Evidence That Alternate Foldings of the Hepatitis δ RNA Confer Varying Rates of Self-Cleavage†

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ABSTRACT: The pentapurine sequence GGAGA, located between 80 and 84 nucleotides downstream of the cleavage site in the self-cleaving antigenomic RNA of hepatitis δ virus, is necessary for highly efficient cleavage and for stability in up to 20 M formamide. Yet much of the cleavage activity lost upon its removal from the 3′ end of an 84-nucleotide RNA can be restored by elongation of the 5′ end of the RNA with the polypyrimidine sequence found in the virus. We propose that this sequence alteration causes a refolding of the RNA, resulting in a "structural compensation" of the active core of the molecule. Restoration of the self-cleavage activity did not restore the ability to cleave in high concentrations of formamide. Deletion mutagenesis was carried out and supported the alternate RNA folding. The ability to assume more than one active conformation and for one RNA structure to compensate for another in supporting ribozyme activity may be unique to RNA enzymes and could be a useful adaptation in viruses or in prebiotic RNAs.

The catalytic activity of a ribozyme is directly related to the three-dimensional structure of the RNA. In attempting to define the essential elements required for activity, self-cleaving or self-splicing RNAs have been reduced in size to active minimal structures. This approach has been successfully used with the group I intron (Price et al., 1985), the hammerhead RNA (Forster & Symons, 1987), the self-cleaving hairpin (Hampel & Tritz, 1989), and the hepatitis δ RNA (Kuo et al., 1988; Rosenstein & Been, 1991; Prasad et al., 1992a,b). In defining such a minimal structure, there is at times a trade-off between the size of the RNA molecule and its activity, and a decision must be made as to when the activity of the RNA molecule becomes unacceptable as a result of the truncation. Further, in assessing biological molecules which may have more than one catalytic activity or may express different manifestations of an activity under different conditions, exclusion of an essential sequence element may occur. The inability of the hammerhead ribozymes to religate following self-cleavage may reflect such a loss. Finally, the position of the ribozyme RNA in the context of a larger fragment of RNA or the conditions at which cleavage was carried out, such as the nature and concentration of ions or the temperature of incubation, may exert a notable positive or negative effect on the enzymatic activity (Belinsky & Dinter-Gottlieb, 1991). This latter point is critical for defining the potential *in vivo* activity of the ribozyme and for the rational design and targeting of the δ ribozyme for therapeutic use.

We have examined the self-cleavage activity of the genomic and antigenomic RNAs from hepatitis δ virus (HDV) under various conditions (Belinsky & Dinter-Gottlieb, 1991; Smith & Dinter-Gottlieb, 1991; Prasad et al., 1992a,b). The antigenomic RNA from HDV is particularly interesting because of its ability to self-cleave to completion at 37 °C even in the presence of 18 M formamide (Smith & Dinter-Gottlieb, 1991; Smith et al., 1992). The pentanucleotide sequence GGAGA at the 3′ end of a 94-nucleotide (nt) molecule is necessary for cleavage in a highly denaturing environment (Smith et al., 1992). In addition, this RNA molecule, now termed Ag5–89 could, in the absence of denaturant, completely self-cleave in 15 s or less at 37 °C. This one molecule had both a high degree of stability and an extremely rapid rate of self-cleavage.

In this work we show that loss of activity due to the removal of 3′ GGAGA can be partially reversed by the restoration of a polypyrimidine sequence found originally 5′ of the cleavage site in the antigenomic RNA. We propose that the reemergence of catalytic activity resulted from the formation of an alternative structure, in which an internal polypurine sequence can pair with the added polypyrimidine stretch. This putative structure allowed self-cleavage to proceed at a slower rate when compared to the original self-cleavage activity of Ag5–89, and this molecule was unable to cleave in the presence of as little as 2 M formamide. While definitive proof of the existence of this structure is beyond the scope of this paper, the deletion mutagenesis approach we have taken allows us to rule out interactions such as the pseudoknot (Perrotta & Been, 1991). In previous work (Smith et al., 1992), a model was proposed in which the interconversion between an active and an inactive structure was sufficient to accommodate experimental observations. Our new data support the existence of two sequence–dependent catalytically active forms as well as at least one inactive form.

MATERIALS AND METHODS

Construction of the Deletion Mutants. A series of exonuclease III/mung bean nuclease deletions was created in the stem IV of Ag5–89, as previously described (Prasad et al., 1992a,b). These same deletions were utilized in the same plasmid constructs now cut with *Dde*I for the transcription runoff, eliminating the 3′ GGAGA and producing Ag5–66 and Ag5–63 RNAs.

Readdition of the 16-nt polypyrimidine run at the 5′ end of the molecule was accomplished by cloning oligonucleotides

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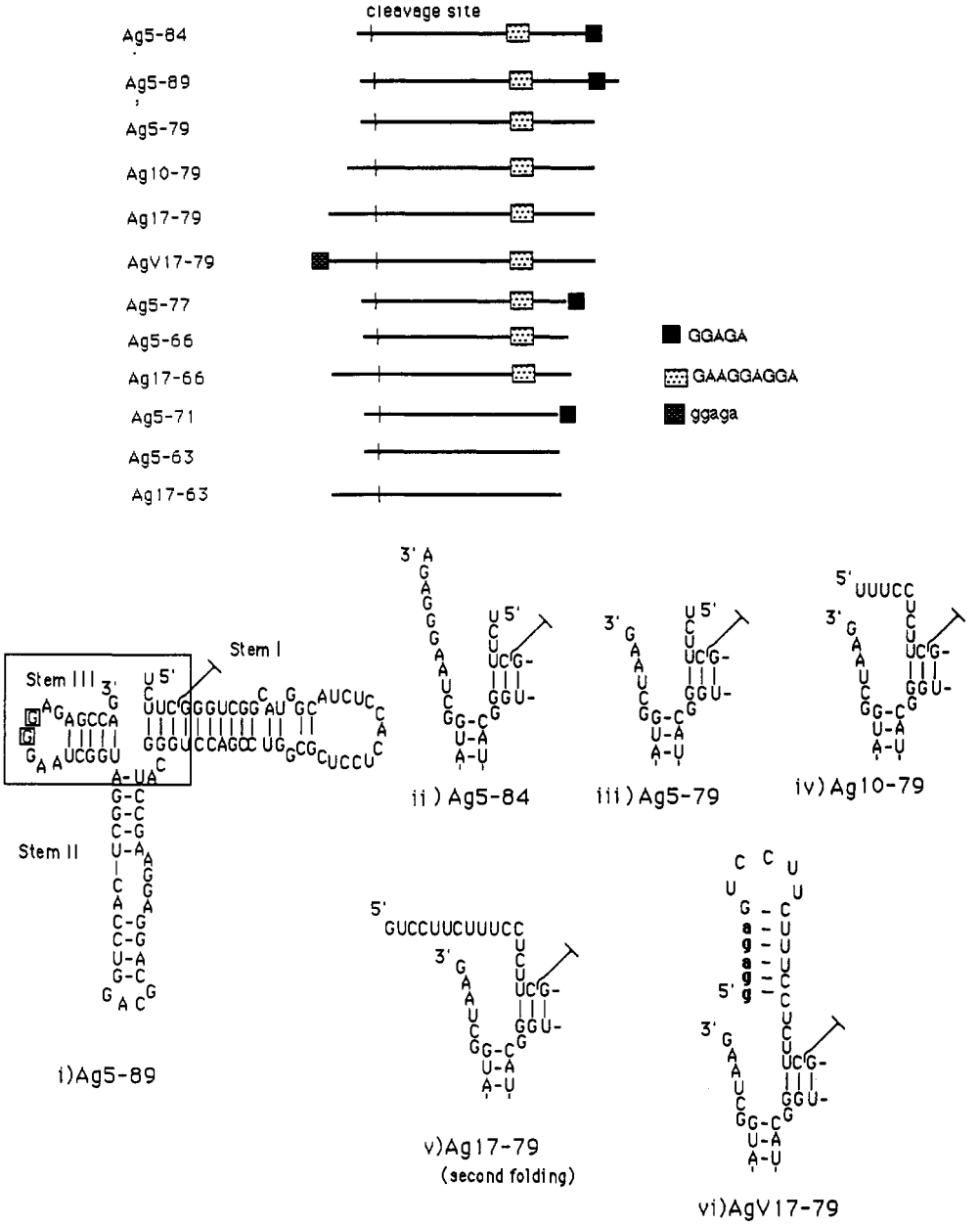

FIGURE 1: (A, Top) Overview of the RNA molecules used in this study. Significant features include the site of self-cleavage, which is the same in all cases, the number of nucleotides upstream and downstream of this site, and the presence or absence of three polypurine regions in the RNAs (boxed). (B, Bottom) Variants on the HDV antigenomic RNA analyzed in this work, folded according to the lowest free energy calculations of the Zuker RNAFOLD program (Zuker, 1989a,b; Jaeger et al., 1989, 1990). The complete folding of the molecule is shown in (i) and has been modified from our earlier model (Prasad et al., 1992b; Smith et al., 1992). The changes are reflected in the stem I region, which is now elongated and somewhat resembles the model proposed by Branch and Robertson (Branch & Robertson, 1991). This caused a relabeling of what had been stem II to stem I, a refolding of stem IV and relabeling to stem II, and creation of a different stem III at the 3′ end of the molecule. The name of each of the RNAs is based on the number of δ RNA nucleotides before and after the cleavage site, separated by a hyphen. In the cases where these numbers are the same but there are still sequence differences, an added descriptor is used (e.g., AgV17–79). The nucleotides in bold type are derived from the vector sequence.

containing the selected deletions into plasmid pJB1789 (Smith, J. B., Thesis). The *Rsr*II–*Eco*RI fragments from pOM2, containing a 12-nt deletion, and pEM4, containing a 15-nt deletion, were used to replace these segments in the original plasmid. The alterations in sequence were confirmed at the DNA level by a sequencing reaction using Sequenase 2.0 and the USB protocol. The resulting RNAs from a *Dde*I cut transcription runoff were termed Ag17–63 and Ag17–66.

Transcription of the RNA in Vitro and Self-Cleavage Analysis. Transcription of the δ RNAs and self-cleavage analysis in the presence and absence of formamide were carried out as previously described (Smith & Dinter-Gottlieb, 1991). The T7 RNA polymerase was purified from an overexpressing clone, pAR1219/BL21, a generous gift of F. W. Studier, and purified according to the protocol of Davanloo et al. (1984). All experiments were repeated at least three times, and the values derived from densitometry readings of the autoradiographs were averaged. The averaged values differed by <10%.

RESULTS

Addition of Sequence Elements 5′ of the Cleavage Site Restored Activity. When 5 nucleotides were removed from the 3′ terminus of Ag5–89 to produce Ag5–84 (Figure 1A,Bi, ii), no effect on cleavage was seen at 37 °C, and the initial rate of cleavage was now 6 min^{-1} (Table 1), compared to 10 min^{-1}. However, when an additional 5 nucleotides, GGAGA, containing the $G_{80}G_{81}$ sequence were removed (Ag5–79, Figure

Table 1: First-Order Rate Constants for Cleavage at 37 °C[a]

RNA	k (min^{-1})	k_{rel}	k_{rel2}	k_{rel3}	k_{rel4}
Ag5–84	6	1			
Ag5–79	<0.01	<0.01			
Ag10–79	0.07	<0.01			
Ag17–79	0.77	0.13			1
AgV17–79	3.2	0.53			
Ag5–77	0.62	0.1	1		
Ag5–66	<0.01	<0.01	<0.01		
Ag17–66	0.77	0.13	1.2		1
Ag5–71	0.71	0.12		1	
Ag5–63	<0.01	<0.01		<0.01	
Ag17–63	0.09	<0.01		0.13	0.12

[a] Initial rates of cleavage calculated for the various RNAs in this study. Four different k_{rel} sets are shown, based on the relative cleavage of the RNA compared with the original molecule from which mutants were derived. The k_{rel} reflects the initial rate of one RNA divided by the initial rate or a comparison RNA. Thus, all RNAs are compared with Ag5–84, and subsets are compared with Ag5–77, Ag5–71, and Ag17–79. The debilitating effect of the internal deletion removing the purine stretch in Ag17–63 was only seen when the GGAGA at the 3′ end was removed, leaving the RNA with no purine-rich stretch at all.

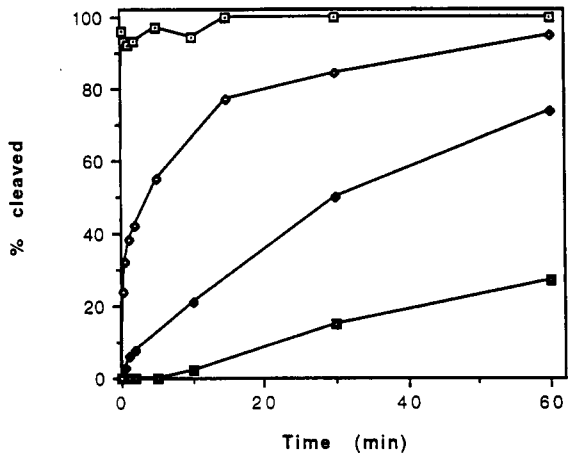

FIGURE 2: Self-cleavage of the antigenomic RNA increases with increasing length of the 5′ end of the molecule: Ag5–79 (⊡); Ag10–79 (◆); Ag17–79 (◇); Ag5–89 (□) (Smith et al., 1992).

1Biii), severe loss of the self-cleavage activity at 37 °C occurred (Figure 2, Table 1). No cleavage was seen until the precursor was incubated for 15 min; then a slow cleavage reaction was initiated, with a rate estimated at <0.01 min^{-1}, until after 60 min, only 20% of the RNA had cleaved. A longer incubation, up to 120 min, did not result in further cleavage (data not shown). The natural δ virus sequence immediately upstream of the cleavage site in the hepatitis δ RNA consists of a run of 17 pyrimidine nucleotides. When 5 nucleotides (–6 to –10 upstream of the cleavage site) from this polypyrimidine stretch were added back to the 5′ end of the Ag5–79 RNA transcript, creating Ag10–79 (Figure 1A,Biv), the rate of the self-cleavage was noticeably affected (Figure 2), reaching 80% cleavage with an initial rate of 0.07 min^{-1} (Table 1). Restoring an additional 6 pyrimidines and a G residue (–11 to –17, Ag17–79, Figure 1Bv) further enhanced the rate and efficiency of cleavage, with cleavage of Ag17–79 reaching 100% in 60 min (Figure 2) with the initial rate of cleavage at 0.77 min^{-1} (Table 1) and a $t_{1/2}$ of 5 min.

The Internal GAAGGAGGA Might Substitute for the Deleted 3′ GGAGA. It was initially puzzling that readdition of 12 pyrimidines at the 5′ end of the RNA should partially restore the activity lost by removal of 5 purine residues at the 3′ end. However, an examination of the RNA structure of Ag17–79 revealed that a purine-rich sequence, GAAGGAGGA, was also present in the stem II region (Figure 1Bv). Elongating the 5′ end to 10 and 17 nucleotides might allow

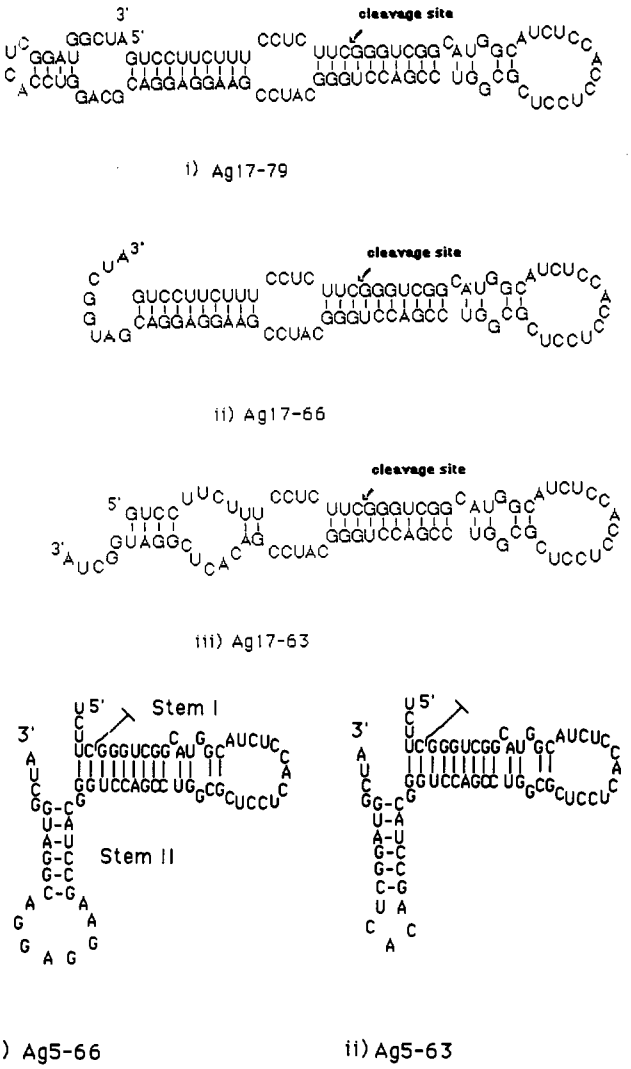

FIGURE 3: (A, Top) Refolded structures generated by elongating the 5′ end of the RNA. The stem I region is identical to the previous folding. The stable pairing of the sequences at the 5′ end of the molecule with sequences within the Figure 1 stem II region can only occur when the 5′ end contains more than 10 pyrimidines. The internal deletion in Ag17–66 (ii) does not affect this alternate pairing, while the deletion in Ag17–63 (iii) weakens it. (B, Bottom) With only 5 nucleotides upstream, the alternate folding cannot form, and a structure resembling the original RNA is proposed. The plasticity of the molecule in achieving alternate foldings may be due to its high GC content. The original antigenomic RNA is 69% GC, and the Ag17–79 molecule is still 62% GC.

pairing of the 5′ pyrimidines with these internal purines, resulting in a different folding of the RNA, altering stem II (Figure 3Ai). Particularly striking was the pairing elicited by elongating the 5′ end to 17 nucleotides in Ag17–79, creating a possible 10 base pair region (Figure 3Ai), which was potentially more strongly paired than the original stem II (Figure 1Bv).

When the RNAFOLD program of Zuker was used to calculate the free energy of the Ag17–79 RNA (Zuker, 1989a,b; Jaeger et al., 1989, 1990), the most stable structure, with a free energy calculated at –29.5 kcal/mol (Table 2), was indeed that shown in Figure 3Ai. The calculated $\Delta G°'$ for the folding of the molecule containing stem II (Figure 1Bv) was –28.9 kcal/mol, supporting the possibility of this alternate folded structure.

Deletion Mutagenesis of the Alternate Folding. To test the hypothesis that the internal polypurine sequence was essential for efficient cleavage in the absence of the terminal polypurine sequence, deletion mutants were produced, shown

Table 2: Calculated Lowest Free Energy Values for the RNA Foldings Shown[a]

RNA	ΔG (kcal/mol)	RNA	ΔG (kcal/mol)
Ag5–89	−31.9	Ag17–79	−29.5
Ag5–84	−28.9	Ag17–63	−24.1
Ag5–79	−28.9	Ag17–66	−25.6
Ag5–63	−24.1	AgV17–79	−33.8
Ag5–66	−21.9		

[a] The lowest free energy values were calculated for the RNAs, using Zuker's RNAFOLD program, as described. It is interesting that the addition of the **ggaga** vector sequence at the 5′ end of the AgV17–79 molecule increases the stability overall, even above that of the molecule with the GGAGA at the 3′ end.

in Figure 3Aii,iii. An approach using deletion of segments of the RNA was intentionally chosen over site-specific mutagenesis for the following reasons. First, since the 10 base pairing is stable, it is not obvious that a change of one or two base pairs would in fact weaken the interaction. Second, it is not clear whether the purine run paired with a pyrimidine run is an important feature of the structure. Altering the sequence might have a deleterious effect in and of itself. Even in the case of the large deletion we introduced, the RNA could realign (see Figure 3Biii) and form a base-paired region. Third and finally, since the δ RNAs can form numerous conformers in solution (Prasad et al., 1992a), the establishment of one definitive form for the RNA is at present beyond our technical abilities. We have sought in our experimental approach to present a reasonable model for the refolding, based upon the free energy measurements, and to take the global approach of preventing the proposed interaction by deletion mutagenesis. The subtleties of the interaction and the sequence requirements will be examined in the future.

We have created a series of mung bean nuclease deletions of the stem II region which retained cleavage activity with 5 nts at the 5′ end and the GGAGA sequence at the 3′ end (Prasad et al., 1992b). The initial rates of cleavage of these deletion mutants, Ag5–77 and Ag5–71 (Figure 1A), were calculated to be, respectively, 0.62 and 0.71 min^{-1} (Table 1). Thus the activity of the RNAs containing the two deletions in the presence of the 3′ GGAGA was comparable, and any difference in activity in the absence of the GGAGA would be due to factors other than the deletion itself.

These deletions were now introduced into RNAs with 17 nts at the 5′ end, and runoff transcripts removing the terminal GGAGA were transcribed and assayed. In Ag17–66, derived from Ag5–77, an internal deletion of 12 nts would have no effect on the pairing with the internal purine sequence (Figures 1A and 3Aii). On the other hand, in Ag17–63 (Figure 1A and 3Aiii), the strong alternate pairing would be disrupted by the 14-nt deletion, which removed most of the internal purine region. RNAs containing only 5 nts upstream of the cleavage site (Figures 1A and 3B, Ag5–66 and Ag5–63) were also transcribed without the 3′ terminal GGAGA. These latter RNAs could not fold into the alternate structure because of the short 5′ terminus but still retained stems resembling stem II (Figure 1Bi). The lowest free energies calculated for all the mutant RNAs are shown in Table 2.

The Internal GAAGGAGGA Could Partially Compensate for Removal of the 3′ GGAGA. Restoration of the polypyrimidine tract upstream of the cleavage site in Ag17–79 restored self-cleavage activity (Figure 2). In mutant Ag17–66, in which the internal pairing is maintained, the cleavage profile is almost identical to that of Ag17–79 (Figure 4), and the initial rates of cleavage were identical at 0.77 min^{-1}. On the other hand, in Ag17–63 with the weakened internal pairing (Figure 3Aiii) activity was diminished, with only 50% cleavage

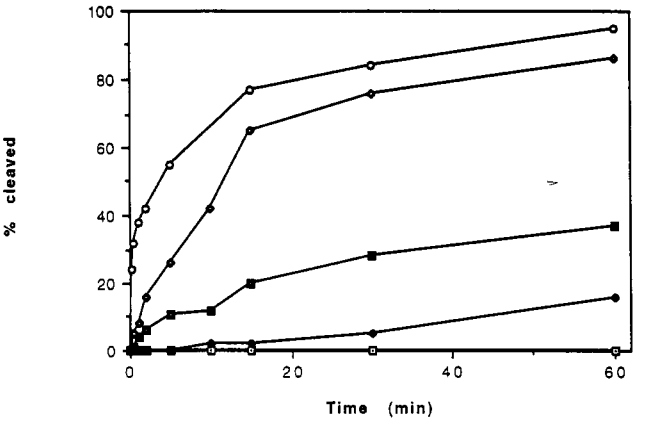

FIGURE 4: Effect of deletion mutations on Ag17–79: Ag5–63 (▣); Ag5–66 (◆); Ag17–63 (□); Ag17–66 (◇).

instead of 100% after 60 min (Figure 4). The effect on the initial rate of cleavage is even more striking, with a reduction to 0.09 min^{-1} and a k_{rel} of 0.12 (Table 2).

Our model for the alternative folding of Ag17–79 proposed that the presence of only 5 nucleotides upstream of the cleavage site would not be sufficient to allow the refolding to occur. The internal mutants with 5 nucleotides at the 5′ end (Ag5–63 and Ag5–66, Figure 3B) showed severely diminished cleavage, with the former not cleaving at all in a 60-min incubation. Initial rates of cleavage were too low to be measured (Table 2), corroborating our prediction.

The hierarchy of structures for δ cleavage can then be summarized. The RNA with 5 nts upstream and the 3′ GGAGA cleaved most rapidly, with an initial rate of 6 min^{-1}. Placing the GGAGA at the 5′ end of the molecule, flanking at 17-nt region 5′ of the cleavage site, produced a rate of 3.2 min^{-1}, even in the absence of the 3′ GGAGA [Figure 1A,Bvi and Smith et al. (1992)]. An internal GAAGGAGGA could substitute for the 5′ or 3′ GGAGA but only in the presence of 17 upstream pyrimidines, yielding an initial rate of 0.77 min−1. Finally, removal of the 3′ GGAGA as well as the internal purine-rich region abolished self-cleavage, and the initial rate could not be measured.

Stability in Formamide Is Independent of Self-Cleavage. We now examined the new series of mutants with respect to their stability in formamide. Ag5–63 and Ag5–66 (Figure 3B) were completely unable to cleave in the presence of the denaturant (Figure 5). On the other hand, the elongation of the 5′ end of the molecule to 17 pyrimidine nts did restore some stability, arguing for interaction between these nts and some portion of the molecule. Ag17–79, as we previously described [Figure 1A and Smith et al. (1992), aka Ag94–V] showed cleavage to 55% in the absence of formamide (Figure 5), but cleavage of 20% was still seen in the presence of 15 M denaturant. Ag17–63 cleaved to 40% in the absence of denaturant, but in 5 M formamide, cleavage was reduced to 10%, and cleavage was eliminated above that concentration. Ag17–66, on the other hand, cleaved to 80% in the absence of denaturant, but inactivation in formamide was rapid, and cleavage in 7.5 M formamide was reduced to 5%, with subsequent loss of activity. No increase in activity of any of these RNAs was detected with addition of denaturant, just a diminishing of the cleavage and, presumably, the structural stability of the catalytic core of the molecule. The formamide stability appeared to be highly sensitive to the presence of the terminal GGAGA element at either the 3′ or 5′ end and could not be replaced by elongation of the 5′ end of the RNA or by the internal purine element.

Altering the GG of the 3′ GGAGA Produced the Same Activity as Elongating the Polypyrimidine Stretch. We

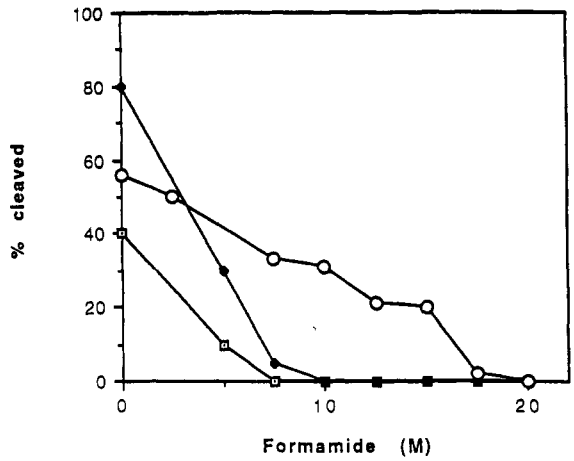

FIGURE 5: The presence of denaturant critically affected the self-cleavage of the mutants. Ag17–63 (⊡); Ag17–66 (◇). For comparison, the data on Ag17–79 (○) are reproduced from Smith et al. (1992). Ag5–63 and Ag5–66 showed no cleavage at any concentration of formamide, nor in this time frame, in the absence of the denaturant and so are not shown on the graph.

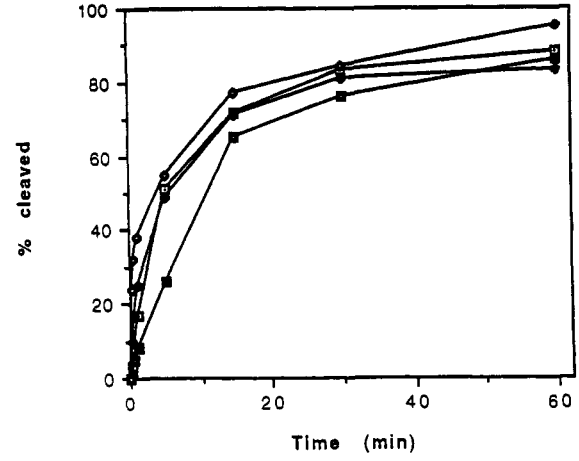

FIGURE 6: Comparison of Ag17–79 (◇) and Ag17–66 (■) with mutants Ag94gag (□) and Ag94dbl (◆). None of the mutants can form a tertiary interaction proposed for the most rapid cleavage and stability in formamide, yet all can cleave to completion in 1 h at 37 °C.

previously demonstrated that mutating $G_{80}G_{81}$ in the 3′ terminal GGAGA sequence (see Figure 1B) to $C_{80}C_{81}$ adversely affected the stability of the RNA in formamide (Ag94gag, Smith et al., 1992). When the $U_{-2}C_{-1}$ just upstream of the cleavage site was changed to GG, cleavage in formamide was poor, except in the presence of a 10 M concentration of the denaturant. However, when both mutations were introduced into the same RNA, activity in formamide was partially restored to the level of that seen with just the $G_{80}G_{81}$ to $C_{80}C_{81}$ alteration (Ag94dbl, Smith et al., 1992). We now tested the self-cleavage of the Ag94gag and Ag94dbl mutants in the absence of the denaturant and compared the cleavage profiles to the Ag17–79 and the internally deleted Ag17–66 RNAs. Figure 6 shows that the profiles of all four RNAs were virtually identical, with similar initial rates of cleavage [Table 1 and Smith et al. (1992)]. All four RNAs reached 70% cleavage in ca. 15 min and cleaved to completion in 60 min.

DISCUSSION

Readdition of a Pyrimidine Tract Supports an Alternate RNA Folding. The folding of an RNA molecule proceeds through the assembly of secondary structures which can then be organized into higher-ordered interactions (Banerjee et al., 1993). Because RNA self-cleaving molecules that have been analyzed are initially embedded within larger RNA fragments, the border that defines the catalytic "core" RNA molecule from the extraneous RNA in many cases is not clear. While these flanking sequences may not directly contribute to a given catalytic function, they nonetheless may influence the activity by providing sequences that structurally compete by forming alternate foldings. The practice of reducing structures of their "minimal" size by sequential removal of RNA, while reducing the number of competing structures, may also result in loss of critical functions which bear on the activity of the self-cleaving RNA.

For the hepatitis δ virus (HDV) self-cleaving RNA, the effort to define a minimally active structure may have resulted in loss of information about the self-cleaving RNA in the context of its biological function. The approach taken in this work has been to remove or add blocks of nucleotides to the self-cleaving HDV RNA and to measure the effect of these changes on self-cleavage activity. The nucleotides that were added were derived from the HDV genome in an attempt to assess the influence of flanking sequences in the self-cleavage reaction.

The unusual distribution of nucleotides in hepatitis δ virus makes this experimental approach more relevant for the HDV self-cleaving RNA than for other ribozymes that have been investigated. The hepatitis δ virus has extensive tracts of purines and pyrimidines throughout, which are reflected in these truncated RNA molecules as well. Particularly striking are a run of 17 pyrimidine nts immediately upstream of the cleavage site in the antigenomic RNA and a stretch of 9 purines in the stem II region (see Figure 1). The presence of these tracts has implications for the folding of this RNA molecule and catalytic function. The removal of one of these purine tracts as positions 80–84 downstream of the cleavage site, for example, destroyed the rapid and efficient cleavage of the HDV (compare Ag5–84 and Ag5–79, Figure 1A and Table 1). The cleavage half-life, which was initially 15 s at 37 °C, was reduced 166-fold.

The sequential addition of pyrimidine blocks to the 5′ end of the Ag5–79 eventually led to the formation of Ag17–79 (Figure 1Bv) and resulted in a self-cleaving RNA molecule which was initially 10-fold faster than the Ag5–79 molecule but was still slower than the Ag5–84 (FIgure 1Bi, Table 1). While Ag17–79 does not have the GGAGA sequential element at the 3′ end, secondary structure predictions support an alternate folding of the RNA which is more stable than any of the other potential foldings (Figure 3Ai). This alternate folding is driven by the presence of the added pyrimidine segments which now pair with the internal polypurine stretch between nucleotide positions 47–54. Recent work by Banerjee et al. (1993) supports our assumption that secondary structure dominates the free energy of RNA folding and adds credibility to these structural predictions.

Deletions That Weaken the Alternate Pairing Diminish the Cleavage Reaction. Since the pairings of either the original stem II (Figure 1Bi) or the alternate stem II′ (Figure 1Bi) or the alternate stem II′ (Figure 3Ai) with the 17 nts 5′ of the cleavage site involve specific runs of nucleotides, site-specific mutations might not affect these crucial interactions. The 12-nt internal deletion in RNA Ag17–66, which left the stem II′ pairing intact, had little effect on the self-cleavage or on the initial rate of cleavage (Figure 4B and Table 1), and its $k_{rel\,4}$ when compared with Ag17–79 was 1. On the other hand, the internal deletion of the polypurine AGGAGGA sequence, producing Ag17–63, resulted in significantly lower self–cleavage activity, and a $k_{rel\,4}$ of 0.12. Ag5–63 RNA was unable to cleave at 37 °C, even with an extended incubation, although there was residual activity in the Ag5–66 RNA with

prolonged incubation. Some subtle interaction must still differentiate these two RNAs, allowing some cleavage in one and none in the other. One possible interaction might be the formation of a pseudoknot between the looped-out purine run in stem II and the pyrimidine stretch in the stem I terminal loop (Figure 3Bii).

The traditional approach of site-specific mutagenesis can lead to ambiguous results with these RNAs. For example, when Perrotta and Been (1991) changed two nucleotides in their proposed pseudoknot, little effect was seen on the cleavage activity. Yet the compensatory mutation produced an RNA whose cleavage was far superior to the original. Similarly, we were able to restore the self-cleavage by changing a GG in the 3′ terminal GGAGA to a CC, to complement the change of a $U_{-2}C_{-1}$ near the cleavage site to a GG (Smith et al., 1992), but the activity was not completely restored, nor was the formamide stability. These earlier results argue for the existence of conformers of the RNAs and make obvious the difficulties in using a site-specific mutagenesis approach to define the RNA structure.

Using nuclease protection to define a structure is also difficult in the presence of multiple conformers. The difference in structure, defined by single-stranded versus base-paired regions in the alternate foldings of the Ag17–79 RNA, centers on ten nucleotides upstream of the cleavage site. Since both conformers we propose, as well as others, may exist at any point, results in either direction would not prove conclusive.

Stability in Formamide and Self-Cleavage Can Be Separated from One Another. Both self-cleavage at the fastest rate and the ability to cleave in 18–20 M formamide depended on the presence of the GGAGA sequence +80 to +84 nt downstream of the cleavage site. Both activities are lost or severely diminished with truncation or alteration of this sequence element (Smith et al., 1992). Self-cleavage, albeit at a slower rate, can be restored by elongation of the 5′ terminus. Yet this RNA is highly sensitive to any formamide in the buffer at all. Our proposed model for Ag17–79 (Figure 3Ai) defines a new 10 base pair association between the elongated 5′ end and an internal purine-rich sequence. This extensive, competing pairing would only be possible with 17 nts upstream, not with 5 or 10, and, if the stability in formamide were dependent merely upon the presence of a strong stem, this new stem is more stable than the original stem II (Figure 1Bi). The basis for stability in formamide is as yet unclear. Mutation of only two bases, $G_{80}G_{81}$, near the 3′ end destroyed that stability (Smith et al., 1992), and now the possible addition of a highly base-paired region did not restore it. As previously proposed (Smith et al., 1992), the stability in denaturant must lie in tertiary interactions within the molecule as well as in the canonical Watson–Crick interactions. This is supported by our ongoing work in which substitution with 7-deazaadenosine has a deleterious effect on the stability in formamide (Gottlieb et al., in preparation).

Inability To Achieve a Tertiary Configuration Correlates with a Slower Rate of Self-Cleavage. The four mutant RNAs in which the 3′ GGAGA sequence is altered or missing self-cleave with almost identical profiles, as seen in Figure 6 and in their initial rates of cleavage in Table 1 and Smith et al. (1992). We had earlier proposed that the Ag94gag and Ag94dbl RNAs, in which the $G_{80}G_{81}$ was replaced by a CC, were unable to form a crucial tertiary interaction conferring stability in formamide (Smith et al., 1992). Removal of the GGAGA would have a similar effect, and the internal deletion in Ag17–66 did not alter this. In these four RNAs, it appears that a secondary interaction supports a reasonable rate of self-cleavage, but the most rapid rate, as well as the formamide stability, is precluded. In sense, we may have trapped intermediate stages in the assembly of the ribozyme, while preventing the final assembly of the most active structure.

Earlier it was proposed that the biphasic nature of cleavage profiles at a given temperature might be due to the necessity of some RNAs within the population to reassort into the catalytically active conformation (Prasad et al., 1992a; Been et al., 1992). The possible exception to this was the Ag5–89 molecule, in which the entire population cleaved in under 15 s. While a structural rearrangement to achieve the active form may still be one of the rate-limiting steps in the cleavage reaction, this work suggests that other self-cleaving conformations may coexist with the most rapid form. Our results indicate that the HDV RNAs may adopt at least two active conformations, both of which retain self-cleavage activity. In the case of the slower cleaving RNA molecule, Ag17–79, the stabilization of the transition state for enzymatic activity is not as great as that seen in the most active form, in Ag5–84. For example, the inability to form the tertiary interaction may impede the proper coordination of the magnesium ion. RNA enzymes may be unique in their ability to form alternate configurations through the availability of complementary sequences, leading to such structural compensation.

At Least One Polypurine Stretch Must Be Present for Activity. We have identified three polypurine regions in truncated forms of the antigenomic δ RNA which are important for self-cleavage (see Figure 1). The GGAGA located 80–84 nucleotides downstream of the cleavage site in Ag5–84 is preferred and offers the most favorable conformation for self-cleavage, even in high formamide. Interestingly, a vector **ggaga** introduced 5′ of the cleavage site during cloning could, in the absence of the original GGAGA, in AgV17–79 (Figure 1A,1Bvi) still support a rapid cleavage reduced only 3-fold and confer some stability in formamide (Smith et al., 1992). A third polypurine region, GAAGGAGGA, located internally in the molecule in Ag17–79 and in Ag17–66, can apparently substitute in the absence of the first two, but only if there is an elongated polypyrimidine stretch 5′ of the cleavage site. While this structure allows self-cleavage at a 10-fold reduced rate, it is unable to support cleavage in formamide. Thus a critical interaction, seen in Ag5–84, has been eliminated, although cleavage is maintained. Removal of all three polypurine regions, as in Ag17–63, produces an inactive molecule, unable to assemble the catalytic core structure (see rates in Table 1).

Structural Considerations. The preferred RNA structure for the Ag17–79 RNA is shown in Figure 3Ai, although the structure in Figure 1Bv is thermodynamically less favorable by only 0.6 kcal/mol (Table 2). Alternate forms of a hammerhead ribozyme have recently been reported (Miller & Silver, 1991) in which the formation of a pseudoknot may decrease the rate of cleavage. Further, a conformational switch in the small ribosomal subunit through alternative pairing of 5′ and 3′ terminal sequences has been proposed (Hernandez & Flores, 1992). Evidence for alternate enzymatic forms in protein enzymes has yet to be reported, perhaps because the complexity of protein folding does not admit to variations. The simplicity and plasticity of ribozymes may more easily allow such compensation to occur. Similarly, the ability of the group I intron from *Tetrahymena* to carry out the functions of an endoribonuclease, a phosphatase, and a polymerase (Zaug et al., 1986; Zaug & Cech, 1986) may also reflect an evolutionary time when multiple functions were inherent in one molecule.

The putative variations in the folding may be an essential feature in the function of the HDV cycle. Alternative

structures which dictate hydrolysis rates can have important biological effects on the processing of the HDV molecule. In an HDV-infected cell, proteins could favor the folding of one form over the other at different stages in the replication cycle of the virus. It has been shown that a segment of the antigenomic RNA serves as the mRNA for the δ antigen (Hsieh et al., 1990), while the full-length RNA is a replicative template for production of infectious genomic RNA. Thus, different rates of cleavage might be mandated at different points in the replication cycle of the virus and mediated through alternate foldings of the RNA.

The Alternate Folding May Define an Alternate Ribozyme. We have previously described a *trans*-cleavage reaction in which a 67-nt ribozyme derived from the antigenomic RNA of hepatitis δ virus cleaved a 13-nt substrate RNA, containing 5 nts 5′ and 8 nts 3′ of the cleavage site (Prasad et al., 1992a). Similarly, Perrotta and Been cut an 8-nt substrate with a 73-nt δ ribozyme (1992), while Branch and Robertson (1991) and Wu et al. (1992) designed enzymes which were smaller in size than the substrate molecule, with ribozymes of 26 and 16 nts and substrates of 65 and 80 nts, respectively. It is clear that the rational design of the HDV ribozyme must take into account structural effects in both the enzyme and the substrate RNAs. While it is possible to incorporate changes in the region that binds a potential substrate, there will be limitations which are dictated by competing structures within a given nucleotide sequence. The possibility of an alternative folding for an active self-cleaving δ RNA may now allow the design of an even smaller δ ribozyme/substrate pair. In this case, maximally, the ribozyme of 55 nt would be targeted against a substrate of 24 nt. Although truncation of the δ RNA to 69 contiguous nts in length 3′ of the cleavage site resulted in a loss of activity in the self-cleaving RNA (Smith & Dinter-Gottlieb, 1991), further truncation may be possible in this altered form. Similarly, the precise sequence requirements of the stem II′ base-paired region must be delineated. In the hairpin ribozyme Fedor and Uhlenbeck (1990) have shown that the efficiency of the cleavage reaction varies with the sequence of the substrate RNA, yet the essential feature for cleavage is the base-pairing of ribozyme and substrate. Perrotta and Been (1992) have shown that the stem I region, which remains virtually unchanged in this new folding, can tolerate base changes, as long as the base pairing is maintained. Sequences essential for ribozyme activity appear to lie in the looped-out region of stem I and, in the genomic RNA, in the GGGCA sequence now partially paired with the cleavage site (Kumar et al., 1992). Experiments to test this new ribozyme and its parameters are now in progress.

Initial interest in the use of ribozymes for antisense therapy centered around their enzymatic activity, with cleavage of multiple substrate molecules by one ribozyme molecule a particularly appealing feature (Cech, 1988). Whether or not turnover is possible in a living cell system, ribozymes still offer the possibility of destroying their target molecules, thus permanently preventing their structural or functional activities. Thus, even a ribozyme with a reduced rate of cleavage and little or no turnover, but which is precisely targeted to a substrate sequence, could find therapeutic use. These studies outline the first steps in designing such a new ribozyme.

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