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Probing the hammerhead ribozyme structure with ribonucleases

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ABSTRACT

Susceptibility to RNase digestion has been used to probe the conformation of the hammerhead ribozyme structure prepared from chemically synthesised RNAs. Less than about 1.5% of the total sample was digested to obtain a profile of RNase digestion sites. The observed digestion profiles confirmed the predicted base-paired secondary structure for the hammerhead. Digestion profiles of both *cis* and *trans* hammerhead structures were nearly identical which indicated that the structural interactions leading to self-cleavage were similar for both systems. Furthermore, the presence or absence of Mg^{2+} did not affect the RNase digestion profiles, thus indicating that Mg^{2+} did not modify the hammerhead structure significantly to induce self-cleavage. The base-paired stems I and II in the hammerhead structure were stable whereas stem III, which was susceptible to digestion, appeared to be an unstable region. The single strand domains separating the stems were susceptible to digestion with the exception of sites adjacent to guanosines; $G^{L2.1}$ in the stem II loop and G^{12} in the conserved GAAAC sequence, which separates stems II and III. The absence of digestion at $G^{L2.1}$ in the stem II hairpin loop of the hammerhead complex was maintained in uncomplexed ribozyme and in short oligonucleotides containing only the stem II hairpin region. In contrast, the G^{12} site became susceptible when the ribozyme was not complexed with its substrate. Overall the results are consistent with the role of Mg^{2+} in the hammerhead self-cleavage reaction being catalytic and not structural.

INTRODUCTION

The hammerhead RNA self-cleavage reaction has been extensively studied in two viroids as well as several other small, circular, pathogenic, satellite RNAs which infect plants in the presence of helper virus (for reviews, 1–6). Although much is known about the mechanism of bond breakage at the site of self-cleavage (7–14), there is still little information on the conformation of the active structure which undergoes self-cleavage. Nucleotide mutations and chemical modifications (13,15–25), phosphodiester bond substitution (11,12,26), UV-induced cross-linking (27), NMR studies (28–30) and computer modelling (31–33) have given only limited information on the possible tertiary structure of self-cleaving hammerhead RNAs. Recently, crystals of the Group I self-spicing intron from *Tetrahymena* have been produced to examine the tertiary structure of this RNA complex (34), while crystals have been obtained of an in *trans* hammerhead complex of RNA ribozyme and all DNA substrate which appear appropriate for structure determination (35).

We have investigated the structural aspect of the mechanism of self-cleavage by assessing which internucleotide phosphodiester bonds in the active hammerhead structure are susceptible to limited RNase digestion. This enzymic digestion strategy, which utilises single strand and nucleotide specific RNases, is a well defined system for examining structural features of RNA species (36–38). RNase digestion has been successfully used to show the secondary and tertiary nucleotide interactions in tRNA species (37,38) and the susceptibility of modified hammerhead ribozymes to digestion (17–19,26). An important consideration in these reactions is to aim for a maximum of one single strand digestion per molecule. This removes the likelihood of exposing additional digestion sites made available only when any structural constraints are relaxed due to digestion at the first site.

In this paper we report RNase susceptible sites in a hammerhead ribozyme based on the sequence of (+) tobacco ringspot virus satellite RNA (sTRSV)(39). Hammerhead conformation in *cis* and in *trans*, as well as the presence or absence of Mg^{2+} did not appreciably alter the profile of RNase susceptible sites. The digestion profiles also confirmed the predicted base-paired secondary structure of the hammerhead ribozyme. However, digestion at sites in stem III and the absence of digestion at sites adjacent to two unpaired guanosines were unexpected.

MATERIALS AND METHODS

Oligonucleotide synthesis and labelling

The RNA oligonucleotides were synthesised on an Applied Biosystems DNA/RNA synthesiser Model 392 using Milligen phosphoramidites and Applied Biosystems deoxycytidine (dC) FOD columns. In this way the 3′ residue in all the

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oligonucleotides was always dC. Synthesis was on a 0.2 μmol scale using a primer cycle. Cleavage from support, deprotection and purification of the crude RNA sample followed the protocols described by Applied Biosystems except that there was a 14 minute coupling time (40). The average coupling efficiency was 98% as determined by analysis of the trityl fraction. During deprotection, 0.1 mM EDTA was included in all aqueous solutions.

The crude RNA oligonucleotides were 5′-labelled with γ-^{32}P-ATP and purified on a denaturing gel (20% w/v polyacrylamide, 8 M urea, 89 mM Tris, pH 8.3, 89 mM boric acid and 2 mM EDTA). The gel band representing full length product was excised and the RNA was eluted into 0.1 mM EDTA at 37°C for 16 h followed by precipitation in 0.3 M sodium acetate, pH 5.2, with 2.5 volumes of ethanol/acetone (1:1). The purified RNA was resuspended in 0.1 mM EDTA for use in self-cleavage reactions and RNase digestion experiments. Unlabelled RNA was isolated from gels stained with toluidine blue.

Hammerhead self-cleavage reaction

Self-cleavage reactions followed previously described methods with slight modification (1,3). Self-cleavage in *trans* involved mixing approximately 80 nM 5′-labelled 32-mer ribozyme (R1, 5′ GGA GUC*UGAUGAGUCCGUGAGGACGAAACAGdC 3′) with approximately 80 nM 5′-labelled 11-mer substrate (S1, 5′ GCUGUC*ACUCdC 3′), or an alternate 11-mer substrate (S2, 5′ GCUG UdC*ACUCdC 3′), which contained a dC at the conserved ...GUC*A... cleavage site sequence. This S2 molecule was used as a non-cleavable substrate. Ribozyme and substrate were combined in 1 mM EDTA then heated to 80°C for 90 sec followed by rapid cooling on ice for 15 min before adding ice cold buffer (50 mM Tris pH 7.5, 10 % PEG 8000 ± 25 mM $MgCl_2$). Samples were subsequently transferred to 25°C for 2 h and the cleavage reaction was stopped with an equal volume of EDTA-formamide loading dye (95% v/v deionised formamide, 0.25 M EDTA, 0.1% w/v bromophenol blue and 0.1% w/v xylene cyanol). These samples were heated to 80°C for 90 sec followed by rapid cooling on ice before electrophoresis on a denaturing 20% polyacrylamide gel. Percentage cleavage was calculated by comparing the radioactivity in substrate and product bands from these gels. Self-cleavage in *cis* was demonstrated using a 44-mer ribozyme-substrate sequence (RS1, 5′ UGUC*A-CUCCUGCUGGAGUCUGAUGAGUCCGUGAGGACGAAA-CAdC 3′). The RS1 molecules were not 5′-^{32}P-radioactively labelled because the labelling reaction induced self-cleavage of the RS1 sequence. Therefore, RS1 was detected in gels by staining with toluidine blue. A corresponding RS2 sequence (5′ UGUdC*A..... 3′) contained a dC substitution at the cleavage site and did not self-cleave; hence it could be 5′-labelled.

RNase digestion of the hammerhead ribozyme complex

RNA oligonucleotide samples containing the dC substitution at the cleavage site were preincubated under self-cleavage conditions in the presence or absence of $MgCl_2$ to ensure maximum probability of forming the hammerhead conformation. For the hammerhead *trans* system only the ribozyme or substrate RNA was 5′ labelled and the ratio of labelled to unlabelled RNA was 1:10 to ensure all the labelled sample was incorporated into a hammerhead complex. RNase digestion of internucleotide phosphodiester bonds was then initiated by adding appropriate enzymes to the preincubated RNA mixture for 10 min at 25°C in a total volume of 15μl. RNase T1 (0.2 to 2×10^{-2} U/μl; Sigma; *Aspergillus oryzae*, cuts downstream of unpaired guanosines — G), RNase T2 (5×10^{-4} to 5×10^{-5} U/μl; Sigma; *Aspergillus oryzae*, cuts downstream of all unpaired residues — C,U,A,G), RNase A (4×10^{-5} to 10^{-6} U/μl; Boehringer Mannheim; bovine pancreas, cuts bonds downstream of unpaired pyrimidines — C,U) and RNase U2 (5 to 1 U/μl; Calbiochem; cuts downstream of unpaired purines - A,G), were used to give a limited digestion profile for the hammerhead ribozyme structure.

RESULTS AND DISCUSSION

Hammerhead base-paired structure and self-cleavage *in cis* and *in trans*

RNA oligonucleotides, which contain the sequence elements of the (+) sTRSV hammerhead self-cleavage structure (2, 39), were synthesised chemically to provide hammerhead structures in *cis* and in *trans*; the features of the hammerhead base-paired

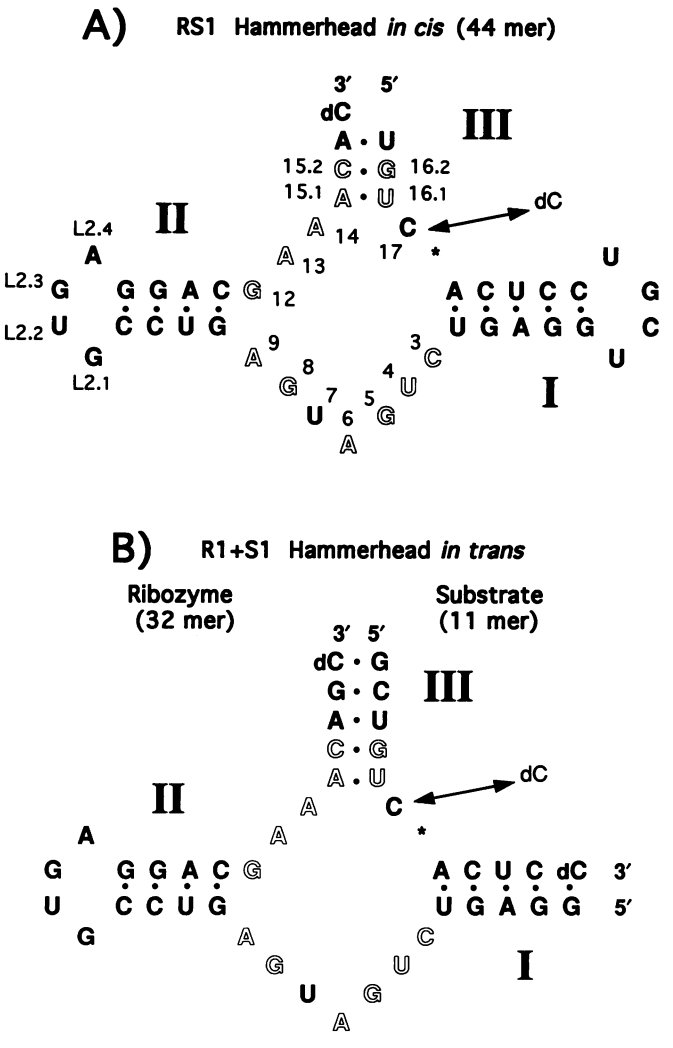

Figure 1. Hammerhead structures used in the RNase digestion studies. **A.** RS1, hammerhead structure *in cis*. The dC^{17} analogue is called RS2. **B.** R1 + S1, hammerhead structure *in trans*. The dC^{17} analogue is called R1 + S2. The self-cleavage site is indicated by *. The 13 conserved nucleotides are given as open letters. The numbering of nucleotides is according to (41).

secondary structures and the numbering of nucleotides in the structure (41) are shown in Figure 1. The structure of the *cis* acting 44-mer ribozyme-substrate hammerheads (RS1 and RS2, the dC^{17} analogue) contained hairpins at stems I and II and an open stem III with adjacent 5′ and 3′ ends. In the *trans* hammerhead system (R1+S1 and R1+S2, the dC^{17} analogue) there was only one hairpin, at stem II in the ribozyme, and open stems I and III where the ribozyme base-paired with the substrate.

In the presence of Mg^{2+}, the *cis* RS1 and the *trans* R1+S1 associated hammerhead structures self-cleaved to the expected product oligonucleotides (Figure 2). The 44-mer RS1 molecule cleaved to produce 40-mer and 4-mer species; however, only the larger of these products became visible with toluidine blue staining (Figure 2A). Cleavage of RS1 appeared complete within the 2 hour incubation. In contrast, the RS2 hammerhead, which was identical to RS1 except for the dC^{17} substitution at the conserved ...GUC*A... cleavage site sequence, showed no self-cleavage in the presence of Mg^{2+}, even with extended incubation. For the *trans* system, the 11-mer substrate S1 was cleaved to yield a 6-mer 5′ species and a 5-mer 3′ species. Because S1 was 5′-labelled, only the 6-mer product was seen in the autoradiographs (Figure 2B). For the *trans* reaction, greater than 96% of the 5′-labelled substrate was cleaved within 2 hours ($t_{1/2}$= 5 min). Substrate S2 was not cleaved by the R1 ribozyme even though S2 was identical to S1 except for the dC^{17} substitution at the cleavage site.

The efficiency of self-cleavage of RS1 and of S1 by R1 indicated that the active hammerhead conformation had been formed. Furthermore, since substitution of dC^{17} into the substrate sequence at the conserved ...GUC*A... sequence

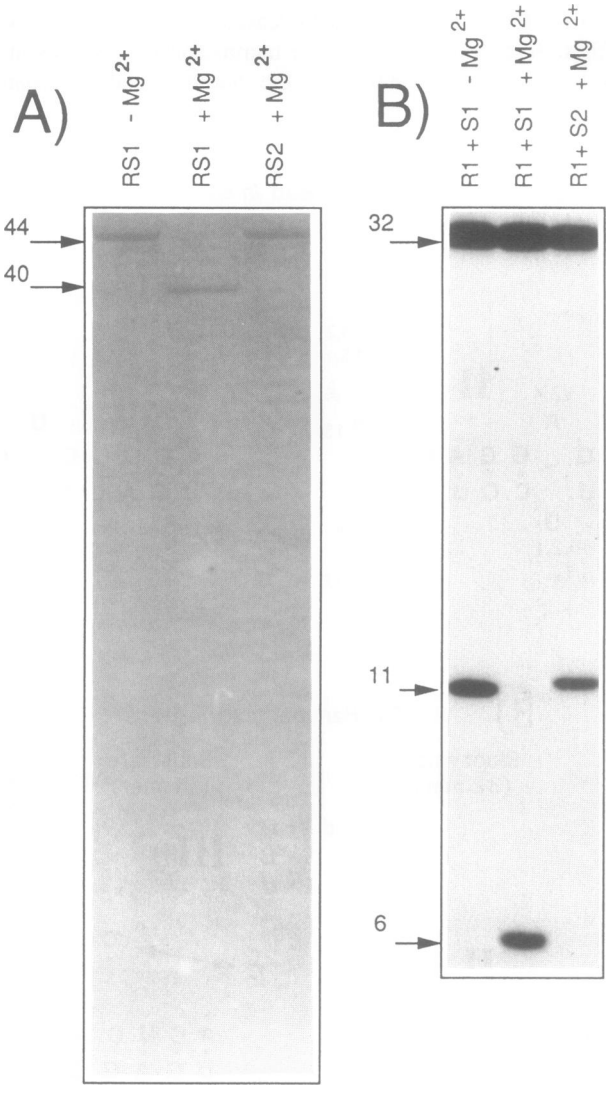

Figure 2. Self-cleavage of chemically synthesised hammerhead RNA sequences and fractionation of reaction mixtures by denaturing polyacylamide gel eletrophoresis **A:** Toluidine blue stained gel showing cleavage of the 44-mer ribozyme-substrate (RS1) to a 40-mer 3′-product in the presence of $MgCl_2$. The 4-mer 5′-product did not become visible with the toluidine blue. The deoxycytidine dC^{17} substituted 44-mer RS2 hammerhead showed no self-cleavage in the presence of $MgCl_2$. **B:** Autoradiograph showing cleavage of the 5′-labelled 11-mer substrate (S1) to a 6-mer 5′-product in the presence of the 32-mer ribozyme (R1) and $MgCl_2$. No cleavage occurred under these conditions in the deoxycytidine dC^{17} substituted 11-mer substrate S2.

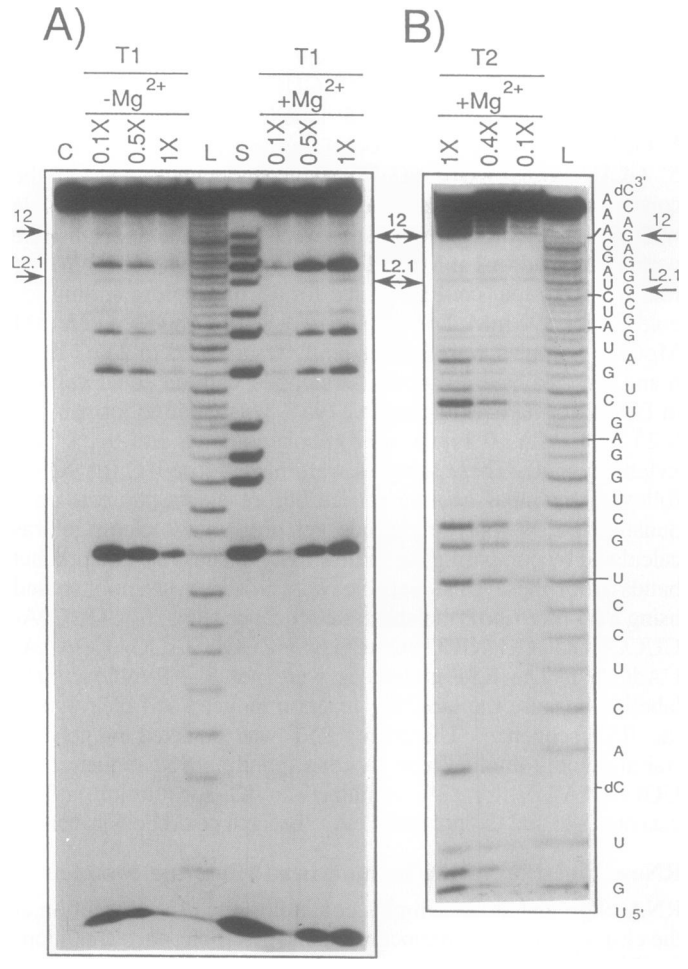

Figure 3. RNase digestion profiles of the 44-mer RS2 hammerhead structure. **A:** Limited RNase T1 digestion in the absence and presence of 25 mM $MgCl_2$ and **B:** Limited RNase T2 digestion. The nucleotide sequence corresponding to gel bands is shown adjacent to the partial alkaline ladder (L). The control (C) is undigested RS2 and a T1 sequencing (S) lane is used to show positions of susceptible phosphodiester bonds 3′ of guanosine residues. For RNAse T1 and T2 the 1×concentration was 0.2 U/μl and 5×10^{-4} U/μl respectively. Arrows at the side of each gel show the $G^{L2.1}$ and G^{12} sites not susceptible to digestion.

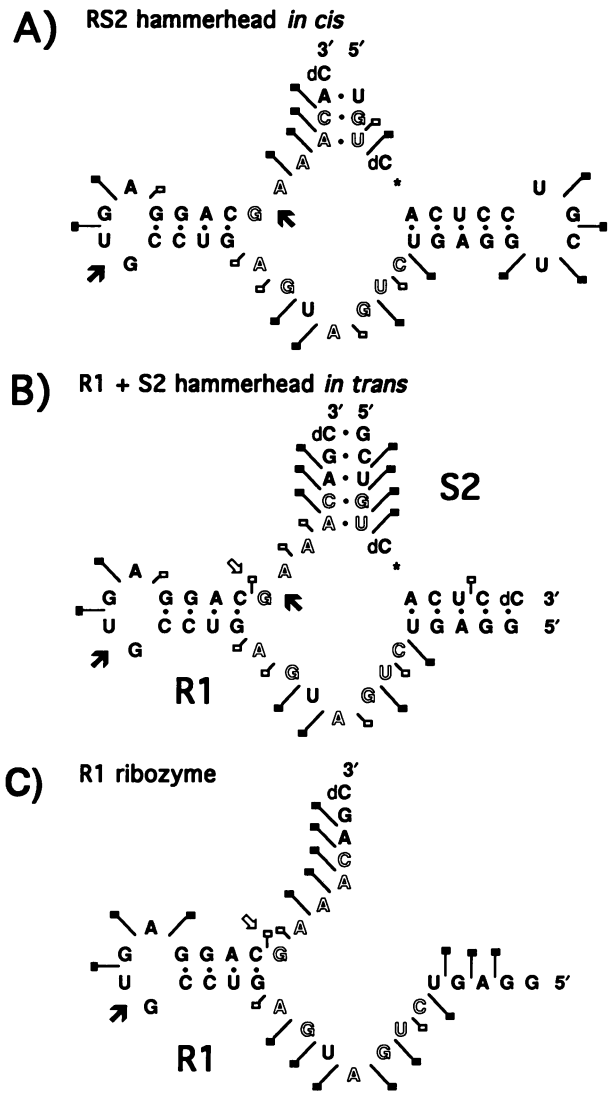

Figure 4. The map of susceptible sites to RNase T1, T2, U2 and A in **A:** RS2, **B:** R1+S2 and **C:** R1 alone. The conserved central core nucleotides are open letters. Strong digestion sites are represented by closed points and weak digestion sites by open points. Absence of digestion at $G^{L2.1}$ and G^{12} is indicated by a solid arrow, while the weak digestion between $C^{11.1}G^{12}$ is shown with an open arrow. Self-cleavage at the phosphodiester bond (*) of the substrate sequence was inhibited by a dC^{17} substitution.

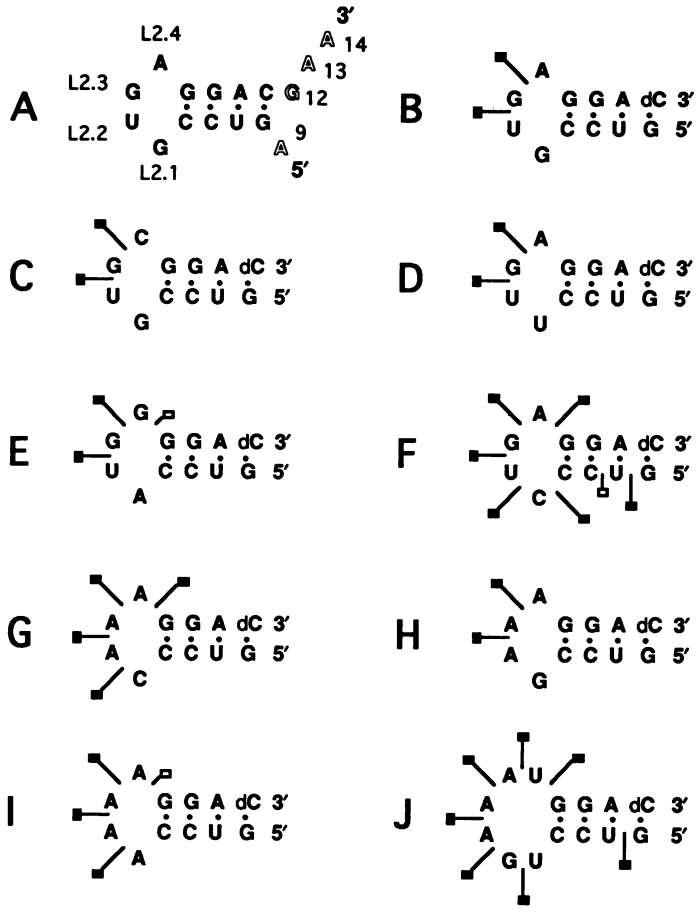

Figure 5. Sequences of different stem II hairpins and the maps of their respective RNase T1, T2, U2 and A susceptible sites. **A:** The reference hairpin loop region from the hammerhead structures shown in Figure 4. **B:** Hairpin oligonucleotide base on the reference sequence, C:, D: E: and F: variants of the reference hairpin loop sequence, and F:, G:, H: and I: hairpin oligonucleotides based on a ...GA-AA... loop sequence. Susceptibility to digestion is as described in Figure 4.

inhibited cleavage, but still allowed formation of the hammerhead structure (14,16,17), the RS2 and R1+S2 oligonucleotides were also expected to form the hammerhead structure. These dC^{17}-substituted species have therefore been used for the hammerhead structural analysis by RNase digestion.

The hammerhead ribozyme sequence of R1, with one additional nucleotide ($G^{16.4}$), was also produced by transcription from a short DNA template (8). This R1 transcript showed no differences to the chemically synthesised ribo-oligonucleotides for self-cleavage of S1 in *trans* (data not shown). Since chemical synthesis produced large quantities of product and was a flexible procedure for incorporating modified nucleotides, we chose to produce all the RNA molecules by chemical synthesis.

RNase digestion profiles of the hammerhead structure

RNase digestion profiles of dC^{17} substituted hammerhead structures were accumulated for the RNases T1,T2, A and U2. The results are illustrated for RNase T1 and T2 digestion of RS2 (Figure 3) but were consistent for all RNases (data not shown). The results combined from all RNase digestion profiles produced a map of the susceptible digestion sites in the hammerhead structure (Figure 4). The presence or absence of Mg^{2+} did not alter the RNase digestion profile, as demonstrated for T1 digestion (Figure 3A). Furthermore, in the absence of Mg^{2+}, RNase digestion profiles were identical for *trans* acting hammerheads containing either the all RNA substrate S1 or the dC^{17} substrate S2 with ribozyme R1 (data not shown). Digestion profiles were virtually identical for hammerhead structures formed in *cis* or in *trans* (Figure 4). The only consistent difference was the weak digestion of the phosphodiester bond between $C^{11.1}G^{12}$ at the 3′ end of stem II in the hammerhead formed in *trans* (Figure 4B) but not in *cis* (Figure 4A).

The phosphodiester bonds in the regions which correspond to the predicted base-paired stems I and II were not susceptible to RNase digestion even at high enzyme concentration (Figure 3B). The limited T2 digestion of RS2 showed these stable stems as

gaps surrounded by a run of bands which represent the single stranded regions. Stem II appeared particularly stable since it was maintained in the R1 ribozyme alone (Figure 4C). This agreed with previous findings that demonstrated the importance of stem II in stabilising the ribozyme structure (22,25,28,31). In contrast to stems I and II in the hammerhead structure, phosphodiester bonds in stem III were susceptible to RNase, which suggested that this stem was unstable. The same conclusion was made from an NMR examination of the hammerhead structure (30). Thus, since both the *cis* and *trans* hammerhead structures self-cleaved with high efficiency (Figure 2), the RNase susceptibility and therefore instability of the stem III region suggests that an unstable stem III correlates with efficient self-cleavage; this is consistent with the results of others (14,30,32).

The single stranded regions of the hammerhead stuctures were particularly susceptible to digestion although some sites consistently showed weak or negligible digestion (Figure 4). It could not be determined if the weaker digestion was the result of conformational distortion around the sites or whether some additional interaction was involved to partially protect the sites. It is of interest that the central core region, which contains several of the weaker digestion sites, has been implicated as the site coordinating Mg^{2+} within the hammerhead structure (11,12,17,20,21,23,31). An unexpected, consistently strong, digestion site occurred at the 3'-end of stem I between $U^{2.1}$ and C^{3} (Figure 4). The reason for the susceptibility of this site to both RNase A and T2, is not clear, but it could be a consequence of an unstable $A^{1.1} \cdot U^{2.1}$ base pair at the end of the stem.

Two sites in predicted single strand regions of the hammerhead structure were not susceptible to RNase digestion (Figures 3 and 4). The absence of digestion at these two sites, adjacent to $G^{L2.1}$ in stem II loop and G^{12} in the conserved GAAAC sequence indicated that some unusual interaction inhibited RNase digestion. The possible reason for the effect in the stem II loop is discussed in the next section. The G^{12} has been shown by NMR to form a $G^{12} \cdot A^{9}$ base pair (30); this base pairing could explain why the phosphodiester bond adjacent to the G^{12} was resistant to digestion (Figure 4A and B). The reason for the observed weak digestion adjacent to A^{9} is unclear, since it would also be expected to be resistant to digestion if in a $G^{12} \cdot A^{9}$ base pair. Digestion of the ribozyme R1 sequence alone showed that all sites in the single strand regions were susceptible to RNase with the exception of the loop of stem II (Figure 4C). This suggested that the $G^{12} \cdot A^{9}$ base pair at the end of stem II formed only as a consequence of substrate binding to the ribozyme. This proposal is supported by the results from a NMR examination of both a hammerhead structure in *trans* and a ribozyme alone; an NMR resonance could be assigned only to a $G^{12} \cdot A^{9}$ base pair at the end of stem II in the hammerhead structure (28,30). The issue remains as to why RNAse cleavage on the 3'-side of G^{12} is inhibited. The possibility of $G^{12} \cdot A^{9}$ and $A^{13} \cdot G^{8}$ mismatched base pairs has been recognised (30, 42, 43) while results of mutation data have been used to argue against the presence of such base pairs (44–46) and also to suggest (45) that G^{8} and G^{12} might be part of a hydrogen-bonded network, but this network was not defined. Obviously the actual situation is yet to be resolved.

RNase digestion profile of stem-loop II region

To determine whether the lack of digestion adjacent to $G^{L2.1}$ in the stem II loop was the result of some tertiary interaction in the hammerhead structure, several small ribo-oligonucleotides which contain only the stem II hairpin sequence were subjected to limited RNase digestion. The hairpin region from the R1 and RS1 sequence is shown for reference (Figure 5A). The different loop sequences in the hairpin oligonucleotides and the digestion maps of RNase susceptible sites in these sequences are shown in Figure 5. No digestion occurred adjacent to $G^{L2.1}$ or $A^{L2.4}$ in the hairpin oligonucleotide containing the stem II loop sequence found in the R1 and RS1 molecules (Figure 5B). This result is consistent with the presence of a $G^{L2.1} \cdot A^{L2.4}$ base pair in the hairpin loop, as was also suggested from an NMR analysis of a hairpin loop by Heus and Pardi (47). The difference in the susceptibility of $A^{L2.4}$ to RNAse digestion in the hairpin oligonucleotide (Figure 5), in the ribozyme (Figure 4C) and the hammerhead complex (Figure 4A, B) is not known.

The loop sequences ...GUGA... and ...GAAA..., with their base-paired stem II (Figures 5B, H), conform to a previously described unusually stable hairpin structure (48,49) which was shown to contain a $G^{L2.1} \cdot A^{L2.4}$ nucleotide interaction (47); such an interaction could reduce the RNase susceptibility of the phosphodiester bonds associated with these nucleotides. This proposal was examined, and confirmed, by the susceptibility of different hairpin stem II sequences to RNase digestion (Figure 5). Where the hairpin loops contained G..A, G..C, U..A or A..G as the L2.1 and L2.4 nucleotides, RNase digestion adjacent to these nucleotides was inhibited (Figure 5B, H; C; D and E, respectively). However, with UG..AU, C..A or A..A nucleotide subdtitutions then all loop phosphodiester sites were susceptible to RNase digestion (Figure 5J; F, G and I, respectively). These latter loop sequences did not conform to the predicted sequence composition for an unusually stable hairpin structure (48,49). In particular, the addition of a uridine 5' and 3' of the ...GAAA... sequence at L2.1 and L2.6 (Figure 5J) allowed RNase digestion at all loop phosphodiester sites which indicated that these uridine additions abolish the stable nature of the hairpin containing only ...GAAA... in the loop sequence (Figure 5H).

Hence, the RNase digestion maps of these different hairpins showed that if G·A, G·C, A·U base interactions occur between the loop L2.1 and L2.4 nucleotides and the first base pair closing the stem was $G^{10.4} \cdot C^{11.4}$, then phosphodiester sites adjacent to the loop L2.1 and L2.4 nucleotides were resistant to RNase digestion. This indicated that a stable hairpin structure, as suggested by (48,49) was formed if the L2.1 and L2.4 nucleotides established any of the G·A, G·C or A·U base interactions. The effect of possible G·U interactions was not examined. Thus, the sequence for the stem II hairpin in the hammerhead ribozyme structure (Figure 1) conforms to predictions for an unusually stable hairpin. Stabilisation of stem II may be a critical aspect to prepare the ribozyme for binding the substrate and/or for allowing the self-cleavage reaction to proceed.

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