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Structural similarities between viroids and transposable genetic elements

(tomato planta macho viroid/tomato apical stunt viroid/RNA sequence analysis/molecular cloning)

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ABSTRACT **The primary structures of the tomato planta macho and tomato apical stunt viroids have been determined, and probable secondary structures are proposed. Both viroids can assume the rodlike conformation with extensive base-pairing characteristic of all known viroids. Sequence homologies between the two viroids (75%) and with members of the potato spindle tuber viroid group (73–83%) indicate that they both belong to this group. Comparative sequence analysis of all members of the group reveals striking similarities with the ends of transposable genetic elements. These similarities, the presence of inverted repeats often ending with the dinucleotides U-G and C-A, and flanking imperfect direct repeats suggest that viroids may have originated from transposable elements or retroviral proviruses by deletion of interior portions of the viral (or element) DNA.**

Viroids are a unique group of plant pathogens that cause several important diseases in cultivated plants (1). Each viroid consists of a single-stranded covalently closed circular RNA species that is infectious and unencapsidated (2). The general belief that viroids do not encode translational products (1) suggests that they are replicated by host enzymes and that host range and pathogenicity involve direct interaction between the viroid RNA (or its complement) and host nucleic acids or other cellular components.

The complete nucleotide sequences of five viroids have previously been determined (3–7); each viroid can form a highly base-paired rodlike secondary structure. Comparative sequence analysis suggests that three viroids, potato spindle tuber viroid (PSTV), citrus exocortis viroid (CEV), and chrysanthemum stunt viroid (CSV), belong to the same "PSTV group," sharing 60–70% sequence homology (8). More distantly related is coconut cadang-cadang viroid (CCCV), which shares a central conserved region with the PSTV group (6), while avocado sunblotch viroid (ASBV) (5) is further removed.

In this paper we present primary and proposed secondary structures for two viroids causing severe symptoms in tomato: tomato planta macho viroid (TPMV) (9) and tomato apical stunt viroid (TASV) (10). Comparative sequence analysis shows that TPMV and TASV belong to the PSTV group and that striking similarities exist among all group members and the ends of transposable elements or retroviral proviruses. These similarities suggest a possible origin of viroids.

MATERIALS AND METHODS

Purification of Viroids. TPMV (obtained from J. Galindo A., Chapingo, Mexico) and TASV (obtained from B. Walter, Colmar, France) were purified from infected tomato tissue (*Lycopersicon esculentum* Miller, cv. Rutgers) as described (11).

Direct Sequence Analysis of Viroid RNA. Purified TPMV and TASV were partially cleaved with RNase T1, RNase U2, or RNase A (Calbiochem), 5′-labeled by incubation with [γ-^{32}P]ATP [3,000 Ci/mmol (1 Ci = 3.7×10^{10} Bq)] and phage T4 polynucleotide kinase (P-L Biochemicals), and fractionated by polyacrylamide gel electrophoresis (4). The sequences of the labeled viroid fragments were then determined by the partial enzymatic cleavage method (12), using RNase T1 (12), RNase U2 (13), RNase Phy M (14), and *Bacillus cereus* extracellular RNase (15) (P-L Biochemicals).

Isolation of Plasmid DNA. The plasmid pUC9 was isolated from chloramphenicol-amplified cultures of *Escherichia coli* strain JM83 (16) and purified through two successive ethidium bromide/cesium chloride equilibrium gradients. *E. coli* strain JM83 and the pUC9 plasmid have been described (17).

Synthesis and Cloning of Double-Stranded (ds) Viroid cDNA. Exact conditions for the synthesis and cloning of ds viroid cDNA have been described (18). In general, standard techniques (19) were used, with several modifications: (*i*) Linear viroid molecules were generated from the circular form by limited treatment with S1 nuclease (20) before polyadenylylation (21). (*ii*) Second-strand cDNA synthesis with *E. coli* DNA polymerase I (large fragment) was performed at 37°C rather than 15°C (11) and was followed by an additional incubation with reverse transcriptase (19). (*iii*) pUC9 was chosen as a cloning vector rather than pBR322 because of its versatile multiple cloning site. Insertion into this site allowed rapid one-step screening of hybrid transformants (17) and efficient 3′-^{32}P-labeling of either strand of the viroid cDNA insert (22) prior to sequence analysis by the method of Maxam and Gilbert (23).

Double-stranded TPMV cDNA was inserted into the *Pst* I site of pUC9 by a standard G·C tailing procedure (19). The ds cDNA was digested for 1 hr at 37°C with S1 nuclease (200 units/ml) to remove non-base-paired regions, extracted with phenol/chloroform, and chromatographed on Sephadex G-100. Radioactive material eluting in the excluded volume was pooled and precipitated with ethanol. Oligo(dG) and oligo(dC) tails were added to *Pst* I-cleaved pUC9 and S1-treated ds TPMV cDNA, respectively, by incubation with terminal deoxynucleotidyltransferase (P-L Biochemicals) (24). The tailed products were combined (50:1, wt/wt), annealed (19), and used to transform *E. coli* strain JM83 (25).

Restriction fragments of ds TASV cDNA were inserted into *Bam*HI/*Hin*dIII-digested pUC9 by *in vitro* ligation. The ds TASV cDNA was digested with *Bam*HI plus *Hin*dIII, extracted with phenol/chloroform, and precipitated with ethanol before frac-

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Abbreviations: ASBV, avocado sunblotch viroid; CCCV, coconut cadang-cadang viroid; CEV, citrus exocortis viroid; CSV, chrysanthemum stunt viroid; PSTV, potato spindle tuber viroid; TASV, tomato apical stunt viroid; TPMV, tomato planta macho viroid; ds, double-stranded; LTR, long terminal repeat.

tionation by gel electrophoresis (23). Two radioactive bands of the predicted size were excised, eluted, and twice precipitated with ethanol. The two ds TASV cDNA fragments were combined with *Bam*HI/*Hin*dIII-digested pUC9 (1:100, wt/wt), incubated at 16°C for 20 hr with phage T4 DNA ligase (P-L Biochemicals), and used to transform JM83 (25).

Isolation, Characterization, and Sequence Analysis of Cloned Viroid cDNAs. Hybrid plasmids were isolated from JM83 by a rapid isolation procedure (26). Putative viroid cDNA inserts were sized and mapped by single or multiple restriction endonuclease digestions followed by gel electrophoresis. cDNA inserts were 3′-labeled with [α-^{32}P]dATP (400 Ci/mmol) and *E. coli* DNA polymerase I (large fragment) at either the *Hin*dIII or the *Eco*RI site of pUC9 (22). The inserts were then excised from the vector with either *Eco*RI or *Hin*dIII, purified by gel electrophoresis, and sequenced by the base-specific chemical cleavage method (23). All experiments involving recombinant DNAs were carried out in compliance with current National Institutes of Health guidelines for recombinant DNA research.

RESULTS

Determination of TPMV and TASV Primary Sequences. There was sufficient overlap among the viroid RNA fragments generated by RNase T1, U2, and A to obtain a full representation of both viroid genomes. Sequence determination proved rapid and in most cases reliable. However, the presence of stable base-paired hairpin structures created band compressions in small regions of the gel and complicated sequence determination (4, 7). In addition, near one hairpin region of TPMV (Fig. 1; nucleotides 331–343) virtually no enzymatic cleavage occurred. Another problem was the occasional lack of specificity/recognition of the pyrimidine-cleaving enzymes at various sites throughout the molecule (not only at hairpin structures). This made the determination of pyrimidines less reliable than that of purines.

Because Cress and Owens (27) have cloned the entire PSTV genome and showed that its sequence could be determined reliably by the partial chemical cleavage method, this strategy was adopted to confirm the nucleotide sequences of TPMV and TASV. Sequence analysis of ds cDNA clones synthesized from the S1-generated templates showed that the entire genome of both viroids was represented. Independent cDNA clones derived from the same region of each viroid had identical DNA sequences. Furthermore, the RNA and DNA sequences were in agreement.

Secondary Structures of TPMV and TASV. Secondary structure models for TPMV and TASV (Fig. 1) were constructed from the nucleotide sequences with the aid of a computer program designed to fold RNA molecules into a secondary structure of minimal free energy (28). Additional minor modifications were made to provide maximal structural homology with the proposed secondary structure of PSTV (3). Both TPMV and TASV can assume the rodlike conformation with extensive base-pairing characteristic of all viroids studied so far (3–8). The computer program was also used to search for stable secondary hairpins that have been observed during *in vitro* thermal denaturation of viroid RNA and are thought to serve an important yet unknown function *in vivo* (8). Both TASV and TPMV can form two (nos. I and II) of the three secondary hairpin structures previously observed with PSTV, CEV, and CSV (8).

Values for the nucleotide composition of TPMV and TASV, percentage of nucleotides base-paired, and number and type of base pairs are all quite similar to those previously reported for PSTV, CSV, and CEV (8). For example, the G-to-C, A-to-U, and purine-to-pyrimidine ratios are near 1, there are almost twice as many G·C as A·U base pairs, and 9–13% of the base pairs are G·U.

TPMV shows 83%, 72%, and 74% sequence homology with PSTV, CSV, and CEV, respectively, while TASV displays 73%, 77%, and 78% homology with the same three viroids. TPMV and TASV show 75% sequence homology. Both TPMV and TASV contain the highly conserved central region (Fig. 1, nucleotides 88–105 and 252–274, 89–106 and 252–274, respectively) found in PSTV, CSV, CEV, and CCCV. As previously noted with PSTV, CSV, and CEV (8), the left halves of the TPMV and TASV molecules show more sequence homology than the right halves.

Possible Translation Products of TPMV and TASV. TASV and its putative complementary RNA contain three AUG initiation codons and could encode polypeptides with approximate M_rs of 800, 1,900, and 5,500. Neither TPMV nor its putative complementary RNA contain AUG initation codons. Only three members of the PSTV group (CSV, CEV, and TASV) contain AUG initiation codons, and the lack of similar open reading frames suggests that viroid-specific proteins are not involved in viroid replication (4).

DISCUSSION

Comparison of the primary structures of TPMV and TASV with those of the other members of the PSTV group (Fig. 2) discloses previously noted viroid features. Common to all are the precisely conserved central region (nos. 92–119 and 313–342) and a nearby uninterrupted stretch of 11 to 18 purines (nos. 54–67). Further examination shows that the upper portion of the conserved (and therefore presumably essential) central region of each viroid displays the following characteristics: (*i*) Its

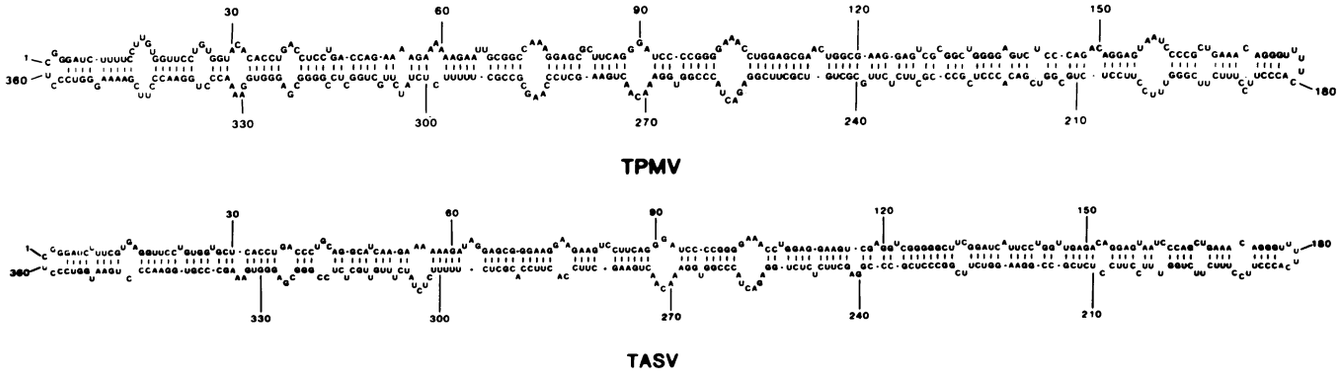

FIG. 1. Nucleotide sequences and proposed secondary structures of TPMV and TASV. Nucleotides are numbered according to the published sequence of PSTV (3).

FIG. 2. Comparison of nucleotide sequences of TASV, CEV (8), PSTV (3), TPMV, and CSV (8). Sequences are aligned for maximal homology. Each column containing at least one nucleotide is numbered, beginning with the first nucleotide of the PSTV sequence (3). Boxed areas contain the conserved central region, adjacent dinucleotides, and the polypurine region of the five viroids.

18-base center (nos. 97 to 114) is an imperfect (5/9 bases) direct repeat (G-G-G···C-C); (*ii*) this center is directly bordered by almost-perfect inverted repeats (6/7 bases for three of the five viroids) (nos. 89 to 96 and 115 to 122) U-C-C-U-·-C-A···U-G-·-A-G-G-A); (*iii*) the 3′ end of the left portion of the inverted repeat is C-A, and the 5′ end of the right portion is U-G.

These features resemble those occurring at the ends of certain moveable genetic elements (29) and retroviral proviruses (30) (Fig. 3). Many moveable genetic elements such as *copia*, TY*1*, the DNA of bacteriophage Mu, and all retroviral proviruses end with the dinucleotides T-G···C-A (30). The termini of all retroviral proviruses and of certain transposable elements, such as TY*1*, contain long terminal repeats (LTRs) ending in the dinucleotides T-G···C-A (31, 32). Retroviral proviruses, but not TY*1*, contain short inverted repeat sequences (ending with T-G and C-A at their 5′ and 3′ termini, respectively) at the ends of each LTR. Short direct repeats occur in some retroviral proviruses (for example, spleen necrosis virus) and certain transposable elements (for example, TY*1*) in the viral or element sequences directly adjoining the 3′ end of the left LTR and 5′ end of the right LTR (31, 32). Short direct repeats are also generated from cellular sequences during the integration process and flank the inserted element or provirus (29–31).

Although resemblance with the ends of retroviral proviruses or transposable elements is most clearly recognizable in the upper portion of the central conserved viroid region, the lower portion of the central conserved region also displays such resemblances to a lesser degree. Its 23-base central portion (nos. 313 to 335) is an imperfect (5–6/11–12 bases) direct repeat, and this central region is bordered on the 3′ end by U-G, as is the case with the upper central region. Beyond these regions, however, the similarities begin to break down. Thus, on the 5′ end, the lower central region is not bordered by C-A, but by C-U, U-C, or U-U. Inverted repeat sequences to the left and right of the lower central region are clearly recognizable only with CSV (G-A-A-G-C-U-U-C···G-A-A-G-C-U-U-C), although the other four viroids contain remnants of such sequences at the corresponding positions.

Examination of the remaining regions of each viroid reveals what appear to be unusually large numbers of C-U-U-C or G-A-A-G sequences or variations thereof. Statistical analysis shows that the viroids contain 4–5 times as many C-U-U-C sequences as would be expected for a random sequence having the same base composition. Indeed, the inverted repeat sequences bordering the upper and lower central viroid regions are mostly composed of C-U-U-C and G-A-A-G sequences. Several areas of the viroid structures containing an abundance of C-U-U-C and G-A-A-G sequences (or variations thereof) may therefore represent remnants of inverted repeats similar to the ones still recognizable in the center of each viroid. In this view, each viroid consists of the following: (*i*) a central region that bears striking similarities to the ends of transposable elements, (*ii*) a polypurine stretch, (*iii*) remnants of sequences similar to those present in the center, and (*iv*) unique viroid sequences. Of these four regions, *i* and *ii* are clearly defined (boxed areas in Fig. 2), whereas borders between *iii* and *iv* cannot be accurately defined.

What could be the significance of such an unusual RNA structure? *A priori* three explanations are possible: (*i*) similarities between viroids and transposable elements could be fortuitous; (*ii*) the similarities could be a consequence of convergence because of similar function; or (*iii*) they could be due to descent from a common ancestor.

Could viroids represent the functional equivalent of moveable genetic elements, that is, RNA elements with the capacity to integrate into cellular RNA? No evidence for RNA integra-

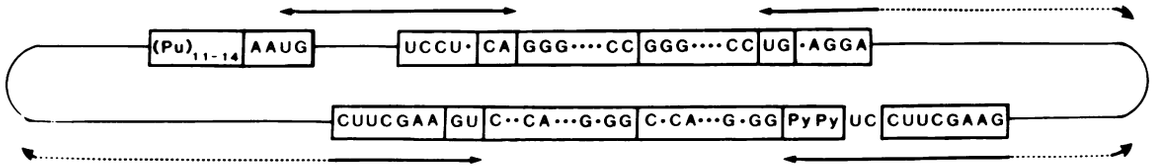

FIG. 3. Diagram of the structural relationships between viroids and moveable genetic elements. Pu, purine; Py, pyrimidine. Boxed areas contain the direct repeats (G-G-G···C-C and G-G-·G···A-C-·C) and adjacent dinucleotides (C-A, U-G, Py-Py) of the central conserved region, flanking inverted repeats (U-C-C-U···A-G-G-A and G-A-A-G-C-U-U-C···A-A-G-C-U-U-C), and the polypurine region [$(Pu)_{11-14}$]. Arrows indicate remnants of LTRs whose interior sequences have been deleted.

tion mechanisms exists, but a speculative scheme has been suggested recently (33). This scheme postulates that eukaryotic cells contain a system of genetic elements consisting of two interacting classes of RNA molecules: (*i*) mobile, circular, viroid-like "signal RNAs" that can be excised from or inserted into other RNAs, and (*ii*) linear "antenna RNAs," which are the targets of the signal RNAs. This system is postulated to exercise genetic control during development, and the RNAs involved are thought to be capable of evolving into viroids and RNA viruses (33). Only future work will determine whether such mechanisms exist. If they do, the transposable element-like structural features of viroids might be required for integration of the viroid into antenna RNAs.

The similarity of viroids to moveable genetic elements is more likely to be a consequence of descent from a common ancestor, and it is not difficult to visualize mechanisms by which viroid-like structures might have evolved from moveable genetic elements. The initial steps of viroid evolution could have been similar to those postulated by Temin for the evolution of retroviruses from moveable genetic elements (30). As with retroviruses, a small moveable genetic element that contained (or generated) control sequences for transcription may have been the ancestor of viroids. Two or more such elements might have become integrated side-by-side into DNA.

In contrast to retroviruses, which are postulated to have evolved by the incorporation of host genes into transposable elements (30), viroids could have evolved by deletion of most of the interior regions of the elements, as well as of intervening host sequences.

If the elements contained short direct repeats flanking the inside of LTR-like sequences (as is the case, for example, in TY*1* and certain retroviral proviruses, such as that of spleen necrosis virus), deletion of element sequences between the direct repeats would bring these next to each other, as is observed in the central viroid region. Such a derivation of the direct repeats would explain why these are flanked by sequences resembling the ends of LTRs. Deletion of internal portions of several such elements integrated side-by-side would leave structures mostly composed of the inverted and direct repeats of the original elements.

Other similarities between viroids and retroviral proviruses are evident. The long uninterrupted stretch of purines present in all five viroids is reminiscent of the putative reverse transcriptase primer sequence for (+) strand DNA synthesis located at the U3 boundary of proviruses (31). Because sequences immediately to the right of the polypurine stretch resemble those observed at the U3 boundary of proviruses, sequences adjoining and including the polypurine stretch may represent remnants of a retroviral provirus. Indeed, the purine stretch in CSV is flanked on the 3′ side by A-A-U-G, as is the case with proviruses (31). These structural similarities suggest that viroid evolution may have involved reverse transcription and integration into DNA. Evolution from retroviral proviruses could readily account for initial transcription of the ancestral viroid.

Because present-day viroids replicate autonomously, they are presumably no longer required to pass through a DNA stage. Indeed, host cell DNA does not contain sequences complementary to PSTV (34–36). In order to replicate autonomously, viroids must contain a recognition site for a host RNA-dependent RNA polymerase. It is conceivable that a primer site for reverse transcriptase (the long purine tract or possibly the short direct repeats of the central conserved region) has evolved into such a recognition site.

If viroids no longer pass through an integrated DNA stage, conservation of sequences required for integration of the postulated ancestral elements into DNA could be due to a new set of selective pressures. Retention of the inverted and direct repeats coupled with deletion (splicing out) of the interior, unique sequences from the moveable elements would result in extensive intramolecular complementarity. This would permit formation of the highly base-paired secondary structures typical of viroids and required for their stability.

Clearly, other interpretations of the remarkable similarities between viroids and transposable genetic elements are possible. For example, resemblance of the long purine tract in each viroid to a reverse transcriptase primer sequence may be fortuitous, in which case functional comparisons with retroviral proviruses would be less plausible. Even so, reverse information flow could have occurred during viroid evolution. One possibility would involve self-primed reverse transcription of an ancestral viroid by mechanisms similar to those postulated to occur with certain small nuclear RNA pseudogenes (37).

Regardless of the exact interpretation one favors, deletion of unique interior regions of putative transposable elements would have to be a prominent feature of any hypothetical scheme postulated. Conceptually, such a deletion process resembles the splicing out of intervening sequences (introns) that occurs in eukaryotic cells during RNA maturation (processing), and it is conceivable that structural requirements of the two processes are similar. In this sense, if not in the sense previously suggested (8, 38, 39), there may be evolutionary or functional links between viroids and introns.

Splicing processes may also be essential features of present-day viroid replication. Synthesis of replicative intermediates containing oligomeric viroid complements (40, 41) and probably oligomeric progeny viroids‡ is believed to involve a rolling-circle mechanism. If so, final steps in viroid replication would entail specific cleavage of oligomeric viroid forms into molecules of monomeric length, followed by ligation of their ends to form covalently closed circular molecules. Structural requirements of all these splicing processes could be similar and might involve some of the conspicuous remnants of inverted repeats flanked by direct repeats present in each viroid. Most full-length linear PSTV molecules isolated from infected plants start with either nucleotide 182 or nucleotide 349 (42) (positions 222 and 423 in Fig. 2). Interestingly, both of these presumed

‡ Branch, A. D., Willis, K. K., Odell, J. T. & Robertson, H. D., Fourth International Conference on Comparative Virology, Oct. 17–22, 1982, Banff, AB, Canada, p. 196 (abstr.).

splicing/joining sites fall within remnants of inverted repeats ending in U-G···C-A that are flanked by imperfect direct repeats.

Although we have mentioned only viroids of the PSTV group, these include all but two of the known viroids, CCCV and ASBV. CCCV displays pertinent features of viroids of the PSTV group, including the conserved central region and a (somewhat shorter) polypurine stretch (6). Furthermore, both the upper and lower central regions are flanked by near-perfect inverted repeats (6/6 and 6/7 bases, respectively) ending in C-A and U-G. However, in contrast to the viroids of the PSTV group, the central regions of CCCV are flanked by U-G at their 5′ ends and C-A at their 3′ ends. ASBV greatly differs from the other viroids (5) and does not appear to fit our model.

The evolutionary implications of similarities between viroids and transposable genetic elements cannot be tested directly. *In vitro* mutagenesis of infectious cloned viroid cDNAs (43) and construction of chimeric cDNAs containing specific regions from different viroids will permit some of the functional consequences of our model to be tested.

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