant but much smaller difference in the cleavage patterns around P1 and P1.1 regions might be due to the difference in the folding conformation. In the case of the S24′-ribozyme complex, S24′ may be partially cleaved under the conditions for the limited digestion experiments resulting a mixture of the substrate-ribozyme and product-ribozyme complexes. In the case of the S24′(P5-)-ribozyme complex, S24′(P5-) may be not cleaved and the substrate-ribozyme complex remains intact. It is known that the cleavage reaction by HDV ribozymes causes a considerable change in the folding conformation of the ribozymes.[21,27,30]

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CONCLUSIONS

To overcome the problem that HDV ribozymes are likely to cleave non-specific RNA because of the short substrate-recognition sequence (7 nt), we constructed newly designed HDV ribozymes with an additional substrate-binding site (P5) and characterized their catalytic properties. By partial disruption of the P2 or P4 stem, we succeeded in preparation of HDV ribozymes that can cleave the target RNA, depending on the formation of P5 stem.

However, the sequence specificity at the P5 site was not high enough to completely exclude a substrate with one-base mismatch. By shortening the substrate-recognition sequence at P1 site from 7 nt to 6 nt to reduce the thermal stability of the resulting P1 stem, one-base mismatch in the P1 stem caused complete loss of the cleavage activity of the ribozyme (see the results for S24′-1(U10C) and S24′-2 in Figure 4). These results suggest difference in the roles of the two substrate-binding sites. The P5 stem formation may play a minor role to select the target RNA but play an important role to keep the ribozyme structure in an active one together with the P1 stem formation. The P1 site may play a main role to capture the substrate RNAs to cleave with higher sequence-specificity than that of P5.

After completion of this work, we became aware of papers reporting similar HDV ribozyme systems that contain a similar P5 stem and one more stem for the cleavage activity.[31,32]

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