

binding pocket in the vicinity of the active site, whereas for both X-ray and *MC-Sym* structures two binding pockets can be observed, as described from the results of NMR experiments.³⁸ Molecular dynamics simulations were performed using the X-ray and *MC-Sym* structures in the presence of lead ions. Both simulations showed very stable structures, and the geometry of the catalytic phosphodiester link was studied. The X-ray structure does not exhibit a geometry suitable to an S_N2 reaction, whereas the *MC-Sym* structure remains close to it. At this time, more experimental data are needed to discriminate between those two conformations. Although the *MC-Sym* structure is the most different, it is able to explain the catalytic activity of the leadzyme.

Acknowledgment

We thank Allen W. Nicholson for useful comments and suggestions. We thank Michael Gilson for providing an access to the Cambridge Structural Database at CARB/NIST, and to Mining Minima. This work was supported by a grant from the Medical Research Council of Canada (MT-14604) to FM. PG holds a M.Sc. scholarship from the Fonds pour la Formation de Chercheurs et l'Aide a la Recherche du Quebec.

[34] Hammerhead Ribozyme Structure and Function in Plant RNA Replication

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Introduction

Evidence that there are biological entities with a lower complexity than viruses has been accumulating for the past 30 years. The most conspicuous representatives of this subviral world are viroid and viroid-like satellite RNAs from plants, which are solely composed of a small [250–450 nucleotide (nt)] single-stranded circular RNA with a high secondary structure content and without any apparent messenger capacity.¹ This makes the replication cycle of these molecular parasites, proposed to occur through a rolling circle mechanism, extremely dependent on enzymes provided by the host in the case of viroids, or by the host and the helper virus in the case of viroid-like satellite RNAs. However, three viroids and all viroid-like satellite RNAs code, in an ample sense, ribozymes catalyzing self-cleavage of their oligomeric strands generated in replication and, in some cases, self-ligation

¹ T. O. Diener, *FASEB J.* 5, 2808 (1991).

of the resulting linear monomers. The hammerhead structure²⁻⁴ is the most widely distributed ribozyme in these small plant RNAs,⁵ and it will be the subject of the present review. To provide a more general outlook we will also consider, in addition to plant viroid and viroid-like satellite RNAs, other small plant and animal RNAs with less-defined biological roles but also exhibiting catalytic activity through hammerhead ribozymes.

Structure of Hammerhead Ribozymes

Catalog of Natural Hammerhead Structures and Conserved Sequence and Structural Motifs

The hammerhead, ribozyme is a small RNA motif that self-cleaves in the presence of a divalent metal ion, generally Mg^{2+} , at a specific phosphodiester bond producing 2',3'-cyclic phosphate and 5'-hydroxyl termini.^{2,4,6} Figure 1 shows the known natural hammerhead structures in panels corresponding to viroid-like satellite RNAs grouped according to their helper virus (B to D),^{2,4,7-11} viroids (E),^{3,12,13} one viroid-like RNA from cherry whose biological nature remains undetermined (F),¹⁴ and three small RNAs, one from carnation (G)¹⁵ and two of animal origin (H),^{16,17} which have a homologous DNA counterpart; the consensus hammerhead structure (A) is displayed as originally proposed⁶ (Fig. 1, right), and in a more recent representation derived from crystallography^{18,19} (Fig. 1, left). It can be observed that these RNAs have hammerhead structures in their plus or in both polarity strands.

² G. A. Prody, J. T. Bakos, J. M. Buzayan, I. R. Schneider, and G. Bruening, *Science* **231**, 1577 (1986).

³ C. J. Hutchins, P. D. Rathjen, A. C. Forster, and R. H. Symons, *Nucleic Acids Res.* **14**, 3627 (1986).

⁴ A. C. Forster and R. H. Symons, *Cell* **49**, 211 (1987).

⁵ G. Bruening, *Methods Enzymol.* **180**, 546 (1989).

⁶ K. J. Hertel, A. Pardi, O. K. Uhlenbeck, M. Koizumi, E. Ohtsuka, S. Uesugi, R. Cedergren, F. Eckstein, W. L. Gerlach, R. Hodgson, and R. H. Symons, *Nucleic Acids Res.* **20**, 3252 (1992).

⁷ J. M. Kaper, M. E. Tousignant, and G. Steger, *Biochem. Biophys. Res. Commun.* **154**, 318 (1988).

⁸ L. Rubino, M. E. Tousignant, G. Steger, and J. M. Kaper, *J. Gen. Virol.* **71**, 1897 (1990).

⁹ P. Keese and R. H. Symons, in "Viroids and Viroid-like Pathogens" (J. S. Semancik, ed.), p. 1. CRC Press, Boca Raton, FL, 1987.

¹⁰ R. F. Collins, D. L. Gellatly, O. P. Seghal, and M. G. Abouhaidar, *Virology* **241**, 269 (1998).

¹¹ W. A. Miller, T. Hercus, P. M. Waterhouse, and W. L. Gerlach, *Virology* **183**, 711 (1991).

¹² C. Hernández and R. Flores, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3711 (1992).

¹³ B. Navarro and R. Flores, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 11262 (1997).

¹⁴ F. Di Serio, J. A. Daròs, A. Ragozzino, and R. Flores, *J. Virol.* **71**, 6603 (1997).

¹⁵ C. Hernández, J. A. Daròs, S. F. Elena, A. Moya, and R. Flores, *Nucleic Acids Res.* **20**, 6323 (1992).

¹⁶ L. M. Epstein and J. G. Gall, *Cell* **48**, 535 (1987).

¹⁷ G. Ferbeyre, J. M. Smith, and R. Cedergren, *Mol. Cell. Biol.* **18**, 3880 (1998).

¹⁸ H. W. Pley, K. M. Flaherty, and D. B. McKay, *Nature* **372**, 68 (1994).

¹⁹ W. G. Scott, J. T. Finch, and A. Klug, *Cell* **81**, 991 (1995).

Inspection of natural hammerhead structures shows that they are characterized by a central core with a cluster of strictly conserved nucleotide residues flanked by three double-helix regions (I, II, and III) with loose sequence conservation except positions 15.2 and 16.2, which in most cases form a C-G pair, and positions 10.1 and 11.1, which in most cases form a G-C pair (Fig. 1). X-ray crystallography of two model hammerhead structures has revealed a complex array of noncanonical interactions between the residues forming the central core, thus providing clues as to why they are strictly conserved in all natural hammerhead structures.^{18,19} Prominent in this array are three non-Watson–Crick pairs (involving A9 and G12, G8 and A13, and U7 and A14 that extend helix II) and a uridine turn motif, the tetranucleotide CUGA (positions 3–6), which along with the active site residue forms the catalytic pocket.

Unusual Features of Some Hammerhead Structures

Deviations from the consensus model have been observed in some natural hammerhead structures (Fig. 1). Apart from some intrinsic flexibility these deviations are most probably the consequence that because of their smallness, the genetic information in viroid and viroid-like RNAs is very compressed and the involvement of specific nucleotide residues in additional functions other than self-cleavage can be presumed. In such cases, a compromise must be reached that may not be optimal for the individual functions.

The pair between C15.2 and G16.2 is substituted by U15.2 and A16.2, respectively, in the plus and minus hammerhead structures of sRPV and csc RNA1 (for abbreviations see Fig. 1). The common C17 residue preceding the self-cleavage site is A in both polarity hammerhead structures of csc RNA1, as well as in those corresponding to the plus strand of sRPV, the minus strand of sLTSV, and in a sequence variant of the minus strand of ASBVd. The other two possible substitutions are excluded, most likely because an U17 could base pair with the conserved A14 extending helix III, and a G17 could base pair with the conserved C3 extending helix I; as mentioned above, conserved residues of the central core are involved in a series of noncanonical interactions that are crucial for the catalytic activity. The C7 or U7 is A in the plus hammerhead structures of sLTSV and csc RNA1, and in the minus hammerhead structures of ASBVd and CarSV RNA; a G at position 7 has never been found. It has already been indicated that the base pair of helix II closest to the central core is generally G10.1 and C11.1. However, there are two exceptions to this rule. First, the plus and minus hammerhead structures of sRPV and CarSV RNA, respectively, have two extra residues, a C between A9 and G10.1, and an A between C11.1 and G12 (Fig. 1); whether or not these two extra residues form a noncanonical base pair that lengthens helix II remains to be determined. And second, between A9 and G10.1, there is an extra U in the plus hammerhead structures of sLTSV and sArMV, and an extra A in the plus hammerhead

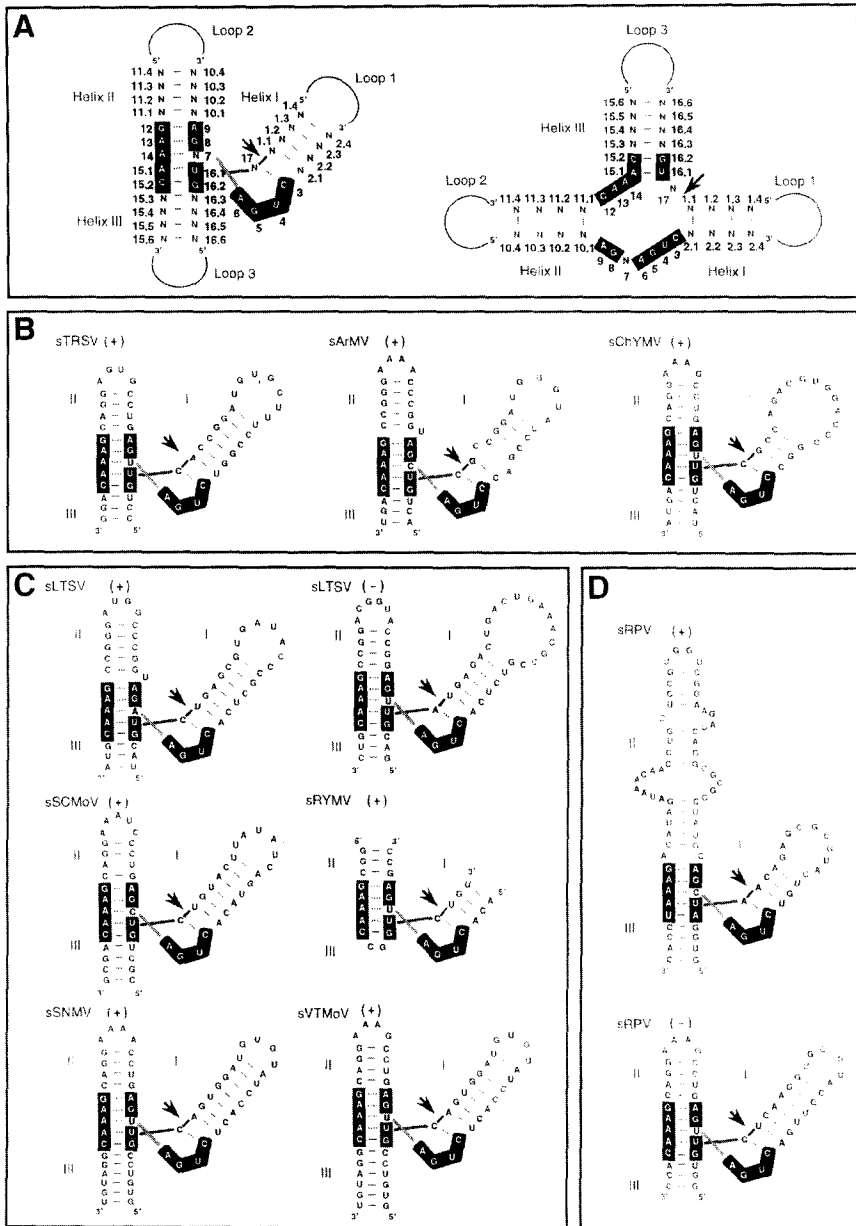
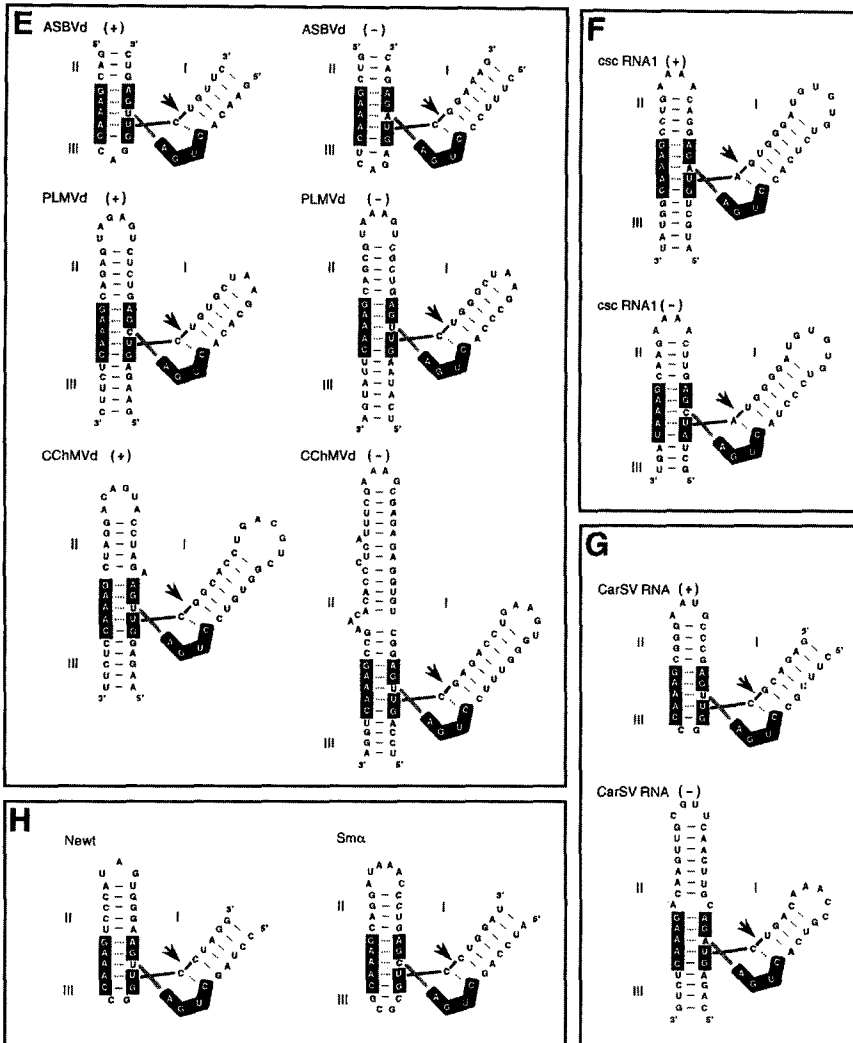


FIG. 1. Hammerhead structures found in nature. (A) Consensus hammerhead structure schematically represented according to X-ray crystallography (left) and as originally proposed with its numbering system⁶ (right). Letters on a dark background refer to absolutely or highly conserved residues in all natural hammerhead structures. Arrows indicate self-cleavage sites. Watson-Crick base pairs and



noncanonical interactions are denoted with continuous and broken lines, respectively. (B) Viroid-like satellite RNAs from nepoviruses: sTRSV (tobacco ringspot virus), sArMV (*Arabidopsis* mosaic virus) and sChYMoV (chicory yellow mottle virus).^{2,7,8} (C) Viroid-like satellite RNAs from sobemoviruses (also called viroids): sLTSV (lucerne transient streak), sSNMV (*Solanum nodiflorum* mottle), sSCMoV (subterranean clover mottle), sVTMoV (velvet tobacco mottle), and sRYMV (rice yellow mottle).^{4,9,10} (D) Viroid-like satellite RNAs from luteoviruses: sRPV (cereal yellow dwarf virus-RPV).¹¹ (E) Viroids: ASBVd (avocado sunblotch), PLMVd (peach latent mosaic), CChMVd (chrysanthemum chlorotic mottle).^{3,12,13} (F–H) Other small RNAs with hammerhead structures: csc RNA1 (cherry small circular RNA1),¹⁴ CarSV RNA (carnation small viroid-like RNA),¹⁵ and Newt and Sm α (transcripts of the newt satellite DNA II and of the schistosome satellite DNA).^{16,17}

structure of CChMVd. The extra U or A, which are compatible with extensive *in vitro* self-cleavage, could either induce a rearrangement of the junction between helix II and the three adjacent noncanonical interactions of the central core, or be accommodated as a bulging residue.

Similarities between Hammerhead Structures of Some Catalytic RNAs

The plus and minus hammerhead structures of sLTSV are more closely related to each other than to any of the other known hammerhead structures.⁴ This is a relatively frequent situation as illustrated by the cases of both polarity hammerhead structures of PLMVd,¹² CChMVd,³ and *csc* RNA1.¹⁴ with the sequence similarity being particularly noticeable in the domain formed by helix I and loop 1 (Fig. 1). These intramolecular similarities may have resulted from template switching by an RNA polymerase in the course of evolution.⁴ More specifically, the sequences forming the hammerhead structures of one polarity in these RNAs could have served as the templates for synthesis of the corresponding hammerhead structure of the opposite polarity.¹⁴

There are also limited sequence similarities between the hammerhead structures of different catalytic RNAs. This is the situation found between the residues of helices I and II close to the central core of both PLMVd hammerhead structures, and the corresponding residues of the hammerhead structures of sSCMoV, sLTSV (plus and minus), and ASBVd (plus).¹² Also in this line, the octanucleotide GGAU-GUGU forming part of helix I and loop 1, and the hexanucleotide CAAAAG forming part of helix II and loop 2 in both *csc* RNA1 hammerhead structures, are found in equivalent positions in the hammerhead structures of sArMV and sSNMV.¹⁴ This can be regarded as support for a common phylogenetic origin of these RNAs, although other possibilities cannot be dismissed.

Computed-Assisted Search for Novel Hammerhead Structures in Databases

On the basis of the consensus structural and sequence features of the hammerhead (Fig. 1), a program was developed to look for domains of this kind in databases. This search not only retrieved the hammerhead structures described previously in plant viroid and viroid-like RNAs as well as in the satellite DNA II sequences of several newt species but, additionally, it uncovered a new hammerhead structure in the satellite DNA sequences of the human blood fluke *Schistosoma mansoni*.¹⁷ PCR (polymerase chain reaction) amplification with primers flanking the repeated unit led mainly to a 335-bp fragment that was found to contain a hammerhead domain preceded by a region with RNA polymerase III promoter elements and followed by a 3'-terminal region. The hammerhead domains of some of the clones fulfill the criteria for activity and self-cleave *in cis* during *in vitro* transcription. The presence of the RNA polymerase III promoter elements suggested that the schistosome repeat was also transcribed *in vivo*.

analysis of total cellular RNA by Northern blotting, RT-PCR, and primer extension confirmed this prediction and showed that self-cleavage occurs also *in vivo*. Moreover, sequence analysis identified in schistosome DNA a potential *trans* cleavage target within a gene coding for a synaptobrevin-like protein, and the corresponding RNA transcript was efficiently cleaved *in vitro*. The discovery of this novel hammerhead structure underscores the potential of bioinformatics for mining in databases functional RNA domains once their key structural motifs have been identified.¹⁷

In Vitro Selection of Hammerhead Ribozymes

The importance of the conserved nucleotide residues forming the central core of the hammerhead structure was initially analyzed by introducing single mutations by site-directed mutagenesis.²⁰ With the advent of *in vitro* selection methods it has been possible to apply this new tool to isolate (i) hammerhead ribozymes with increased AUA cleavage activity from an RNA pool randomized at three positions 7, 10.1 and 11.1,²¹ (ii) *trans*-acting hammerhead ribozymes from an RNA pool in which the helix II-loop 2 sequence and most of the central core were randomized,²² and (iii) *cis*-acting hammerhead ribozymes from an RNA pool in which positions 10.1 and 11.1 and most of the central core were randomized.²³ Interestingly, the active ribozymes recovered in all cases predominantly contain the central core of the natural consensus hammerhead structure, thus showing that it offers the optimal catalytic efficiency for this motif.

The hammerhead ribozyme has been optimized by natural selection for cleavage after GUC (Fig. 1), and it is assumed that the inability to cleave after a G17 results from forming a pair with C3 that impairs the participation of these two residues in alternative interactions required for catalysis. *In vitro* selection has been used to investigate the nature of hammerhead-like domains capable of cleaving after AUG. Such catalytic domains do exist in the sequence space, and one of the ribozymes selected cleaves *in cis* and *trans* after this triplet with rates comparable to that of the GUC-cleaving hammerhead.²⁴ Nuclease probing indicates that the selected ribozyme has an overall secondary structure similar to that of the consensus hammerhead ribozyme, although there are differences that include substitutions and two deletions (positions 3 and 9) in the central core, and an altered helix II-loop 2 sequence. These results expand the application of hammerhead ribozymes for specific RNA cleavage and underline the power of *in vitro* selection.

²⁰ D. E. Ruffner, G. D. Stormo, and O. C. Uhlenbeck, *Biochemistry* **29**, 10695 (1990).

²¹ K. L. Nakamaye and F. Eckstein, *Biochemistry* **33**, 1271 (1994).

²² M. Ishizaka, Y. Ohshima, and T. Tani, *Biochem. Biophys. Res. Commun.* **214**, 403 (1995).

²³ J. Tang and R. R. Breaker, *RNA* **3**, 914 (1997).

²⁴ N. K. Vaish, P. A. Heaton, O. Fedorova, and F. Eckstein, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2158 (1998).

Function of Hammerhead Ribozymes in Their Natural Habitat

Rolling Circle Mechanism for Replication of Viroid and Viroid-like Satellite RNAs

The available data indicate that replication of these small RNAs occurs through a rolling-circle mechanism with exclusively RNA intermediates.^{25,26} Two variants of the mechanism, symmetric and asymmetric, have been proposed, their main difference being the existence and absence, respectively, of the circular monomeric minus RNA acting as a replicative intermediate. Detection of this RNA species in tissue infected by ASBVd²⁶⁻²⁸ and by PLMVd²⁹ is taken as evidence that these viroids, and most likely CChMVd and the viroid-like satellite RNAs with ribozymes in both polarity strands, follow the symmetric pathway.^{30,31} In contrast, nonhammerhead viroids and other viroid-like satellite RNAs with ribozymes only in one polarity strand seem to replicate via the asymmetric alternative.^{32,33} In this asymmetric variant of the rolling circle mechanism, the infecting monomeric plus circular RNA is transcribed into linear multimeric minus strands that are directly used as a template for the generation of the linear multimeric plus strands. In the symmetric variant, the linear multimeric minus strands are processed and ligated to the minus circular monomer that in the second half of the cycle, symmetric to the first half, serves as the template for synthesis of the multimeric plus linear RNAs. In both cases, the plus multimeric forms are cleaved to unit-length molecules that are then ligated to yield the circular progeny. The rolling circle mechanism, therefore, requires a highly specific processing activity to excise precisely the monomeric forms from the oligomeric intermediates. Such specificity is achieved in some viroid and viroid-like satellite RNAs by ribozymes embedded in one or in both polarity strands, thus circumventing the need to rely on host factors for this critical replication step. The ribozymes are of the hammerhead type, except in the case of the minus polarity strands of the viroid-like satellite RNAs from nepoviruses, which self-cleave through hairpin ribozymes.³⁰

²⁵ A. D. Branch and H. D. Robertson, *Science* **223**, 450 (1984).

²⁶ C. J. Hutchins, P. Keese, J. E. Visvader, P. D. Rathjen, J. L. McInnes, and R. H. Symons, *Plant Mol. Biol.* **4**, 293 (1985).

²⁷ J. A. Daròs, J. E. Marcos, C. Hernández, and R. Flores, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12813 (1994).

²⁸ J. A. Navarro, J. A. Daròs, and R. Flores, *Virology* **253**, 77 (1999).

²⁹ F. Bussière, J. Lehoux, D. A. Thompson, L. J. Skrzeczkowski, and J. P. Perrault, *J. Virol.* **73**, 6353 (1999).

³⁰ G. Bruening, B. P. Passmore, H. Van Tol, J. Buzayan, and P. E. Feldstein, *Mol. Plant-Microbe Interact.* **4**, 219 (1991).

³¹ R. H. Symons, *Nucleic Acids Res.* **25**, 2683 (1997).

³² A. D. Branch, B. J. Benefeld, and H. D. Robertson, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9128 (1988).

³³ P. A. Feldstein, Y. Hu, and R. A. Owens, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6560 (1998).

In Vivo Functional Significance of Hammerhead Ribozymes

Distinct lines of evidence support the involvement of hammerhead structures in the *in vivo* processing of oligomeric viroid and viroid-like RNAs containing these catalytic domains. For sTRSV,² sRPV,¹¹ ASBVd,^{27,34} CChMVd,¹³ PLMVd (Hernández, unpublished data), and CarSV RNA (Daròs, unpublished data), linear monomeric RNAs of one or both polarities with 5' termini identical to those produced in the corresponding *in vitro* self-cleavage reactions have been isolated from infected tissue. Moreover, compensatory mutations or covariations that preserve the stability of the hammerhead structures are frequently found in sequence variants of PLMVd^{12,35} and CChMVd,^{13,36} and the observed *in vivo* reversion of mutations introduced *in vitro* to eliminate self-cleavage of the hammerhead structure of a viroid-like satellite RNA³⁷ further supports the *in vivo* role of these ribozymes, as does the correlation existing between the infectivity of different PLMVd and CChMVd variants and the extent of their self-cleavage during *in vitro* transcription.^{35,36} On the other hand, the presence of a 2'-phospho monoester, 3',5'-phospho diester bond at a unique position of the plus circular sVTMoV and sSNMV RNAs has been interpreted as the signature of an RNA ligase at the ligation site.³⁸ Since such a position is coincidental with the self-cleavage sites predicted by the hammerhead structures contained in these RNAs, this can be considered as more indirect proof in favor of their *in vivo* significance. Data suggesting the existence of an extra 2'-phospho monoester at the nucleotide preceding the predicted self-cleavage/ligation site have also been obtained for other viroids^{35,36} and viroid-like satellite RNAs.^{11,37}

With the exception of PLMVd, for which certain degree of self-ligation of the linear monomers resulting from self-cleavage of the dimeric transcripts has been reported,³⁹ no significant reversibility of the cleavage reaction mediated by hammerhead structures has been detected.² Most likely this is because the *in vitro* folding of PLMVd linear molecules into a conformation in which the close proximity of the 5' and 3' termini favors their spontaneous circularization. However, since the phosphodiester bonds produced are mostly 2',5' instead of the 3',5' usually found in RNA,³⁹ the *in vivo* significance of this reaction is unclear. Intriguingly, the only report that disagrees with the *in vivo* functioning of a hammerhead structure concerns a nonpathogenic RNA, the newt transcript, for which the *in vitro* self-cleavage site has been mapped 46–47 residues downstream of the *in vivo* cleavage site in ovarian tissue.¹⁶ Because of the very distinct biological nature of

³⁴ J. A. Navarro and R. Flores, *EMBO J.* **19**, 2662 (2000).

³⁵ S. Ambrós, C. Hernández, J. C. Desvignes, and R. Flores, *J. Virol.* **72**, 7397 (1998).

³⁶ M. de la Peña, B. Navarro, and R. Flores, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 9960 (1999).

³⁷ C. C. Sheldon and R. H. Symons, *Virology* **194**, 463 (1993).

³⁸ P. A. Kibertis, J. Haseloff, and D. Zimmern, *EMBO J.* **4**, 817 (1985).

³⁹ F. Côte and J. P. Perrault, *J. Mol. Biol.* **273**, 533 (1997).

this molecule with respect to most hammerhead RNAs, this discrepancy in their processing mechanisms should not be surprising.

Regulation of Hammerhead Ribozymes in Vivo

The activity of these catalytic domains must be finely tuned during replication of viroid and viroid-like RNAs in which they are embedded; hammerhead ribozymes must catalyze processing of oligomeric RNAs to monomers but at the same time their activity should be regulated to preserve a certain level of monomeric circular RNAs to act as templates in the rolling circle replication. Two different strategies have been proposed to achieve this. Some of the hammerhead structures, such as those found in both ASBVd RNAs,³ in the plus sRYMV and CarSV RNAs,^{10,15} and in the newt and schistosoma transcripts,^{16,17} are thermodynamically unstable, having a stem III of only two or three base pairs closed by a small loop of two or three residues (Fig. 1). In line with this instability, *in vitro* self-cleavage of these monomeric RNAs is very inefficient. However, in the corresponding dimeric or multimeric replicative intermediates, the sequences of two single-hammerhead structures can form a stable double-hammerhead structure with an extended helix III that promotes efficient self-cleavage⁴⁰. A different situation within this same scheme has been observed in the plus sRPV RNA in which adoption of an active single-hammerhead structure in the monomeric form is prevented by a pseudoknot between residues in loop I and in a G+C-rich bulge of helix II; in a multimeric context, a double-hammerhead structure that lacks the pseudoknot can be formed and mediate efficient self-cleavage.⁴¹

A distinct strategy has been proposed for sLTSV, PLMVd, CChMVd, and esc RNA1, in which stable single-hammerhead structures can be potentially adopted by the monomeric plus and minus strands. However, the formation of these hammerhead structures is impeded in their predicted conformations of lowest free energy because the conserved sequences of both polarity hammerhead structures, because of their extensive complementarity, are involved in an alternative folding that does not promote self-cleavage of the monomeric RNAs. The catalytically active hammerhead structures are probably only formed transiently during transcription, promoting self-cleavage of the multimeric RNAs.^{4,12} Therefore, there seems to be an interplay between two conformations, one with the hammerhead structure and promoting self-cleavage, and another favoring circularization, mediated probably by a host RNA ligase.

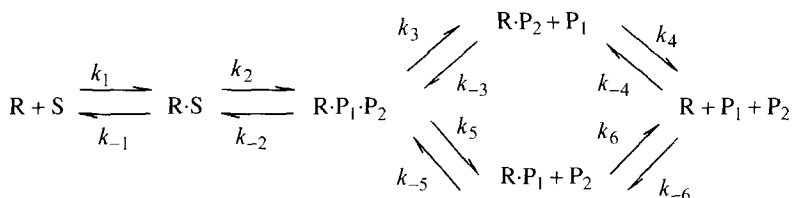
⁴⁰ A. C. Forster, C. Davies, C. C. Sheldon, A. C. Jeffries, and R. H. Symons, *Nature* **334**, 265 (1988).

⁴¹ S. I. Song, S. L. Silver, M. A. Aulik, L. Rasochova, B. R. Mohan, and W. A. Miller, *J. Mol. Biol.* **293**, 781 (1999).

In Vitro Assays to Test Hammerhead-Mediated RNA Cleavage

Kinetic Pathway for Hammerhead Ribozyme

A major effort has been made over the past few years to understand the mechanism that governs the hammerhead cleavage reaction, which can occur *in cis* and *in trans*. In their natural context, hammerhead ribozymes act *in cis* and, therefore, they catalyze a single turnover intramolecular cleavage. Initial studies were performed in the *cis* format, and the extent of RNA self-cleavage under standard conditions (see below) was found to be extremely rapid (completed in less than 1 min) but no further kinetic analysis was done.^{4,42} To gain a deeper insight, the hammerhead was divided into two separate fragments, the ribozyme itself that remains unchanged after the reaction, and the substrate that is cleaved *in trans* when combined with the ribozyme in the presence of Mg^{2+} . This intermolecular format, in which the ribozyme may proceed through multiple rounds of substrate binding, cleavage, and product release, has permitted a detailed kinetic dissection.^{43,44} A minimal kinetic pathway has been proposed for the intermolecular reaction:



in which the five main species are the ribozyme (R), substrate (S), ribozyme-substrate complex ($R \cdot S$), ribozyme-products complex ($R \cdot P_1 \cdot P_2$), and products (P_i).⁴⁵ The reaction starts with the binding between ribozyme and substrate to form the $R \cdot S$ complex, a step governed by the rate constant k_1 . At this point, if Mg^{2+} or other divalent metal ion is present, cleavage occurs, giving the $R \cdot P_1 \cdot P_2$ complex with a rate constant k_2 . These steps are reversible and elemental dissociation rate constants for the complexes (k_{-1} , k_{-3} , k_{-4} , k_{-5} and k_{-6}), and even a rate constant for ligation (k_{-2}), can be defined.

Methods for Testing RNA Cleavage Using a Cis Format

The hammerhead RNA is obtained by *in vitro* transcription of a linearized recombinant plasmid ($0.1 \mu g/\mu l$) containing, under the control of a phage RNA polymerase promoter, either a minimal hammerhead motif⁴² or a larger sequence in which the hammerhead motif is embedded.⁴⁶ Alternatively, the template for

⁴² C. C. Sheldon and R. H. Symons, *Nucleic Acids Res.* **17**, 5679 (1989).

⁴³ O. C. Uhlenbeck, *Nature* **328**, 596 (1987).

⁴⁴ T. K. Stage-Zimmermann and O. C. Uhlenbeck, *RNA* **4**, 875 (1998).

⁴⁵ M. J. Fedor and O. C. Uhlenbeck, *Biochemistry* **31**, 12042 (1992).

hammerhead RNA synthesis can also be prepared by hybridization of a pair of oligodeoxyribonucleotides (5 ng/ μ l), one containing the transcription promoter followed by the hammerhead motif, and the other complementary to the promoter sequence in order to obtain a partially duplex DNA.⁴⁷ In both cases, *in vitro* transcription reactions containing 1 U/ μ l T7 RNA polymerase, 40 mM Tris-HCl (pH 7.5), 6 mM Mg²⁺, 0.1 μ g/ μ l bovine serum albumin (BSA), 10 mM dithiothreitol (DTT), 0.5 mM each of ATP, CTP, and GTP, 0.025 mM UTP, and 1 μ Ci/ μ l [α -³²P]UTP are incubated at 37° for 1 h. The extent of self-cleavage during transcription (fraction of cleaved versus total RNA) is determined by electrophoresis of the transcription products on 1 \times TBE polyacrylamide gels (5–15% w/v) containing 7 M urea and, occasionally, 40% formamide to improve the separation of RNAs with a high secondary structure content. A method to obtain intramolecular cleavage rates during *in vitro* transcription has been devised.⁴⁸ The fraction of transcribed RNA that remains uncleaved (F) is independent of the transcription rate as long as the latter stays constant. Therefore, F can be plotted versus time (t) and the experimental data fitted to the equation

$$F = (1 - e^{-kt})/(kt)$$

in which k is the rate constant for intramolecular cleavage. Values obtained for this constant (around 1 min⁻¹) agree well with those derived from assays *in trans*, indicating that no fundamental kinetic distinction exists between the intra- and the intermolecular reactions.⁴⁸

To study the self-cleavage reaction in a protein-free environment, the uncleaved transcript is eluted in the absence of divalent metal ions and then is incubated under standard self-cleavage conditions: 50 mM Tris-HCl (pH 8), 5 mM MgCl₂, and 0.5 mM EDTA at 25° or 40° for 1 h.⁴⁶

Methods for Testing RNA Cleavage Using a Trans Format

RNAs are obtained by *in vitro* transcription (see above) or chemically synthesized. The substrate is 5'-end-labeled with T4 polynucleotide kinase and [γ -³²P]ATP⁴⁶. The reaction can be studied under two alternative conditions: single turnover in which the ribozyme is in excess (μ M to nM) over the substrate (nM to pM) and a single cleavage event occurs, and multiple turnover in which consecutive rounds of substrate binding (in excess over the ribozyme), cleavage, and product release take place. The ribozyme and substrate are heated together at 95° for 2 min in 50 mM Tris-HCl (pH 7.5) without Mg²⁺ and allowed to cool to 25°. A zero time point is taken and MgCl₂ (10 mM final concentration) is then

⁴⁶ A. C. Forster, C. Davies, C. J. Hutchins, and R. H. Symons, *Methods Enzymol.* **181**, 583 (1990).

⁴⁷ J. P. Milligan and O. C. Uhlenbeck, *Methods Enzymol.* **180**, 51 (1989).

⁴⁸ D. M. Long and O. C. Uhlenbeck, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6977 (1994).

added to initiate the cleavage reaction. Aliquots are removed at different times and quenched immediately with a 5-fold excess of stop solution (9 M urea in 50 mM EDTA), and the substrate and products from each aliquot are separated on denaturing polyacrylamide gels (15–20%) and quantitated by an image analyzer. Under single-turnover conditions data can be fitted to the equation

$$F_t = F_\infty(1 - e^{-kt})$$

where F_t and F_∞ are the fractions of product at time t and at the end point, respectively, and k the rate constant of cleavage. For a hammerhead behaving ideally, every substrate molecule is bound to a ribozyme molecule, and the observed rate of cleavage is determined by the balance between the forward (k_2) and the reverse rate constants (k_{-2}). For the hammerhead ribozyme $k_2 \gg k_{-2}$ and, therefore, the observed rate of cleavage corresponds to k_2 . For the multiple turnover reaction, steady-state rates are measured over a range of substrate concentrations, and the rate of cleavage, normalized to the ribozyme concentration, is plotted versus substrate concentration. The resulting data are fitted to the Eadie–Hofstee equation to obtain the rate constant of cleavage and the Michaelis constant.⁴⁴

Conclusions and Perspectives

Discovery of ribozymes has produced a scientific revolution with deep functional, biotechnological, and even evolutionary implications. We know now that processing of some cellular and some invading RNAs is mediated by ribozymes, which likely represents a relic of the RNA world presumed to have existed on Earth before the advent of DNA and proteins. Most of the ribozymes have been found in viroid and viroid-like satellite RNAs from plants as well as in other small RNAs of animal and fungal origin, suggesting that further research on these RNAs may lead to discovery of new ribozymes. All ribozymes found in nature behave as RNases (and some, additionally, as RNA ligases). Certain ribozymes, including the hammerhead, have been manipulated to act *in trans* against specific target RNAs. This has opened the possibility of engineering “restriction RNases,” a tool with a great potential impact in medicine and industry. The hammerhead ribozyme, because of its extreme structural simplicity, has served as a model system for extensive structural and functional analysis, and it is expected to continue playing a capital role in future developments of ribozymology.

Acknowledgments

Work in the laboratory of R.F. has been partially supported by Grants PB95-0139 and PB98-0500 from the Comisión Interministerial de Ciencia y Tecnología de España. C.H., J.-A.D., and A.V. were recipients of postdoctoral contracts and M. de la P. of a predoctoral fellowship from the Ministerio de Educación y Cultura de España.