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Peach Latent Mosaic Viroid is Locked by a 2′,5′-Phosphodiester Bond Produced by *in Vitro* Self-ligation

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Although some viroid-like satellite RNAs possess self-cleavage and self-ligation activities, we show that the peach latent mosaic viroid (PLMVd) is unique among all known viroids since it also has such activities. These catalytic activities should have important roles in the rolling circle replication of PLMVd. According to this proposed mechanism, self-cleavage of the multimeric strands occurs *via* hammerhead structures producing monomers possessing 2′,3′-cyclic phosphate and 5′-hydroxyl termini. In the most stable predicted secondary structure for PLMVd these two extremities are juxtaposed, in order for self-ligation to occur. To establish the nature of the phosphodiester bond produced by self-ligation, we followed the classical procedure of complete enzymatic RNA hydrolysis coupled with thin layer chromatography fractionation. Using this procedure, we report that the self-ligation of PLMVd transcripts produces almost exclusively the 2′,5′ isomer (>96%). Primer extension assays also revealed that reverse transcriptase can read througth this 2′,5′ linkage, suggesting that it does not prevent further replication of the viroid. Moreover, we have observed that this 2′,5′ linkage is resistant to the debranching activity contained in nuclear extracts, as well as being capable of preventing further viroid self-cleavage. Thus, if viroids do indeed self-ligate *in vivo*, the resulting 2′,5′-phosphodiester bond could contribute to the stability of these RNA species. Finally, an analysis of both the sequence and the structural requirements for hammerhead self-cleavage and self-ligation suggests that these two RNA processes may be interrelated. We hypothesize that the intramolecular self-ligation which produces circular conformers may contribute to the circularization step of the rolling circle replication, while the intermolecular non-enzymatic ligation is a potential mechanism for the sequence reassortment of viroids and viroid-like species.

Keywords: viroids; rolling circle replication; self-ligation; catalytic RNA; RNA stability

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Introduction

Viroids are small, single-stranded, circular RNAs that infect higher plants, causing diseases in crop species which result in important economic losses in agriculture (reviewed by Diener, 1993). Along with the viroid-like plant satellite RNAs and the *delta* satellite RNA (HDV), of the human hepatitis B virus these RNA pathogens are proposed to constitute a brotherhood of small infectious circular RNAs (Branch *et al*., 1990). Sharing several structural characteristics, these RNA species have been suggested to have a monophylogenic origin (Elena *et al*., 1991). These RNAs have been proposed to replicate in a DNA-independent manner *via* a rolling circle mechanism (Branch & Robertson, 1984). According to this model, these species are copied to give multimeric minus strands which are then cleaved and ligated into circular monomeric strands. From these circular monomeric minus strands, the same three steps of synthesis, cleavage

Abbreviations used: AMV, avian myeloblastosis virus; cDNA, complementary deoxyribonucleic acid; HDV, hepatitis *delta* virus; hnRNP, heterogeneous nuclear ribonucleoprotein; PLMVd, peach latent mosaic viroid; PSTVd, potato spindle tuber viroid; RNase, ribonuclease; TLC, thin layer chromatography.

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and ligation are repeated in order to produce progeny of plus polarity. Alternatively, the multimeric minus strands can be directly copied into multimeric plus strands before being cleaved and circularized into progeny of plus polarity.

Several observations obtained from *in vitro* experiments have shed some light on the crucial ligation step which converts the linear monomeric strands into circular conformers. It has been shown that a wheat germ RNA ligase known to participate in tRNA intron splicing can circularize linear unit-length transcripts of potato spindle tuber viroid (PSTVd; Branch & Robertson, 1982) and peach latent mosaic viroid (PLMVd; Lafontaine *et al.*, 1995). In addition, *in vitro* experiments have demonstrated that T1 ribonuclease cleaves and circularizes PSTVd transcripts of greater than unit-length to monomeric molecules (Tsagris *et al.*, 1991; Steger *et al.*, 1992). More recently, PSTVd transcripts have been shown to be enzymatically cleaved and ligated in a nuclear extract from non-infected potato suspension cells (Baumstark & Riesner, 1995). Besides these examples of the protein catalysis of RNA ligation, the RNA itself has been proposed to be responsible for the ligation of some viroids and satellite RNAs. The circularization of the minus strand of the satellite RNA of tobacco ringspot virus was shown to be catalyzed by its ''hairpin'' autocatalytic structure (Van Tol *et al.*, 1991; Feldstein & Bruening, 1993). It has also been suggested, that the corresponding strand of plus polarity may self-ligate at a reduced level (Prody *et al.*, 1986). Additionally, non-enzymatic self-ligation has been proposed to occur with HDV, as small transcripts corresponding to the HDV sequences adjacent to the ligation site have been shown to self-ligate when juxtaposed by complementary oligonucleotides (Sharmeen *et al.*, 1989).

Recently, it has been shown that monomeric PLMVd transcripts of both polarities may be circularized by a mechanism of non-enzymatic self-ligation (Lafontaine *et al.*, 1995), and that PSTVd transcripts are capable of performing a similar self-ligation reaction (Baumstark *et al.*, 1997), suggesting that this mechanism can occur with at least some viroids. Previously, we have studied the kinetics of the reaction and determined both the optimal conditions and the substrate requirements of the PLMVd self-ligation (Lafontaine *et al.*, 1995). We proposed that self-ligation occurred by a mechanism involving a nucleophilic attack by the 5′-hydroxyl group on a 2′,3′-cyclic phosphate group. Both of these reacting termini are generated from the preceding hammerhead self-cleavage. In the predicted most stable secondary structure for PLMVd, these termini are juxtaposed owing to base-pairing with the complementary strand (see Figure 1). These features were shown to be necessary and sufficient for the non-enzymatic ligation to occur. The PLMVd intramolecular self-ligation producing circular conformers was proposed to contribute to the circularization step of the rolling circle replication of viroids. In contrast, the intermolecular non-enzymatic ligation of various PLMVd transcripts was suggested as a mechanism for sequence reassortment in viroids and viroid-like species. Thus, non-enzymatic ligation may be an important chemical reaction for both the replication and the evolution of viroids and related satellite RNAs. Here, we report that the self-ligation of PLMVd primarily produces a 2′,5′-phosphodiester bond, and further analyze the consequences of the presence of such an unusual chemical bond in a viroid *in vitro*.

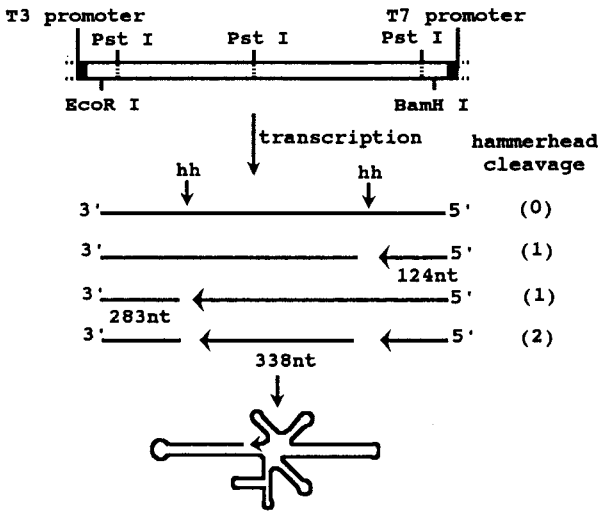

Figure 1. Schematic representation of the plasmid construction, minus polarity transcriptional products, and non-enzymatic intramolecular self-ligation of PLMVd. Details of the construction of the pPD1 plasmid have been reported (Beaudry *et al.*, 1995). The numbers in parentheses are the number of hammerhead (hh) self-cleavages required to produce the various transcripts, with the actual cleavage sites being indicated by arrows. The arrowheads on the transcripts indicate the presence of a 2′,3′ phosphocyclic group at that terminus. The lengths in nucleotides (nt) of the cleaved fragments used here are indicated. The schematic is a representation of the lower energy secondary structure of the PLMVd monomeric transcripts as predicted by computer analysis. This structure was previously shown to support the intramolecular self-ligation of PLMVd (Lafontaine *et al.*, 1995).

Results

Production of transcripts for self-ligation

The pPD1 clone used here contains an insert of two tandemly repeated PLMVd sequences (Figure 1). This construction permits the synthesis of transcripts of either plus or minus polarity (see Materials and Methods). During *in vitro* transcription, RNAs of both polarities possessing hammerhead sequences are produced and self-cleave efficiently (Beaudry *et al.*, 1995). From a dimeric PLMVd insert, self-cleavage at both hammerhead

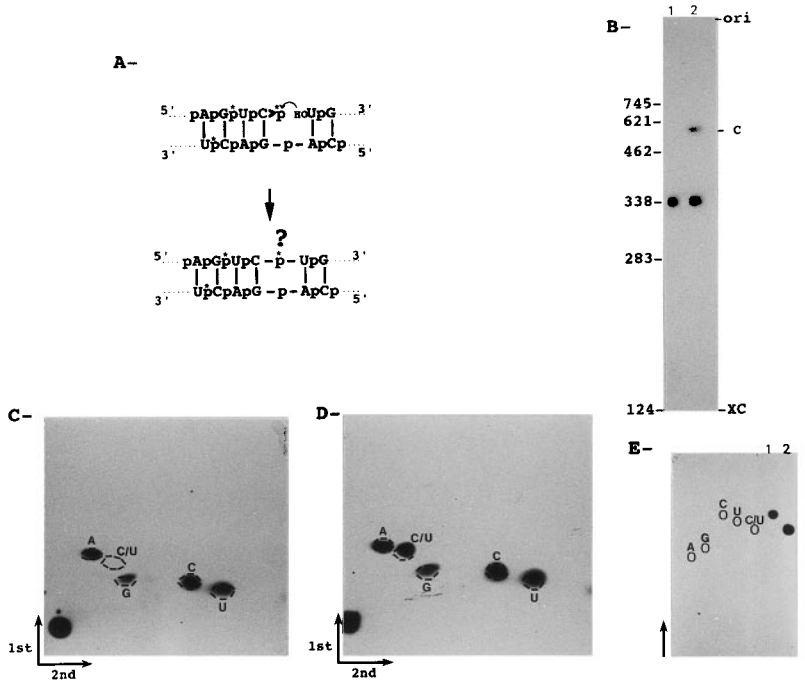

Figure 2. Biochemical characterization of the nature of the phosphodiester bond produced by PLMVd intramolecular self-ligation. a, Representation of the intramolecular self-ligation of PLMVd transcripts. Only the sequence and secondary structure near the self-ligation site are shown. The attack of the 5′ hydroxyl group on the 2′,3′-cyclic phosphate is represented by the arrow. Stars (*) indicate the position of the radioactive phosphate groups incorporated by *in vitro* transcription. b Autoradiogram of a 5% PAGE gel of self-ligated monomeric transcripts. Self-ligation was performed for zero and 16 hours (lanes 1 and 2, respectively). Adjacent to the gel, the positions of several PLMVd transcripts are indicated for size reference purposes. C indicates circular product; Ori and XC indicate the position of the origin and the xylene cyanol migration, respectively. c and d, Autoradiograms of two-dimensional TLCs of the 283 nt transcript and the circular transcripts (produced by self-ligation) digested by RNase T2 for 15 minutes, respectively. Broken circles represent the migration of commercial monophosphate-nucleotides and the C/U dinucleotide detected by UV shadowing. e, Autoradiogram of one-dimensional TLC performed in order to confirm the identity of the dinucleotide produced at the self-ligation site. The dinucleotide was digested overnight with either RNase T2 or nuclease P1 (lanes 1 and 2, respectively).

sites produces 338 nucleotide (nt) linear monomers that have the 2′,3′-cyclic phosphate and 5′-hydroxyl termini required for further self-ligation (Lafontaine *et al*., 1995).

Self-ligation produces a 2′,5′-phosphodiester bond

The nature of the phosphodiester bond produced by the non-enzymatic ligation of PLMVd remains unknown. In order to establish the nature of the phosphodiester bond produced by self-ligation, we followed the classical procedure of complete enzymatic RNA hydrolysis coupled with thin layer chromatography (TLC) fractionation originally developed for the study of modified tRNA nucleotides (Silberklang *et al*., 1979).

Minus-strand, PLMVd monomer transcripts internally labelled with [α-^{32}P]UTP were synthesized, purified, and self-ligated under protein-free conditions (Figure 2b). This generated ~10% circular conformers possessing a slower electrophoretic mobility (Figure 2b). Self-ligated transcripts were purified in order to permit their biochemical characterization. PLMVd transcripts of 283 nt that have hydroxyl groups at both termini were used as controls. The transcripts were incubated for 15 minutes in the presence of ribonuclease T2 (RNase T2) followed by two-dimensional TLC fractionation (Figure 2c and d). RNase T2 specifically hydrolyzes 3′,5′-phosphodiester bonds in ribonucleic acids producing 3′-monophosphate nucleotides. Since the radioactive phosphate groups were randomly introduced at the 74 possible U residues (see Figure 2a), RNase T2 cleavage should release those phosphate groups with any of the upstream nucleotides. For the 283 nt transcripts, four spots of mobility corresponding to the 3′-monophosphate nucleotides were observed (Figure 2c). In contrast, the digestion of the self-ligated transcripts produced five spots: four 3′-monophosphate nucleotides and an additional species of mobility corresponding to a non-radioactive cytidylyl-2′,5′-uridine used as a control (Figure 2d). The autoradiograms shown here were over-exposed in order to allow the detection of any trace products. For this reason, these autoradiograms do not reflect the actual proportion of each product. As shown by the radioactive spot at the origin, a short incubation did not allow for complete hydrolysis. In contrast, overnight incubation of self-ligated transcripts with the same preparation of RNase T2, which, according to the manufacturer, contains trace amounts of other ribonucleases, deoxyribonucleases and phosphodiesterases, fully hydrolyzed the RNA into four mononucleotides (data not shown).

The presence of a 2′,5′-phosphodiester bond formed by self-ligation receives additional support from nuclease P1 digestions. This enzyme, which specifically cleaves 3′,5′-phosphodiester bonds releasing 5′-monophosphate nucleotides, produced

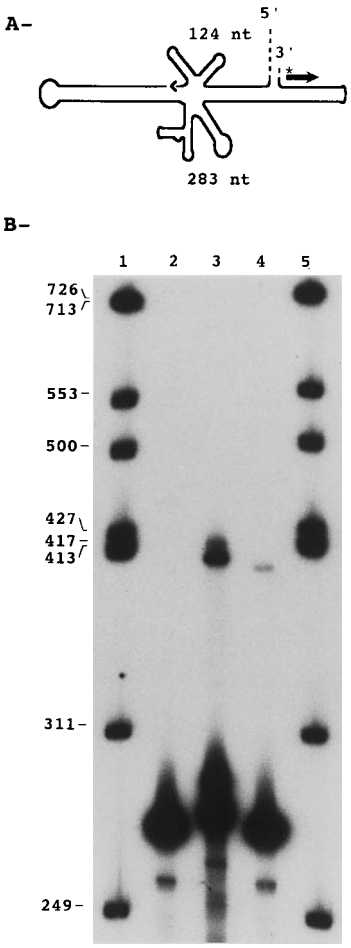

Figure 3. Primer extension assays. a, Representation of the intermolecular self-ligation coupled with the reverse transcriptase assay. The continuous line and the broken line represent PLMVd and vectorial sequence, respectively, of both transcripts. The arrowhead at the 124 nt transcript terminus indicates the presence of a 2′,3′ cyclic phosphate at this end. The primer hybridizing at the 3′ end of the 283 nt nucleotide transcript is represented by a bold arrow. The star indicates the radioactive phosphate present at the 5′ end of this primer. b, Analysis of both intermolecular self-ligation and primer extension assays. Lane 3 is a 16 hour self-ligation performed with radioactive 283 nt transcripts. Lanes 2 and 4 are the radioactive reverse transcriptase assays performed with non-radioactive 283 nt transcripts alone and self-ligation mixture, respectively. Lanes 1 and 5 are positions of radioactive, single-stranded, DNA molecular mass markers (sizes are indicated adjacent to the gel). The samples were fractionated by 5% PAGE in the presence of 50% formamide.

only one spot with control linear transcripts (i.e. 5′-monophosphate uridine), and two spots with self-ligated circular molecules (i.e. 5′-monophosphate uridine and 5′-monophosphate cytidylyl-2′,5′-uridine dinucleotide; data not shown). From four experiments, the quantification of several TLC plates of hydrolyzed mixtures of self-ligated circular transcripts with a PhosphorImager indicated that self-ligation resulted in the formation of a 2′,5′-phosphodiester bond in at least 96% of the transcripts. In order to confirm the identity of the nucleotide linked by a 2′,5′-phosphodiester bond, which should be $C^{32}pUp$, we extracted the corresponding TLC spot after RNase T2 hydrolysis. This spot was submitted to various hydrolysis procedures and the reaction mixtures analyzed by one-dimensional TLC (Figure 2e). Overnight incubation with ribonuclease T2 released radioactive cytosine ($C^{32}p$, lane 1), confirming that the dinucleotide was $C^{32}pUp$, because the radioactive phosphate was introduced as part of the uridine. Alkaline hydrolysis of the dinucleotide yielded the same result (data not shown). Finally, an overnight incubation in the presence of nuclease P1 did not hydrolyze the phosphodiester bond (lane 2), thereby confirming that only the 2′,5′-phosphodiester-linked dinucleotide was present. In conclusion, these results demonstrate that the regiospecificity of the PLMVd self-ligation has a great preference for the 2′,5′ isomer over the 3′,5′ isomer.

Reverse transcriptase read through of the 2′,5′-phosphodiester bond

If *in vivo* self-ligation occurs producing a 2′,5′-phosphodiester bond, this linkage should not limitate further replication of the resulting viroid. In order to verify this hypothesis, we performed primer extension assays. The self-ligation between two minus-polarity PLMVd transcripts (i.e. 124 and 283 nt) that hybridize together was used as model system for further primer extension assays with an oligonucleotide complementary to the 3′ end of the 283 nt transcripts as primer (Figure 3a). Utilizing radioactive 283 nt transcripts, we detected the presence of a novel 407 nt RNA species when ligation occurred (Figure 3b, lane 3). When a similar reaction was performed with unlabelled transcripts to permit a subsequent primer extension step, a cDNA of 397 nt length was detected compared to a cDNA of 273 nt from the 283 nt transcripts alone (compare lanes 4 and 2, respectively). Considering that most of the phosphodiester bonds produced by the self-ligation are 2′,5′-linkages, these results indicate that a reverse transcriptase can read through the novel bond. These results confirm the previous report of the ability of reverse transcriptases to read through a large proportion of 2′,5′-phosphodiester bonds introduced into small, single-stranded, synthetic RNA templates (Lorsh *et al.*, 1995), even though PLMVd is highly structured as compared to the species used in this earlier study. This latest report also demonstrated that a proportion of the reverse transcriptase reaction terminated one nucleotide beyond a 2′,5′-phosphodiester bond, and hence did not have the ability to complete further the elongation of the resulting cDNA. In our assays, the ability of a reverse transcriptase to read through the novel bond was estimated to be greater than 50% of the time (see Materials and Methods). The use of self-

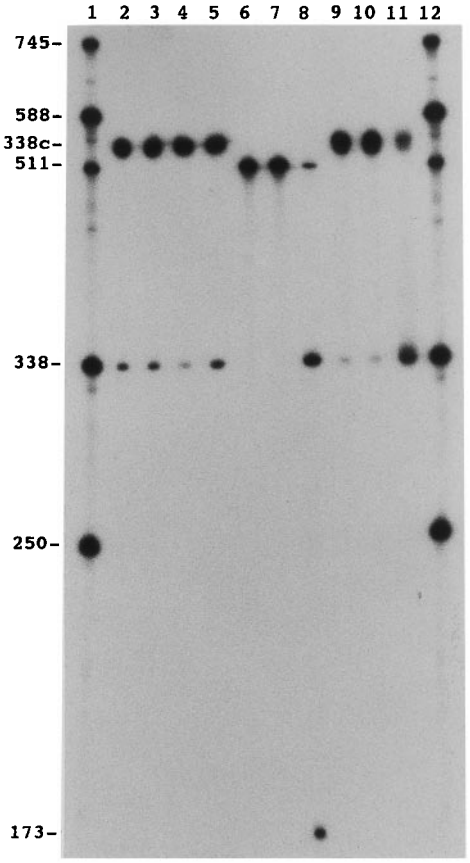

Figure 4. Self-cleavage assays analyzed by 5% PAGE. Lanes 2 to 5 are assays with circular transcripts produced by self-ligation; in lanes 2 and 3 the samples were incubated for zero and ten minutes without $MgCl_2$ and prior heat denaturation-renaturation, while in lanes 4 and 5 the heat denaturation-renaturation was performed and $MgCl_2$ then added prior to incubation for 10 and 60 minutes, respectively. Lanes 6 to 8 are assays performed with linear 511 nt PLMVd transcripts of plus polarity, with the conditions being similar to those described for lanes 2 to 4, respectively. Lanes 9 to 11 are self-cleavage assays of circular PLMVd transcripts prepared enzymatically (i.e. including a 3′,5′-phosphodiester bond at the self-cleavage/self-ligation site), with the reaction conditions as described for lanes 2 to 4. Finally, lanes 1 and 12 are RNA molecular mass markers whose sizes are indicated on the left. The position of the monomeric circular PLMVd transcripts is indicated by 338c.

ligated transcripts of plus-polarity as templates, different oligonucleotides as primers, or different reverse transcriptases, gave similar results, albeit with different elongation efficiencies. Thus, these results suggested that self-ligated viroid producing a 2′,5′-phosphodiester bond can be read by a polymerase.

The 2′,5′-phosphodiester bond prevents the self-cleavage of PLMVd transcripts

The presence of a 2′,5′-phosphodiester bond at the self-cleavage/self-ligation site did not significantly reduce the ability of PLMVd transcripts to act as templates. However, it remains unknown whether the presence of such a linkage confers any advantage to the viroid. Using a minimal model system to study hammerhead ribozyme catalysis, it has been shown that the 2′-hydroxyl group of the ribose adjacent to the cleavage site is absolutely required for the reaction to occur (Perreault *et al.*, 1990). Therefore, we hypothesized that a 2′,5, phosphodiester bond at the self-cleavage/self-ligation site could contribute to the stabilization of the circular transcripts by preventing further viroid self-cleavage. In order to verify this hypothesis, we performed self-cleavage assays of transcripts containing either a 2′,5′ or 3′,5′-phosphodiester bond at the self-ligation site (Figure 4). Circular transcripts produced by self-ligation were snap-cooled, and then incubated in the presence of magnesium. At 10 and 60 minutes after the addition of the magnesium the proportion of linear conformers was found to increase to 0.04 and 6%, respectively (lanes 2 to 5). However, it remains unclear whether the linearization resulted exclusively from hammerhead self-cleavage, or also from the nonspecific hydrolysis of phosphodiester bonds (i.e. RNA nicking). In contrast, circular PLMVd RNA synthesized *via* an enzymatic method (Beaudry & Perreault, 1995) so that it contains only 3′,5′-phosphodiester bonds, was found to self-cleave efficiently within ten minutes after the addition of magnesium (~50%; lane 11). Finally, greater than unit-length, linear, PLMVd transcripts were observed to self-cleave twice as well as the circular conformer under similar conditions (88%; lane 8), clearly indicating that circularity contributes to preventing hammerhead self-cleavage. The presence of a 2′,5′-phosphodiester bond produced by self-ligation prevents further self-cleavage, thereby stabilizing the circular conformer and allowing it to adopt an alternative structure without any risk of being linearized.

The 2′,5′-phosphodiester bond is resistant to the intron-debranching enzyme

If a 2′,5′ phosphodiester bond has a role to play in viroid stability, it is important that this linkage cannot be altered or cleaved by host enzymes, especially the intron debranching enzyme that specifically recognizes and subsequently cleaves 2′,5′ phosphodiester bonds. In order to evaluate such a possibility, we incubated circular or linear PLMVd transcripts, or an intron from the heterogenous nuclear ribonucleoprotein A1 (hnRNP A1), with a S100 soluble extract from isolated nuclei (Figure 5). Under these conditions, lariat introns from hnRNP A1 transcripts were efficiently debranched (lanes 1 to 3). In contrast, circular PLMVd transcripts produced either by self-ligation (lanes 4 to 6), or by our enzymatic procedure so as to include only 3′,5′-phosphodiester bonds (lanes 7 to 9), as well as linear 338 nt transcripts (lanes 10 to 12), were not altered by the debranching activity of

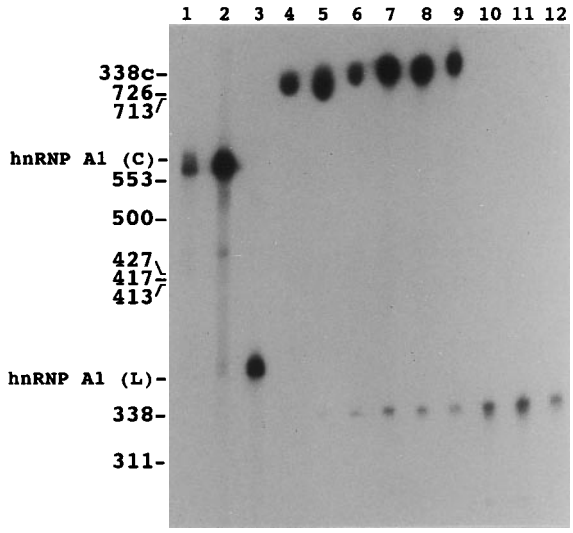

Figure 5. Autoradiogram of the debranching intron enzyme assays. The hnRNP A1 introns (lanes 1 to 3), circular PLMVd transcripts produced by either self-ligation (lanes 4 to 6) or enzymatic synthesis (lanes 7 to 9), and monomeric linear PLMVd transcripts were not incubated (lanes 1,4,7 and 10), or were incubated for one hour either in absence (lanes 2, 5, 8 and 11) or the presence (lanes 3, 6, 9 and 12) of an S100 nuclear extract. Adjacent to the gel the positions of several PLMVd transcripts are indicated as molecular mass markers as well as both the circular (C) and linear (L) hnRNP A1 conformers.

the protein extract. Thus, the viroid 2′,5′-phosphodiester bond produced by self-ligation is resistant to the debranching enzyme. This type of resistance has been reported for a 2′,5′-phosphodiester bond introduced into synthetic ribonucleic acids (Ruskin & Green, 1985; Arenas & Hurwitz, 1987). The debranching enzyme was proposed to recognize a junction composed of three strands, one of which is linked by a 2′,5′-phosphodiester bond. Our results indicate that any other ribonuclease activity present in the protein extract was sufficient to hydrolyze the RNA molecules present, although this does not exclude the possibility that other cellular nucleases can specifically degrade a 2′,5′-linkage.

Discussion

Regiospecificity and efficiency of PLMVd self-ligation

Previously, we reported that transcripts of sequence corresponding to PLMVd have the ability to self-ligate (Lafontaine *et al.*, 1995). Here, we report the nature of the phosphodiester bond formed by this non-enzymatic self-ligation, and further characterize its properties. PLMVd self-ligation produces primarily 2′,5′-phosphodiester bonds (>96%). Even allowing for any imprecision in the quantification of the different TLC spots in the assay, the predominance of the 2′,5′ *versus* 3′,5′ isomer was unequivocal. This regiospecificity is in agreement with previous reports of the non-enzymatic, template-directed condensation of nucleotides and oligonucleotides (Renz *et al.*, 1971; Usher & McHale, 1976). For example, the non-enzymatic joining of both adenosine and short poly(A) oligonucleotides, which harbour 2′,3′-cyclic phosphate groups, on poly(U) templates were shown to yield at least 97% of 2′,5′ isomer (Renz *et al.*, 1971; Usher & McHale, 1976). It has been suggested that the almost complete absence of the 3′,5′ isomer can be attributed to the intrinsic lack of activity of the 3′-hydroxyl group. Moreover, the orientation of the RNA helix favours the formation of the 2′,5′-isomer; thus, factors which alter the relative orientation of template-bound reactants may influence both the efficiency and regiospecificity of the condensation.

The self-ligation reaction that we characterized involved a 2′,3′-phosphocyclic cytosine and a 5′-hydroxyl uridine as reactants juxtaposed on the template. We propose that the most likely reaction mechanism involves a nucleophilic attack of the 5′-hydroxyl group on the phosphate of the 2′,3′-cyclic phosphate, resulting in the formation of a new phosphodiester bond. A six hour self-ligation reaction was found to produce ~10% circular conformers. Subsequent heat denaturation and slow renaturation of the transcripts to favour folding into the most stable secondary structure was not observed to increase the percentage of circular products (P. Marquis & J.-P. P., unpublished results). These results suggest that under the *in vitro* reaction conditions used, only a small proportion of the 2′,3′-cyclic phosphate groups present as reactants for the production of phosphodiester bonds, while the majority are simply hydrolyzed into either 2′ or 3′-phosphate groups. *In vivo* it is possible that a cofactor, such as a protein, contributes to the enhancement of the self-ligation reaction. To date, no 2′,5′-phosphodiester bond has been characterized in viroids and related satellite RNAs isolated from infected plants, although the presence of a 2′,5′-phosphodiester linkage was not specifically investigated.

The presence of 2′,5′-phosphodiester bonds is not restricted to the *in vitro* self-ligation of viroids. For example, the branched RNA strands in introns involve a 2′,5′-phosphodiester bond. In these examples the formation of the 2′,5′-phosphodiester bond occurs at a site where a 3′,5′-phosphodiester bond is already present. Moreover, an RNA ligase that catalyzes the formation of a 2′,5′-phosphodiester bond was purified from *Escherichia coli* and shown to have the ability to ligate tRNA half-molecules, although the natural substrates of this enzyme remain unidentified (Arn & Abelson, 1996). In addition, the occurrence of 2′,5′-oligoadenylates in both eukaryotes and prokaryotes has been reported (Trujillo *et al.*, 1987). Even if 2′,5′-phosphodiester bonds are frequently qualified as being unnatural and a remnant of a prebiotic

chemistry, they are represented in nature today and are important biologically.

2′,5′-Phosphodiester bonds in viroids and related satellite RNAs

It has been suggested that the intramolecular self-ligation of viroids yielding circular conformers could contribute to the circularization step of rolling circle replication, while the intermolecular non-enzymatic ligation is a potential mechanism for the sequence reassortment of viroids and viroid-like species (Robertson, 1992). In order for this hypothesis to be valid, the presence of a 2′,5′-phosphodiester bond in these species of RNA must not be a limitation to further replication; rather, these molecules should conserve their ability to act as templates. Primer extension assays have shown that the presence of a 2′,5′-phosphodiester bond in templates does not necessarily interrupt the reverse transcriptase elongation; in fact approximately one-half of the synthesized cDNAs are extended beyond the self-ligation site. Thus, the presence of such a linkage does not constitute an important obstacle to polymerase progression, rather the template ability of these species is conserved. The reduced potential of these molecules to act as templates is probably compensated by the important gain in stability conferred by the 2′,5′-linkage. This observation supports the idea that self-ligation is a mechanism involved in both the replication and the evolutionary processes of viroids and related satellite RNAs. Regardless of whether or not self-ligation and self-cleavage occur *in vivo*, the only crucial host component for PLMVd replication is a polymerase, thereby making its rolling circle replication largely an RNA-based mechanism (Bussière *et al*., 1995).

Depending on the RNA species, the cleavage step of the rolling circle replication is either catalyzed by a host endoribonuclease or involves RNA self-cleavage. Viroid-like plant satellite RNAs, HDV and two viroids (avocado sunblotch viroid and PLMVd) undergo specific self-cleavage *via* autocatalytic ''hammerhead'', ''hairpin'' or ''delta'' structures (Symons, 1992). In contrast, several mechanisms, including protein catalysis and RNA self-ligation, have been proposed for the ligation step, but none has yet been confirmed *in vivo*. For PLMVd, the most stable predicted secondary structures of strands of both polarities include double-stranded helices in which both termini are juxtaposed. We predicted, using computer modelling, the most likely stable secondary structures of each of the known self-cleaving strands of viroids and related RNA (i.e. viroid-like plant satellites RNA and HDV). In general, among the ten most stable structures (i.e. less than 5% of difference in terms of free energy), at least one localized the self-ligation site into a double-stranded helix (data not shown). This result confirms that the structural requirements for self-ligation are respected in all RNA strands known to self-ligate. Only the minus

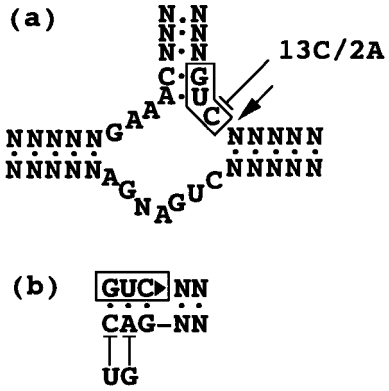

Figure 6. Schematic representation of the sequence and secondary structure requirements for hammerhead self-cleavage (a) and self-ligation (b). Only highly conserved nucleotides are defined. For the hammerhead structure, the natural nucleotide variants of the GUC triplet (boxed), and their frequency of occurrence, are indicated. For the self-ligation structure, the possible nucleotide variants that can effectively base-pair with the GUC triplet are shown.

strands of the satellite RNA of the arabis mosaic virus, the chicory yellow mottle virus and the tobacco ringspot virus do not fold into a structure to allow self-ligation (i.e. the termini were located in a single-stranded region). However, unlike the other RNA species, these three self-cleave by a ''hairpin'' structure in which the catalytic domain has been shown to catalyze both the cleavage and ligation reactions (Van Tol *et al*., 1991; Feldstein & Bruening, 1993; Berzal-Herranz *et al*., 1992). In contrast, hammerhead structures largely favour cleavage over ligation (Hertel & Ulhenbeck, 1995). Therefore, hammerhead self-cleaving strands may require an alternative mechanism of non-enzymatic self-ligation for circularization of the RNA species, as shown to occur with PLMVd transcripts.

Self-cleavage and self-ligation dependency

The hammerhead structure is responsible for the self-cleavage in most self-cleaving viroids and related RNAs. This domain consists of three helices and a catalytic center formed by highly conserved nucleotides (Figure 6a). Co-variations of hammerhead base-paired nucleotides occur between RNA species, as well as between variants of the same species, suggesting a selective pressure in favour of the self-cleavage activity (Bussière *et al*., 1996; Hernandez & Flores, 1992). A compilation of the natural hammerhead sequences shows that the triplet adjacent to the self-cleavage site is a GUC in 13 cases, while GUA is found in only two instances. In addition, an AUA triplet is present at that position in the plus polarity strands of barley yellow dwarf virus satellite RNA, a species in which this hammerhead motif includes an unusual pseudoknot structure whose contribution to the mechanism remains unclear (Miller *et al*., 1991).

With the exception of the latter case, all hammerhead structures include the first two nucleotides that are always base-paired, and a third nucleotide located adjacent to the cleavage site which does not form a base-pair. Sequence requirements of the hammerhead RNA self-cleavage reaction have been studied with a minimal model structure (Ruffner *et al*., 1990). The first base-pair (GC) can be replaced by UA or AU with efficient self-cleavage conserved. The identity of the second base-pair (UA) appears to be essential for efficient self-cleavage, indicating a contribution to the catalytic mechanism. Finally, the single-stranded C can be replaced by an A without a significant decrease in activity. However, substitution by either a G or a U drastically decreases self-cleavage. These data from a model system only partly explain the naturally occurring sequence variations. For example, it is clear that the selection pressure is strong in favour of a U at the second position of the triplet. In contrast, the possibility of having different base-pairs at the first position of the triplet, and of having an A or G at the cleavage site, are not reflected in nature. With the high mutation rates observed in these species, these results are unexpected. In contrast to self-cleavage, the self-ligation reaction has minimal sequence and structural requirements. Specifically, all that is required is a complementary strand that juxtaposes both of the reacting termini (Figure 6(b)). If self-ligation does indeed occur *in vivo*, we believe that C is favoured over A 13:2 at the third position because the resulting GC base-pair is more stable than an AU base-pair, and that this yields a more efficient self-ligation. The preference for G at the first position of the triplet may be due to its contribution to the stabilization of the self-ligation helix, even though A and U have been shown to be efficient for self-cleavage. In contrast, the selection pressure on the second nucleotide is strictly from the requirements for the self-cleavage mechanism to be efficient. It is important to note that in general the helix supporting self-ligation is relatively small; hence, the more stable it is (i.e. the preference for GC base-pairs) the more likely self-ligation is. Following such a rationale, self-cleavage and self-ligation appear to be dependent on each other for their sequence requirements. Either the naturally occurring sequences surrounding the ligation site support a mechanism of self-ligation, or self-ligation activity contributes a selective pressure on the sequence including the highly conserved GUC triplet of the hammerhead domain.

2′,5′-Phosphodiester bonds and viroid stability

In addition to showing that the 2′,5′-phosphodiester bond produced by self-ligation is not an obstacle to further replication, we have shown that it is not specifically hydrolyzed by the intron debranching enzyme. We believe that the self-ligated bond was not recognized by this enzyme because it is not part of a three-strand junction as is found in introns. Searching to see if such a 2′,5′ linkage provided any advantages to the circular species, we demonstrated that it prevents further self-cleavage when the RNA strand is incubated under conditions allowing this reaction. Thus, the presence of a 2′,5′-phosphodiester bond at the self-cleavage/self-ligation site allows the viroid to adopt alternative conformations comparable to the rod-like most stable structure without becoming linear. In fact, this novel bond acts as a padlock protecting the integrity of the circular RNA, a significant advantage for the viroid.

Based on their self-cleavage ability, it has been suggested that viroids and related satellite RNAs could induce host pathogenesis by expressing this catalytic property in *trans* in order to cleave cellular RNA in a manner reminiscent of ribozymes. This hypothesis has not as yet received any support from experiments, partly because viroid-like transcripts made completely of 3′,5′-phosphodiester bonds will favour intramolecular self-cleavage over the intermolecular reaction. We have demonstrated that the self-ligated circular transcripts lose their ability to self-cleave. By analogy to the *trans* model hammerhead structure, production of a 2′,5′-phosphodiester bond by self-ligation modified the substrate portion of the viroid, while the catalytic core remained unchanged. We postulate that regardless of whether self-ligated viroids can fold so as to allow for the formation of the hammerhead catalytic core, it is possible that they cleave RNA substrates in *trans*. If such a mechanism occurs, the presence of a 2′,5′-phosphodiester bond at the self-cleavage/self-ligation site would have a key role in both viroid integrity and pathogenesis. Considering that self-cleaving viroids are very old, perhaps even a relic of a precellular world, the fact that self-ligation can still occur today *via* the same mechanism as in ancient times suggests that it has been positively selected for.

Materials and Methods

In vitro transcription and purification of PLMVd RNA

The synthesis and purification of all the linear transcripts used were as described (Beaudry *et al*., 1995). Briefly, *in vitro* transcriptions were performed using the plasmid pPD1 construction as template. This construction possesses two tandemly repeated PLMVd sequences cloned into the *Pst*I restriction site of pBluescript II KS (+/−) (cf. Figure 1). The insert is flanked by T3 and T7 promoters for the production of plus and minus polarity transcripts, respectively. For random internal labelling, 50 μCi of [α-^{32}P]UTP (3000 Ci/ mmol, Amersham) was added to the transcription reaction. After transcription, DNase (RNase free) treatment, and precipitation of nucleic acids, 0.5 volume of stop buffer (0.3% (w/v) each of bromophenol blue and xylene cyanol, 10 mM EDTA (pH 7.5), 97.5% (v/v) deionized formamide) was added to the resuspended transcript solutions, and the resulting mixtures denatured for two minutes at 65°C prior to electrophoresis through a 5% (w/v) polyacrylamide gel (PAGE) in 100 mM Tris-borate (pH 8.3), 1 mM EDTA, 7M urea buffer. Transcripts were detected by UV shadowing, excised, eluted, precipitated, purified by

passage through Sephadex G-50 spun columns (Pharmacia), lyophilized and stored at −70° C. Circular PLMVd transcripts, which include exclusively 3′,5′-phosphodiester bonds, were synthesized as described (Beaudry & Perreault, 1995).

Self-ligations

The intramolecular self-ligation of unit-length PLMVd transcripts (338 nt, ~500,000 cpm) resuspended in ultrapure water was performed by adding concentrated reaction buffer to a final concentration of 4 mM Tris-HCl (pH 7.9), 100 mM $MgCl_2$ in a final volume of 15 µl (Lafontaine *et al*., 1995). The samples were then incubated for 6 to 12 hours at 16° C. The ensuing radioactive transcripts were ethanol precipitated and washed, prior to lyophilization. The resulting pellets were either stored at −70°C for subsequent analysis, or resuspended in a small volume of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA solution and analyzed by 5% PAGE. The intermolecular reactions were performed with 1 µg of non-radioactive 124 nt transcripts incubated with either ~ 500,000 cpm of radioactive or 1 µg of non-radioactive transcripts of 283 nt. Both the 124 and 283 nt transcripts are of minus polarity, and were produced by self-cleavage during *in vitro* transcription (cf. Figure 1). Both transcripts were incubated together under the self-ligation conditions described above, and then precipitated with ethanol and either conserved for further analysis, or purified by 5% PAGE.

RNase digestion and analysis by thin layer chromatography

The purified circular, radioactive, 338 nt transcripts produced by self-ligation were resuspended in 10 µl of either 150 mM ammonium acetate (pH 5.3) containing 2 mg of nuclease P1 (Boehringer Mannheim), or 10 mM sodium acetate (pH 4.5) containing 0.5 unit RNase T2 (Gibco BRL), and then incubated for either 15 minutes or overnight (~12 hours) at 37°C. After the reaction, the samples were analyzed by two-dimensional TLC on cellulose plates with UV indicator (Mandell) according to the solvent system described by Nishimura (1979). Non-radioactive mononucleotides and dinucleotides were purchased (Sigma), and were also fractionated. The resulting dried plates were analyzed by both UV shadowing and autoradiography, and the spots quantified with a PhosphorImager (Molecular Dynamics). The radioactive spot corresponding to the dinucleotide was recovered according to the procedure of Houssier *et al*. (1988). Dinucleotides were digested with either nuclease P1 or RNase T2 under the conditions described above, or by alkaline hydrolysis. The resulting samples were analyzed on PEI-cellulose with UV indicator (Mandell) according to the solvent system described previously (Yang *et al*., 1995).

Primer extension assays

Non-radioactive transcript mixtures from intermolecular self-ligations were analyzed by primer extension assays. In the example illustrated in Figure 3, the oligonucleotide primer (5′ GTTCCCGATAGAAAGGC 3′) was complementary to a sequence near the 3′ end of the 283 nt transcript (cf. Figure 3). The oligonucleotide had been previously labelled by polynucleotide kinase treatment in presence of [γ-^{32}P]ATP (3000 Ci/ mmol, Amersham) as recommended by the enzyme's manufacturer (Pharmacia Biotech). Equimolar amounts (10 pmol) of 5′ end labelled primer and transcript mixture were resuspended together in a solution of 100 mM Tris-HCl (pH 8.3) in a final volume of 4.5 µl. The mixtures were heated at 80°C for two minutes, incubated at 55°C for three minutes and then kept on ice for five minutes. The samples were then incubated for two hours at 37°C in the presence of 0.3 mM of each dNTP (Pharmacia Biotech), 1X AMV reverse transcriptase extension buffer (Boehringer Mannheim), 0.125 mM actinomycin, 2.5 mM Na_4P2O_7, 30 units of RNA guard (Pharmacia Biotech) and 200 units of SuperScript reverse transcriptase (Gibco BRL) in a final volume of 20 µl. After the incubation, 40 µl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 1 µl each of glycogen (20 mg/ml; Boehringer Mannheim) and phenol saturated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA were added and the nucleic acids precipitated with ethanol, washed with ethanol and lyophilized. The resulting pellet was resuspended in stop buffer and analyzed by 5% PAGE through a gel containing 50% formamide. The ability of the reverse transcriptase to read through the novel bond was estimated using several similar experiments as presented in Figure 3b. The quantifications were performed with a Phosphorimager (Molecular Dynamics). We estimated that the proportion of the 397 nt cDNA (fully elongated) was 4% compared to 96% for the 273 nt (either terminated at the self-ligation site or from the unreacted 283 nt transcripts; Figure 3b, lane 4). Taking into account that the efficiency of self-ligation was 8% of the 283 nt transcripts (lane 3), we deduced that the reverse transcriptase read through about half of the 2′,5′ phosphodiester bonds. Appropriate self-ligation and primer extension controls were performed.

Self-cleavage assays

Various purified transcripts, both subjected and not subjected to a preliminary self-ligation step, were tested for self-cleavage ability. These transcripts (~3000 cpm) were resuspended in a volume of 9 µl of 10 mM Tris-HCl (pH 8.0) 1 mM EDTA and then heated at 90°C for one minute prior to snap-cooling on ice for 30 seconds so as to favour the adoption of the hammerhead structure. Self-cleavage of the transcripts was initiated by adding 1 µl of 1 M $MgCl_2$, and the reaction incubated at 37°C for different times. The reactions were stopped by the addition of 0.5 volume stop buffer, kept on ice, denatured for two minutes at 65°C, and finally purified on 5% PAGE gels.

Incubation of transcripts with S100 nuclear extract

Both circular and linear transcripts possessing either 2′,5′ or 3′,5′-phosphodiester bonds at the self-cleavage/ self-ligation site were produced as described above. The lariat introns between exon 7 and 7b of hnRNP A1 transcripts and S100 nuclear extract were kindly provided by S. Hutchison and B. Chabot (Université de Sherbrooke). Both the splicing reaction and the preparation of the nuclear extract, which possesses the intron debranching enzyme, were performed as described (Krainer *et al*., 1984; Dignam *et al*., 1983). Briefly, transcripts (~250 cpm) were incubated for one hour at 37°C with 10 µl of nuclear extract in a final volume of 25 µl containing 10 mM Hepes-NaOH (pH 7.9), 50 mM KCl, 10% glycerol, 8 mM EDTA, 0.25 mM phenylmethylsulphonyl fluoride (PMSF) and 0.25 mM DTT. The reactions were stopped by phenol/chloroform extraction (1:1, v/v), and the ribonucleic

acids precipitated with ethanol, washed with ethanol, lyophilized and fractionated on 5% PAGE gels.

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