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Current Review

The Intriguing Viroids and Virusoids: What Is Their Information Content and How Did They Evolve?

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The low molecular weight, circular, single-stranded pathogenic RNAs of plants are creating considerable interest because of their small size of 246 to 388 nucleotides, their unique structural features, and their ability to often produce dramatic symptoms as well as no symptoms on infected plants. In addition, many are important pathogens of agricultural crops. Their apparent inability to code for any proteins indicates that all interactions with cellular components must be by their sequence and their secondary and tertiary structures. Characterization of such interactions is likely to contribute to our understanding of the relation of structure to function of RNA in all cells. For general reviews, see Diener (1979, 1983, 1987), Riesner and Gross (1985), Keese and Symons (1987), and Keese *et al.* (1988).

These RNAs can be divided into two groups: the viroids, which replicate independently because they do not require a helper virus, and the encapsidated viroidlike satellite RNAs or virusoids, which require a helper virus. The 16 viroids that have been characterized so far (Table 1) can be divided into two main groups on the basis of comparative sequence homology. Avocado sunblotch viroid (ASBV) is the only member so far of one group, whereas the remainder of the viroids can be divided into two subgroups based on related sequences in the central part of the rodlike viroid molecules (see below; Keese and Symons 1985, 1987; Koltunow and Rezaian 1989).

Only four virusoids have been discovered so far, all in Australasia (Table 2; Francki 1985, 1987; Keese and Symons 1987; Symons, in press), although a different isolate of lucerne transient streak virusoid (vLTSV) has been found in Canada (Abouhaidar and Paliwal 1988). The virusoids in infected plants exist almost solely as circular molecules, either free or encapsidated within virions of the helper virus.

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This is in contrast to the similarly sized satellite RNA of tobacco ringspot virus (sTRSV) that is found as both linear and circular single-stranded molecules *in vivo*, but only the linear form is encapsidated (Kiefer *et al.* 1982; Bruening 1990).

Although it is generally considered that viroids and virusoids do not code for any polypeptides, the evidence is only circumstantial and definitive proof is lacking (Sänger 1987; Keese and Symons 1987). Despite the inability of circular RNAs to be translated by eukaryotic ribosomes (Kozak 1979), the possibility cannot be dismissed that subgenomic fragments of viroids and virusoids may act as messenger RNAs *in vivo* and that initiation of peptide synthesis may occur at codons other than AUG (Dasso and Jackson 1989; Kozak 1989).

The major problem in relating sequence and structure of viroids and virusoids to function is the scarcity of marker functions. When using mutants for this type of work, these can be obtained in two ways, either by isolating naturally occurring mutants of a viroid or virusoid (sequence variants) or by preparing them by site-directed mutagenesis of cDNA clones (Owens and Hammond 1988, 1990). However, assay of the variation of the pathogenic effect on inoculated plants is one of the few ways available that have yielded definitive data (Visvader and Symons 1986). More recently, preliminary data that indicate an effect on movement from cell to cell of potato spindle tuber viroid (PSTV) by mutations in the right-hand terminal loop of the viroid molecule should help to identify structural features that determine viroid host range (Owens and Hammond 1990).

In the area of replication, *in vitro* analysis of the processing of oligomeric precursors of ASBV and the four virusoids has identified a specific self-cleavage structure known as the hammerhead and has shown that about 20–30% of each molecule is required for the formation of these structures. This area is discussed below, together with sequence domains in viroids in terms of function and providing evidence for the evolution of viroids, and possibly virusoids, by RNA recombination.

Sequence domains in viroids and virusoids indicate evolution by RNA rearrangement. Comparative pairwise sequence analysis of members of PSTV subgroup B1 (Table 1) indicated the presence of five domains (Fig. 1), the boundaries of which were defined by very sharp changes in sequence homology, from high to low or vice versa (Keese and Symons 1985, 1987; Keese *et al.* 1988). Different pairwise comparisons were always consistent in defining the

exact position of the boundaries. The domain model was developed using viroid sequences only from PSTV subgroup B1 (Table 1); these were the only ones available at that time. However, the viroids of apple scar skin viroid (ASSV) subgroup B2 have the same domains as shown by pairwise comparison of sequences (Koltunow and Rezaian 1989).

The PSTV group of Table 1 is divided into two subgroups on the basis of the sequences in the central conserved (C) domain. Within the C domain of each subgroup, there are sequences of about 30 nucleotides that are highly conserved among all members of each subgroup but which are quite different from those of the other subgroup (Visvader *et al.* 1985; Koltunow and Rezaian 1989). These central conserved sequences make up about one third of the C domain in each case. However, a common feature of both subgroups is the presence of a short inverted repeat sequence within the conserved nucleotides, which is shown by arrows in Figure 1.

This domain model led to the proposal that the evolution of viroids involved the rearrangements of domains between viroids infecting the same cell followed by further evolution (Keese and Symons 1985). Experimental support of such a model is difficult to obtain, but new viroid sequences that continue to appear provide strong indirect evidence. For example, in the recently sequenced columnea latent viroid (Hammond *et al.* 1989), the left-hand terminal (T1) and right-hand terminal (T2) domains show high sequence homology to the same domains in PSTV and tomato apical stunt viroid, respectively, with boundaries sharply defined and consistent with other pairwise comparisons (Fig. 2). The presence of subdomain lengths of sequences of tomato planta macho viroid in the pathogenic (P) domain and of PSTV and of hop stunt viroid (HSV) in the C domain (Fig. 2) indicate that rearrangements can occur within a domain as well as at the boundaries.

An even more scrambled viroid is Australian grapevine viroid (AGV) of ASSV subgroup B2 (Table 1) in which nearly all the 370 nucleotides appear to be derived from segments of citrus exocortis viroid (CEV), PSTV, ASSV, and grapevine yellow speckle viroid (Rezaian 1990). The sequence similarity of the segments in AGV varies from 52 to 100% of the corresponding sequences in the putative parent viroids (Koltunow and Rezaian 1989).

Only one example has been found so far in the virusoids of such RNA rearrangements. Four separate isolates of subterranean clover mottle virus (SCMoV) contained two different-sized virusoids, which could occur together or separately (Francki 1985, 1987). Sequence analysis of the virusoids showed the remarkable situation where the left-

Table 1. Classification of viroids[a] according to groups and subgroups

Designation	Abbreviation	Length (nucleotides)
A, ASBV group		
ASBV subgroup		
Avocado sunblotch viroid	ASBV	246–251
B, PSTV group		
B1, PSTV subgroup		
Chrysanthemum stunt viroid	CSV	354 and 356
Citrus exocortis viroid	CEV	370–375
Coconut cadang-cadang viroid	CCCV	246 and 247
Coconut tinangaja viroid	CTiV	254
Columnea latent viroid	CLV	370
Cucumber pale fruit viroid[b]	CPFV	303
Hop latent viroid	HLV	256
Hop stunt viroid	HSV	297–303
Potato spindle tuber viroid	PSTV	359
Tomato apical stunt viroid	TASV	360
Tomato planta macho viroid	TPMV	360
B2, ASSV subgroup		
Apple scar skin viroid	ASSV	330
Australian grapevine viroid	AGV	369
Grapevine yellow speckle viroid	GYSV	367
Grapevine viroid 1B	GV1B	363

[a] Classification scheme of Koltunow and Rezaian (1989) for viroids that have been sequenced.
[b] Cucumber pale fruit viroid is really a sequence variant of hop stunt viroid.

Table 2. Virusoids[a] discovered to date

Helper virus	Virusoid abbreviation	Length (nucleotides)
Lucerne transient streak virus	vLTSV	324
Solanum nodiflorum mottle virus	vSNMV	377
Subterranean clover mottle virus	vSCMoV	332 and 388
Velvet tobacco mottle virus	vVTMoV	365 and 366

[a] Encapsidated, viroidlike, satellite RNAs.

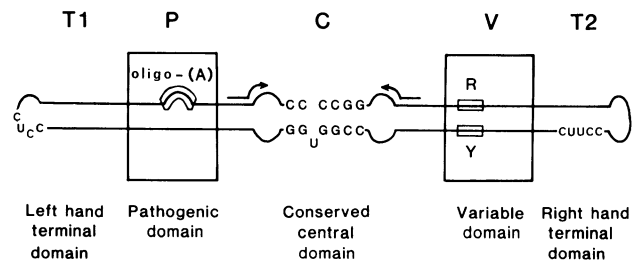


Fig. 1. Model of viroid domains for the potato spindle tuber viroid (PSTV) group of viroids. The five domains, T1, P, C, V, and T2, were determined from sequence homologies between the viroids. The arrows depict an inverted repeat sequence that can form a stem loop. Sequence elements are those of PSTV subgroup B1 (see Table 1). R and Y indicate a short oligopurine-oligopyrimidine helix. The same domains exist in apple scar skin viroid subgroup B2 (Koltunow and Rezaian 1989). This figure is modified from Keese and Symons (1987). (Reprinted with permission from Viroids and Viroid-like Pathogens, pages 1-47. Copyright CRC Press, Inc., Boca Raton, FL.)

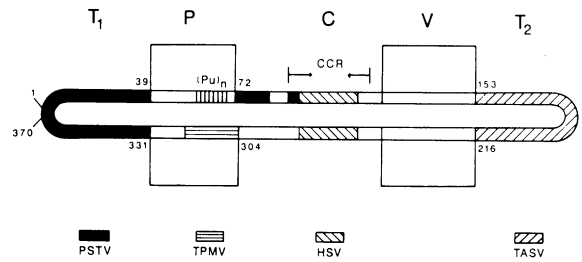


Fig. 2. Schematic diagram of columnea latent viroid showing the sequence homologies to other viroids. Domains (abbreviations as given in Fig. 1) are shown together with residue numbers at the boundaries of each domain. The central conserved region (CCR) contains the inverted repeat sequence. This figure is adapted from Hammond *et al.* (1989). (Reprinted by permission of Oxford University Press.)

hand part of each molecule was almost identical, whereas the right-hand parts differed in size and were completely different except for some short sequences (Fig. 3; Davies *et al.* 1990). Presumably, the two virusoids arose from recombination between three parents that have yet to be discovered.

The mechanism of these RNA rearrangements is unknown. One feasible mechanism is discontinuous transcription where an RNA polymerase copying one viroid or virusoid template changes over to copy a juxtapositioned second template at some point, most probably determined by the tertiary structures of the two templates (Keese and Symons 1985, 1987).

Examples of recombination between plus strand RNA viruses are accumulating (King 1988). In the case of plant viruses, perhaps the most definitive example is that provided by Allison *et al.* (1990) for the tripartite cowpea chlorotic mottle virus where full infection requires the simultaneous inoculation of the three genomic RNAs. RNAs 1 and 2 are each monocistronic whereas RNA 3 is dicistronic, coding for protein 3a (probably responsible for virus movement in the plant) and the coat protein. Inoculation of plants with normal RNAs 1 and 2 and two RNAs 3, one with a deletion in the 3a cistron and the other with a deletion in the coat protein cistron, led to normal infection and the recovery of wild-type RNA 3. No systemic infection resulted when each of the mutant RNAs 3 was inoculated separately with RNAs 1 and 2. Hence, the RNA recombination event must have occurred with high frequency in many of the inoculated cells. Such a result should encourage workers in the viroid and virusoid field to conduct similar experiments to test viroid and virusoid evolution by RNA rearrangements.

P domain of CEV plays a role in pathogenicity. Naturally

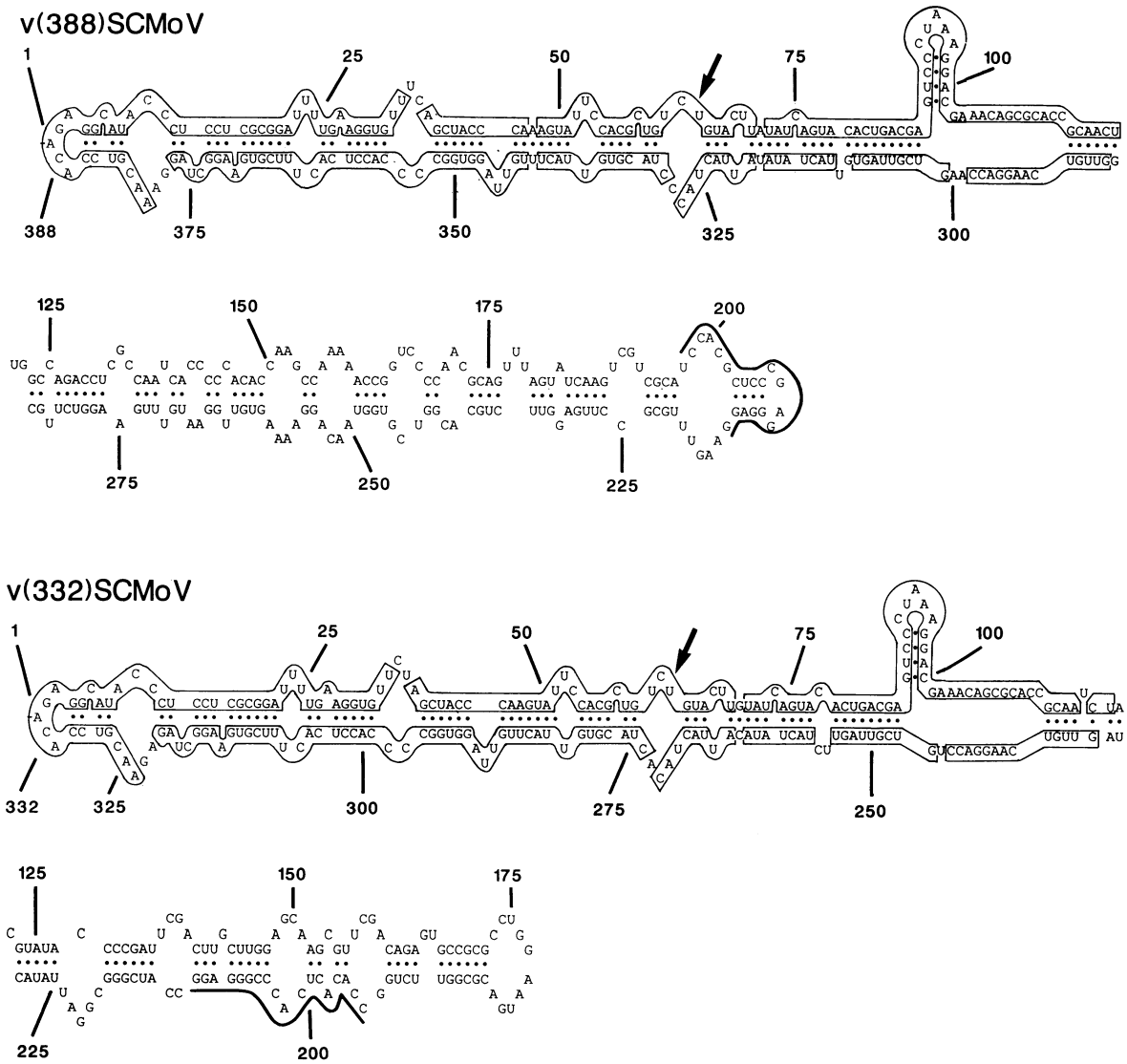
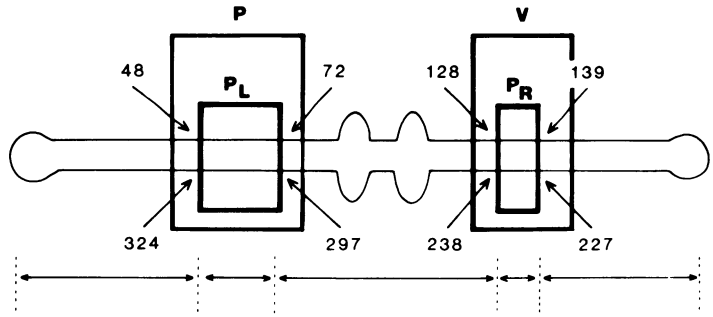


Fig. 3. Proposed secondary structures for v(388)SCMoV and v(332)SCMoV RNAs (vSCMoV, subterranean clover mottle virusoid). Nucleotides conserved between both RNAs at similar positions in the secondary structures are boxed. An 18-nucleotide sequence in which 14 of the nucleotides are common in both RNAs is indicated by a thick line. The site of self-cleavage in each RNA is shown by an arrow. This figure is reproduced from Davies *et al.* (1990).



Total number of nucleotides in each domain	96	49	116-117	20-24	89
Maximum number of nucleotide differences between any two variants in each domain	0	12	4	17	0
Maximum variation (%) between any two variants	0%	25%	3%	77%	0%

Fig. 4. Summary of sequence analysis of 17 sequence variants of citrus exocortis viroids (CEVs) (Visvader and Symons 1985). Most nucleotide changes occur in the P_L and P_R domains within the normal pathogenic (P) and variable (V) domains of CEV. This figure is reproduced from Keese *et al.* (1988). (Reprinted with permission from RNA Genetics, Vol. III, Variability of RNA Genomes, pages 71-98. Copyright CRC Press, Inc., Boca Raton, FL.)

occurring isolates of viroids, prepared from a single plant, often contain more than one sequence variant of a viroid, and these can be separated and sequenced by the preparation of full-length cDNA clones from the viroid mixture (Visvader and Symons 1985; Rakowski and Symons 1989). Since cDNA clones of viroids and their RNA transcripts that are infectious when inoculated onto susceptible plants (Cress *et al.* 1983) can be prepared, this offers a unique opportunity to relate sequence, and hence structure, with pathogenicity.

Sequence analysis of PSTV, CEV, and HSV variants showed that almost all sequence differences for each viroid are located within the P and variable (V) domains (Schnölzer *et al.* 1985; Visvader and Symons 1985; Shikata 1990). This indicates that variation in severity of symptom expression of these sequence variants is most likely determined by variation of sequence in one or both of these domains. Most data are available for CEV, and the results showed that, in 17 sequence variants, essentially all sequence variation occurred within the P and V domains in regions called P_L and P_R (Fig. 4, Visvader and Symons 1985; Keese *et al.* 1988).

To define whether the P or V domain or both were responsible for modulating symptom expression, chimeric infectious cDNA clones were prepared in which approximately one half of one sequence variant was joined through the C domain with the other half of another variant (Fig. 5). Two sequence variants were used, one which induced severe symptoms on tomato seedlings and the other which induced very mild symptoms. cDNA constructs were conveniently prepared using the *Bam*HI and *Hin*dIII sites in the C domain. Infectivity results clearly indicated that the P domain determined symptom expression, while the V domain may have had an effect on the level of viroid that develops in infected plants (Visvader and Symons 1986). It was confirmed that the sequence of the progeny viroids was always the same as that of the clone used for inoculation. However, the results provide no information on the molecular basis of the induction of the pathogenic response.

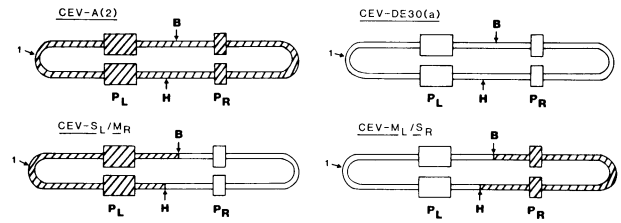


Fig. 5. Schematic diagram of two parental citrus exocortis viroids (CEVs) and two chimeric viroids in circular form. Parent CEV-A(2) induces severe symptoms on tomato seedlings, whereas the other parent, CEV-DE30(a), induces very mild symptoms. P_L and P_R domains (Fig. 4) and nucleotide 1 are indicated. B indicates *Bam*HI site and H indicates *Hin*dIII site in cDNA clones; these were used to construct the two chimeras (CEV-S_L/M_R and CEV-M_L/S_R). This figure is reproduced from Visvader and Symons (1986). (Reprinted by permission of Oxford University Press.)

Periodicity of sequences in viroids indicates origin by duplication. Most viroids exhibit a structural periodicity characterized by repeat units of 11 or 12 nucleotides for the PSTV subgroup, 60 nucleotides for ASSV, and 80 for ASBV (Juhasz *et al.* 1988). Although the repeat sequence was found in only roughly one third of the total sequence of members of the PSTV subgroup, the longer repeats of ASSV and ASBV occupied the full length of each molecule (Table 3).

The periodicities of Table 3 appear to be specific for viroids, since none were found in other small RNAs, such as small nuclear RNAs and the virusoids, or in random sequences generated when the same base composition as that of some of the viroids was used (Juhasz *et al.* 1988). However, a conspicuous periodicity was not found for HSV or its sequence variant, cucumber pale fruit viroid, both members of the PSTV subgroup, which remains a puzzle. It was suggested by Juhasz *et al.* (1988) that the observed periodicity of viroids may play a role in the interaction

of the DNA-like viroid RNA with proteins, since one of the characteristics of the protein-binding ability of DNA is structural periodicity (Travers 1987).

An additional possibility is that this structural periodicity of most viroids reflects their evolutionary origin where sequence duplication followed by mutation allowed an increase in size and the development of infectious molecules (Diener 1989). An excellent example of such partial duplication of a viroid molecule is shown during the infection of coconut palms by coconut cadang-cadang viroid (CCCV). The 246-nucleotide viroid appears early in infection but, as symptoms develop, new higher molecular weight forms arise and eventually dominate the viroid population as the disease progresses (Imperial *et al.* 1981; Mohamed *et al.* 1982). Duplication of the whole T2 domain occurs and is initiated at three separate sites within the V domain to give extra sequences of 41, 50, 55, or 2 × 50 nucleotides (Fig. 6; Haseloff *et al.* 1982; Keese and Symons 1987; Keese *et al.* 1988).

Table 3. Examples of structural periodicity in viroids[a,b]

Viroid[c]	Repeat unit (nt)	Consensus
PSTV	12	CNGRRGRRAYCN (nt 69–172)
CCCV	12	CNGRRGRRAYCN (nt 244–129)
ASSV	60	Repeated 4.5 times in 330 nt
ASBV	80	Repeated 3 times in 247 nt

[a] Data from Juhasz *et al.* (1988).
[b] R, purine (A or G); Y, pyrimidine (C or U); N, a nonconserved nucleotide (A, C, G, or U); and nt, nucleotide.
[c] Abbreviations are given in Table 1.

The functional implications of the development of these higher forms of CCCV during disease progression are not known. Obviously, they must provide some replication advantage, for example, by providing increased competition for binding to these repeated sequences of some host component important for replication but which is in limited supply (Keese *et al.* 1988). It has been suggested that this duplication of the T2 domain of CCCV occurs by discontinuous transcription by an RNA polymerase switching or jumping from one template to another (Keese and Symons 1985), a model analogous to that proposed for the generation of defective interfering RNAs of influenza virus (Jennings *et al.* 1983). Presumably, the sequence duplication in the other members of the PSTV group of viroids could have occurred in a similar way to eventually build up the full-length molecule.

Replication pathway for viroids and virusoids occurs via a rolling circle mechanism. It is generally agreed that these covalently closed circular RNAs are replicated by a rolling

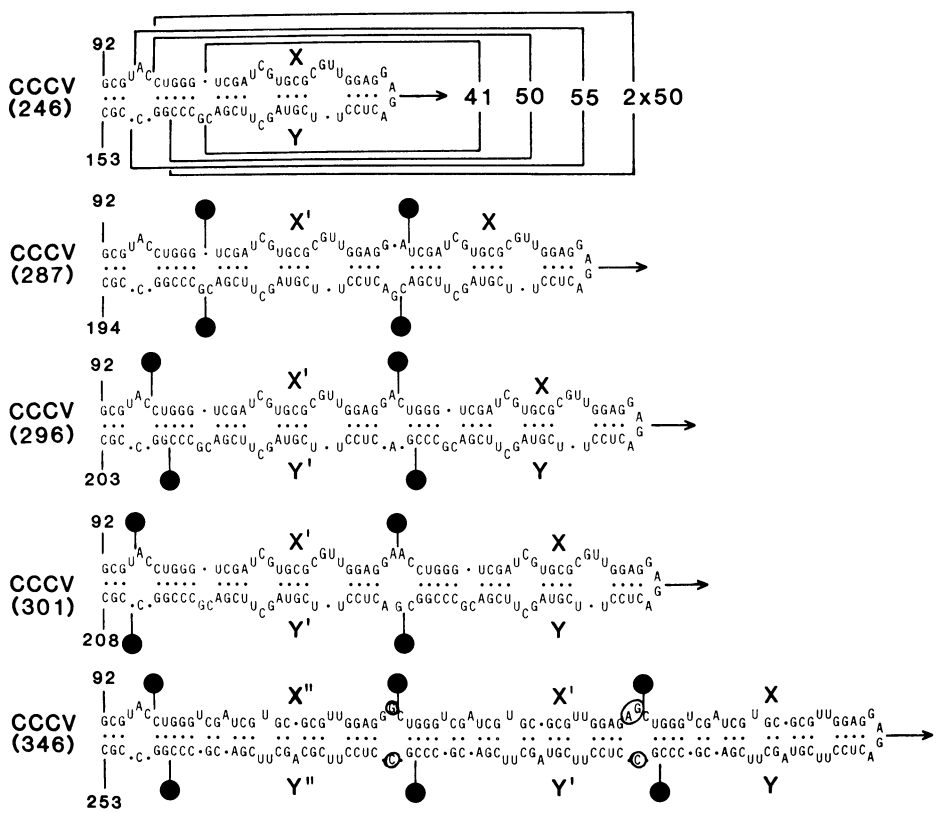


Fig. 6. Partial sequence duplications in coconut cadang-cadang viroid (CCCV). The two sequences, X and Y, with a total of 41, 50, 55, or 2 × 50 nucleotides are duplicated as indicated. The arrows pointing to the right indicate the boundaries of the X and Y sequences, while the filled circles mark the boundaries of the duplicated sequences. Circled nucleotides are sites of mutation in sequence variants. This figure is reproduced from Keese *et al.* (1988). (Reprinted with permission from RNA Genetics, Vol. III, Variability of RNA Genomes, pages 71-98. Copyright CRC Press, Inc., Boca Raton, FL.)

circle mechanism (Fig. 7; Branch and Robertson 1984; Symons 1989), although many aspects of their replication are uncharacterized at the molecular level. In one of the two variations of the rolling circle mechanism (Fig. 7A), the infectious circular plus RNA is copied continuously by an unidentified RNA-dependent RNA polymerase to form a concatameric minus strand (step 1). Specific cleavage of this strand produces monomers (step 2) that are circularized by a host RNA ligase and then copied by the same or a different RNA polymerase (step 4). Specific cleavage of the long linear plus strand produces plus monomers (step 5) that are circularized (step 6) to give the progeny RNA. ASBV and vLTSV most likely follow this pathway (Hutchins *et al.* 1986; Forster and Symons 1987a, 1987b).

In the other variation (Fig. 7B), the linear minus strand (step 1) is not cleaved but copied directly to give a linear plus strand (step 3) that is cleaved to give plus monomers and, finally, circular progeny. Most viroids and three of the four virusoids most likely follow this route (Branch *et al.* 1988; Hutchins *et al.* 1985; Davies *et al.* 1990).

There are many unanswered questions about these two related pathways. What RNA polymerase is involved in the replication? Does the same enzyme copy both the plus and minus strands? In the case of viroids, the enzyme must be host encoded, but there is no direct evidence to indicate whether it is RNA polymerase I, II, or III or some variant of their multicomponent complexes. In the case of the satellite virusoids, it is generally assumed that the helper viral-coded RNA polymerase, with or without host components, is responsible but no direct proof is available. The possibility that a host-derived enzyme similar to that required for viroid replication is needed cannot be dismissed. There is no information for a specific promoter sequence on the circular RNAs that defines the site of initiation of transcription.

The sites of viroid and virusoid synthesis in the cell are also unknown. In the case of PSTV and CEV, Riesner and his colleagues have clearly shown that the nucleolus is a major site of viroid accumulation (Riesner 1987; Harders *et al.* 1989), but nothing is known about the actual site of synthesis. A comprehensive analysis by *in situ* hybridization is required to provide more definitive data on these aspects for both viroids and virusoids.

Mechanism for processing precursors of the viroids of the PSTV group is still to be resolved. A major aspect of the rolling circle mechanism is the highly specific cleavage reaction required to produce monomers from concatameric RNA. As considered below, a unique self-cleavage reaction that is mediated by RNA in the absence of protein and demonstrated *in vitro* most likely operates *in vivo* for ASBV and the four virusoids. However, for all viroids of the PSTV group, no such definitive data exist, and resolution of the processing mechanism remains a major challenge in the viroid field.

So far, attempts to detect a nonenzymatic processing event for oligomeric PSTV have shown only very low (Robertson *et al.* 1985) or negligible (Tsagris *et al.* 1987a) activity. However, Tsagris *et al.* (1987b) appear to have achieved reasonably specific cleavage of PSTV precursors and the production of circular monomers in low yield when greater-than-unit-length precursors were incubated with extracts of plant nuclei. The results were taken to indicate that *in vivo* processing of PSTV precursors requires the action of specific plant RNases to give linear monomers which are then ligated to the circular form. As the authors state, the possibility that proteins in the nuclear extract may have a structural rather than a catalytic role in the processing of the precursor RNA cannot be eliminated.

The results are not quite as convincing as may first appear, because most RNases cleave single-stranded regions to give 5′-hydroxyl and 2′,3′-phosphate termini, which are those required for covalent coupling of RNA by plant RNA ligases (Branch *et al.* 1982). Furthermore, given that the precursor oligomeric RNA consistently folds into one or more configurations, a nonspecific RNase could nick exposed single-stranded regions at approximately monomeric lengths with the subsequent RNA ligase coupling of juxtapositioned 5′- and 3′-termini. Obviously much more specific data are needed to provide further support for or against a specific nuclease cleavage of precursor RNAs.

It seems feasible that the *in vivo* mechanism for the viroids of the PSTV group could turn out to be some type of self-cleavage. Already three types of self-cleavage have been identified in only 12 self-cleaving RNAs that have been characterized so far: nine via the hammerhead reaction (see below), a different and still to be fully characterized structure for the minus sTRSV (Bruening *et al.* 1988; Bruening 1990), and an unknown structure for the plus and minus RNAs of hepatitis delta virus (Taylor 1990; Branch *et al.* 1990). It is eminently feasible that more types of self-cleavage will be found, one of which could account for the processing of precursors of all members of the PSTV group.

Site-directed mutagenesis defines a potential site for CEV processing. Monomeric cDNA clones of PSTV and CEV are infectious on tomato seedlings when cloned at the *Bam*HI site (see Fig. 8A for CEV) in certain *Bam*HI-cut plasmid or phage vectors, as are the RNA transcripts and excised monomers (Tabler and Sänger 1984; Visvader and Symons 1985). However, monomeric cDNA clones prepared at other sites in the viroid molecule, for example the other site in Figure 8A, are not infectious unless they can be excised as monomers with sticky ends. Presumably, the excised monomers are ligated to monomeric circles *in vivo* to initiate the rolling circle transcription from the circular DNA.

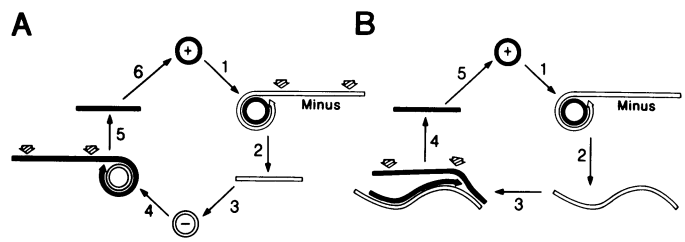


Fig. 7. Rolling circle models for the replication of viroids and virusoids and the satellite RNA of tobacco ringspot virus. **A**, Pathway where both plus and minus RNAs cleave. **B**, Pathway where only the plus RNA cleaves. Sites of cleavage are indicated by arrows. This figure is reproduced from Forster and Symons (1987a). (Reprinted with permission of Cell Press.)

By remarkable coincidence, infectivity of the intact monomeric cDNA clones is correlated with the presence of an 11-nucleotide sequence 3′ to the *Bam*HI site in the viroid sequence that is repeated after the *Bam*HI cloning site in the vector (black bar in Fig. 8B, wild-type sequence contains a G at the site of circled A and U) (Tabler and Sänger 1984; Visvader and Symons 1986). Since monomeric clones without a repeat sequence or with only a six-nucleotide repeat are not infectious, this indicates that the processing of RNA transcripts occurs within the 11-nucleotide repeat sequence.

Site-directed mutagenesis of G97 of the CEV sequence to U or A (Fig. 8B) resulted in an infectious clone, but a noninfectious excised insert (Visvader *et al.* 1986). Surprisingly, the infectious CEV progeny from the infectious mutated cDNA clone contained the wild-type sequence. The result was explained by the *in vivo* processing of the RNA transcripts, generated *in vitro* or *in vivo*, at one of

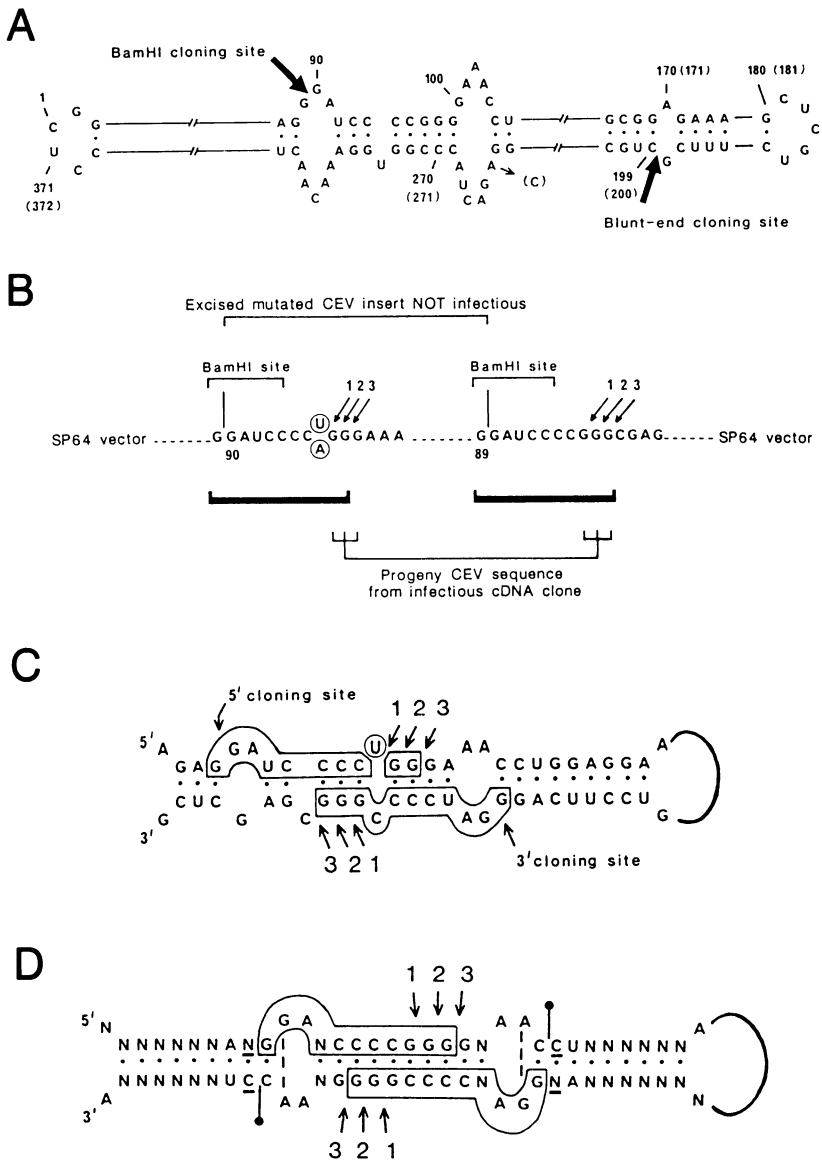


Fig. 8. Summary of data indicating site of cleavage of plus citrus exocortis viroid (CEV) and other potato spindle tuber viroid (PSTV) subgroup B1 viroids during rolling circle replication. **A**, Sites of the CEV molecule used in the construction of full-length cDNA clones. **B**, Potential *in vivo* processing sites of RNA transcripts derived from two point mutant cDNA clones of CEV; the circled nucleotides represent the point mutations introduced at G97 to give either A97 or U97. The wild-type cDNA clone as well as the excised insert were infectious when inoculated on tomato seedlings. In contrast, only the intact mutant clones were infectious. The 11-nucleotide repeat sequence correlates with infectivity and the proposed sites of processing at positions 1, 2, or 3, which are indicated. **C**, Palindromic sequences and structures that may form at the proposed 5′- and 3′-processing sites of the RNA transcripts of the U97 mutant cDNA clone. The 11-nucleotide repeat sequence is boxed. **D**, Conserved nucleotides and potential structures in the conserved central domain of all PSTV subgroup B1 viroids. N is a nonconserved nucleotide. The 11-nucleotide sequence is boxed, and the proposed processing sites are indicated by arrows. In hop stunt viroid, a U is inserted at the position of the filled circle. This U could pair with an A that is represented by N, leaving an unpaired C which is marked C. Potential A:G base pairs are joined by broken lines. This figure is adapted from figures in Visvader *et al.* (1985). (Reprinted by permission of Oxford University Press.)

the three phosphodiester linkages on the 3′ side of the point mutation (identified as 1, 2, or 3 in Fig. 8B). It is approximately the same site identified by Tsagris *et al.* (1987b) as one of two possible processing sites in PSTV from their experiments using nuclear extracts as described above.

Given that processing occurs at one of the three proposed sites in CEV and that some other processing mechanism is not operating, then one possible two-dimensional processing structure is possible and is given in Figure 8C (Riesner *et al.* 1979; Visvader *et al.* 1985; Diener 1986). The boxed nucleotides are the 11-nucleotide repeats of Figure 8B, while the seven nucleotides (CGAGCUC) near the 3′ end are vector sequences that provide limited base pairing. Processing would require excision of monomeric RNA by cleavage at two positions corresponding to sites 1, 2, or 3 (indicated by arrows) and its subsequent enzymatic ligation to a circular form. Alternatively, the excision mechanism may directly produce a circular monomer.

During continuous rolling circle transcription of a circular minus template, the structure analogous to that of Figure 8C is given in Figure 8D. All nucleotides are derived from the top strand of the C domain (Fig. 1), and those shown are conserved in all viroids of the PSTV subgroup. It is possible that the double cleavage considered in Figure 8C is replaced by a more complex sequence of events for oligomeric precursors considered in Figure 8D. For example, if the palindromic structure is the actual structure required for cleavage, then cleavage may only occur on the top strand to release a linear monomer followed by the rearrangement of the structure to place the next cleavage site in an active structure. In this way, there would be sequential release of linear monomers.

There is no real evidence for any of these models. Diener (1986) has considered models analogous to those in Figure 8D, while Riesner (1990) has described structural models with similar hybridization in the central conserved region for an oligomer of nearly four units. Despite the limited data, considerable speculation, and two-dimensional model building, we really cannot be confident about any aspect of the processing of oligomeric precursors of the PSTV group of viroids.

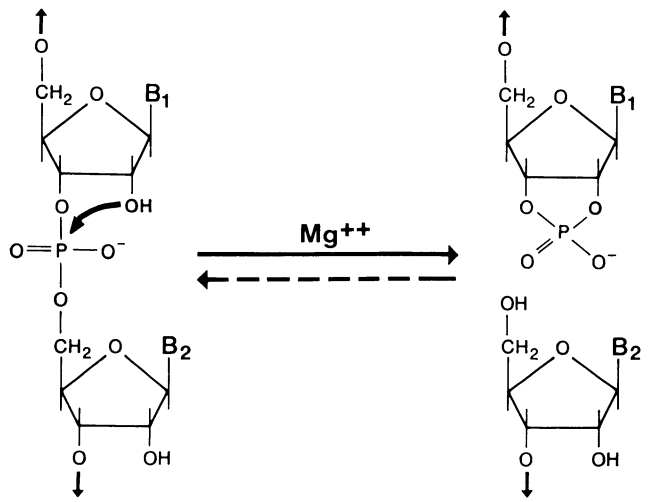


Fig. 9. The self-cleavage reaction that involves a phosphoryl transfer reaction from the 5′-hydroxyl of the 3′ nucleotide residue to the 2′-hydroxyl of the 5′ residue. This figure is reproduced from Symons (1989).

Hammerhead self-cleavage reaction in ASBV and the four virusoids. This area has been extensively reviewed recently (Symons 1989, 1990, in press; Sheldon *et al.* 1990; Forster *et al.* 1990; Davies *et al.* 1990), and only key aspects will be considered here.

Plus and minus RNA transcripts prepared from cDNA clones of ASBV and vLTSV self-cleave in a highly specific manner both during transcription and after isolation in the complete absence of protein. The reaction involves a Mg^{2+}-catalyzed phosphoryl transfer reaction that cleaves the RNA to give termini with a 2′,3′-cyclic phosphate and a 5′-hydroxyl (Fig. 9). On the basis of the specific sites of cleavage, a conserved two-dimensional hammerhead structure was developed (Fig. 10) that could be applied to the self-cleaving RNAs of plus and minus ASBV, plus and minus vLTSV, plus solanum nodiflorum mottle virusoid, plus velvet tobacco mottle virusoid, and plus vSCMoV, as well as the plus sTRSV.

The hammerhead structure of Figure 10 consists of three base-paired stems (I, II, and III) around an open single-stranded region with 13 nucleotides (boxed) that are conserved in nine self-cleaving RNAs identified so far which cleave via this structure. Although definitive evidence has yet to be obtained, it seems likely that the self-cleavage reaction characterized *in vitro* is also responsible for processing of oligomeric precursors *in vivo*.

The two-dimensional hammerhead structure of Figure 10 obviously does not explain why there is a nonenzymatic cleavage of RNA at the specific site. It is considered that, in the presence of Mg^{2+}, the hammerhead assumes an active tertiary structure which lowers the activation energy sufficiently and specifically at the internucleotide bond of the self-cleavage site to allow the phosphoryl transfer of the self-cleavage reaction. When cleaved, the structure is considered to relax and thus prevent the reverse reaction from occurring, since the overall reaction is theoretically reversible.

In the case of ASBV and the four virusoids, the sequences involved in the hammerhead reaction provide information

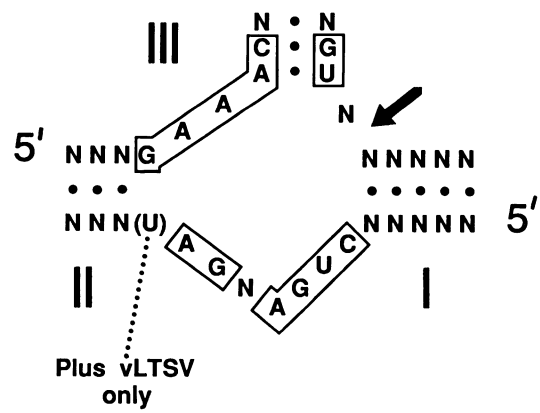


Fig. 10. Consensus hammerhead structure around the self-cleavage site (indicated by arrows) of avocado sunblotch viroid, the four virusoids, and the plus satellite RNA of tobacco ringspot virus (vLTSV, lucerne transient streak virusoid). The 13 conserved nucleotides are boxed, and the nonconserved nucleotides are given as N.

for a key step in the replication cycle. They are also the only sequences in these RNA pathogens for which we have information indicating their functional role. Thus, in the case of ASBV, the plus and minus self-cleavage sites are 14 nucleotides apart, and the sequences of the two hammerhead structures involve approximately the central one third of the total molecule (Hutchins *et al.* 1986; Forster *et al.* 1987). Whether this part of the ASBV molecule has any other functional role is unknown.

The plus and minus self-cleavage sites of vLTSV are only six nucleotides apart, and the sequences that make up the plus and minus self-cleavage structures overlap and constitute the right-hand one third of the vLTSV molecule (Forster and Symons 1987a, 1987b). Regarding ASBV, we do not know if this region has any other function, and we do not have any data on the information content of the rest of the molecule.

Only the plus RNAs of the other three virusoids self-cleave. About 60 nucleotides are required for the hammerhead structures, and these are derived from the top strand on the left-hand side of the rodlike molecules (Keese and Symons 1987; Davies *et al.* 1990). Taken together with the opposite, partially base-paired strand of each virusoid, the total accounts for about one third of each molecule. No other functional information is known for these RNAs, except for specificity in relation to their helper viruses (Francki 1985, 1987; Symons, in press).

Elements of tertiary structure are present in viroids and virusoids. It seems feasible that in the rodlike structures of viroids and virusoids additional elements of tertiary structure occur both in the native state as well as during various stages of their replication. Such structural elements would most likely play a functional role in the life cycle of these pathogens as well as in the initiation of pathogenic effects.

One well-defined example of local tertiary structure in PSTV is the UV-induced covalent cross-linking between G98 and U260 in the highly conserved region of the C domain (Fig. 11; Branch *et al.* 1985). Presumably this region of the viroid is in an activated state that allows specific and efficient cross-linking to occur when irradiated with UV light. Similar cross-linking was also found in the conserved bulged helix of HeLa cell 5S RNA (Fig. 11; Branch *et al.* 1985) as well as in a viroidlike domain of hepatitis delta virus RNA (Branch *et al.* 1989) and 7SL RNA, which is a component of the signal recognition particle (Zwieb and Schüler 1989).

The site of cross-linking in HeLa 5S RNA is in a region of the molecule analogous to that of the protein TFIIIA binding site on Xenopus 5S RNA and near the binding site for ribosomal protein L5, while 7SL RNA binds a number of specific proteins. Thus, the UV-sensitive element in PSTV could form a binding site for one or more proteins involved in replication and transport (Branch *et al.* 1990). The numerous structural forms possible in PSTV and related viroids, as has been well-documented by Riesner and his colleagues (Riesner 1990), could also provide multiple opportunities *in vivo* for the binding of host protein and for the interaction with other cellular components.

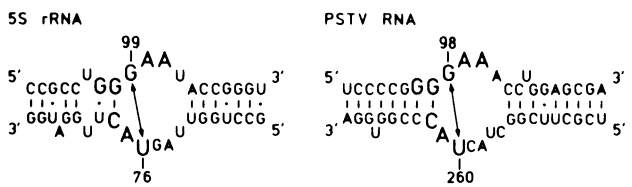


Fig. 11. Schematic diagram of UV-induced cross-linked sites in HeLa 5S rRNA and potato spindle tuber viroid (PSTV). Double-headed arrows indicate positions of covalent attachment. This figure is reproduced from Branch *et al.* (1985).

Another type of tertiary element is the hammerhead structure implicated in the *in vivo* processing of oligomeric precursors of ASBV, the four virusoids, and the plus sTRSV, as already considered above. Likewise, processing in the PSTV group of viroids may eventually be characterized as another activated tertiary structure.

Putative conserved elements of introns in viroids and virusoids most likely have nothing to do with common origins and RNA processing. During the past nine years, it has become popular to speculate on the possibility that viroids and virusoids contain the conserved elements of group I introns, that these indicate a common origin, and, by strong implication, are involved in the processing of the multimeric precursors during rolling circle replication (e.g., Diener 1981; Dinter-Gottlieb 1986, 1987). Features that are usually absent from such sequence comparisons are a critical statistical analysis of the probability of the observed partial sequence homologies occurring by chance and the presence of such homologies in other RNAs which would be considered to be completely unrelated. It is a sobering thought to consider that the comparison of the sequence of PSTV with 25 computer-generated random sequences of the same size and base composition gave sequence homologies of 29.5 ± 4.2% (Fig. 2 in Keese *et al.* 1988).

In the case of the virusoids, processing of oligomeric precursors most likely occurs *in vivo* via a self-cleavage reaction involving the hammerhead structure, a reaction extensively characterized *in vitro* (Fig. 10; Forster and Symons 1987a, 1987b; Forster *et al.* 1987; Sheldon and Symons 1989; Davies *et al.* 1990). As Diener (1989) points out, the absence of these group I-type sequence elements in the hammerhead structure effectively destroys any consideration that they play a role in RNA processing in these pathogenic RNAs. As already considered above, if a similar type of self-cleavage reaction is involved in the processing of precursors of the PSTV group of viroids, then this would provide further evidence against any role for these various group I intronlike related sequences.

Given that these group I intronlike sequences in viroids and virusoids do have some functional role, it is feasible that they could be involved in some aspect of tertiary structure, for example in a way similar to proline that is usually involved at bends in protein molecules. Our understanding of the role of various sequence motifs in the tertiary structure of RNA is increasing; one recent example would be the structural and functional roles played by pseudoknots (Pleij 1990).

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