Constructing an efficient *trans*-acting genomic HDV ribozyme

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Abstract We have engineered a genomic HDV ribozyme to construct several *trans*-acting ribozymes for use in *trans* to cleave target RNAs. Among the 10 different combinations attempted, only HDV88-Trans had cleavage activity on the 13-nucleotide substrate, R13, in vitro. To improve the cleavage efficiency, at least in vitro, of the HDV88-Trans ribozyme ($k_{clv} = 0.022$ min^{-1}), we have constructed several variants that differ in forming stem II (length) in the pseudoknot secondary structure model. When cleavage rate constants were analyzed and compared among variants of HDV88-Trans, HDV88-Trans-4 yielded $k_{clv} = 1.7$ min^{-1}. HDV88-Trans-4 thus represents the highest active genomic HDV ribozyme that functions in *trans* thus far constructed, and has activity under physiological conditions (pH 7.1 at 37°C with 1 mM of $MgCl_2$).

Key words: HDV ribozyme; Pseudoknot structure; Ribozyme, *trans*-acting

1. Introduction

Genomic and antigenomic hepatitis delta virus (HDV) RNAs have self-cleavage activity both in vivo and in vitro. As observed with hammerhead and hairpin ribozymes, the self-cleavage activity of HDV ribozymes requires divalent cations and results in products with 2′,3′-cyclic phosphate and 5′-OH group at their cleavage sites. These ribozymes are completely dissimilar, however, in primary and secondary structures. It has been reported that both antigenomic and genomic ribozymes play a role in RNA processing during replication of the human hepatitis delta virus [1] and HDV RNA may thus represent the first clear example of a ribozyme that, in its natural form, functions in human cells.

Several models of its secondary structure have been proposed [2]. To elucidate the functional structure of the HDV ribozyme and to evaluate the role of bases, we have used in vitro mutagenic analyses [3–5] and experiments with chemical probes [6]. Our results indicate that important bases lie within three single-stranded regions (SSrA, 726-731; SSrB, 762-766; and SSrC, 708-715 in Fig. 1A), and these can be drawn in a pseudoknot-like model of the secondary structure. Ribozymes are expected to form functional antisense reagents and, thus, to cleave target RNAs, *cis*-acting (intramolecular) ribozymes were dissected into substrates and enzymes. Earlier, efficient *trans*-acting hammerhead and hairpin ribozymes were constructed and showed that they function very efficiently both in vitro and in vivo [7]. Similarly, both genomic and antigenomic HDV ribozymes have been dissected into substrates and enzymes, but not yet analyzed as extensively as hammerhead and hairpin ribozymes. To increase their activity, non-physiological conditions, such as high temperature and denaturants, have been used in reaction conditions.

In the present study, we engineered the genomic HDV ribozyme to construct several *trans*-acting ribozymes to use in *trans* to cleave target RNAs. We succeeded in increasing their activity under physiological reaction conditions.

2. Materials and methods

2.1. Oligonucleotides

Oligonucleotides were synthesized by the phosphoramidite method using a DNA/RNA synthesizer [model 392; ABI]. All reagents necessary for DNA and RNA synthesis were obtained from ABI, ABN and Glen Research. Purification, quantitative analysis and 5′ end labels of these oligonucleotides were as described elsewhere [5].

2.2. Preparation of trans-acting genomic HDV ribozymes

Vectors containing the sequence of mutated genomic HDV ribozyme shown in Fig. 2 were prepared using mutagenesis primers d(GAGGAGGCTGGGACCTCGAGCCTATAGT) for separation A (pUHD88-*trans*), d(GCGAGGAGGCTGGGCTCGAGCCTATAGT) for separation B, d(GCCAGCGAGGAGGCCTCGAGCCTATAGT) for separation C, d(CCAGCCGGCGCCAGCCTCGAGCCTATAGTG) for separation F, d(GTTGCCCAGCCGGCCTCGAGCCTATAGT) for separation G and H, d(GCCATTACCGAGGGGCTCGAGCCTATAGTG) for separation I and J with pUHD88 (for separation A, G, I and J) or pUHD88DI3 (for separation B, C, F and H) as templates. Ribozymes of separation D (52 nts) and E (45 nts) were synthesized chemically. Derivatives of HDV88-*trans* were prepared by using d(TAGGCTCGAGTCCCAGCC) for Trans-0, d(TAGGCTCGAGGTCCCAGCC) for Trans-1, d(TAGGCTCGAGTGGTCCCAGCC) for Trans-2, d(TAGGCTCGAGATGGTCCCAGCC) for Trans-3, d(ATTACGAATTCTAATACGACTCACTATAGGAATCTCCCAGCCTCCTCG) for Trans-4 and d(ATTACGAATTCTAATACGACTCACTATAGGATCTCCCAGCCTCCTCG) for Trans-5 from pHDV86 (for Trans-0, -1, -2 and -3) or pUHD88 (for Trans-4 and -5).

2.3. Preparation of substrates

r(GAUGGCCGGCAUG), namely R13, which was used for the substrate of separation A, B, C, D, E and derivatives of HDV88-trans were synthesized chemically. Other substrates were prepared by in vitro run-off transcription. Templates for substrates were constructed by in vitro mutagenesis from pUHD88 (for F, G and I) or pUHD88-DI3 (for H and J) with mutagenesis primers, d(ACTCTAGAGGATCCGAGGAGGCTGGGAC) for separation F, d(ACTCTAGAGGATCCGCCAGCGAGGAGGC) for separation G and H, d(ACTCTAGAGGATCCACGGTCCCCTCGGA) for separation I

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and d(ACTCTAGAGAATCCTCGGAATGTTGCCC) for separation J.

2.4. Plasmid DNA

All plasmids used in this study included the T7 promoter and part of the HDV genomic sequence in the *Eco*RI-*Bam*HI site of pUC118. Some had a *Xho*I site between the T7 promoter and the HDV sequence [5]. All experiments were conducted using *E. coli* MV1184 as host cells. Plasmid DNA was prepared from an overnight culture by alkaline lysis and purified using QIAGEN-Tip 20 (DIAGEN, Germany). DNA sequencing was conducted double-stranded DNA as a template on a DNA sequencer (Model 373A; ABI) using dideoxy chaintermination with the fluorescence Taq DyeDeoxy Terminator system (ABI).

2.5. Transcription in vitro

Transcription in vitro was conducted using the AmpliScribe T7-Specific Transcription Kit (Epicentre Technologies, USA). A linearized vector with *Bam*HI was used for a template of transcription in vitro. After the transcription reaction, an equal volume of stop solution containing 50 mM EDTA and 9 M urea was added to stop transcription. After denaturation at 90°C for 2 min and quick chilling on ice, the transcript was isolated by extraction from denaturing 8% PAGE containing 7 M urea. The average yield of *trans*-acting ribozyme was 700 pmol (20 μg) per 1 pmol (2.2 μg) of template DNA.

2.6. Cleavage reactions

For single turnover reactions, 75 pmol of unlabeled *trans*-acting ribozyme and 0.075 pmol of radiolabeled substrate were mixed in 5 μl of 75 mM Tris-HCl (pH 7.1). After denaturation at 90°C for 2 minutes, the mixture was placed on ice for 30 min. Preincubation was conducted at the reaction temperature (37°C) for 10 min and then 2.5 μl of prewarmed 5 mM of $MgCl_2$ was added to start the cleavage reaction. The final concentrations of components in this mixture were 10 μM *trans*-acting ribozyme, 0.01 μM substrate, 50 mM Tris-HCl and 1 mM $MgCl_2$. Control reactions contained equal amounts (ca. 2.14 μg) of tRNA instead of ribozyme. To compare the activities of combination A to J, final concentrations of ribozyme and substrate were both 0.1 μM. Cleavage reactions were conducted in 50 mM Tris-HCl (pH 7.1) with 25 mM of $MgCl_2$ at 37°C or 55°C for 1 h. Samples (1 μl) were withdrawn at the indicated time and mixed with equal volumes of stop solution containing 50 mM EDTA and 9 M urea, and were fractionated in denaturing 20% polyacrylamide gel electrophoresis. The amounts of cleaved products were quantified based on radioactivity intensity by use of a bioimaging analyzer (BA100; Fuji Film, Japan). Cleavage activity (k_{clv}) was indicated by the rate of formation of the cleaved product. For kinetic analysis, we used simple pseudo-first-order equations and experimental data was curve-fitted [Kawakami et al., unpublished results].

3. Results and discussion

3.1. Comparison of trans-acting systems

Several different types of *trans*-acting genomic and antigenomic HDV ribozymes were constructed by Been [8,9], Branch and Robertson [10] and Wu et al. [11]. We have dissected the *cis*-acting genomic HDV RNA at different sites on the pseudoknot model for constructing ten different *trans*-acting HDV ribozymes (Fig. 2) and conducted cleavage reactions with high concentrations of Mg^{2+} ions (25 mM), 50 mM of Tris-HCl (pH 7.1) at 37°C or 55°C for 1 h. We used the above high concentrations of Mg^{2+} ions to detect even low activity. Among these sets, only the separation A molecule, a similar construct made in an antigenomic one [8], was active. No other constructs showed activity (data not shown). The A *trans*-acting system, separated between 698 and 699, is composed of HDV88-Trans (699-770) as the enzyme and substrate R13 (686-698) as the substrate. To compare activity quantitatively, we conducted all experiments under the same physiological conditions, i.e. pH 7.1 at 37°C with 1 mM of Mg^{2+} ions, and obtained the rate constant of cleavage (k_{clv}) and the ratio of ribozymes with active form (end point value; EP)

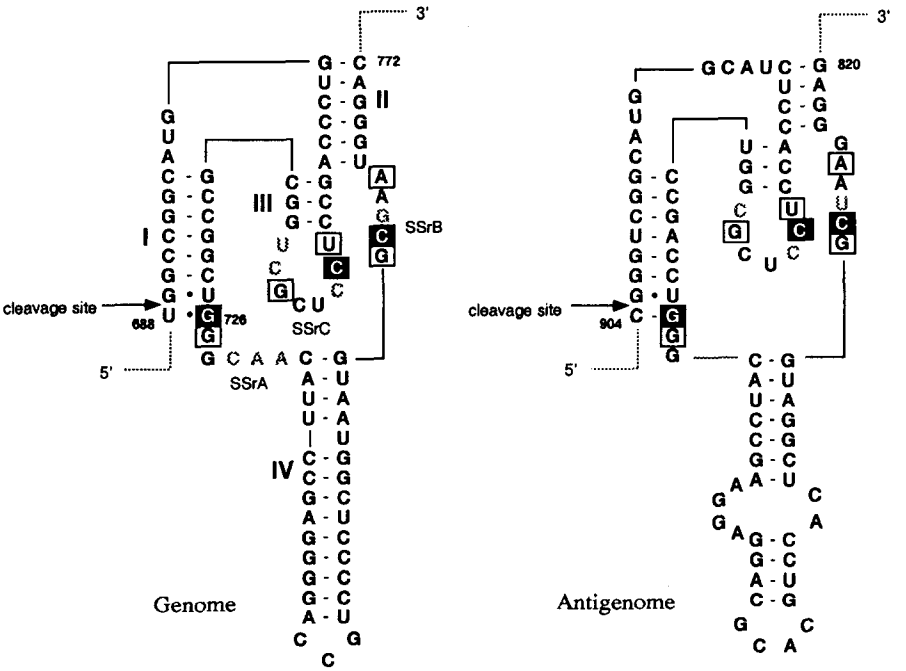

Fig. 1. Important bases in single-stranded regions (SSrA, B and C) of the genomic HDV ribozyme (HDV88) and antigenomic ribozyme are shown in pseudoknot secondary structure models. These data are based on our previous in vitro mutagenesis studies [3,5]. The most important residues (ones which cannot be substituted in any other bases) are shown by white letters against black squares. The next important residues (partially substitutable to some bases) are shown by letters in boxes. Unmarked letters can be substituted in any other base. Stem regions are shown as I to IV.

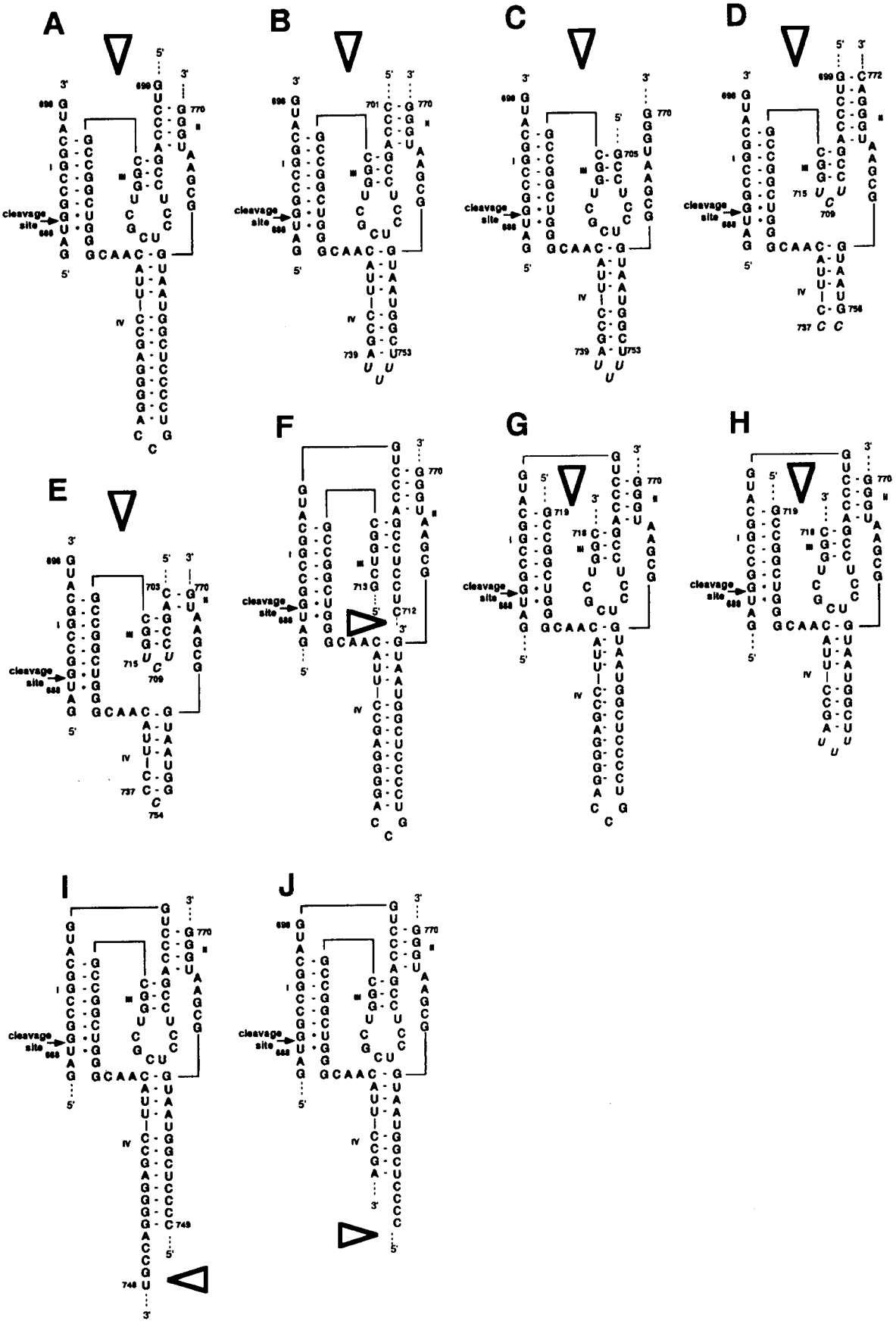
A
B
C
D
E
F
G
H
I
J
cleavage site

Fig. 2. Construction of ten types of *trans*-acting genomic HDV ribozymes. The nucleotide number is according to Makino et al. [23]. Sequences in italics are not original HDV sequences. Dotted lines indicate vector-derived sequences. White arrows indicate disconnected positions for the *trans*-acting HDV ribozyme.
←

values. The k_{clv} value of HDV88-Trans was 0.022 min^{-1} and the EP was 51%.

Table 1 summarizes several k_{clv}values observed in both antigenomic and genomic HDV ribozymes. These results suggest the following: (1) A appears to be more active than I or J, and (2) antigenomic ribozyme activity seems to be higher than the genomic. Since both genomic and antigenomic HDV ribozymes are represented as pseudoknot-like structures [12] and important residues identified from our mutagenic analyses [3–5] are all conserved between genomic and antigenomic HDV ribozymes (Fig. 1), low genomic HDV ribozyme activity could be due either to stabilization of an inactive conformation or to interference in forming an active structure, as opposed to an antigenomic one.

3.2. *Improving HDV88-Trans activity*

Wu et al. previously reported that a *trans*-acting genomic HDV ribozyme (683-728 and 760-770) has activity [11] and corresponds to our I (or J), but this type showed no activity under our reaction conditions (they recently designed an improved, different construct [13]). The difference in construct from ours is that their ribozyme has a nonnatural stem IV sequence and different vector sequences at both 5′ and 3′ ends. It was also reported that vector sequences at both 5′ and 3′ ends of *cis*-acting HDV ribozyme enhance its self-cleavage activity [14]. Given this evidence, we focused on the extra sequences at both 5′ and 3′ ends. To improve the cleavage activity of HDV88-Trans, we either increased or substituted sequences at the 3′ end so that stem II (in the pseudoknot secondary structure model) could be extended from 5 to 9 base-pairs (Fig. 3, Trans-0 to Trans-5) and compared their activities in terms of the rate of catalysis (k_{clv}). By extending stem II, we assume that the stability of the pseudoknot structure will be increased.

As shown in Fig. 3, k_{clv} values increased according as stem II became longer, from 5 to 8 base-pairs. Among the several

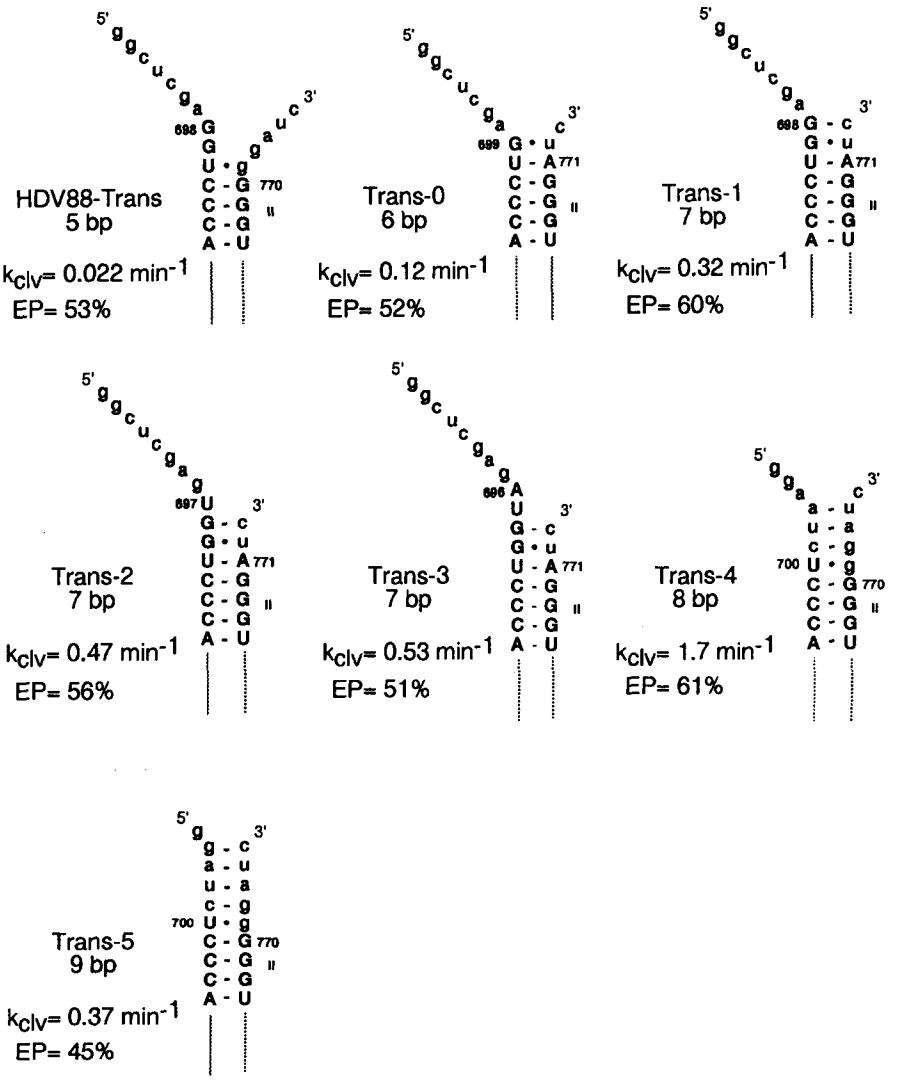

Fig. 3. Variants of the stem II region and their kinetic activities. Small letters indicate vector-derived sequences.

Table 1
Comparison of k_{clv} values of several types of *trans*-acting HDV ribozymes

HDV ribozymes	kinetic constants (k_{clv})	
Separation A		
Genomic	0.022 min^{-1}	(pH 7.1 at 37°C, in this paper)
Antigenomic	~2.8~ min^{-1}	(pH 7.4 at 55°C, Perrota and Been [8])
	1.5 min^{-1}	(pH 8.0 at 37°C, Been et al. [9])
Separation I or J		
Genomic	–	(pH 7.1 at 37°C, in this paper)
	0.011 min^{-1}	(pH 7.2 at 50°C, Wu et al. [11])
Antigenomic	0.52 min^{-1}	(pH 8.0 at 37°C, Been et al. [9])

constructs we made, Trans-4 (8 base-pairs) showed the highest activity. When the rate constants were compared for HDV88-Trans versus Trans-4, the later construct has nearly 80-fold higher k_{clv} values. The stabilities of stem II at 37°C in 1 M NaCl were calculated based on the nearest neighbor parameters [15,16]. The free energy changes by formation of stem II were −5.5 kcal·mol^{-1} (HDV88-Trans), −7.2 kcal·mol^{-1} (Trans-0), −8.5 kcal·mol^{-1} (Trans-1, -2 and -3), −10.2 kcal·mol^{-1} (Trans-4), and −12.3 kcal·mol^{-1} (Trans-5), respectively. These values may not be accurate by dangling ends of stem II and the effect of stacking with stem III, but seem to correlate well with the logarithm of k_{clv} values (data not shown). This result indicates that stabilization of the local structure at stem II might affect fairly directly the activity of HDV ribozyme. Been and Perrotta reported an interaction between SSrA and SSrB in antigenomic HDV ribozyme [17]. This interaction should bring stem II to near the active site. In the case of *Tetrahymena* group I intron, a local structure (P5abc) is indispensable to change the core structure into the active form [18]. Furthermore in the cleavage of tRNA by Pb^{2+} ions, sufficient Mg^{2+} ions to ensure proper folding of tRNA are required, and the cleavage rate correlates with the structure of the active site [19]. In our case, a stable stem II structure may have an influence of the core structure and of arrangement or distance of the functional groups at the active site. By further extending the stem II structure (beyond 8 base-pairs, Trans-5), we observed a 5-fold decrease in the k_{clv} value. This result suggests that stem II of the *trans*-acting genomic HDV ribozyme plays an essential role in maintaining the pseudoknot structure. Too rigid a stem structure, however, makes the entire molecule inflexible and, subsequently, it inhibits structural changes necessary for expressing its ribozyme activity. This speculation agrees with previous results showing that stem II is an important region for formation of active core [9,20] and the GGAGA sequence at the 3′-end of the HDV ribozyme is important in expressing its activity under denaturation conditions with urea or formamide [21,22] because, in the pseudoknot structure model, these residues are located in stem II, meaning stable stem II structure is important to ribozyme activity under denatured conditions.

The construct isolated here, Trans-4, obtained k_{clv} activity comparable to the hammerhead and hairpin ribozymes. HDV88-Trans-4 represented the highest active genomic HDV ribozyme (1.7 min^{-1}) that functions in *trans* under physiological conditions (pH 7.1 at 37°C with 1 mM of $MgCl_2$). Taking these results together, we are now constructing several minimal *trans*-acting HDV ribozymes derived from Trans-4.

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