Effects of Circular Permutation on the Cis-Cleavage Reaction of a Hepatitis Delta Virus Ribozyme: Application to Trans-Acting Ribozyme Design†

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ABSTRACT: In this study we investigated the effects of the relocation of the wild type termini on the folding of a cis-cleaving RNA Rz 1 that was modified from the autolytic domains of hepatitis delta virus (HDV) RNA. Ten circularly permuted (CP) isomers of this ribozyme were synthesized. The structure homogeneity of RNA molecules, the molar ratio of the active species that undergoes cis-cleavage, and the rate of cis-cleavage were examined for each construct. CP isomers with new termini in H1 or at the junction of H2−H3, H1−$J_{1/4}$, $J_{1/4}$−H4, or H4−$J_{4/2}$ of the proposed pseudoknot-like structure were inactive. The single breaks of phosphodiester bond in H2, $J_{1/2}$, Lp4, and at the 3′-end of Lp3 decreased but did not abolish autolytic activity. The structural heterogeneity of RNA molecules may account for the limited cis-cleavage of the latter three isomers. The findings of circular permutation analysis were used as the basis for designing an active trans-cleaving ribozyme by dividing the cis-cleaving ribozyme into two subdomains at $J_{1/2}$ and Lp4. The ribozyme subdomain catalyzed the site-specific cleavage of the circularly permuted composite substrate RNA *in trans*. Thus, the structure of HDV autolytic domain could be re-formed after two subdomains were associated through the base-pairing interactions of H1, H2, and H4.

The genomic and antigenomic senses of hepatitis delta virus (HDV)[1] RNA undergo cis-cleavage reaction *in vitro* (Sharmeen et al., 1988; Wu et al., 1989). The site-specific cleavage activity requires the presence of magnesium ion or other divalent cations (Wu et al., 1989; Suh et al., 1993). Like other self-cleaving RNAs, the cleavage products of HDV RNAs contain a 5′-hydroxyl group and a 2′,3′-cyclic phosphate (Wu et al., 1989). The boundaries of the autolytic domains of both senses of HDV RNA have been defined (Sharmeen et al., 1988; Wu et al., 1989; Perrotta & Been, 1990, 1992; Wu & Huang, 1992), and the sequence requirement and secondary structure of each domain have been investigated extensively. These two autolytic domains derived from complementary senses of HDV RNA have many common features. Each domain contains four base-pairing regions that fold into a pseudoknot-like structure (Been et al., 1990; Perrotta & Been, 1991; Wu et al., 1992, 1993; Wu & Huang, 1992; Kummar et al., 1994). The sequence requirements of several single-stranded regions of these two domains are also quite similar (Wu & Huang, 1992; Kummar et al., 1992; Wu et al., 1993; Kawakami et al., 1993; Thill et al., 1993; Tanner et al., 1994). Although the understanding of the tertiary structure associated with the pseudoknot-like catalytic RNA is limited, several variants of HDV autolytic domains that cis-cleave efficiently have been constructed [the variants in Wu and Huang (1992) and in Wu et al. (1993)]. These RNA molecules, termed HDV ribozymes, retain most, if not all, of the essential elements of the natural HDV autolytic domains.

Circularly permuted (CP) RNAs, which are structural isomers of an RNA molecule, have the wild type termini connected and the new termini created at alternative sites. These CP RNAs allow the study of the effect of the break of particular ribose phosphate backbone on the folding pathway and the tertiary structure of an RNA [reviewed in Pan and Uhlenbeck (1993)]. Recently, the effects of circular permutation on the folding of tRNA (Pan et al., 1991) and the binding of the RNA moiety of RNase P to its pre-tRNA substrate (Pan & Zhong, 1994) as well as the interaction of R17 coat protein and its binding site have been investigated (Gott et al., 1993).

In this study, we synthesized CP isomers of a cis-cleaving HDV ribozyme and investigated the effects of the relocation of 5′-terminus to 10 selected locations on RNA folding. Moreover, we divided cis-cleaving ribozymes into subdomains by choosing different pairs of permissive locations as the termini of each bimolecular RNA construct and four trans-cleavage systems have been constructed. The site-specific cleavage reaction of the substrate RNA catalyzed by the corresponding trans-acting ribozyme was investigated and the ability to reconstitute HDV autolytic domain was evaluated.

MATERIALS AND METHODS

Plasmid Construction. Rz 1 is a self-cleaving RNA which was previously named DG-5-7 [one of the variants in Wu et al. (1993)]. In construct Rz 1, the cDNA of ribozyme sequence was placed downstream of a T7 promoter and between a *Kpn*I site and a *Hind*III site. The *Hind*III runoff

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[1] Abbreviations: CP, circularly permuted; DTT, dithiothreitol; E, extent of cleavage; EDTA, ethylenediaminetetraacetic acid; HDV, hepatitis delta virus; HEPES, 2-[4-(2-hydroxylethyl)-1-piperazinyl]-ethanesulfonic acid; *k*, cis-cleavage rate; PCR, polymerase chain reaction; PEI, polyethylenimine cellulose; TLC, thin layer chromatography.

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Table 1: Summary of Synthetic DNA Primers and Templates of PCR Reactions

name	sequence (5′ → 3′)	PCR 5′-primer	PCR 3′-primer[a]	template
Rz 1	(−10 to −1)[b] + (1 to 66)			
CP−1	(2 to 67) + (−10 to 1)	T7P[c] + (2 to 16)	(1 to −10) + (67 to 53)	Rz 1-T7
CP-2	(6 to 67) + (−10 to 5)	T7P + (6 to 22)	(5 to −10) + (67 to 48)	Rz 1-T7
CP-3	(10 to 67) + (−10 to 9)	T7P + (10 to 28)	(9 to −10) + (67 to 45)	Rz 1-T7
CP-4	(28 to 67) + (−10 to 27)	T7P + (28 to 52)	(27 to 1) + (-1 to −6)	Rz-CP
CP-5	(38 to 67) + (−10 to 37)	T7P + (38 to 52)	(37 to 1) + (-1 to −6)	Rz-CP
CP-6	(41 to 67) + (−10 to 40)	T7P + (41 to 55)	(40 to 1) + (-1 to −6)	Rz-CP
CP-7	(48 to 67) + (−10 to 47)	T7P + (48 to 67) + (−10 to 5)	(47 to 26)	Rz 1-T7
CP-8	(53 to 67) + (−10 to 52)	T7P + (53 to 67) + (−10 to 5)	(52 to 35)	Rz 1-T7
CP-9	(59 to 67) + (−10 to 58)	T7P + (59 to 67) + (−10 to 5)	(58 to 44)	Rz 1-T7
CP-10[d]	(17 to 67) + (−10 to 16)	T7P + (17 to 42)	(16 to −10) + (67 to 45)	Rz 1-T7

[a] The sequence of each 3′ primer is complementary to the ribozyme sequence. [b] The numbering system of nucleotides follows that of Figure 1. [c] T_7P has the sequence of 5′-TAATACGACTCACTATA-3′. [d] The sequence of CP-10 was different from other CP isomers of Rz 1 at H3 (see Figure 1 for details).

transcript of construct Rz 1 contains 10 nucleotides upstream and 66 nucleotides downstream of the cleaving point. Construct Rz 1-T7 was derived from construct Rz 1 by deleting the T7 promoter. For the construction of Rz 1-CP, DNA oligos 5′-TTAAG GATCC GGGGG ACTTC GGTCC GCGAA TGGGA AGCTT GGTAC-3′ and 5′-AATTA AGCTT ATCCG ACCCA TCAGG TACCA AGCTT CCCAT TC-3′ were annealed and extended by Taq polymerase (Promega). The double-stranded DNA was completely digested by *Bam*HI, followed by partial digestion by *Hind*III and then cloned to pUC19. The sequence of each recombinant construct was confirmed by DNA sequencing (Sanger et al., 1977). Constructs Rz 1-T7 and Rz 1-CP were used as the templates for PCR amplification.

Templates for RNA Synthesis. The template for the synthesis of each CP isomer of Rz 1 RNA was generated by PCR amplification. The primers and templates for each PCR are summarized in Table 1. The 5′-primer of PCR contained a 17-nucleotide T7 promoter followed by the ribozyme sequence, and the 3′-primer of PCR contained nucleotides that were complementary to the ribozyme sequence. Usually, a 100 μL polymerase chain reaction contained ~200 pmol of each primer. The templates of RNA 37 and RNA 73 were synthesized by PCR as well. The PCR products were directly used as templates for RNA synthesis without further purification.

Preparation of RNAs. The internally ^{32}P-labeled RNA was synthesized by T7 RNA polymerase runoff transcription reaction in the presence of ~20 μCi [α-^{32}P]CTP, 0.3 mM CTP, 1 mM of each ATP, GTP, and UTP, and PCR products or *Hind*III-digested construct Rz 1 as template. Typically the *in vitro* transcription reaction was carried out in the presence of 12 mM $MgCl_2$ at 37 °C for 1 h, and the reaction was stopped by addition of an equal volume of 50 mM EDTA in 7 M urea as described [see Wu et al. (1989) for details]. For the synthesis of Rz 1, the concentration of $MgCl_2$ was decreased to 4 mM, the reaction temperature was changed to 10 °C, and the reaction time was elongated to 12 h . The full-length transcript was purified from polyacrylamide gel containing 7 M urea as described (Wu et al., 1989). The concentrations of RNA 37 and RNA 73 were determined from the specific activity of CTP, the CTP contents of each RNA and the radioactivity of the RNA fragment.

To obtain RNA molecules that contained a 5′-monophosphate, transcription reactions were carried out using 3 mM GMP in addition to 1 mM of each NTP. To prepare circular form RNA, the gel-purified, internally ^{32}P-labeled CP-1 RNA that contained 5′-monophosphate was head-to-tail ligated by T4 RNA ligase. The reaction was conducted in 40 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 25 units of T4 RNA ligase at 14 °C for 5 h. The circular RNA and the unligated linear RNA were resolved by polyacrylamide/urea gel electrophoresis.

Cis-Cleavage Reactions. RNA was denatured at 90−95 °C for 1.5 min, cooled down to room temperature, and incubated at 50 °C for at least 5 min. A solution of Tris-HCl, pH 7.5, at 50 °C, and $MgCl_2$ that was prewarmed at 50 °C for 5 min was then added to the RNA to initiate cis-cleavage. The final concentrations of Tris-HCl and $MgCl_2$ were 40 mM and 12 mM, respectively, in most of the cases. The cis-cleavage reaction was quenched by the addition of an equal volume of 50 mM EDTA in 7 M urea. The full-length RNA and cleavage products were resolved by polyacrylamide−7 M urea denaturing gel. Since RNA molecules were labeled by [α-^{32}P]CTP, the relative number of moles of the remaining full-length RNA and cleavage product was calculated by dividing the radioactivity (cpm) of each RNA fragment with the number of C residues in that RNA species. The extent of the cis-cleavage reaction E is defined as the (moles of cleavage product)/[(moles of cleavage product) + (moles of remaining precursor RNA)]. The E value represents the molar ratio of the full-length RNA molecules that undergo cis-cleavage. E_{max} is the E value when the cis-cleavage reaction levels off. E_{max} represents the reactive species of each RNA, and its value varied between 20% and 90% for the cis-cleaving RNAs in this report. In general, the reaction follows the pseudo-first-order kinetics for the first 4−5 half-lives of cis-cleavage reaction. The cis-cleavage rate (k) was the slope of the plot of ln ($1 - E/E_{max}$) vs time.

Two-Dimensional Gel Electrophoresis Analysis. The RNA molecules were denatured−renatured and preincubated with 40 mM Tris-HCl at 50 °C for 30 min prior to electrophoresis. The electrophoresis on the first dimension was carried out in a native 10% polyacrylamide gel (acrylamide:bisacrylamide = 19:1) in 50 mM Tris-HOAc, pH 7.5, and 10 mM $Mg(OAc)_2$ at 12 W for 4.5 h at room temperature. The non-denaturing gel slice was then turned by 90° and mounted in a 10% polyacrylamide gel containing 7 M urea. The electrophoresis of the second dimension was conducted in 1 × TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.0).

Identification of Cleavage Site. About 1000 cpm of the purified [α-^{32}P]GTP or [α-^{32}P]CTP-labeled 5′-cleavage product of CP isomer was digested by 0.37 unit of nuclease P1 (Sigma) in 0.1 M NH_4OAc, pH 5.0, at 37 °C for 30 min. The reaction products were then applied to a polyethylenimine cellulose (PEI) plate (Sigma). The thin layer chromatography (TLC) was conducted in 1.1 M NH_4OAc, pH 7.5.

The location of the cleaving point was confirmed by primer extension. The purified 3′-cleavage product was annealed with 10 000 cpm of the ^{32}P-labeled PCR 3′-primer of CP-7 (Table 1) in 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.75 mM $MnCl_2$ containing 2 mM dNTP's. The primer extension reaction was conducted at 72 °C for 30 min with 1 unit of retrotherm reverse transcriptase (Epicentre Technologies). In addition, dideoxy sequencing of construct Rz 1 with the same 5′-end-labeled primer was conducted in parallel to serve as size markers.

Trans-Cleavage Reaction. RNA 73 (substrate) and RNA 37 (enzyme) were mixed, heated at 90−95 °C for 1.5 min, cooled down to room temperature, and preincubated at 50 °C for at least 5 min to promote annealing. The trans-cleavage reaction was then initiated by adding a stock solution of Tris-HCl, pH 7.5, and $MgCl_2$ to final concentrations of 40 and 12 mM, respectively. Alternatively, the substrate and enzyme RNA's were de- and renatured separately; substrate RNA and enzyme RNA were preincubated with Tris-HCl/$MgCl_2$ solution at 50 °C for more than 5 min individually. Two RNA solutions were mixed to initiate the trans-cleavage reaction. The trans-cleavage reaction was conducted at 50 °C, and the reactions were stopped by the addition of 2 volumes of 50 mM EDTA in 7 M urea.

RESULTS

Rz 1. Rz 1 is an HDV ribozyme that possesses the characteristics of the autolytic domains of both senses of HDV RNA. In this study, Rz 1 was the wild type molecule containing 76 nucleotides (residue −10 to residue 66) that may fold into a pseudoknot-like structure (Figure 1). It is an active cis-cleaving RNA, since more than 90% of the Rz 1 RNA molecules underwent cis-cleavage at the rate of ∼0.4 min^{-1} in the presence of 12 mM $MgCl_2$ at 50 °C (Figures 3A and 4). In Rz 1, the 5′-GGUA-3′ at the 5′-terminus and the 5′-AAGCU-3′ at the 3′-terminus are non-HDV sequences to allow high efficiency of the RNA synthesis and to provide the sequence for the runoff transcription reaction. In addition, a well-characterized hairpin loop of non-HDV RNA (Cheong et al., 1990), i.e., H4 and loop 4, has substituted the self-complementary sequences located at the 3′-half of the autolytic domain of each sense of HDV RNA (Wu & Huang, 1992; Wu et al, 1993). The H1 and $J_{1/4}$ of Rz 1 have the same sequences as the corresponding regions of the autolytic domain of HDV antigenomic RNA. The remaining sequences of Rz 1 were derived from the autolytic domain of HDV genomic RNA.

Circularly Permuted and Circular Isomers. Circularly permuted (CP) RNA of Rz 1 was individually synthesized by transcribing the corresponding DNA template obtained from polymerase chain reactions. The residues upstream of the cleavage site as well as the single-stranded nucleotides near the normal 3′-terminus are not required for the cis-cleavage reaction of HDV ribozymes (Perrotta & Been 1990). Residues −10 to −2 and residues 63−67 may serve as the linkers to connect the 5′- and 3′-boundaries of the cis-cleaving domain. Ten positions in the interior of the ribozyme molecule were selected for circular permutation. Three of them were located in double-stranded regions (H1 for CP-1 and CP-2, and H2 for CP-9), two of them were in single-stranded regions ($J_{1/2}$ for CP-3 and Lp4 for CP-7), and the remaining five were at the junctions of different structural/sequence domains (Figure 1A,B). In addition, the circular isomer of Rz 1 was synthesized by connecting the termini of one of the CP isomer by T4 RNA ligase. Since a correctly folded structure is required to maintain the activity of a catalytic RNA, the effect of the ligation of the wild type termini and the relocation of the 5′-terminus to a particular location on the overall folding of ribozyme Rz 1 could be monitored by assaying the cis-cleaving activity of circular isomer and CP isomers. The cis-cleavage reaction was typically carried out in the presence of 40 mM Tris-HCl and 12 mM $MgCl_2$ at 50 °C after the RNA was heat-denatured and then renatured. The activity of each isomer was evaluated by the kinetics of cis-cleavage reaction as well as the fraction of the active full-length RNA molecules that undergoes cis-cleavage.

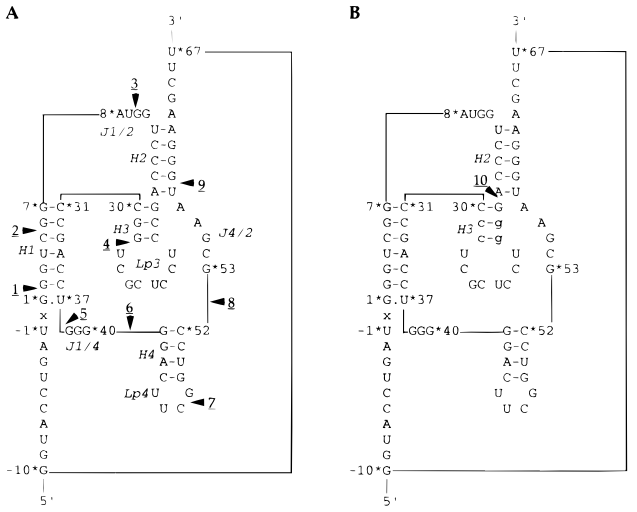

FIGURE 1: The sequences of different RNAs. (A) Rz 1 is a *Hind*III runoff transcript. Its sequence starts at G-10 (5′) and terminates at U66 (3′). Each CP isomer contains an extra U residue (U67) in comparison to Rz 1. G-10 and U67 are ligated in each CP isomer. The residues downstream and upstream of each arrowhead are the 5′- and 3′-termini, respectively. "×" represents the cleaving point. H1−H4 are the four double-stranded regions. $J_{1/2}$, $J_{1/4}$, and $J_{4/2}$ are the single-stranded regions connecting different helices. (B) The sequence of CP-10. The sequence of the H3 of this isomer is changed to enhance the efficiency of transcription reaction.

The cis-cleavage of the circular isomer could not be detected under standard assay conditions (data not shown), but the elevation of [$MgCl_2$] from 12 to 20 or 40 mM significantly enhanced its cis-cleavage activity (Figure 2B). Nevertheless, the circular RNA cleaved at a substantially reduced rate as compared to the linear RNA even in the presence of higher [$MgCl_2$] (Figure 2A,B). It is likely that the ligation of the wild type 5′- and 3′-termini may generate certain steric constrains that destabilize H1. Consequently, the spacial arrangement of the cleaving point is disturbed, and certain essential functional groups associated with the first base pair of H1 and $J_{1/4}$ are misaligned. Moreover, the magnesium binding site of the circular isomer may be altered.

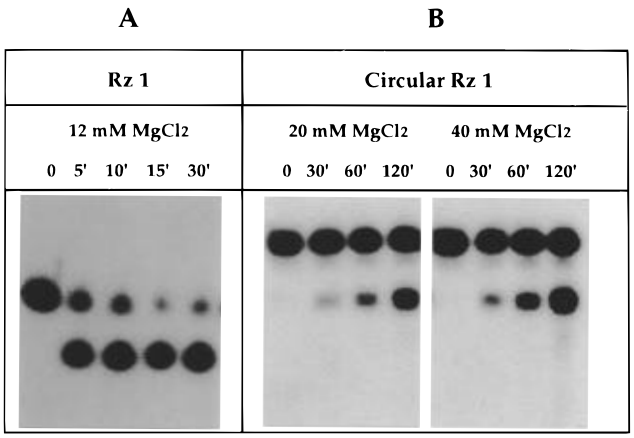

FIGURE 2: The cis-cleavage reactions of Rz 1 its circular and CP isomers. (A) Rz 1 was incubated at 50 °C with 40 mM Tris-HCl for 30 min (0), or with 40 mM Tris-HCl and 12 mM $MgCl_2$ for 5, 10, 15, and 30 min. The upper bands and lower bands are the full-length RNA and the 3′-cleavage product, respectively. The 5′-cleavage product ran off the gel. (B) The circular isomer was incubated at 50 °C with 40 mM Tris-HCl for 120 min (0) or with 40 mM Tris-HCl and 20 or 40 mM $MgCl_2$ for 30, 60, and 120 min. The upper bands are the circular RNA, and the lower bands are the linearized RNA.

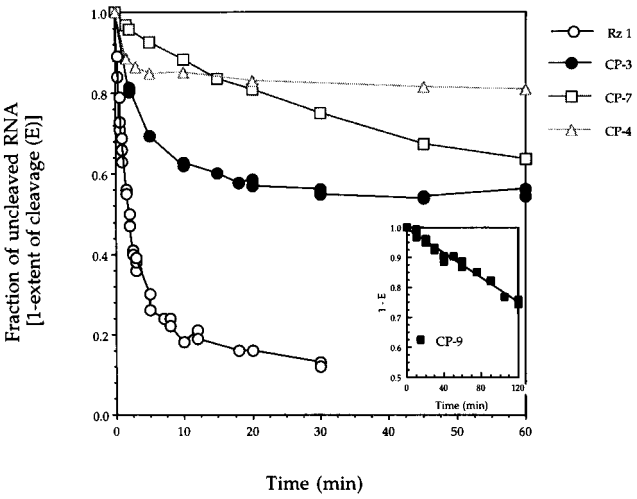

FIGURE 3: Time course of the cis-cleavage reaction of Rz 1 and different CP isomers. The reactions were conducted in the presence of 40 mM Tris-HCl and 12 mM $MgCl_2$ at 50 °C.

It is possible that the elongation of the linker between the 5′- and 3′-boundaries of the autolytic domain may relieve some unfavorable constraints associated with the circular molecule. This possibility was not examined.

Four CP isomers cis-cleaved, but their cis-cleavage activities varied. Only ~20% of CP-4, <50% of CP-3, and <50% of CP-7 underwent cis-cleavage at 50 °C in the presence of 12 mM $MgCl_2$. Prolonged incubation under the same condition did not elevate the extent of cleavage (Figure 3). The low extent of cleavage suggests that a large amount of the RNA molecules of these isomers are irreversibly trapped in certain inactive conformation(s). The kinetics of cis-cleavage of the reactive RNA molecules of each CP isomer was investigated. CP-3 and CP-4 cleaved at a rate comparable to that of Rz 1 (Table 2). The reactive CP-7 molecules cleaved at a rate ~10-fold slower than that of Rz 1 (Table 2). The destabilization of the superstable hairpin loop due to the disconnection of Lp4 and H4 may account for the reduction of cis-cleavage rate. Unlike the other CP isomers, the cis-cleavage reaction of CP-9 did not level off during the 2-h incubation period (Figure 3), and its cis-cleavage rate was estimated to be 100-fold slower than that of Rz 1 (Table 2). Presumably, the conformational rearrangement instead of the cleavage process is rate limiting. Therefore, the single breaks in $J_{1/2}$ (CP-3), in Lp4 (CP-7), at the 3′-end of Lp3 (CP-4) as well as near the bottom of H2 (CP-9) may not disrupt the catalytic core since each of these CP isomers undergoes cis-cleavage. In the presence of 12 mM $MgCl_2$, each active CP isomer is more reactive than the circular isomer. The results reveal that a single break of the phosphodiester bond at an appropriate location may overcome the perturbation of the catalytic core caused by the ligation of wild type termini. Moreover, the breaks in $J_{1/2}$ (CP-3) and Lp4 (CP-7) had the most significant effect. Nevertheless, the relief of the alteration of folding geometry was not complete since none of these cis-cleaving CP isomers was as reactive as Rz 1.

Table 2: Rate of Cis-Cleavage of Different RNAs in the Presence of 12 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.5) at 50 °C

RNA	k (min^{-1})[a]	E_{max} (%)[b]
Rz 1	0.43	90%
CP-3	0.23	50%
CP-4	0.80	20%
CP-7	0.04	40%
CP-9	0.0024[c]	~30% (2 h)

[a] The rate of cis-cleavage of the active species of Rz 1 or CP isomer was determined as described (Materials and Methods). [b] E_{max} represents the active RNA species of each variant. [c] The cis-cleavage reaction of CP-9 did not level off after 2 h of incubation. E_{max} of CP-9 was assumed to be 100% for the determination of k.

The CP isomers with a single break of the phosphodiester bond in H1 (CP-1 and CP-2) or at the junction of H2−H3 (CP-10), H1−$J_{1/4}$ (CP-5), $J_{1/4}$−H4 (CP-6), or H4−$J_{4/2}$ (CP-8) did not cleave in the presence of 12 mM $MgCl_2$ (data not shown). However, unlike the circular isomer, these CP isomers remained inactive when they were incubated with higher concentration of $MgCl_2$ (data not shown). Since these CP RNAs have the breaks either in certain structural domains or at the junction of two structural/sequence domains that are essential for autolytic reaction, the loss of cis-cleavage activity of these isomers is not surprising. The results confirm that HDV ribozyme has a compact structure. Moreover, the folding geometry of the catalytic core may be severely altered by the single break of the phosphodiester bond in each of these CP isomers.

The RNA molecules synthesized by a standard transcription reaction contain a 5′-triphosphate. It is likely that the highly negatively charged triphosphate group affects the folding of each CP isomer. To examine this possibility, CP RNAs with a 5′-monophosphate were synthesized by the transcription reactions that contained excess GMP (described in Materials and Methods). Since GMP may compete with GTP for the first nucleotide of transcript but cannot be incorporated elsewhere, the full-length transcripts contained primarily 5′-monophosphate. For each CP isomer, the cis-cleavage activity of the mixture of 5′-monophosphate RNA and 5′-triphosphate RNA was indistinguishable from that of the pure 5′-triphosphate RNA (data not shown). Thus, the triphosphate to monophosphate replacement at the 5′-terminus of each CP isomer had no detectable effect on the folding of ribozyme molecule (data not shown).

We performed cis-cleavage reactions under different conditions known to affect RNA folding to examine whether

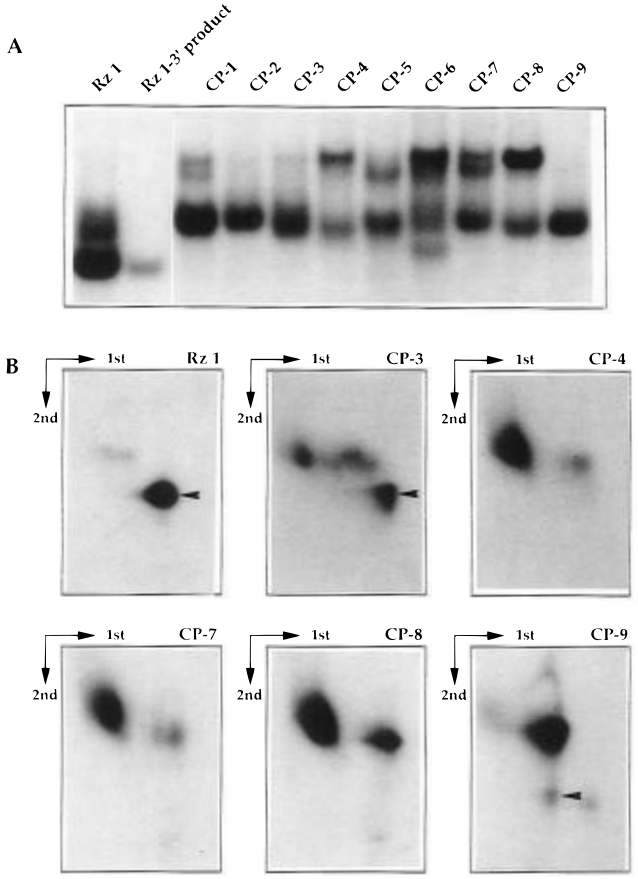

FIGURE 4: Two-dimensional gel electrophoresis analysis. (A) Electrophoresis on the first-dimensional non-denaturing gel. Each RNA was denatured at 90−95 °C for 1.5 min, cooled down to room temperature, and then incubated with 40 mM Tris-HCl at 50 °C for 30 min. After the addition of a 1/5 volume of 30% glycerol containing 0.05% bromophenol blue and 0.05% xylene cyanole, RNA was analyzed by electrophoresis on a 10% non-denaturing gel. (B) Electrophoresis on the second-dimensional denaturing gel. "→ 1st" and "→ 2nd" illustrate the orientations of the first and the second dimensions, respectively. The longer products of the cis-cleavage reactions of Rz 1, CP-3, and CP-9 are indicated by arrowheads.

inactive isomers can be activated or the activity of active isomers can be enhanced. The variation of [$MgCl_2$] between 12 and 40 mM alone or together with the change of incubating temperature to 37 or 60 °C did not affect the cis-cleavage reactions (data not shown). Repeated cycles of heat de- and renaturation (one cycle means 90−95 °C for 1.5 min and then 50 °C for 5 min) (Wu & Lai, 1990) elevated slightly the molar ratio of the reactive RNA molecules (the extent of cleavage) of the cis-cleaving CP isomers (data not shown). In contrast, the addition of 2.5−10 M formamide or 1−5 M urea severely decreased the extent of cleavage of these CP isomers (data not shown). However, none of these treatments activated any of the inactive CP isomer (data not shown).

Structural Homogeneity of Different CP Isomers. A two-dimensional gel electrophoresis analysis was developed to examine the structural homogeneity of Rz 1 as well as its CP RNAs (described in Materials and Methods). Rz 1 migrated as two species on the first-dimensional non-denaturing gel (Figure 4A). The minor species migrated slightly slower, while the major species, which represented ∼90% of the materials, comigrated with the 3′-cis-cleavage product of Rz 1. The nature of these two species was resolved by electrophoresis on the second-dimensional denaturing gel. The results showed that the minor species represented the residual full-length RNA whereas the major species was the 3′-cleavage product (Figure 4B). These findings suggest that the presence of 10 mM magnesium ion in the non-denaturing gel of the first dimension allowed the cis-cleavage of Rz 1 to occur.

Each CP isomer that lost cis-cleavage activity had a distinct migration pattern on non-denaturing gel (Figure 4A). In the case of CP-1 and CP-2, most of the RNA molecules migrated together, with a migration rate slightly slower than that of Rz 1. Thus, the RNA molecules containing a single break of the phosphodiester bond in H1 adopt relatively compact structure(s). In contrast, CP-5, CP-6, and CP-8 RNAs were more heterogeneous in structure. About 40% of CP-5, >50% of CP-6, and ∼80% of CP-8 existed as a very slow-migrating form on non-denaturing gel, suggesting a less compact structure. This result implies that certain interactions that are important for folding have been disrupted by the relocation of 5′- and 3′-termini to the junction of H1−$J_{1/4}$, $J_{1/4}$−H4, or H4−$J_{4/2}$. Furthermore, preincubation with 12 mM $MgCl_2$ for 30 min at 50 °C prior to the non-denaturing gel electrophoresis did not alter the migration pattern, including the total number and relative amount of each species, of any inactive CP isomer (data not shown).

CP-3, CP-4, and CP-7 each migrated as more than one species on non-denaturing gel. Moreover, ∼50% of CP-3 cis-cleaved in the process of two dimensional gel electrophoresis. In contrast, CP-4 or CP-7 did not undergo cis-cleavage (Figure 4A,B). The structural heterogeneity of CP-3, CP-4, and CP-7 may account for the low extent of cleavage of each CP isomer, although it is not clear which species of each isomer folds correctly for cis-cleavage. CP-9 migrated as a single species on non-denaturing gel, and the mobility of this species was very similar to that of Rz 1 (Figure 4A). The CP-9 RNAs appear to have structures similar to those of CP-1 and CP-2 which are relatively compact; however, at least some of the CP-9 RNAs cis-cleaved in the non-denaturing gel (Figure 4B).

Identification of the Cleaving Point. Experiments were conducted to examine whether the cleavage sites of the cis-cleaving CP isomers remained unaltered. The sequence of the residues around the cleaving point was determined by nearest neighbor analysis for CP-3, CP-7, and CP-9. The [α-^{32}P]GTP-labeled 5′-cleavage products of each CP RNA released ^{32}P-labeled pU>p after nuclease P1 digestion (Figure 5A). Thus, the cleaving points of these CP isomers were between a U residue (5′) and a G residue (3′). Since there are many 5′-UG-3′ in the RNA molecule, the exact location of the cleavage site was further confirmed by primer extension of the 3′-cleavage product. The results indicated that the cleaving points of CP-7 and CP-9 were between U − 1/G + 1 (Figure 5B). Thus, the cleavage sites of the active CP isomers remain unaltered.

Design of Trans-Cleaving Ribozymes. The information obtained from the cis-cleaving CP isomers provides a rational basis for the design of trans-cleaving ribozymes. Four bimolecular constructs were made by dividing the cis-cleaving ribozymes into substrate and enzyme subdomains from permissive locations. In addition, two RNA molecules of each construct associate via base-pairing interactions to reconstitute the pseudoknot-like structure of HDV autolytic

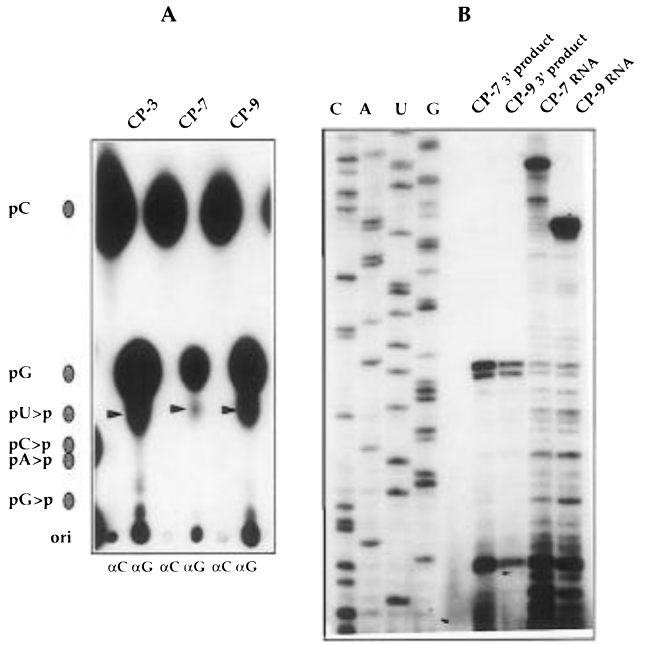

FIGURE 5: The identification of the cleaving point of different CP isomers of Rz 1. Nearest neighbor analysis of the 5′-cleavage products of CP-3, CP-7, and CP-9 (A) and primer extension of the 3′-cleavage products of CP-7 and CP-9 (B) were conducted as described in Materials and Methods. RNAs were internally labeled by [α-^{32}P]CTP (αC) or [α-^{32}P]GTP (αG). In addition, pC, pG, and four ^{32}pN>p's were run in parallel to serve as markers.

domain. The catalytic efficiency of each trans-cleaving ribozyme was determined.

A ribozyme molecule (RNA 37) that contains the 5′-strand of H2, the entire H3 and Lp3, the 3′-strand of H1, $J_{1/4}$, and the 5′-strand of H4 was constructed (Figure 6A). The corresponding substrate molecule (RNA 73) has a circularly permuted composite: it contains the complementary sequences for the formation of H1, H2, and H4, the $J_{4/2}$ region, and the residues upstream of cleaving point (Figure 6A). The autolytic domain conceivably can be reconstituted by the formation of H1, H2, and H4. This system 73/37 represents the division of CP-3 RNA at Lp4 (or the division of CP-7 RNA at $J_{1/2}$) into two parts, whereas the H4 of RNA 73/RNA 37 bimolecular construct was extended in comparison to that of Rz 1 RNA (Figure 1A and 6A). When RNA 37 and RNA 73 were mixed in the presence of Mg^{2+}, RNA 37 catalyzed the cleavage of RNA 73 *in trans* (Figure 6B). When RNA 37 (2.5 nM) was incubated with excess amount of RNA 73 (50 or 100 nM), RNA 37 behaved like a true enzyme since it was capable of multiple rounds of cleavage (Figure 6C). Steady state rates of cleavage were measured at several substrate concentrations that were at least 4-fold greater than ribozyme concentration in the presence of 12 mM Mg^{2+} at 50 °C (Figure 6D). A K_m of 58 nM and k_{cat} of 0.66 min^{-1} were obtained from these data (Figure 6E). The second-order rate constant (k_{cat}/K_m) was 1.1×10^7 M^{-1} min^{-1}, which was within the range 10^7-10^8 M^{-1} min^{-1} for the association of oligonucleotides to form the duplex (Nelson & Tinoco, 1982) and for the binding of substrate RNAs to the corresponding ribozymes (Fedor & Uhlenbeck, 1990; Herschlag & Cech, 1990). Nevertheless, the extended H4 in the RNA 73/RNA 37 bimolecular construct seems to be important for the formation of substrate/ribozyme complex. The catalytic efficiency (k_{cat}/K_m) was enhanced 100-fold (the k_{cat} was increased ~20-fold, and the K_m was decreased ~5-fold) when the H4 of this trans-cleavage system was elongated from the 4-bp Rz 1 sequence to the 11-bp RNA 73/RNA 37 sequence (data not shown).

The other three trans-cleavage systems were constructed by interrupting the sequences of Rz 1 from $J_{1/2}$ (system 03/30), Lp3 (system 04/40), or Lp4 (system 07/70). For each system, the substrate contains the cleavage site while the corresponding ribozyme possesses trans-cleaving activity. Bimolecular constructs similar to those of systems 03/30 and 07/70 have been made previously (Been et al., 1993; Puttataju et al., 1993; Branch & Robertson 1991; Wu et al., 1992).

For system 07/70, the formation of the pseudoknot-like structure requires the base-pairing interactions in H2 and H4. However, similar to that of system 73/37, the efficiency of the trans-cleavage reaction of system 07/70 was significantly affected by the size of H4. The catalytic efficiency could be enhanced ~100-fold when the 4-bp H4 of Rz 1 sequence was extended by 2 base pairs, and further extension of H4 to 12 bp elevated the catalytic efficiency another 10-fold (data not shown). A modified version of system 07/70 has the k_{cat}/K_m of $\sim 1 \times 10^8$ M^{-1} min^{-1}; nevertheless, the affinity between the substrate and ribozyme RNAs of this bimolecular construct was so tight that the ribozyme can hardly turn over (data not shown).

The reconstitution of the autolytic domain of system 03/30 relies on the formation of the 7-bp H1. The trans-cleavage reaction of this system occurred, even though the k_{cat}/K_m was estimated to be 200−500-fold lower than that of system 73/37 [the data was derived from a single-turnover reaction in which 5 nM of substrate was incubated with 50 nM of ribozyme, and the value of k_{cat}/K_m was obtained by dividing the rate of cleavage that follows the first-order kinetics by the ribozyme concentration (50 nM)]. The low efficiency of trans-cleavage of system 03/30 may be due to the less stable bimolecular complex. However, although the assay conditions differed, the catalytic efficiency of system 03/30 seemed to be lower than that of a similar system constructed by Been's group (1992) in which the substrate molecule was shorter from the 5′-end. The formation of complex with alternative conformations and/or the presence of intramolecular interactions because of the extra residues associated with the substrate molecule may account for the lower efficiency of trans-cleavage of system 03/30. The details of the kinetics of the trans-cleavage reaction have not been investigated.

In the case of system 04/40, the cleavage of substrate could be detected when the trans-cleavage reaction was performed under conditions of ribozyme excess. Nevertheless, the catalytic efficiency of system 04/40 was lower than that of system 03/30 even though the bimolecular complex of the former contains more base pairs than that of the latter (data not shown). Thus, the base-pairing interaction energy in H1, H2, and H3 is not high enough to compensate the alteration of RNA folding caused by the break of the phosphodiester bond at the 3′-end of Lp3.

DISCUSSION

We studied the effects of single breaks of the phosphodiester bond in the interior of a cis-cleaving HDV ribozyme Rz 1. The wild type termini of Rz 1 were ligated, and 10 locations in this cis-cleaving RNA were selected to be the new 5′-terminus. The autolytic activity of each CP isomer

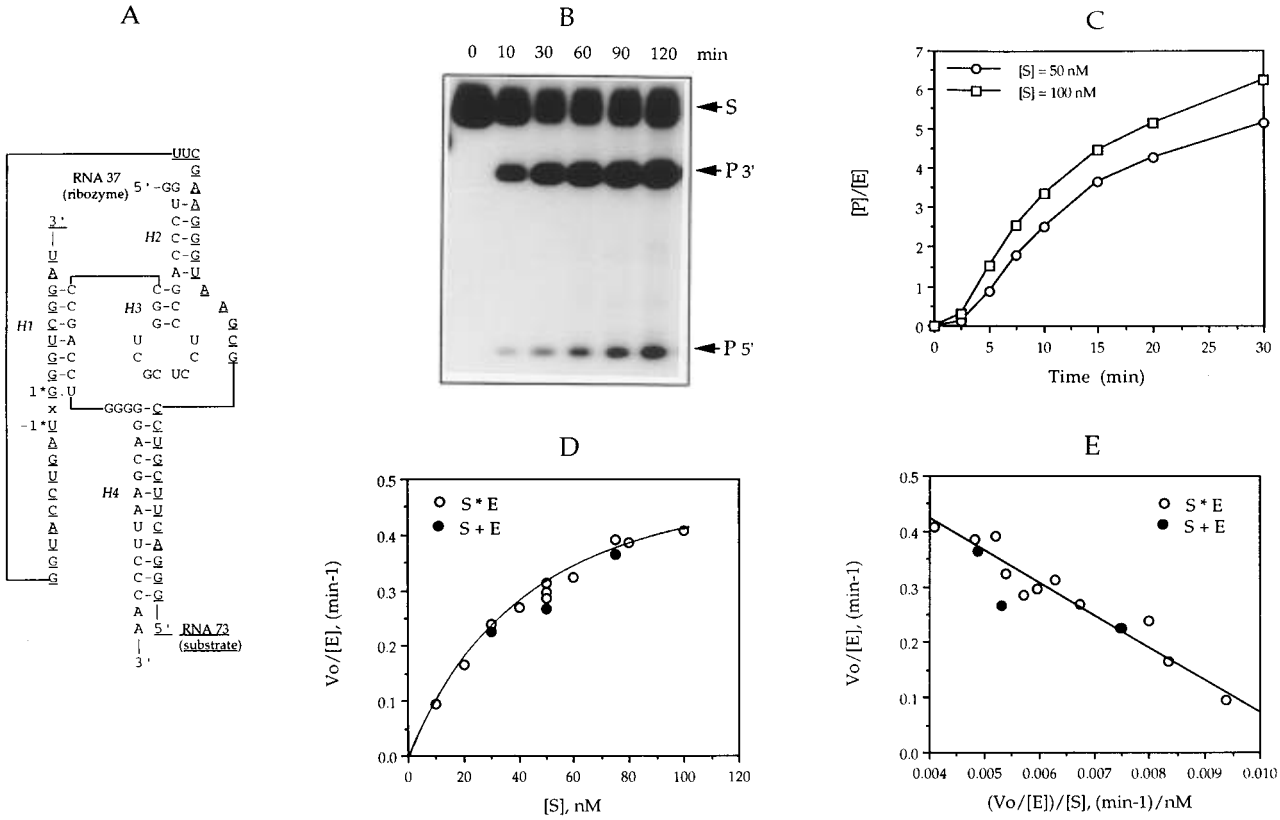

FIGURE 6: Trans-cleavage of RNA 73 (substrate) catalyzed by RNA 37 (enzyme). (A) The sequence and proposed secondary structure of a circularly permuted trans-acting ribozyme (RNA 37) and its substrate (RNA 73). The residues of RNA 73 are underlined. H4 of this bimolecular construct was extended in comparison to that of Rz 1. There is a UC mismatched pair in H4, which was incorporated accidentally. (B) Cleavage of 5 nM ^{32}P-labeled substrate by 10 nM enzyme. The substrate (S) and cleavage products (P5′ and P3′) were separated by electrophoresis on a 10% polyacrylamide−7 M urea denaturing gel. (C) Cleavage reaction with excess substrate. The reaction mixture contains 50 or 100 nM of ^{32}P-labeled substrate and 2.5 nM of enzyme. Moles of product generated per mole of enzyme ([P]/[E]) as a function of time are plotted. RNA 73 and RNA 37 were preannealed for the experiments of B and C. (D) Multiple-turnover kinetics of cleavage reaction. The cleavage of different concentrations of substrate (10−100 nM) catalyzed by 2.5 nM of enzyme. The steady state rate of cleavage (V_o, nM/min) was divided by the concentration of enzyme ([E] = 2.5 nM), and the value of V_o/[E] (with the unit of min^{-1}) is plotted vs substrate concentration ([S], nM). Data of open circles were derived from the experiments with preannealed substrate and enzyme (S * E). For the data of closed circle, the two RNA molecules were not preincubated (S + E). (E) Eadie−Hofstee plot of the data to generate K_m (slope) and k_{cat} (Y-intercept), $Y = 0.66 - 58X$.

was examined to evaluate the effect of single breaks on RNA folding.

The structural probing and mutational analysis studies have illustrated that HDV ribozymes contain four double-stranded regions (H1−H4). The secondary structure rather than the sequences of helices H1, H2, and H4 is critical for cis-cleavage. Moreover, two helices (H2 and H3) may stack coaxially and the framework of HDV ribozymes has a pseudoknot-like structure (Perrotta & Been 1991; Been et al., 1992; Kummar et al., 1992; Wu et al., 1992, 1993; Wu & Huang, 1992). The previous thermodynamic studies have indicated that a break in an RNA helix can notably stabilize the helical structure (Walker et al., 1994). In addition, a large amount of the single breaks in the helical regions of tRNA, RNase P RNA, or the R17 coat protein binding site did not affect the tertiary structure of each RNA molecule (Pan et al., 1991; Pan & Zhong, 1994; Gott et al., 1993). However, in this report, we showed that breaks at the 5′ strand of H1 were not tolerated and a break in H2 severely decreased cis-cleavage activity. It is likely that certain catalytically important residues or functional groups, such as the first base pair of H1 of CP-1 and CP-2, and the $J_{4/2}$ region of CP-9 may be misplaced when the helical structure of H1 or H2 was altered. Alternatively, the stabilization of the helical structure of H1 or H2 is not sufficient to overcome the deleterious effect of the ligation of the wild type termini.

The $J_{1/4}$, $J_{4/2}$, and Lp3 regions of HDV ribozyme have stringent sequence requirements (Wu & Huang, 1992; Kumar et al., 1992; Kawakami et al., 1993; Wu et al., 1993; Thill et al., 1993; Tanner at al., 1994). It has been postulated that some residues of these regions are directly involved in the cleavage process or Mg^{2+} binding. In addition, certain residues may participate in sequence-specific interactions that are critical for maintaining the tertiary structure of catalytic core. Results of circular permutation analysis illustrate that the single breaks at the 5′-end of $J_{1/4}$ (CP-5), the 3′-end of $J_{1/4}$ (CP-6), and the 5′-end of $J_{4/2}$ (CP-8) completely abolished cis-cleavage activity. Besides, the single break at the 3′-end of Lp3 (CP-4) prevented a large portion of the RNA molecules (∼80%) from adopting correct conformation for cis-cleavage. Thus, both the integrity of the phosphodiester backbone and the identity of the catalytically important residues in $J_{1/4}$, $J_{4/2}$, and Lp3 are critical for the overall folding and/or the stabilization of HDV ribozyme.

In contrast, the CP isomers that have single breaks in $J_{1/2}$ and Lp4 cis-cleaved reasonably well despite of the detrimental effect of the ligation of the wild type termini. Thus, the relocation of the 5′-terminus to either region has a relatively small effect on the tertiary structure of the HDV autolytic domain. The result is fully agreeable with the three-

dimensional model of HDV ribozyme proposed by Tanner et al. (1994) in which both $J_{1/2}$ and Lp4 project away from the catalytic core. Moreover, it is also consistent with the previous findings that the HDV autolytic domain can be reconstituted after the cis-cleaving HDV ribozyme was separated at $J_{1/2}$ (Been et al., 1992; Puttataju et al., 1993) or at Lp4 (Branch & Robertson, 1991; Wu et al., 1992; Perrotta & Been, 1993) into two subdomains.

Furthermore, we tried to construct trans-cleaving ribozymes by dividing the cis-cleaving RNAs into a substrate subdomain and an enzyme subdomain from permissive locations that were identified by circular permutation studies. Four trans-cleavage systems have been made, and each enzyme subdomain catalyzed the site-specific cleavage of the corresponding substrate subdomain with varying efficiency. Thus, the tertiary structure of HDV autolytic domain could be reconstituted through the base-pairing interactions in each bimolecular construct, although the ability of each construct varied.

The ribozyme molecule of a bimolecular construct with the termini at $J_{1/2}$ and Lp4 possessed high catalytic efficiently. The result provides additional evidence that the integrity of $J_{1/2}$ and Lp4 is not essential for the folding of HDV autolytic domain. The novel trans-acting ribozyme (RNA 37) interacts with its substrate (RNA 73) that contains circularly permuted sequences through the formation of H1, H2, and H4. Although this bimolecular construct contains more intermolecular base pairs than the other constructs (H1 for system 03/30; H1, H2, and H3 for system 04/40; H2 and H4 for system 07/70), the stronger base-pairing interaction did not prevent the turnover of the ribozyme molecule. It is likely that the steric constraints associated with the ligation of the wild type termini in the substrate molecule (RNA 73) may decrease the energy of folding that facilitates turnover after trans-cleavage reaction. Previous studies have indicated that H2, H4, and base pairs 2−7 of H1 do not have a stringent sequence requirement (Wu et al., 1992, 1993). In addition, H2 can be elongated from 5 to 10 bp (unpublished observation of this laboratory) and H4 can be variable in size as long as the bimolecular complex is stable. Therefore, the sequences of the 5′-strand of H2, the 3′-strand of H1, and the 5′-strand of H4 of this trans-acting ribozyme (RNA 37) can be custom-made. Moreover, the targeted RNA can be flexible in sequence since only the $J_{4/2}$ region and the residue 3′ to the cleaving point have stringent sequence requirements (Wu et al., 1993; Kawakami et al., 1993).

REFERENCES

Been, M., Perrotta, A. T., & Rosenstein, S. P. (1992) *Biochemistry 31*, 11843−44852.

Branch, A. D., & Robertson, H. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10163−10167.

Cheong, C., Varani, G., & Tinoco, I., Jr. (1990) *Nature* (*London*) *346*, 680−682.

Fedor, M. J., & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1668−1672.

Gott, J. M., Pan, T., LeCuyer, K. A., & Uhlenbeck, O. C. (1993) *Biochemistry 32*, 13399−13404.

Herschlag, D., & Cech, T. R. (1990) *Biochemistry 29*, 10159−10171.

Kawakami, J., Kummar, P. K. R., Suh, Y. A., Nishikawa, F., Kawakami, K., Taira, K., Ohtsuka, E., & Nishikawa, S. (1993) *Eur. J. Biochem. 217*, 29−36.

Kummar, P. K. R., Suh, Y. A., Miyashiro, H., Nishikawa, F., Kawakami, J., Taira, K., & Nishikawa, S. (1992) *Nucleic Acids Res. 20*, 3919−3924.

Kummar, P. K. R., Taira, K., & Nishikawa, S. (1994) *Biochemistry 33*, 583−592.

Nelson, J. R., & Tinoco, I., Jr. (1982) *Biochemistry 21*, 5289−5295.

Pan, T., & Uhlenbeck, O. C. (1993) *Gene 125*, 111−114.

Pan, T., & Zhong, K. (1994) *Biochemistry 33*, 14207−14212.

Pan, T., Gutell, R. R., & Uhlenbeck, O. C. (1991) *Science 254*, 1361−1364.

Perrotta, A. T., & Been, M. (1990) *Nucleic Acids Res. 23*, 6821−6827.

Perrotta, A. T., & Been, M. (1991) *Nature (London) 350*, 434−436.

Perrotta, A. T., & Been, M. (1992) *Biochemistry 31*, 16−21.

Perrotta, A. T., & Been, M. (1993) *Nucleic Acids Res. 21*, 3959−3963.

Puttaraju, M., Perrotta, A. T., & Been, M. D. (1993) *Nucleic Acids Res. 21*, 4253−4258

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1074−1078.

Sharmeen, L., Kuo, M. Y. P., Dinter-Gottlieb, G., & Taylor, J. (1988) *J. Virol. 62*, 2674−2679.

Suh, Y. A., Kumar, P. K. R., Taira, K., & Nishikawa, S. (1993) *Nucleic Acids Res. 21*, 3277−3280.

Tanner, N. K., Schaff, S., Thill, G., Petit-Koskas, E., Crain-Denoyelle, A.-M., & Westhof, E. (1994) *Current Biol. 4*, 488−498.

Thill, G., Vasseur, M., & Tanner, N. K. (1993) *Biochemistry 32*, 4254−4262.

Walker, A. E., Turner, D. H., Kim, J., Lyttle, M. H., Muller, P., Mathews, D. H., & Zuker, M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9218−9222.

Wu, H. N., & Lai, M. M. C (1990) *Mol. Cell. Biol. 10*, 5575−5579.

Wu, H. N., & Huang, Z. S. (1992) *Nucleic Acids Res. 20*, 5937−5941.

Wu, H. N., Lin, Y. J., Lin, F. P., Makino, S., Chang, M. F., & Lai, M. M. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1831−1835.

Wu, H. N., Wang, Y. J., Hung, C. F., Lee, H. J., & Lai, M. M. C. (1992) *J. Mol. Biol. 223*, 233−245.

Wu, H. N., Lee, J. Y., Huang, H. W., Huang, Y. S., & Hsueh, T. G. (1993) *Nucleic Acids Res. 21*, 4193−4199.

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