Random mutations to evaluate the role of bases at two important single-stranded regions of genomic HDV ribozyme

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ABSTRACT

In elucidating function of two important single-stranded regions (SSrA (726–731 nt) and SSrB (762–766 nt)) derived mainly from three secondary structure models in genomic hepatitis delta virus (HDV) ribozyme possessing self-cleavage activity, we have constructed several random mutants at those two regions on the HDV88 molecule (683–770 nt) by oligonucleotide-directed mutagenesis. When self-cleavage activities were compared among mutants, at the region SSrA, G726 was found to play an important role during cleavage reaction since substitutions of the base to A (mutant A20) or C (mutant A16) or U (mutant A23), reduced the ribozyme activity to very low levels suggesting the importance of G726 position. C763 at SSrB region was found to play a more significant role during catalysis than G726 (at region SSrA) since any substitutions at C763 completely inactivated the ribozyme. Other bases located in these two regions could be substituted to other bases at the expense of some self-cleavage activity. The results presented here together with our previous deletion analysis indicate that these two regions may play an important role during cleavage process.

INTRODUCTION

Human hepatitis delta virus (HDV) is known to cause fulminant hepatitis or chronic hepatitis with linear cirrhosis by hepatitis B virus (HBV) or by superinfecting the chronic HBV carriers (1). The genome is a single-stranded circular RNA consisting of about 1700 nucleotides. It appears to replicate through a rolling circle mechanism (2) and resembles plant pathogenic RNA viruses in many features including presence of self-cleaving sequences (3, 4). The fact that both genomic and antigenomic RNA strands of HDV possess self-cleaving activity at positions 688/689 in genome and 903/904 in antigenome may be responsible in RNA processing during viral replication (2–4), therefore, HDV RNA may represent a first clear example of a self-cleaving RNA (ribozyme) that in its natural form functions in human cells. As with other self-cleaving RNAs, self-cleavage activity of HDV ribozyme requires a divalent cation and produces products having 2', 3'-cyclic phosphate and 5'-OH group (2, 4). However, the self-cleaving sequence in the HDV ribozyme resembles neither hammerhead-type (5) nor hairpin-type ribozymes (6) suggesting that HDV ribozyme would have to represent a different structural motif from other known ribozyme types (6–9), thus, HDV ribozyme represents an unique type of self-cleavage RNA (4, 10). Evidence relating to distinct structural motifs of HDV ribozyme has been presented mainly by three groups (Fig. 1A, B and C; 4, 11–14). It was also observed that HDV ribozyme undergoes extremely rapid kinetics and is active under wide pH range (pH 5 to 9) (15).

Recently, the genomic HDV ribozyme sequence was truncated from both termini and internal regions were also deleted by a few investigators to define catalytic center and to create a minimal molecule possessing self-cleavage activity. Wu & Lai (15) reported that the molecule which has 88 nts (683–770) was cleaved more efficiently than several other longer molecules. The structural motifs have been generated initially for the HDV88 ribozyme using RNAFOLD program of the Zuker and it was later modified using ribonuclease analysis (4, 11) (Fig. 1C). Perrotta and Been (12) and Rosenstein and Been (13) showed a molecule consisting of 85 nt (∼1 to +84 relative to the cleavage site) which appears to be sufficient for efficient self-cleavage activity under wide variety of conditions (12, 16). Secondary structures for both genomic and anti-genomic HDV ribozymes have suggested a pseudoknot-like motif based on nuclease probing data and mutagenesis (Fig. 1A; 12, 13). Earlier studies have elucidated that an active conformer is not the most stable structure but is a partially loosened structure (15–18).

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A self-cleaving HDV ribozyme was modified to produce a ribozyme capable of catalyzing the cleavage of RNA in an intermolecular (trans) reaction by Branch and Robertson (14) and assigned an ‘axehed’ structure to both genomic and anti-genomic HDV ribozymes (Fig. 1B). The ‘axehed’ structure does not include some of the bases on the enzyme subdomain which are needed to form the pseudoknot helix in Perrotta and Been model (Fig. 1A; 12). Moreover, an ‘axehed’ model is devoid of interactions between segments 699—704 and 767—772 nt in trans-cleavage reaction where these bases are shown to be involved in favouring stem region to stabilize the HDV ribozyme in pseudoknot motif. Subsequently, trans-cleavage reaction has been also shown to occur with other structural motifs like pseudoknot molecule (19) and Wu et al.’s (11) structure. Although intermolecular cleavage reaction has been demonstrated with three structural motifs, the catalytic center and the role of bases in the HDV ribozyme remain obscure.

In order to elucidate the functionally important single-stranded loop regions, we have constructed several internal deletion variants of the HDV133 and HDV88 molecules using oligonucleotide-directed mutagenesis, since single-stranded RNA regions appear to provide a potential catalytic core in the case of hammerhead-type ribozyme, and also found to be essential for the function of other RNA molecules (20, 21). Our deletion mutagenesis results showed that stem-loop regions are structurally important but are not the catalytic core because variants HDV133DI-1 [deletion of bases between 701—718, numbering of bases used in this communication is according to the Makino et al. (22)] and HDV88DI-3 (deletion of bases between 740—752) continue to possess self-cleavage activity (23). These results are in agreement with genomic HDV71 ribozyme reported recently by Thill et al. (24) where deletion of bases between 741—756 tolerate self-cleavage activity and possess about 50% cleavage activity compared to HDV88 (longer form). Thus, these results suggest that other single-stranded regions derived from the three recent models are important for interaction with the Mg$^{2+}$ ions in the HDV ribozyme. Therefore, to evaluate the role of other single-stranded regions on the genomic HDV ribozyme and the base requirements in catalysis, two such single-stranded regions (726—731 and 762—766) were selected in the present studies. The bases between 726—731 (referred as SSrA region) and bases 762—766 (referred as SSrB region) appear to form single-stranded regions in all three recent models (Fig. 1A, B and C). These two regions form the hinge region in model 1B and C and Wu et al. (11) had suggested as important region for the catalytic activity of HDV ribozyme.

Here we present results of random mutations at two single-stranded regions (SSrA and SSrB). Random mutants were constructed using oligonucleotide-directed mutagenesis and screened based on their incapacity to self-cleave the precursor RNA as efficiently as the wild type. These mutagenesis results showed that a few bases at these regions are found to play a key role in the self-cleavage activity.

**MATERIALS AND METHODS**

**Plasmid DNA**

The plasmid pUHD88 was used in the present studies and the construction of the vector has been reported previously (23). It contains the genomic HDV sequence from 683 to 770 nt possessing self-cleavage activity. All experiments were carried out using *E. coli* MV1184 as host cell. The plasmid DNA was prepared from the overnight grown culture.

**Oligonucleotide primers**

The following deoxyoligonucleotides for the generation of random mutants were synthesized by the phosphoramidite method on an automated DNA synthesizer (Applied Biosystems, Model 380A) as d(TCCCCCTCGGAATG77GCCAGCGGGCCAG) for SSrA (726—731 nt); d(AGAGGCACCCCATTTCCGAGGGGG) for SSrB (762—766 nt) mutants (italics indicate the randomized bases during synthesis). The synthesized oligonucleotides were used to random mutagenesis at a level of 37.5% (that is 62.5% of correct nucleotide and 12.5% of non-wild type bases each).

**In vitro transcription and screening of mutants**

Each mutant was subjected to *in vitro* transcription and evaluated for their self-cleavage activity during transcription process. For this, the plasmid DNA was isolated by alkaline lysis method and linearized with *Bam* HI. The linearized DNAs were extracted twice with buffer phenol and recovered by ethanol precipitation. The DNAs were further treated with 20% PEG (6000 MW) in order to remove small RNAs. The conditions used for transcription were 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-32p] CTP; 2 μg linear plasmid DNA and 7 units of T7 RNA polymerase/μg of DNA. After 60 min at 42°C equal volume of the stop solution containing 50 mM of EDTA and 9 M of urea was added to stop the transcription. The resultant RNA as fractionated by electrophoresis on a 8% (W/V) polyacrylamide gel containing 7 M urea. RNA as located by autoradiography. The self-cleaved transcripts in 6 M MgCl$_2$ could generate a 3' cleavage product that was resolved from the precursor during polyacrylamide gel electrophoresis. The extent of self-cleavage during transcription for each mutant was then calculated by comparing the level of precursor RNA with that of 3' cleavage product using Bioimaging analyzer, BA 100 (Fuji Film). The mutants which cannot process the precursor RNA as efficiently as wild type during transcription (low or no self-cleavage activity) were selected to determine their nucleic acid sequence.

**DNA sequencing**

To identify substitution of bases at desired places among mutants, single-stranded DNAs isolated from transformants using procedures of Vieira and Messing (26) were sequenced in their entirety on DNA sequencer (Applied Biosystems Model, 373A) using the dideoxy chain-termination method with fluorescence Taq Dye Primer (-21mer) system (Promega).

**RESULTS**

In order to investigate the role of two important single-stranded regions (SSrA and SSrB) and to identify functionally important bases located within these regions on the self-cleavage activity of genomic HDV ribozyme, several random mutants were constructed using oligonucleotide-directed mutagenesis. These regions are highlighted in the recent three models as shown in Fig. 1A, B and C. The pUHD88 vector containing active genomic HDV ribozyme sequence was chosen as parent plasmid to introduce mutations at SSrA (bases between 726—731) and...
SSrB (bases between 762–766) regions. The self-cleavage activity of each mutant was calculated by comparing the level of 3’-product with that of transcription precursor during in vitro transcription process (Fig. 2). Mutants were screened primarily by self-cleavage activity during in vitro transcription and potential candidates selected on the basis of reduced or absent self-cleavage activity compared to the wild type ribozyme. The minor bands which were appeared on the autoradiogram (Fig. 2) other than the precursor and 3’-product position might have derived from the minor contaminant of template DNAs since rapid purification method was adopted. The DNA was sequenced from all potential candidates in order to confirm the substitutions of bases in their RNA molecules.

Self-cleavage activity of different random mutants at SSrA region of genomic HDV ribozyme

The self-cleavage activities during the transcription process of different single or multiple mutants at SSrA region are shown in Table 1. In order to compare the importance of each base on the self-cleavage activity in the region SSrA, the mutants which have single or multiple substitutions were compared based on their self-cleavage activities and the results are summarized in Fig. 3A. Among six bases at SSrA region G726 position was found to have a role on the self-cleavage activity since substitution for A (mutant A20 in Table 1), or C (mutant A16) or U (mutant A23), reduced considerably the self-cleavage activity of ribozyme regardless of whether it was a point or multiple mutation (mutants A1, A3, A12, A14, A15 and A19) suggesting the importance of G726 position. Substitution of G728 to C leads to complete loss of self-cleavage activity (mutant A5) however, when substituted to A (mutant A7) it retained about 60% cleavage activity which could be deduced possibly from double mutations (mutant A7) since our earlier point mutation results showed that A730 could be substituted to U without altering on self-cleavage activity. When base A730 was replaced with C (mutant A13), the cleavage activity was reduced to 32%. Substitutions of bases for C (mutant A21) at G727 and A731 (mutant A22) were found to possess 0 and 39% self-cleavage activities, respectively. Mutations at the position C729 and other substitutions at aforementioned bases could not be realized by our screening method since only the mutants which are shown to possess no or reduced self-cleavage activity compared to the wild type during in vitro transcription were selected as potential candidates.
Presented here are relative self-cleavage activities with that of HDV88 (wild type) ribozyme. Fig. 3. Effects of various base substitutions at regions SSrA and SSrB. The values comparing the levels of precursor RNA with that of 3'-cleavage product by among variants, their DNA was sequenced.

RNA (low or no self-cleavage activity) as efficiently as wild type during transcription have either single or multiple in vitro. The mutants which were screened based on their self-cleavage activity during in vitro transcription have either single or multiple substitutions at the region SSrB as shown in Table 2. Fig. 3B summarizes the effects of base substitutions at region SSrB. Position C763 was found to be one of the important bases at SSrB region of the HDV ribozyme since when the base was substituted either for A (mutant B8) or G (mutant B19) or U (mutant B18), the catalytic activities were completely abolished regardless of whether it was a point or multiple mutation (B2, B5, B12 and other mutants). In the variant which contains base substitution at position A765 for U, the cleavage activity was about 79% (mutant B7). When G762 was substituted for U (mutant B3) the percentage of self-cleavage activity was 78%, but the activity was further reduced by replacing with A (B9 and B11 mutants). When A766 was substituted for G (mutant B10) or C (mutant B9) or U (mutant B18), the self-cleavage activity was abolished. However, the cleavage activities shown in Fig. 3B for mutants at A766 were further reduced by replacing with A (B9 and B11 mutants). The role of A766 in the catalytic activity of genomic HDV ribozyme compared to other bases.

**DISCUSSION**

Recently many types of ribozymes have been found in nature and among them hammerhead, hairpin and HDV RNA molecules belong to the same group based on the generation of products having 2', 3'-cyclic phosphate and 5'-OH groups. Previously, in our molecular orbital calculations of analogous phosphorous compound for elucidating the action of ribozymes (26–31), it
was observed that Mg$^{2+}$ plays a crucial role in catalysis (27). Consensus sequences of hammerhead and hairpin-type ribozymes appear to form a cavity for Mg$^{2+}$ ion, however such a region has remained obscure in HDV ribozyme.

In elucidating functionally important single-stranded loop regions in genomic HDV ribozyme, we constructed several internal and/or terminal deletion variants of the HDV133 (654–786 nt on genomic RNA) and the HDV88 molecule (683–770 nt) by oligonucleotide-directed mutagenesis (23). Those results suggest that other single-stranded regions such as bases from 726–731 and 762–766 are the potential candidates to interact with Mg$^{2+}$ ions.

In view of this, we constructed several mutants which were randomly substituted for other bases at two regions (SSrA and SSrB) on the HDV RNA molecule. For this purpose we screened a number of colonies based on their self-cleavage activities during transcription and defined their sequences. The mutants which have either no or low self-cleavage activity compared to the wild type were selected as potential candidates in an attempt to identify the role of bases at the two regions which contribute significantly during catalysis. Among six bases at SSrA region, G726 position was found to play an important role since substitutions for A (mutant A20), or C (mutant A16) or U (mutant A23), reduced the self-cleavage activity of ribozyme to very low levels. Other bases located in this region (SSrA) could also influence the activity by some extent depending upon the base substitutions. Interestingly, the effect of substitution at position C729 could not be realized by our screening procedure, suggesting that substitutions for other bases at this position could tolerate the self-cleavage activity. C763 at SSrB region played a more important role on catalytic activity since when this was substituted for either A (mutant B8) or G (mutant B19) or U (mutant B18), the catalytic activity was completely abolished. Bases other than C763 at region SSrB could also influence the activity by substitutions. B4 mutant was isolated during DNA sequence analysis of some mutants which were exhibiting similar self-cleavage activity with that of wild type. A few mutants may regain the catalytic activity to that of wild type ribozyme by compensation mutations. In the mutant B3, G762 has been substituted for U resulting in 78% cleavage activity, however when base substitutions took place simultaneously at G762 to U and G764 to U (mutant B4) the cleavage activity was returned to the level of wild type. However, the mutants which were isolated so far at base positions G726 and C763 in SSrA and SSrB regions, respectively, could not be compensated by base substitutions at other positions suggesting that these two bases may interact with Mg$^{2+}$ ions during self-cleavage activity. These two regions are involved in forming hinge regions in both models (Fig. 1B and C). It was also suggested by Wu et al. (11) that the bases present at the hinge region together with two stem structures in their model may represent the core structure of the HDV catalytic RNA.

Several secondary structure models have been assigned to both genomic and anti-genomic HDV ribozymes, such as pseudoknot-like (12), axehead (14) and modified clover-leaf model (11). Perrotta and Been (12) demonstrated by point mutations that base pairs at stem II region in model A of Fig. 1 are important for stabilizing the self-cleaving structure as well as in forming the major pseudoknot helix and these base pairs at this region are conserved in genomic and anti-genomic HDV ribozyme. Nevertheless, the genomic enzyme subdomain used by Branch and Robertson (14) lacks two of the six base pairs which are needed to form the major pseudoknot helix. Further SSrA and SSrB regions (single-stranded regions) are separated by a stem-loop region and are included in the enzyme molecule in the recent trans-cleavage reaction proposed by Perrotta and Been (19). Our point mutation results are in agreement with pseudoknot model (12) for the genomic HDV ribozyme where 3 base pairs are essential (between bases G705–C718; C706–G717 and C707–G716) for the self-cleavage activity, since disruption of base pairings resulted in very low levels of activity (unpublished results).

Although an axehead model showed that the bases C729 and C730 involved in base pairing with G761 and U760, respectively, our random mutation results suggest that they are not forming base pairs, since the mutants in which A730 substituted for C (mutant A13) or U (mutant A7) still possess self-cleavage activity. Wu et al. (11) modified recently their clover-leaf structure (4) for genomic HDV ribozyme and presented a newer model. The bases G762 and C729, and C763 and G728 are shown to be involved, in Watson–Crick base pairings, however our mutation results suggested that these are not involved in forming base pairings with respective bases since G762 tolerates substitutions for U or A and G728 tolerates substitution for A.

The random mutagenesis results presented here describes the importance of two bases G726 and C763 on two single stranded regions SSrA and SSrB, respectively, along with other substitutions. It is possible that these two bases are involved in interactions with Mg$^{2+}$ ions either directly or indirectly during self-cleavage activity of genomic HDV ribozyme. Interestingly, the bases located at single-stranded regions (SSrA and SSrB) resemble the consensus sequence of the hammerhead structure to some extent. As in the case of hammerhead ribozyme in which two bases (G10 and A8) are the part of consensus sequences (located at single-stranded regions) and HDV ribozyme also possesses two such bases at regions SSrA (G726) and SSrB (C763) and are found to play an important role during cleavage process. Our random mutation results presented here together with our earlier deletion mutational results (23) indicate that these two regions may play an important role during cleavage process. The bases which are present in SSrA and SSrB regions are largely conserved in genomic and anti-genomic HDV ribozymes. Further, ongoing point mutation analysis may reveal the importance of bases located in these two regions and other regions in order to obtain the structure-functional relationship of genomic HDV ribozyme.

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