JOURNAL OF VIROLOGY, Oct. 2008, p. 10321–10325 Vol. 82, No. 20
0022-538X/08/$08.00+0 doi:10.1128/JVI.01229-08
Copyright © 2008, American Society for Microbiology. All Rights Reserved.

Monomeric Linear RNA of *Citrus Exocortis Viroid* Resulting from Processing In Vivo Has 5′-Phosphomonoester and 3′-Hydroxyl Termini: Implications for the RNase and RNA Ligase Involved in Replication▽

María-Eugenia Gas, Diego Molina-Serrano, Carmen Hernández, Ricardo Flores, and José-Antonio Darròs*

Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV), Avenida de los Naranjos, 46022 Valencia, Spain

Received 13 June 2008/Accepted 4 August 2008

Members of the family *Pospiviroidae*, like *Citrus exocortis viroid* (CEVd), replicate through an RNA-based asymmetric rolling-circle mechanism in which oligomeric plus-strand [(+)] RNA intermediates are cleaved to monomeric linear (*ml*) RNA and then circularized. Here we show, by rapid amplification of 5′ and 3′ cDNA ends and in vitro ligation assays, that *ml* CEVd (+) RNA resulting from cleavage of a dimeric transcript transgenically expressed in *Arabidopsis thaliana* contains 5′-phosphomonoester and 3′-hydroxyl termini. The nature of these termini and the double-stranded structure previously proposed as the substrate for cleavage in vivo suggest that a type III RNase catalyzes cleavage and an RNA ligase distinct from tRNA ligase promotes circularization.

Viroids are plant pathogens consisting of a non-protein-coding small circular RNA (246 to 401 nucleotides [nt]) (5, 8, 13, 26). The approximately 30 viroid species known are classified into two families, *Pospiviroidae* and *Avsunviroidae*, whose members replicate in the nucleus and chloroplast, respectively (13). Viroid replication occurs through an RNA rolling-circle mechanism characterized by the reiterative RNA-RNA transcription of circular templates and processing of the resulting oligomeric RNAs of one or both polarities (2). In members of the family *Pospiviroidae*, like *Potato spindle tuber viroid* (PSTVd), the most abundant circular RNA in vivo to which plus-strand [(+)] polarity is assigned arbitrarily, is transcribed into complementary oligomeric minus-strand [(−)] RNAs. These strands serve directly as templates for the synthesis of oligomeric (+) RNAs that are processed into monomeric linear (*ml*) and monomeric circular (*mc*) RNAs (1). In members of the family *Avsunviroidae*, like *Avocado sunblotch viroid*, the oligomeric (−) RNAs are processed into *mc* (−) RNAs, which through a second rolling circle direct the synthesis of oligomeric (+) RNAs, with subsequent processing producing the *mc* (+) RNAs (7). Whereas in the family *Avsunviroidae*, cleavage of the oligomeric RNAs of both polarities is mediated by hammerhead ribozymes that generate *ml* RNAs with 5′-hydroxyl (5′-OH) and 2′,3′-cyclic phosphodiester (2′,3′>P) termini (11), how this step happens in the family *Pospiviroidae* is unclear. Previous work showed that viroid RNA processing occurs accurately in transgenic lines of the viroid nonhost *Arabidopsis thaliana* expressing dimeric transcripts of representative members of the family *Pospiviroidae* (6). With this experimental system, the processing site of oligomeric (+) RNA intermediates has been mapped at a position within a conserved double-stranded structure that two consecutive hairpin I motifs can promote (Fig. 1) (14).

Here, using transgenic *A. thaliana* expressing a dimeric transcript of *Citrus exocortis viroid* (CEVd) (genus *Pospiviroid*, family *Pospiviroidae*), we have determined the termini of the *ml* (+) RNA accumulating in vivo by rapid amplification of 5′ and 3′ cDNA ends (5′- and 3′-RACE) (25) and by in vitro ligation assays. The nature of these termini, 5′-phosphomonoester (5′-P) and 3′-OH, has deep implications concerning the host enzymes that mediate processing of the replicative intermediates.

5′- and 3′-RACE of the *ml* CEVd (+) RNA. An RNA preparation enriched in viroid RNAs was obtained by chromatography on nonionic cellulose CF11 (21) from an *A. thaliana* transgenic line expressing constitutively a dimeric head-to-tail CEVd (+) transcript (sequence variant with GenBank nucleotide sequence accession number M34917 with a deleted G between positions 70 and 74) starting at position 40 and ending at position 39 (6). The preparation was separated by double polyacrylamide gel electrophoresis (PAGE), first in native conditions and then in denaturing conditions (12), and the fraction of RNAs comigrating in the region of a *ml* CEVd (+) RNA standard purified from CEVd-infected gynura (*Gynura aurantiaca* DC) was eluted. Northern blot hybridization revealed that the *ml* (+) RNA was the only CEVd species present in the fraction (data not shown). To characterize the 5′ terminus of the *ml* CEVd (+) RNA, three aliquots of this fraction were ligated to the adaptor oligoribonucleotide PI [5′-r(CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA)-3′, where r indicates that the sequence corresponds to an oligoribonucleotide] with T4 RNA ligase 1 (Epicentre) that requires 5′-P and 3′-OH termini. The first aliquot did not receive any previous treatment, while the second was pretreated with T4 polynucleotide kinase (Roche) and the third was pretreated with calf alkaline phosphatase (Roche). The ligation products were used as templates in reverse transcription (RT) reactions with Superscript III reverse transcriptase

* Corresponding author. Mailing address: Instituto de Biología Molecular y Celular de Plantas (CSIC-UPV), Avenida de los Naranjos, 46022 Valencia, Spain. Phone: 34 963877893. Fax: 34 963877859. E-mail: jadaros@ibmcp.upv.es.
▽ Published ahead of print on 13 August 2008.

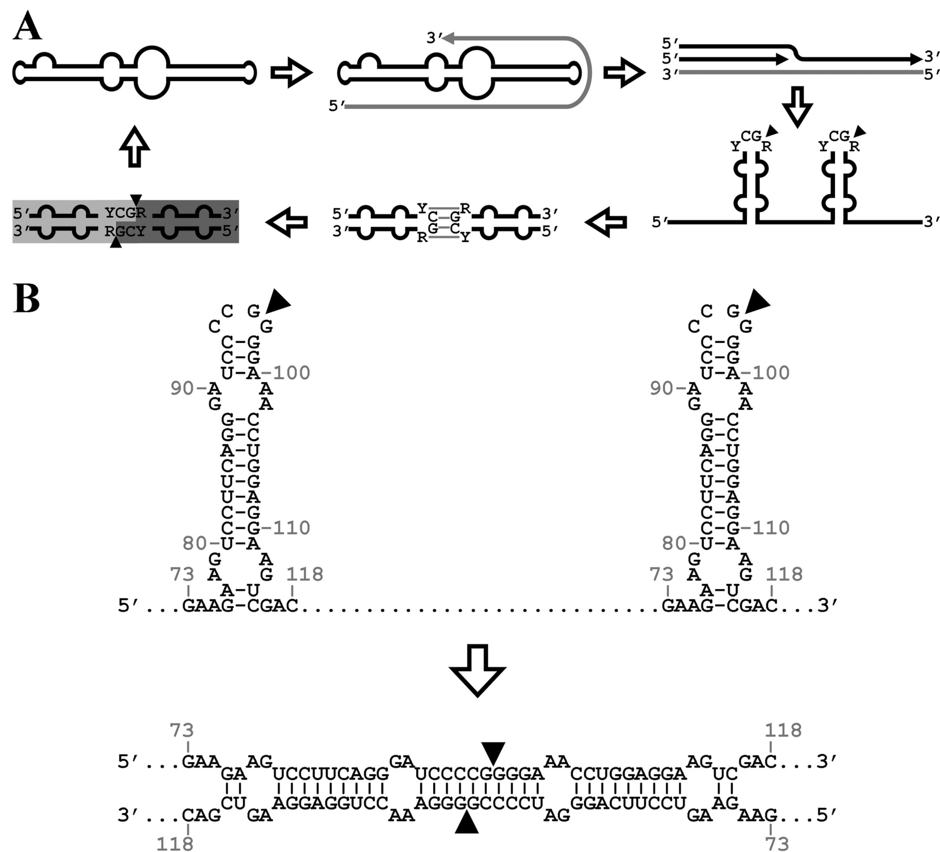

FIG. 1. (A) Asymmetric rolling-circle replication cycle followed by members of the family *Pospiviroidae* that includes the kissing-loop interaction between two contiguous hairpin I motifs and the subsequent formation of a double-stranded structure proposed to be the substrate for cleavage (14). (B) Hairpin I and double-stranded structure corresponding to CEVd (+) RNA. Black arrowheads indicate the processing sites located 2 nt apart in each strand. Nucleotide numbering refers to CEVd sequence variant (with GenBank sequence number M34917) with a deleted G between positions 70 and 74.

(Invitrogene) and the viroid-specific primer PII (5′-GCTTCA GCGACGATCGGATGTGGAGCC-3′) complementary to CEVd sequence between positions 203 and 229 and then subjected to PCR amplification with the High Expand DNA polymerase mixture (Roche), an adaptor-specific primer PIII (5′-GGAGCACGAGGACACTGACATGG-3′) with the same sequence as that underlined in PI and a nested viroid-specific primer PIV (5′-TTCTCCGCTGGACGCCAGTGATCCGC-3′) complementary to CEVd sequence between positions 147 and 172 (Fig. 2A, left). Separation of RT-PCR products by PAGE showed amplification of a single prominent cDNA in the reaction mixture containing the untreated aliquot and in the mixture pretreated with polynucleotide kinase (Fig. 2B, lanes 1 and 3). Conversely, no amplification products were detected in the reaction mixture containing the aliquot pretreated with alkaline phosphatase (Fig. 2B, lane 2). The amplification products resulting from the untreated and polynucleotide kinase-treated aliquots were eluted from the gel and cloned in plasmid pTZ57R/T (Fermentas). Sequencing of several clones corresponding to each product showed that they were identical and consisted of a 115-bp DNA that, in addition to the adaptor, included the CEVd sequence from G97 to A172. This result confirmed that processing of the dimeric CEVd (+) RNA occurs between positions G96 and G97 as previously determined by primer extension (14), and it also showed that the resulting *ml* (+) RNA intermediate has a 5′-P terminus, since it was ligated to the adaptor without pretreatment with polynucleotide kinase but not after pretreatment with alkaline phosphatase.

This approach was extended to two additional members of the family *Pospiviroidae* expressed in transgenic *A. thaliana* (6): *Apple scar skin viroid* and *Hop stunt viroid*, the type species of the genera *Apscaviroid* and *Hostuviroid*, respectively (13). The results showed that the 5′ termini of the purified *ml* viroid (+) RNAs also have a 5′-P group and confirmed the processing sites between positions G90 and A91 (*Apple scar skin viroid*, sequence variant with GenBank accession number AF421195) and G82-G83 (*Hop stunt viroid*, sequence variant with GenBank accession number D13764) (data not shown) as previously identified by primer extension (14). The same strategy was finally applied to the *ml* CEVd (+) RNA purified from infected gynura. In this case, six or seven cDNAs were amplified, indicating that the population of *ml* CEVd (+) RNA is more heterogeneous in an infected plant. However, this population included the 115-bp product, thus confirming the presence of an RNA starting and ending at positions G97 and G96, respectively, and with a 5′-P terminus.

To characterize the 3′ terminus of the *ml* CEVd (+) RNA

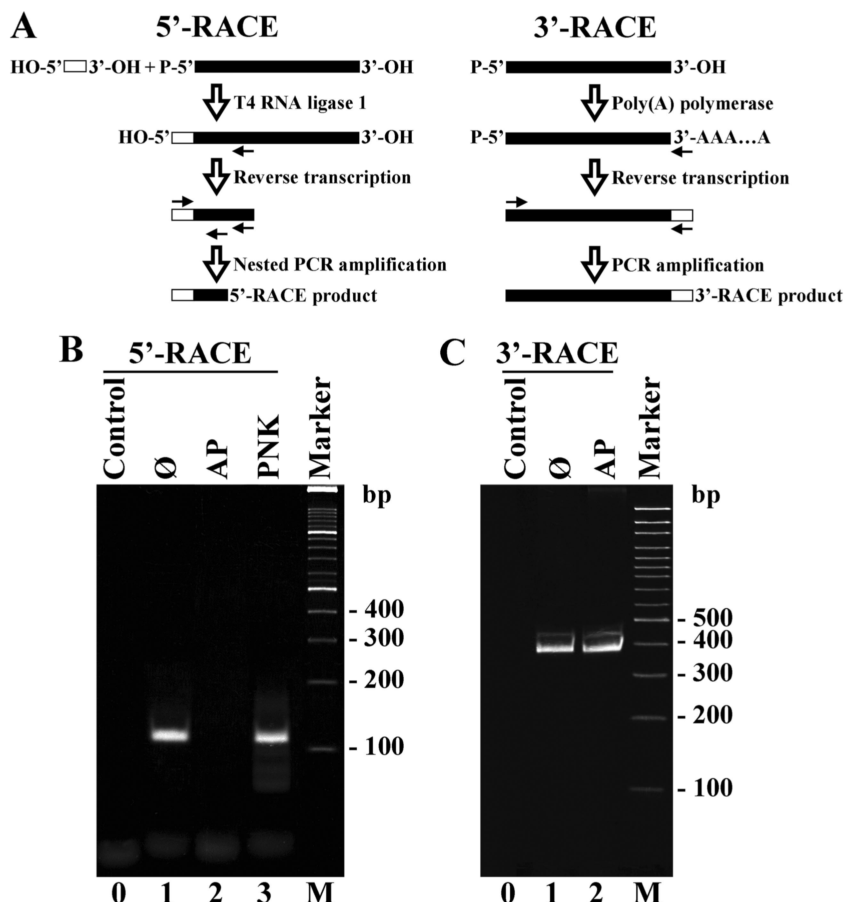

FIG. 2. Analysis of the 5′ and 3′ termini of the *ml* CEVd (+) RNA purified from transgenic *A. thaliana* expressing a dimeric CEVd (+) transcript. (A) Scheme depicting 5′- and 3′-RACE procedures. (B and C) RNA aliquots not treated or subjected to different pretreatments were subjected to 5′-RACE (B) and 3′-RACE (C), with the resulting products being separated by PAGE. Lane 0, control without RNA; lane 1, untreated RNA (Ø); lanes 2 and 3, RNA treated with alkaline phosphatase (AP) and polynucleotide kinase (PNK), respectively; lane M, DNA markers (M) with sizes (in base pairs) indicated to the right of the gels.

purified from transgenic *A. thaliana*, two aliquots of the preparation were 3′ polyadenylated with yeast poly(A) polymerase (USB) that adds AMP residues to an RNA with a terminal 3′-OH group. The first aliquot did not receive any previous treatment, while the second was pretreated with alkaline phosphatase. The polyadenylation products were subjected to RT with a poly(A) tail-complementary primer PV (5′-CCGGATC CTCTAGATCGGCCGCT$_{17}$V-3′, where V is A, C, or G) and to PCR amplification with this same primer and the CEVd-specific PVI primer (5′-GGAAACCTGGAGGAAGTCG-3′) homologous to the CEVd sequence between positions 98 and 116 (Fig. 2A, right). PAGE separation of the resulting RT-PCR products showed the amplification of the same two DNAs in both reaction mixtures (Fig. 2C, lanes 1 and 2). The cDNAs amplified from the untreated RNA aliquot were eluted from the gel and cloned. Sequencing of several clones revealed that the largest and less abundant product was a 408-bp DNA including the CEVd sequence from G98 to U370 and from C1 to G96 and that the smaller and most abundant product was a 362-bp DNA including the CEVd sequence from G98 to U370 and from C1 to G50. The sequence of the largest amplification product confirmed again that processing of oligomeric CEVd (+) RNA in transgenic *A. thaliana* occurs between positions G96 and G97 (14). Moreover, since the *ml* CEVd (+) RNA was polyadenylated without any pretreatment, this RNA must contain a 3′-OH free terminus, together with the already characterized 5′-P. On the other hand, the sequence of the smaller amplification product predicted a second 3′-OH free terminus at position G50 that had not been detected in previous experiments (14). The observation that CEVd contains a poly(A)-rich sequence (positions 51 to 61) next to G50 suggests that the smaller amplification product most likely resulted from hybridization of the poly(dT) 3′ tail of primer PV with this poly(A)-rich region and that, consequently, position G50 does not actually correspond to a *ml* CEVd (+) RNA terminus.

In vitro ligation assays. Next, the susceptibility of the *ml* CEVd (+) RNA purified from transgenic *A. thaliana* to be ligated by two enzymes with distinct specificities was assayed: the T4 RNA ligase 1 (Rnl1) that requires 5′-P and 3′-OH termini (9, 28, 29) and the *A. thaliana* tRNA ligase that requires 5′-OH and 2′,3′>P termini (10) (Fig. 3A). Additionally, *ml* CEVd (+) RNA purified from CEVd-infected gynura, as well as RNA synthesized in vitro by transcription and subsequent treatment with tobacco acid pyrophosphatase (Epi-

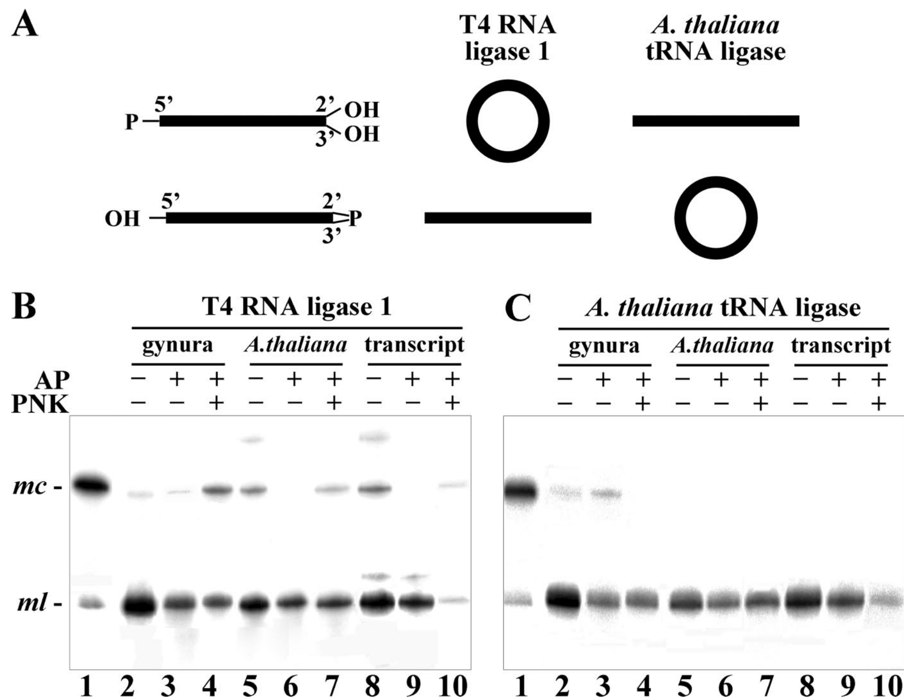

FIG. 3. (A) Diagram depicting results of ligation by T4 RNA ligase 1 and *A. thaliana* tRNA ligase of two RNAs with different termini. (B and C) Ligation in vitro of *ml* CEVd (+) RNA purified from infected gynura and transgenic *A. thaliana* expressing a dimeric CEVd (+) transcript. Products generated by T4 RNA ligase 1 (B) and *A. thaliana* tRNA ligase (C) were separated by denaturing PAGE and analyzed by Northern blot hybridization. Prior to ligation, aliquots of *ml* CEVd (+) RNA from infected gynura (lanes 2 to 4) or transgenic *A. thaliana* (lanes 5 to 7) and *ml* CEVd (+) RNA synthesized in vitro with 5′-P and 3′-OH termini (lanes 8 to 10) were not treated (−) (lanes 2, 5, and 8) or pretreated (+) with alkaline phosphatase (AP) (lanes 3, 6, and 9) or pretreated (+) with alkaline phosphatase and polynucleotide kinase (PNK) (lanes 4, 7, and 10). Lane 1, RNA from infected gynura containing *mc* and *ml* CEVd (+) RNAs with their positions indicated to the left of the gels. The product with the lowest mobility in lanes 5 and 8 most likely corresponds to the dimeric linear CEVd RNA.

centre)—resulting in a full-length *ml* CEVd (+) RNA from positions G97 to U370 and from C1 to G96 with 5′-P and 3′-OH termini—were used as a controls. Aliquots of these three RNAs that were either untreated, pretreated with alkaline phosphatase, or pretreated first with alkaline phosphatase and then with polynucleotide kinase were assayed for ligation with T4 RNA ligase 1 and *A. thaliana* tRNA ligase. For this purpose, a recombinant version of the latter (GenBank accession number NP_172269) with a carboxy-terminal His tag was expressed in *Escherichia coli* and purified. Ligation products were separated by denaturing PAGE and examined by Northern blot hybridization with a cRNA probe labeled with ^{32}P. The *ml* CEVd (+) RNA from infected gynura was circularized by the T4 RNA ligase 1 in all instances (Fig. 3B, lanes 2 to 4), whereas that from transgenic *A. thaliana* was ligated when untreated (Fig. 3B, lane 5) or pretreated with alkaline phosphatase plus polynucleotide kinase (Fig. 3B, lane 7), but not when pretreated only with alkaline phosphatase (Fig. 3B, lane 6). The *ml* CEVd (+) RNA control synthesized in vitro with 5′-P and 3′-OH termini behaved similarly to the RNA from *A. thaliana* (Fig. 3B, lanes 8 to 10).

On the other hand, *A. thaliana* tRNA ligase circularized, although with low efficiency, the *ml* CEVd (+) RNA from infected gynura (Fig. 3C, lane 2). Circularization of this RNA also occurred after pretreatment with alkaline phosphatase (Fig. 3C, lane 3), but not when additionally pretreated with polynucleotide kinase (Fig. 3C, lane 4). Neither the *ml* CEVd (+) RNA from transgenic *A. thaliana* (Fig. 3C, lanes 5 to 7) nor the CEVd (+) RNA synthesized in vitro with 5′-P and 3′-OH termini (Fig. 3C, lanes 8 to 10) were circularized by the *A. thaliana* tRNA ligase. Altogether, these results indicate that the *ml* CEVd (+) RNA from transgenic *A. thaliana* contains essentially 5′-P and 3′-OH termini, whereas its counterpart from infected gynura is more heterogeneous and composed by molecules with 5′-P and 3′-OH termini and with 5′-OH and 2′,3′>P termini. The 5-P termini of some molecules from gynura may be buried within the secondary structure, thus explaining their circularization with T4 RNA ligase 1 after pretreatment with alkaline phosphatase (Fig. 3B, lane 3).

Termini of the native *ml* (+) RNAs from the family *Pospiviroidae*: comparison with previous data. The identification of 5′-P and 3′-OH termini in the *ml* (+) RNA intermediate processed in transgenic *A. thaliana* expressing a dimeric CEVd (+) transcript is consistent with data from an early report with *Chrysanthemum stunt viroid*, indicating that 72% of *ml Chrysanthemum stunt viroid* (+) RNA purified from infected plants contains a 5′-P terminus (23). In addition, *ml* CEVd (+) RNA with 5′-P and 3′-OH termini obtained by in vitro transcription and treatment with alkaline phosphatase and polynucleotide kinase was shown to be highly infectious (24). However, subsequent studies on the nature and biological significance of the *ml* PSTVd (+) RNA based on the 5′-end labeling of this species isolated from infected tissue (22), on its ligation in vitro by wheat germ and *Chlamydomonas reinhardtii* RNA ligases (3, 19), and on the infectivity of *ml* PSTVd (+) RNA produced by

artificially nicking the circular forms (16) led to the proposal that the *ml* PSTVd (+) RNA intermediate has 5′-OH and 2′,3′>P termini. However, the present results and those from a previous work aimed at characterizing the in vivo processing site of the oligomeric (+) RNAs of CEVd and two other members of the family *Pospiviroidae* (14) indicate that the *ml* CEVd (+) RNA population in transgenic *A. thaliana* is mostly homogeneous and representative of the product resulting from the processing in vivo of the dimeric transcript, possibly because viroid replication in this nonhost plant is low and RNA-RNA amplification of the *mc* CEVd (+) RNA is inefficient (6, 14). In contrast, in a host plant like gynura, the *mc* CEVd (+) RNA serves as a template for efficient RNA-RNA amplification and the population of the replicative intermediate *ml* CEVd (+) is contaminated with nicked by-products of the most abundant *mc* CEVd (+) RNA (14). Therefore, the experimental system based on transgenic *A. thaliana* offers the advantage that it can be programmed with a replication intermediate (6), and the fate of the intermediate can be monitored with little interference resulting from degradation of the *mc* viroid (+) RNA in vivo or in vitro during extraction and purification. Despite the fact that the present results suggest that the *ml* CEVd (+) RNA forms with 5′-OH and 2′,3′>P termini from gynura arise from nicking of the most abundant *mc* CEVd (+) RNA, the possibility that a fraction of these molecules may participate in the replication cycle cannot be discarded.

Host factors involved in viroid RNA processing. In the family *Avsunviroidae*, hammerhead ribozymes catalyze self-cleavage of the oligomeric RNAs of both polarities in vivo (11). However, ribozyme-mediated RNA processing does not seem to operate in the family *Pospiviroidae* (27), and accordingly, the current consensus is that cleavage and ligation of the oligomeric (+) RNA intermediates are catalyzed by host enzymes. A good candidate to mediate cleavage is a type III RNase, because the 5′-P and 3′-OH termini identified here in the *ml* CEVd (+) RNA are those characteristically produced by these enzymes that act on double-stranded or highly structured RNA substrates (20). Moreover, some type III RNases cleave both strands of their substrates, generating products of approximately 20 bp with 2-nt 3′-protruding termini (15). A conserved double-stranded structure, formed by a kissing-loop interaction between two hairpin I motifs contiguous in the oligomeric (+) RNA, has been proposed as the substrate for cleavage, with the cleavage sites in each strand being 2 nt apart (Fig. 1) (14). To date, in *A. thaliana*, seven type III RNases have been described, including four Dicer-like proteins (DCL1 to DCL4) and three additional ones (RTL1 to RTL3) (4, 17). Regarding ligation, the resulting *ml* CEVd (+) RNA with 5′-P and 3′-OH termini is not a substrate of the tRNA ligase, the only plant RNA ligase described so far (10). Therefore, unless these termini are later modified, the present results suggest the existence of a second plant RNA ligase that would mediate joining of the same 5′-P and 3′-OH termini as those required by the T4 RNA ligases 1 and 2 (18, 24, 28, 29).

We thank A. Ahuir for excellent technical assistance. Work in the J.-A.D. and R.F. laboratories was supported by the Ministerio de Educación y Ciencia (MEC) of Spain (grants BFU2005-06808/BMC and AGL2004-06311-C02-01) and by the Generalitat Valenciana (ACOMP07/268). M.-E.G. received predoctoral fellowships from MEC and CSIC-Bancaja, and D.M-S. received a predoctoral fellowship from MEC.

REFERENCES

1. **Branch, A. D., B. J. Benenfeld, and H. D. Robertson.** 1988. Evidence for a single rolling circle in the replication of potato spindle tuber viroid. Proc. Natl. Acad. Sci. USA **85:**9128–9132.
2. **Branch, A. D., and H. D. Robertson.** 1984. A replication cycle for viroids and other small infectious RNAs. Science **223:**450–455.
3. **Branch, A. D., H. D. Robertson, C. Greer, P. Gegenheimer, C. Peebles, and J. Abelson.** 1982. Cell-free circularization of viroid progeny RNA by an RNA ligase from wheat-germ. Science **217:**1147–1149.
4. **Comella, P., F. Pontvianne, S. Lahmy, F. Vignols, N. Barbezier, A. DeBures, E. Jobet, E. Brugidou, M. Echeverria, and J. Sáez-Vásquez.** 2008. Characterization of a ribonuclease III-like protein required for cleavage of the pre-rRNA in the 3′ETS in *Arabidopsis*. Nucleic Acids Res. **36:**1163–1175.
5. **Daròs, J. A., S. F. Elena, and R. Flores.** 2006. Viroids: an Ariadne's thread into the RNA labyrinth. EMBO Rep. **7:**593–598.
6. **Daròs, J. A., and R. Flores.** 2004. *Arabidopsis thaliana* has the enzymatic machinery for replicating representative viroid species of the family *Pospiviroidae*. Proc. Natl. Acad. Sci. USA **101:**6792–6797.
7. **Daròs, J. A., J. F. Marcos, C. Hernández, and R. Flores.** 1994. Replication of avocado sunblotch viroid: evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. Proc. Natl. Acad. Sci. USA **91:**12813–12817.
8. **Ding, B., and A. Itaya.** 2007. Viroid: a useful model for studying the basic principles of infection and RNA biology. Mol. Plant-Microbe Interact. **20:**7–20.
9. **El Omari, K., J. Ren, L. E. Bird, M. K. Bona, G. Klarmann, S. F. J. LeGrice, and D. K. Stammers.** 2006. Molecular architecture and ligand recognition determinants for T4 RNA ligase. J. Biol. Chem. **281:**1573–1579.
10. **Englert, M., and H. Beier.** 2005. Plant tRNA ligases are multifunctional enzymes that have diverged in sequence and substrate specificity from RNA ligases of other phylogenetic origins. Nucleic Acids Res. **33:**388–399.
11. **Flores, R., J. A. Daròs, and C. Hernández.** 2000. The Avsunviroidae family: viroids containing hammerhead ribozymes. Adv. Virus Res. **55:**271–323.
12. **Flores, R., N. Duran-Vila, V. Pallás, and J. S. Semancik.** 1985. Detection of viroid and viroid-like RNAs from grapevine. J. Gen. Virol. **66:**2095–2102.
13. **Flores, R., C. Hernández, A. E. Martínez de Alba, J. A. Daròs, and F. Di Serio.** 2005. Viroids and viroid-host interactions. Annu. Rev. Phytopathol. **43:**117–139.
14. **Gas, M. E., C. Hernández, R. Flores, and J. A. Daròs.** 2007. Processing of nuclear viroids in vivo: an interplay between RNA conformations. PLoS Pathog. **3:**1813–1826.
15. **Hammond, S. M.** 2005. Dicing and slicing—the core machinery of the RNA interference pathway. FEBS Lett. **579:**5822–5829.
16. **Hashimoto, J., K. Suzuki, and T. Uchida.** 1985. Infectivity of artificially nicked viroid molecules. J. Gen. Virol. **66:**1545–1551.
17. **Hiraguri, A., R. Itoh, N. Kondo, Y. Nomura, D. Aizawa, Y. Murai, H. Koiwa, M. Seki, K. Shinozaki, and T. Fukuhara.** 2005. Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in *Arabidopsis thaliana*. Plant Mol. Biol. **57:**173–188.
18. **Ho, C. K., and S. Shuman.** 2002. Bacteriophage T4 RNA ligase 2 (gp24.1) exemplifies a family of RNA ligases found in all phylogenetic domains. Proc. Natl. Acad. Sci. USA **99:**12709–12714.
19. **Kikuchi, Y., K. Tyc, W. Filipowicz, H. L. Sänger, and H. J. Gross.** 1982. Circularization of linear viroid RNA via 2′-phosphomonoester, 3′,5′-phosphodiester bonds by a novel type of RNA ligase from wheat-germ and *Chlamydomonas*. Nucleic Acids Res. **10:**7521–7529.
20. **MacRae, I. J., and J. A. Doudna.** 2007. Ribonuclease revisited: structural insights into ribonuclease III family enzymes. Curr. Opin. Struct. Biol. **17:**138–145.
21. **Pallás, V., A. Navarro, and R. Flores.** 1987. Isolation of a viroid-like RNA from hop different from hop stunt viroid. J. Gen. Virol. **68:**3201–3205.
22. **Palukaitis, P., and M. Zaitlin.** 1987. The nature and biological significance of linear potato spindle tuber viroid molecules. Virology **157:**199–210.
23. **Palukaitis, P., and R. H. Symons.** 1980. Purification and characterization of the circular and linear forms of chrysanthemum stunt viroid. J. Gen. Virol. **46:**477–489.
24. **Rigden, J. E., and M. A. Rezaian.** 1992. In vitro synthesis of an infectious viroid: analysis of the infectivity of monomeric linear CEV. Virology **186:** 201–206.
25. **Schaefer, B. C.** 1995. Revolutions in rapid amplification of cDNA ends: new strategies for polymerase chain-reaction cloning of full-length cDNA ends. Anal. Biochem. **227:**255–273.
26. **Tabler, M., and M. Tsagris.** 2004. Viroids: petite RNA pathogens with distinguished talents. Trends Plant Sci. **9:**339–348.
27. **Tsagris, M., M. Tabler, and H. L. Sänger.** 1987. Oligomeric potato spindle tuber viroid (PSTV) RNA does not process autocatalytically under conditions where other RNAs do. Virology **157:**227–231.
28. **Uhlenbeck, O. C., and R. I. Gumport.** 1982. T4 RNA ligase, p. 31–58. *In* P. Boyer (ed.), The enzymes. Academic Press, New York, NY.
29. **Wang, L. K., B. Schwer, and S. Shuman.** 2006. Structure-guided mutational analysis of T4 RNA ligase 1. RNA **12:**2126–2134.