Citrus Cachexia Disease: Molecular Characterization of its Viroid Agent

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ABSTRACT. The Group II of citrus viroids (CVd-II) can be considered generically as a group of viroid variants related to the hop stunt viroid (HSVd) by molecular structure and sequence. Five field cachexia isolates and a non-cachexia source were subjected to retrotranscription and DNA amplification, cloning and sequencing. SSCP analysis demonstrated the existence of variability occurring among and within cachexia isolates. The variability was confirmed by sequence analysis of selected clones. The variable domain V was extremely conserved in all the cachexia isolates. Five nucleotide differences affecting both the upper (three nucleotides) and lower (two nucleotides) strands of the V domain were identified as the most characteristic feature discriminating cachexia from non-cachexia isolates. A sub-optimal branched secondary structure has been identified as characteristic of sequences from cachexia isolates.

Index words. SSCP, HSVd, variability, secondary structure.

The cachexia disease is characterized by the formation elongated pits on the wood underneath the bark of sensitive species (3). Usually these pits are full of gum deposits and when the disease is severe, gum may show in the surface of the bark. Mandarins, clementines, satsumas and their hybrids grown as commercial varieties and the alemow, used as a rootstock, are susceptible.

The viroid etiology was demonstrated in 1988 as specific components of the citrus viroid II (CVd-II) group (4, 25). At least two types of variants (CVd-IIa and CVd-IIb) with subtle differences in electrophoretic mobilities in sPAGE analysis were identified. In spite of the sequence homology revealed by molecular hybridization, only the fast migrating variants (CVd-IIb) induced symptoms when inoculated on the cachexia indicator Parson's Special mandarin (25). Similarly, only CVd-IIb variants induced cachexia symptoms in field grown susceptible hosts (Orlando tangelo and alemow) (Palacio and Duran-Vila, unpublished data).

Sequencing demonstrated that CVd-II viroids were actually variants of HSVd, a viroid that has been found as naturally occurring in many cultivated crops (1, 15, 26). Differences in the nucleotide sequence of pathogenic (CVd-IIb) and non-pathogenic (CVd-IIa) variants appears to be very subtle (14).

The objective of this work was the molecular characterization of citrus variants of HSVd and the identification of pathogenicity motifs.

MATERIALS AND METHODS

Viroid sources and nucleic acid extraction procedures. Six CVd-II isolates from different sources were selected from the viroid collection maintained at IVIA: X-701 (Cachexia 114, Corsica), X-704 (Old-line Navel orange, California), X-707 (Prior Lisbon lemon, California), X-712 (Salzara Satsuma, Spain), X-715 (Cajel sour orange, Spain) and CVd-IIa-117 (Nules clementine, Spain). These isolates had been maintain in citron for at least 10 yr.

Samples (5 g) of young leaves and stems were homogenized in 5 ml volume of extraction medium (0.4M Tris-HCl, pH 8.9; 1% (w/v) SDS; 5 mM EDTA, pH 7.0; 4% (v/v) mercaptoethanol) and 15 ml of water saturated phenol (23). The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10mM Tris-HCl; 10mM KCl;

0.1mM $MgCl_2$; pH 7.4). Aliquots of the nucleic preparations were analyzed by sPAGE analysis and slot-blot hybridization against a cRNA-HSVd probe to confirm infection.

Bioassay. Parson's Special mandarin plants grafted on Rough lemon rootstock were used as indicators (20). At least three indicator plants were used in each bioassay. Inoculated plants and uninoculated controls were kept in the greenhouse at 28° to 32°C. Parson's Special mandarin plants were graft inoculated with citron bark tissue. For symptom evaluation, gumming and wood staining symptoms at the bud union were recorded 12 mo after inoculation.

DNA synthesis and cloning. DNA was synthesized from the LiCl-soluble fraction essentially as described by Astruc et al. (1). First strand synthesis was performed using the HSVd-specific 26-mer oligonucleotide VP-19 (5'-GCCCCGGGCTCCTTTCTCAGGTAAG-3') complementary to bases 60 to 85 of HSVd using Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) (Promega Corp., Madison, WI). Second strand synthesis and amplification of dsDNA was performed with the oligonucleotides VP-19 and the 27-mer VP-20 (5'-CGCCCGGGGCAACTCTTCTCAGAATCC-3') homologous to residues 78 to 102 of HSVd. PCR was performed in 1.5 mM $MgCl_2$, 0.12 mM dNTPs, 0.5M of each primer and 1 U of Taq DNA polymerase. PCR parameters were: denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 40 sec, 60°C for 40 sec and 72°C for 1 min, to finish with extention at 72°C for 5 min. The size of the DNA product was determined by electrophoresis in 2% agarose gels and its homology with HSVd was confirmed by slot-blot hybridization.

The purified DNA was digested with the endonuclease *Sma* I (see underlined, the *Sma* I site in primers sequences) and the resulting fragment was ligated to the *Sma* I site of the dephosphorylated pUC 18 plasmid (Pharmacia). Plasmids from transformed cells were subjected to restriction analysis to verify the presence of an insert of the expected size.

Single-stranded conformation polymorphism (SSCP) analysis. Cloned viroid DNA was recovered from the plasmids by PCR amplification using the same conditions described above. Aliquots of 2 l of the PCR products were mixed with 20 µl of the denaturing solution (90% formamide, 25 mM EDTA, 0.05% xylene-cyanole and 0.05% bromophenol blue), heated for 10 min. at 100°C and chilled on ice. Denatured DNA was subjected to 14% PAGE ($8.5 \times 7.0 \times 0.1$cm gels) in TBE buffer (89mM Tris-Borate, 2mM EDTA; pH 8.3) at 200V constant voltage until the xylene cyanol dye had migrated off gel (18). The DNA bands were viewed by silver staining (11).

Sequence analysis. Inserts from cloned viroid DNA were sequenced with the ABI PRISM DNA sequencer 377 (Perkin-Elmer). Multiple alignment of sequences was performed using the program Clustal V (10). Secondary structure analysis were obtained with the program MFOLD (circular version) from the GCG package (27).

RESULTS

Analysis of viroid sources. Analysis of nucleic acid preparations by sPAGE and silver staining showed that all the isolates contained an RNA band with the characteristic mobility of CVd-II (Table 1). Only slight differences in mobility were observed among different isolates. All the samples except the uninoculated control showed a positive hybridization reaction against a DIG-labelled cRNA probe (Table 1). All the isolates, except CVd-IIa-117 produced the characteristic symptoms of cachexia on the Parson's Spe-

TABLE 1
BIOCHEMICAL AND BIOLOGICAL INDEXING OF CITRUS VIROID II ISOLATES

	Biochemical indexing		Biological indexing
Isolate	sPAGE	Hybridization	Parson's[z]
X-701	+	+	3.2
X-704	+	+	3.0
X-707	+	+	2.6
X-712	+	+	3.0
X-715	+	+	2.8
CVd-IIa-117	+	+	—

[z]The symptoms on Parson' Special mandarin were rated as severe (4), moderate (3), mild (2) and very mild (1). The table shows the average rating of the reactions recorded on three indicator plants.

cial mandarin indicator (Table 1). When the nucleic acid preparations were subjected to retrotranscription and PCR amplification, a monomeric DNA which showed a positive hybridization against a HSVd-cRNA probe was obtained (data not shown). SSCP analysis of the RT-PCR products revealed major differences in the migration of the single stranded DNA bands indicating variations on their conformation and, therefore, demonstrating that the viroid sources were not identical (Fig. 1A).

Identification of variability. The DNA obtained from each isolate was ligated to the pUC18 vector to recover clones containing full length viroid inserts. SSCP analysis of the clones recovered from each cachexia isolate showed differences in the electrophoretic mobility of the ssDNAs, thus illustrating the heterogeneity of all of them (Fig. 1B). Conversely, the non-cachexia isolate CVd-IIa-117 appeared to be very homogeneous (Fig. 1C). In all instances, the most frequent SSCP profile was identical to that observed when the DNA source (RT-PCR product) used for cloning had been subjected to SSCP analysis. This indicated the existence of a "master sequence" as the most frequent variant in the population.

The variability identified by SSCP analysis was confirmed by sequence analysis of selected clones (Table 2). From 30 clones of CVd-IIa-117, all showing identical SSCP profiles, 6 were sequenced and presented the same nucleotide sequence (Fig. 2 A), thus confirming the homogeneity of this isolate. Conversely, several sequence variants were identified by SSCP analysis in all the cachexia isolates (Table 2) and confirmed by sequencing selected clones. The "master sequences" and the changes found among and within isolates are summarized in Fig. 3. Isolates X-701, X-712 and X-715, all from Mediterranean sources, share very high sequence homologies ranging from 98.3% (between X-701 and X-712) to 99.7% (between X-712 and X-715). Isolates X-701 and X-707, from Corsica and California, respectively, appear to be the most distant with 94.6% of sequence homology.

The variable domain (V) was very conserved among the sequences from cachexia inducing isolates. Similarly, the variable domain of the non-cachexia isolate CVd-IIa-117 was identical to that of two sequences reported elsewhere as non-cachexia inducing sources: CVd-IIa-1 (14) and CVd-IIa-2 (24). Five nucleotide differences, affecting both the upper (3 nucleotides) and lower (2 nucleotides) strands of the V domain, were identified as the most characteristic feature discriminating cachexia and non-cachexia isolates (shown as shaded in Fig. 3).

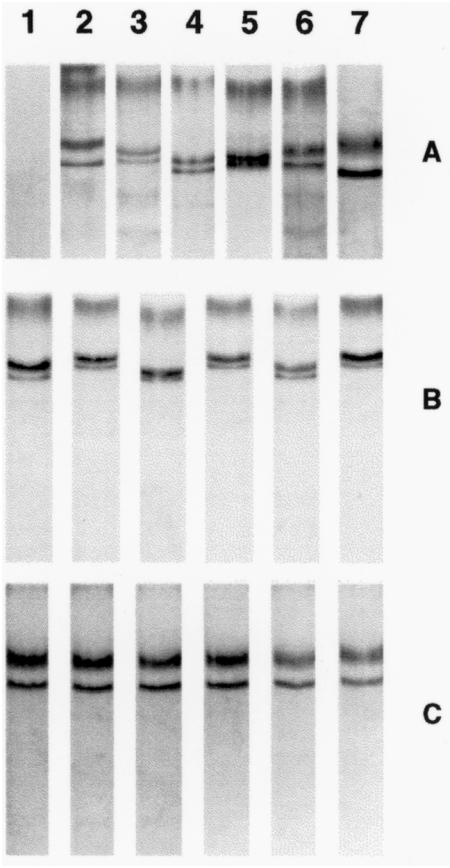

Fig. 1. A) SSCP analysis of RT-PCR products: Uninoculated control (lane 1) and isolates X-701, X-704, X-707, X-712, X-715 and CVd-IIa-117 (lanes 2-7). B) SSCP analysis of six clones from isolate X-704. C) SSCP analysis of six clones from isolate CVd-IIa-117.

Secondary structures. The conformation of minimal free energy of the 6 "master sequences" is a rod-like structure with around 70% base pairing (Fig. 2A and 2B). The non-cachexia isolates always presented values of minimal free energy slightly lower than those of the cachexia isolates (Table 3). The five changes discriminating cachexia from non-cachexia sequences appeared to be highly compensatory in the rod-like structure offered by the MFOLD program when the temperature choice was set at 32ºC (a temperature adequate for symptom expression). Within the V domain the secondary structure appears to be more relaxed in the cachexia sequences as result of: a) Two mismatched nucleotides (positions 105 and 193) in the upper and lower strands respectively (Fig. 2B) versus a single mismatched nucleotide (position 106) in the upper strand (Fig. 2A); b) An arm of seven paired nucleotides (underlined in Fig. 2B) versus 8 (underlined in Fig. 2A); c) An arm of five paired nucleotides (underlined in Fig. 2B) versus 6 (underlined in Fig. 2A).

The "master sequences" of all the cachexia isolates were able to fold into a characteristic branched suboptimal secondary structure (Fig. 2C) in which around 60% of the nucleotides are paired. This suboptimal structure had values of free energy higher than the rod-like structure of minimum free energy. The sequences of the non cachexia isolates (CVd-IIa-117, CVd-IIa-1, CVd-IIa-2) did not fold into this suboptimal branched structure.

TABLE 2
CITRUS VIROID II VARIANTS IDENTIFIED BY SSCP ANALYSIS AND SEQUENCING

Isolates	Number of clones analyzed		Variants (#)	Size range (nucleotides)	Homology (%)
	SSCP	Sequencing			
CVd-IIa-117	30	6	1	303	100
X-701	10	5	4	296-299	98.0 - 99.7
X-704	30	7	4	299	98.7 - 99.7
X-707	30	8	5	296-297	99.0 - 99.7
X-712	10	5	4	300-301	98.7 - 99.7
X-715	10	4	3	299-300	99.3 - 99.7

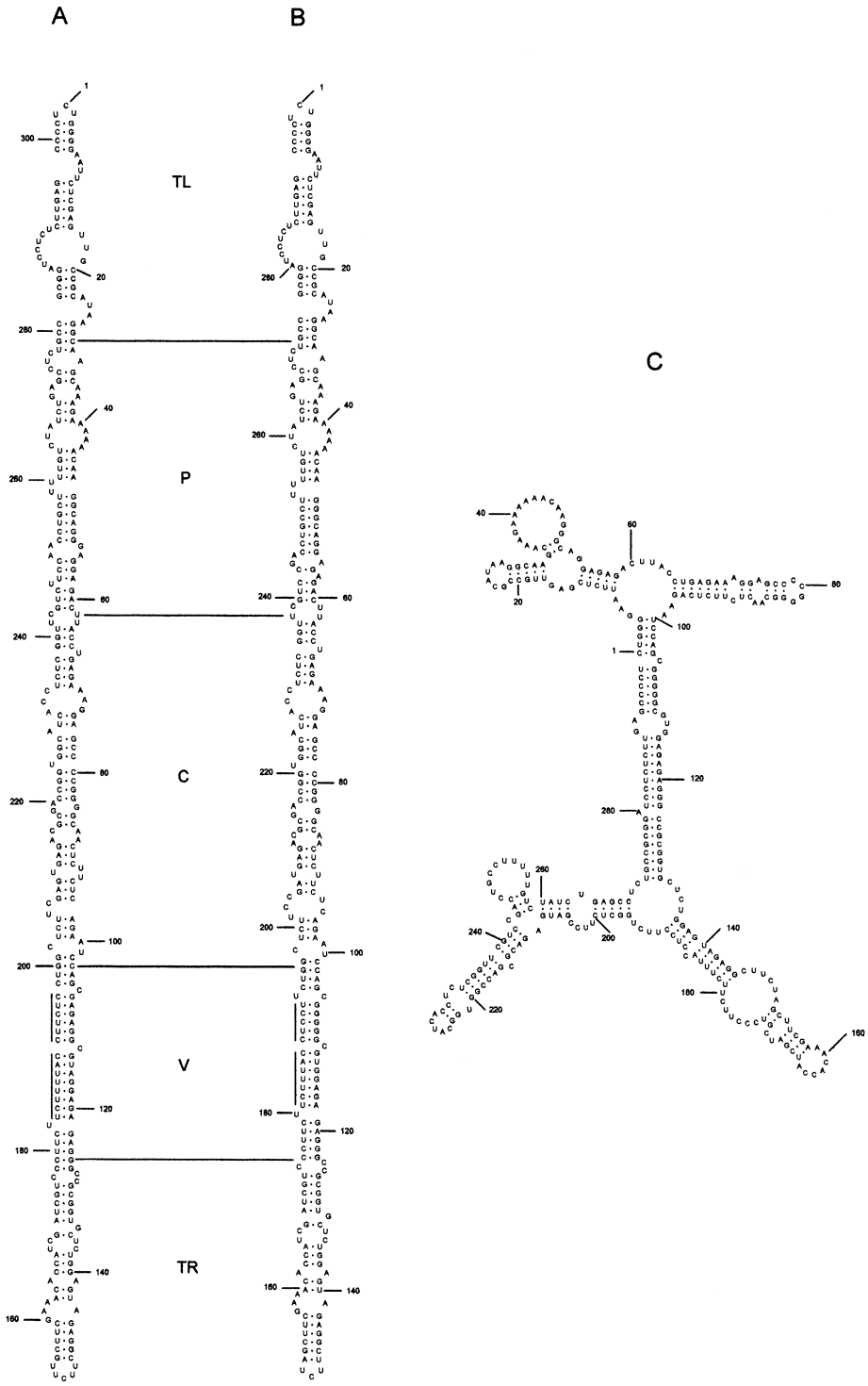

Fig. 2. A) Sequence and secondary structure of CVd-IIa-117. B) Sequence and secondary structure of minimum free energy of the "master sequence" of the cachexia isolate X-707. C) Sequence and suboptimal branched secondary structure of the master sequence of isolate X-707.

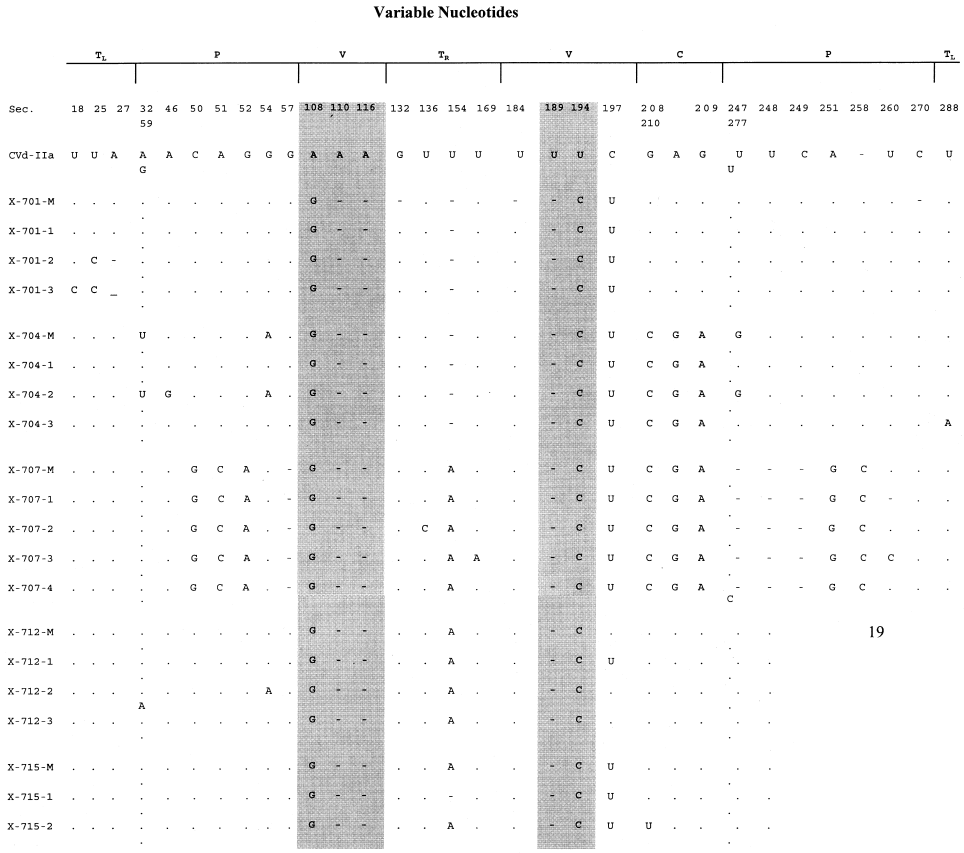

Fig. 3. Summarized alignment of "master" and variant sequences of each isolate (only the variable nucleotides are shown). Structural domains: TL (terminal left), P (pathogenic), C (central), V (variable) y TR (terminal right). M = Master sequence. The characteristic changes in the V domain discriminating sequences from cachexia and non-cachexia isolates are shaded.

DISCUSSION

SSCP analysis of viroid sources revealed differences in electrophoretic profiles differentiating pathogenic from non-pathogenic isolates. These differences indicated that the DNA recovered from different field isolates presented conformational changes and, therefore, variations in their nucleotide sequences (16). Sequencing demonstrated that the pathogenic isolates contained a population of variants with homologies above 98%. Conversely, the non-pathogenic isolate contained a single or highly dominant sequence.

Populations of sequence variants have already been described for several viroids including HSVd (12). The reported heterogeneity of field isolates has been considered to be the result of either co-infection with different viroid sources or as the accumulation of mutants arising *de novo* during replication in absence of proof-reading mechanisms (6). Our results indicate that HSVd in citrus conforms a "quasispecies" model as a complex and dynamic distribution of non-identical but related replicons (5), with a "master sequence" representing the best fit individual within the viroid population replicating in a given environment.

TABLE 3
SECONDARY STRUCTURES AND FREE ENERGIES OF MASTER SEQUENCES[z]

Isolates	Rod-like		Sub-optimal	
	Base pairing (%)	Free energy (kcal mol^{-1})	Base pairing (%)	Free energy (kcal mol^{-1})
X-701	68.9	-85.0	59.5	-80.3
X-704	69.6	-92.2	60.2	-88.6
X-707	68.7	-92.3	60.6	-85.9
X-712	69.3	-95.1	58.0	-86.2
X-715	69.3	-95.3	57.3	-85.1
CVd-IIa-117	69.3	-97.9	—	—
CVd-IIa-1[y]	68.9	-96.2	—	—
CVd-IIa-2[x]	70.2	-99.5	—	—

[z]Observations provided by MFOLD analysis at 32°C.
[y]CVd-IIa-1 (14).
[x]CVd-IIa-2 (24).

Sequencing analysis showed that the V domain is extremely conserved in pathogenic as well as in non-pathogenic isolates. Independently of the changes observed in other regions of the viroid molecule, a five nucleotide motif located in the V domain allows the discrimination between pathogenic and non-pathogenic variants. This is compatible with previous observations showing that non-cachexia inducing isolates could be discriminated by molecular hybridization using an oligomeric probe complementary to the sequence of the upper strand of the V domain which contained the three nucleotides characteristic of this type of isolates (13).

Since viroids are non coding genomes, its activity, including pathogenicity, must be mediated by structural features. Although it has been widely accepted that the P domain is responsible for the pathogenicity of many viroids, studies with artificial chimeras suggest that several structural domains may be involved in the regulation of pathogenesis (21, 22). Phylogenetic analysis of HSVd variants recovered from different crops predicted that the covariations found in the P domain may result in local conformation and stability changes probably affecting pathogenicity (12). However, sequence analysis of the citrus HSVd variants presented here indicates that conformational changes in the P domain are found indistinctly in pathogenic and non-pathogenic variants and, therefore, must be unrelated to the cachexia disease. The observation that the V domain is highly conserved among pathogenic variants suggests that, instead of the P domain, the five nucleotide motif in the V domain of the cachexia isolates is probably involved in the conformational differences responsible of symptom induction.

MFOLD analysis showed that the rod-like secondary structure of the pathogenic variants had a minimum free energy higher than the non-pathogenic ones and, therefore, was less stable. In spite of the similarity between the rod-like structure of both type of isolates, the V domain of the pathogenic variants appears to be more relaxed. This structural difference, although very subtle, may be directly responsible for differences in pathogenicity as already reported for variants of PSTVd differing only in a few changes of the VM region (7, 8). However, site direct mutagenesis studies in PSTVd indicated that there was not a consistent correlation between base pairing in the VM

region and modifications in symptom expression (9, 17). Therefore, the structural differences found in the V domain may not account for the biological differences between cachexia and non-cachexia variants.

Gruner et al. (8) suggested that changes in a given region of the viroid molecule may not conform the primary target for the pathogenic reaction, but regulate the accessibility of another region of the molecule which then would act as the primary target. Our results indicate that all the pathogenic variants may fold as a branched secondary structure as a result of the complete reorganization of the base pairing observed in the minimum free energy rod-like structure. Since the non pathogenic variants do not appear to be able to adopt this alternative branched structure, it is very tempting to speculate that conformations other than the rod-like structure may provide the target for the primary pathogenic interaction. Theoretically, the lower stability of the rod-like structure of pathogenic variants would favor the transition from the rod-like to alternative structures of higher free energy and, therefore, the availability of hypothetical binding sites with host factors not accessible in the rod-like structure (8). Based on computer analysis, structures other than the rod-like, considered as characteristic of viroids, have also been described for many viroids (2) but unfortunately, the conformation that viroids adopt *in vivo* still remains unknown.

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Note: The results of this work are in agreement with a recent publication by Reanwarakorn and Semancik (19) in which the implication of the V domain in pathogenicity was further demonstrated by infectivity studies of artificial chimeras.

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