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Cleavage of Oligoribonucleotides by a Ribozyme Derived from the Hepatitis δ Virus RNA Sequence†

Anne T. Perrotta and Michael D. Been*
Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
Received September 25, 1991; Revised Manuscript Received November 5, 1991

ABSTRACT: A self-cleaving RNA sequence from hepatitis δ virus was modified to produce a ribozyme capable of catalyzing the cleavage of RNA in an intermolecular (trans) reaction. The δ-derived ribozyme cleaved substrate RNA at a specific site, and the sequence specificity could be altered with mutations in the region of the ribozyme proposed to base pair with the substrate. A substrate target size of approximately 8 nucleotides in length was identified. Octanucleotides containing a single ribonucleotide immediately 5′ to the cleavage site were substrates for cleavage, and cleavage activity was significantly reduced only with a guanine base at that position. A deoxyribose 5′ to the cleavage site blocked the reaction. These data are consistent with a proposed secondary structure for the self-cleaving form of the hepatitis δ virus ribozyme in which a duplex forms with sequences 3′ to the cleavage site, and they support a proposed mechanism in which cleavage involves attack on the phosphorus at the cleavage site by the adjacent 2′-hydroxyl group.

Hepatitis δ virus (HDV)[1] is a small single-stranded RNA virus that has been found in certain patients who are also infected with hepatitis B (Taylor, 1990). A self-cleaving sequence present in both the genomic RNA and the complementary antigenomic RNA may act to process the RNA during rolling circle replication of the viral RNAs (Kuo et al., 1988; Sharmeen et al., 1988; Wu et al., 1989). The HDV RNA, therefore, may be the first clear example of an autocatalytic RNA (ribozyme) that in its natural form functions in human cells. With the identification of self-cleaving sequences in the HDV RNAs (Kuo et al., 1988; Sharmeen et al., 1988; Wu et al., 1989), it was suggested that the HDV self-cleaving structure would have to represent a structural motif distinct from other self-cleaving RNAs (Hutchins et al., 1986; Forster & Symons, 1987; Hampel et al., 1990; Feldstein et al., 1990). Evidence in support of a potential secondary structure that is common to both the genomic and antigenomic self-cleaving sequences has been presented (Perrotta & Been, 1991; Rosenstein & Been, 1991). As with other self-cleaving RNAs, self-cleavage activity of the HDV RNA requires a divalent cation, and cleavage generates products containing a 5′-hydroxyl group and a 2′,3′-cyclic phosphate (Kuo et al., 1988; Wu et al., 1989).

Studies on the structure, substrate specificity, and kinetic mechanism of other autocatalytic RNAs have been expedited by converting the intramolecular reaction to intermolecular forms (Zaug & Cech, 1986; Zaug et al., 1986; McSwiggen

† This research was supported by a grant from the NIH (GM-40689). M.D.B. received support from the American Cancer Society (JFRA-233).

[1] Abbreviations: HDV, hepatitis δ virus; nt, nucleotide or nucleotides; PEI, poly(ethylenimine); TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid.

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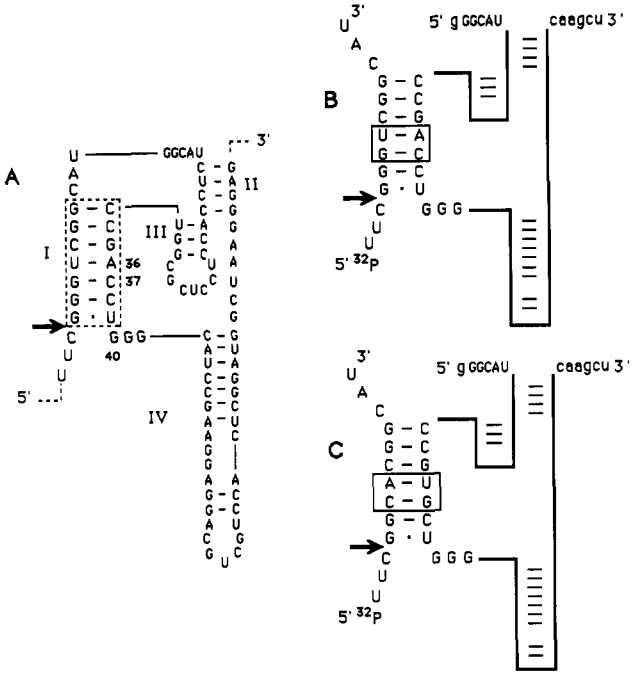

FIGURE 1: Sequences and potential secondary structures of HDV ribozymes. The sites of cleavage are indicated by the arrows. The self-cleaving sequence SA1-2 (A) is drawn as previously proposed (Perrotta & Been, 1991; Rosenstein & Been, 1991). Ribozymes ADC1 (B) and ADC2 (C) are drawn base paired with the substrates DHS1 and DHS3, respectively. The boxed region in (B) and (C) marks the regions where the sequences varied. In (B) and (C), lower-case letters are used to show sequences present in the transcripts that were contributed by the promoter or vector and are not considered to be part of the ribozyme.

& Cech, 1989; Herschlag & Cech, 1990a,b; Uhlenbeck, 1987; Ruffner et al., 1990; Fedor & Uhlenbeck, 1990; Dahm & Uhlenbeck, 1990; Feldstein et al., 1990; Hampel et al., 1990; Perreault et al., 1991). If the proposed model for the HDV self-cleaving structure (Figure 1A) is correct, it should be possible to design a trans-acting ribozyme in which substrate binding is specified by the duplex adjacent to the cleavage site (boxed region, Figure 1A). In this paper we show that a catalytic form of the hepatitis δ RNA, generated by removing the 5′ side of stem I, is capable of cleaving oligoribonucleotides at defined sequences. Using substrates of various sizes and sequence, we provide evidence that an intermolecular form of the stem I interaction, the cleavage-site duplex, is required for the trans reaction. The trans reaction was used to examine base and sugar requirements for the nucleotide directly 5′ to the site of cleavage.

Materials and Methods

Enzymes, Reagents, and Chemicals. T7 RNA polymerase was purified from an overexpressing clone kindly provided by W. Studier (Davanloo et al., 1984). Modified T7 DNA polymerase (Sequenase) was purchased from U.S. Biochemicals (Cleveland, OH). Restriction endonucleases and other enzymes used for the plasmid constructions, nucleotides, ^{32}P-labeled nucleotides, and chemicals were purchased from commercial sources. Formamide was recrystallized before using.

Plasmid Construction. The plasmids pSA1-2 and pSI5′3′ (Perrotta & Been, 1991) contained synthetic versions of the antigenomic self-cleaving element inserted downstream of a T7 promoter. pADC1 and pADC2 were generated from pSA1-2 and pSI5′3′, respectively, by oligonucleotide-directed deletion mutagenesis using a uracil-containing single-stranded form of the plasmids as the template (Kunkel et al., 1987; Vieira & Messing, 1987). The oligonucleotide (5′AGGAGGTGGAGATGCC-CTATAGTGAGTCGT) was complementary to a portion of the antigenomic sequence and to a portion of the T7 promoter. It was designed to delete the region from +2 relative to the T7 promoter to +10 relative to the cleavage site in the sequence of the self-cleaving element, thus removing the 5′ side of stem I in the proposed structure. Plasmids with the proper deletion were identified by sequencing miniprep DNA by primer extension with modified T7 DNA polymerase (Tabor & Richardson, 1987) and dideoxynucleotide chain terminators (Sanger et al., 1977). Following a second round of transformation and sequencing to ensure segregation of the deleted form, plasmid DNA was prepared from overnight cultures by boiling lysis and purified by CsCl equilibrium density centrifugation in the presence of ethidium bromide (Maniatis et al., 1982).

Preparation of Precursor RNA. Plasmid DNA was linearized with restriction endonuclease *Hin*dIII using conditions recommended by the supplier. The conditions used for transcription were 40 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, ribonucleoside triphosphates at 1 mM each, 0.1 mg/mL linear plasmid DNA, and 50 units of T7 RNA polymerase/μg of DNA. After 60 min at 37 °C, EDTA was added to 50 mM and formamide to 50% (v/v), and the RNA was fractionated by electrophoresis on an 8% (w/v) polyacrylamide gel containing 7 M urea. RNA was located by UV shadowing, excised, eluted overnight at 4 °C [in 10 mM EDTA, 0.1% (w/v) sodium dodecyl sulfate], and recovered by ethanol precipitation. Concentrations were estimated from the base composition and extinction coefficients at 260 nm.

Chemically Synthesized Oligonucleotide Substrates. The substrate RNAs (DHS1, UUC^GGGUCGGCAU; DHS2, UUC^GGGUCGG; DHS3, UUC^GGCACGGCAU; DHS4, C^GGGUCGG; DHS5, U^GGGUCGG; DHS6, A^GGGUCGG; DHS7, G^GGGUCGG) and the mixed oligonucleotide (DHS8, dC^rGGGUCGG) were supplied by U.S. Biochemicals, where they were chemically synthesized and deprotected and the bases checked for deprotection by HPLC. In our hands, each was gel purified and the sequence confirmed by enzymatic sequencing of 5′ ^{32}P-labeled material (Donis-Keller et al., 1977). Alkaline hydrolysis of DHS8 did not release a 5′-labeled mononucleotide, which was consistent with the presence of a 5′-deoxyribose, although the base at that position was not identified. Substrate oligonucleotides were radiolabeled in a 10-μL reaction containing 25 pmol of oligonucleotide, 25 pmol of [γ-^{32}P]ATP (7000 Ci/mmol), 50 mM Tris-HCl (pH 8.9 at 24 °C), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 10 units of T4 polynucleotide kinase; following incubation for 30 min at 37 °C, EDTA was added and the labeled oligonucleotide was gel purified. For some experiments, trace amounts of the labeled substrates were mixed with a known amount of the unlabeled oligonucleotide. The unlabeled substrate contained a 5′-OH group.

Gel Electrophoresis. Products were fractionated by electrophoresis on 20% polyacrylamide (bisacrylamide:acrylamide, 1:29) gels (0.7 mm thick × 19 cm wide × 22 cm high) containing 7 M urea, 0.1 M Tris–borate, pH 8.3, and 1 mM EDTA. Following electrophoresis, the gel was transferred to an acetate sheet and covered with plastic wrap and an autoradiogram prepared at −70 °C. To quantify results from gels, bands were located using the autoradiogram, excised, and quantified by measuring Cerenkov scintillation.

Thin-Layer Chromatography. PEI plates from EM Science (sold by VWR), were prewashed with H_2O and dried imme-

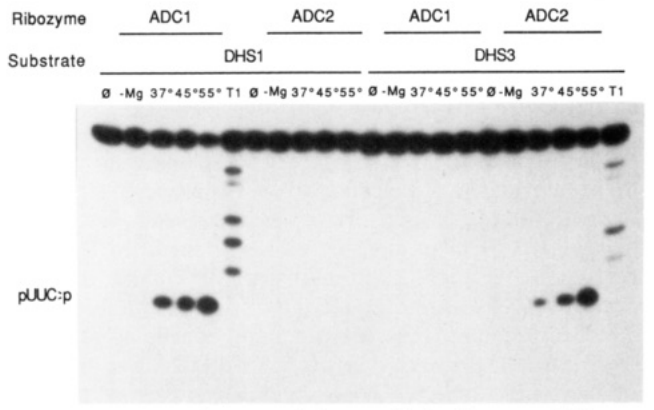

FIGURE 2: Trans cleavage of the matched substrate. Substrate oligonucleotides DHS1 and DHS3 (5′ end labeled) were incubated with either ADC1 or ADC2 at 37, 45, or 55 °C, as indicated. The reactions, containing 40 mM Tris-HCl (pH 8.0 at 25 °C), 1 mM EDTA, 11 mM $MgCl_2$, and 1.5 μM substrate, were initiated by the addition of ribozyme to 0.3 μM and then incubated at the indicated temperatures. The pH of the complete reaction varied from 7.7 at 37 °C to 7.4 at 55 °C. Reactions were terminated after 30 min by the addition of 10 μL of formamide containing 25 mM EDTA and fractionated by electrophoresis on a 20% polyacrylamide gel. Control reactions were incubated for 30 min at 55 °C in the absence of either the ribozyme (ϕ) or $MgCl_2$ (−Mg). Marker lanes (T1) contained T1 partial digests of the substrate oligonucleotide. The position of the end-labeled cleavage product (pUUC>p) is indicated.

diately before using. Samples (2 μL) were spotted 2 cm from the bottom edge of the plate. The solvent was 1 M LiCl. Quantitation was done using a Bioscan single-wire detector.

RESULTS

Sequence Specificity of a HDV-Derived Ribozyme. Using a plasmid containing a cloned synthetic version of the antigenomic self-cleaving sequence (pSA1-2) (Perrotta & Been, 1991), the portion of the sequence forming the 5′ end of the element was deleted, generating pADC1. In vitro synthesis with T7 RNA polymerase generated a *Hin*dIII runoff RNA lacking the 5′ side of stem I (nucleotides 5′ to position 10 were replaced by a single G in this transcript) (Figure 1B). A second version of the truncated sequence, pADC2, incorporated a mutation in the 3′ side of stem I (A36U, C37G; Figure 1C) (Perrotta & Been, 1991). RNAs transcribed from pADC1 and pADC2 (ADC1 and ADC2) were purified and tested for cleavage activity with two oligoribonucleotide substrates. Substrate DHS1 was a 13-mer; it contained the wild-type sequence from nt position −3 to +10 relative to the cleavage site and had the potential to form the postulated cleavage-site duplex with ADC1 RNA (Figure 1B). DHS1 would contain two mismatches in a similar interaction with ADC2. The substrate DHS3, relative to DHS1, contained two base changes, a G to C at position 3 and a U to A at position 4 so that it would contain two mismatches with ADC1 but could form a cleavage-site duplex with ADC2 (Figure 1C). Each substrate was 5′ end labeled with ^{32}P and incubated with either ADC1 or ADC2. Cleavage of either substrate at the correct site would release a 5′-end-labeled trinucleotide, [^{32}P]UUC. In 10 mM Mg^{2+} at 37, 45, and 55 °C, DHS1 was cleaved by ADC1 but not by ADC2, while DHS3 was cleaved by ADC2 but not by ADC1 (Figure 2). Thus, under these conditions, each form of the ribozyme cleaved only the "matched" substrate with which it could form Watson–Crick base pairs. The accuracy of the cleavage reaction was confirmed by analyzing the cleavage products on a sequencing gel adjacent to T1 and alkaline hydrolysis ladders of the end-labeled substrates (data not shown; however, see Figure 4 below). With an internally

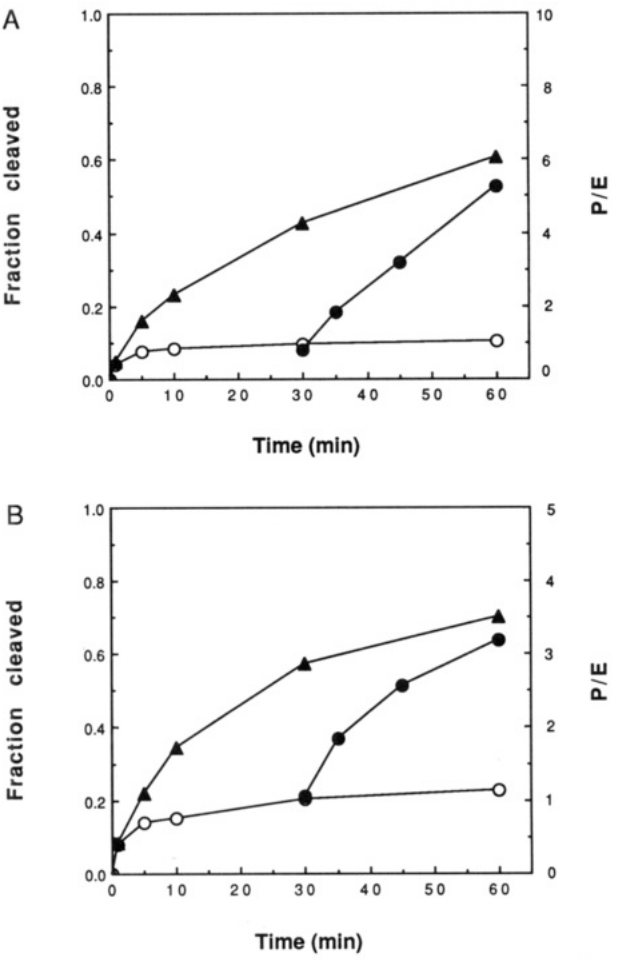

FIGURE 3: Ribozyme turnover at 55 °C. (A) Substrate ([5′-^{32}P]-DHS1) and ribozyme (ADC1) were preincubated separately for 3 min at the reaction temperature in 40 mM Tris-HCl, 1 mM EDTA, and 11 mM $MgCl_2$ (pH 7.7 at 37 °C, pH 7.4 at 55 °C) and then mixed to start the reaction. After mixing, the concentration of DHS1 was 2 μM and the concentration of ADC1 was 0.2 μM. Samples (5 μL) were removed at the indicated times, quenched with an equal volume of formamide containing 25 mM EDTA, and fractionated by electrophoresis on a 20% polyacrylamide gel. Labeled substrate and product bands were quantified and the results expressed both as the fraction of the total radioactivity in each lane present in the product and as the moles of product generated per mole of ribozyme (P/E). At 55 °C, 90–92% of the substrate can be cleaved; the data have not been corrected for this end point. Symbols: (▲) reaction at 55 °C; (○) reaction at 37 °C; (●) reaction incubated at 37 °C and then shifted to 55 °C after 30 min. (B) The experiment was done as in (A), except the ribozyme concentration was increased to 0.3 μM and the substrate concentration was reduced to 1.5 μM.

labeled substrate, made by transcription from synthetic templates (Milligan et al., 1987), both 5′ and 3′ products were observed (data not shown).

Ribozyme Turnover. With a 10-fold excess of substrate (DHS1) to ribozyme (ADC1), approximately 60% of the substrate was cleaved in 60 min at 55 °C (Figure 3A, solid triangles), indicating that 6 mol of substrate/mol of ribozyme was cleaved. However at 37 °C, the portion of substrate that was cleaved plateaued at about 10% (open circles). The extent of the reaction at 37 °C could represent a single-cleavage event per molecule of ADC1. Consistent with that interpretation, increasing the ratio of ADC1 to DHS1 at 37 °C resulted in a larger fraction of the substrate being cleaved, but it still plateaued at approximately 1 mol of product/mol of ribozyme (Figure 3B, open circles). If, following a 30-min incubation at 37 °C, the reaction was shifted to 55 °C, cleavage activity resumed (Figure 3, closed circles), indicating that the ribozyme

had not been inactivated during the incubation at 37 °C. Addition of free ribozyme after 30 min at 37 °C resulted in additional cleavage (data not shown), indicating that the substrate was still in an available form. Preincubation of the ribozyme at 55 °C or denaturation of the ribozyme by heat prior to addition of Mg^{2+} (Walstrum & Uhlenbeck, 1990) did not result in increased activity at 37 °C (data not shown).

Preferred Substrate Target Size Is Consistent with the Proposed Cleavage Site Duplex. To evaluate the extent to which the proposed cleavage-site duplex (stem I) might contribute to substrate binding, the effect of varying substrate size was examined. DHS1 was 5′ end labeled with ^{32}P, gel purified, and then subjected to partial hydrolysis to generate a ladder of end-labeled fragments when displayed on a sequencing gel. Incubation of the mixture of end-labeled fragments with excess ADC1 in 10 mM Mg^{2+} resulted in cleavage of the fragments which were 10 nt or longer (Figure 4A, lanes 9 and 10), indicating that at least 7 nt 3′ to the cleavage site were required under these conditions. Raising the Mg^{2+} concentration to 50 mM did not reduce the size requirement (data not shown), but lowering the Mg^{2+} concentration to 2 mM (lanes 7 and 8) or adding urea to 2 M (lanes 11 and 12) reduced activity. This experiment identified those substrate fragments which were cleaved rapidly; it would not reveal a low level of cleavage of the smaller fragments. However, because the experiment was done in ribozyme excess, it is unlikely that the shorter fragments were simply competed from the binding site by the longer fragments, and therefore it should present a fairly accurate picture of the requirements 3′ to the cleavage site.

The requirements 5′ to the cleavage site were examined in a similar manner; a 10 nt long substrate, DHS2, was 3′ end labeled with [5′-^{32}P]pCp and the analysis repeated (Figure 4B). The labeled substrate (5′UUC^GGGUCGGp*Cp, where p* is the labeled phosphate) contained 8 nt 3′ to the cleavage site, and in the presence of Mg^{2+}, substrates which were 9 nt or longer were cleaved by ADC1 to generate an 8 nt long labeled product (lanes 7–9). These data indicated that a single nucleotide 5′ to the cleavage site was sufficient for substrate binding and cleavage. This was consistent with an earlier finding with the genomic self-cleaving sequence which demonstrated that one nucleotide 5′ to the cleavage site was sufficient for self-cleavage (Perrotta & Been, 1990).

Base and Sugar Requirements for the Nucleotide 5′ to the Cleavage Site. Octanucleotides of the sequence 5′N^GGGUCGG, where N was either riboC, U, A, G, or deoxyC, were 5′ end labeled with ^{32}P and tested for cleavage by ADC1. Release of [5′-^{32}P]nucleoside 5′-phosphate 2′,3′-cyclic phosphate was monitored by thin-layer chromatography (Figure 5A). Oligonucleotides with 5′ rC, rU, and rA were cleaved at similar rates and to similar extents under the conditions tested (Figure 5B). The oligonucleotide with rG was cleaved less efficiently, approximately 10-fold slower, even when 4-fold higher ribozyme concentrations were used (Figure 5B). With a deoxyribose at the −1 position, no cleavage was detected (Figure 5A).

Discussion

For the HDV-derived ribozyme and substrates used in this study, the data indicate a target size of 7–8 nt under the conditions tested. This is slightly longer than the 4–6-nt target for the *Tetrahymena* intron-derived endoribonucleases (Zaug et al., 1986; Murphy & Cech, 1988) but shorter than the 12–15-nt targets generally used for hammerhead and hairpin ribozymes (Haseloff & Gerlach, 1988; Fedor & Uhlenbeck, 1990; Hampel et al., 1990; Feldstein et al., 1990; Chowrira & Burke, 1991). It is not yet clear to what extent this target

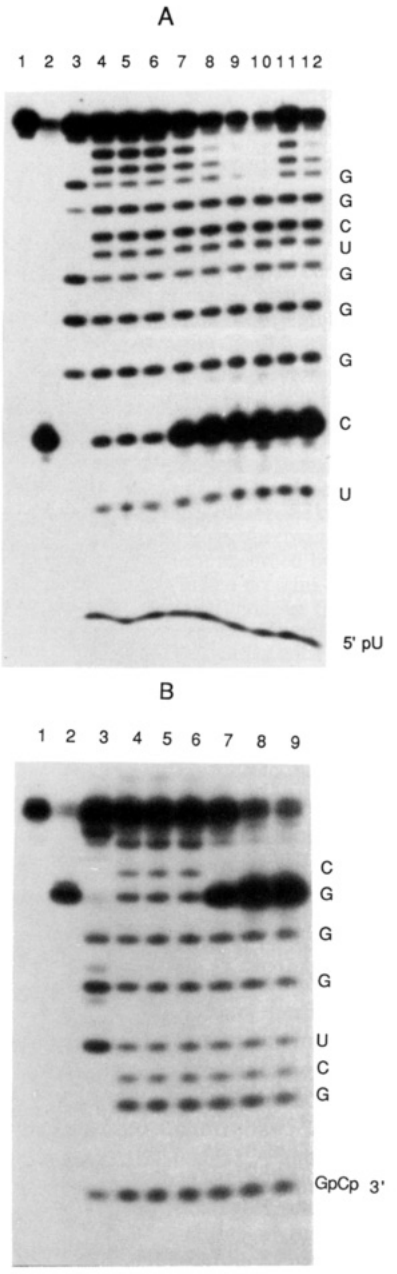

FIGURE 4: Estimation of the substrate target size. (A) Requirements 3′ to the site of cleavage. An alkaline hydrolysis generated partial digest of the 5′-end-labeled substrate oligonucleotide, DHS1 (lane 4), was incubated with 0.3 μM ADC1 at 50 °C in 0 mM (lane 6), 2 mM (lanes 7 and 8), or 10 mM Mg^{2+} (lanes 9–12) for 5 min (lanes 7, 9, and 11) or 30 min (lanes 8, 10, and 12). In addition to the Mg^{2+}, which was used to initiate the cleavage, reactions shown in lanes 5–12 contained 30 mM Tris-HCl/7 mM sodium bicarbonate (pH 7.5) and 0.5 mM EDTA; reactions shown in lanes 11 and 12 also had 2 M urea. The amount of total substrate in each reaction was estimated to be less than 25 nM. Samples were prepared for electrophoresis by mixing 5 μL of the reaction with an equal volume of formamide containing 25 mM EDTA. Products were fractionated on a 20% polyacrylamide/7 M urea gel; an autoradiogram of the gel is shown. Markers and controls: lane 1, labeled DHS1 untreated; lane 2, DHS1 cut by ADC1 in 10 mM Mg^{2+} for 10 min at 50 °C; lane 3, T1 partial digest of DHS1; lane 5, the alkaline digest incubated at 50 °C for 30 min in 10 mM Mg^{2+} without ribozyme. (B) Requirements 5′ to the site of cleavage. An alkaline generated partial digest of 3′-end-labeled DHS2 (UUC^GGGUCGGp*Cp) (lane 4) was incubated at 50 °C with 0.3 μM ADC1 in 0 mM (lane 6), 2 mM (lane 7), 10 mM (lane 8), or 20 mM Mg^{2+} (lane 9). The reactions were terminated after 5 min by the addition of an equal volume of formamide containing 25 mM EDTA. Reaction conditions were otherwise as described for (A). Markers and control lanes 1–3 and 5 were the equivalent of those described above. The conditions used for 3′ labeling and partial digestion by alkali or T1 have been described previously (Perrotta & Been, 1990, 1991).

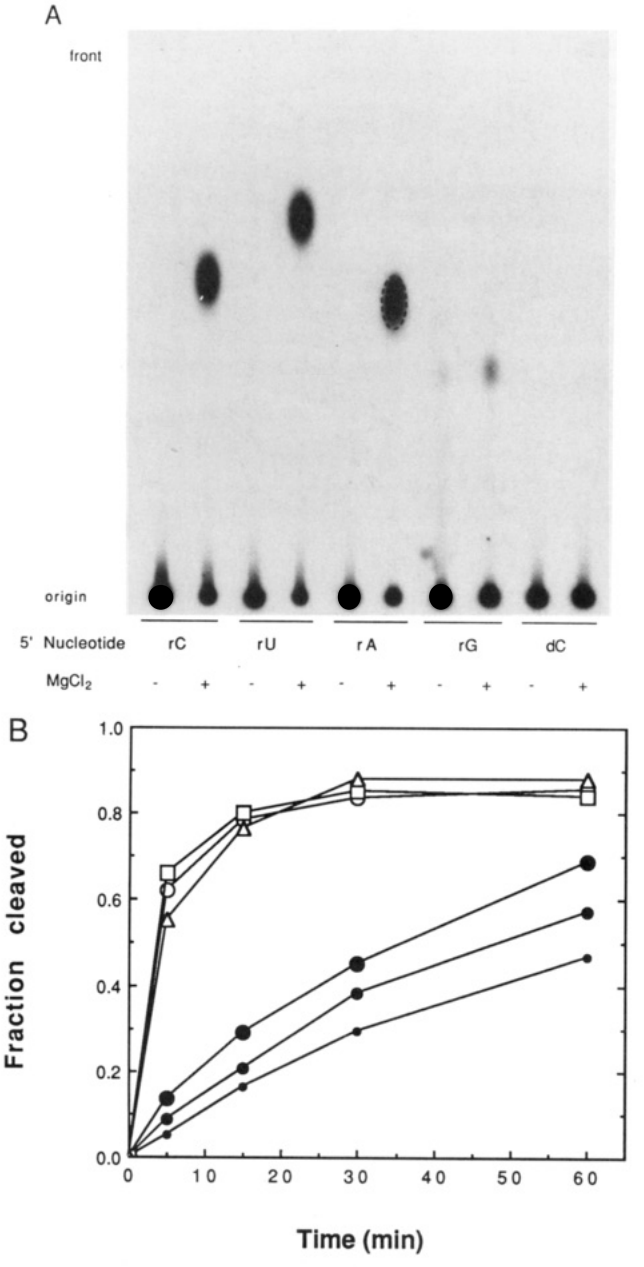

FIGURE 5: Effect of changes to the nucleotide at the position 5′ to the cleavage site. (A) Trace amounts of oligonucleotides of the sequences [5′-^{32}P]pN^GGGUCGG, where N is the nucleotide indicated in the figure, were incubated in 10-μL reactions at 55 °C in 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 μM ADC1, with and without 11 mM $MgCl_2$ as indicated. The ribozyme was added last. After 5 min, 2.5 μL of 0.1 M EDTA was added to stop the reaction, and 2 μL from each reaction was fractionated on PEI plates. An autoradiogram of the plate is shown. The position of the adenosine 5′-phosphate 2′,3′-cyclic phosphate marker is indicated by the dashed oval. (B) Reactions were as described above except the concentration of ADC1 was varied in the reaction with DHS8 (5′G). The PEI plate was prespotted with 2.5 μL of 0.1 M EDTA at the origins, and at the indicated times, 2 μL of the reaction was removed and spotted directly onto the PEI plate to stop the reaction. Symbols: (○) DHS4 (5′C) with 1 μM ADC1; (□) DHS5 (5′U) with 1 μM ADC1; (△) DHS6 (5′A) with 1 μM ADC1; (·, •, ●) DHS8 (5′G) with 1, 2, or 4 μM ADC1. Values were not adjusted for the final extent of the reaction.

size can be modified by changes in the reaction conditions or by alterations to the ribozyme. The data would indicate that specificity is strongly influenced by Watson–Crick base pairing between the substrate and the ribozyme. As such, it may be expected that reaction conditions or sequence changes within the cleavage-site duplex, which would affect the stability of that interaction, could influence target size as well. Structural constraints may also limit target size. A 7–8 base-pair duplex would require more than half a turn in an A-form helix; this may, depending on the overall structure and folding (or assembly) pathway of the ribozyme and substrate, present a barrier to binding long substrates. While such an effect has yet to be demonstrated, it could provide a mechanism for enhancing the fidelity of sequence-specific cleavage by increasing the K_d for substrates (Herschlag, 1991).

Evidence for base pairing at two positions within the cleavage-site duplex (position 3 with 37 and position 4 with 36) has been presented (Figures 1 and 2). The results with the trans reaction are consistent with those obtained by mutagenesis of the equivalent positions in the self-cleaving RNA (Perrotta & Been, 1991). The potential for a GU base pair (1G:39U) at the base of the duplex is suggested but not proven; mutations at either position reduced self-cleavage activity and substitutions that might generate Watson–Crick base pairs do not restore full self-cleavage activity (Perrotta and Been, unpublished results). For either the antigenomic sequence (Figure 1A) or the genomic sequence, in which there is a U at position −1, it would be possible to extend stem I to include a base pair (CG or UG) involving the nt at position −1 and a G at position 40. Results from the trans reaction indicate that only a G at position −1 substantially decreased cleavage. These data were consistent with results obtained from mutagenesis of the self-cleaving form of the ribozyme, in which a G at the −1 position also resulted in slow cleavage (Perrotta and Been, unpublished data), but do not answer questions concerning the nature of potential interactions with the base at that position. In a study on sequence requirements in the hammerhead (Ruffner et al., 1990), a G immediately 5′ to the cleavage site also resulted in the least activity.

Turnover of the HDV-derived ribozyme with the substrate DHS1 is essentially nonexistent at 37 °C and is even low at 55 °C.[2] With the *Tetrahymena* intron-derived ribozyme, Herschlag and Cech (1990a,b) have shown that turnover is limited by product dissociation. A similar explanation for the low turnover with ADC1 would be consistent with a stable cleavage-site duplex. However, alternative models, such as one in which conformational changes in the ribozyme were required between successive cleavages, might also slow turnover. Experiments are underway to distinguish between these possibilities.

The trans reaction was used to test a prediction of the model for the mechanism of cleavage. Self-cleavage of HDV RNA generates a 2′,3′-cyclic phosphate and a 5′-OH (Wu et al., 1989), suggesting that cleavage occurs by a transesterification mechanism, involving attack on the phosphorus at the cleavage site by the adjacent 2′-OH or O^- (Wu et al., 1989). If that mechanism is correct, it predicts that removal of the hydroxyl group from that 2′ position would prevent cleavage. The lack of cleavage of the substrate missing the 2′-hydroxyl group therefore provides additional evidence for the transesterification mechanism. These results are consistent with findings for other self-cleaving RNAs (Dahm & Uhlenbeck, 1990; Yang et al., 1990; Chowrira & Burke, 1991). We expect that the trans form of the cleavage reaction will facilitate additional studies

[2] With ADC1 and DHS1 at 55 °C, the second-order rate constant measured under single-turnover conditions [k_c/K_m = 4.7 (±0.2) × 10^6 M^{-1} min^{-1}] is about 10-fold higher than that measured for multiple turnover [k_{cat}/K_m = 0.46 (±0.05) × 10^6 M^{-1} min^{-1}] (Perrotta, Fierke, and Been, unpublished data). Within experimental error, the K_m was the same, 0.5–0.7 μM. This suggested that, even at 55 °C, there is a rate-limiting step which follows cleavage.

on the structure and mechanism of the δ ribozyme.

ACKNOWLEDGMENTS

We thank S. Rosenstein and A. Kendall for their comments on the manuscript and C. Fierke for comments and discussion.

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