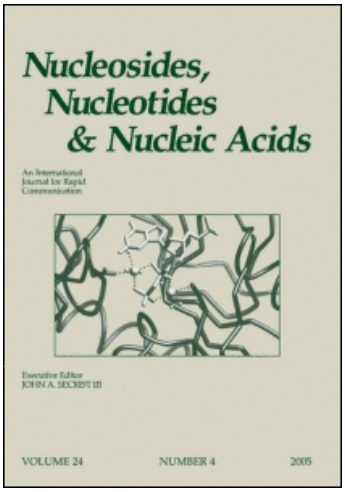

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Properties of Antigenomic Hepatitis Delta Virus Ribozyme Cis- and Trans- Analogs

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ABSTRACT

A series of permuted variants of antigenomic HDV ribozyme and trans-acting variants were constructed. The catalytic activity study of the ribozymes has shown that all the variants were capable of self-cleaving with equally biphasic kinetics. Ribonuclease and Fe(II)-EDTA cleavage have provided evidence that all designed ribozymes fold according to the pseudoknot model and the conformations of the initial and cleaved ribozyme are different. A scheme of HDV ribozyme self-cleavage reaction was suggested. The role of hydrogen bonds in the reaction was evaluated by substitution of ribose in the ribozyme for deoxyribose. It was found that the 2′-OH group of U23 and C27 is critical for the reaction to occur; the 2′-OH group of U32 and U39 is important, while 2′-OH groups of other nucleotides of loop 3, stem 4 and stem 1 are unimportant for the cleavage activity.

Key Words: HDV ribozyme; Connectivity isomers; Ribo/deoxy substitutions.

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INTRODUCTION

In the genomic and antigenomic RNAs of hepatitis delta virus (HDV) found RNA sequences (ribozymes) are capable of site-specific self-cleavage under physiological conditions.[1,2] The proposed functional parts of the ribozyme associated with its secondary structure are: five helices (*s1-s4* and *s1.1*), two loops (*l3* and *l4*) and three

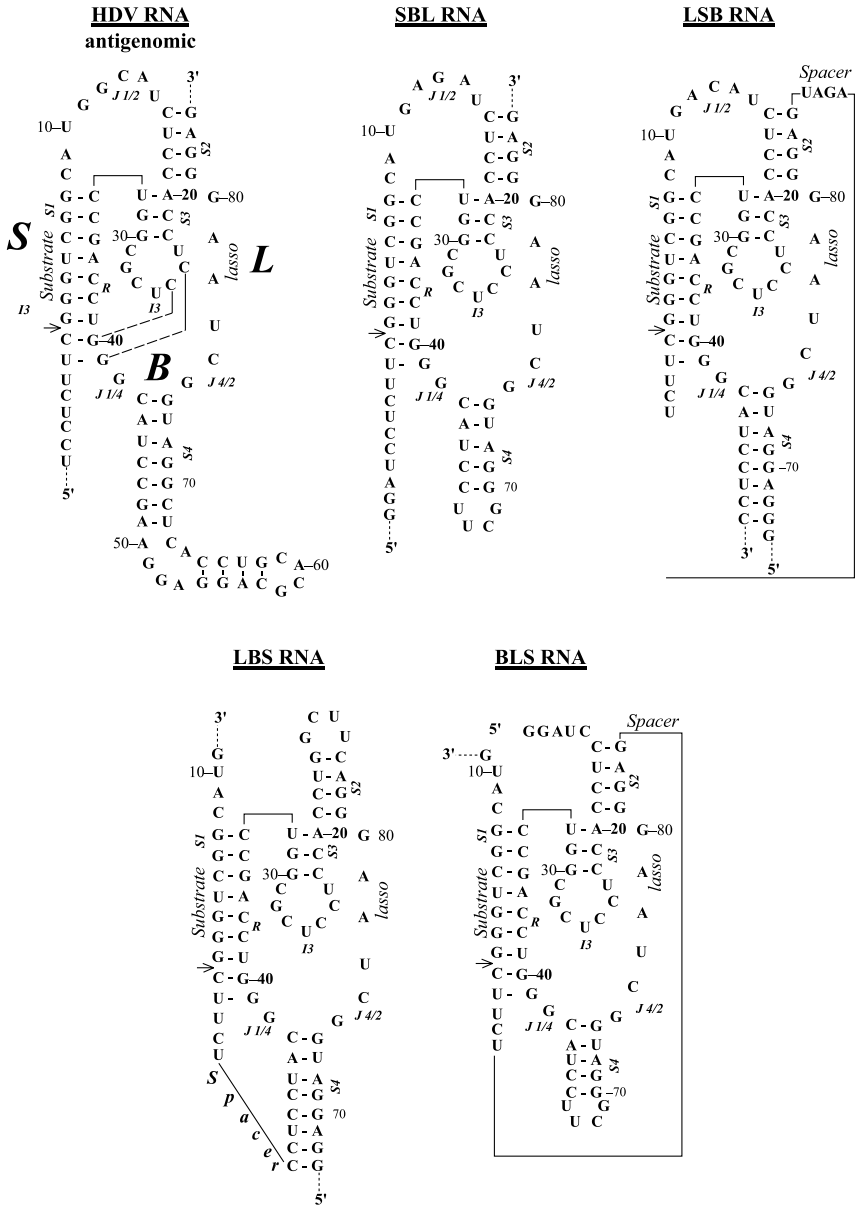

Figure 1. Nucleotide sequence and proposed secondary structure of antigenomic HDV ribozyme variants: natural ribozyme, its permuted variants SBL, LSB, LBS and BLS.

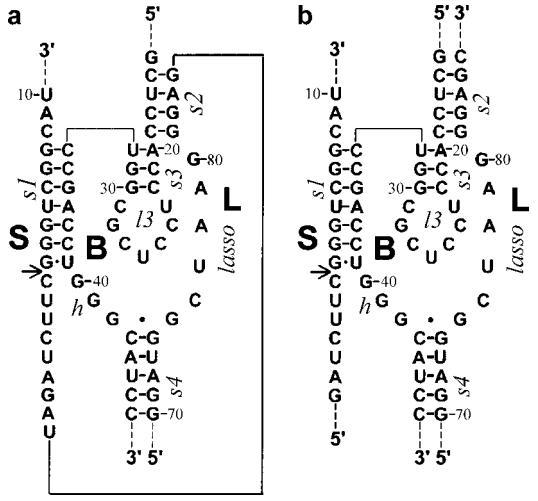

Figure 2. Nucleotide sequence and proposed secondary structure of antigenomic HDV ribozyme trans-variants: bimolecular LS:B (a) and trimolecular L:S:B (b).

joining regions (*J1/2, J1/4* and *J4/2*).[3] They can be combined in three subchains: Substrate chain (S), Substrate binding chain (B) and the Lasso (L) (Fig. 1). The fragments can associate by base pairing in *s1, s2* and *s4* to generate the cleavable structure. The S, B and L subchains can be left unconnected (thus forming three-chain trans-ribozyme) or joined in an arbitrary order forming either two-chain trans-ribozymes of different connectivities (Fig. 2), or several single-chain cis-ribozymes of different connectivities (SBL, LSB, BLS, LBS, Fig. 1). Any two of the S, B, L chains can be joined in an arbitral combination using an appropriate joining oligonucleotides spacer. Being mixed with the third part, they form an active two-chain trans-ribozyme. Recently we have undertaken a thorough kinetic study of different noncirculary permuted variants of the ribozyme: three-chain, two-chain and single-chain with different connectivities of the parts.[4–6] It was found that all of them were capable of self-cleavage, being almost equally active under optimal spacer and media conditions. The self-cleavage reaction was found to be irreversible with equally biphasic kinetics.

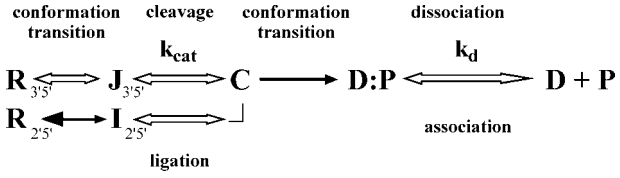

Figure 3. Schematic representation of HDV ribozyme self-cleavage reaction. R, original ribozyme; J, ribozyme capable of self-cleavage but with the intact phosphodiester bond; C, ribozyme with the cleaved phosphodiester bond; D:P, two-component ribozyme form capable of dissociation; D + P, dissociated ribozyme components; D and P, long and short self-cleavage products; I, a part of the ribozyme inactivated during incubation. Wide and narrow arrows indicate the fast- and slow-running processes, respectively.

Different ways of reaction initiation, kinetics under suboptimal conditions, and jumpwise change in reaction conditions from nonpermissive to optimal have been used, and the temperature dependence has been studied. Different ways for producing nonpermissive conditions were employed: low pH value, very high or low temperatures, high concentration of formamide to start reaction with deliberately different ribozyme conformations far from the optimal for the reaction initiation. Partial restoring of the initial reaction characteristics has been shown by the reinitiation of reaction stopped after completing of the first phase. On the basis of all findings, the following scheme of the self-cleavage reaction is proposed (Fig. 3): 1) activation of the ribozyme with energy of 40–50 kcal/mol and the characteristic time of several deciminutes at optimal reaction conditions; 2) fast and reversible reaction of the phosphodiester bond cleavage (cleavage with formation of 2′3′ cyclophosphate and ligation); 3) the ligation reaction leading to isomerization of the 3′,5′-phosphodiester bond to the 2′,5′ bond in the self-cleavage site with the characteristic reaction time of tens of minutes; and 4) practically irreversible conformational change leading to

Table 1. Catalytic activity of HDV ribozyme variants.

	#	Linker between subfragments	k1 (1/min.)	EP (%)	Ea kcal/mol	Tm (°C)
50′C			Linker between B & S			
LBS	5	G	0			
	1	GUAGA	0.39 ± 0.2	63 ± 10	43 ± 4	246 ± 2
	4	GAAU	0.59 ± 0.3	62 ± 6		
	2	GUUGAGC	1.20 ± 0.5	45 ± 3		
	3	GAAUUC	2.45 ± 0.3	28 ± 3	36 ± 4	37 ± 2
60′C						
LBS	5	G	0			
	1	GUAGA	0.9 ± 0.3	55 ± 4	43 ± 4	46 ± 2
	4	GAAU	0.5 ± 0.2	62 ± 6		
	2	GUUGAGC	0.8 ± 0.5	54 ± 3		
	3	GAAUUC	2.4 ± 0.3	34 ± 3	36 ± 4	37 ± 2
60′C			Linker between L & S			
BLS	1	UUGAGC	0.12 ± 0.02	53 ± 4		54 ± 3
	2	UAGA	0.27 ± 0.02	44 ± 4		50 ± 2
	3	AAU	0.05 ± 0.01	51 ± 3		
	4	AAUC	0.13 ± 0.01	46 ± 3		
	5	UCGAC	0.39 ± 0.2	43 ± 6		
	6	C	0.007 ± 0.002	80 ± 20		
50′C			2 Linkers between L & S and S & B			
LSB		UAGA & CAUGACAU	1.02 ± 0.02	17 ± 1	40.5 ± 1	56.5 ± 0.5
50′C			Linker between S & B			
SBL		CAUGACAU	1.25 ± 0.1	25 ± 2	50 ± 2	37 ± 2

k1, the apparent constant of the first reaction phase; EP, a part of ribozyme molecules, whose cleavage rate is not subjected to the first order kinetics of the initial reaction phase; Ea, activation energy of the reaction; Tm, temperature of half-maximum of reaction rate.

fixation of the cleavage due to immobilization of the 5′-terminal nucleotide of the product in the center of the formed structure and displacement of the 3′-terminal nucleotide to the periphery. The latter process has the characteristic time of tens of minutes and relatively low activation energy. 5) Product dissociation,which is being highly different in different permuted variants, does not practically influence the overall reaction efficiency.

Overall reaction parameters are given in Table 1 for different connectivity isomers of the ribozyme. Data obtained are not sufficient to estimate values of all intrinsic reaction constants for the scheme shown in Fig. 3, but it is easy to find several sets of the constants that help to fit the experimental data into the model. As a result of the study it was suggested that the reaction proceeds through at least 3 states different in structure: pre-reactive, reaction competent and post-reactive. We have made ribonuclease and Fe(II)-EDTA cleavage assays for prereactive and postreactive states for all different connectivity isomers LBS, BLS, SBL and LSB as well as their partial complexes SB, LB. We have concluded that the structure of pre-reactive and post-reactive state of the ribozyme are very different with respect to *s1.1, s2* and *s3* stems. Similar conclusions were made on the basis of different experimental approach in Ref. [7].

In an attempt to define the essential structural elements required for activity and spatial organization of HDV ribozyme we have constructed several trans-variants of antigenomic HDV ribozyme with a series of 2′-OH replacements for 2′-H within the numerous elements of the ribozyme. Here we present short summary of our recent study of HDV ribozyme analogs.

RESULTS AND DISCUSSION

To analyze the role of 2′-hydroxyl groups, deoxynucleotides were incorporated both in the LS and B ribozyme fragments (Table 2). The LS:B trans-ribozyme was selected among other possible trans-ribozymes for chemical modification study due to the high stability of the complex between LS and B chains under all reaction conditions (up to 90°C). The self-cleavage product of this ribozyme is also sufficiently stable under the reaction conditions to provide ''single-turnover situation'' without the necessity of using a large excess of unlabeled components of the reaction. As a consequence one can treat the trans-ribozyme as its cis-analog using the formal two-term pseudo-first-order reaction equation:

$$A/Ao = EP*\exp(-k2*t) + (1 - EP)*\exp(-k1*t)$$

Here we summarize briefly the data obtained with ribo/deoxy substitutions in some selected parts of the ribozyme. Together with the similar data of Nishikawa et al.[8] and Fiola and Perreault[9] our deoxy modifications cover almost all nucleotides of the ribozyme. Comparing the results obtained by analysis of the reaction kinetics, we considered a two-fold difference of the reaction constants as meaningless because this difference can be readily attributed to minor changes in the reaction conditions. Indeed, experimental systems used in different studies differ not only in reaction conditions but in the structure of the molecule. The major difference is that the S:BL trans-ribozyme

Table 2. Deoxyribonucleotide derivatives of LS:B ribozyme.

	(1-EP)*k1		EP			k1 (1/min.)		
Original B	0.84	100%	0.4	±	0.08	1.4	±	0.7
Modifications in loop */3*								
dU23	0.039	5%	0.85	±	0.1	0.25	±	0.01
dT23	0.018	2%	0.79	±	0.2	0.09	±	0.00
dC24	0.14	17%	0.63	±	0.1	0.38	±	0.6
dC25	0.55	65%	0.55	±	0.1	1.21	±	1.4
dU26	0.43	50%	0.50	±	0.1	0.9	±	0.8
dT26	0.045	5%	0.77	±	0.1	0.20	±	0.10
dC27	0.030	4%	0.80	±	0.0	0.15	±	0.02
dG28	1.26	150%	0.42	±	0.1	2.16	±	1.4
dC29	0.106	13%	0.49	±	0.1	0.21	±	0.16
Modifications in *J1/4*								
dG40	0.80	96%	0.45	±	0.06	1.46	±	0.53
dG41	0.63	75%	0.47	±	0.07	1.18	±	0.57
dG42	0.91	108%	0.47	±	0.05	1.71	±	0.72
dG40dG41	0.35	42%	0.59	±	0.03	0.87	±	0.23
Modifications in stems *s3* and *s1*								
dU32	0.01	1%	0.97		–	0.27		
dU39	0.19	22%	0.56	±	0.07	0.43	±	0.20
Modifications in *J4/2*								
dG75	0.006	1%	0.35			0.01		
dC76	0.062	7%	0.54	±	0.05	0.13	±	0.04
Multiple modifications in stems *s1*, *s2* and *s4*								
dN15-dN19	0.060	7%	0.55	±	0.11	0.13	±	0.05
dN43-dN47	0.321	38%	0.45	±	0.06	0.58	±	0.21
dC33, dC34, dC37, dC38, dN43-dN47	0.303	36%	0.41	±	0.03	0.52	±	0.09
dG35, dA36	0.35	41%	0.44			0.62		
dG35, dA36, dN43-dN47	0.00	<1%						
dN43-dN47, N70-N74, N81-84,J2/1	0.54	64%	0.20	±	0.1	0.68	±	0.1
N70-N74, N81-84,J2/1	0.39	46%	0.29	±	0.1	0.55	±	0.1
dN15-dN19, N70-N74, N81-84,J2/1	0.03	3%	0.02		0.02	0.03		0.02

k1 and EP as in Table 1; (1-EP)*k1 is initial rate of reaction.

is composed of loosely bound S and BL components, however, the components of our B:LS trans-ribozyme do not dissociate and even do not exchange their parts at temperatures up to 90°C. Thus neither ribo/deoxy substitutions influenced the assembly of our ribozyme.

Kinetic analysis of these ribozyme variants has demonstrated a slight reduction in k1 caused by substitution of the 2′-OH group for nucleotides (nt) C25 and U26 of loop *l3* and all nucleotides of *J1/4* (Table 2). A marked reduction in catalytic activity has been noticed when C24 and C29 were replaced by deoxy derivatives of nucleotides. A drastic reduction was produced by substitutions U23/dU23 and C27/dC27 in l3,

U32/dU32 at the top of *s3* and U39/dU39 in *s1*. The substitution G28/dG28 exhibited increased cleavage activity. U26 was insensitive to 2′ oxygen removal but the ribozyme was almost inactive when a methyl group were introduced in 5th position on the base. (Table 2, U26/dT26). In loop *l3* we observed the highest effect at U23 which agrees with Ref. [8] and contradicts Ref. [9] who have found U26 to be the most sensitive to ribo/deoxy substitution in this region.

Since stems *s2* and *s4* were proposed to play solely structural roles, one could expect that the nucleotides in both stems can be replaced with their deoxy analogs without marked influence on the self-cleavage reaction. In agreement with Ref. [9], complete replacement of ribonucleotides by the corresponding deoxy derivatives in *s4* (nt 43–47 and 70–74) does not influence self-cleavage reaction efficiency. The downstream strand of *s2* (nt 81–84) and joining sequence of *s2* and *s1* (nt 85–89 and −5 and −3) can also be modified without a marked decrease in the reaction efficiency. At the same time incorporation of deoxynucleotides in the upstream strand of *s2* (nt 15–19) decreased the reaction efficacy by at least one order of magnitude in contrast to the data.[9]

In agreement with data Ref. [8] and [9] C76 and particulary G75 for our ribozyme were very sensitive to 2′-oxygen removal, while none of the three nucleotides of *J1/4* (nt 40–42) was responsive to the modification. The last finding agrees with Ref. [9] but contradicts Ref. [8].

Modification of *s1* led us to results partially conflicting with the data of Ref. [9]. Deoxynucleotides at 35th and 36th positions together with 43–47th of *s4* caused inactivation of our ribozyme while deoxy- substitution at nt 33, 34, 37 and 38 also in combination with 43–47th of *s4* caused a two-fold decrease in the reaction rate. However, deoxy- groups at nt 35 and 36 without modifications in *s4* exert minor influence on the reaction efficiency. By contrast, the U39/dU39 mutation, which is almost neutral according to Ref. [9], decreased our ribozyme activity five-fold (Table 2).

To sum up the results obtained by ribo/deoxy substitution study we need to rationally explain the apparently conflicting data for numerous modifications. In our opinion the reason is in the use of a formal pseudo-first-order reaction equation for a multi-step process devoid of a definite rate limiting reaction step. In this case there is no intrinsic reaction constant that may be associated with any formal reaction constant.

We consider the ribozyme self-cleavage reaction as multi-step process passing through at least three different conformations. At least one of the conformational transition is temperature-sensitive and requires activation energy in the scale of 20–40 kcal/mol. Both Tm value and the activation energy depend on the structure of the original molecule (Table 1). All three conformations may involve 2′-OH groups of different nucleotides at different steps of the reaction. Thus, even if the reaction proceeds under different conditions (or different spatial organization) through the same conformational steps the final apparent reaction constants of the formal pseudo-first-order reaction equation may be differently sensitive to the modification, unless the modification completely blocks the process. We thus conclude that the 2′-OH groups of G75, C76, G30 and U32 have a crucial role in the cleavage reaction or stabilization of the active conformation. It seems probable that 2′-OH groups of nt 35 and 36 of *s1*, 23, 26 and 27 of l3, 22 of *s3*, 15–19 of *s2* and 78, 79 of *J4/2* participate in stabilization of any of the three conformations of the ribozyme through which the reaction proceeds but the bonds that they form are dispensable for the reaction until they are not modified

simultaneously. It seems that all other ribonucleotides of the ribozyme can be replaced with deoxynucleotides without marked influence on the reaction rate.

EXPERIMENTAL SECTION

Synthesis of the cis-ribozyme analogs and analysis of self-cleavage activity was done as previously described.[5] Synthesis, deprotection and purification of trans-ribozyme analogs was performed using solid-phase phosphite triester approach as described in Ref. [10].

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