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Assessment of disparate structural features in three models of the hepatitis delta virus ribozyme

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ABSTRACT

Three models for the secondary structure of the hepatitis delta virus (HDV) antigenomic self-cleaving RNA element were tested by site-directed mutagenesis. Two models in which bases 5′ to the cleavage site are paired with sequence at the 3′ end of the element were both inconsistent with the data from the mutagenesis. Specifically, mutations in the 3′ sequence which decrease self-cleavage activity could not be compensated by base changes in the 5′ sequence as predicted by these models. The evidence was consistent with a third model in which the 3′ end pairs with a portion of a loop within the ribozyme sequence to generate a pseudoknot structure. This same pairing was also required to generate higher rates of cleavage in trans with a 15-mer ribozyme, thus ruling out a proposed hammerhead-like 'axehead' model for the HDV ribozyme.

INTRODUCTION

Self-cleaving RNA sequences (ribozymes) are found in both the genomic and antigenomic strands of hepatitis delta virus (HDV) (1–3). In vitro, self-cleavage requires only Mg^{2+}, or another divalent cation, and generates a 2′,3′ cyclic phosphate and a 5′ hydroxyl group (1–3). The HDV ribozymes cleave efficiently in the presence of moderate to high levels of denaturants (4–8), and both the genomic and antigenomic RNAs are highly structured in the presence of 5 M urea at 37° provided Mg^{2+} is present (9). Identification of the exact positions of the self-cleavage sites (1–3) revealed that neither sequence could be folded into either of the two well defined self-cleaving ribozyme structural motifs, the hammerhead or the hairpin/paperclip (10–13).

Several secondary structure models and variations have been proposed for both the RNA of the genomic and antigenomic HDV ribozymes (3,6,9,14–17). Some of the proposed models contain structural elements unique to either the genomic or the antigenomic ribozyme sequence (3,15–17). We have proposed a secondary structure which could be adopted by both in which each of the paired elements contain both covariant basepairs and common sequences (6,9). That structure contains four paired regions (stems I–IV, Fig. 1A) with the cleavage site positioned at the 5′ end of an 84 nucleotide (nt) sequence required for rapid and efficient cleavage. The 5′ sides of both stems I and II precede the 3′ sides of both along the linear sequence so that together the two pairings could form a pseudoknot (18). The other two pairings (III and IV) form stem and loop structures. In this model, stem I defines the cleavage site and the 5′ end of the ribozyme, and stem II defines the 3′ end of the minimal highly-active self-cleaving domain. The proposed pairings in this model have been tested and confirmed by mutagenesis and by generating active trans-acting ribozymes that are composites of genomic and antigenomic sequences, chosen to maintain structural elements (19).

The sequence requirements 5′ to the cleavage site appear to be minimal. A single nucleotide 5′ to the cleavage site is sufficient for both self-cleavage of the genomic sequence (5), and trans-cleavage with antigenomic and composite ribozyme sequences (19,20). While such data demonstrate that a single nucleotide is sufficient, it leaves open the question of how sequences 5′ to the cleavage site, when they are present, may affect the cleavage reaction. In sharp contrast to the above model, secondary structures have been proposed in which sequences 5′ to the cleavage site form a 4 or 5 basepair duplex with sequences from the 3′ end of the element (14–17). For the antigenomic sequence, two alignments of these sequences have been suggested (Fig. 1B and 1C, top structures) (14,16). For the genomic sequence, several models contain pairings between the 3′ end of the self-cleaving domain and sequences 5′ to the cleavage site (3,14,15,17). With the alignment proposed by Branch and Robertson (Fig. 1B) (14), the structure can accommodate both the genomic and antigenomic ribozyme sequence in a motif they refer to as an axehead (14).

Base changes near the 3′ end of the antigenomic element (nt 82 and 83, ref 19; nt 81 and 82, ref 16) reduce the rate of cleavage under a variety of conditions (19) and can have a dramatic effect on the ability of the ribozyme to self-cleave in denaturants (6,16,19). Three of the models (6,14,16) (Fig. 1) make different specific predictions as to what additional base changes would be required to complement mutations at the 3′ end of the element and restore efficient self-cleavage. We have previously shown that mutations in the 3′ sequence could be complemented by mutations at nucleotide positions 17 and 18 within loop I (Fig. 1A) (6,19). However, in those plasmid constructs (pSA1-2

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and derivatives) the sequence 5′ to the cleavage site had been altered to accommodate a restriction site used in the construction, so the potential for the two alternative pairings (Fig 1B and 1C) would have been compromised. For the work reported here, we restored HDV sequences 5′ to the cleavage site so all 3 models could be tested by mutagenesis and the effect of mutations directly compared. The same mutation at the 3′ end of the element could now be used to test each model and we find no evidence for pairing with sequences 5′ to the cleavage site. In addition, we extended the analysis to trans-cleavage by 15-mer ribozymes and find the data is consistent with the pseudoknot model rather than the proposed 'axehead' secondary structure of the HDV ribozymes (14).

MATERIALS AND METHODS

Enzymes and reagents

T7 RNA polymerase was purified from an over-expressing clone provided by W.Studier (21). Modified T7 DNA polymerase (Sequenase) was purchased from US Biochemicals (Cleveland). Other enzymes used in preparing plasmids or labeled RNA (restriction endonucleases, T4 DNA polymerases, T4 DNA ligase, T4 polynucleotide kinase, and calf intestinal phosphatase), nucleotides, ^{32}P-labeled nucleotides, and chemicals were purchased from commercial sources.

Plasmids and ribozymes

The plasmids used as templates for RNA synthesis were prepared from pSA1-2, pSII3′, pSII5′, and pSII5′3′ (6) by oligonucleotide directed mutagenesis of uracil containing single-stranded plasmid DNA using methods adapted from Kunkel (22) as previously described (6). Sequence changes were introduced 5′ to the cleavage site using oligonucleotides of the following sequences: 5′ dGCCGACCCGA AGAGCCCTAT AGTGA (pAG6, pAGSII5′, pAGSII3′, and pAGSII5′:SII3′), 5′ dTGCCGACCCG AAGCT CCCTATAGTG A (pAG-5-6, pAG-5-6:SII3′), and 5′ dCATGCCGACC CGCTGAGCCC TATAGTGA (pAG-2-3, pAG-2-3:SII3′). pAG79C80U and pAG-2-3:79C80U were prepared from pAG6 and pAG-2-3, respectively, using the oligonucleotide, 5′ dGCTTGCTCCA GTAGCCATCC G. Each mutation was identified by sequencing miniprep DNA (23), the miniprep DNA was retransformed to ensure segregation, and the DNA was resequenced after isolating DNA from a 250 ml culture by a boiling method and purifying it by equilibrium centrifugation in CsCl with ethidium bromide (24).

The plasmids used to prepare substrates D4B and D4C were made by replacing the stem IV sequence in pSA1-2 and pSII5′ (6) with the sequence 5′ CATGGATCCATG by the same methods described above using the oligonucleotide, 5′ dCCTTAGCCAT GGATCCATGC CCAGGTC. A shortened form of stem IV can still form but it contains a *Bam*HI site in the sequence corresponding to the loop. A precursor containing this ribozyme sequence was fully capable of self-cleavage (unpublished results), consistent with earlier results on the deletion of stem IV (19).

The 15 nucleotide (nt) long ribozymes (15B, 15C, and 15D) were synthesized and deprotected at US Biochemicals (Cleveland). Each was purified by gel electrophoresis before using.

Transcriptions and substrate preparation

To prepare the self-cleaving form of the ribozymes, plasmid DNA was linearized with *Hin*dIII, extracted with phenol and chloroform and ethanol precipitated. Transcription reactions (50 μl) contained 15 mM $MgCl_2$, 40 mM TrisHCl pH 7.5, 5 mM DTT, 2 mM spermidine, 1 mM each ATP, UTP, CTP and GTP, 50 μCi [α^{32}P]CTP, 2.5 μg DNA, and 300 units of T7 RNA

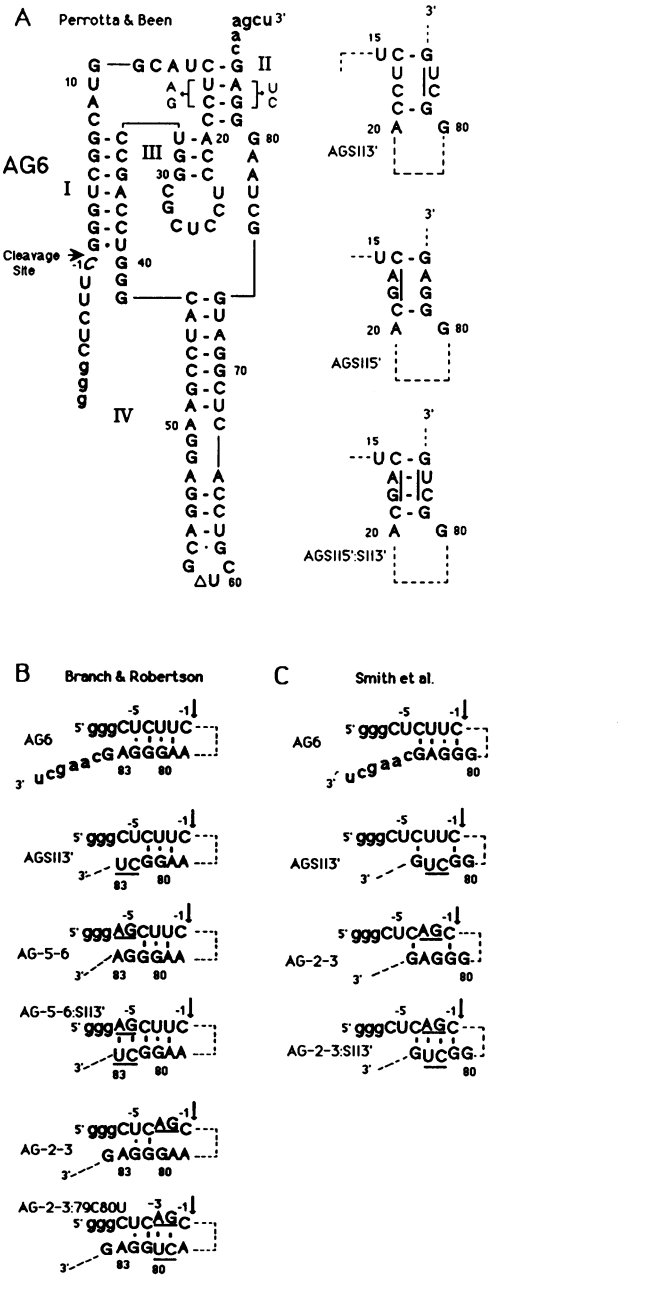


Figure 1. AG6, the sequence and alternative secondary structures involving sequences at the 3′ end of the antigenomic HDV self-cleaving RNA. (**A**) The 3′ sequence, GGAG (nt 81 to 84), sequence is shown paired with CUCC (nt 16 to 19) to generate stem II (5). The positions of base changes (vertical bars) that generate AGSII3′ (G82C, A83U), AGSII5′ (U17A, C18G), and AGSII5′:SII3′ (G82C, A83U, U17A, C18G) are shown to the right. (**B**) Alternative pairing according to Branch and Robertson (14). The 3′ sequence, AGGG (nt 79 to 83), is shown paired with the 5′ sequence UCUU (nt −5 to −2) (14). The positions of base changes (underlined) expected to disrupt or restore this structure are shown for AGSII3′, AG-5-6, and AG-5-6:SII3′. Dashed lines indicate sequence omitted. (**C**) Alternative pairing according to Smith et al. (16). The 3′ sequence, GGAG (nt 81 to 84), is shown paired with the sequence 5′ CUUC (nt −4 to −1) (16). The positions of base changes (underline) expected to disrupt or restore this structure are shown for AGSII3′, AG-2-3, and AG-2-3:SII3′. Dashed lines indicate sequence omitted.

polymerase. Incubation was for 60 min at 37°C after which an equal volume of formamide/50 mM EDTA was added and the products fractionated by electrophoresis on a 6% polyacrylamide gel containing 7 M urea. RNA was located in the gel by autoradiography and eluted from an excised gel slice into 0.1% SDS and 10 mM EDTA. RNA was recovered by ethanol precipitation. Transcriptions of *Bam*HI cut pD4B and pD4C were similar except the label was omitted during transcription, the reaction was scaled up 10-fold and the RNA was located by UV shadowing. Fifty pmoles of D4B and D4C RNA was dephosphorylated with calf intestinal phosphatase and 5′ end-labeled using polynucleotide kinase and [γ^{32}P]ATP. The RNA was repurified on a polyacrylamide gel under denaturing conditions.

RESULTS

The effect of 3′ mutations on self-cleavage

The possibility for sequences near the 3′ end of the element to base pair with 4 to 5 nucleotides 5′ to the cleavage site was restored to a synthetic version of the antigenomic self-cleaving sequence (SA1-2) (6) by replacing the sequence GGGAAUUC, 5′ to the cleavage site, with GGGCUCUUC, generating AG6 (Fig 1A). About 44% of the AG6 precursor cleaves during transcription (Fig 2); similar to what was seen for SA1-2 (6,19). The effect of base changes in sequences proposed to form stem II of SA1-2 have been characterized previously, but to rule out the possibility that those results were influenced by the absence of appropriate HDV sequences 5′ to the cleavage site, the same changes were introduced into AG6. AGSII3′ contains a G to C change at position 82 (G82c) and an A to U change at position 83 (A83u). This change in SA1-2 had resulted in a 15-fold decrease in the rate constant for self-cleavage (19) and when introduced into AG6 (AGSII3′) it reduced the amount of precursor that cleaved during transcription to 13% (Fig 2).

Testing potential compensatory mutations

For each of the three models, distinct predictions are made as to which other base changes would compensate for the effect of mutations at positions 82 and 83. In the pseudoknot model (Fig 1A), U17a and C18g (AGSII5′) should restore basepairing in stem II. In the Branch and Robertson model (14) (Fig 1B), changes 5′ to the cleavage site, U-5g and C-6a (AG-5-6; negative numbering is used 3′ to 5′ from the cleavage site), would be predicted to extend a shortened 3 basepair duplex to 5 basepairs. In the Smith et al. model (16) (Fig 1C), U-2g and U-3a (AG-2-3), would be predicted to restore a 4 basepair duplex with the sequence 5′ to the cleavage site. All three pairs of mutations were made, both without (generating AGSII5′, AG-5-6, and AG-2-3) and with the changes at positions 82 and 83 at the 3′ end (generating AGSII5′:SII3′, AG-5-6:SII3′, and AG-2-3:SII3′). Labeled transcripts prepared from each of the plasmids fractionated on polyacrylamide gels under denaturing conditions revealed various levels of cleavage during transcription (Fig. 2). Cleavage of AGSII5′ was barely detectable (1% cleavage) whereas cleavage of precursors with base changes 5′ to the site of cleavage appeared to be comparable to AG6 (AG-2-3, 43% cleaved; AG-5-6, 56% cleaved). Of the base change combinations intended to test the potential for pairing, only AGSII5′:SII3′ cleaved to an appreciable extent during transcription (56% cleaved vs 6% for AG-5-6:SII3′ and 2% for AG-2-3:SII3′).

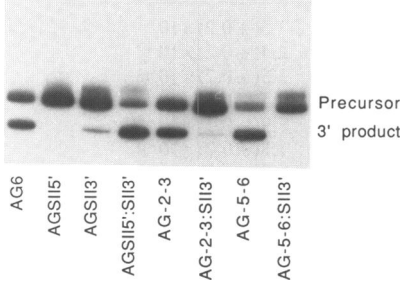


Figure 2. Extent of cleavage during transcription of the antigenomic ribozyme variants used in this study. Plasmid DNA containing a sequence for each of the ribozymes indicated was cut with *Hin*dIII and transcribed with T7 RNA polymerase as described in the methods section. After 1 hr, the reaction was terminated with EDTA and 10 μl of each was fractionated by electrophoresis under denaturing conditions; migration is top to bottom. A print of an autoradiograph is shown. The positions of precursor and 3′ product bands are indicated to the right, and the short 5′ product migrated off the gel under the conditions used. To estimate the percent cleaved as reported in the text, a digitized CCD image of the autoradiogram was quantified with NIH-Image software.

Kinetics of the self-cleavage reactions

The extent of cleavage during transcription could reflect either the rate of cleavage, the fraction of RNA that is folding into an active conformation, or some combination of both along with other factors. To examine the ability of each sequence to act as a catalyst for its own cleavage, the precursor RNA was isolated from the gel, and the rate constants for self-cleavage were measured at 37° in 10 mM Mg^{2+} (Fig 3A−3D, Table I). Rates were also determined with 10 M (40%) formamide added to the reaction (Table I). The rate constants for different precursors vary over about 3 orders of magnitude, with the highest being on the order of 1/min. Unlike SA1-2 for which cleavage rates were slightly stimulated (~2-fold) in 10 M formamide (19), the rate constants for cleavage of AG6 decreased slightly with denaturant (0.84 to 0.33/min). In the absence of any other changes, the mutations G82c and A83u, resulted in a 25-fold decrease in rate (Fig 3A) which decreased another factor of 10 with the addition of denaturant. Mutations at position −5 and −6 had no effect on the rate (Fig 3C) whereas mutations at −2 and −3 resulted in a 10-fold decrease (Fig 3D). The addition of formamide had no effect with AG-5-6 and may have a small stimulatory effect on cleavage of AG-2-3 (~2-fold). The changes at positions 17 and 18 (AGSII5′) (Fig 3B) caused a dramatic 10^3-fold decrease in the rate constant.

When each of the 5′ mutations (AGSII5′, AG-2-3, and AG-5-6) were combined with AGSII3′, only one (AGSII5′) restored the higher rate of cleavage. The AGSII5′:SII3′ combination cleaved about 2-fold faster than the parental sequence in both the absence and presence of formamide and thus was significantly faster than either AGSII5′ or AGSII3′. The AG-5-6:SII3′ combination cleaved at about the rate of the AGSII3′ mutant and slower than AG-5-6, a result consistent with the lack of effect seen with the −5 and −6 mutations alone. The AG-2-3:SII3′ precursor cleaved slower than either AG-2-3 or AGSII3′.

One other possibility for 5′ pairing was tested. Full activity of the AG-2-3 mutant was not restored by the SII3′ mutation, however the −2-3 mutation would be predicted to also disrupt pairing in the Branch and Robertson model (Fig 1B). In that model, bases at positions −2 and −3 pair with nt 79 and 80. Mutations at positions −2 and −3 would disrupt this interaction

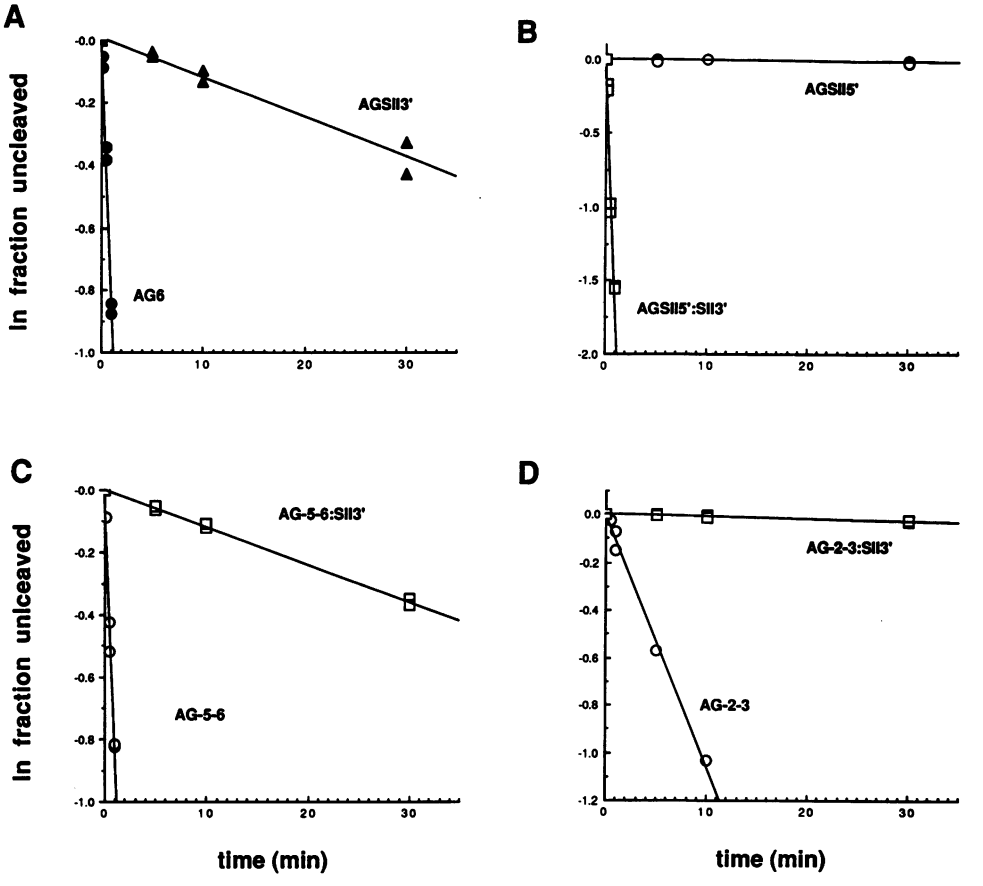


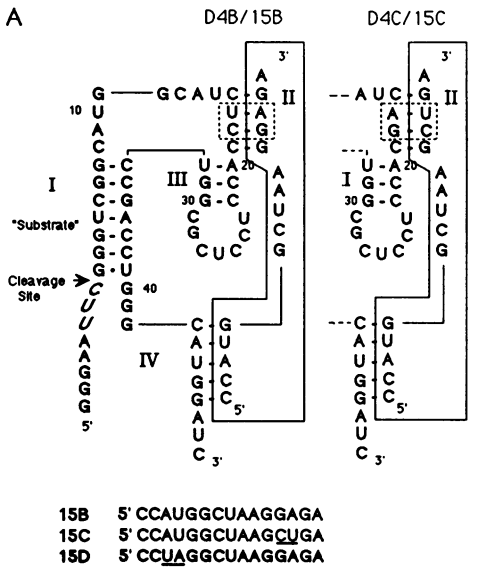
Figure 3. Kinetics of self-cleavage. (**A**) Cleavage of AG6 and AGSII3'. (**B**) Cleavage of AGSII5' and AGSII5':SII3'. (**C**) Cleavage of AG-5-6 and AG-5-6:SII3'. (**D**) Cleavage of AG-2-3 and AG-2-3:SII3'. Precursor RNA (^{32}P-labeled) was pre-incubated at 37° in 40 mM Tris−HCl (pH 8.0) and 1 mM EDTA for 5 minutes. The reaction was initiated with the addition of $MgCl_2$ to 11 mM and incubation was continued at 37°. At defined times, aliquots were removed and mixed with equal volumes of formamide containing 50 mM EDTA to stop the reaction. The RNA was fractionated by electrophoresis on a 6% polyacrylamide gel in urea, and the precursor and 3' product fragment were located by autoradiography, excised and quantified by counting Cherenkov scintillation. The natural log of the uncleaved fraction was plotted against time to obtain first order rate constants (see Table I).

Table I. Rate constants for self-cleavage

RNA precursor	Genotype	Cleavage rates* k (min^{-1})	k_{rel}(%)	$k^f(min^{-1})$
AG6	'wt'	$8.4(\pm0.4)\times10^{-1}$	**100**	$3.3(\pm0.2)\times10^{-1}$
AGSII3'	G82c,A83u	$3.3(\pm1.8)\times10^{-2}$	**4**	$2.4(\pm0.2)\times10^{-3}$
AGSII5'	U17a,C18g	$3.7(\pm0.6)\times10^{-4}$	**0.04**	$3.5(\pm0.2)\times10^{-4}$
AGSII5':SII3'	compensatory	$1.6(\pm0.2)$	**190**	$9.5(\pm0.3)\times10^{-1}$
AG-5-6	U-5g,C-6a	$1.0(\pm0.2)$	**120**	$7.9(\pm0.3)\times10^{-1}$
AG-5-6:SII3'	'compensatory'	$1.7(\pm0.5)\times10^{-2}$	**2**	$1.8(\pm0.2)\times10^{-3}$
AG-2-3	U-2g,U-3a	$9.0(\pm0.2)\times10^{-2}$	**11**	$1.8(\pm0.2)\times10^{-1}$
AG-2-3:SII3'	'compensatory'	$1.0(\pm0.2)\times10^{-3}$	**0.12**	$3.0(\pm1.0)\times10^{-4}$
AG79C80U	A79c,G80u	$< 1\times10^{-4}$	**0**	$< 1\times10^{-4}$
AG-2-3:79C80U	'compensatory'	$< 1\times10^{-4}$	**0**	$< 1\times10^{-4}$

* First order rate constants obtained at 37°C in 40 mM Tris−HCl, 1 mM EDTA, 11 mM $MgCl_2$ (pH7.5) (k) and with the addition of formamide to 10 M (k^f). Values given are the average of three independent determinations with the standard deviation from the mean. The relative rates (k_{rel}, no formamide) are $k_{mutant}/k_{wt}\times100$.

(Fig 1B) and would not be complemented by the changes made at positions 81 and 82. However, this possibility was tested with mutations at nt 79 and 80 (A79c,G80u). Precursor AG79C80U cleaved very inefficiently during transcription (<2% cleaved, data not shown) and there was no detectable cleavage of the purified precursor after 2 hours (k <0.0001/min; Table I). When the mutations at −2 and −3 were combined with the mutations at 79 and 80, cleavage activity was not reestablished even though, by the model of Branch and Robertson (14), pairing should be restored (Fig 1B). As with AG79C80U, during transcription of AG-2-3:79C80U less than 2% cleaved (data not shown) and no cleavage of the purified precursor was detected after two hours.



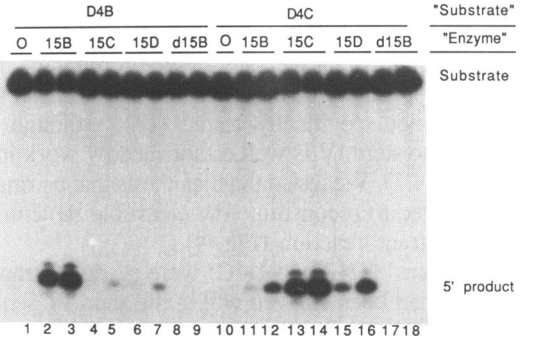


Figure 5. Trans-cleavage by 15-mer ribozymes. A trace amount (~1–5 nM) of 5′ end-labeled substrate (D4B or D4C) and 2 μM 15-mer were pre-incubated separately in 11 mM $MgCl_2$, 1 mM EDTA, 40 mM Tris–HCl (pH 8.0) at 37° for 5 minutes and then mixed to start the reaction (final 15-mer concentration was 1 μM). Aliquots were removed at 5 minutes (lanes 2,4,6,8,11,13,15,17) and 30 minutes (lanes 3,5,7,9,12,14,16,18) and quenched as described above for the self-cleavage reactions. The samples labeled O enzyme (lanes 1 and 10) were incubated for 30 min 37° in the absence of 15 mer. The products were fractionated on a 15% polyacrylamide gel and an autoradiogram is shown.

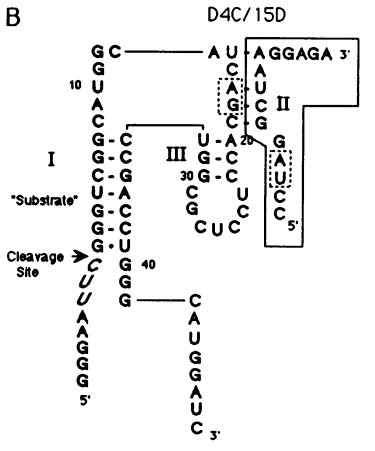


Figure 4. Proposed secondary structure for the trans-cleavage complex involving stem II and stem IV pairings. (**A**) The matched combinations, D4B/15B and D4C/15C. The dashed-line box indicates bases changed in stem II; the solid line box is the 15-mer 'ribozyme'. (**B**) An alternative alignment of D4C and 15D that could account for higher rates of cleavage of this mismatched combination than was seen with D4B/15D or D4C/15B.

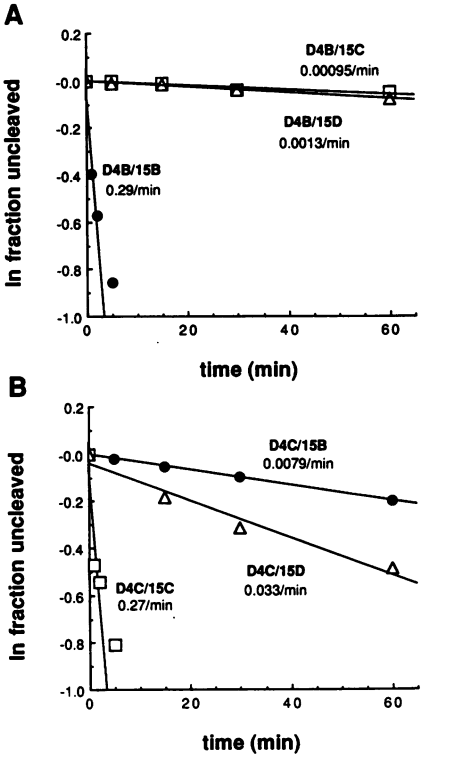


Figure 6. Pseudo first-order kinetics of trans-cleavage by 15-mer ribozymes. (**A**) Cleavage of D4B by matched ribozyme 15B, and mismatched ribozymes 15C and 15D. (**B**) Cleavage of substrate D4C by matched ribozyme 15C, and mismatched ribozymes 15B and 15D. Reactions were as described in the legend to Fig. 5 except that additional time points were taken. Substrate and product were fractionated on a 15% polyacrylamide gel, located by autoradiography and quantified. The substrate/ribozyme combination is indicated along with the negative of the slope of the line shown. The points shown are not corrected for the final extent of cleavage.

Thus the 10-fold decrease in cleavage rate caused by mutations at −2 and −3 could not be accounted for by any of the three models and the data is inconsistent with specified pairings in two of the models. In the pseudoknot model, nt 79 and 80 fall in the non-paired region joining stems IV and II. In deletions studies we have found that G80 is not required for cleavage activity in either cis or trans but that deletion of A79 (A78) resulted in almost complete loss of activity in trans and an A79c mutation in another antigenomic precursor sequence (SA1−2) resulted in at least a 200-fold decrease in self-cleavage activity (unpublished results). This suggests that the defect in AG79C80U is due to the change at position 79, but the reason for the effect is not known.

Trans-cleavage reactions

Dividing the self-cleaving sequence within the region corresponding to stem-loop IV (Fig. 1A) generates two fragments which when combined will result in the longer (5′) fragment being cleaved (14,15, Perrotta and Been, unpublished data). Although this reconstitution has been interpreted as evidence for the

'axehead' structure and pairing 5′ to the cleavage site (14) (Fig. 1B), trans-cleavage with these fragments, in the absence of other data, is consistent with any of the proposed models. The two pairings proposed to be necessary for the axehead structure in trans were not specifically tested (14), although the one corresponding to stem IV is well established by work in several labs (15,19,25–27). We tested the hypothesis that pairing in stem II is also required to reconstitute the cleavable structure in this version of the trans reaction (Fig. 4).

Two 'substrates' (D4B and D4C) were made by transcribing a plasmid that had been cleaved within the stem IV sequence at an engineered *Bam*HI site. These differed from each other only in that D4B was wild type at positions 17 and 18, while D4C had the same changes at these positions as the AGSII5′ mutants (Fig 4A). Four 15 nt-long 'ribozymes' were tested. 15B had the potential to provide one strand of both stems II and IV when paired with D4B, but it would generate two mismatches in stem II with D4C. 15C could potentially pair with D4C to form both stems, but two mismatches would destabilize the stem II pairing with D4B. 15D would contain mismatches in stem IV with both substrates, and would contain additional mismatches in stem II with D4C. An oligodeoxyribonucleotide with the sequence of 15B (d15B) was also tested. The 5′-end labeled substrates were incubated with excess 15-mer ribozymes for 5 and 30 minutes (Fig 5). The deoxy form of the 15-mer had no activity and the others cleaved to various extents, with the combinations of D4B/15B and D4C/15C cleaving to the greatest extent. The rates of cleavage with the 15-mer in excess were measured (Fig 6). The fastest cleavage reactions were with the combinations able to form both stems II and IV (D4B/15B, D4C/15C) (Fig. 6A and 6B). The pseudo-first order rate constant for cleavage of D4B decreased 200-fold with either 15C or 15D. For D4C the rate decreased 30-fold with 15B and 10-fold with 15D. The relatively high activity of D4C/15D, which should have mismatches in both stems, may be due to a realignment of the sequences to regenerate a cleavable structure (Fig 4B). This was not tested but would be consistent with results indicating that stem IV is not an essential catalytic component of the ribozyme (19,25–27).

DISCUSSION

Self-cleaving ribozymes from HDV cleave with as few as one nucleotide 5′ to the cleavage site (5,19,20). This observation leaves open the possibility that sequences 5′ to the cleavage sites may influence the reaction. Although in our model, the potential for such interactions is left undefined (6,9), other models have been presented in which sequences 5′ to the cleavage site are Watson–Crick basepaired to the 3′ end of the self-cleaving domain (14,16). In regards specifically to the Branch and Robertson model (14), mutations at positions −5 and −6 did not decrease the rate or extent of self-cleavage of an HDV antigenomic derived self-cleaving sequence. In contrast, mutations at positions 82 and 83 (SII3′) did have an effect, reducing cleavage by a factor of 25. In this model the combination of these changes in a single precursor would replace two non-Watson–Crick basepairs (a GU and perhaps an AC) with CG and a UA basepairs, changes that would be predicted to stabilize that pairing, yet the cleavage rate is no faster than the SII3′ mutations alone. In addition, mutations at positions −2 and −3 together with mutations at 79 and 80 do not appear to interact through Watson–Crick pairing. This would suggest that the 5′ pairing as presented in this model is not required for ribozyme activity.

With regards to the Smith et al. model (16), mutations at positions −2 and −3 decreased the rate of cleavage. The rate constant for cleavage was down about 10-fold. We have observed a 10-fold decrease in the rate also when the C at −1 is changed to a G, but not when it is changed to a U or A (Perrotta and Been, unpublished results). Smith et al. (16) report a 10^4 -fold decrease in rate with mutations at positions −1 and −2 (C-1g and U-2g; Ag94cs, ref 16). Thus, the effect with both sets of mutations 5′ to the cleavage site was to decrease the rate of cleavage, although the magnitude of the effect appeared to be greatly enhanced for the changes immediately adjacent to the cleavage site. Smith et al. (16) also made mutations at the 3′ end of the element at positions 81 and 82 (AG94gag) and observed a 50-fold decrease in the rate of cleavage, thus our results are in good agreement on the effect of mutations at the 3′ end. However, our results, and interpretation of the results, differ with the double mutations. Smith et al. (16) report a small (2-fold) increase in activity with the double mutant (4% activity, compared to the parent precursor, AG96) over the 3′ mutation alone (2% activity), while we observed a 30-fold decrease with the combination (AG-2-3:SII3′) relative to AGSII3′ (0.12% and 4% of AG6 activity, respectively). Either combination would be expected to restore the 5′ pairing according to this model, but neither combination restored the original higher level of activity, indicating that if the effect of mutations 5′ to the cleavage site is due to loss of a structure then that structure is not the one proposed. Smith et al. (16), however, attributed the lack of full cleavage activity in their double compensatory mutant to specific sequence requirements at the cleavage site which cannot be compensated for by second-site mutations. This argument cannot account for lack of activity in the double mutant that we used. The mutations at −2 and −3 resulted only in a 10-fold drop in the cleavage rate but when combined with the mutations at positions 82 and 83, activity dropped another 90-fold. This additional decrease cannot be due to specific sequence requirements at 82 and 83 because in AGSII5′:SII3′ those same mutations resulted in enhanced rates of cleavage relative to AG6 and AGSII5′. Thus either the mutations at positions −2/−3 and 82/83 act independently to slow cleavage or, if these positions can basepair as proposed in the model by Smith et al (16), they must generate a less active structure.

The data from site-specific mutagenesis is incompatible with both models that propose a specific pairing 5′ to the cleavage site. Therefore, any potential interactions with sequences 5′ to the cleavage site still remain to be defined. On the other hand, the same 3′ mutations which could not be compensated by mutations 5′ to the cleavage site were more than adequately compensated by base changes at positions 17 and 18 (3′ to the cleavage site). In this case the double mutant, AGSII3′:SII5′, cleaved 2-fold faster than AG6, 50-fold faster than SII3′, and 4000-fold faster than SII5′. A similar effect was seen previously with the precursor SA1-2 (19). Compared to the other double mutants, AGSII5′:SII3′ cleaved 100-fold faster than AG-5-6:SII3′ and 1600-fold faster than AG-2-3:SII3′. Thus, the stem II pairing which, with stem I, results in the pseudoknot folding (Fig 1A) is required for optimal ribozyme self-cleavage whether or not sequences 5′ to the cleavage site are involved in additional interactions.

Two forms of a trans reaction have been described for the HDV ribozyme (14,16). In one of these, the RNA can be separated into two pieces by interrupting the sequence in stem IV (Fig. 1A and 4A) (14,15). The two RNAs, when combined, result in cleavage of the larger (5′) fragment at the normal site. The pairing which corresponds to stem IV in a bimolecular form of the genomic ribozyme was shown to be required for this reaction (15), and our data with base changes on the 3′ side of stem IV in the antigenomic sequence is consistent with that result. We have now shown that, in the antigenomic sequence, the stem II pairing also contributes to the formation of the cleavable structure when the ribozyme is separated in stem IV. We propose that the combination of stem II and stem IV bring the joining region (JIV/II), with key nucleotides necessary for ribozyme activity, into proper position. Thus the trans reaction described by Branch and Robertson (14) would be consistent, not with the axehead model as they proposed (14), but with the pseudoknot model (6,9).

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