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SHORT COMMUNICATION

Intra- and Intermolecular Nonenzymatic Ligations Occur within Transcripts Derived from the Peach Latent Mosaic Viroid

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We report here the nonenzymatic self-ligation of transcripts corresponding to the peach latent mosaic viroid (PLMVd). This is the first description of this process with viroid sequences, although it has been reported to occur with human hepatitis delta virus RNA. Self-ligation occurs when the 5′-hydroxyl and the 2′,3′-cyclic phosphate termini produced by the hammerhead self-cleavage of the viroid RNA are juxtaposed by the viroid rod-like structure, and a phosphodiester bond is formed between the two following hydrolysis of the cyclic phosphate. Unit-length transcripts undergo intramolecular folding, and their subsequent self-ligation produces circular molecules. The self-ligation observed *in vitro* may contribute to PLMVd circularization during rolling circle replication; however, this does not exclude the possibility that a host RNA ligase catalyzes the ligation steps *in vivo*. Like self-cleavage, self-ligation is probably an ancestral reaction, and the enzyme-catalyzed ligation most likely evolved from this primitive mechanism. Furthermore, the intermolecular self-ligation of annealed transcripts derived from PLMVd is demonstrated, suggesting a possible mechanism for sequence reassortment in viroids.

According to the hypothesis that viroids replicate by a rolling circle mechanism (*1–4*), viroids are copied to give multimeric minus strands which are then cleaved and circularized into monomeric strands. The process is then repeated to produce progeny viroids of plus polarity. Alternatively, the multimeric minus strands can be directly copied into multimeric plus strands before being cleaved and circularized into progeny viroids. Several mechanisms have been proposed for the ligation step which converts the linear monomeric strands into circular conformers; however, none has been confirmed *in vivo*. It has been shown that a wheat germ RNA ligase can circularize linear unit-length viroids (*5–7*), and, more recently, *in vitro* experiments have demonstrated that T1 ribonuclease cleaves and circularizes potato spindle tuber viroid transcripts from more than one unit length to monomeric molecules (*8*). Besides these examples of the protein catalysis of RNA ligation, the RNA by itself was proposed to be responsible for the ligation of some satellite RNAs. The circularization of the minus strand of the satellite RNA of tobacco ringspot virus was shown to be catalyzed by the "hairpin" autocatalytic structure, while it has been suggested (*9, 10*), but not yet demonstrated, that the corresponding strand of plus polarity may self-ligate at a reduced level (*11*). Finally, the human hepatitis delta virus (HDV), a satellite-like RNA of the hepatitis B virus, has been shown to undergo nonenzymatic self-ligation when the linear monomer adopts a rod-like structure where the 5′ and 3′ ends are juxtaposed (*12*). Nevertheless, self-ligation activity has not been demonstrated in viroid sequences.

Peach latent mosaic viroid (PLMVd), a viroid of 338 nt, is the infectious agent responsible for peach latent mosaic disease (*13*). Transcripts of both polarities derived from PLMVd self-cleave exclusively by single hammerhead structures (*14, 15*). Prior to this finding, only satellite RNAs were known to undergo self-cleavage by single hammerhead structures. In light of this observation, and considering that only satellite RNAs have been shown to undergo self-ligation, PLMVd was a candidate of choice to investigate whether self-ligation might occur within a viroid sequence. The pPD1 clone used in the present study includes an insert of two tandemly repeated PLMVd sequences (Fig. 1A, ref. *14*). Upon digestion with restriction enzyme *Eco*RI or *Bam*HI (Pharmacia), these constructions allowed the synthesis of either plus or minus transcripts, depending upon the RNA polymerase used (T3 or T7, Promega). During *in vitro* transcription RNA of both polarities possessing hammerhead sequences were both produced and observed to self-cleave efficiently at both the 5′ and 3′ sites (Fig. 1A, ref. *14*). The self-cleavage permitted the isolation of monomeric PLMVd of 338 nt with both 2′,3′-cyclic phosphate and 5′-hydroxyl termini.

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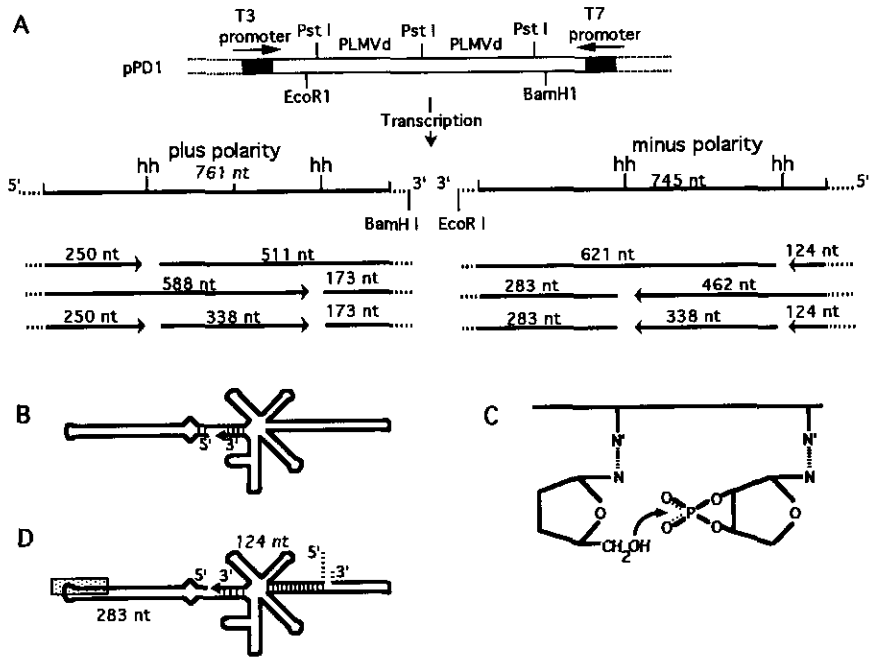

FIG. 1. Schematic representation of plasmid construction, transcriptional products, and nonenzymatic self-ligations of PLMVd. (A) pPD1 construction and RNA fragments produced by *in vitro* transcription. Details of the construction of pPD1 plasmid were reported previously (*14*). For the preparation of plus-strand RNA, the pPD1 clone was digested with *Bam*HI and transcribed with T3 RNA polymerase, while minus-strand RNA was obtained by *Eco*RI digestion followed by T7 RNA polymerase-catalyzed transcription. Full lines indicate sequences from PLMVd, and dashed lines sequences from vector. hh indicates hammerhead self-cleavage sites, and the length of each cleaved fragment in nucleotides (nt) is indicated. An arrow at the transcript end denotes a 2′,3′-cyclic phosphate. (B) Schematic representation of the lower energy secondary structure of the plus-polarity monomeric RNA of PLMVd (*14*) during intramolecular self-ligation. Only the base-paired regions flanking the ligation site are represented in detail. The more stable secondary structure adopted by the both plus- and minus-polarity transcripts have been predicted by computer analysis using the program MulFold. (C) Schematic representation of the proposed molecular mechanism for self-ligation. The arrow shows the nucleophilic attack of the 5′-hydroxyl group on the 2′,3′-cyclic phosphate. N and N′ represent hybridized bases. (D) Schematic representation of the lower energy secondary structure of minus-polarity transcripts of 283 and 124 nt hybridized together for intermolecular ligation. As in A, full lines indicate sequences from PLMVd, dashed lines sequences from vector, and only the intermolecular base-paired regions are represented by ladders. The dotted box indicates the sequence complementary to the oligonucleotide used to determine the proportion of 2′,5′ and 3′,5′ isomer produced by nonenzymatic intermolecular ligation (see text).

The incubation of purified minus-strand PLMVd monomer transcripts with wheat germ RNA ligase, the enzyme initially suggested as responsible for viroid ligation (*5, 7*), produced approximately 50% circularization. This was visualized by gel retardation of the circular RNA (C) in comparison to the linear conformers (L) (Fig. 2A, lane 2). When unit-length minus transcripts were incubated in protein-free conditions at 16° for 6 hr in 20 m*M* Tris–HCl, pH 7.9, and 100 m*M* $MgCl_2$, 5% of the RNA with the same slower electrophoretic mobility as that produced by the wheat germ ligase was detected (Fig. 2A, lane 3). When the magnesium was replaced by 1 m*M* EDTA, only 0.3% of the same product was detectable (Fig. 2A, lane 4). Analysis of the protein-free ligation mixture by native 5% PAGE revealed that the reaction products have the same length as the linear reactants (Fig. 2B) and, therefore, the difference observed by gel retardation resulted from the presence of two conformations, namely, the circular and linear conformers. In addition, the end labeling of the reaction products by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP, or by T4 RNA ligase in the presence of [α-^{32}P]pCp (after the dephosphorylation of nonradioactive purified self-ligation products), was not possible, thereby supporting the hypothesis that the newly formed molecules are circular (data not shown). Taken together these results prompt the conclusion that self-ligation occurs intramolecularly to 5% within monomeric transcripts *in vitro* (Fig. 1B). The monomeric transcripts of plus polarity also self-ligated with a similar efficiency (Fig. 2B, lane 6; 5.0%). Under the conditions used, self-ligation time course experiments showed a linear increase in the amount of product with time (~0.9%/hr, Fig. 3A), and the reaction was observed to be substrate concentration independent (data not shown). The self-ligation observed *in vitro* may contribute to PLMVd circularization during rolling circle replication; however, this does not exclude the possibility that a host RNA ligase catalyzes the ligation steps *in vivo*. Like self-cleavage, self-ligation is probably an ancestral reaction, and the enzyme-catalyzed ligation evolved from this primitive mechanism.

As mentioned previously, the unit-length transcripts of

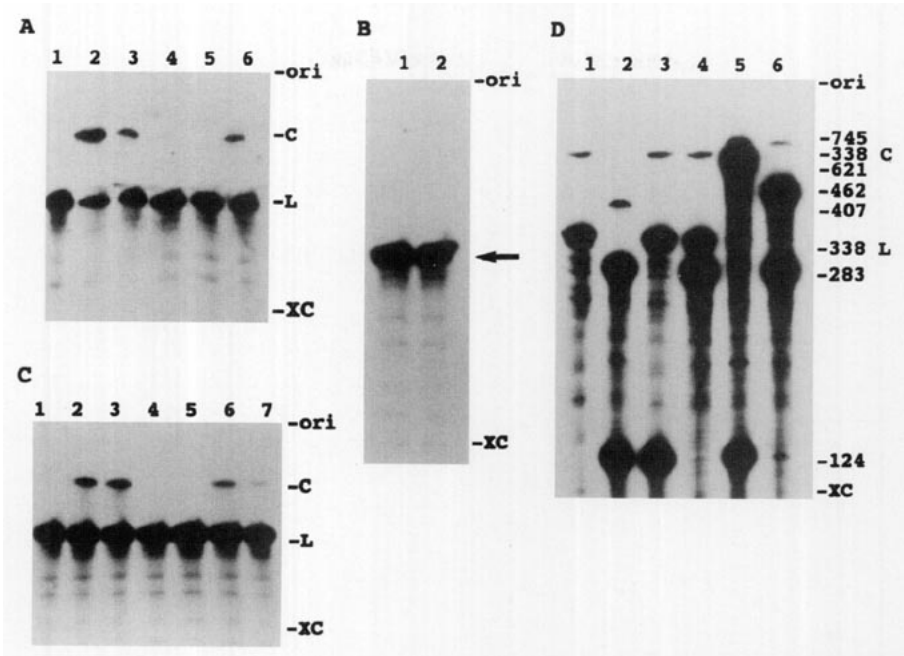

FIG. 2. Analysis of intra- and intermolecular nonenzymatic ligation. (A) Ligation of both monomeric minus- and plus-polarity transcripts of PLMVd analyzed on a denaturing 5% PAGE gel. Lanes 1–4 are the minus-polarity transcripts while lanes 5 and 6 are the plus-polarity transcripts. Lanes 1 and 5 are the control lanes representing the untreated transcripts. *In vitro* transcription of pPD1 in the presence or absence of [α-^{32}P]UTP (3000 Ci/mmol, Amersham) was performed as reported previously (*14*). After transcription, RNAs were ethanol precipitated and resuspended in 10 μl H_2O plus 10 μl stop buffer (0.3% each of bromophenol blue and xylene cyanol, 10 m*M* EDTA, pH 7.5, and 97.5% deionized formamide), denatured for 2 min at 65°, and separated on a 5% polyacrylamide gel containing 100 m*M* Tris–borate, pH 8.3, 1 m*M* EDTA, and 7 *M* urea buffer. Transcripts were detected by autoradiography or UV shadowing, excised, eluted, ethanol precipitated, passed twice through Sephadex G-50 spun columns, and lyophilized. Lane 2, ligation catalyzed by RNA ligase purified from raw wheat germ (according to the procedure reported in ref. *17*) for 6 hr at 31° in 20 m*M* Tris–HCl, pH 7.9, 6 m*M* $MgCl_2$, 1 m*M* ATP, 20 μ*M* DTT supplemented with 34 U RNA guard (Pharmacia) in a final volume of 50 μl. Lanes 3 and 6, self-ligation of transcripts via incubation for 6 hr at 16° in a final volume of 15 μl containing 20 m*M* Tris–HCl, pH 7.9, and 100 m*M* $MgCl_2$. Lane 4, self-ligation as in lane 3 but with the 100 m*M* $MgCl_2$ replaced by 1 m*M* EDTA. The ligations were stopped and analyzed on denaturing 5% PAGE. ori, origin; XC, xylene cyanol. C and L indicate the migration position of the circular and linear transcripts. (B) Self-ligation of PLMVd monomeric minus-polarity transcripts, performed as described in Fig. 2A but analyzed on a native 5% PAGE (i.e., without urea, at 4°). Lane 1 is the untreated transcripts while lane 2 is the self-ligation in presence of 100 m*M* $MgCl_2$. The arrow indicates the monomeric transcripts. The reaction was stopped by adding 0.5 vol of 50% glycerol, 0.4% bromophenol blue, and 0.4% xylene cyanol solution and then electrophoresed. (C) Investigation of the self-ligation mechanism within the monomeric plus-polarity transcripts. All ligation reactions were performed 16° for 6 hr in 20 m*M* Tris–HCl, pH 7.9, and 100 m*M* $MgCl_2$. Lane 1, untreated transcripts. Lane 2, self-ligation. Lane 3, self-ligation in the presence of 1 m*M* ATP. Lanes 4 and 5, transcripts were either 5′ kinased or 3′ dephosphorylated, respectively, by T4 polynucleotide kinase (Pharmacia), phenol extracted, ethanol precipitated, ethanol washed, dried, and then tested for self-ligation. Lanes 6 and 7, transcripts were snap-cooled in the absence or presence, respectively, of 67% formamide before the self-ligation. (D) Assays of intermolecular ligation with minus-polarity transcripts and analysis by denaturing 5% PAGE. In lane 1 the 338-nt transcripts were incubated alone, while in lane 2 (124 and 283 nt), lane 3 (124 and 338 nt), lane 4 (338 and 283 nt), lane 5 (124 and 621 nt), and lane 6 (462 and 283 nt) the indicated transcript pairs were incubated together under self-ligation conditions as in C. Adjacent to the gel are the positions of each fragment (in nt) determined by comigration of purified PLMVd dimeric RNA incubated under self-cleavage conditions. The fraction of ligated transcripts was determined by analysis of dried gels using a PhosphorImager (Molecular Dynamics).

plus polarity were also capable of self-ligation to 5% (Fig. 2A, lane 6, and Fig. 2C, lane 2). The addition of ATP (1 m*M* final) did not increase the observed product amounts (Fig. 2C, lane 3), suggesting the involvement of the phosphate from the 2′,3′-cyclic phosphate in the formation of the phosphodiester bond. Neither 5′-phosphorylated or 3′-dephosphorylated linear unit-length transcripts, produced by the action of T4 polynucleotide kinase in the presence or absence (*16*) of ATP, respectively, self-ligated (Fig. 2C, lanes 4 and 5). This suggests a requirement for both the 5′-hydroxyl and the 2′,3′-cyclic phosphate groups in self-ligation. As proposed by Sharmeen *et al.* (*12*), most likely the 5′-hydroxyl group is involved in a nucleophilic attack on the phosphate of the 2′,3′-cyclic phosphate, which then undergoes hydrolysis producing a phosphodiester bond (Fig. 1C). The 2′,3′-cyclic phosphate is highly strained and will undergo rapid hydrolysis with a large negative standard enthalpy change (*18*).

The hammerhead self-cleavage of PLMVd transcripts of both polarities required a preliminary heat denaturation followed by snap-cooling to allow conformational transition from the native to the autocatalytic structure (*15*). This transition was enhanced by the addition of formamide (*15*). When unit-length transcripts were submitted to preliminary heat denaturation and snap-cooling (90° for 1 min followed by ice for 1 min), the self-ligation efficiency was reduced by half compared to self-ligation

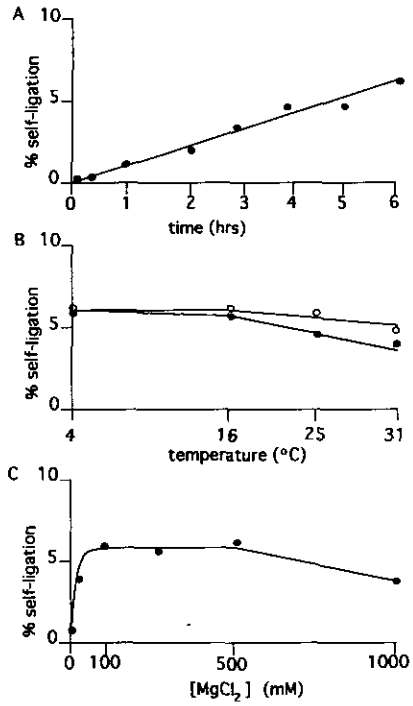

FIG. 3. Kinetic analysis of the self-ligation of the minus-polarity transcripts. (A) Time course. (B) Temperature dependence. Closed circles are for transcripts which were not subjected to any procedure of denaturation and renaturation before the self-ligation (usual treatment), while open circles indicate that transcripts were denatured (90° for 2 min) and then renatured by slowly decreasing the temperature until 16° before the self-ligation. (C) $MgCl_2$ dependence. Self-ligation was performed as indicated for Fig. 2, lane 3. The average values of triplicate experiments are reported.

without pretreatment (Fig. 2C, lane 6; 3%). In the presence of 67% formamide during the pretreatment only a trace of the self-ligation products were detectable (Fig. 2C, lane 7; 0.2%). In contrast, when the transcripts were denatured (90° for 2 min), and then renatured by slowly decreasing the temperature to 16° before the self-ligation in order to favor the adoption of the most stable structures, the reaction efficiency was slightly increased (Fig. 3B). Taken together, these results suggest that the most stable structure supports the self-ligation process (see Fig. 1B). In this structure, both donor and receptor ends are juxtaposed by a complementary strand that plays a structural role and must not be considered as a ribozyme (Figs. 1B and 1C).

Investigation of the self-ligation reaction's magnesium dependence showed increasing amounts of products, up to a maximum of 5% for 6 hr of incubation, with an increase in $MgCl_2$ concentration from 0 to 100 m*M* (Fig. 3C). The level of products then remained constant at 5% from 100 to 500 m*M* and decreased by about half at 1 *M* $MgCl_2$. In Fig. 2A, the mixture analyzed in lane 4 suggested that in the absence of magnesium, no self-ligation occurred. However, overexposure of the autoradiogram revealed a small amount of product (0.3%), indicating that the magnesium cation is not absolutely required. Several other biochemical parameters of the reaction were investigated, including the temperature (Fig. 3B) and the pH dependence (data not shown). All data supported the proposal of a nucleophilic attack from the 5′-hydroxyl group on the 2′,3′-cyclic phosphate group after both strands were juxtaposed by the complementary strand (Fig. 1C); these features are necessary and sufficient for the nonenzymatic ligation to occur.

In order to investigate intermolecular ligation (Fig. 1D), various RNA species of minus polarity generated by transcription from pPD1 were incubated together in protein-free conditions (Fig. 2D). Intermolecular ligation always occurred when a 2′,3′-cyclic phosphate group and a 5′-hydroxyl terminus were juxtaposed by a complementary strand. The minus-polarity transcripts of 462, 338, and 124 nt can be considered phosphate donor strands since they carry a 2′,3′-cyclic phosphate group. The 462- and 124-nt transcripts cannot self-ligate intramolecularly, even though they are phosphate donors, due to their secondary structure which will not juxtapose the end groups. Unlike the intramolecular self-ligation, linear RNA products are generated by the intermolecular reaction (Fig. 2D). Incubation of 338-nt transcripts with either 124- or 283-nt transcripts showed the intramolecular process to be favored over the intermolecular reactions (Fig. 2D, lanes 3 and 4). These observations were expected because of the intrinsic folding capacity of the monomeric transcripts (*14*) which played a phosphate donor role for the ligation with the transcripts of 283 nt and an acceptor role when ligated with the 124-nt transcripts. The intermolecular ligation was also examined with PLMVd transcripts of plus polarity and similar results were obtained (data not shown). The intermolecular self-ligation might occur with some frequency in nature and could therefore contribute to viroid sequence evolution by a process of reassortment. Such a mechanism would allow the integration of exogenous genetic information into viroids during their rolling circle replication (*19*).

In order to examine the nature of the phosphodiester bond formed during this ligation, we evaluated the proportion of 2′,5′ and 3′,5′ isomers produced in accordance with the procedure of Sharmeen *et al.* for HDV (*12*). Intermolecular ligation was performed with nonradioactive 124- and 283-nt transcripts. The ligation products (407 nt) were then hybridized to an oligonucleotide (5′CATCAAAAGTTTCGCCGCATTTCAG3′) complementary to positions 25 to 49 downstream of the ligation site (Fig. 1D, dotted box). The resulting complex was then primer extented using avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim, Canada) as described (*20, 21*), and the products were analyzed by denaturing 5% PAGE (data not shown). Under these conditions, for reasons which are not clear (*12*), the reverse transcriptase cannot read through the 2′,5′ isomer and extension terminates at the ligation site or one nucleotide

beyond. Concurrently, primer extension using the transcripts of 462 nt produced by T7 RNA polymerase, which catalyzed exclusively 3′,5′-phosphodiester bond formation, was performed as control. The analysis of several assays established a ratio of ~30% 3′,5′:~70% 2′,5′-phosphodiester bonds being produced by self-ligation. This is in agreement with another report showing the prevalence of 2′,5′-phosphodiester bond formation over 3′,5′-phosphodiester bonds in RNA self-ligation (*22*). Furthermore, when the self-ligated PLMVd is snap-cooled (95° for 1 min followed by ice for 1 min) and then incubated at 37° for 15 min (i.e., hammerhead self-cleavage conditions), only 9% of the circular RNA self-cleaved compared to 54% for the linear RNA of 462 nt produced by *in vitro* transcription (data not shown). These results support the presence of mostly 2′,5′ isomer, which is not hammerhead self-cleaved in the circular RNA. To date, no 2′,5′-phosphodiester bond has been characterized in viroids isolated from plant hosts, leading us to two hypotheses. On one hand, it is possible that a cofactor, such as a protein, could sterically block the formation of the 2′,5′-phosphosdiester bond *in vivo* (*12*). On the other hand, the presence of a 2′,5′-phosphodiester bond at the hammerhead cleavage site may prevent linearization of the newly formed circular RNAs. However, this latest hypothesis requires that the viroid replicase activity is allowed to read through the 2′,5′ isomer, which appears highly improbable, but not impossible.

In accordance with the role of the complementary strand to juxtapose both reactional termini, the efficiency of the reaction could be regulated by the stability (sequence composition and length) of both of the double-stranded helices adjacent to the ligation site; the more stable the helices, the more efficient the ligation. For PLMVd, these helices are relatively small (i.e., 2 and 4 base pairs in the rod-like secondary structure, Fig. 1B) and may therefore limit self-ligation efficiency. In contrast, the fact that the small adjacent base-paired regions in PLMVd allow for little self-ligation may be considered as an advantage for sequence reassortment via the intermolecular process. Only a reduced sequence complementarity would be required between two RNA molecules for the intermolecular reaction to occur. In the example in Fig. 1D, the substrates are hybridized by a 4-base pair helix adjacent to the ligation site and a 12-base pair helix in the right arm region.

In conclusion, the self-ligation observed *in vitro* may contribute to PLMVd circularization during rolling circle replication *in vivo;* however, this does not exclude the possibility that a host RNA ligase catalyzes the ligation steps *in vivo*. The self-ligation may not be restricted to PLMVd and HDV, because other viroids and related satellite RNAs have a similar predicted secondary structure for the region surrounding the ligation site.

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