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Systematic substitution of individual bases in two important single-stranded regions of the HDV ribozyme for evaluation of the role of specific bases

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To elucidate the role of specific bases in the self-cleavage activity of the human hepatitis delta virus (HDV) ribozyme, systematic substitutions of individual bases in two important single-stranded regions [between nucleotides 726–731 (SSrA region) and 762–766 (SSrB region)] were carried out by oligonucleotide-directed point mutagenesis. Among the mutants obtained, 12 mutants (G726 variants, G727A, G727C, G728C, G762A, G762C, C763 variants and A766C) could not tolerate the respective base-substitutions and self-cleavage activities were reduced to very low levels (10%), suggesting a requirement of the respective bases. In particular, G726 in the SSrA region and C763 in the SSrB region were found to be essential for the ribozyme activity. We could determine the preferred sequences, 5′-G-G-(G/A/U)-N-(A/U/G)-Pu-3′ for SSrA and 5′-(G/U)-C-N-(A/G/U)-A-3′ for SSrB regions, respectively.

HDV ribozyme; Self-cleavage activity; Pseudoknot structure; Single-stranded region; Point mutation

1. INTRODUCTION

Human hepatitis delta virus (HDV) contains a small, covalently closed, circular RNA that can be considered as a naturally occurring satellite of human hepatitis B virus [1]. Like other subviral pathogenic circular RNAs, such as viroids and some plant viral satellite RNAs, HDV is thought to replicate into longer-than-unit length progeny by an RNA-to-RNA rolling-circle pathway [2]. Moreover, sequence and structural analysis of the HDV self-cleaving domains have revealed no homologies to well-characterized ribozymes, such as those of the hammerhead type [3] or hairpin type [4]. Hence, HDV is believed to be a distinct new type of ribozyme with self-cleavage activity [5,6].

Recently, secondary structural motifs flanking the self-cleavage site of both genomic and antigenomic HDV ribozymes have been proposed (Fig. 1A, [7,8]; 1B, [9]; 1C, [10]). Although a limited number of mutagenesis experiments has confirmed the requirement for complementary base-pairings in the helices of genomic [10] and antigenomic [7,11,12] HDV ribozymes, the catalytic center and the roles of bases in single-stranded regions have not been characterized.

In order to elucidate the relationship between function and structure of the genomic HDV ribozyme, we constructed several deletion and random variants in two HDV molecules (HDV133 and HDV88). Results of deletion mutagenesis showed that stem-loop regions between bases 701–718 and 740–752 are structurally important but are not the catalytic core since variants HDV133DI-1 (deletion of bases 701–718; the numbering of bases used here is that of Makino et al. [13]) and HDV 88DI-3 (deletion of bases 740–752) continue to exhibit self-cleavage activity [14]. Our results of point and compensation mutations at stem regions on pseudoknot structure of HDV ribozyme suggested that stem I and stem III are essential for catalytic activity. These mutagenic results strongly support a pseudoknot-like structure for genomic HDV ribozyme [15]). Our results from an analysis of the effects of random mutations in the two single-stranded regions between positions 726–731 (SSrA region) and 762–766 (SSrB region) on the secondary structure of pseudoknot model, confirmed that these two single-stranded regions are involved in the self-cleaving activity and a few bases located in these regions may play a critical role in the catalysis [16].

The intent of this study was to determine the preferred sequneces at two important single-stranded regions, SSrA and SSrB, by changing each nucleotides in

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Abbreviations: HDV, hepatitis delta virus; PEG, polyethylene glycol; BPB, Bromophenol blue; XCFF, xylene cyanol FF.

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these regions to each of the other possible nucleotides and then examining the capacities of mutants to self-cleave the precursor RNA.

2. MATERIALS AND METHODS

2.1. *Plasmid DNA*

The plasmid pUHD88 was used in the present study and the construction of the vector has been reported previously [14]. It contains the genomic HDV sequence from nucleotide (nt) 683 to 770, which has self-cleavage activity. All experiments were carried out using *E. coli* MV1184 as the host. The plasmid DNA was prepared from an overnight culture. For construction of various point variants of HDV88, single-stranded DNA (template) was isolated from pUHD88 by the procedure of Viera and Messing [17].

2.2. *Construction of point variants*

For introduction of point variations in two single-stranded regions [SSrA (nt 726–731) and SSrB (nt 762–766)] of the genomic HDV ribozyme, we used oligonucleotide-directed mutagenesis. Oligonucleotides were designed with systematic substitutions of each base in SSrA and SSrB by other bases and were synthesized by the phosphoramide method on an automated DNA synthesizer (model 380A, Applied Biosystems; CA USA). Each oligomer had nine complementary bases (respective to the template DNA) on either side of the site of the point mutation .

To confirm the nature of the systematic substitution of each base in SSrA and SSrB regions, single-stranded DNAs isolated from transformants were sequenced on a DNA sequencer (model 373A; Applied Biosystems) by the dideoxy chain-termination method with a fluorescent Taq Dye Primer (21mer) system (Applied Biosystems; CA USA).

2.3. *Preparation of precursor RNAs and the extent of self-cleavage activity*

Plasmid DNA was isolated by the alkaline lysis method, with subsequent treatment with 20% PEG (M_r 6,000), and CsCl density gradient ultracentrifugation. The DNA was linearized with *Bam*HI and used for transcription in vitro. The reaction mixture for transcription contained 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 0.01% bovine serum albumin, ribonucleotides at 5 mM each, 0.5 mCi/ml [α-^{32}P]CTP, 2 μg of linear plasmid DNA and 7 units of T7 RNA polymerase/μg of DNA. After 60 min at 42°C, an equal volume of stop solution (9 M urea, 50 mM EDTA, 0.1% BPB, 0.1% XCFF) was added and then heat-denatured RNA was fractionated by electrophoresis on an 8% (w/v) polyacrylamide gel (PAGE) that contained 7 M urea. The transcripts and 3′-products were located by autoradiography. The extent of self-cleavage activity during transcription for each variant was calculated with a Bio-imaging analyzer (BA100; Fuji Film; Tokyo Japan).

2.4. *Inefficient self-cleavage reactions of some point variants*

Variants that could not process the precursor RNA as efficiently as the wild type (very low or no self-cleavage activity) were isolated from the gel for further analysis of their self-cleavage activity. The transcripts were excised and extracted from the gel by overnight incubation with 0.1 mM EDTA, 0.3 M NaOAc and 20 mM Tris-HCl (pH 8.0) and recovered by ethanol precipitation. The self-cleavage reaction was performed at 50°C for 17–3 h in the presence of 25 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0).

3. RESULTS

To identify the functional role of bases located within two important single-stranded regions (SSrA, nt 726–731, and SSrB, nt 762–766) in the self-cleavage activity of the genomic HDV ribozyme (HDV88), each base in these regions was replaced sequentially to other bases to oligonucleotide-directed mutagenesis. These regions are shadowed in the diagrams of the three recently proposed models,which are shown in Fig. 1. The point mutants were used for transcription reactions in vitro and products of reaction were separated by PAGE on denaturing gel (Fig. 2). The self-cleavage activity of each variant was calculated by comparing the amount of 3′-product with that of the product of transcription. Depending upon their self-cleavage activities during the transcription reaction in the presence of 6 mM $MgCl_2$, variants could be divided into three groups. The first

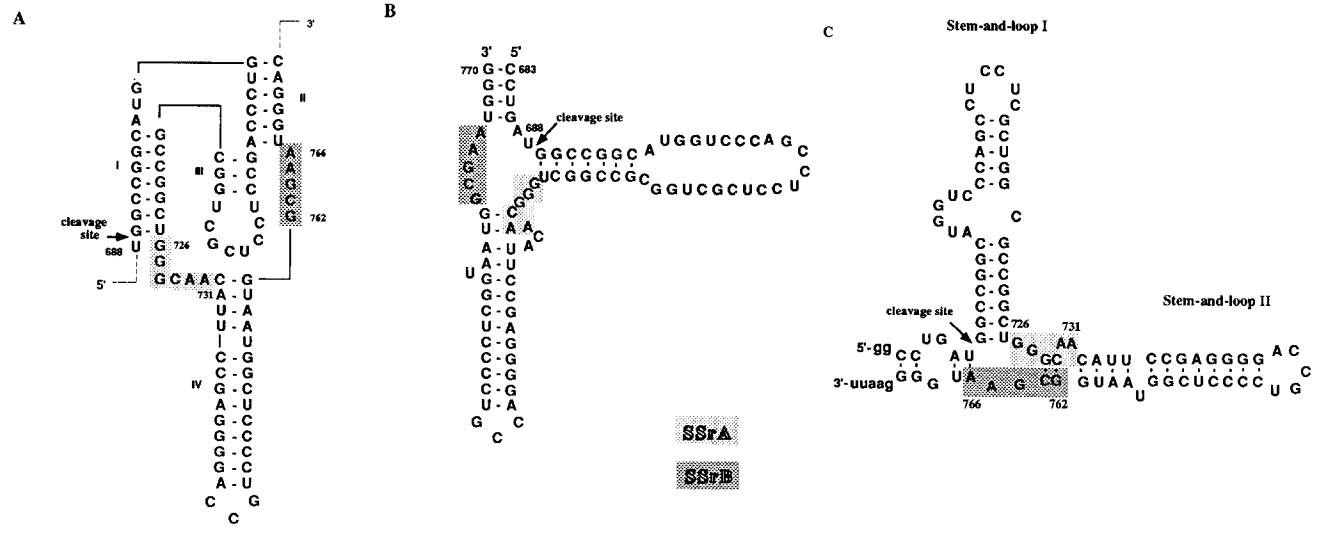

Fig. 1. Potential secondary structures of the genomic HDV ribozyme. Two regions in which substitution were introduced systematically (SSrA and SSrB) are shadowed. The genomic sequence for HDV is numbered according to Makino et al. [13]. Base pairs are indicated by dashes, continuity of sequence by the solid lines. (A) The HDV85 molecule in a pseudoknot-like structure, as proposed by Perrotta and Been [7]. (B) The HDV88 molecule in an axehead model, as proposed by Branch and Robertson [9]. (C) The HDV88 molecule, as proposed by Wu et al. [10].

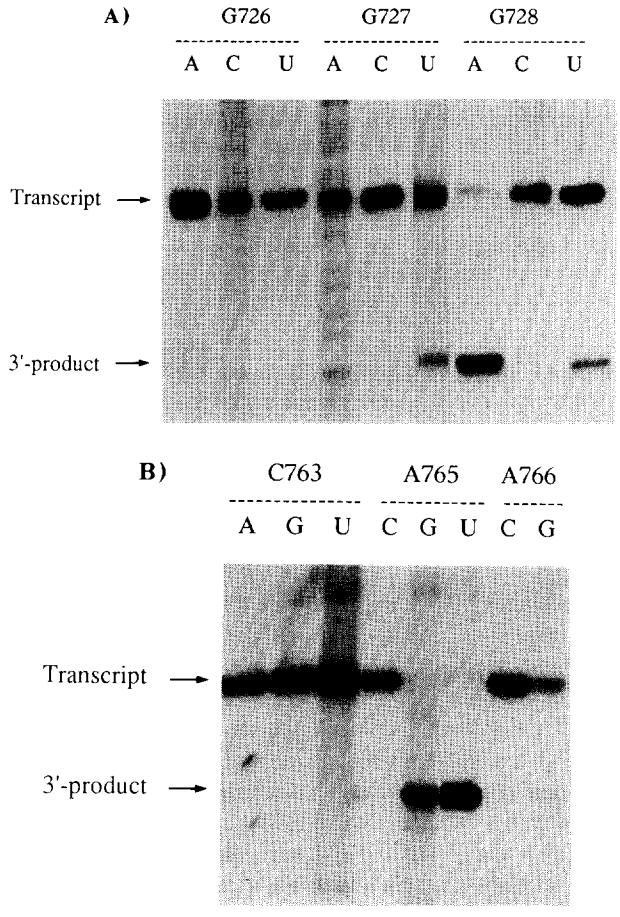

Fig. 2. Analysis of self-cleavage activities of several variants in the SSrA (A) and SSrB (B) of the genomic HDV ribozyme, during in vitro transcription, on an 8% polyacrylamide gel that contained 7 M urea. The mutated position and nucleotide are indicated above each lane. The conditions used for transcription and PAGE are described in section 2.

group retained almost the same activity as that of wild type (HDV88, > 70%) and the second group had intermediate activity (10–50%). The third group had lost all the activity (< 10%). In order to clarify the important residues in these regions, transcripts of the third group were recovered from the gel and self-cleavage activities were analyzed in the presence of the 25 mM $MgCl_2$ at 50°C. The various self-cleavage activities are summarized in Figure 3.

3.1. *Self-cleavage activity of variants with point mutations in the SSrA region*

Self-cleavage activities associated with various point mutations in the SSrA region are summarized in Fig. 3A. All three mutants at G726 had almost no cleavage activity, suggesting the important role of this base in ribozyme activity. Mutants G727A, G727C and G728C also had very low activities, like the G726 mutants. Other variants in the SSrA region, G727U, G728U and all mutants at A730, had different catalytic activities which depended upon the nature of the base substitutions. However, in the case of C729, mutants retained self-cleavage activity regardless of base substitutions. Mutant G728A retained activity at the level of the wild type.

3.2. *Self-cleavage activity of variants with point mutations in the SSrB region*

The effects of point mutagenesis in the SSrB region are summarized in Fig. 3B. Among the bases in the SSrB region, C763 was found to play a critical role during self-cleavage reaction since substitutions by other bases, namely A, G or U, completely abolished the self-cleavage activity. Similarly, G762A, G762C and A766C mutants self-cleaved at substantially lower rates (< 10%). However, when A765 was substituted by G, the self-cleavage activity was similar to that of the wild-type (HDV88) ribozyme. In the case of G762U, A765C, A765U, A766G and A766U mutants, the cleavage rates of mutants were lower than that of the wild-type, although the magnitude of the effect varied considerably depending on the position substituted and the nature of the substitution. The mutants with lower self-cleavage activity (< 10%) were analyzed at 50°C with a higher concentration of Mg^{2+} ions. In the case of C763 mutants no cleavage product was observed even after incubation for 3 h with 25 mM $MgCl_2$.

4. DISCUSSION

Accumulating evidence suggests that some single-stranded loop regions of RNA molecules play a pivotal role in catalysis. The possible role of the essential nucleotides in ribozymes can be considered either to involve interactions with metal ions suggested as real catalyst recently [18,19] and/or maintenance the tertiary structure of the molecule, both of which are required for formation of the active conformation. Our previous results of internal deletion mutants [14], point and compensation mutations at stem regions [15] and random mutations [16] on HDV genomic ribozyme suggested that single-stranded regions, bases from 726–731 (SSrA) and 762–766 (SSrB), in the pseudoknot-like model (Fig. 1A), play an important role for its self-cleavage reaction.

In order to analyze the requirement for each specific base located in these regions (SSrA and SSrB) of HDV88, we systematically substituted by site-specific mutagenesis. The results of point mutations in the SSrA region showed that, among six bases, G726 plays an important role since substitutions by A, C or U reduced the self-cleavage activity of the ribozyme to very low levels even after incubation for as long as 3 h. Substitution by A was the greatest impediment with respect to self-cleavage activity. C763 in the SSrB region plays an important role in the catalytic activity since, when this C was substituted by any other base, the catalytic activ-

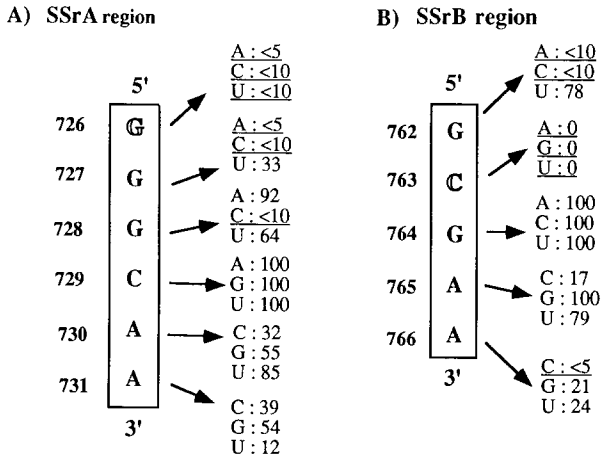

Fig. 3. Self-cleavage activities of variants with various point mutations in the SSrA and SSrB regions. The values presented here were calculated by comparing the amount of processed product with that of the transcript during the transcription process in vitro (in the presence of 6 mM $MgCl_2$) or during the self-cleavage reaction (underlined values were obtained in the presence of 25 mM $MgCl_2$). (A) Bases in the SSrA region and (B) bases in the SSrB region. N indicates the base which abolished the self-cleavage activity by base substitutions.

ity was completely abolished and the mutants could not achieve self-cleavage even after incubation for 3 h in the presence of 25 mM $MgCl_2$. Presumably, the essential nucleotides in catalysis participate in the tertiary interactions that are required to maintain the structure, as well as providing functional groups for the ion-binding pocket and catalytic mechanism [20]. The base pairings of stem II are clearly important for activity because, in the presence of denaturants, the activity of the antigenomic HDV ribozyme is reduced [7]. In view of this result, attention should be focused on C763 in the SSrB region together with G726 in the SSrA region, which may play a critical role in catalysis. Wu et al. also suggested that the bases present in the hinge region between two stem-and-loop structures in their model (Fig. 1C) may represent the core structure of the HDV catalytic RNA [10].

Three models of secondary structure have been proposed for both the genomic and the antigenomic HDV ribozymes, such as pseudoknot-like (Fig. 1A; [7,8]), axehead (Fig. 1B; [9]) and modified cloverleaf (Fig. 1C; [10]). Among three structural models the stem II and III regions of the model A are characteristic for each model (paring partners are different and characteristic to each motif; Fig. 1). Our previous [15,16] and present results are mostly in agreement with the pseudoknot-like model, but several point and compensation mutants at A704 and U767 retained self-cleavage activity even when base pairs were disrupted, suggesting that Watson-Crick base pairs are not essential in this region for self-cleavage activity [15]. However, V1 nuclease probing data suggest that stem II region exists despite the disruption of a base-pair between A704:U767 [15].

Inefficiently self-cleaving variants at these regions could not regain activity even when incubated with other metal ions such as $CaCl_2$ and $MnCl_2$, which activate the wild-type HDV88 ribozyme to a similar or greater extent than $MgCl_2$ (unpublished result). It may suggest that bases in these two regions are involved in interactions with metal ions either directly or indirectly during the self-cleavage of the genomic HDV ribozyme. The bases in the SSrA and SSrB regions are largely conserved in sigle-stranded region of the genomic and antigenomic HDV ribozymes [7,12]. To our knowledge there are no other studies of systematic substitutions of bases in the genomic HDV ribozyme for evaluation of their importance. By analyzing self-cleavage activities of various mutants of SSrA and SSrB regions we could determine the preferred sequences for these regions and the sequences are 5′-G-G-(G/A/U)-N-(A/U/G)-Pu-3′ and 5′-(G/U)-C-N-(A/G/U)-A-3′ for SSrA and SSrB regions, respectively. Recently, we employed in vitro selection proceedure [21,22] to find the important bases at a remained single-stranded region, SSrC (U708-U715) and could find a few bases that are important for the cleavage activity (unpublished data). Thus, several bases in the SSrC region, together with C763 and other bases in the SSrA and the SSrB regions, appear to represent the core structure of the genomic HDV ribozyme. Furthermore, studies of tertiary interactions for evaluation of the folding of mutant RNAs may clarify the relationship between structure and function of the genomic HDV ribozyme.

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