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Accelerated Publications

Self-Cleavage of Hepatitis Delta Virus Genomic Strand RNA Is Enhanced under Partially Denaturing Conditions†

Sarah P. Rosenstein and Michael D. Been*
Department of Biochemistry, Box 3711, Duke University Medical Center, Durham, North Carolina 27710
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ABSTRACT: Self-cleavage of a polyribonucleotide containing an autocleaving sequence from the genomic strand of hepatitis delta virus was enhanced by conditions that destabilized RNA structure. Self-cleavage of the transcripts used in this study required Mg^{2+} (or another divalent cation), and in the absence of denaturants, maximum cleavage was observed at very low Mg^{2+} concentrations (0.05–0.1 mM). However, at 37 °C and in the presence of 2–10 mM Mg^{2+} the rate of cleavage was increased as much as 50-fold with the addition of urea to 5 M or formamide to 10 M. Cleavage was prevented by higher concentrations of the same reagents (9.5 M urea or 22.5 M formamide), presumably because a structure required for self-cleavage is disrupted by strongly denaturing conditions. In contrast to a previous report [Wu, H.-N., & Lai, M. M. C. (1989) *Science 243*, 652–654], we find that chelating Mg^{2+} with EDTA terminates the cleavage reaction without promoting measurable amounts of ligation of the cleavage products. The ability of denaturants to promote rapid self-cleavage in vitro raises the possibility that an unidentified factor could have a similar effect in vivo.

Specific sequences of RNA can fold to mediate both intramolecular catalysis, such as self-splicing (Kruger et al., 1982; Cech & Bass, 1986; Peebles et al., 1986; van der Veen et al., 1986) or self-cleavage (Prody et al., 1986; Hutchins et al., 1986; Buzayan et al., 1986; Forster & Symons, 1987; Epstein & Gall, 1987), and the catalytic cutting or joining of other RNA substrates (Guerrier-Takada et al., 1983; Zaug & Cech, 1986; Zaug et al., 1986). Self-cleaving domains of RNA may represent examples of the smallest and least embellished RNA structures that mediate cleavage reactions (Forster & Symons, 1987). Several self-cleaving RNAs have been harbingers of small catalytic RNAs which have been engineered to site specifically cleave substrate RNAs (Uhlenbeck, 1987; Haseloff & Gerlach, 1988; Hampel & Tritz, 1989). A common and well-characterized motif of secondary structure for self-cleaving RNAs is the hammerhead (Hutchins et al., 1986), a three-stemmed structure that can be drawn for sequences flanking the cleavage sites of certain single-stranded RNA plant pathogens (Forster & Symons, 1987) and the transcript of a newt satellite DNA (Epstein & Gall, 1987). However, many other RNA structures are also

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capable of self-cleavage. Well-characterized examples include certain tRNAs (Werner et al., 1976; Brown et al., 1983) and the minus strand of the satellite of tobacco ringspot virus (sTRSV)[1] (Forster & Symons, 1987; Hampel & Tritz, 1989). Most recently, self-cleaving sequences which do not conform to the common hammerhead motif have been found in the hepatitis delta virus (HDV) genomic RNA (Kuo et al., 1988a; Wu et al., 1989) and antigenomic RNA (Sharmeen et al., 1988).

It has been hypothesized that RNA self-cleavage and possibly autoligation function in the replication of certain small infectious RNAs of plants (Branch & Robertson, 1984). It has been suggested that HDV may replicate by a mechanism similar to that of the infectious RNAs of plants (Kuo et al., 1988b; Makino et al., 1987; Wang et al., 1986). As with many self-cleaving RNAs, cleavage of the HDV RNAs results in the formation of a 5′-hydroxyl group and a 2′,3′-cyclic phosphate at the break (Kuo et al., 1988a; Sharmeen et al., 1988; Wu et al., 1989), suggesting that cleavage of the phosphodiester backbone occurs by a transesterification reaction. Therefore, the energy necessary to regenerate the 3′,5′-phosphodiester linkage has been conserved, and autoligation may be possible. However, outside of the self-splicing RNAs, demonstration of RNA-mediated religation of cleavage products is rare. Autoligation that may be physiologically relevant has been demonstrated for the minus strand of sTRSV (Buzayan et al., 1986) but not for most of the hammerhead self-cleaving RNAs. In one exception, hammerhead RNA cleavage products incubated in the presence of Zn^{2+} for extended times at 4 °C did produce some ligated product (Prody et al., 1986). Under conditions in which oligo(adenylic acid) containing 2′,3′-cyclic phosphate and 5′-hydroxyl groups will ligate in the presence of poly(uridylic acid) (Usher & McHale, 1976), Sharmeen et al. (1989) demonstrated religation of the cleavage products generated from the antigenomic HDV sequence. This reaction required incubation at 4 °C in 1 M ethylenediamine in the presence of a partially complementary polyribonucleotide and may have resulted in a mixture of products containing 2′,5′- and 3′,5′-phosphodiester bonds. Recently, Wu and Lai (1989) reported interesting results which were interpreted to demonstrate the rapid and efficient reversal of self-cleavage with HDV genomic strand RNA. In their experiments, chelating Mg^{2+} with excess EDTA, prior to denaturing the RNA in the presence of urea, resulted in less cleavage than if the sample was similarly treated but without addition of EDTA. The authors suggested that the RNA was cleaved in the reaction prior to denaturation and that chelating the Mg^{2+} with EDTA altered the structure of the RNA and promoted rapid religation. A distinctly different explanation is that additional cleavage occurred in the presence of urea if Mg^{2+} was present but not if it was chelated. We have examined conditions that promote self-cleavage and present evidence that, in the presence of Mg^{2+}, denaturants such as urea and formamide can significantly increase the rate of self-cleavage of this particular RNA sequence. Therefore, it is likely that the question of religation remains open.

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). Restriction endonuclease *Eco*RI was a gift from P. Modrich. Other restriction endonucleases, nucleotides, and ^{32}P-labeled nucleotides were purchased from commercial sources. Formamide was recrystallized before use.

Plasmid DNA. The plasmid pDM1X was constructed and kindly provided to us by M. Belinsky and G. Dinter-Gottlieb. It contains 135 nucleotides (nt) of HDV sequence (Kuo et al., 1988b), positions 651–786 on the map of Wang et al. (1986), inserted into the *Pst*I/*Xba*I sites of pGEM4Z (Promega) such that transcription with T7 RNA polymerase yields a transcript with 19 nt of vector-derived sequence at the 5′ end followed by the genomic HDV sequence and vector-derived sequence at the 3′ end, the length of which depends on the restriction enzyme site used. *Escherichia coli* (strain JM83) was transformed with pDM1X and 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 (*Xba*I, *Bam*HI, or *Eco*RI), and the reaction was stopped by addition of EDTA to 20 mM. The products were extracted once with buffered phenol and once with chloroform, and the DNA was recovered by ethanol precipitation. The conditions used for transcription were 40 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 2 mM spermidine, ribonucleoside triphosphates at 1 mM each, 0.5 mCi/mL [α-^{32}P]GTP, 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 a 6% (w/v) polyacrylamide gel containing 7 M urea. RNA bands were located by UV shadowing or autoradiography, and the precursor band was excised (10–30% of the RNA cleaved during the transcription). The RNA was eluted overnight at room temperature in an elution mix containing 0.75 M ammonium acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate, and the RNA was recovered from the eluate by ethanol precipitation. Plasmid DNA cut with *Xba*I yields a transcript with no 3′ vector sequence, *Bam*HI cutting adds 6 nt, and *Eco*RI cutting adds 27 nt of vector sequence at the 3′ end. This precursor RNA is very similar to HN3-24 used by Wu and Lai (1989) but may differ in the flanking vector-derived sequences and at one position (underlined below), which is a G in the Wu and Lai construct (Makino et al., 1987). Self-cleavage occurs between nucleotides at position 685/686 (Kuo et al., 1988a), which corresponds to position 688/689 in the numbering system used by Wu et al. (1989) (Makino et al., 1987). The sequence and site of cleavage have been confirmed by sequencing the DNA, the precursor RNA, and RNA cleavage products (Perrotta and Been, unpublished experiments). Products of self-cleavage are a 54-nt 5′ fragment and a 3′ fragment that is 100, 106, or 127 nt long, depending on the runoff. The complete sequence of the *Eco*RI runoff is 5′ GGGAGACAAG CUUGCAUGCC UGCAGGGUCC GCGUUCCAUC CUUUCUUACC UGAU∧ GGCCGGCAUG GUCCCAGCCU CCUCGCUGGC GCCGGCUGGG CAACAUUCCG AGGGGACCGU CCCCUCGGUA AUGGCGAAUG GGACCCACAA AUCUCUCUAG /AGGAUC/CCCG GGUACCGAGC UCGAAUU/, where small capitals indicate vector sequence, capitals are HDV sequence, the caret (∧) is the site of self-cleavage, and the slashes are the ends of the transcripts made from *Xba*I-, *Bam*HI-, or *Eco*RI-cut DNA, respectively.

Gel Electrophoresis. All products were fractionated by electrophoresis on 6% polyacrylamide [bis(acrylamide):acrylamide, 1:29] gels (0.7 mm thick × 19 cm wide × 22 cm

[1] Abbreviations: HDV, hepatitis delta virus; sTRSV, satellite of tobacco ringspot virus; nt, nucleotides; EDTA, ethylenediaminetetraacetate.

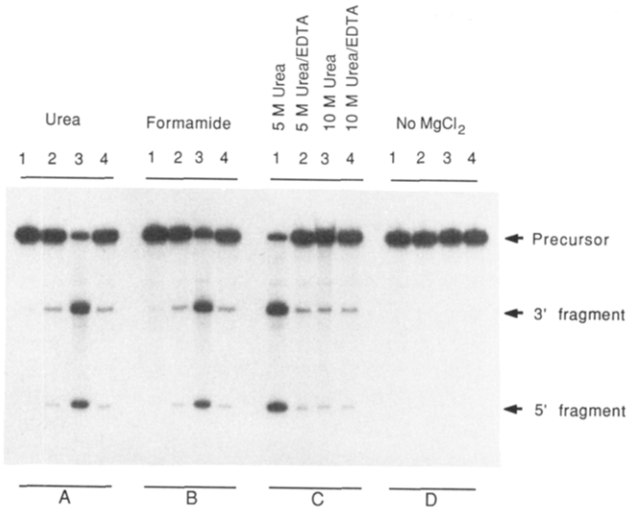

FIGURE 1: Self-cleavage of HDV genomic RNA fragment. (A and B) Gel-purified *Bam*HI runoff RNA, internally labeled with ^{32}P, was incubated at 37 °C in 40 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 11 mM $MgCl_2$. Aliquots of 10 μL were removed at 10 s (A1 and B1) and at 5 min (A2 and B2) and mixed with 10 μL of either 10 M urea and 25 mM EDTA (set A) or 24 M (95% v/v) formamide and 25 mM EDTA (set B). Also after 5 min 10-μL samples were mixed with 10 μL of 10 M urea (A3) or 24 M formamide (B3) without EDTA or with 1 μL of 0.1 M EDTA and incubated for an additional 5 min before the denaturant was added (A4 and B4). All samples were heated to 95 °C for 3 min after the denaturant was added. (C) Reaction conditions were as described above except that, after 5 min at 37 °C, aliquots were diluted 1:20 into either 5 M urea (C1), 5 M urea containing 25 mM EDTA (C2), 10 M urea (C3), or 10 M urea containing 25 mM EDTA (C4). (D) Incubation of RNA in the absence of $MgCl_2$. Reaction conditions were as described above except that no $MgCl_2$ was added. Samples D1–D4 were treated as described for A2, A3, B2, and B3, respectively. In all cases samples were reheated to 95 °C prior to electrophoresis. An autoradiogram of the gel is shown. Bands were excised, and the amount of radioactivity was quantified. For each sample lane the percent cleavage [calculated as (sum of the cpm in the products)/(sum of the cpm in products and precursor) × 100] was as follows: A1, 1%; A2, 5%; A3, 72%; A4, 3%; B1, 1%; B2, 4%; B3, 61%; B4, 6%; C1, 81%; C2, 6%; C3, 10%; C4, 6%.

high) containing 7 M urea, 50 mM Tris–borate, pH 8.3, and 0.5 mM EDTA. Following electrophoresis the gel was transferred to filter paper, dried, and exposed to X-ray film. Bands were located by use of the autoradiogram, excised, and quantified by measurement of Cherenkov scintillation.

Results

Religation or Absence of Cleavage? Although conditions for self-cleavage of the genomic HDV sequence have been reported (Kuo et al., 1988a; Wu et al., 1989; Wu & Lai, 1989), we felt it was important to reevaluate them to establish efficient quenching conditions for the self-cleavage reaction. Conditions similar to those used by Wu and Lai (1989) were examined. A ^{32}P-labeled polyribonucleotide containing the self-cleaving sequence from the genomic strand of HDV was synthesized in vitro and purified. This RNA was incubated in the absence or presence of Mg^{2+}, and aliquots were subjected to various conditions as described below. No cleavage was seen if the RNA was incubated in the absence of $MgCl_2$ (Figure 1, set D) or if EDTA was present in molar excess to Mg^{2+} (data not shown). In the presence of 11 mM $MgCl_2$ and 1 mM EDTA, cleavage resulted in two fragments that were readily resolved from the precursor and each other by electrophoresis in a polyacrylamide gel under denaturing conditions (Figure 1, sets A–C). Aliquots of the reaction were removed at 10 s (lanes A1 and B1) and 5 min (lanes A2 and B2) and mixed with an equal volume of loading mix containing either 10 M urea and 25 mM EDTA (lanes A1 and A2) or 24 M (95% v/v) formamide and 25 mM EDTA (lanes B1 and B2). To approximate the conditions of sample preparation used by Wu and Lai (1989), all samples were heated to 95 °C for 3 min following addition of the denaturant. For aliquots removed at 10 s, less than 2% of the precursor RNA was cleaved (lanes A1 and B1 and the legend to Figure 1). For those aliquots removed at 5 min, the amount of cleaved RNA had increased but was still less than 10% of the total (lanes A2 and B2). Adding EDTA (50 mM final) after 5 min, but prior to addition of the denaturants, also resulted in less than 10% cleavage (lanes A4 and B4). However, more than half of the RNA was cleaved (72%, lane A3; 61%, lane B3) if the addition of urea or formamide was not accompanied by the addition of EDTA.[2] Therefore, extensive cleavage was seen only if the EDTA was omitted from the samples to which denaturant was added, results that are consistent with those of Wu and Lai (1989). Those authors interpreted the difference in extents of cleavage as evidence for religation after the Mg^{2+} was chelated. However, to see a decrease in the amount of cleavage, it was not necessary to add excess EDTA prior to addition of the urea; a comparable small fraction of RNA was cleaved independently of whether the EDTA was added prior to or concurrent with the denaturant (compare lanes A2 and A4 or lanes B2 and B4).

Nevertheless, the following question could be raised: In those reactions where extensive cleavage was seen, did most of the cleavage precede the addition of denaturant (in which case chelation of Mg^{2+} promoted the ligation, even in the presence of urea or formamide) or follow the addition of denaturant (in which case chelation of Mg^{2+} inhibited cleavage)? In 10 mM Mg^{2+} it is quite possible that the concentrations of urea and formamide used were not adequately denaturing to terminate the cleavage reaction. Therefore, higher concentrations of urea and formamide were tested. When precursor RNA was incubated for 5 min in buffer containing 10 mM Mg^{2+} and aliquots were rapidly mixed into 19 volumes of a loading mix containing 10 M urea (~9.5 M urea final concentration), 10% or less of the precursor was cleaved in both the absence and presence of EDTA (Figure 1, set C). In the same experiment we observed 81% cleavage when the sample was similarly diluted 1:20 into 5 M urea without EDTA and less than 10% cleavage when EDTA was included. Stopping the reaction with high concentrations of formamide (22.5 M final), cold 5% trichloroacetic acid, or EDTA resulted in extents of cleavage qualitatively judged to be similar (data not shown). From these data we concluded the following: (i) under the conditions used, less than 10% of the RNA was cleaved in the 5 min prior to addition of the denaturant, while extensive cleavage observed in the absence of added EDTA occurred after the addition of denaturant; (ii) EDTA or a combination of EDTA and denaturant terminated the cleavage reaction, while in the absence of EDTA high concentrations of denaturants or 5% trichloroacetic acid was required to quench the reaction; and (iii) adding excess EDTA alone did not promote any measurable amount of religation. It was clear that this RNA molecule had some unusual properties, and it warranted additional investigation.

[2] The extent of cleavage seen in the presence of free Mg^{2+} and 5 M urea was time and temperature dependent and could be increased by repeated cycles of heating and cooling. As a result, variability in the extent of cleavage is seen as a function of the time and treatment of the sample prior to loading it onto the gel. In the presence of excess EDTA the fraction of cleaved RNA in a comparably treated sample is constant (data not shown).

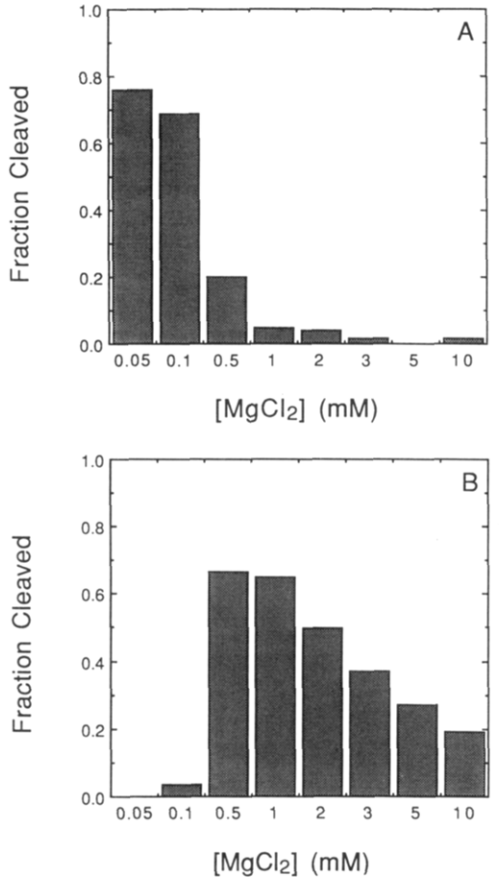

FIGURE 2: Self-cleavage as a function of $MgCl_2$ concentrations. Precursor RNA (*Bam*HI runoff) was preincubated for 5 min at 37 °C in either the absence (A) or presence of 5 M urea (B), and then $MgCl_2$ was added. The final conditions were 40 mM Tris-HCl (pH 8.0) with $MgCl_2$ and urea as indicated; the EDTA concentration was below 1 μM. Incubation was allowed to proceed for 15 min (A, no denaturant) or for 3 min (B, 5 M urea) and then terminated by the addition of EDTA to 25 mM. Products were quantified as described for Figure 1.

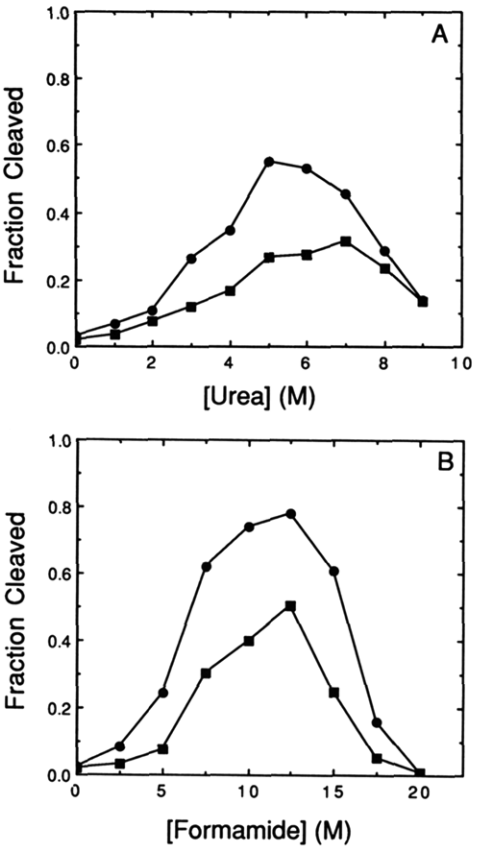

FIGURE 3: Self-cleavage as a function of urea or formamide concentration. Precursor RNA (*Bam*HI runoff) was preincubated in the absence of $MgCl_2$ at 37 °C in a reaction mix containing increasing amounts of denaturant for 5 min, and then $MgCl_2$ was added to initiate the cleavage reaction; the final reaction conditions were 40 mM Tris-HCl (pH 8.0), 1 mM EDTA, $MgCl_2$ at either 3 or 11 mM, and formamide or urea as indicated. Reactions were terminated after 5 min by addition of 0.5 volume of 25% sucrose and 50 mM EDTA. Products were quantified as described for Figure 1.

High Mg^{2+} Concentrations Inhibit Cleavage. The requirement for Mg^{2+} or other divalent cations in the HDV RNA self-cleavage reaction (Kuo et al., 1988a; Wu et al., 1989) was further characterized. With the transcripts used in this study, efficient cleavage required a divalent cation, but the effect of varying the cation concentration was complicated. In the absence of denaturants, very low concentrations of magnesium ion (<0.1 mM) were found to result in the most cleavage (Figure 2A). However, in the presence of 5 M urea the optimal $MgCl_2$ concentration increased (Figure 2B). (Note that the reactions done in the absence of urea were incubated 5 times longer than those containing urea.) From this it appears that cations at least have a role in stabilizing structure and that optimal metal ion concentrations will be very dependent on other reaction conditions that affect structure. In related experiments, it was found that both Mn^{2+} and Ca^{2+} had effects very similar to that of Mg^{2+} (data not shown).

The addition of monovalent salts, which can displace Mg^{2+} bound to RNA (Jack et al., 1977), are likely to affect the stability of an RNA structure. Adding NaCl, KCl, or ammonium acetate to 0.2 M did stimulate the rate of cleavage in 10 mM Mg^{2+} about 2-fold. However, in the absence of Mg^{2+}, no cleavage was observed in NaCl at concentrations up to 1 M (data not shown).

Urea and Formamide Enhance Cleavage. The extent of cleavage at early time points showed a clear dependence on denaturant concentration. Precursor RNA was preincubated in the presence of increasing amounts of urea or formamide, and the reaction was initiated by the addition of $MgCl_2$. The cleavage reaction was allowed to proceed for 5 min at 37 °C and then stopped with EDTA, and the extent of cleavage was quantified (Figure 3). Although more cleavage was seen in 2 mM Mg^{2+} than in 10 mM Mg^{2+}, the effects of the denaturants were similar. The amount of cleavage increased with urea concentrations up to 5–7 M and formamide concentrations up to 12.5 M (50% v/v). Above those concentrations the amount of cleavage that occurred in 5 min decreased. However, a significant amount of cleavage did occur at concentrations as high as 8 M urea and 15 M formamide. Thus, even relatively high concentrations of those denaturants not only failed to terminate the reaction but actually appeared to stimulate cleavage. Addition of urea to prepare the samples for electrophoresis, as was done by Wu and Lai (1989), could have increased the amount of cleavage. Urea-enhanced cleavage during the sample preparation may also explain why Wu et al. (1989) observed no change in the kinetics of the cleavage reaction as they varied the reaction temperature and $MgCl_2$ concentration.

Kinetics of the Cleavage Reaction. Extended reaction times indicated that the variations in the amount of cleavage seen in the above experiments largely reflect differences in initial rates of cleavage. Therefore, factors that affect the kinetics of self-cleavage were examined. Under a given set of conditions, the fraction of RNA cleaved as a function of time was found to be independent of RNA concentrations (data not

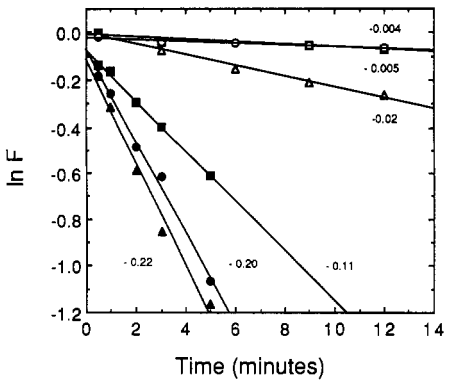

FIGURE 4: Kinetics of cleavage in the absence and presence of 5 M urea. Precursor RNA was preincubated at 37 °C for 5 min, and the reaction was initiated by the additon of $MgCl_2$: final conditions were 40 mM Tris-HCl (pH 8.0), 1 mM EDTA, 3 mM $MgCl_2$, and either no urea (open symbols) or 5 M urea (closed symbols). Aliquots of 10 μL were removed at various times and mixed with an equal volume of 10 M urea containing 30 mM EDTA to terminate the reaction. The products were fractionated by denaturing polyacrylamide gel electrophoresis and quantified. The natural log of the fraction of precursor RNA remaining (ln *F*) as a function of time is plotted; the slope of each line (negative of the first-order rate constant, min^{-1}) is given. Circles, *Xba*I runoff; squares, *Bam*HI runoff; triangles, *Eco*RI runoff.

Table I: Effect of Temperature and Formamide Concentration on Rate of Cleavage

temp (°C)	*k* (0 M)[a]	*k* (5 M)[a]	*k* (10 M)[a]
30	0.001	0.016	0.06
40	0.01	0.21	0.55
50	0.15	(1.9)	(1.5)
60	(2.2)	(2.1)	0.2

[a] First-order rate constants (min^{-1}) for cleavage of the *Bam*HI runoff RNA in 0, 5, and 10 M formamide. Rate constants were obtained from graphs such as those shown in Figure 4, except for those shown in parentheses which, due to the extent of cleavage at the earliest time points, were estimated from the first time point.

shown). In 2 mM Mg^{2+}, variables that were found to affect the rate of cleavage included the length of the transcript, the urea of formamide concentrations, and the temperature. A moderate but consistent effect of extra, non-HDV, sequence was observed with three versions of the precursor: runoff transcripts which differed in length at the 3′ end. Rate constants for cleavage were determined from the slope of a plot of the natural log of the fraction of precursor RNA remaining at various times (Figure 4). These plots were consistent with a first-order reaction. At 37 °C and in the absence of denaturant, the observed rate constants for cleavage were low: 5×10^{-3} min^{-1} for the *Xba*I runoff, 4×10^{-3} min^{-1} for the *Bam*HI runoff, and 2×10^{-2} min^{-1} for the *Eco*RI runoff. In 5 M urea the rate constants for cleavage increased to 0.11, 0.20, and 0.22 min^{-1} for the *Xba*I, *Bam*HI, and *Eco*RI runoffs, respectively.

Variations in formamide concentration and temperature also affected cleavage (Table I). Rate constants, as a function of these variables, were determined for the *Bam*HI runoff as in the above experiment. Temperature has a dramatic effect; the rate of cleavage increased as much as 10–15-fold when the temperature was raised 10 °C. Taylor and colleagues noted this and used elevated temperatures (50 °C or higher) to stimulate the amount of cleavage of both genomic and antigenomic HDV RNA (Kuo et al., 1988a; Sharmeen et al., 1988). At 40 °C the addition of 10 M formamide increased the rate of cleavage 50-fold. However, there is a threshold; in 10 M formamide the rate of cleavage began to decrease when the temperature was raised from 50 to 60 °C, suggesting that an essential structure for cleavage was being melted out.

Discussion

Self-cleavage of the HDV genomic strand autocleavage site appears to be stimulated by conditions that are partially denaturing. It is unlikely that the urea and formamide are acting directly to cleave the RNA because the enhancement effect is reduced at high concentrations of the denaturants. More plausibly, the main effect of the denaturants is to destabilize the structure of the RNA, which in turn increases self-cleavage activity by an unknown mechanism. There is precedent for low concentrations of urea and formamide increasing both the rate and the specificity of ribozyme-catalyzed RNA cleavage reactions (Zaug et al., 1986, 1988; Murphy & Cech, 1989), and Zaug et al. (1988) have discussed possible explanations for the rate enhancement. In the case of HDV RNA self-cleavage, the rate effect is more dramatic and occurs at higher denaturant concentrations.

It is possible that destabilizing conditions could expedite folding of the RNA into a conformation competent for self-cleavage. One possibility is that inactive and active conformations of the RNA are both present in the reaction, and destabilizing conditions could either increase the amount of the active form by destabilizing the inactive forms or increase the rate of interconversion between the various conformations. Pretreating the RNA to a heat-denaturation step followed by either slow or rapid cooling to various temperatures prior to addition of $MgCl_2$ has not altered the subsequent rate of self-cleavage (data not shown), but this does not rule out the possibility that inactive conformations or structures merely re-form under the conditions that have been tested. Although a gross structural change, such as disruption of extensive base pairing, may be required, there could also be subtle changes in flexibility that have a small positional effect, perhaps improving the orientation of the 2′-hydroxyl group for nucleophilic attack on the phosphorus. Alternatively, if an RNA–metal complex exists in rapid equilibrium between an uncleaved and cleaved form, destabilizing conditions may facilitate either a dissociation of the products or some other structural change which favors cleavage.

Destabilization will also be one effect of lowering the Mg^{2+} concentration and may account for the increased cleavage at lower cation concentrations. At very low Mg^{2+}, inhibition of cleavage by 5 M urea could be due to either a loss of an essential structure or a lower effective concentration of the metal ion. Grosshans and Cech (1989) have shown that divalent cations may have multiple roles in RNA-catalyzed reactions, and further studies will be necessary for a clearer understanding of the function of cations in self-cleavage of the HDV genomic RNA.

It is hypothesized that cleavage (and ligation) of genomic and antigenomic HDV RNA is required in the replication of the viral genome and that the RNA self-cleavage sites might fulfill this requirement (Kuo et al., 1988a; Wu et al., 1989; Sharmeen et al., 1988). The in vitro rates of self-cleavage at 37 °C (in the absence of denaturants) would appear to be exceptionally slow for replication of a viral RNA which is only 1700 nt long. Variations in the length of the precursor fragment do affect the rate of cleavage (Figure 3), so it is possible that the slow rate of cleavage is due to the sequence context of the self-cleaving fragment. However, it appears that cleavage of HDV RNA isolated from infected tissue is no more capable of rapid cleavage in vitro under physiological conditions than the transcripts made in vitro (Kuo et al., 1988a). If cleavage at the self-cleavage site is physiologically relevant, it could be that, in vivo, RNA cleavage is facilitated by a viral

or cellular factor, perhaps an RNA-binding or unwinding activity, and that, in vitro, denaturants somehow mimic this activity.

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