Viroids and virusoids are related to group I introns

(*Tetrahymena* intervening sequence/potato spindle tuber viroid/mitochondrial conserved sequences/group II introns)

GAIL DINTER-GOTTLIEB

Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309

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ABSTRACT Group I introns are found in nuclear rRNA genes, mitochondrial mRNA and rRNA genes, and chloroplast tRNA genes. The hallmarks of this intron class are a 16-nucleotide consensus sequence and three sets of complementary sequences. The viroids (circular pathogenic plant RNAs) and the virusoids (plant satellite RNAs) also contain the consensus sequence and the three sets of complementary bases. Pairing of the complementary bases would generate a viroid structure resembling a group I intron, which might be stabilized *in vivo* through interactions with proteins. The *Tetrahymena* self-splicing rRNA intron further has sequences homologous with regions of potato spindle tuber viroid associated with the severity of viroid symptoms.

The coding regions of many eukaryotic genes are interrupted by apparently extraneous segments of DNA, termed *introns*. In order for the gene to be properly expressed, the introns must be removed from the RNA transcripts and the residual exons must be ligated, a reaction known as RNA splicing. After excision, the intron may exist in a lariat structure (1, 2), as a linear molecule (3-5), or as a circular RNA (5, 6).

One intron class, termed group I, is recognized by conserved sequence and structural features and is found among mitochondrial messenger and ribosomal RNA genes, chloroplast transfer RNA genes, and nuclear ribosomal RNA genes, a surprisingly broad distribution (3, 7-14). The hallmark of this intron class is a 16-nucleotide phylogenetically conserved sequence (3), the group I consensus sequence (Fig. 1 *Upper*). Twelve bases of this sequence, termed box 9L, form a region of *cis*-dominant mutations in the splicing of the yeast mitochondrial cytochrome *b* (*cob*) intron 4. Elsewhere in the intron is box 2, which is complementary to box 9L. Another pair of sequence elements, called box A and box B (or box P and box Q), is less conserved as to sequence but is located upstream of box 9L and is rich in G+C (13). Although no splicing-defective mutants have yet been found *in vivo*, mutations introduced into this region in the *Tetrahymena* intervening sequence cause defective splicing of the RNA transcript (13). A third pair of sequences, box 9R and box 9R', although not conserved as to sequence, are conserved in location. Box 9R is located just downstream of 9L, and 9R' is just upstream of A. The ability of 9R and 9R' to base pair has been implicated in yeast *cob* mRNA splicing, since a point mutation at the base of the helix destroys splicing, while a double mutation, restoring pairing, also restores splicing of the intron (14).

The pairing of the complementary boxes, and the order in which they occur within the intron, 9R', A, B, 9L, 9R, and 2, force the RNA into a characteristic structure (Fig. 1 *Upper*). In this case, the box 9L-box 2 pairing would be at the level of tertiary structure.

The phenomenon of self-splicing *in vitro* has been described for several group I introns, the nuclear ribosomal

RNA intron of *Tetrahymena thermophila* (15-17), *Neurospora* mitochondrial *cob* intron 1 (4), and the large ribosomal RNA and two messenger RNA introns from yeast mitochondria (18, 19). No enzymes or other proteins are required for these reactions *in vitro*, the sole requirements being GTP and a divalent cation, usually magnesium, and in some cases monovalent cations or polyamines. A series of transesterification reactions occurs, first as guanosine becomes covalently bonded to the 5' end of the excised intron, then exon ligation, and the 3' hydroxyl of the intron's 3'-terminal guanosine attacks a specific bond 4 to 19 bases from the 5' end of the intron, thus creating a covalently closed circle and releasing a short oligonucleotide. Both the *Tetrahymena* and the yeast ribosomal RNA introns cyclize, but no circle form has been found for the *Neurospora* intron. On the basis of similarities in intron structure, it seems reasonable that all group I introns splice by a similar mechanism (20). In some cases, however, proteins may be necessary for stabilizing the structure and maintaining the base pairing of the boxes.

Since viroids are single-stranded circular RNAs, similar in size to some of the circular introns, comparisons have been drawn between the two types of molecules. Diener (21) proposed an intron origin for viroids, and subsequently, noting the similarities between the negative strand of the viroid and the U1 small nuclear ribonucleoprotein particle, proposed that the viroids might be escaped introns (22). Other homologies with the positive and negative strand viroids were noted by Dickson (23). As additional viroids have been sequenced, further comparisons between the viroids and the various intron types have become possible.

RESULTS

A search of the viroid sequences revealed that the 16-nucleotide group I consensus sequence is present in all of the viroids.* In fact, this sequence represents the lower portion of the "central conserved region" of the viroids (24). This region is centrally located when the structure is written in the canonical rod form (ref. 25; Fig. 2 *Upper*). As shown in Fig. 2 *Lower*, the sequence is present even in viroids such as CCCV, which has only 11% sequence homology with the PSTV group (24). Surprisingly, it is found in virusoids as well.

Virusoids are covalently closed, single-stranded, circular RNA molecules of 300-400 nucleotides, which are found encapsidated with other, much larger, single-stranded, linear RNAs in some viruses. The RNA of such a virus is termed RNA 1, while the accompanying satellite RNA is RNA 2. Two of the virusoids that have been sequenced, velvet tobacco mottle virus RNA 2 (= VTMoV) and *Solanum*

Abbreviations: PSTV, potato spindle tuber viroid; CCCV, coconut cadang-cadang viroid; CEV, citrus exocortis viroid; TASV, tomato apical stunt viroid; TPMV, tomato planta macho viroid; VTMoV, velvet tobacco mottle virusoid; SNMV, *Solanum nodiflorum* mottle virusoid.

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FIG. 1. (Upper) Structure of a group I intron (exemplified by the self-splicing intron of *Tetrahymena*) generated by pairing box 9R with box 9R' and box A with box B in the secondary structure, and box 9L with box 2 at the level of tertiary structure [after Michel and Dujon (7)]. On the right, 16N indicates 16 nucleotides not shown. (Lower) Potato spindle tuber viroid (PSTV) structure derived from pairing the conserved sequence elements that are also found in group I introns.

nodiflorum mottle virus RNA 2 (= SNMV) (26) contain the group I consensus sequence and box sequences (Fig. 2 Lower), although the virusoids have little sequence homology with the viroids.

The conserved sequence elements of group I introns also occur in the viroids.* Box 9L, a portion of the consensus sequence, is present in all viroids examined, although there is one base difference in all viroids except for TASV and TPMV. The initial G is instead a U or A. A box 2 region has been located, and in some cases it contains a single changed base such that its ability to form five base pairs with box 9L is preserved (Fig. 2 Lower). Such compensatory base changes provide evidence that sequence elements are paired in folded RNA structures (27). Boxes 9R and 9R' are also present, as well as the G+C-rich boxes A and B. Significantly, the 5'-to-3' order of the sequence elements, 9R', A, B, 9L, 9R, and 2 is the same as in the group I introns.

This order is important, because the pairing of the sequence elements determines the structure of the group I intron. In the model shown in Fig. 1 Upper, the secondary structure involves pairing 9R-9R' and A-B, while 9L and 2 would pair at the tertiary level. When the boxes in PSTV are

paired, a structure is generated in the viroid which is strikingly similar to that of group I introns in the region of the boxes (Fig. 1 Lower). The calculated free energy for folding of this molecule, approximately -100 kcal/mol ($1 \text{ cal} = 4.184 \text{ J}$), is not nearly as favorable as that calculated for the rod structure, -209.8 kcal/mol (M. Zuker, personal communication), yet it is possible that a structure such as this might be stabilized by proteins *in vivo*.

All group I introns terminate in a guanosine in the region following box 2. In the circular form of the *T. thermophila* intron, this guanosine is located between box 2 and box 9R'. Recently Visvader *et al.* (28) determined the site of processing of a cloned monomer of CEV and concluded that the three processing sites all followed a guanosine residue in the region corresponding to the viroid central conserved region (CC-CCGGG), which lies between box 2 and box 9R' (in the "D stem" of Fig. 1 Lower).

The homologies between group I introns and viroids prompted a search for other similarities with the self-splicing intron of *T. thermophila*. The two circular RNAs are similar in size: PSTV is 359 bases in length, and the *Tetrahymena* intron is 399 bases in its circular form. The similarities

85	GGA	GAAAC	109
	AG	UCC.CCGGG	CUGGAGC
277	UC	AGGUGGCC	GGCUUCG
	AACAA	AUCAUC	249
	GA	Box 9L	Box 9R
	(CGU)YUCAAACGACUACANG	Box 2	Box 9R'
		Box A	Box B
<i>Tetrahymena</i>	GUUCACAGACUAAUG	GACUA	UGUGGGUC
IVS			UGCGGG
PSTV	CUUCGGCUACUACCCG	CUGAU	ACUGCCAG
		UACUA	GGUGGAAA
CEV	CUCUGGAUACUACCCG	UUGGU	CCACUUU
		UACUA	GGUGGAAA
CSV	CUUUGGCUACUACCCG	UUGGU	CUUCCUCU
		AACUA	GGUGGAAA
TPMV	CUUCGGAGACUACCCG	UUGGU	CCACUUU
		GACUA	GGUGGAAA
TASV	CUCUGGAGACUACCCG	CUAGG	CCACUUU
		GACUA	CCUCCAAA
CCCV	CUUUGGAGACUACCCG	UUGGU	CCACUUU
		GACUA	GGUGGAAA
VTMoV	GUAAAGGUACUACAGA	UUGGU	CUAGUGAU
		UACUA	CACUCC
SNMV	GUAAAGGUACUACAGA	UUGGU	CUAGUGAU
		UACUA	ACUUC

FIG. 2. (Upper) Central conserved region of PSTV as written in the conventional rod form. Dots indicate Watson-Crick or wobble base pairing. Of the 19 nucleotides in positions 90–108, 15 are identical with the D stem of the *Tetrahymena* self-splicing intron. Of the 16 nucleotides in positions 250–265, 12 are identical with the group I consensus sequence, found in all group I introns. (Lower) Sequence alignments. Homology with the group I consensus sequence (topmost sequence; Y, pyrimidine; N, any nucleoside; GA may replace AC) is present in both viroids and virusoids. Underlines indicate identity with the group I consensus. Conserved sequence elements (the "boxes") which pair and delineate a characteristic structure for the group I introns are also found in viroids and virusoids, in the same 5'-to-3' order. IVS, intervening sequence; CEV, citrus exocortis viroid; CSV, chrysanthemum stunt viroid; TPMV, tomato planta macho viroid; TASV, tomato apical stunt viroid; CCCV, coconut cadang-cadang viroid; VTMoV, velvet tobacco mottle virusoid; SNMV, *Solanum nodiflorum* mottle virusoid.

between the two classes of RNAs in their secondary and tertiary structures as group I introns have already been noted. Surprisingly, a strong homology between the D stem of the *Tetrahymena* intron and the viroid central conserved region, 15/19 bases identical, was found (A. Hadidi, personal communication; Fig. 3 Top). This is the portion of the viroid that is shown paired, in the central conserved region of the rod structure, in Fig. 2 Upper. The lower viroid strand in that figure has 12 nucleotides identical with the 16-nucleotide consensus sequence found in all group I introns. Resemblances between this region, movable genetic elements, and retroviral proviruses have been noted (29). It has also been proposed as a signal for initiation, elongation, or termination of replication (30). The virusoids, which contain the 16-nucleotide region, but contain a degenerate D stem sequence (Fig. 3 Top), are unable to replicate without another RNA virus present (26). It is interesting to note, furthermore, that this region in the *Tetrahymena* intron, PSTV, and the plant virus satellite RNA PARN 5 (31) shows homology with the consensus sequence for the conserved stem and loop structure near the 3' end of group II introns (ref. 7; Fig. 3 Middle). Experiments in our laboratory have shown that the D stem portion of the *Tetrahymena* intron can be deleted with little or no effect on the self-splicing activity of the molecule (G.D.-G., L. A. H. Dokken, and T. R. Cech, unpublished results). While not involved in the self-splicing of group I introns, its conservation in the group II introns may indicate

<i>Tetrahymena</i> rRNA intron	AGUCUCAGGGGAAACUUUGAGA
Potato spindle tuber viroid	GAUCCCGGGGAAACUUGAGC
<i>Vicia faba</i> tRNA intron	AGCCUUGGUAUGGAAACAUUAAG
Velvet tobacco mottle virusoid	AGUCCGAAAGGACGAAACGGAUGUA
Group II Consensus	QAGCYGUAUQ Q GAAACU YACGUACGUUY
<i>Tetrahymena</i> d stem	AGUCUCAGGGGAAACUUUGAGA
PSTV	GAUCCCGGGGAAACUUGAGC
PARN 5	CGUC GGGGGGAAACCCCUUG
VTMoV	AAAAGGACGAAACGGAUG
I. PSTV (49–54)	GAAAAG
IVS (27–32)	GAAAAG
II. PSTV (114–122)	UGGCAUAUAG
IVS (179–188)	UGGUAUAUAG
III. PSTV (303–314)	UAUCUUUUUUUG
IVS (10–22)	UAUUUACUUUUG

FIG. 3. (Top) Homologies with a region of the *Tetrahymena* intron termed the D stem are seen in viroids, virusoids, and the two plant chloroplast tRNA group I introns that have been sequenced. Underlines indicate identity with the consensus sequence. (Middle) The D stem region of *Tetrahymena* shows homology with the conserved stem and loop near the 3' end of the group II introns. Q, purine; Y, pyrimidine. Similar, though less well conserved, homologies are found in PSTV and with peanut stunt virus-associated RNA 5 (PARN 5) and VTMoV RNA 2. (Bottom) Homologies of PSTV pathogenicity modulating (PM) regions with the *Tetrahymena* intron (intervening sequence, IVS).

that the D stem has a function in the splicing of this class of introns.

Finally, comparisons were made between the *Tetrahymena* intron and the pathogenicity modulating (PM) regions of PSTV. The differences in symptom severity caused by mild, intermediate, and severe strains of PSTV depend upon the nucleotide sequences in three regions of the molecule (32, 33). These three regions are located at nucleotides 49–54, 114–122, and 303–314 in PSTV. Fig. 3 Bottom reveals homologies between the *Tetrahymena* linear intron and the PM regions of the mild PSTV strain. The region of homology in the intron from nucleotide 10 to nucleotide 22 contains the site of cyclization of the linear molecule (17). Since even minor changes in the sequence of PSTV may eliminate replication and infectivity, it is difficult to ascertain whether these similarities have any significance, but they further reinforce the relationship between the two molecules and emphasize the conservation of significant sequences across species.

DISCUSSION

In summary, the hypothesis that viroids are related to introns has been further supported by numerous sequence and structural homologies between group I introns as a class, and specifically, with the self-splicing intron of *Tetrahymena thermophila*. In fact, on the basis of the crucial sequence similarities, the viroids and virusoids appear to be closely related to group I introns. The question still remains as to whether viroids evolved from introns or whether both evolved from a common ancestor molecule. The consensus sequence of 16 nucleotides appears to be an integral part of the group I intron structure, and its phylogenetic conservation attests to its importance.

Fortunately, it is possible to approach some of the pertinent questions on an experimental level. To begin, can viroids self-cleave and autocyclize?

Intermediates of viroid replication have been detected *in vivo*. Concatemer linear forms of the PSTV complementary (minus) strand have been reported (34, 35), while plus strand concatemers have been detected for CCCV (24), CEV (36), and avocado sunblotch viroid (ASBV) (37), as well as for the virusoids of VTMoV and SNMV, all of which resemble group I introns. On the basis of the presence of minus-strand PSTV linear concatemers, a model for viroid replication has been proposed (38). The circular plus strand would be copied by an unknown plant cell RNA polymerase via a rolling-circle form of replication to form minus-strand linear concatemers. Either these might be copied into plus-strand concatemers that then are cleaved and cyclized by plant enzymes, or the minus-strand concatemer might first be cleaved, then copied into plus-strand monomers and cyclized. Although the enzymes for these reactions have not been identified, an RNA ligase activity from wheat germ can cyclize the natural PSTV linear monomers (39).

Yet linear concatemers can be formed in a nonenzymatic fashion. The linear *Tetrahymena* intron is capable of concatemerization *in vitro*. In this case, the 3' hydroxyl group of the 3'-terminal guanosine attacks the junction between nucleotides 15 and 16 in another linear molecule, covalently attaching, and releasing an oligonucleotide of length 15, in a reaction analogous to the cyclization reaction. Dimer, trimer, and larger linear molecules may be produced, as well as their circle forms. Under cyclization conditions, these will then form monomer circles (40). Cloned viroid transcripts might be assessed for such activity as well. A similar model for minus-strand concatemerization and plus-strand processing for the peanut stunt virus-associated RNA 5 (PARNA 5), which also contains intron-like box sequences, as well as a noncoded 3'-terminal guanosine in the minus strand, has recently been proposed (31). The cloning of PSTV cDNA (41) allows *in vitro* transcription of viroid molecules, and precursor molecules have been isolated and placed under splicing and cyclization conditions, in reactions analogous to those seen for the *Tetrahymena* self-splicing intron (5, 17). Robertson *et al.* have recently reported that a monomer-length PSTV containing a 2',3'-cyclic phosphate was produced under such conditions (42). A similar result has also been reported for satellite tobacco ringspot virus RNA (43). These results, should they be corroborated by *in vivo* findings, would indicate a cleavage and ligation mechanism different from that seen for the *Tetrahymena* intron.

The self-splicing intron of *Tetrahymena* rRNA requires no proteins for splicing or maintaining its structure, but other introns, with less stable base pairing or less complementarity in the boxes, might well require proteins to maintain an active structure. PSTV oligomeric *in vitro* transcripts can be specifically cleaved and circularized when incubated in cell extracts (H. L. Sanger, personal communication). This may be due to the presence of enzymes in the extract or to the formation of stable viroid ribonucleoproteins using cell proteins, and their subsequent autocatalytic splicing. Such a situation appears to occur with the nuclear rRNA intron of *Neurospora crassa*. Although the six boxes are present, they do not pair to give the core structure in deproteinized RNA because stronger alternative base pairing forces the region into a rod structure. *In vivo*, however, the intron exists as a ribonucleoprotein, and psoralen cross-linking studies indicate a structure that is consistent with pairing of the conserved sequence elements (4, 44).

Recent evidence indicates that PSTV is found in a ribonucleoprotein complex *in vivo* (45, 46), and this offers the intriguing possibility that viroids, found as rods in the

deproteinized state (47, 48), may assume other foldings when stabilized by proteins in the cell.

A possible drawback here concerns the *in vitro* structure of the viroid transcript, which, once deproteinized, will be in the rod form, and presumably inactive. However, the existence of metastable forms might contribute a population of active molecules with base pairing similar to that of group I introns.

Another question concerns the natural linear viroid molecules arising from the viroid circles upon storage (49). Attempts have been made to identify the ends of these natural linear molecules, but the results have been only partially in agreement (50, 51). When the circular *Tetrahymena* intron is incubated in a Mg²⁺-containing buffer, a unique bond is broken, at the same site at which the circle was formed. This has been termed "autoreopening" or "site-specific hydrolysis" (52). The reaction rate increases rapidly with pH in the pH range 7.5–9.5 (52). It should be possible to subject the viroid circles to similar conditions, to see if such a specific bond might be selected.

The discovery that the central conserved region of the viroids is not unique to viroids, but that one portion of it exists in all group I introns as the group I consensus, and another portion is found in some introns as the d stem (Fig. 2 Upper), raises further questions about the function of this region. So far the d stem region has been found to be well conserved in the *Tetrahymena* intron and to be present in degenerate form in the virusoids and in the two plant chloroplast group I tRNA introns whose sequences have been determined (refs. 11 and 12; Fig. 3 Top). In the *Tetrahymena* intron, the d stem is found in the region bounded by the A and B boxes, while the homologous region in viroids is located upstream of box A and box 9R'. In the plant tRNA group I introns it is located near the 5' end of the intron. This may be an example of the intermolecular RNA rearrangement recently proposed for viroid and virusoid evolution (53). While this region is not necessary for self-splicing of the *Tetrahymena* intron, it may serve as a recognition site for structural proteins or, as previously suggested, be essential for replication of the viroids.

In the case of the viroids, it appears that an intron can display selective pathogenic activity. Is it possible that other introns may behave as pathogens? The senescence plasmids in *Podospora* contain group I introns, a number of which are very similar to the *Tetrahymena* intron (54, 55). The possibility exists that the introns themselves are involved in causing senescence in the organism, thus acting as a pathogen. Variations in pathogenicity in viroids appear to be caused by minor mutations of specific regions. It would be interesting to determine whether the senescence functions of the *Podospora* plasmid introns can be thus altered.

Finally, it is not yet clear whether nuclear, mitochondrial, or chloroplast group I introns are ever released from these organelles into the cytoplasm. Their containment may prevent unscheduled activity and pathogenesis. In this sense, the viroids may be true "escaped introns."

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