

# Detailed analysis of base preferences at the cleavage site of a *trans*-acting HDV ribozyme: a mutation that changes cleavage site specificity

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## ABSTRACT

In our previous attempt at *in vitro* selection of a *trans*-acting human hepatitis delta virus (HDV) ribozyme, we found that one of the variants, G10-68-725G, cleaved a 13 nt substrate, HDVS1, at two sites [Nishikawa, F., Kawakami, J., Chiba, A., Shirai, M., Kumar, P.K.R. and Nishikawa, S. (1996) *Eur. J. Biochem.*, 237, 712–718]. One site was the normal cleavage site and the other site was shifted 1 nt toward the 3'-end. To clarify the interactions between nucleotides around the cleavage site of the *trans*-acting HDV ribozyme, we analyzed the efficiency of the reaction for every possible base pair between the substrate and the ribozyme at positions -1 (-1N:726N) and +1 (+1N:725N) relative to the cleavage site using the genomic HDV ribozyme, TdS4(Xho), and derivatives of the most active variant, G10-68. These mutagenesis analyses revealed that the +1 base of the substrate affects the structure of the catalytic core in the complex with G10-68-725G, substrate and divalent metal ions, and it shifts the cleavage site. In a comparison with other variants of the *trans*-acting HDV ribozyme, we found that this cleavage site shift occurred only with G10-68-725G.

## INTRODUCTION

The genome of the human hepatitis delta virus (HDV) is a single-stranded circular RNA of ~1700 nt, which appears to replicate through a rolling circle RNA-to-RNA pathway, as do some plant pathogenic RNA viruses (1–3). Both genomic and antigenomic HDV RNAs have self-cleavage activity (ribozyme) in the presence of divalent metal ions, producing a 2',3'-cyclic phosphate and 5'-OH group (1–2) as do other known ribozymes, such as hammerhead and hairpin ribozymes. However, the primary sequence does not resemble those of the ribozymes of the latter two types and details of the reaction mechanism are unknown.

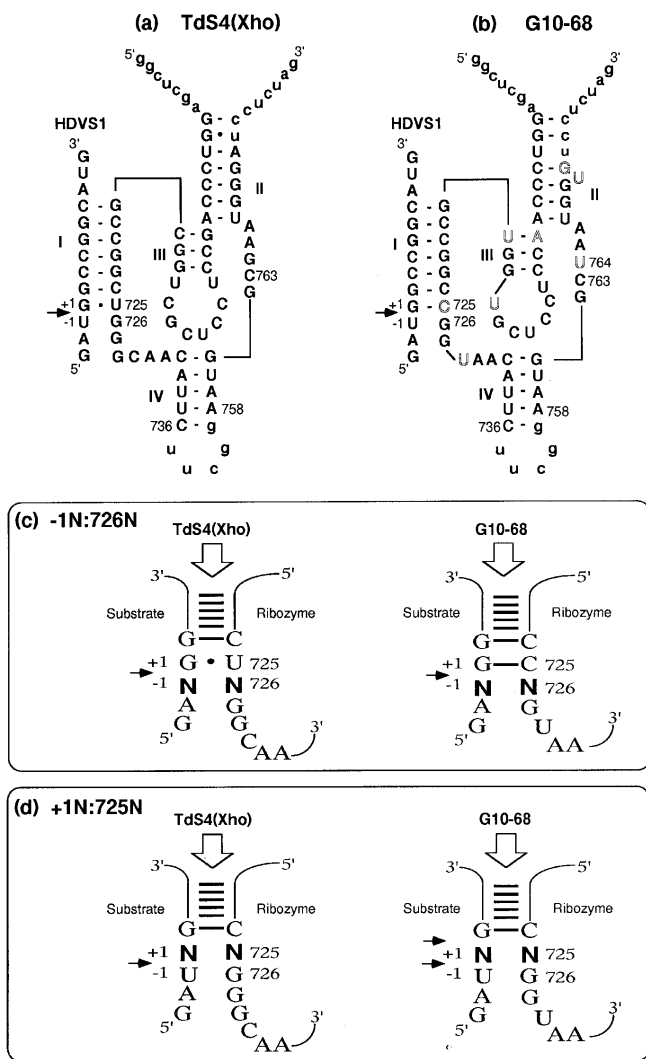
Several models of the secondary structure have been proposed for genomic and antigenomic HDV ribozymes (4–7). Many attempts have been made to understand the roles of different bases

and to define the structure of the catalytic core of the HDV ribozyme (reviewed in 8,9). We also constructed several variants with point and random mutations in single-stranded and stem regions (10–14). Results of further chemical probing studies (15) and an analysis of the interference by phosphorothioate substitutions (16) have elucidated important bases and phosphates. A model of the tertiary structure of the HDV ribozyme has been proposed based on the pseudoknot structure (17,18). It was recently suggested that this pseudoknot structure probably exists and functions *in vivo* (19). In attempts to design a *trans*-acting ribozyme capable of the site-specific cleavage of a substrate, the *cis*-acting HDV ribozyme has been truncated and divided into substrate and ribozyme portions (7,20–22).

In our previous *in vitro* selection study of *trans*-acting HDV ribozymes (23), we obtained a dominant clone, designated G10-68, from the random RNA pool based on the truncated *trans*-acting HDV ribozyme, TdS4(Xho). TdS4(Xho) is constructed from the original sequence by shortening stem IV, which is not important for activity (Fig. 1a). Further investigations indicated that the G10-68-725G variant, with a change at 725C to 725G in G10-68 (Fig. 1), cleaved the substrate at two sites and yielded two types of products. The new product was generated because the cleavage site was shifted to the +1 position relative to the original site due to the mutation. Several analyses involving the mutagenesis of the *cis*-acting HDV ribozyme around the cleavage site have been previously reported, but cleavage site shifting has not been studied thoroughly (8,24,25).

In this study, we focused on the base specificity at the cleavage site. To study the relationship between base pairing and the cleavage site shift, we constructed derivatives of the substrate (HDVS1) and the ribozyme (TdS4(Xho) and G10-68), and measured cleavage efficiency. We compared results for every combination of bases in the substrate and ribozyme at a position designated -1 (-1N:726N) and +1 (+1N:725N) relative to the normal cleavage site. In this study, we find that, in the case of the G10-68-725G ribozyme, the +1 base of the substrate determines the cleavage site specificity. We further find that this phenomenon is characteristic of the reaction catalyzed by this mutant ribozyme.

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**Figure 1.** Secondary structures of genomic *trans*-acting HDV ribozyme TdS4(Xho) (a) and a variant, G10-68 (b), and representations of mutations at cleavage sites, -1N:726N (c) and +1N:725N (d). Numbering is based on that of Makino *et al.* (32). Open letters indicate bases different from those in TdS4(Xho). Small letters do not originate from HDV. Both 5'- and 3'-sequences of small letters indicate vector derived sequences. To truncate the original stem IV, the wild-type sequence from nt 736 to 758 was changed to include UUCGG sequence by small letters. Arrows indicate cleavage sites. HDVS1 is a 13mer substrate.

## MATERIALS AND METHODS

### Synthesis of substrates and ribozymes

All oligonucleotides were synthesized on an automated DNA/RNA synthesizer (model 392 or 394; Applied Biosystems). DNA and RNA phosphoramidites were purchased from Glen Research. Products were purified as described in the user bulletin from ABI (no. 53; 1989) with minor modifications.

Substrates sequences (-1N HDVS1, +1N HDVS1) were as follows. -1N HDVS1, 5'-GA<sub>-1</sub>(A,U,C,G)GGCCGGCAUG-3'; +1N HDVS1, 5'-GAU<sub>+1</sub>(A,U,C,G)GCCGGCAUG-3'. All ribozymes were prepared by run-off transcription (AmpliScribe T7 transcription kit; Epicentre Technologies) of the appropriate

plasmids after digestion with *Xba*I, and purified on 8% polyacrylamide denaturing gel that contained 7 M urea.

### Mutants of *trans*-acting ribozymes [TdS4(Xho) and G10-68]

The *trans*-acting ribozymes TdS4(Xho) and G10-68 (Fig. 1) were constructed as described previously (23). Mutant variants were prepared using a system for oligonucleotide-directed *in vitro* mutagenesis (Amersham) after isolation of the single-stranded DNA. Primer sequences were as follows (mutated bases are underlined):

- G10-68-725N, d[GAATGTTACC(A.C.T)GCCGGCACCA];
- G10-68-726N, d[AGAATGTTAC(A.G.T)GGCCGGCACC];
- a-1, d(AATGTTGCCCCCGCCGGCGCCA);
- b-1, d(CCGAAGAATGCCCGCCGGCGCA);
- b-2, d(CCGAAGAATGCCCCCGCCGGCACCA);
- b-3, d(GAATGTTACCGCCGGCACCA).

### Cloning and vectors

Vector pUCT7 was a modified version of pUC118; it included the promoter for T7 RNA polymerase and a *Xho*I site at the *Eco*RI-*Bam*HI site (14). All experiments were conducted with *Escherichia coli* MV1184 as the host. Plasmid DNA was prepared from an overnight culture and purified with QIAGEN-tip 20 (QIAGEN).

### Cleavage activity of ribozymes

The 5'-end of each substrate was labeled with [ $\gamma$ -<sup>32</sup>P]ATP by T4 polynucleotide kinase (Takara). The cleavage reaction was conducted under ribozyme saturating (single-turnover) conditions as follows: 5 or 10  $\mu$ M ribozyme, 0.01  $\mu$ M substrate, 10 mM Mg<sup>2+</sup> in 50 mM Tris-HCl (pH 7.4) at 37°C. The reaction solution containing the ribozyme and substrate in Tris-HCl solution without MgCl<sub>2</sub> was denatured at 90°C for 2 min and then the solution was cooled on ice. The solution was then incubated at 37°C for 10 min and the reaction was initiated by addition of MgCl<sub>2</sub> solution. At appropriate times, aliquots of the reaction mixture were removed and the reaction was stopped by addition of an equal volume of stop solution (9 M urea, 50 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol) on ice. After electrophoretic fractionation on a gel that contained 20% polyacrylamide and 7 M urea, the radioactivity of the bands (substrate, 13 nt; product, 3 or 4 nt) on the gel was determined with a Bioimaging Analyzer (BAS2000; Fuji Film).

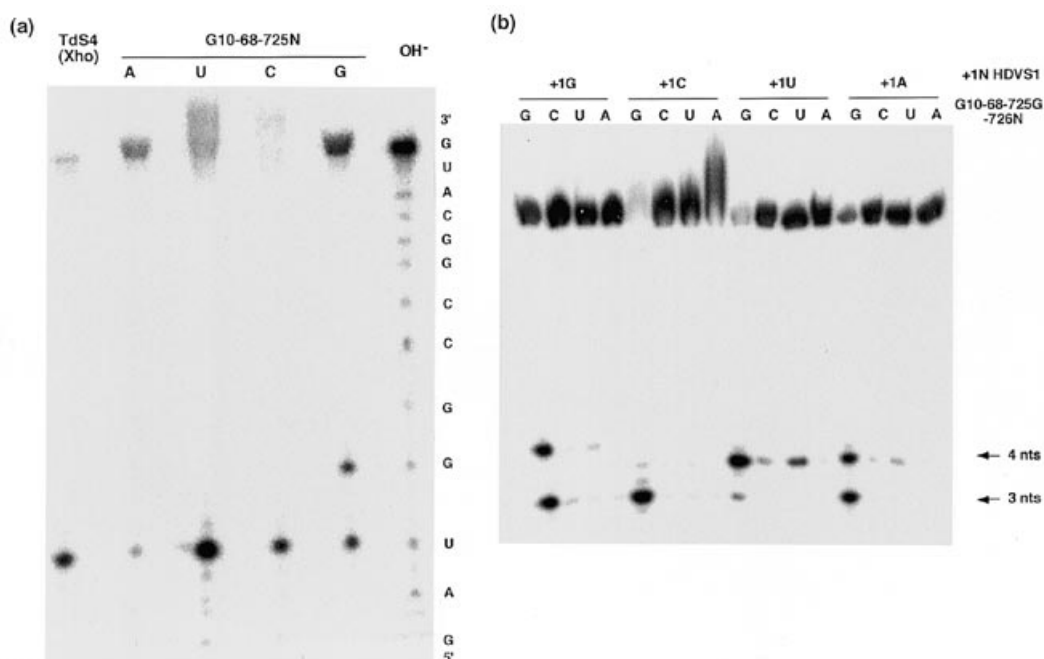
Cleavage activity was indicated by the rate of cleaved product formation. For kinetic analysis, we used a simple pseudo-first order equation: cleaved yield (%) = [EP] · (1 - e<sup>-kt</sup>) (Table 1) and experimental data were fitted to a curve (23). This is based on the assumption that under the excess ribozyme condition, substrates are saturated for the ribozyme at time zero. The reverse rate constants for the formation of active and inactive complexes are very much smaller than the rate constant for cleavage. Thus, substrate cleavage would proceed as a first order reaction. The cleavage reaction consists of two steps, namely conformational change and chemical reaction. From our kinetic analysis, we cannot conclude that *k*<sub>obs</sub> reflects either the conformational change or chemical step.

Alkaline hydrolysis ladders of HDVS1 were generated by incubating the 5' labeled HDVS1 in 50 mM NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> (pH 9.2) containing 1 mM EDTA and 5  $\mu$ g carrier tRNA for 9 min at 90°C. The partially digested HDVS1 was loaded as a size marker.

**Table 1.** Cleavage efficiency for base pair combinations that involve the -1 position

-1N HDVS1	Rz	TdS4(Xho)-726N					G10-68-726N				
		A	U	C	G	EP (%)	A	U	C	G	EP (%)
		$k_{\text{obs}} (\text{min}^{-1})$					$k_{\text{obs}} (\text{min}^{-1})$				
-1A		nd	nd	nd	0.430	[83]	nd	nd	nd	0.207	[66]
-1U		nd	nd	nd	1.90	[77]	nd	nd	<1 %	1.05	[81]
-1C		nd	nd	nd	0.674	[81]	nd	nd	<1 %	0.049	[61]
-1G		nd	nd	nd	0.092	[49]	nd	nd	nd	0.074	[69]

Ribozyme, G10-68-726N or TdS4(Xho)-726N; substrate, -1N HDVS1. [Ribozyme], 10  $\mu\text{M}$ ; [substrate], 0.01  $\mu\text{M}$ ;  $[\text{Mg}^{2+}]$ , 10 mM and 50 mM Tris-HCl (pH 7.4) at 37°C. Values in brackets are end points (EP) of reactions calculated from the pseudo-first order equation: fraction cleaved (%) =  $[\text{EP}] \cdot (1 - e^{-kt})$ . nd, no cleavage reaction was detected in 60 min. In cases of low cleavage activity, percentage cleavage after 60 min is shown.



**Figure 2.** (a) Cleavage of HDVS1 by ribozymes [TdS4(Xho), G10-68-725N] and the alkaline-generated ladders of HDVS1, as analyzed by 20% PAGE containing 7 M urea. (b) Cleavage of substrates (+1N HDVS1) by ribozymes (G10-68-725G-726N), as analyzed by 20% PAGE containing 7 M urea. Cleavage reactions were conducted under the standard conditions (see Materials and Methods) for 60 min and reaction products were subjected to denaturing PAGE. (b) The 4 nt product in lanes 1-4 (from the left) migrates higher than in other lanes, which is caused by the sequence difference and it was confirmed by the alkaline generated ladder of each substrate (data not shown).

## RESULTS AND DISCUSSION

The pseudoknot secondary structures of genomic and antigenomic HDV ribozymes consist of two stems (I, II), two stem-loops (III, IV) and three single-stranded regions in the *cis*-acting structure (5,13,17,26). Based on this pseudoknot secondary structure, the *trans*-acting HDV ribozyme TdS4(Xho) was designed (Fig. 1a) by separation of the structure into two molecules at the junction between stem I and II, and truncation of stem IV (23). A variant G10-68 (23) which was obtained by *in vitro* selection has several base changes compared with TdS4(Xho) (open letters, Fig. 1b). When we examined the effect of mutation on G10-68, we found that one of the variants (G10-68-725G), in which 725C was

changed to 725G, produced two cleavage products from a 13mer substrate, HDVS1. Cleavage patterns on a 20% polyacrylamide gel containing 7M urea are shown in Figure 2a. Cleavages by this ribozyme were observed at two sites, the original one and a site one base closer to the 3'-end, as was confirmed by partial alkaline hydrolysis of the substrate. This two site cleavage seemed specific to some sequences around the cleavage site. In the present study, to obtain more details about the base specificity around the cleavage site of the *trans*-acting HDV ribozyme, we modified TdS4(Xho), G10-68 and the substrate HDVS1 (Fig. 1; Materials and Methods) and examined their effect on cleavage reactions. Schematic representations of sites of cleavage of substrates by ribozymes are shown in Figure 1c and d.

**Table 2.** Cleavage activity for combinations that involve base pairs at the +1 position

Rz		$k_{\text{obs}}$ (min <sup>-1</sup> )				[% cleavage]
		G10-68-725N				TdS4(Xho)
+1N	HDVS1	725A	725U	725C	725G	725U
+1A		0.003 [9]	0.279 [55]	0.137 [63]	0.027 [32]	0.661 [66]
		*nd	*nd	*nd	*0.026 [22]	*nd
+1U		0.011 [35]	0.006 [18]	0.002 [<5]	<4	<2
		*nd	*0.003 [<7]	*<1	*0.094 [68]	*<1
+1C		<1	0.004 [11]	0.009 [<6]	0.516 [70]	<1
		*nd	*0.013 [<6]	*0.002 [<4]	*nd	*nd
+1G		0.008 [25]	0.890 [85]	1.05 [81]	0.011 [26]	1.90 [77]
		*nd	*nd	*nd	*0.011 [19]	*nd

Ribozyme, G10-68-725N or TdS4(Xho); substrate, +1N HDVS1. [Ribozyme], 10  $\mu\text{M}$ ; [substrate], 0.01  $\mu\text{M}$ ; [ $\text{Mg}^{2+}$ ], 10 mM and 50 mM Tris-HCl (pH 7.4) at 37°C. An asterisk indicates the 4 nt product (+1 shift in the cleavage site). Values in brackets show the percentage cleavage after 60 min.

### Changes in cleavage efficiency with various base pairs at the -1 position (-1N:726N)

The position designated -1N corresponds to the 5' base of the cleavage site and it is linked to stem I, in which 7 bp are required for cleavage by the HDV ribozyme (24). To clarify the specificity of this base and the site of action of the *trans*-acting HDV ribozyme, we investigated all combinations of base pairs between the -1N nucleotide in the substrate, HDVS1, and the ribozymes, TdS4(Xho)-726N and G10-68-726N (Fig. 1c). The cleavage efficiencies are summarized in Table 1. As clearly shown in Table 1, 726G in both the TdS4(Xho)-726N and G10-68-726N ribozymes is indispensable to cleavage reactions. In contrast, the -1N position in the substrate could accommodate any base, although the rate of cleavage differed among combinations of bases. This result closely agrees with that observed with the *cis*-acting HDV ribozyme (27). The cleavage rate was highest with the wobble base pair -1U:726G, followed by C:G > A:G > G:G in TdS4(Xho) and A:G > G:G > C:G in G10-68. In the case of G10-68, the +1 position forms a stable G:C Watson-Crick base pair. Thus, when stem I is extended by an additional canonical base pair (-1C:726G), the stem I structure becomes more stable, especially near the cleavage site, by changing from a wobble base pair to a Watson-Crick base pair. It is probably not suitable for local conformation of the cleavage site, as a result the rate of the cleavage reaction is reduced. The original sequence around the cleavage site (+1 or -1) has a G:U wobble base pair and this wobble base pair appears to be important for the cleavage reaction (27).

Experimental data for TdS4(Xho) demonstrated the low activity associated with -1G:726G, as reported for the antigenomic HDV ribozyme (8,24). A:G can easily form a wobble base pair (28), and G:G can also interact. However, steric hindrance reduces the efficiency of the cleavage reaction. In conclusion, the base (726N) opposite of the substrate base at the -1N site must be G. We previously reported that 726G was essential to the *cis*-acting HDV ribozyme as shown in a mutagenesis study (11). Our previous chemical probing analysis also indicated that N-7

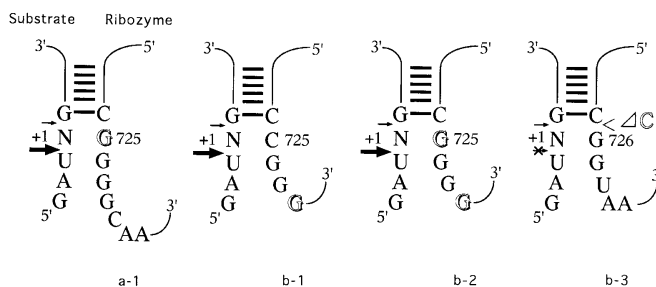
of 726G might be involved in some interactions with the substrate or other parts (15).

### Changes in cleavage efficiency with various base pairs at the +1 position (+1N:725N)

To clarify the base specificity and its effect on site-specific cleavage, we investigated all combinations between +1N of HDVS1 and G10-68-725N or TdS4(Xho) (Fig. 1d) in terms of the cleavage reaction rate (Table 2). In both the G10-68 and TdS4(Xho) ribozymes, the +1G:725U (original form) wobble base pair allows significant cleavage activity, as it does in the antigenomic HDV ribozyme (8,27). The activities of G10-68-725N with various substrates can be ranked as follows: (+1N:725N) G:Py > C:G > A:Py >> A:G > N:A, Py:Py, G:Pu >> U:G. A similar tendency was observed with wild-type TdS4(Xho): Pu:Py (G:U > G:C > A:U) >> Py:Py (U:U, C:U).

With the G10-68-725G variant, distinct cleavage reactions occurred at two neighboring phosphates—one at the original cleavage site and the other at an adjacent site—to yield products of 3 and 4 nt as a result of labeling the 5'-phosphate. With the +1U:725G wobble base pair, the 4 nt product was generated almost exclusively (Fig. 2b, 9th lane from the left). In contrast, the stable C:G Watson-Crick base pair generated only a 3 nt product (Fig. 2b, lane 5). In the case of the (G, A):G base pair, both products were produced in equal amounts (Fig. 2b, lanes 1 and 13). It is noteworthy that only one base, +1N, affected the environment around the cleavage site and caused a +1 shift in this site.

The results on the effect of the -1 position (-1N:726G) in Table 1 indicated that the counter base on the 5' side of the cleavage site, 726G, was essential to normal cleavage (3 nt product). Was this observation allowed for in the G10-68-725G-726N variant? When the 4 nt product was generated (+1N:725G), the counter base of +1N was settled to 725G. Whereas when a 3 nt product was produced (-1U:726N), the counter base of -1U should be 726G. The results shown in Figure 2b confirm this hypothesis. For normal cleavage by the *trans*-acting HDV ribozyme, 727G is also important (data not shown), as observed in the *cis*-acting HDV ribozyme (11). The predicted duplex stability of stem I,



**Figure 3.** Comparison of secondary structures of ribozyme derivatives with changed SSrA (I–IV junction) sequence that catalyzed two cleavage reactions and HDVS1 +1N that formed stem I. All variants are based on TdS4(Xho) and G10-68. a-1, the 725 substituent of TdS4(Xho); b-1, a G10-68 variant possessing the antigenomic-like junction sequence; b-2, the 725 substituent of b-1; b-3, the 725C deletion mutant of G10-68. Arrows indicate cleavage sites and their sizes represent the relative efficiencies of cleavage reactions. Open letters indicate changed nucleotides from original sequences (see the text). Junction sequences between stems I and IV are shown for the *trans*-acting HDV ribozyme.

which contains the terminal base pair +1N:725G, is not significantly altered. Calculations of the free energy ( $\Delta G^{\circ}_{37}$ ) of the duplex stability with various base pairs (for +1N:G) yielded  $-12.8$  (U:G),  $-13.1$  (G:G or A:G) and  $-13.2$  kcal/mol (C:G) (29).

Our thiophosphate modification interference experiment (16) suggests that the phosphate at +1 position is involved in both the catalytic core as well as the cleavage phosphate. At this moment, the mechanism of cleavage shift by G10-68-725N is not yet clear, but at least in the case of the normal Watson–Crick base pair at (+1N:725N), 3 nt products were always obtained (Table 2). This suggests that non-Watson–Crick base pairs including wobble base pairs at the +1 position might have some key role for the cleavage site shift.

### Comparison with other ribozymes

In both genomic and antigenomic HDV ribozymes, this type of shift of cleavage site observed with G10-68-725G has not been reported. To evaluate the site specificity of this reaction, we examined interactions between +1N HDVS1 and other variants of HDV ribozymes (a-1, b-1, b-2 and b-3) as shown in Figure 3 and Table 3. The derivative ribozyme a-1, the 725G substituent of TdS4(Xho), cleaved the substrate normally, although the rate of cleavage was low compared with that of TdS4(Xho). Cleavage efficiency was ranked as follows, +1C:725G (C:G) > G:G > A:G >>> U:G. The second derivative, b-1, is an antigenomic-like mutant of G10-68, in which the junction region between stems I and IV (SSrA) is shortened to GGG (5). G10-68 has a great homology with antigenomic HDV ribozymes other than SSrA, that is the same stem III, loop III is of a similar length and sequence except with a U at its 3'-end instead of C; junction II–IV is of similar sequence lacking only the extra G at its 3'-end (23). With Py:Py, a +1 shift in cleavage was observed but product yield was very low. The third derivative, b-2, is the 725G mutant of b-1. b-1 had high cleavage activity at the normal cleavage site. b-2 (C:G) was also active at the normal cleavage site, but with (U:G) two cleaved products (3 and 4 nt) were generated with low yields. The fourth derivative b-3 lacks 725C of G10-68; it has 6 bp in stem I and the same junction sequence between stems I and IV as G10-68. This derivative had no cleavage activity. Even though

**Table 3.** Comparison of cleavage efficiencies by various derivative ribozymes

+1N HDVS1	Rz	a-1	b-1	b-2	b-3
		% cleavage	% cleavage	% cleavage	% cleavage
+1A		9	79	10	nd
		* << 1	* nd	* < 1	* < 1
+1U		<< 1	< 1	3	nd
		* < 1	* 4	* 6	* 2
+1C		27	5	84	nd
		* nd	* 5	* nd	* nd
+1G		13	87	7	nd
		* nd	* nd	* nd	* nd

Ribozymes, a-1, b-1, b-2 and b-3; substrate, +1N HDVS1; [ribozyme], 5  $\mu$ M; [substrate], 0.01  $\mu$ M; [ $Mg^{2+}$ ], 10 mM and 50 mM Tris–HCl (pH 7.4) at 37°C. The percentage cleavage after 60 min is shown. An asterisk indicates the 4 nt product (+1 shift in the cleavage site).

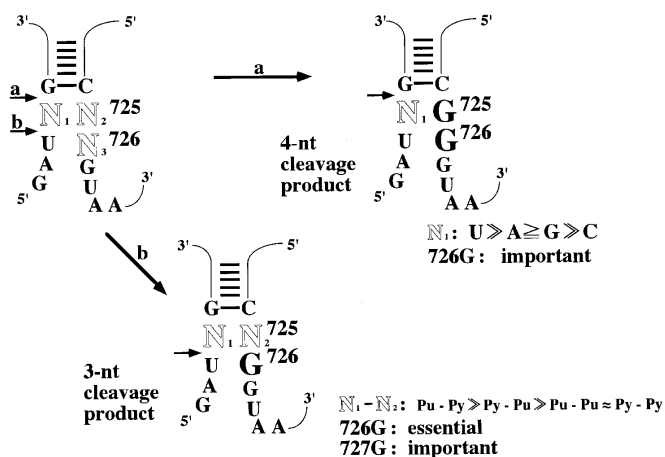
the yields were very low, a shift of cleavage was observed only in the non-Watson–Crick base pairs at (+1N:725G/C), as described in the previous section (Table 2).

If the +1 shift in the cleavage site can be explained by the structures of base pairs at the –1 and +1 positions with  $725G726G$ , any HDV ribozyme that contains  $725G726G$  may be able to cleave at a shifted site. However, this remarkable change in the cleavage site was observed only with the G10-68-725G variant out of several variants. This result suggests that the newly formed  $5'-725GGGAAA-3'$  in the single-stranded region between stems I and IV may play an important role in the catalytic core of the +1 shift cleavage. The size of the catalytic core around the cleavage site with bound divalent metal ions, which is affected by the conformation of base pairs at the –1 and +1 positions and the length or sequence of the junction between stems I and IV, must also influence the rate and site of cleavage.

For both genomic and antigenomic HDV ribozymes, the residue 763C in junction II–IV is essential to cleavage activity (11,17). In addition, cleavage analysis by  $Pb^{2+}$  ions suggested that the 3' phosphate of 763C is close to the catalytic core (30) and this corresponds with our results (16,33; Jeoung *et al.*, unpublished results). From these points of view, we conducted the mutational studies with G10-68-725G at residues 763C and 764U, because they are very close to the catalytic site in the 3-D models, especially 763C (17,18,31). With any +1N HDVS1, 763C was essential. The cleavage role of G10-68-725G was devoted to any base at 764, except the combination of +1U:725G and 764C, which produced two cleavage products in low yield, but the 4 nt product was not dominant (data not shown). These results suggest that 763C is probably very close to the catalytic site in the case of G10-68-725G but base 764 does not affect the cleavage site environment between the substrate and G10-68-725G.

### Comparison of rates of cleavage by G10-68-725G at different concentrations of $Mg^{2+}$ ions and with other divalent metal ions

The exact role and binding site of divalent metal ions have not been clarified in HDV ribozymes. If the metal binding site is located in the catalytic core, it may be possible to detect differences in the characteristics of cleavage due to differences in



**Figure 4.** Schematic representation of cleavage routes that produced 3 and 4 nt products. Ribozyme, G10-68-725G-726N; substrate, +1N HDVS1. Reaction conditions were the same as those described in legends of Tables 1 and 2.

the site of metal ions when a minor structural change is introduced by +1N to the catalytic core.

We analyzed the effects of different metals and concentrations of  $Mg^{2+}$  ions under the same cleavage reaction conditions as described in the Materials and Methods (30 min incubation with metal).  $Mg^{2+}$  ions were tested in 1, 10, 50 and 100 mM but the characteristic patterns of cleavage by G10-68-725G did not change under any concentration. The 4 nt product yield remained unchanged above 10 mM  $Mg^{2+}$  ions, but the level of the 3 nt product increased (by 20–40% in 100 mM) above that at 10 mM  $Mg^{2+}$  ions in +1A/G HDVS1.

$Mg^{2+}$  and  $Mn^{2+}$  ions yielded the same cleavage patterns. With  $Ca^{2+}$  ions, +1U:G did not produce the 4 nt product predominantly but two cleavage products were generated as observed with +1G:G or +1A:G. Pauling's ionic radius of  $Ca^{2+}$  ions (0.99 Å) is larger than that of  $Mg^{2+}$  ions (0.65 Å). Thus, it may be harder for  $Ca^{2+}$  ions to fit in the metal binding pocket, and the specificity of  $Ca^{2+}$  ions would be expected to be lower than that of  $Mg^{2+}$  ions.

The characteristic shift in the cleavage site was also confirmed within the *cis*-acting HDV ribozyme upon conversion from *trans* to *cis* [+1U:725G,  $k_{obs} = 1.54/\text{min}$  (+1 shift); +1A:725G, 0.899/min (normal), 0.826/min (+1 shift); +1G:725G, 0.240/min (normal), 0.240/min (+1 shift); +1C:725G, 8.54/min (normal)]. It is of interest that changes at the +1 base caused a shift in the reaction site in the mutual relationship among G10-68-725G, the +1N substrate and the metal. Further studies such as NMR spectra are expected to produce information about base interactions around the cleavage site, including the metal ion site.

## CONCLUSIONS

In studying the base specificity of the *trans*-acting HDV ribozyme cleavage site, we introduced base substitutions at the +1 position (+1N:725N), the -1 position (-1N:726N) and SSrA region (the junction region between stems I and IV) of TdS4(Xho) and G10-68. In the characteristic cleavage patterns by the G10-68-725G ribozyme (Fig. 4), the pathway that produces each product is shown (absolute rates of cleavage not shown). Clearly, (i) 726G, the counterpart of the base at the cleavage site is essential; (ii) as the next 3' base, 727G is favoured for cleavage; and (iii) the G10-68-725G ribozyme shifts the cleavage site of the

substrate because of a change in the +1 base. This phenomenon appears to be unique to the G10-68 variant that was obtained by selection *in vitro*.

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