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Minireview

Viroids with Hammerhead Ribozymes: Some Unique Structural and Functional Aspects with Respect to Other Members of the Group

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Viroids, subviral pathogens of plants, are composed of a single-stranded circular RNA of 246 – 399 nucleotides. Within the 27 viroids sequenced, avocado sunblotch, peach latent mosaic and chrysanthemum chlorotic mottle viroids (ASBVd, PLMVd and CChMVd, respectively) can form hammerhead structures in both of their polarity strands. These ribozymes mediate self-cleavage of the oligomeric RNAs generated in the replication through a rolling circle mechanism, whose two other steps are catalyzed by an RNA polymerase and an RNA ligase. ASBVd, and presumably PLMVd and CChMVd, replicate and accumulate in the chloroplast, whereas typical viroids replicate and accumulate in the nucleus. PLMVd and CChMVd do not adopt a rod-like or quasi rod-like secondary structure as typical viroids do but have a highly branched conformation. A pathogenicity determinant has been mapped in a defined region of the CChMVd molecule.
Key words: Catalytic RNAs / Hammerhead structures / Rolling circle replication / Self-cleavage.

Introduction

The paradigm established at the turn of the 19th century considering viruses as the lowest step on the biological scale, based on pioneering studies on the causal agent of the tobacco mosaic disease, remained unchallenged for more than seventy years. It was not without some difficulty that the first violation of this paradigm, which also came from the plant world, was accepted in the late sixties/early seventies when the existence of a first class of subviral pathogens was proposed: the viroids (Diener, 1971). As with other biological systems, the powerful physicochemical techniques combined with those of molecular biology have allowed rapid progress in the structural characterization of these minimal pathogens that are exclusively composed of a small circular single-stranded RNA of 246 to 399 nt with a high content of secondary structure (Flores *et al.*, 1998). Advances from a functional perspective have been much more modest, but all the available information clearly indicates that viroids and viruses, contrary to what their similar names may suggest, are very different biological entities. It should be sufficient in this respect to note that viroids, as opposed to viruses:

(i) do not code for any protein,
(ii) some of them contain ribozyme domains,
(iii) have presumably a very old monophyletic origin, going back to the precellular RNA world postulated to have preceded to our present world based on DNA and proteins (Diener, 1989; Elena *et al.*, 1991).

Taking into account the conservation of a series of sequence motifs and structural domains, as well as the results of the phylogenetic analyses, the 27 viroids of known sequence have been classified into two families, *Pospiviroidae* and *Avsunviroidae*, the respective type members of which are potato spindle tuber viroid (PSTVd) and avocado sunblotch viroid (ASBVd) (Flores *et al.*, 1998). Most viroids belong to the first family and are characterized by a central conserved region (CCR) within the proposed rod-like secondary structure (Gross *et al.*, 1978; Keese and Symons, 1985). Members of the second family, composed only by ASBVd (Symons, 1981; Hutchins *et al.*, 1986), peach latent mosaic viroid (PLMVd) (Hernández and Flores, 1992) and chrysanthemum chlorotic mottle viroid (CChMVd) (Navarro and Flores, 1997), do not have a CCR and both of their polarity strands can self-cleave through hammerhead structures. The initial discovery of the hammerhead ribozyme in ASBVd (Hutchins *et al.*, 1986) and in a satellite RNA structurally similar to viroids but functionally dependent on a helper virus (Prody *et al.*, 1986), represents a hallmark in molecular biology with major functional, evolutive and even biotechnological implications.

We concentrate in this review on the second viroid family, *Avsunviroidae*, to which we will also refer to as hammerhead viroids paying particular attention to their most prominent peculiarity.

Hammerhead Viroids: Unique Structural Features

The characterization of ASBVd (Symons, 1981) provided an unexpected molecular picture for what was thought at that time to be a viroid. ASBVd was unusually small (247 nt) but, more importantly, it displayed neither the CCR preserved in all known viroids, nor any significant sequence similarity. An hexanucleotide in the upper strand of the proposed rod-like structure was the only remnant that could remind of the CCR of typical members of the group. Later on when a model that divided the rod-like secondary structure of viroids into five domains was advanced (Keese and Symons, 1985), ASBVd was considered as the exception to the rule. Moreover, the finding that dimeric ASBVd RNAs of both polarities were able to self-cleave *in vitro* through hammerhead structures (Hutchins *et al.*, 1986), as was the plus polarity strand of the satellite RNA of tobacco ringspot virus (Prody *et al.*, 1986), casted doubts on whether ASBVd was a viroid or a viroid-like satellite RNA. The discovery of PLMVd (Hernández and Flores, 1992) and more recently of CChMVd (Navarro and Flores, 1997), the second and third hammerhead viroids, settled the question particularly because cDNA clones of both viroids were infectious and pathogenic. A detailed comparison between these three viroids reveals that although no extensive sequence similarity exists between them, apart from the short sequences conserved in all hammerhead structures, PLMVd and CChMVd are more closely related on the basis of their G+C content (above 50%), predicted branched secondary structures of lowest free energy and morphology of their hammerhead structures, as well as on a physico-chemical basis such as their insolubility in 2 M LiCl. ASBVd remains a singular viroid because of its low G+C content (36%), predicted rod-like or quasi-rod-like secondary structure, unstable single hammerhead structures (see below), and solubility in 2 M LiCl. On this basis it has been proposed that the Avsunviroidae family contains two genera, *Avsunviroid* and *Pelamoviroid*, whose type members are ASBVd and PLMVd, respectively (Flores *et al.*, 1998).

The hammerhead structures fall clearly into two classes (Figure 1). The monomeric strands of PLMVd and CChMVd can form stable hammerhead structures and self-cleave accordingly *in vitro*. Conversely, the monomeric ASBVd RNAs can only adopt thermodynamically unstable hammerhead structures, particularly in the plus polarity strand with a stem III of only two base pairs closed by a loop of three residues, and their self-cleavage is very much restricted. However, the oligomeric ASBVd RNAs that are the replicative intermediates of the rolling circle mechanism can form stable double hammerhead structures (Forster *et al.*, 1988), that very likely catalyze their self-cleavage to linear monomers. This may represent a way to regulate the activity of the ribozymes.

ASBVd: a Model for Replication Studies of Hammerhead Viroids

There is another aspect for which ASBVd is a unique viroid: it can accumulate in infected avocado leaves to very high levels that in some cases parallel those of the 5S ribosomal RNA (Figure 2). This makes the system ASBVd-avocado very attractive and permits to tackle some fundamental questions related to the subcellular localization and replication of this viroid. Previous cell fractionation studies using tomato tissue infected with PSTVd revealed that it accumulates essentially in the nuclei and particularly in the nucleoli (Harders *et al.*, 1989). Similar results were

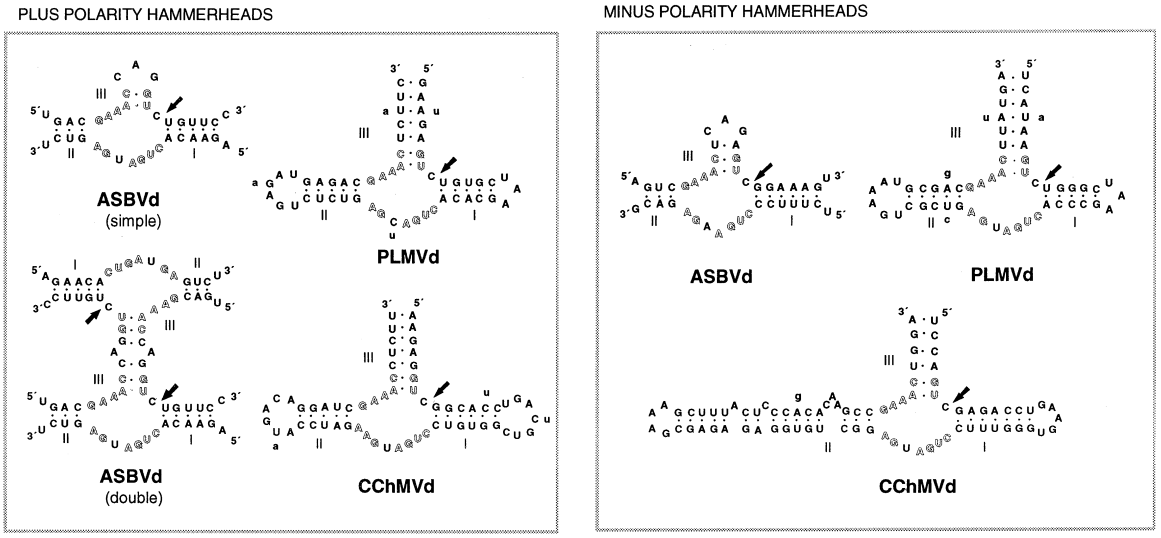

Fig. 1 Schematic Representation of the Hammerhead Structures that Can Be Formed by ASBVd, PLMVd and CChMVd RNAs. Outlined letters indicate nucleotides conserved in most natural hammerhead structures, and substitutions found in some PLMVd and CChMVd variants that do not affect the stability of the ribozymes are in lowercase letters. The self-cleavage sites are denoted by arrowheads. Both the single and the double hammerhead structures of the plus polarity strand of ASBVd are presented.

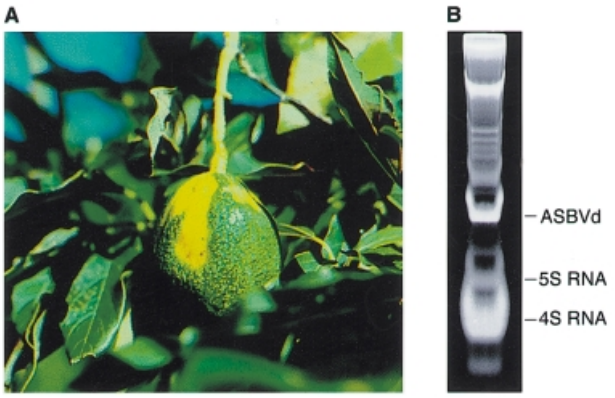

Fig. 2 ASBVd and Its Characteristic Symptomatology.
(A) Avocado fruit displaying the symptoms (yellow areas) induced by avocado sunblotch viroid (ASBVd). (B) Analysis by polyacrylamide gel electrophoresis of a partially purified nucleic acid preparation from ASBVd-infected avocado leaves showing the very high accumulation level of the viroid; for comparative purposes other abundant small cellular RNAs are also indicated.

obtained when this question was re-examined with other typical members of the *Pospiviroidae* family by *in situ* hybridization of thin sections of tissue combined with confocal laser scanning and electron microscopy (Bonfiglioli *et al.*, 1996). This approach is less prone to generate artifacts but demands relatively high concentrations of the RNA to be detected. However, when this latter methodology was applied to ASBVd, most of the viroid was localized in the chloroplasts (Bonfiglioli *et al.*, 1994; Lima *et al.*, 1994), emphasizing again another major difference between ASBVd (and probably PLMVd and CChMVd), and members of the other viroid family.

Although the replication and accumulation sites of a viroid do not necessarily have to be the same because it can be synthesized in one cell compartment and then translocated to another, the replicative intermediates of PSTVd have been detected in the nuclei (Spiesmacher *et al.*, 1983), indicating that they are also the organelles where replication of this viroid occurs. Regarding ASBVd, its replicative intermediates have been recently identified in chloroplasts (Navarro *et al.*, 1999), indicating that the replication and accumulation sites of this viroid also coincide with but are different to those of PSTVd.

The longer-than-unit nature of some of the replicative intermediates found in viroid-infected tissues, and the circular structure of the template that initiates the replication cycle support the view that viroids replicate through a rolling-circle mechanism (Branch *et al.*, 1984; Hutchins *et al.*, 1985). In this model the infecting, most abundant monomeric circular RNA, to which the (+) polarity is arbitrarily assigned, is recognized as a template by an RNA-dependent RNA polymerase that, after several transcription rounds, leads to oligomeric (–) strands that are then processed to unit-length and ligated to the monomeric (–) circular RNA, *via* an RNase and an RNA ligase, respectively. This latter RNA species serves as the intial template for the second half of the cycle which is a symmetric version of the first one (Figure 3). There is considerable evidence supporting that ASBVd follows this symmetric rolling circle model because the monomeric (–) circular RNA has been found in infected avocado leaves (Daròs *et al.*, 1994; Navarro *et al.*, 1999). Conversely, repeated attempts to identify the equivalent RNA molecule in PSTVd-infected tomato have failed and therefore this viroid is assumed to follow an alternative asymmetric pathway of the rolling replication mechanism in which the oligomeric (–) strands, resulting from the first transcription step, serve directly as the template for synthesis of the complementary RNAs which, after processing and ligation, lead to the final product of the cycle, the monomeric (+) circular RNA (Branch *et al.*, 1988).

Considering the different cell compartments in which replication of ASBVd and PSTVd occurs, distinct catalytic activities can be anticipated to take part in the process. The available evidence indicates that this is indeed the case and that the differences are of a fundamental nature, particularly in the processing reaction that is ribozymatic and mediated by hammerhead structures in the *Avsunviroidae* family (Figure 1). In the *Pospiviroidae* family, however, the reaction is generally assumed to be catalyzed by a host RNase (Baumstark *et al.*, 1997), although there is not an unanimous consensus in this respect (Liu and Symons, 1998). The hammerhead structures are not only active *in vitro* but also *in vivo* as revealed by the isolation of ASBVd- and CChMVd-specific linear RNAs of one or both polarities with 5′-termini identical to those produced in the corresponding *in vitro* self-cleavage reactions

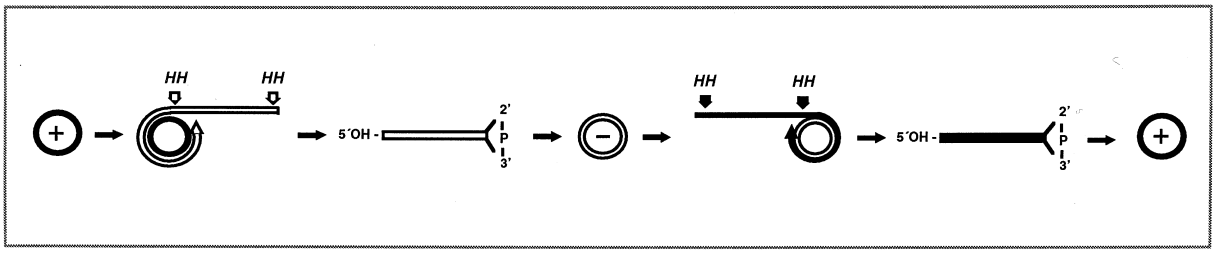

Fig. 3 Symmetric Rolling-Circle Model for Replication of Members of the *Avsunviroidae* Family.
The plus and minus polarity strands are indicated by solid and open lines. Cleavage of multimeric strands of both polarities is mediated by hammerhead ribozymes (HH), which generate linear monomeric RNAs with 5′-hydroxyl and 2′-3′-cyclic phosphate termini. Arrowheads denote the self-cleavage sites.

(Daròs *et al.*, 1994; Navarro and Flores, 1997), as well as by the identification in some PLMVd and CChMVd variants of compensatory mutations or covariations in the hammerhead structures that preserve their stability (Figure 1) (Hernández and Flores, 1992; Navarro and Flores, 1997).

There are also important differences in the enzymatic activities catalyzing the first step of the replication cycle. Studies on RNA synthesis of typical members of the *Pospiviroidae* family with *in vivo* and *in vitro* approaches have revealed that this step is sensitive to the low levels of α-amanitin that characteristically inhibit the nuclear DNA-dependent RNA polymerase II (Mühlbach and Sänger, 1979; Flores and Semancik, 1982; Schindler and Mühlbach, 1992), suggesting the involvement of an enzyme of this class. Conversely, this same step is insensitive to α-amanitin in the case of ASBVd (Marcos and Flores, 1992), indicating the participation of an enzyme which is not RNA polymerase II. For more than twenty years, the only really well characterized chloroplastic RNA polymerase was the plastid-encoded polymerase (PEP) with a multi-subunit structure similar to the *E. coli* enzyme (Bottomley *et al.*, 1971). However, a new single-subunit nuclear-encoded polymerase (NEP) resembling phage RNA polymerases has been recently described (Hedtke *et al.*, 1997). Both PEP and NEP RNA polymerases are resistant to α-amanitin that, therefore, cannot discriminate which of them is involved in ASBVd replication. The use of another antibiotic, tagetitoxin, may help to solve this intriguing question (Navarro, Vera and Flores, unpublished data).

Finally, distinct RNA ligases can also be anticipated to mediate the third step of the replication cycle in *Pospi-* and *Avsunviroidae* if, as assumed but not proved, the replication site is a common feature shared by all members within each family. Nuclear extracts from potato cells are able to process PSTVd transcripts with a short repetition of the CCR upper strand and to ligate the resulting product to the monomeric circular form (Baumstark *et al.*, 1997). The involved RNA ligase may have properties similar to the enzyme from wheat germ (Konarska *et al.*, 1981) that requires 5′-hydroxyl and 2′-3′-cyclic phosphate termini consistent with those presumably generated by the processing nuclear RNase. This is further supported by the observation that wheat germ RNA ligase catalyzes the *in vitro* circularization of PSTVd monomeric linear forms isolated from infected tissue. Since the same 5′-hydroxyl and 2′-3′-cyclic phosphate termini are produced in RNA self-cleavage mediated by hammerhead ribozymes in members of the *Avsunviroidae* family, the participation of a similar RNA ligase, in this case with a chloroplastic localization, can be presumed. On the basis of the observed self-ligation of PLMVd RNA *in vitro* the possibility that this may also be the mechanism operating *in vivo* has been advanced (Côté and Perrault, 1997). However, this protein-free pathway is unlikely because most of the phosphodiester bonds produced by self-ligation are 2′-5′ instead of 3′-5′, and also because the structure of the ligation site of one viroid-like satellite RNA that self-cleaves also through a hammerhead structure contains a 2′-phosphomonoester, 3′-5′ phosphodiester group (Kiberstis *et al.*, 1985), a mark consistent with the involvement of an RNA ligase but not with self-ligation. Moreover, the monomeric linear strands of PSTVd, obtained by incubation of a transcript with a short repetition of the CCR upper strand with a nuclear extract from potato, are also able to self-ligate *in vitro* in the absence of proteins (Baumstark *et al.*, 1997), indicating that this reaction is not limited to hammerhead viroids.

PLMVd and CChMVd: Models for Investigating Structure-Function Relationships in Hammerhead Viroids

One additional peculiarity of hammerhead viroids is that they have a very narrow host range: each one replicates only in its natural host or in very closely related plant species. In other words, it has not been possible so far to transmit these viroids to more convenient experimental hosts, and this imposes important restrictions on their study. For example, different ASBVd sequence variants have been associated with a distinct symptomatology (Semancik and Szychowski, 1994), but establishing a direct relationship is not feasible in the ASBVd-avocado system because successful mechanical inoculations are difficult to achieve, and a long assay period of at least one year is usually required to observe the symptoms. Therefore, although the ASBVd-avocado system has facilitated the study of the properties of the replicative intermediates, which like the viroid itself accumulate to very high levels (Daròs *et al.*, 1994), it is of limited use for other purposes.

The discovery of PLMVd, the second hammerhead viroid (Hernández and Flores, 1992), opened new possibilities for the study of additional aspects of the viroid-host interaction within the *Avsunviroidae* family. PLMVd accumulates to low levels in the infected tissue but it can be mechanically inoculated on GF-305 peach seedlings which may develop the symptoms in a relatively short period of time (2–3 months). Furthermore, since there are severe and latent strains of PLMVd depending on whether or not they induce leaf symptoms on peach seedlings grown in the greenhouse, cloning and sequencing of the corresponding RNAs may cast some light on the molecular determinants of pathogenicity in this viroid. An analysis of 29 different sequence variants derived from a severe and two latent PLMVd isolates has revealed a size of 335 to 338 nt and a large number of polymorphic positions in the viroid molecule (Ambrós *et al.*, 1998), indicating that PLMVd, like other RNA replicons, propagates in its host as a population of closely related but not identical variants forming what is known as a quasi-species. On the basis of the variability pattern found, three types of structural constraints limiting the genetic divergence of PLMVd sequences can be distinguished: formation of stable ham-

merhead structures in both polarity strands, conservation of a similar branched secondary structure of minimal free energy, and preservation of a potential pseudoknot-like element between two loops of the secondary structure proposed for PLMVd. When the biological properties of the individual PLMVd variants were explored by inoculating their cDNAs, infections induced by clones from the latent isolates were always symptomless. By contrast, the biological effects produced by the cDNAs from the severe isolate were variable: most of them induced the onset of symptoms but some incited either symptomatic or non symptomatic infections in different plants. Therefore, the PLMVd severe isolate appears to be formed by a mixture of variants with different pathogenicity. These results provide a molecular framework that explains the pattern usually observed in PLMVd natural infections which are phenotypically stable when caused by latent isolates but exhibit symptomatic fluctuations when originated by severe isolates. These fluctuations may be the result of different balances reached in the course of the infection between variants with different pathogenicity co-existing in the severe isolates. In fact, analyses of the populations generated *de novo* by inoculating individual PLMVd cDNA clones have shown that the high genomic variability of PLMVd is not a consequence of repeated inoculations of the same individual field trees, a feasible situation in woody hosts such as fruit trees with a long productive life, but rather from the unusual ability of PLMVd to evolve rapidly (Ambrós *et al.*, 1999).

The extreme sequence variability of PLMVd has precluded the assignment of the pathogenic effect of some variants to a defined structural motif. The identification and characterization of CChMVd (398–399 nt), the third member of the *Avsunviroidae* family (Navarro and Flores, 1997), has permitted us to address some of these questions. Although CChMVd, like PLMVd, accumulates *in vivo* to very low levels, its natural host chrysanthemum is also a convenient experimental system with an elapsing time of only 10–12 days between inoculation and the onset of symptoms. CChMVd shares with PLMVd a computer-predicted branched conformation but in the case of CChMVd this conformation is additionally supported by the distribution of the variability found in the sequence variants. This variability is accommodated in loops or, when occurring in hairpin stems, their stability is preserved by co-variations or compensatory mutations (Navarro and Flores, 1997; De la Peña, Navarro and Flores, submitted for publication). Therefore, there is now solid evidence indicating that not all viroids adopt a rod-like or quasi-rod like secondary structure as initially presumed. Moreover, a close inspection of the sequence heterogeneity found in sequence variants from latent and severe isolates of CChMVd, combined with bioassays of these variants and experiments of site-directed mutagenesis, has enabled us to map a determinant of pathogenicity in a defined region of the molecule, the first determinant of this class found in a hammerhead viroid (De la Peña, Navarro and Flores, submitted for publication).

Future Prospects

Whereas autolytic processing through hammerhead ribozymes of both polarity RNAs is well established in the known members of the *Avsunviroidae* family, there are still unsolved issues on viroid replication awaiting to be addressed that include the characterization of the RNA polymerase and RNA ligase involved in the first and third steps of the replication cycle, respectively. Other intriguing questions that are presently *terra incognita* concern the molecular determinants targeting ASBVd (and presumably PLMVd and CChMVd) to the chloroplast, the nature of the host component with which the viroid RNA initially interacts and the signal transduction pathway leading to the onset of symptoms, and whether transcription of viroid strands is initiated at defined positions in the molecule that are determined by specific sequences (promoters), or at random, a feasible alternative from a theoretical perspective considering the circular nature of the template. The solution to these questions will certainly provide interesting clues on how hammerhead viroids recruit host RNA polymerases and other cell factors needed for their replication and movement, as well as for inducing their pathogenic effects.

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