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Ribozyme cleavage of a 2′,5′-phosphodiester linkage: Mechanism and a restricted divalent metal-ion requirement

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ABSTRACT

The natural substrate cleaved by the hepatitis delta virus (HDV) ribozyme contains a 3′,5′-phosphodiester linkage at the cleavage site; however, a 2′,5′-linked ribose-phosphate backbone can also be cleaved by both *trans*-acting and self-cleaving forms of the HDV ribozyme. With substrates containing either linkage, the HDV ribozyme generated 2′,3′-cyclic phosphate and 5′-hydroxyl groups suggesting that the mechanisms of cleavage in both cases were by a nucleophilic attack on the phosphorus center by the adjacent hydroxyl group. Divalent metal ion was required for cleavage of either linkage. However, although the 3′,5′-linkage was cleaved slightly faster in Ca^{2+} than in Mg^{2+}, the 2′,5′-linkage was cleaved in Mg^{2+} (or Mn^{2+}) but not Ca^{2+}. This dramatic difference in metal-ion specificity is strongly suggestive of a crucial metal-ion interaction at the active site. In contrast to the HDV ribozymes, cleavage at a 2′,5′-phosphodiester bond was not efficiently catalyzed by the hammerhead ribozyme. The relaxed linkage specificity of the HDV ribozymes may be due in part to lack of a rigid binding site for sequences 5′ to the cleavage site.

Keywords: catalytic RNA; divalent metal ions; HDV; hepatitis delta virus; self-cleavage

INTRODUCTION

Hepatitis delta virus (HDV) is an unusual human pathogen (Rizzetto et al., 1980). The HDV particle contains a single-stranded circular RNA genome, and it is replicated through a double rolling-circle mechanism (Chen et al., 1986; Wang et al., 1986). The only catalytic activity that has been identified as being coded by the virus resides in the RNA. Self-cleaving sequences in both the genomic and antigenomic strands are responsible for releasing unit-length linear RNAs from the replication intermediates (Kuo et al., 1988; Sharmeen et al., 1988; Wu et al., 1989). The linear monomers of either sense are then ligated to form circles. Self-ligation to generate the circular RNAs is chemically feasible, but it is also possible that ligation of the linear monomers is catalyzed by a host RNA ligase (Neel & Robertson, 1997).

The in vitro self-cleavage reaction generates a 2′,3′-cyclic phosphate and a 5′-hydroxyl group (5′-OH displacement), suggesting that the mechanism for HDV cleavage is via nucleophilic attack at the scissile phosphodiester bond by the adjacent 2′-hydroxyl group, similar to the hammerhead and hairpin ribozymes (Sharmeen et al., 1988; Wu et al., 1989). The minimal sequence required for self-cleavage comprises approximately 84 nt 3′ and only 1 nt 5′ to the cleavage site (Perrotta & Been, 1990, 1991). The identity of the 5′ nucleotide is not critical, and there is no evidence to indicate base pairing of any 5′ nucleotides to specific sequences in the 3′ domain.

A variety of divalent metal ions can support HDV ribozyme cleavage activity (Kuo et al., 1988; Sharmeen et al., 1988; Wu et al., 1989; Perrotta & Been, 1990; Suh et al., 1993). With the HDV ribozymes, as with other ribozymes (Chowrira & Burke, 1991; Dahm & Uhlenbeck, 1991; Nesbitt et al., 1997), metal ions are proposed to have either structural or catalytic functions or both. Despite the preponderance of evidence that divalent cations are required, it has been difficult to sort out what function metal ions may serve for the HDV ribozyme reaction. It appears that divalent metal ions have the expected effect of stabilizing the tertiary folding of the HDV RNA (Rosenstein & Been, 1996); however, a more direct involvement of divalent metal ions in the chemical step of HDV catalysis has not been demonstrated. The single phosphorothioate substitution at the reactive phosphoryl group of the HDV ribozymes has failed to demonstrate direct metal-ion coordination to the pro-Rp nonbridging oxygen, as had

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been done with the hammerhead ribozyme (Dahm & Uhlenbeck, 1991; Scott & Uhlenbeck, 1999), because the HDV reaction, though inhibited in Mg^{2+}, is not rescued with a softer metal (Fauzi et al., 1997; A.T. Perrotta & M.D. Been, unpubl. data). Therefore, any evidence of metal-ion involvement in or near the catalytic site would strengthen an argument for a role of metal ions in HDV ribozyme catalysis.

A 2′,5′-linkage at the cleavage site of the HDV ribozyme has been reported in a nonenzymatic religation of the HDV RNA in vitro using a chemical catalyst and a complementary RNA oligonucleotide that spans the cleavage site (Sharmeen et al., 1989). This reaction was predicted to yield both 3′,5′- and 2′,5′-linkages at the ligation junction, and the 2′,5′-linkage was hypothesized to be resistant to cleavage by the genomic HDV ribozyme. In our hands, a purported mixture of linkages at the ligation junction prepared by a similar method was cleaved to completion in the *cis*-acting form of the HDV genomic ribozyme (Shih & Been, unpubl. data). This result suggested that either the chemical ligation yielded predominantly the 3′,5′-linked precursor or the HDV ribozyme was capable of cleaving a 2′,5′-linkage. In this study, we demonstrate that the HDV ribozymes can cleave a 2′,5′-phosphodiester bond located at the cleavage site. Cleavage appears to result from an attack at the phosphorus center by the adjacent 3′-OH, a mechanism analogous to cleavage of the normal 3′,5′-linkage. Efficient cleavage of a 2′,5′-phosphodiester bond was not seen in either the hairpin (Feldstein et al., 1990) or hammerhead ribozyme (this study), both of which also cleaved a 3′,5′-linkage through a 5′-OH displacement mechanism. In addition, the distinct characteristics of HDV cleavage toward a 2′,5′-linkage showed a different pattern of metal-ion preference, suggesting important interactions of divalent metal ions at the active site of the HDV ribozymes.

RESULTS

Cleavage of a 2′,5′-linkage by *trans*-acting ribozyme

The linkage specificity of HDV ribozyme cleavage was tested with a *trans*-acting ribozyme, ADC1, derived from the antigenomic sequence (Perrotta & Been, 1992). Two chemically synthesized oligonucleotide substrates were used, DS3 and DS2, that share the same sequence but differ in their phosphodiester linkage at the cleavage site (a 3′,5′- and a 2′,5′-linkage, respectively) (Fig. 1A). The rate constants for the uncatalyzed degradation of the two linkages in Mg^{2+} or Ca^{2+} were essentially the same (Table 1), indicating that the stability of the two linkages is similar under the conditions used in this study. Site-specific cleavage of both substrates by ADC1 was tested in 10 mM $MgCl_2$, $CaCl_2$, $MnCl_2$, or $CdCl_2$. DS3 was cleaved in $MgCl_2$, $CaCl_2$, and $MnCl_2$ (Fig. 2A). The pseudo first-order rate constants for cleavage of DS3 at 1 μM ADC1 are 0.7 and 0.9 min^{-1} in $MgCl_2$ and $CaCl_2$, respectively (Table 1). ADC1 also cleaved DS2 (2′,5′-linked), but with a different pattern of metal-ion preference. The 2′,5′-linked substrate was cleaved by ADC1 in $MgCl_2$ and $MnCl_2$, but was resistant to cleavage in $CaCl_2$ (Fig. 2A and Table 1). Higher concentrations of $CaCl_2$ (100 mM) did not restore cleavage activity (data not shown).

To test if the inactivity of the HDV ribozymes toward a 2′,5′-linkage in the presence of $CaCl_2$ was due to the

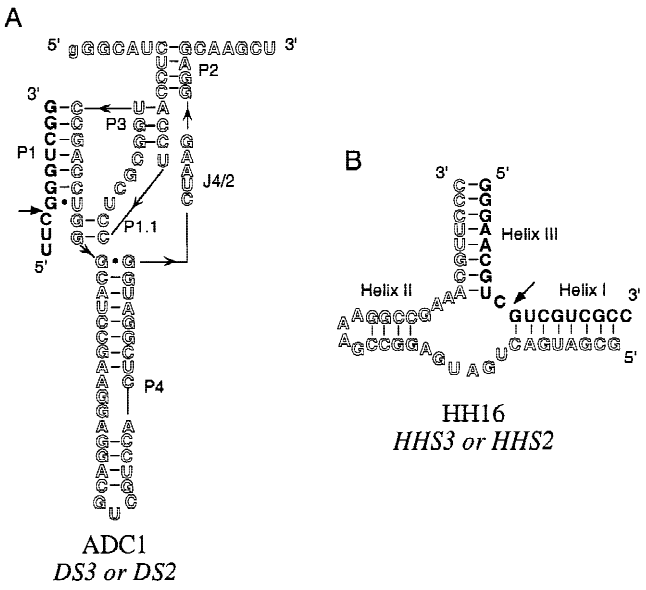

FIGURE 1. Sequences and secondary structures of the HDV and hammerhead ribozymes with their substrates. The sequences of ribozymes and substrates are shown in outlined and solid letters, respectively. The names of the two oligonucleotide substrates listed under each ribozyme (italicized) share the same sequence but differ only in the linkage at the cleavage site, where they are either 3′,5′- or 2′,5′-linked. Cleavage sites are indicated by arrows. **A**: ADC1, an HDV antigenomic *trans*-acting ribozyme, and its 10-mer substrates DS3(3′,5′) or DS2(2′,5′). **B**: HH16, a hammerhead ribozyme (Hertel et al., 1994), and its 18-mer substrates HHS3(3′,5′) or HHS2(2′,5′).

TABLE 1. Cleavage rate constants (k_{obs}) of the *trans*-acting ribozyme ADC1.

Substrate	Metal ion[a]	k_{uncat}[b] (min^{-1})	k_{cat}[c] (min^{-1})	Rate acceleration[d]
DS3	Mg^{2+}	6.8×10^{-6}	7.1×10^{-1}	1.0×10^{5}
DS3	Ca^{2+}	7.9×10^{-6}	9.1×10^{-1}	1.2×10^{5}
DS2	Mg^{2+}	7.2×10^{-6}	8.1×10^{-3}	1.1×10^{3}
DS2	Ca^{2+}	9.2×10^{-6}	6.2×10^{-6}	—

[a]Concentrations of divalent metal ions are 11 mM in 40 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

[b]Average of two determinations, and the deviation is less than 10%.

[c]The deviation of rate constants is less than 20% from at least three independent determinations.

[d]The rate acceleration is calculated as k_{uncat}/k_{cat}.

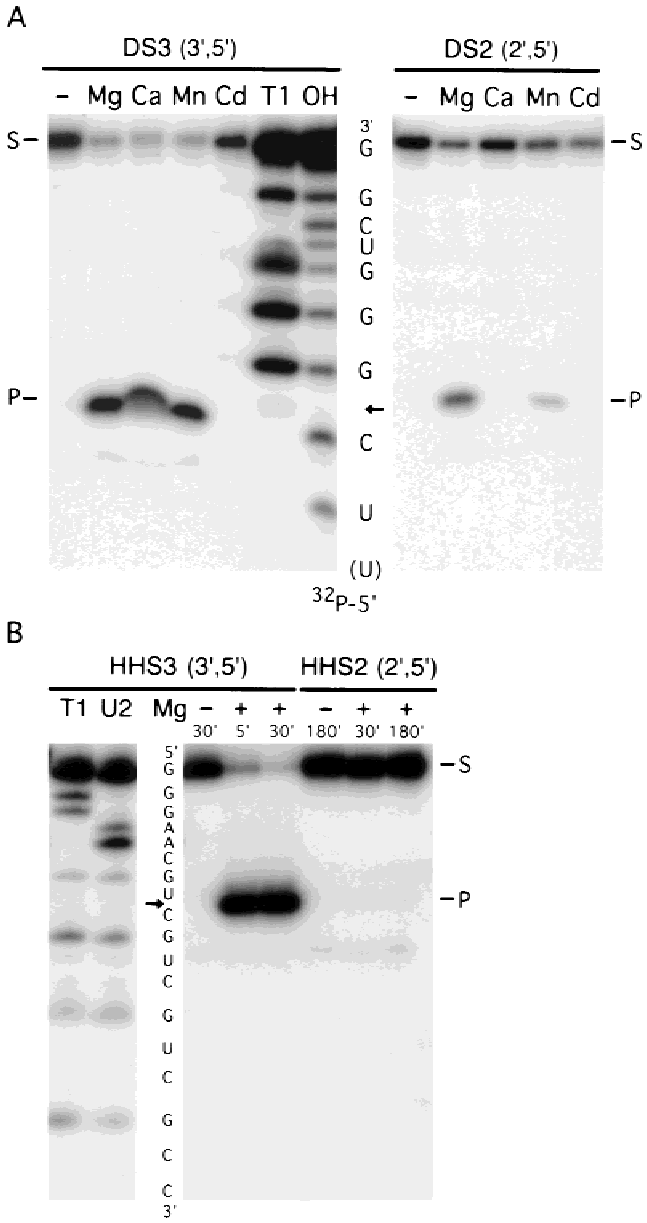

FIGURE 2. Cleavage reactions of *trans*-acting ribozymes ADC1 and HH16. **A**: *Trans* cleavage of DS3 (3′,5′-linked) and DS2 (2′,5′-linked) by ADC1. A trace amount of 5′-^{32}P-labeled DS2 or DS3 was pre-incubated with 1 μM ADC1, and the reactions were initiated by addition of different divalent metal ions as indicated. The cleavage reactions of DS3 and DS2 were terminated after 3 and 60 min, respectively, and fractionated by polyacrylamide gel electrophoresis. Control reactions (–), without any divalent metal ions added, were incubated at 37 °C for 3 or 60 min for DS3 or DS2, respectively. **B**: Cleavage of HHS3 (3′,5′-linked) and HHS2 (2′,5′-linked) by HH16. Single-turnover cleavage reactions of HH16 were initiated by mixing the ribozyme and 3′-end-labeled substrate at 37 °C and terminated at the indicated times. The cleavage sites are indicated by arrows. S and P indicate substrate and product, respectively. Marker lanes: T1 and OH^- contained T1 endonuclease and alkaline partial digests, respectively.

loss of substrate binding, the equilibrium dissociation constant of DS2 was estimated from the K_i measured in a competitive inhibition binding assay (Herschlag & Cech, 1990). The cleavage rate of DS2 is much slower than DS3; therefore, the cleavable substrate DS2 served as an inhibitor in the DS3 cleavage reactions (less than 2% of DS2 was cleaved during the inhibition experiment in $MgCl_2$). The K_i of DS2 was compared to a noncleavable substrate analog, DS10 (5′UUdC^GGG UCGG3′), which has a 3′,5′-linkage but contains a deoxynucleotide at the cleavage site to prevent cleavage. The results indicated that both inhibitors effectively competed with the normal substrate, and, although both have a slightly lower affinity for the HDV ribozyme in the presence of $CaCl_2$ (Table 2), it was apparent that the 2′,5′-linked substrate was binding to the ribozyme in $CaCl_2$ but was not being cleaved. These data suggested that the metal-ion preference in cleavage of the two linkages reflected specific interactions of divalent metal ions with the active site in the ribozyme.

To determine if cleavage of a 2′,5′-linkage could be a common property of small ribozymes, we also tested for hammerhead ribozyme cleavage of a 2′,5′-phosphodiester bond. The sequence of the hammerhead ribozyme used was the same as the well characterized hammerhead 16 (HH16) (Hertel et al., 1994; Fig. 1B). The 3′,5′-linked substrate was cleaved by HH16 with a rate constant of 0.92 min^{-1}, essentially the same as reported in the literature (Hertel et al., 1994; Fig. 2B). However, the maximal rate constants for hammerhead-catalyzed cleavage of the 2′,5′-linked substrate were estimated from a 48-h time course to be 3.5×10^{-5} and 5.7×10^{-5} in the presence of Mg^{2+} and Ca^{2+}, respectively. These rate constants are less than sixfold above the background rate constants for cleavage of these linkages in the absence of ribozymes. It has been reported that the hairpin ribozyme does not cleave a 2′,5′-linkage (Feldstein et al., 1990), so it appears that cleavage of the alternative linkage could be peculiar to the HDV ribozyme.

Self-cleavage of a 2′,5′-linkage

The linkage specificity of the HDV cleavage reaction was also tested in the context of a *cis*-acting ribozyme. Two self-cleaving sequences, LD3 (all 3′,5′-linkages) and LD2 (one 2′,5′-linkage at the cleavage site), were made by splint-facilitated ligation (Moore & Sharp, 1992) of substrates DS3 and DS2 to the 5′ end of the ADC1 ribozyme. These two self-cleaving ribozymes are slightly different from a wild-type ribozyme because of a dele-

TABLE 2. Inhibition constants of substrate and substrate analog.

Ribozyme	Substrate	Inhibitor	K_i in $MgCl_2$ (nM)	K_i in $CaCl_2$ (nM)
ADC1	DS3	DS2	10 ± 2	62 ± 7
ADC1	DS3	DS10	60 ± 4	154 ± 10

tion of three nucleotides: C8, A9, and U10 in the single-stranded region joining the 5′ sides of P1 and P2. The first-order rate constant for LD3 cleavage (0.46 min^{-1}) was close to the pseudo first-order rate constant for DS3 cleavage by ADC1 (0.71 min^{-1}; Fig. 3A and Table 1). In the presence of $MgCl_2$, the 2′,5′-linked LD2 cleaved with a first-order rate constant (0.060 min^{-1}) sevenfold greater than the pseudo first-order rate constant for DS2 cleavage in *trans* (Fig. 3B). Although the cleavage activity of LD2 in the presence of $CaCl_2$ appeared to be slightly greater than the background rate measured in the absence of divalent metal ions, this rate was still at least four orders of magnitude slower than the cleavage rate in $MgCl_2$. Thus, the *cis*-acting ribozymes behaved closely to what would be predicted based on results from the *trans* reactions.

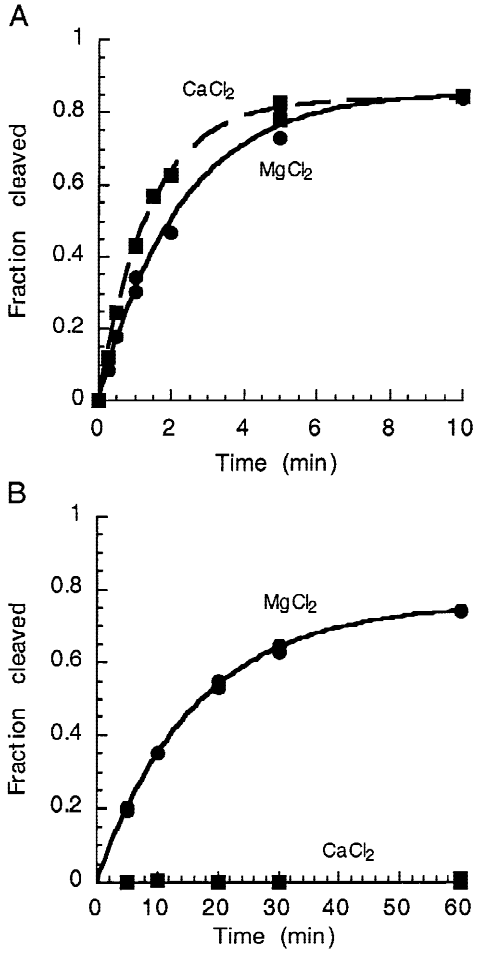

FIGURE 3. Self-cleavage reactions of HDV ribozymes LD3 and LD2. **A**: LD3 (3′,5′-linked) and **B**: LD2 (2′,5′-linked) in $MgCl_2$ (circles) or $CaCl_2$ (squares). Fractions of cleavage product are plotted against time and fit to a single exponential. The cleavage rate constants (k_{obs}, average of three determinations) for LD3 are 0.46 and 0.74 min^{-1} in $MgCl_2$ and $CaCl_2$, respectively. For LD2, the rate constant is 0.06 min^{-1} in $MgCl_2$ and $<10^{-5}$ min^{-1} in $CaCl_2$.

Cleavage products of DS3 and DS2 are the same

The mechanism for HDV cleavage of a 3′,5′-linkage is proposed to be an in-line nucleophilic attack at the scissile phosphodiester bond by the adjacent 2′-hydroxyl group (Kuo et al., 1988; Sharmeen et al., 1988). An analogous model for cleavage of a 2′,5′-linkage would use the 3′-hydroxyl group as the nucleophile for in-line attack and the reaction would still generate a 2′,3′-cyclic phosphate and a 5′-hydroxyl group. The presence of a 2′,3′-cyclic phosphate in the 5′ cleavage product would exclude the possibility that cleavage of a 2′,5′-linkage is a hydrolysis reaction in which a water molecule is the nucleophile. If that were the case, a 3′- or 5′-terminal phosphate should be generated. To verify the mechanism, 5′ cleavage products of both substrates were isolated and treated with HCl to open the predicted 2′,3′-cyclic phosphate to a 2′- or 3′-terminal phosphate. Untreated and acid-treated products were then fractionated by polyacrylamide gel electrophoresis (Fig. 4A) or thin-layer chromatography (Fig. 4B). Under these conditions, trinucleotides bearing a cyclic phosphate versus a terminal phosphate are separated on the basis of charge. Difference in migration of the products after acid treatment indicated that a 2′,3′-cyclic phosphate was generated by HDV cleavage of both the 3′,5′- and the 2′,5′-linkages.

Cleavage of both substrates shows a similar pH dependence

Although the HDV ribozyme did cleave a 2′,5′-linkage, it is also significant that the pseudo first-order rate constant for DS2 cleavage was 100-fold slower than DS3 in 10 mM $MgCl_2$ (Table 1). The rate constants had been measured under ribozyme-saturating conditions, where the rate limiting step was not substrate binding. Both the association and dissociation rate constants for substrate binding were faster than the first-order rate constant for the chemistry step (k_2) in $MgCl_2$ (I-h. Shih & M.D. Been, in prep.). Therefore, the slow cleavage of DS2 indicated either a higher energy barrier for the chemical conversion or a possible conformational change prior to the chemical step. The hypothesis that the chemical conversion step was rate limiting under these conditions was examined by measuring the pH dependence of the single-turnover cleavage reactions of DS3 and DS2 (Fig. 5). The pH profiles of the two substrates show nearly identical bell-shaped curves, suggesting that it is possible that the same protonation and/or deprotonation steps are involved in the rate-limiting step of both substrates. Although the apparent pK_as ($\sim$5.6 and 8.9; Fig. 5) have not been assigned to particular functional groups, this result provided evidence that the pseudo first-order rate constants for both substrates measured the same rate-limiting step.

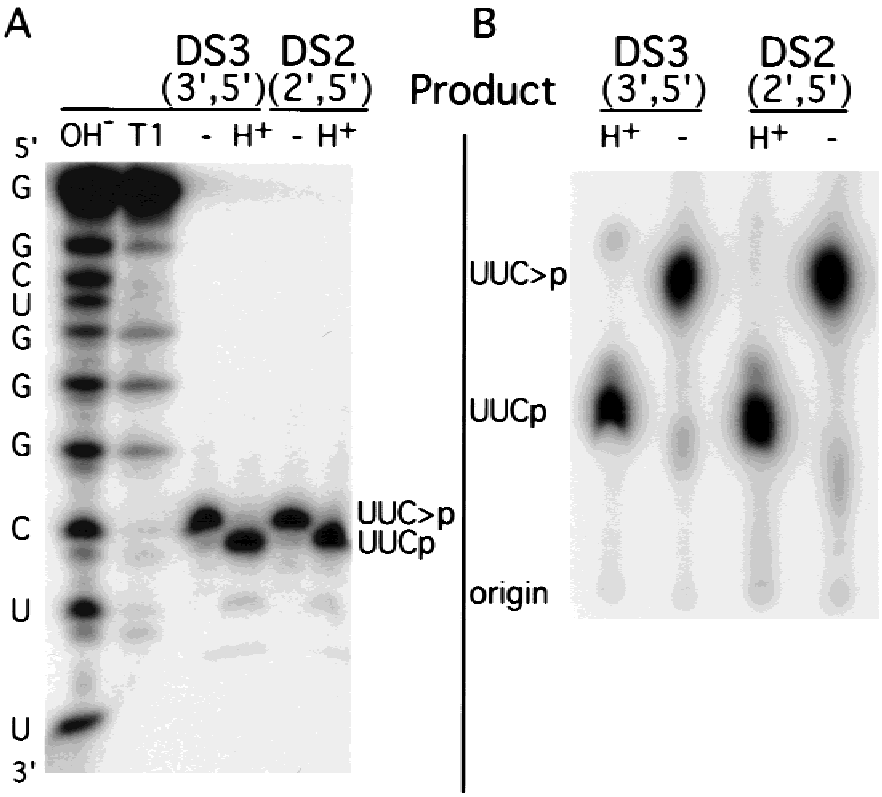

FIGURE 4. Characterization of 5′ cleavage products from DS3 and DS2. The 5′ products of DS3 and DS2 were treated with 0.1 M HCl to open the cyclic phosphate. The products with (H^+) and without (–) the acid treatment were fractionated by electrophoresis on a 20% polyacrylamide gel (**A**) and by thin-layer chromatography (**B**). Terminal phosphate and 2′,3′-cyclic phosphate are indicated as p and >p, respectively. Marker lanes: T1 and OH^- contained T1 endonuclease and alkaline partial digests of DS3, respectively.

DISCUSSION

HDV ribozyme cleavage of a 2′,5′-linkage draws attention to questions concerning the recognition of the scissile phosphodiester bond and the mechanism of cleavage by this ribozyme. We know that a single nucleotide 5′ to the cleavage site is sufficient for cleavage, but the identity of the base is not critical (Perrotta & Been, 1992). As a result, association of the nucleotide 5′ to the cleavage site by the substrate binding site is unlikely to depend on Watson–Crick base pairing for this specific interaction. The recently discovered P1.1 pairing in the genomic (Ferré-D'Amaré et al., 1998) and antigenomic ribozymes (Wadkins et al., 1999; Fig. 1) also argues against the possibility that the base 5′ to the cleavage site could pair to one of the Gs joining P1 to P4. It is possible that lack of a strict base-pairing requirement for the 5′ sequence results in some flexibility of the scissile phosphodiester linkage in forming essential interactions in the active site, and, thus, allows the HDV ribozyme to position a 2′,5′-phosphodiester bond and the adjacent 3′-OH group into a cleavable configuration.

Based on the nature of the cleavage products and the pH profile of the *trans* cleavage reactions, it appears that the mechanisms for cleavage of a 3′,5′- and a 2′,5′-linked substrate are similar. However, the difference in the linkage of the scissile bond does affect catalysis by the HDV ribozyme, in that a slower rate of chemical conversion (k_2) was seen for the 2′,5′-linkage. Thus, because both phosphodiester linkages have similar reactivity under the uncatalyzed conditions, the ribozyme does not reduce the activation energy required

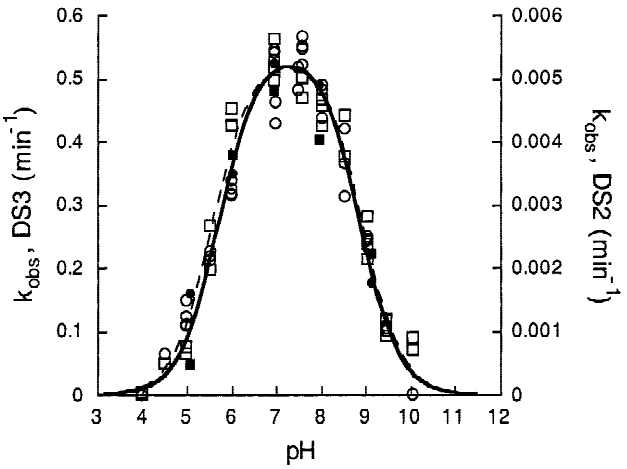

FIGURE 5. pH dependence of the cleavage rate constants of DS3 and DS2. Cleavage reactions of DS3 and DS2 were done in ribozyme-saturating conditions with $MgCl_2$. The pseudo first-order rate constants (k_{obs}) versus pH are plotted at different scales for DS3 and DS2. The pK_{a1} values for DS3 and DS2 are 5.7 (± 0.1) and 5.6 (±0.1), respectively; the pK_{a2} values for DS3 and DS2 are both 8.9 (±0.1). Open circles: DS3 with 1 μM ADC1; closed circles: DS3 with 5 μM ADC; open squares: DS2 with 1 μM ADC1; closed square: DS2 with 5 μM ADC1.

for cleavage of the 2′,5′-linkage to the same extent that it does for the 3′,5′-linkage. This implies that the active site does distinguish a 2′,5′- from a 3′,5′-linkage. How is the 2′,5′-linked substrate accommodated in the cleavage reaction? Of apparent critical importance for the reaction would be the need to activate the 3′-hydroxyl instead of the 2′-hydroxyl group for a nucleophilic attack on the phosphorus center. It is also important to note that, assuming an in-line mechanism for the cleavage reaction, the proposed pentacovalent transition-state intermediate would be different for the two reactions (Fig. 6). The most obvious difference is that the apical position occupied by the attacking nucleophile is the 2′-oxygen in one case and the 3′-oxygen in the other. In addition, the positions of the equatorial oxygens would also be different. Thus with the alternative substrate, the slower cleavage of the 2′,5′-linkage could be caused by a number of factors including those affecting nucleophile activation, shielding of negative charges, or protonation of the leaving group. Despite the 100-fold difference in the rate constants for cleavage, the similar pH dependence of the two reactions suggests that the same functional groups may be involved in catalysis.

The change in divalent metal-ion preference of HDV cleavage upon alteration of the linkage at the scissile bond supports the idea that the active site of the HDV ribozyme is formed in part by bound divalent metal ions. Metal ions could participate directly, either by contributing to binding of the substrate at the active site or by acting as cofactors and promoting chemical catalysis. In $CaCl_2$ the HDV ribozyme binds a 2′,5′-linked substrate as well as a 3′,5′-linked substrate analog containing a single deoxynucleotide at the cleavage site; thus, it appears that Ca^{2+} ions can still promote proper folding of the HDV ribozymes for binding of this substrate but do not support cleavage of the 2′,5′-linkage after binding. For ribozyme catalysis, it has been proposed that metal ions would be particularly useful to activate the nucleophilic attacking group or to stabilize negative charge developed in the transition-state intermediate on the phosphate oxygens and the leaving group (Piccirilli et al., 1993; Pyle, 1993; Weinstein et al., 1997). Several chemical and physical properties of metal ions, for example, pK_a, coordination number and geometry, softness and hardness, and ionic radius, could affect the extent of metal-ion catalysis in ribozymes. The difference in pK_a values between Mg^{2+} (pK_a =

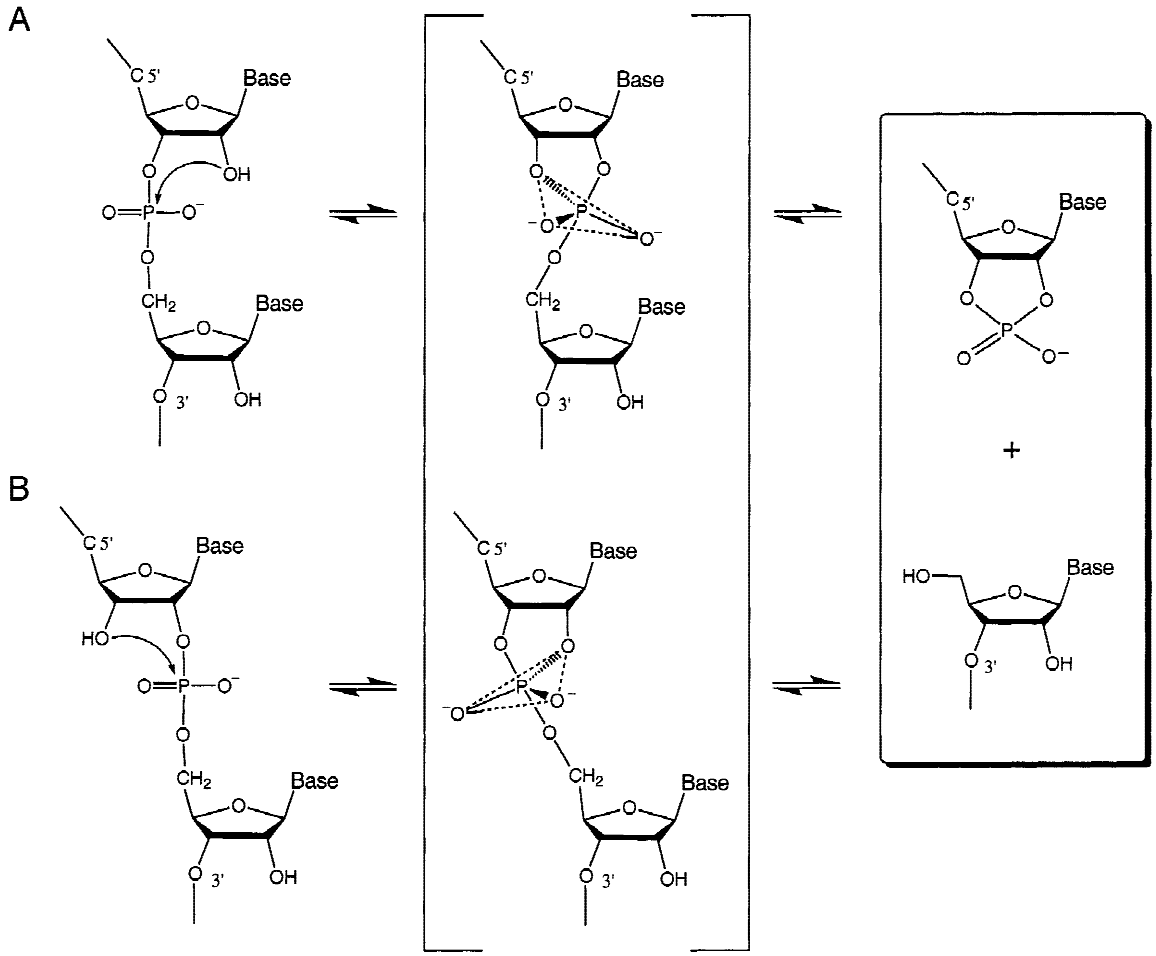

FIGURE 6. Proposed cleavage mechanisms for a 3′,5′-linkage (**A**) and a 2′,5′-linkage (**B**).

11.4) and Ca^{2+} ($pK_a = 12.6$) (pK_a values from Perrin & Dempsey, 1974) would not account for a 10^4-fold difference in the cleavage rate constants for the 2′,5′-linkage in those two metals. Also, the correlation of an increase in the rate constant with a decrease in the pK_a of the metal ion (Dahm et al., 1993) was not seen in the cleavage of the 3′,5′-linkage, where Ca^{2+} worked as well as, or slightly better than, Mg^{2+}. Therefore, assuming the metal ion plays the same role in cleavage of both substrates, the difference in pK_a values alone would not explain why Ca^{2+} ions would fail to activate the 3′-hydroxyl group in cleavage of a 2′,5′-linkage.

We presume that with the 2′,5′-linkage, a nonnative metal-ion binding site may be generated in both the ground state and transition state. As a result, the lack of activity with Ca^{2+} could be due to its different coordination number and geometry or its larger size, such that it cannot orient or fit properly in the putative metal-ion binding pocket to facilitate cleavage of a 2′,5′-linked substrate. This interpretation would be consistent with the finding that Mn^{2+}, as well as Mg^{2+}, can stimulate the cleavage of the 2′,5′-linkage. These same factors could interfere with metal-ion specificity regardless of its specific role in catalysis. Another possibility is that the difference of the metal-ion function in the cleavage mechanism of the two linkages is structural but not catalytic, and, as a result, the two reactions have different metal-ion requirements. One example of this would be if an additional metal-mediated conformational change prior to the chemical step is required for the 2′,5′-linked substrate. Although this explanation cannot be ruled out, the similar pH profiles of the cleavage rate constants for the two different linkages in Mg^{2+} weaken this argument.

Although most evidence has consistently supported a requirement for divalent metal ions in HDV ribozyme catalysis, it has been difficult to unravel a specific role for these metal ions (Suh et al., 1993; Fauzi et al., 1997). In addition, a crystal structure of the 3′ cleavage product of a genomic ribozyme did not reveal a metal ion in the catalytic pocket (Ferré-D'Amaré et al., 1998). The altered divalent metal-ion preference with cleavage of the 2′,5′-linkage, even in the absence of an understanding of the mechanism for the difference, provides strong evidence for an essential divalent metal-ion requirement.

It is unlikely that the HDV ribozyme cleavage of a 2′,5′-linkage is a biologically significant reaction even though 2′,5′ cleavage is not limited to the *trans* reaction. However, demonstration of self-cleavage of a 2′,5′-linkage is biologically important for at least two reasons. First, it argues against the possibility that a 2′,5′-linkage at the ligation junction in the circular viral RNA replication products would, alone, protect HDV genomic and antigenomic circles from recleavage. Second, this cleavage reaction could indirectly provide insight into the mechanism of ligation of linear replication products to form circles in vivo. If the circular viral RNA replication products are generated by an HDV ribozyme-catalyzed ligation of linear replication products through the reverse of the cleavage reaction, one would predict that there are some 2′,5′-linked as well as 3′,5′-linked circles. In our hands, the equilibrium of the cleavage reaction in vitro greatly favors cleavage over ligation; therefore, we cannot test for the production of a 2′,5′-linkage in that reaction. However, if 2′,5′-linked viral RNA circles were detected from infected cells, it could be taken as support for the ribozyme-catalyzed mechanism of circularization of replication products. Currently, the evidence of a cellular RNA ligase that catalyzes ligation of HDV replication products (Neel & Robertson, 1997), together with the evidence that the equilibrium of the ribozyme reaction favors cleavage, makes ribozyme-mediated ligation less attractive than a ligase-catalyzed reaction.

MATERIALS AND METHODS

Enzymes, chemicals, oligonucleotides, and plasmids

T7 RNA polymerase (provided by M. Puttaraju) was purified from an overexpressing clone provided by W. Studier (Davanloo et al., 1984). T4 DNA ligase was kindly provided by S.M. Crary. Restriction endonucleases, nucleotides, ^{32}P-labeled nucleotides, chemical reagents, and other enzymes were purchased from commercial suppliers. The HDV ribozyme sequences were transcribed from plasmids pADC1. Construction of pADC1 was reported previously (Perrotta & Been, 1992). Plasmid DNA was purified by CsCl/ethidium bromide equilibrium centrifugation.

Preparation of RNAs

HDV ribozymes ADC1 were prepared from plasmid DNA linearized with *Hin*dIII. In vitro transcriptions with T7 RNA polymerase were as described previously (Perrotta & Been, 1992). Following transcription, the RNA was purified by electrophoresis on a 6% polyacrylamide gel under denaturing conditions and eluted from gel slices. Concentrations were estimated from the base composition and extinction coefficients at 260 nm. The hammerhead ribozyme HH16 (Hertel et al., 1994) and substrate HHS3 were synthesized by T7 RNA polymerase transcription of partially duplex synthetic DNA templates (Milligan et al., 1987) and purified as described above.

Chemically synthesized oligonucleotide substrates

The RNA substrates DS3 (5′UUC(3′,5′)GGGUCGG3′), DS2 (5′UUC(2′,5′)GGGUCGG3′), and HHS2 (5′GGGAACGUC (2′,5′)GUCGUCGCC3′) were synthesized at Ribozyme Pharmaceuticals, Inc. (Boulder, Colorado). The different linkages were confirmed by enzymatic digestions: phosphodiesterase I (USB) digested DS3 and DS2 to completion; however, DS2 was resistant to cleavage by RNase A at the position of the

alternative linkage. The noncleavable substrate analog DS10 (5′UUdC(3′,5′)GGGUCGG3′), which contains one deoxynucleotide 5′ to the cleavage site, was purchased from Dharmacon Research, Inc. (Boulder, Colorado). The 2′-phosphoramadite was purchased from Glen Research.

The 5′-end labeled substrate RNA was prepared using polynucleotide kinase and [γ-^{32}P]ATP (Perrotta & Been, 1992). The 3′-end labeled substrate RNA was prepared using T4 RNA ligase and [5′-^{32}P]pCp (Perrotta & Been, 1990). The labeled oligonucleotides were purified on 20% polyacrylamide gels.

Preparation of self-cleaving sequences

The *cis*-acting ribozymes, LD3 and LD2, were prepared by ligation of DS3 and DS2, respectively, to the 5′ end of ADC1 with a splint oligodeoxynucleotide spanning 10 nt on each side of the ligation junction (Moore & Sharp, 1992). Equimolar amounts of ADC1, splint oligonucleotide, and DS3 (or DS2) were heated to 95 °C and slowly cooled to room temperature in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 50 mM NaCl to form the ternary complex. The ligation reaction was carried out in 1 mM ATP, 66 mM Tris-HCl (pH 7.5), 0.3 mM EDTA, 16.25 mM NaCl, 11 mM $MgCl_2$, 10 mM dithiothreitol, and 35% glycerol with 1 pmol of T4 DNA ligase at room temperature for 4 h. The ligated RNAs were purified on 4% polyacrylamide gels.

Kinetics

The basal cleavage rate constants of DS3 and DS2 in the absence of the ribozymes were measured at 40 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 11 mM $MgCl_2$ (or $CaCl_2$) at 37 °C from 5-day time courses. Single-turnover reactions for the *trans*-acting ribozymes were measured with saturating ribozyme and trace amounts of 5′-end labeled substrates under standard conditions of 40 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 11 mM $MgCl_2$ or $CaCl_2$ at 37 °C, unless otherwise specified. Solutions containing the ribozyme and substrate in 40 mM Tris-HCl (pH 8.0) and 1 mM EDTA were heated to 95 °C for 1 min, allowed to cool to room temperature, and then preincubated at 37 °C for 10 min. Cleavage reactions were initiated by adding a starting cocktail to yield a final concentration of 40 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 11 mM divalent metal ions ($MgCl_2$, $CaCl_2$). Aliquots were quenched either with an equal volume of stop mix (50% formamide and 50 mM EDTA) and fractionated on a 20% polyacrylamide gel, or with an equal volume of 0.1 M EDTA and fractionated on PEI (poly(ethylenimine)) plates with 1 M LiCl. *Cis*-acting ribozymes were heat treated in 40 mM Tris-HCl (pH 8.0) and 1 mM EDTA, and the cleavage reactions were started by addition of a starting cocktail as described above. Aliquots were terminated and fractionated on polyacrylamide gels or PEI plates for the 3′- or 5′-end labeled precursors, respectively. Radioactivity of substrate and product bands was quantified using a phosphorimager (Molecular Dynamics). Data were fit using KaleidaGraph data analysis/graphics application (Synergy Software). The pseudo first-order rate constants for single-turnover cleavage reactions were obtained by fitting the data to a single exponential: $F_t = F_\infty \times (1 - e^{-kt})$, where F_t and F_∞ are the fractions cleaved at time t and at the end point.

Inhibition constants of DS2 and DS10 in the cleavage reactions of DS3 were measured under single-turnover conditions, in which the ribozyme was in excess, but at a concentration that is subsaturating with respect to the substrate (Herschlag & Cech, 1990). Under these conditions, the second-order rate constant is k_{cat}/K_M. The cleavage reactions of DS3 were challenged by the inhibitor, DS2 or DS10, at various concentrations and the second-order rate constants were measured. The inhibition constants were obtained from the plot of k_{obs}/[ribozyme] versus [inhibitor] fitting with the equation: $(k_{cat}/K_M)_{obs} = (k_{cat}/K_M)/(1 + [\text{inhibitor}]/K_i)$.

Identification of a 2′,3′-cyclic phosphate

The 5′-end labeled substrates DS3 and DS2 were cleaved by ADC1 and fractionated on a 20% denaturing gel. The 5′ cleavage products were eluted and incubated in 0.1 M HCl or water (control) for 3 h at room temperature. The acid hydrolysis reaction was neutralized with an equal volume of 0.1 M NaOH. NaCl was added to the control reactions to a final concentration of 0.05 M. Reaction products were fractionated on a 20% polyacrylamide gel or on a PEI plate with 1 M LiCl and 0.1 M boric acid.

The pH profile of the cleavage reactions

The conditions for the cleavage reactions were the same (with $MgCl_2$) except for the buffer system, which was 25 mM acetic acid/25 mM MES (2-(N-morpholino)ethanesulfonic acid)/50 mM Tris (pH 4.0–8.0) or 50 mM MES/25 mM Tris/25 mM AMP (2-amino-2-methyl-1-propanol) (pH 7.0–10.0) instead of 40 mM Tris-HCl (Ellis & Morrison, 1982). The triple buffer systems were titrated with HCl or NaOH, and the ionic strength was maintained within 10% deviation from 0.5. The pH dependence of the observed cleavage rate constants was fit using the equation:

$$k_{obs} = k_2/(1 + 10^{\text{p}K_{a1}\text{-pH}} + 10^{\text{pH-p}K_{a2}}).$$

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