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Domains in viroids: Evidence of intermolecular RNA rearrangements and their contribution to viroid evolution

(coconut cadang cadang viroid partial duplication/RNA viruses/defective interfering RNAs/discontinuous transcription)

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ABSTRACT **On the basis of sequence homology a model is proposed for five structural and functional domains in viroids. These domains include (*i*) a conserved central region capable of forming two alternative structures that may regulate two phases of the viroid replication cycle, (*ii*) a region associated with pathogenicity, (*iii*) a domain with high sequence variability, (*iv* and *v*) two terminal domains that are interchangeable between viroids. That the evolution of viroids has involved RNA rearrangements of domains is supported by the partial duplication of coconut cadang cadang viroid, which arises *de novo* during each infection. Similar RNA rearrangements have been established for animal viral defective interfering RNAs, which arise by some form of discontinuous transcription. This mechanism could account for the origin of viroids and also RNA viruses, whereby modules of genetic information may have undergone repeated exchange between RNA pathogens and the RNA of their hosts.**

Viroids, the smallest known class of autonomously replicating pathogens, are structurally among the best characterized RNA molecules (1, 2). Functionally, however, these single-stranded, circular RNA molecules of 246 to 375 residues remain an enigma. They apparently rely entirely on host factors for their replication, which proceeds via greater than unit length plus and minus RNA intermediates (3–8), since there is no evidence of any mRNA activity (9, 10). Consequently, all required enzymic functions, including the ability to amplify an RNA template, must potentially exist in the uninfected hosts. At present, these include only the higher plants, where viroids cause several economically important diseases (9, 10).

Eight viroid species and more than thirty variants have been sequenced so far (Table 1). Sequence and structural similarities that have been previously reported include a rod-like secondary structure with characteristic thermodynamic properties (1, 2), a conserved central region (9, 10, 12–14, 16), and an oligopurine tract about 25–50 residues 5′ of this homologous region (10, 12, 21). Only two specific regions have been associated with function. One is a region responsible for variation in pathogenicity of PSTV isolates (19, 20, 22, 23). The second involves the *in vitro*, nonenzymic processing of an ASBV dimeric transcript between residues 55 and 56 (unpublished data).

To understand further how these small RNA molecules are able to manipulate host functions by using only sequence and structural signals, we have developed a model of viroid domains in which function is correlated with five structurally distinguishable regions (Fig. 1). This model not only provides a more rational basis for mutagenic studies of infectious viroid cDNA recombinant clones (refs. 24–26; unpublished data) but also indicates that the evolution of viroids has involved intermolecular rearrangements of viroid sequences. This model can accommodate all viroids except ASBV, because it lacks significant sequence homology with other viroids. Subsequently in this paper the phrase "all viroids" omits ASBV.

Table 1. Viroids and their isolates whose nucleotide sequences have been determined

Viroid	No. of residues	Refs.
Avocado sunblotch viroid (ASBV)	247	11
Chrysanthemum stunt viroid (CSV)	356, 354	12, 13
Citrus exocortis viroid (CEV)	370, 371, 372, 374, 375	13–15, 48
Coconut cadang cadang viroid (CCCV)*	246, 247	16
Hop stunt viroid (HSV) (cucumber pale fruit viroid)	297, 303	17, 18
Potato spindle tuber viroid (PSTV)	359	10, 19, 20
Tomato apical stunt viroid (TASV)	360	21
Tomato planto macho viroid (TPMV)	360	21

We define a viroid isolate as one whose components share greater than 90% sequence homology with a previously published viroid sequence. Cucumber pale fruit viroid, therefore, is an isolate of HSV since it shares 95% sequence homology with HSV (18).
*CCCV infections produce four major RNA components, all derived from an infectious monomeric small form (D-0) of 246 or 247 residues. These include monomers and dimers of both the D-0 form and any one of a set of larger forms (D-41, D-50, or D-55) which contains a duplication involving 41, 50, or 55 residues.

Domains of Viroids: The Model. The five structural domains are depicted schematically in Fig. 1 and more specifically located in Fig. 2. They are summarized together with their hypothesized functions as follows:

C domain. This conserved central core is centered at the strictly conserved bulged helix

$$\begin{array}{l} CC\ \ CCGG \\ GG_{U}GGCC \end{array}$$

It may represent an important control region in viroid replication and signal functional changes through structural alterations.

P domain. This region is associated with pathogenicity (19,

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Abbreviations: See Table 1 for abbreviations of viroids; SCMoV, subterranean clover mottle virus; DI RNA, defective interfering RNA.

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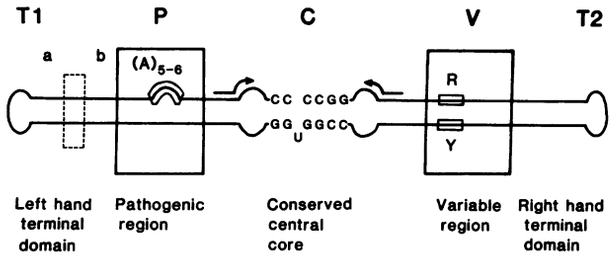


FIG. 1. Model of five viroid domains (T1, P, C, V, and T2) determined from sequence homologies between viroids. The arrows depict an inverted repeat, which can potentially form a nine-base-pair stem (Fig. 3). a and b are segments of the T1 domain that flank a region of RNA exchange (dashed box). R and Y indicate a short oligopurine·oligopyrimidine helix.

20, 22, 23) and is characterized by an $(A)_{5-6}$ sequence present in all viroids.

V domain. This region shows the greatest variability between closely related viroids and may be associated with pathogenicity. More interestingly, it may be involved in RNA rearrangements such as that found for the partial duplication in CCCV (16).

T domains. The terminal regions are considered to have undergone intermolecular RNA exchange between viroids. Although the functional role of these domains is unclear, the evidence for these exchanges suggests a role for RNA rearrangements in the origin and evolution of viroids.

Control Function of the Conserved Central Domain C. In addition to the conserved bulged helix (Fig. 1), an alternative structure is possible (Fig. 3). This stem–loop (1, 2) is deduced from temperature-jump experiments to monitor the thermal denaturation of PSTV, CEV, CSV, and CCCV (1, 2). By such a scheme, the highly conserved CCCCGGGG sequence would form part of the loop atop a nine-base-pair stem (Fig. 3), preventing the conserved bulged helix from forming. Despite many sequence differences, the same nine-base-pair stem can be formed for all viroids, except for a single non-base-paired C residue in HSV.

It is proposed that both mutually exclusive structures are

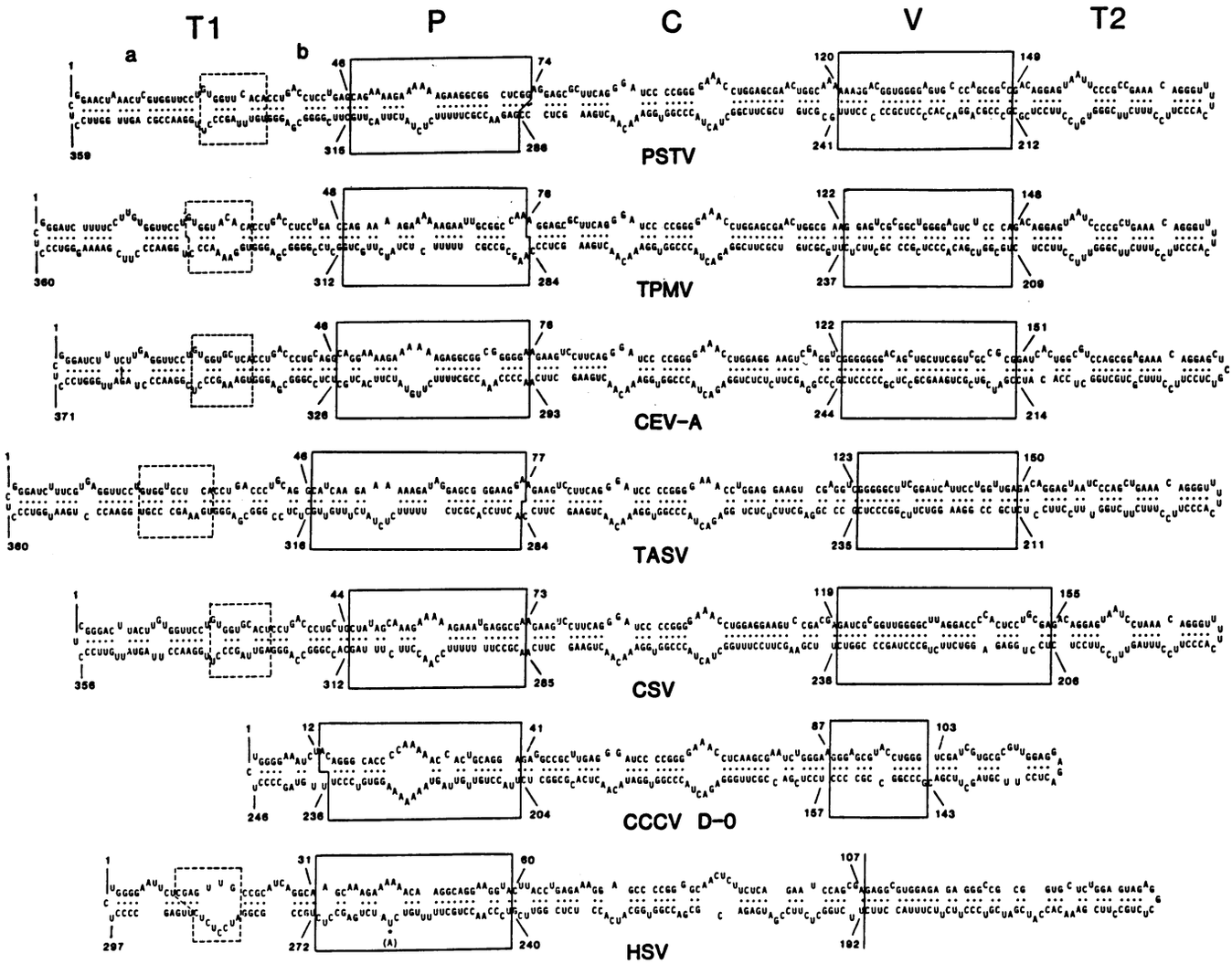


FIG. 2. Domain boundaries for each viroid. In pairwise sequence comparisons of viroids containing highly homologous C domains (e.g., between PSTV, TPMV, and CCCV or between CEV-A, TASV, and CSV) there is significantly less sequence homology in the P and V regions, occurring about 5–9 residues 5′ and about 7–15 residues 3′ of the inverted repeat (Fig. 1). Similarly, in comparisons of PSTV, TPMV, TASV, and CSV, a change from low homology in the V domain to high homology in the T2 domain defines the boundary for these two domains. However, the boundary of the V and T2 domains of HSV cannot be determined due to the present lack of homologous viroid sequences. Therefore, only the boundary between the C and V domains of HSV is given. The P domain, with a conserved oligo(A) sequence flanked by regions with greater variability, has its borders based on homologies between the P region of HSV and other viroids such as PSTV and by certain pairwise comparisons such as CEV-A and TASV in which there is significant change from relatively low sequence homology in the P region to higher homology in the adjacent T1 and C domains. *, Sequence heterogeneity in HSV (17).

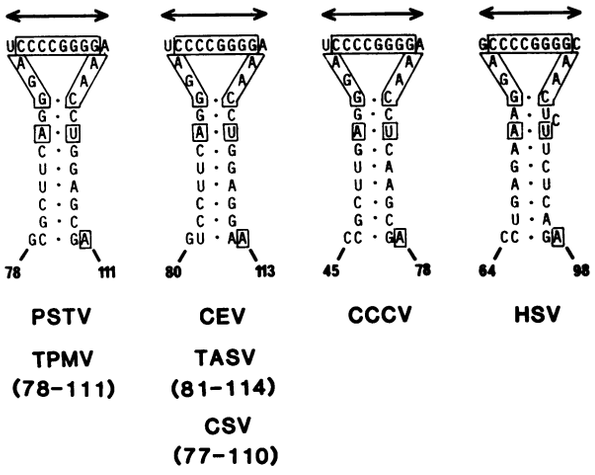


FIG. 3. Alternative secondary structure in the central conserved region. Residue numbers for various viroids are given on structures or in parentheses. A nine-base-pair stem common to all viroids, except ASBV, and a 10-residue self-complementary sequence (↔) in the loop are depicted. The CCCCGG sequence in the proposed loop corresponds to one strand of the strictly conserved bulged helix (Figs. 1 and 2), which would thus be disrupted by the formation of this nine-base-pair stem–loop structure. The boxed residues are conserved in all viroids.

important in viroid function and that the structural switching from the putative native form (1, 2) to the nine-base-pair stem structure controls a switching in function for this region. For example, the strictly conserved bulged helix (Fig. 1) resembles several protein binding sites (27), in particular the ribosomal protein L18 binding site of 5S RNAs (27), and may signal RNA polymerase binding and/or initiation of the synthesis of the minus strand. Thus, when the greater-than-unit-length plus strands are synthesized (3–8), the nine-base-pair stem may then arise transiently, either during transcription or by *inter*molecular base pairing of the 10-residue self-complementary loop $\overset{U}{G}CCCCGGGG\overset{A}{C}$ (Fig. 3). This could induce an alternative function not signalled in the native structure, such as the processing of the multimeric product to the monomeric species.

Association of Pathogenicity with the P Domain. Different isolates of PSTV that vary by a few residues in the P domain can be correlated with changes between mild, intermediate, severe, and lethal symptoms (20). Further support for the association of pathogenicity with this domain comes from studies on CEV isolates (ref. 15; unpublished data). One isolate (CEV-A) produces severe stunting and epinasty on tomato, whereas another isolate (CEV-DE26) elicits only mild symptoms. The two isolates differ by 27 residues, restricted mainly to the P and V domains.

Sequence Variability of the V Domain. This is the most variable region and shows less than 50% sequence homology between otherwise closely related viroids, such as between TASV and CEV-A or TPMV and PSTV (Table 2). Similarly, different sequence isolates of CEV show considerable variation in this region (ref. 15; unpublished data). This may reflect an involvement in pathogenicity but, unlike the P domain, no viroid isolates have yet been found that have sequence differences restricted to the V domain corresponding to differences in symptom expression. The only significant sequence relationship between viroids in the V region appears to be the presence of an oligopurine·oligopyrimidine helix, usually with a minimum of three G·C base pairs (Figs. 1 and 2).

RNA Rearrangements of the T Domains. Indirect evidence has attributed possible functions to these domains, such as the specific binding in both of these regions by purified tomato DNA-dependent RNA polymerase II, a postulated viroid replicase (28). These domains include the termini of PSTV linears as indicated by the presence, in both, of residues with 2′,3′-cyclic phosphates (29).

Table 2. Sequence homology between domains of different viroids

Viroids used for pairwise comparison		Sequence homology, %					
		Domains					
1	2	T1	P	C	V	T2	Overall
TASV	CEV-A	91	54	99	49	46	73
	PSTV	67	59	65	30	90	64
TPMV	PSTV	67	73	94	42	95	76
	CEV-A	80	70	69	29	37	60
CCCV D-0	PSTV	25	14	70	37	27	38
	HSV	52	33	42	ID	ID	39
HSV	PSTV	23	58	35	ID	ID	35
CSV	CEV-A	77	42	82	28	38	59
	PSTV	69	49	71	31	81	61
CEV-A	PSTV	62	71	65	31	38	55

Sequence homology was determined by the best alignment, allowing for additions and deletions, but constrained by the requirement of a match consisting of a minimum of three consecutive residues. Percent sequence homology = (number of matching residues in both sequences ÷ total number of residues compared) × 100. ID, insufficient data.

In addition, sequence data show that three viroids (TASV, TPMV, and CCCV) exhibit unusual relationships with respect to their terminal sequences. For example, TASV shares 73% overall sequence homology with CEV-A but the T2 domains are only 46% homologous (Table 2). In contrast, TASV shares less overall sequence homology with PSTV (64%) but the T2 domains are highly homologous (90%). Therefore, TASV appears to be a recombinant between the T2 domain of a PSTV-like viroid and all but the T2 domain of a CEV-like viroid. TPMV shares 76% overall sequence homology with PSTV but the T1 domains are less homolo-

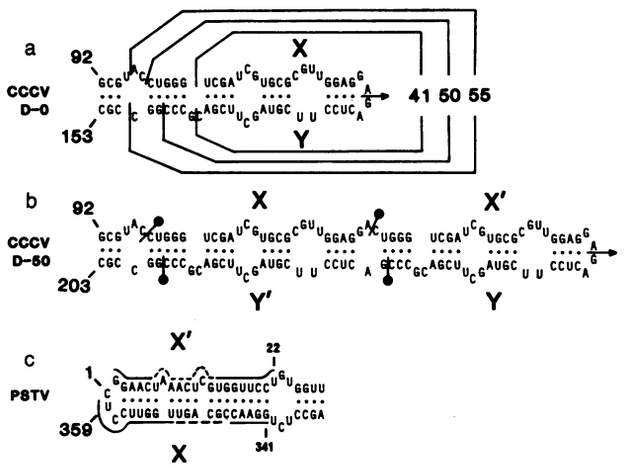


FIG. 4. Viroid sequence duplications (16). (*a*) Sequences involved in the partial duplication of the small form of CCCV (D-0) in which two adjacent sequences (X and Y) of variable size are duplicated, leading to a double duplication of 41, 50, or 55 residues (D-41, D-50, D-55). The arrow depicts the boundary of the X and Y sequences. (*b*) CCCV D-50 duplication, showing conservation of the rodlike secondary structure. The filled-in circles mark the boundaries of the duplicated sequences. (*c*) Possible duplication of section a of the PSTV T1 domain (Fig. 2) in which 13 residues (continuous line) out of 19 in the X segment (residues 341–359) are repeated in the adjacent X′ segment (residues 1–22). Dashed lines indicate nonhomologous residues.

gous (67%). In contrast, TPMV shares less overall sequence homology with CEV-A (60%) but the T1 domains are more homologous (80%). Thus, TPMV appears to be a recombinant between the T1 domain of a CEV-like viroid and all but the T1 domain of a PSTV-like viroid. Finally, CCCV D-0 (Fig. 2) shares 70% sequence homology with the C domain of PSTV but only 23% sequence homology with respect to the T1 domains. In contrast, CCCV D-0 shares low homology with the C domain of HSV (42%), but the T1 domains are more homologous (58%) since the T1 domain of CCCV D-0 is almost identical to section a of the HSV T1 domain (Fig. 2). Therefore, CCCV appears to be a recombinant between a viroid with a PSTV lineage and the T1 domain of a HSV-like viroid.

These sequence relationships are difficult to explain by mutation from a common ancestor. A more feasible hypothesis is that viroid evolution has involved intermolecular recombination between distinct viroid species co-infecting a common host such as tomato (9, 21), leading to RNA exchanges of the T domains. Whereas the RNA exchange involving the 5′ and 3′ residues of the T2 domain of the common ancestor of TASV and CEV presumably occurred at positions in the V domain, RNA rearrangements of the T1 domain appear to involve two regions. These include the P domain and the dashed box depicted in Figs. 1 and 2. This is suggested by related regions of CCCV and HSV, in which section b of CCCV appears to have been deleted (Fig. 2), and by a possible duplication in section a of PSTV (Figs. 2 and 4*c*), where two adjacent sequences of about 20 residues, which border on the dashed box area, have 13 residues in common.

Whereas these above examples of RNA exchange remain hypothetical, the distinctive partial duplications of CCCV (Fig. 4) determined by Haseloff *et al.* (16) provide more direct evidence of RNA rearrangements in viroids. During infection and replication of the CCCV monomeric small form (D-0, Figs. 2 and 4), a second, larger, form of the viroid arises and dominates (30); in it the T2 region and part of the V domain are duplicated (Fig. 4). Three forms (D-41, D-50, and D-55) have been characterized so far in which 41, 50, or 55 residues are duplicated, with only one of these forms found in each infected coconut palm (16).

These duplications in fact are double duplications of the form X,Y → XX,YY, in which X = 21, 26, or 28 residues and Y = 20, 24, or 27 residues. They are found in only three of the nine possible combinations of X and Y: 21 and 20 (CCCV D-41), 26 and 24 (CCCV D-50), and 28 and 27 (CCCV D-55), probably because these three combinations least disrupt the native secondary structure (Fig. 4). Since these exchanges occur at different points in the V domain it may reflect either specific structural signals or merely the low functional constraints that exist in this domain.

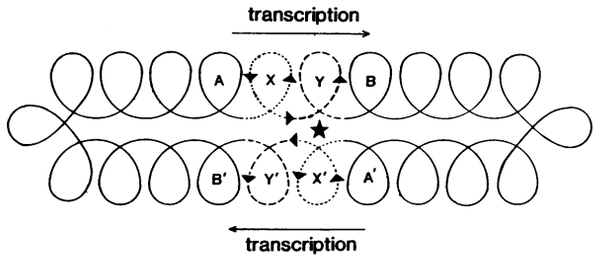
Intermolecular RNA rearrangements have been previously reported for animal viruses such as polio virus (31), foot-and-mouth disease virus (32), and the defective interfering (DI) RNAs of Sindbis virus (33) and influenza virus (34). In the case of plant viruses, the two circular satellites (virusoids) of subterranean clover mottle virus [SCMoV (35)] provide possible evidence of an RNA exchange. Isolates of SCMoV contain either one or two virusoids of 332 and 388 residues in a circular, single-stranded, rodlike structure similar to viroids (ref. 8; unpublished data). The left-hand 218 residues of each virusoid show 95% sequence homology but the rest of each molecule shows only 25% homology (8). All of these examples, together with the extensive intramolecular rearrangements observed for DI RNAs (36) and the CCCV partial duplications, may indicate a general feature of all RNA viruses.

Mechanism of RNA Rearrangements in Viroids. RNA rearrangements may occur either by recombination involving strand scission and ligation or by discontinuous transcription. The former mechanism requires precise sequence or structural signals such as for the processing of mRNA introns by alignment with U1 small nuclear RNA (37) or as in the case of the nonenzymic splicing of *Tetrahymena* precursor rRNA (38). The latter mechanism of discontinuous transcription requires reinitiation of a nascent transcript elsewhere on either a DNA template as suggested for trypanosome variant surface antigen genes (39) or an RNA template as postulated for animal viral DI RNAs (36).

Discontinuous transcription can be readily applied to viroid replication. By analogy with influenza ribonucleoprotein, which is postulated as the template for the generation of influenza DI RNAs (34), the greater-than-unit-length plus or minus RNAs observed for viroid infections (3–8) may occur *in vivo* as helical coils surrounding protein cores. This template could be used for transcription, whereby discontinuous transcription of a jumping RNA polymerase could account more readily than RNA recombination for all postulated viroid rearrangements (Fig. 5). These include (*i*) nonhomologous intermolecular rearrangements as demonstrated by TASV and TPMV, (*ii*) different types of deletions and duplications such as the complex CCCV double duplication that appears to be linked to CCCV replication (16) or a possible simple duplication as in PSTV (Fig. 4*c*), and (*iii*) variability in the site of rearrangement such as the varying crossover points in the V domain of the CCCV partial duplications (Fig. 4 *a* and *b*).

Consequences of the Viroid Domain Model. As yet, we do not know the functional significance of a single residue in viroids. Nevertheless, the extensive sequence and physical data have yielded a model of viroid domains with several important functional implications.

(*i*) Despite the small size and the apparent lack of protein coding capacity of viroids, they appear to be multigenic, with functions corresponding to structurally distinct, interchangeable domains. Due to the lack of mRNA capacity (9, 10), these functions must be directly related to the sequence and secondary structure. Local tertiary interactions may also prove significant.



★ Region of discontinuous transcription by a jumping RNA polymerase

Example 1. CCCV D-50

$$-A \rightarrow -AX \overset{\star}{\rightarrow} -AXX' \overset{\star}{\rightarrow} -AXX'Y \overset{\star}{\rightarrow} -AXX'YY' \rightarrow -AXX'YY'B'$$

Example 2. PSTV

$$-A \rightarrow -AX \overset{\star}{\rightarrow} -AXX' \rightarrow -AXX'Y' \rightarrow -AXX'Y'B'$$

FIG. 5. Proposed mechanism for the generation of RNA rearrangements by discontinuous transcription. Boundaries of RNA exchange are juxtaposed as part of a nucleoprotein replicating complex; the scheme is adapted from Jennings *et al.* (34). Viroid transcription occurs on a helical dimeric RNA template, whereby RNA polymerase jumping in one region may lead to a variety of RNA rearrangements. The complex double duplication that occurs in CCCV D-50 (Fig. 4*b*) could arise by a triple jump in this region (example 1). Two jumps (X → XX′Y′ → XX′Y′Y) in different regions could also give the same result. The postulated PSTV partial duplication (Fig. 4*c*) would require only a single jump (example 2). The star in each of these two examples represents a single jump; letters refer to segments of viroid sequences.

(*ii*) The model will allow a rational approach to *in vitro* mutagenesis studies on viroids. Changes in the V domain may be expected to least affect successful viroid infection but may lead to altered pathogenicity or an altered pattern of replicative intermediates. Pathogenicity could be tested by mutations in the P domain and thus provide data on the interface with host factors whereby viroids, and possibly other viruses, exert their pathogenic effects. Changes in the central region may be accommodated only if compensatory changes are made elsewhere such that the stem formed in the postulated structure of Fig. 3 is maintained. Furthermore, chimeric viroids generated by the exchange of T domains may be viable, while coinfection of two viroids could give rise to novel species.

(*iii*) Despite the lack of obvious sequence homology with viroids, other RNA species such as ASBV, the satellite RNA of tobacco ringspot virus (40), and the virusoids of velvet tobacco mottle virus (41, 42), solanum nodiflorum mottle virus (41, 42), lucerne transient streak virus (43, 44), and SCMoV (8, 35) may share functional homology with the viroids described here. For example, the domain model can be extended to these latter RNAs, which all share a conserved region analogous to the C domain of viroids (unpublished data). Furthermore, as already considered, the virusoids of SCMoV show evidence of a terminal domain exchange similar to TASV and TPMV.

(*iv*) Intermolecular rearrangements have been determined for a number of animal viruses, including influenza (34) and a DI RNA from Sindbis virus, which has a covalently attached cellular tRNA at its 5′ end (33). On the basis of sequence homologies, RNA exchange has been postulated for the evolution of several plant viruses, including alfalfa mosaic virus, brome mosaic virus, and tobacco mosaic virus (45), the virusoids of SCMoV (ref. 8; unpublished data) and now viroids. These examples suggest the possibility of rapid cross species exchange of genetic elements as first proposed for DNA bacteriophages (46) and more recently for RNA viruses (47). In the case of RNA pathogens, one mechanism can account for all rearrangements, namely, some form of discontinuous transcription involving a jumping RNA polymerase, which is presently the favored mechanism for explaining the origin of animal viral DI RNAs (36). Thus, RNA rearrangements could account for a diverse origin of viroids that may have involved multiple exchanges from RNA viruses, satellite or virusoid RNAs, or even host RNAs.

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