VIROIDS

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PART VIII

CHAPTER 56

RIBOZYME REACTIONS OF VIROIDS

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Viroids replicate through a DNA-independent rolling-circle mechanism involving the synthesis of multimeric strands of both polarities and their subsequent cleavage into monomeric fragments, which are then circularized to produce the progeny (Branch and Robertson 1984). Depending on whether or not the minus multimeric strands are cleaved and ligated to unit-length circular strands, which are then used as templates for the second half of the cycle, the viroid RNA is considered to replicate by either a symmetric or asymmetric mode (see Chapter 5 'Replication'). While processing of the multimeric plus RNA intermediates is generally believed to require a host ribonuclease for the members of the family *Pospiviroidae* (formerly known as group B viroids), this step is autocatalytic and mediated by hammerhead ribozymes in members of the family *Avsunviroidae* (formerly known as group A viroids) (reviewed in Symons 1989; Flores *et al.* 1998; see Chapter 8 'Classification'). However, the possibility has been raised that the processing step is RNA-catalyzed in all cases (reviewed in Symons 1997). The three viroid species within the family *Avsunviroidae* known to date, *Avocado sunblotch viroid*, ASBVd (Symons 1981; Hutchins *et al.* 1986), *Peach latent mosaic viroid*, PLMVd (Hernández and Flores 1992; Shamloul *et al.* 1995) and *Chrysanthemum chlorotic mottle viroid*, CChMVd (Navarro and Flores 1997), can adopt hammerhead structures on their plus and minus polarity strands and, as a consequence, they are presumed to replicate according to the symmetric rolling-circle mechanism. The hammerhead structures appear as the only 'homologous molecular characters' shared by these viroids. Briefly, the hammerhead structure is a small RNA motif consisting of three sequence non-specific helices bordering a catalytic core of 11 conserved residues which form a complex array of non-canonical interactions (Prody *et al.* 1986; Hutchins *et al.* 1986; Forster and Symons 1987; Pley *et al.* 1994) (see Figure 56.1). The adoption of this structure in the presence of a divalent cation, usually magnesium, results in the self-cleavage of the RNA chain at a specific phosphodiester bond creating 2',3'-cyclic phosphate and 5'-hydroxyl termini.

Biochemical knowledge in respect to both the detailed structural features and a molecular mechanism of the hammerhead structures has been reviewed recently (Flores *et al.* 2000; Stage-Zimmermann and Uhlenbeck 1998) and, therefore, will not be the main focus of this chapter that primarily aims to present the hammerhead structure as an essential molecular feature of the *Avsunviroidae* members and, particularly, of their replication cycle. We will also consider the potential of this self-cleaving motif to act in *trans* targeting cellular RNA and, more specifically, to contribute to viroid pathogenesis.

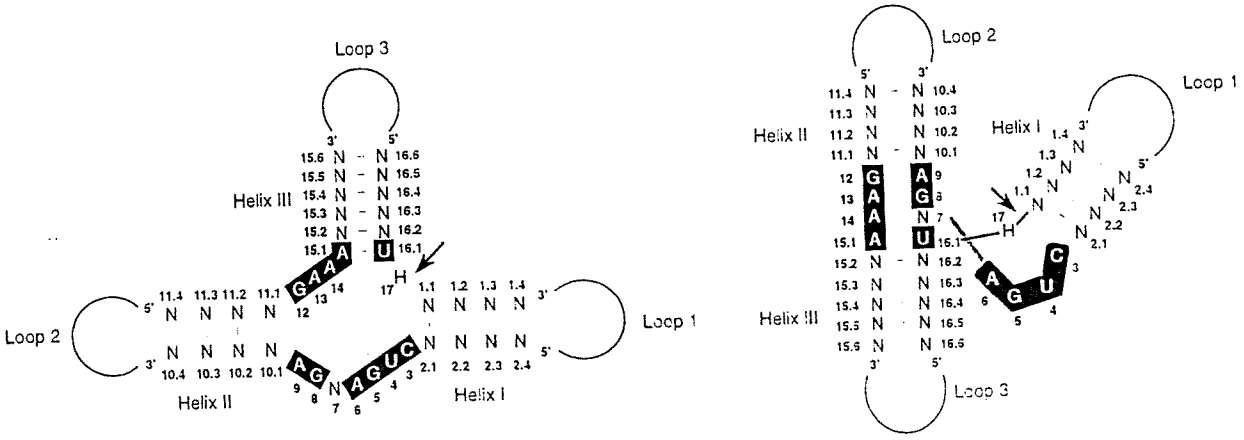

Figure 56.1 Consensus hammerhead structure derived from 23 natural hammerhead sequences, schematically represented as originally proposed with its numbering system and names of helices and loops (Hertel *et al.* 1992) (left), and according to X-ray crystallography data (Pley *et al.* 1994) (right). Letters on a dark background refer to absolutely conserved residues in all natural hammerhead structures and N to residues involved in Watson-Crick base pairs. Arrows indicate self-cleavage sites. Watson-Crick base pairs and non-canonical interactions are denoted with continuous and broken lines, respectively.

HAMMERHEAD STRUCTURES OF VIROIDS: MOLECULAR ARCHITECTURE

Figure 56.2 shows the six hammerhead structures described so far in viroids. In ASBVd, the sequences involved in hammerhead structures of both polarities are found in the upper and lower strands of the central domain of the quasi-rod-like secondary structure proposed for this viroid, with the remaining nucleotides of the genome, referred here as 'extracatalytic' RNA sequences, flanking the central domain (Figure 56.2A). Therefore, the sequences forming the catalytic core are not contiguous but segregated into two subdomains. In contrast, the sequences involved in the hammerhead structures of PLMVd and CChMVd are contiguous and located in an arm of their proposed branched conformation, with the 'extracatalytic' RNA sequences constituting the rest of the genomes (Figure 56.2B and C).

There are two classes of viroid hammerhead structures according to their morphology. The monomeric strands of PLMVd and CChMVd can adopt stable hammerhead structures with helices I and II of five-six base pairs closed by short loops 1 and 2 (the CChMVd minus hammerhead structure is an exception in having an unusually long imperfect helix II), and helices III of six-eight base-pairs (Figure 56.2B and C). Conversely, the hammerhead structures that can form the monomeric ASBVd RNAs are thermodynamically unstable, particularly in the plus polarity strand with a stem III of only two base pairs closed by a loop 3 of three residues (Figure 56.2A). This very different architecture of the viroid hammerhead structures has major implications for their *in vitro* and *in vivo* self-cleavage efficiency (see section below).

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.e. stems I, II and III) with loose sequence conservation except at 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 (Figure 56.2). Some viroid hammerhead structures present unusual features. For example, a transition U to C affecting the conserved U4 in the plus hammerhead structure has been observed in a sequence variant of PLMVd (Ambrós and Flores 1998). On the other hand, the common C1^{-} preceding the minus self-cleavage site is A in a sequence variant of ASBVd, and the common pyrimidine residue at position $^{-}$ is substituted by an A in the minus hammerhead structure of another ASBVd variant (Rakowski and Symons 1989). An extra A between A9 and G10.1 of the plus hammerhead structure of CChMVd has been also reported (Figure 56.2C). This extra residue, which is compatible with extensive *in vitro* self-cleavage, could either induce a rearrangement of the junction between helix II and three adjacent non-canonical interactions of the central core, or be accommodated as a bulging residue. These and other sequence variations in the hammerhead structures retrieved in nature from different self-cleaving RNAs have been compiled recently (see Flores *et al.* 2000). The conservation of the sequences forming the hammerhead structures in the *Avsunviroidae* members, as well as in most other hammerhead structures known so far, extend beyond the strict requirements for self-cleavage, suggesting that additional selective pressures may act on these sequences. However, the identity of any other selective pressure remains unidentified.

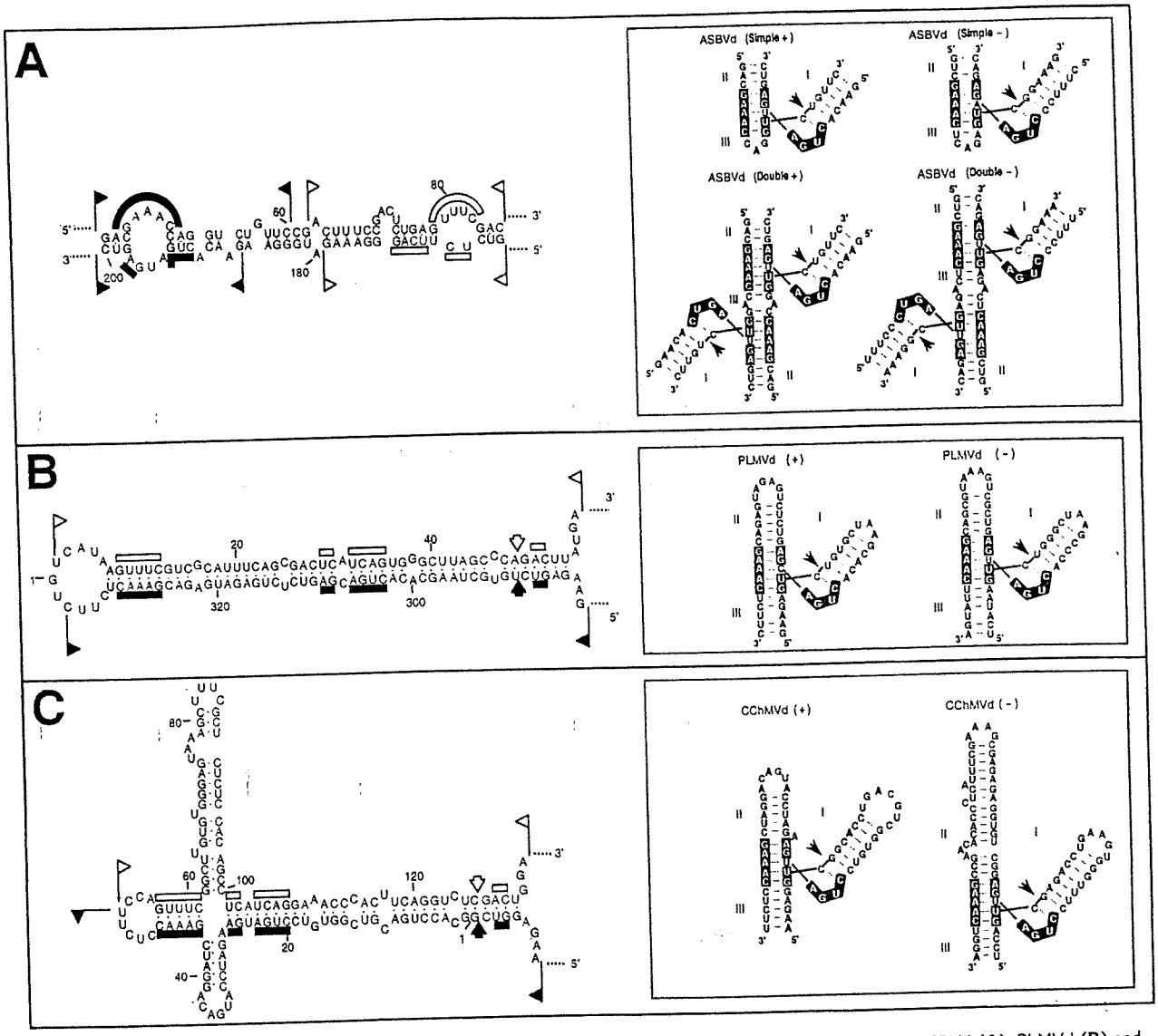

Figure 56.2 Nucleotide sequence and alternative secondary structure of the hammerhead self-catalytic motifs of ASBVd **(A)**, PLMVd **(B)** and CChMVd **(C)**. At the left, delimited by flags, are represented the fragments of the most stable secondary structure proposed for these viroids that contain the sequences involved in forming both polarity hammerhead structures. Extracatalytic sequences are indicated by broken lines, conserved hammerhead residues by bars and self-cleavage sites by arrows. Closed and open symbols refer to plus and minus polarities, respectively. Within the right panels are the plus and minus hammerhead sequences folded into their active secondary structures. Stems I, II and III are shown and the arrowheads indicate self-cleavage sites. Letters on a dark background refer to conserved hammerhead residues. In the case of ASBVd, the double hammerhead structures are also shown. Sequence were retrieved from the viroid and viroid-like database (http://www.callisto.si.usherb.ca/~jpperra; Pelchat *et al.* 2000b). Other details as in Figure 56.1.

CIS-ACTING HAMMERHEAD STRUCTURES OF VIROIDS: *IN VITRO* AND *IN VIVO* FUNCTION

The similarities found between the plus and minus hammerhead sequences and their genomic organization within each viroid most likely have physical and functional consequences. The PLMVd and CChMVd sequences spanning the two hammerhead domains are almost complementary and can fold into structures with long double-stranded regions; this is the typical arrangement found in the most stable secondary structures predicted for the two viroids (Figure 56.2B and C). These stable arrangements, formed by the superposition of the hammerhead sequences of both polarities, have the potential to prevent the

adoption of the active hammerhead foldings, which are alternative structures of higher energy (Hernández and Flores 1992; Beaudry *et al.* 1995; Navarro and Flores 1997). More importantly, self-cleavage inhibition permits the accumulation of certain levels of the viroid monomeric circular forms, which are the templates of the rolling-circle mechanism of replication. In addition, the compact non-self-cleaving structures may contribute positively to the extra- (e.g. during transmission) and intracellular stability of these RNA species. The peculiar organization of the hammerhead sequences may also be informative concerning mechanistic requirements. For example, these RNAs may need to have similar hammerheads in order to perform *in vivo* self-cleavage to essentially the same extent in both strands, as appears to be the case in PLMVd (Bussière *et al.* 1999). Interactions with cellular components (e.g. proteins) enhancing self-cleavage may have promoted conservation of similar hammerheads. Therefore, a complex synergy between the stability of the viroid RNA as a whole, and the mechanisms of self-cleavage regulation, has probably contributed to the emergence of the superimposed hammerhead sequences.

As already indicated, the ability of viroid RNAs that possess autocatalytic sequences to self-cleave depends on their adoption of a conformation different from the most stable structure (Figure 56.2). Self-cleavage of viroid strands occurs at either single or double hammerhead structures depending on whether or not the sequences can form stable helices surrounding the catalytic core. Whereas the six hammerhead structures of viroids have stable helices I and II, this is not the case for helix III. Both polarity hammerhead structures of PLMVd have stable helices III and self-cleave *in vitro*, and most likely *in vivo*, through single hammerhead structures (Hernández and Flores 1992; Beaudry *et al.* 1995) (Figure 56.2B). This is also probably the case with the two hammerhead structures of CChMVd, which also have stable helices III (Navarro and Flores 1997), although the extended helix II of the minus hammerhead structure might facilitate the adoption of alternative foldings inactive for self-cleavage (Figure 56.2C). In contrast, the single hammerhead structures of ASBVd have unstable helices III closed by short loops, and their self-cleavage is assumed to occur via double hammerhead structures involving longer-than-unit RNAs that allow stabilization of the catalytic core (Forster *et al.* 1988) (Figure 56.2A). ASBVd plus strands self-cleave through a double hammerhead structure during *in vitro* transcription and after gel purification, whereas ASBVd minus strands self-cleave via a double hammerhead structure during *in vitro* transcription, but mostly via a single hammerhead structure after gel purification (Davies *et al.* 1991). This is most probably the consequence of the different stability of helix III in both hammerhead structures.

Direct enzymatic sequencing and primer extension experiments have shown that *in vitro* self-cleavage of ASBVd, PLMVd and CChMVd occurs at the positions predicted by the hammerhead structures (Hutchins *et al.* 1986; Hernández and Flores 1992; Navarro and Flores 1997). The efficiency of the corresponding *in vitro* self-cleavage reactions can be high; for example, around 50–60% of PLMVd strands self-cleave under standard conditions (Hernández and Flores 1992; Beaudry *et al.* 1995). However, this efficiency is strongly increased (>95%) when the same RNAs are transcribed under conditions of slow polymerase activity, which favors the adoption of the active hammerhead structures catalyzing self-cleavage reactions (Bussière 1999). The self-cleavage efficiency is also strongly dependent on divalent ions such as Mg^{2+}.

There is also solid evidence supporting the involvement of hammerhead structures in the *in vivo* processing of viroid RNAs with these catalytic domains. For ASBVd (Darós *et al.* 1994; Navarro and Flores 2000), CChMVd (Navarro and Flores 1997), and PLMVd (C. Hernández, unpublished data), linear RNAs of one or both polarities with 5′-termini identical to those generated in the corresponding *in vitro* self-cleavage reactions have been isolated from infected tissues. Moreover, the frequent occurrence in sequence variants of PLMVd (Hernández and Flores 1992; Beaudry *et al.* 1995; Ambrós *et al.* 1998) and CChMVd (De la Peña *et al.* 1999) of compensatory mutations or covariations that preserve the stability of the hammerhead structures, further support their *in vivo* role, as also does the correlation existing between the infectivity of different PLMVd and CChMVd variants and the extent of their self-cleavage during *in vitro* transcription (Ambrós *et al.* 1998; De la Peña *et al.* 1999).

In vivo, self-cleavage of viroid strands should be under regulation, with two different mechanisms appearing to operate for this purpose. In the case of PLMVd and CChMVd, their most stable secondary structures are transiently lost during transcription with the concurrent adoption of the active single hammerhead structures that promote self-cleavage before synthesis is completed and the most stable secondary structures are reformed. In ASBVd self-cleavage of monomeric strands is restricted because the single hammerhead structures are unstable whereas the multimeric replicative intermediates can adopt stable double hammerhead structures and self-cleave to their unit-length strands. Therefore, in both situations the hammerhead ribozymes are active only during replication. Self-cleavage of PLMVd RNAs *in vivo* appears almost optimal reaching near total processing of the multimeric strands into their corresponding monomeric units (Bussière *et al.* 1999). For the reasons stated above, this high efficiency may be the result of slow progression of the host RNA polymerase during replication. The situation seems similar in the case of CChMVd, for which the predominant RNAs accumulating in infected cells are also the monomeric linear strands of both polarities (Navarro and Flores 1997). In contrast, the most abundant ASBVd RNA in infected tissue is the plus circular monomer, a clear indication of the low efficiency of the corresponding single hammerhead structure,

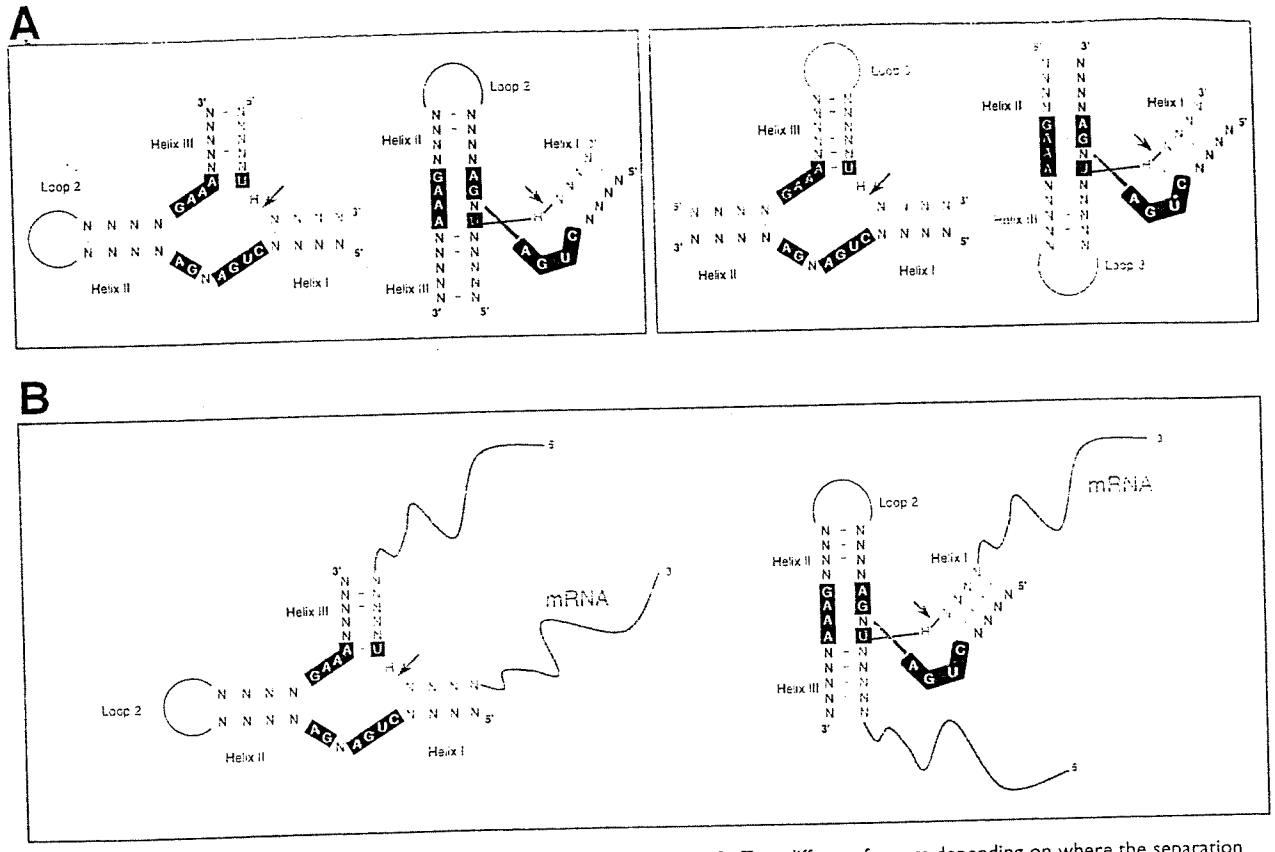

Figure 56.3 Schematic representation of *trans*-acting hammerhead ribozymes. **A.** Two different formats depending on where the separation between the ribozyme itself (black letters) and the substrate (grey letters) is established. **B.** A hammerhead ribozyme targeting a long mRNA substrate. Letters on a dark background refer to conserved hammerhead residues. Other details as in Figures 56.1 and 56.2.

although decreasing levels of multimeric strands up to octamers in size have been also detected (Bruening *et al.* 1982).

VIROID HAMMERHEAD STRUCTURES: POTENTIAL FOR *TRANS*-ACTING FUNCTION

In their natural context, the hammerhead structures of viroids operate in *cis* mediating the self-cleavage of the RNAs in which they are contained. However, active hammerhead structures can also be formed by annealing two different RNA fragments in *trans*, such that one RNA fragment acts as the ribozyme and the other as the substrate (see Figure 56.3A). If the complementary regions between the two RNAs are short enough, the cleavage products will dissociate from the ribozyme, thus permitting the binding of new substrate molecules. Via successive rounds of binding and cleavage a single ribozyme molecule can therefore cleave many substrate molecules, thereby establishing a classic enzyme/substrate relationship (Uhlenbeck 1987). Furthermore, by changing the complementary sequences between the ribozyme and its substrate, it is possible to create a ribozyme with new substrate specificity. A wide variety of RNAs can be targeted for cleavage by such engineered ribozymes (Figure 56.3B). Because of their ability to interact directly with RNA, ribozymes, particularly those of the hammerhead class, are currently being developed as potential therapeutic agents for a wide range of applications based on the specific cleavage of different RNAs of biological relevance including viroids themselves (see the preceding Chapter 'Biotechnological approaches for controlling viroid diseases'). In the coming years, altered forms of these versatile molecules will surely emerge as a new class of drugs.

Apart from these applications, a detailed description of which falls outside the scope of this chapter, we will consider the possibility that viroid pathogenesis of members of the family *Avsunviroidae* could result from *trans* cleavage of host RNAs recognized by the hammerhead ribozymes (Symons 1989). No supporting evidence for such a mechanism has been reported yet. The following discussion is based on experiments performed with PLMVd as a model viroid in an attempt to put this intriguing hypothesis to test (Côté 2000). Minimal artificial hammerhead ribozymes are prefolded into a quasi catalytically

active structure and following the substrate binding, which involves the formation of helixes at both sides of the cleavage site, the break of the scissile bond occurs. Natural hammerhead structures are integral features of viroid RNAs. In the most stable secondary structures of these RNAs, the hammerhead catalytic core is not formed because this is not the most stable structure. For example, active PLMVd hammerhead structures of both polarities are adopted either during the *in vitro* transcription or by a prior heat denaturation coupled to a snap-cooling treatment, that favor these active structures over others more stable but lacking catalytic activity. Therefore, the knowledge acquired from studies with minimal hammerhead ribozymes can not be simply extended to situations in which this catalytic motif is included in full-length viroid RNAs.

In order to compare the cleavage efficiency of a hammerhead catalytic sequence as a model molecule or as part of a viroid, a series of experiments were performed in which four PLMVd-derived transcripts, acting as the ribozyme, were tested for their ability to catalyze the cleavage of a short substrate (Côté 2000). As expected, no cleavage products were detected when the ribozyme was a PLMVd 250-nt transcript lacking the hammerhead sequences, whereas most of the substrate (>85%) was cleaved by a ribozyme with the sequences corresponding to the minimal plus hammerhead structure. This efficient cleavage probably results from the absence of extra sequence interfering with the adoption of the catalytically active folding. When the ribozyme was composed of a full-length PLMVd RNA circularized *in vitro* to have either a 3',5'- or a 2',5'-phosphosdiester bond at the self-cleavage site, only a trace amounts of product (<1%) were detected in both cases. Finally, the ribozyme corresponding to the full-length PLMVd linear RNA that accumulates predominantly in infected peach cells was able to cleave the substrate, although with an extremely low efficiency (<5%). Further experiments showed that cleavage in this latter case was most likely performed by the hammerhead motifs released during the preliminary denaturation-renaturation treatment, because no cleavage was detected when this treatment was omitted. Altogether, these experiments indicate that the possibility that a viroid may function as a *trans*-acting ribozyme triggering a pathogenic cascade is unlikely. The main impediment comes from the catalytic sequences being embedded in very stable secondary structures, reducing considerably their ability to hybridize in *trans* with other RNAs.

As already mentioned, minimal hammerhead structures can efficiently catalyze *trans* cleavage of small substrates. The possibility that such an active ribozyme could be released by specific hydrolysis during the viroid life cycle seems remote because there is no indication supporting the idea that if the viroid is attacked by host RNases, the hammerhead sequences would be protected against degradation. Alternatively, minimal hammerhead structures could be transiently formed during viroid replication. Such a possibility has been evaluated by testing the cleavage of a model substrate during the *in vitro* transcription of a PLMVd dimeric RNA (Côté 2000). In the absence of either the DNA template or the T7 RNA polymerase, no cleavage was observed. However, a small fraction of the substrate was cleaved during transcription, supporting the idea that if any *trans* cleaving activity exists this should occur during viroid replication. Additional experiments performed in the presence of protein A1, which has been previously shown to enhance the *trans* cleavage activity of hammerhead ribozymes (Herschlag *et al.* 1994), have allowed us to increase slightly the extent of cleavage (Côté 2000). In all, these results do not exclude the possibility that *in vivo* a host protein could open the compact PLMVd structure and favor the recognition and cleavage of a host RNA possessing a sequence targeted by the viroid hammerhead ribozymes. To this aim, at least two other requisites need to be met. First, the sequences forming the binding region of the ribozyme (i.e. one strand of helices I and II), should be expected to be conserved between sequence variants, but sequencing of numerous PLMVd natural variants (Hernández and Flores 1992, Ambrós *et al.* 1998; Pelchat *et al.* 2000a) has shown that this is not the case. And second, a natural substrate(s) for the viroid hammerhead ribozymes should exist. PLMVd replication intermediates have been predominantly detected in chloroplasts but attempts with well-established procedures to retrieve chloroplastic sequence(s) which could be potentially cleaved by one or the other PLMVd hammerhead structures have been unsuccessful. Therefore, the hypothesis that PLMVd, and by extension other hammerhead viroids, may exert their pathogenic effect by hammerhead-mediated cleavage of a cellular RNA appears very unlikely.

PERSPECTIVE

The previous discussion points out that in their natural context, hammerhead ribozymes of viroids are not good candidates for targeting cellular RNA of biological relevance. On the other hand, there is evidence that at least two viroids of the family *Avsunviroidae* replicate and accumulate in the chloroplast (see Chapter 5 'Replication') suggesting that, since they can be genetically manipulated and are endowed with the information to be host- and organelle-specific, they could in principle be appropriate vehicles for delivery of nucleic acid-based drugs into chloroplasts. It is clear that there is still a long way to go before such a development could be reached but since the hammerhead motif has to fold into an active conformation to express its catalytic *trans* cleaving potential, one option would be to engineer viroids with pre-folded hammerhead structures. However, whether these modified ribozymes are compatible with normal viroid functions, particularly replication, remains to be discovered.

Acknowledgements

This work was supported in part by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada to J.-P. Perreault and by grants PB95-0139 and PB98-0500

from the Comisión Interministerial de Ciencia y Tecnología de España to R. Flores. F. Côté and M. De la Peña were recipients of predoctoral fellowships from the NSERC and Fonds pour la Formation des Chercheurs et l'Aide à la Recherche (FCAR, Québec), and from the Ministerio de Educación y Cultura (España). J.-P. Perreault is an MRC scientist.

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