Self-cleavage activity of the genomic HDV ribozyme in the presence of various divalent metal ions

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ABSTRACT
To identify the divalent metal ions that can support the self-cleavage activity of the genomic ribozyme of human hepatitis delta virus (HDV), we tested the activity of various divalent metal ions in the ribozyme reactions catalyzed by HDV88 (683 – 770 nt) and 88DI3 (HDV88 with the sequence from 740 – 752 nt deleted). Among various metal ions tested, Mg\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\) and Sr\(^{2+}\) efficiently supported the self-cleavage reactions of the HDV88 and 88DI3 ribozymes. In the case of the 88DI3 ribozyme, other divalent metal ions, such as Cd\(^{2+}\), Ba\(^{2+}\), Co\(^{2+}\), Pb\(^{2+}\) and Zn\(^{2+}\), were also able to support the self-cleavage reaction to some extent (<10%). In the presence of spermidine (0.5 mM), the cleavage reaction was promoted at lower concentrations of effective divalent metal ions. The HDV ribozyme represents the only example of ribozyme to date of a ribozyme that catalyzes the self-cleavage reaction in the presence of Ca\(^{2+}\) ions as efficiently as it does in the presence of Mg\(^{2+}\) ions.

INTRODUCTION
The RNA of human hepatitis delta virus (HDV) contains a self-cleaving domain that is known to play a key role in the viral replication process (1, 2). In order to characterize the structure-function relationship of the self-cleaving domain (HDV ribozyme), we previously performed site-specific mutagenesis of the genomic HDV ribozyme and was able to identify catalytically important regions (3, 4) and bases (5, 6). Furthermore, our results supported the pseudoknot secondary structure for HDV ribozyme that was proposed by Been and Perrotta (7). When the important bases were changed to other bases in our variants, ribozyme activity was not restored by replacement by any other divalent metal ions, indicating that the most effective divalent metal ions interact at similar positions into the genomic HDV ribozyme (unpublished result).

Divalent metal ions are absolutely required for ribozyme activity. It has been suggested that metal ions have two important roles in ribozyme reactions: they are involved in formation of active structures and they participate directly in the chemistry at the active site (8–11). In general, Mg\(^{2+}\), Mn\(^{2+}\) and other divalent metal ions can support the catalytic reactions of different kinds of ribozyme. However, the effectiveness of the different metal ions may vary with the type of ribozyme. Divalent metal ion requirements for various ribozymes have been studied (12–16). In the case of the HDV ribozyme, low levels of Mg\(^{2+}\) ions are sufficient to permit the cleavage reaction (17), but no detailed studies have yet been carried out to examine the reaction of the HDV ribozyme in the presence of other divalent metal ions. We have now investigated the metal ion requirement of the self-cleavage activity of the genomic HDV ribozyme using two efficiently self-cleaving HDV molecules, HDV88 and 88DI3 (3). Our most interesting observation with the HDV ribozyme is that it can perform self-cleavage activity in the presence of Ca\(^{2+}\) ions as efficiently as in the presence of Mg\(^{2+}\) ions at a similar concentration. Other metal ions can also support the self-cleavage activity to some extent, for example, Sr\(^{2+}\) and Cd\(^{2+}\). In addition, the 88DI3 ribozyme (the shorter form) has enhanced cleavage activity with some divalent metal ions, as compared to the HDV88 ribozyme. Only slight improvements in the self-cleavage activity were observed in the presence of spermidine in contrast to the results observed with hammerhead-type ribozymes (14).

MATERIALS AND METHODS
Plasmid DNAs
The plasmids pUHD88 and pUH88DI3 were used in the present studies and the construction of these plasmids has been reported previously (3). HDV88 contains the genomic HDV sequence, from nt 683 to nt 770, that has self-cleavage activity.

Preparation of HDV self-cleaving RNAs
Uniformly labeled transcripts of HDV88 and 88DI3 were prepared by in vitro T7 transcription system. We observed previously that the HDV88 and 88DI3 transcripts have nearly 90% of their maximum cleavage activity under the conditions for in vitro transcription (3). In order to reduce the self-cleavage activity during transcription, we allowed the reaction to proceed at 4°C for 2 days, as suggested by Thill et al. (18). The components of the reaction and the extraction solution for isolating the transcripts were similar to those reported previously (3). After

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transcription in vitro, the products were separated on an 8% (w/v) polyacrylamide gel that contained 7 M urea (PAGE) and the transcript was isolated as described previously (3).

Analysis of self-cleavage activity with different divalent metal ions

The self-cleavage reactions were carried out in 50 mM Tris—HCl (pH 8.0) at 50°C with varying concentrations of divalent metal ions. All components used in cleavage reactions, including water, were treated with diphenylthiocarbazone to remove divalent metal ions, as described by Holmquist (19). In some reactions, spermidine was included in the reaction mixture at a concentration of 0.5 mM. All reactions were incubated at 50°C for 1 hour and were terminated by the addition of 9 M urea, 50 mM EDTA, 0.1% BPB and 0.1% XGFF, and reaction products were separated by denaturing PAGE. Percentages of 3'-cleavage product were determined by use of a Bio-imaging analyzer (BA100, Fuji Film).

RESULTS AND DISCUSSION

To determine which divalent metal ions support the self-cleavage activity of genomic HDV ribozyme, activities of HDV88 and 88D13 (Fig. 1; 3) were examined in 50 mM Tris—HCl (pH 8.0) with varying concentrations of divalent metal ions. Rosenstein and Been (20) observed that, in the case of the longer HDV ribozyme (160 nt), optimal concentrations of divalent metal ions for self-cleavage activity were found to be different in the presence and in the absence of denaturants. Therefore, we selected two shorter forms of the HDV ribozyme, HDV88 (101 nt) and 88D13 (91 nt) for our study of the effects of metal ions. These ribozymes have enhanced self-cleavage activity as compared to several other of our HDV ribozyme constructs (3) and their activities are independent of denaturing conditions (3). When self-cleavage activities were compared for the HDV88 and the 88D13 ribozymes at various concentrations of metal ions, the 88D13 ribozyme had higher activity (Fig. 2A and 2B). However, the optimum concentration of divalent cations for self-cleavage activity was almost the same for both the HDV88 and the 88D13 ribozymes. Therefore, the enhanced cleavage activity observed with 88D13 is probably due to deletion of a part of the sequence of stem IV (733—761 nt) which is not essential for formation of the catalytic core-structure. Among various metal ions tested Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$ and Sr$^{2+}$ can effectively support the self-cleavage reactions of the HDV88 and 88D13 ribozymes (Fig. 2 and 3).

With each divalent metal ion tested, self-cleavage activity of both ribozymes increased dramatically as the concentration was

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Figure 1. Potential secondary structure of the genomic HDV88 ribozyme: the pseudoknot-like structure proposed by Perrotta and Been (20). The genomic sequence for HDV is numbered as in the report by Malrino et al (24). Base pairs are indicated by dashes and continuity of sequence by solid lines. 88D13 molecule has U-U-U in a small box instead of large box of HDV88 (3).

Figure 2. Effects of metal ions on the self-cleavage reactions of the HDV88 (A) and 88D13 (B) ribozymes. The self-cleavage reaction contained 50 mM Tris—HCl (pH 8.0) and varying concentrations of divalent metal ions. The inner box shows the results of the self-cleavage reaction at low concentrations of metal ions (<1.2 mM).
raised from 0.1 to 1 mM and then reached a plateau value. Mn$^{2+}$ was the most effective divalent ion at concentrations below 1 mM when the cleavage reaction of the HDV ribozyme was assayed with the various divalent metal ions. When the concentration of Mn$^{2+}$ ions was higher than 1 mM, nonspecific cleavage was observed. This was realized when summation of counts (radioactivity) of the original transcript and 3' cleaved product decreased with increased concentration of Mn$^{2+}$ above 1 mM resulting in smearing on the gel (Fig. 4). By contrast, Ca$^{2+}$ and Mg$^{2+}$ ions favor the cleavage reactions of both ribozymes equally. This result is unique, since the reaction of no other ribozyme has been reported to be so effectively supported by Ca$^{2+}$ ions. A hammerhead ribozyme has high cleavage activity with Mg$^{2+}$, Co$^{2+}$, Mn$^{2+}$ but weak activity with Ca$^{2+}$ ions (14). The Tetrahymena ribozyme has cleavage activity in the presence of Mg$^{2+}$ or Mn$^{2+}$ ions but Ca$^{2+}$ ions at 0–10 mM fail to facilitate the reaction. In the presence of Mg$^{2+}$ ions at 0.5 mM, only Ca$^{2+}$ ions at 1 mM evoked the activity (8). Recently, Lehman and Joyce (21) isolated a variant of the Tetrahymena ribozyme that is active in the presence of Ca$^{2+}$ ions by an in vitro selection method. In the HDV ribozyme, Ca$^{2+}$ ions alone can mediate the folding and initiate the cleavage reaction as efficiently as Mg$^{2+}$ and Mn$^{2+}$ ions. Our UV-crosslinking studies also support the conclusion that Ca$^{2+}$ ions can fold the HDV ribozyme as well as do Mg$^{2+}$ ions (unpublished data). This unique characteristic is not only useful as we attempt to understand the mechanism of cleavage but it may also be useful for application of the HDV ribozyme in vivo. Sr$^{2+}$ ions also support the reactions of both ribozymes to a lesser extent. Mg$^{2+}$ ions stimulated the cleavage reaction of HDV ribozymes in a somewhat unusual manner. At concentrations above 25 mM, cleavage activity abruptly increased and most substrates were cleaved. Thus, it appears that the HDV ribozyme may shift to another active conformation at higher concentrations of Mg$^{2+}$ ions. On the contrary with increasing concentrations of Ca$^{2+}$ ions, nonspecific cleavages was observed as in the case of Mn$^{2+}$ ions.

Five other divalent metal ions (Cd$^{2+}$, Ba$^{2+}$, Co$^{2+}$, Pb$^{2+}$, Zn$^{2+}$) were tested for their ability to support self-cleavage of the HDV ribozyme. A substantial increase in activity with Cd$^{2+}$ ions at above 10 mM was observed with the 88DI3 ribozyme and about 12% of substrate molecules self-cleaved, but the HDV88 molecule was inactive. Low cleavage activities were observed with Ba$^{2+}$ (4% at 25 mM), Co$^{2+}$ (3% at 25 mM), Pb$^{2+}$ (7% at 0.5 mM) and Zn$^{2+}$ (9% at 0.5 mM) ions with the 88DI3 ribozyme, while none of these ions promoted the self-cleavage reaction of the HDV88 ribozyme. These results suggest that the 88DI3 molecule adopts the active conformation necessary for catalytic activity more easily than the HDV88 molecule, as a result of the deletion of extra sequence. Other investigators have observed that the stem IV region plays a role in stabilizing the
secondary structure of the HDV ribozyme and contains less important bases (22, 23). In the presence of 0.5 mM spermine, which can greatly reduce the charge repulsion of phosphate groups, the cleavage activity of the hammerhead ribozyme is twice that observed with divalent metal ions alone. Thus this alternate cation can participate in the folding of the hammerhead ribozyme (14). In order to determine whether the polyamine can support higher cleavage activity of the HDV ribozyme, 0.5 mM spermidine was included in the cleavage reaction together with the various different metal ions. As shown in Figure 3, in each case, the presence of spermidine promoted the cleavage reaction at a lower concentration of divalent metal ions and broadened the curves of activity versus the concentration of metal ion, suggesting that spermidine can help in the proper folding that is required to generate an active RNA molecule. However, spermidine failed to increase the maximum activity of the self-cleavage reaction. These results suggest that polyamine can take the place of a divalent cation when metal ions are present at insufficient levels for an efficient cleavage reaction, as in the case of the hammerhead ribozyme. The observed differences in the extent of enhancement by the polyamine between the hammerhead and HDV ribozymes may reflect the possibility that the latter ribozyme can easily adopt an active conformation and initiate the self-cleavage reaction in the presence of low levels of metal ions. Earlier, it was noted that salts of monovalent cations, such as NaCl, KCl and ammonium acetate, can double the rate of the self-cleavage reaction of the HDV ribozyme in the presence of 10 mM Mg$^{2+}$ ions. However, NaCl alone at 1 M failed to promote the reaction (20). In the case of other types of ribozyme, monovalent metal ions, such as Na$^+$ and K$^+$ at concentrations from 1–200 mM failed to support activity in the presence of 0.5 mM MgCl$_2$ (8).

Although, the metal ion requirements of the HDV ribozyme are slightly different from those of hammerhead ribozyme, the mechanism of cleavage by metal ions appears to be the same in both cases. The striking metal-ion specificity of the ‘thio effect’ observed with the HDV ribozyme (unpublished results) supports the hypothesis that HDV ribozyme is also a metalloenzyme as are Tetrahymena and hammerhead ribozymes. As we pointed out earlier (25), based on molecular orbital calculations, cations including metal ions never occupy the space between the most negatively charged phosphate oxygens (11, 25). Therefore, the role of metal ions acting merely as counter-ions is unlikely. In fact, the first demonstration of Mg$^{2+}$ ion acting as a Lewis acid has been reported for Tetrahymena ribozyme (10) and there are evidences based on pH-rate profiles that magnesium hydroxide acts as a general base in hammerhead-ribozyme catalyzed reactions (26). Lastly, the trace metal ion-requirement of the HDV ribozyme is suitable for application in vivo. Our ongoing probing studies may reveal the metal-binding sites, as well as details of the facilitation of folding by divalent metal ions of the HDV ribozyme.

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REFERENCES

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