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Identification of a retroviroid-like element from plants

(viroids/viroid-like satellite RNAs/hammerhead structures/pararetroviruses/retroelements)

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ABSTRACT **The biological nature of carnation small viroid-like RNA (CarSV RNA), a 275-nt circular molecule with self-cleaving hammerhead structures in its strands of both polarities, was investigated. The lack of infectivity observed in a series of transmission assays in carnation indicates that CarSV RNA, in spite of sharing structural similarities with viroid and viroid-like satellite RNAs from plants, does not belong to either of these two groups. Additional evidence in this direction comes from the observation that CarSV RNA also exists in carnation plants as DNA tandem repeats. In this respect, CarSV RNA is similar to a small transcript of a tandemly repeated DNA sequence of the newt genome. Moreover, CarSV and newt RNAs have similarities in their sequences as well as in some characteristics of their corresponding hammerhead structures. Further analyses have revealed that CarSV DNA is found directly fused to DNA sequences of carnation etched ring caulimovirus, a pararetrovirus, most likely in the form of an extrachromosomal element. The properties of the CarSV RNA/DNA system are those of a retroviroid-like element having some features in common with viroid and viroid-like satellite RNAs from plants and others with the newt transcript.**

Viroid and viroid-like satellite RNAs from plants (1, 2) and the hepatitis δ virus (HDV) RNA (3) form a group of small, infectious, circular molecules that replicate by a rolling-circle mechanism with only RNA intermediates (4–8). Whereas viroids are endowed with autonomous replication, viroid-like satellite RNAs and HDV RNA are functionally dependent on several plant RNA viruses and on hepatitis B virus, respectively. Carnation small viroid-like RNA (CarSV RNA†) is a 275-nt RNA (10) that resembles the aforementioned subviral plant RNAs in size, circularity, and sequence (10, 11). Moreover, plus and minus CarSV RNAs self-cleave *in vitro* through hammerhead structures (10), as do two viroids and several viroid-like satellite RNAs (12–14). These self-cleaving domains are very probably also operative *in vivo* and mediate processing of oligomeric RNAs, the postulated replicative intermediates of the rolling-circle mechanism.

The structural similarities that CarSV RNA shares with viroid and viroid-like satellite RNAs suggested to us that it could be an additional member of either of these two groups of small pathogenic RNAs (10). More specifically, analysis of trimer frequencies indicated the possibility that CarSV RNA may be related to viroid-like satellite RNAs (11). Here we report a series of studies aimed at defining the biological nature of CarSV RNA. Our results show that this viroid-like RNA is unique in having a DNA counterpart, an observation that has led us to the proposal that the CarSV RNA and DNA represent the two forms of a retroviroid-like element. This type of retroelement, to our knowledge, has been previously undescribed.

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MATERIALS AND METHODS

Purification, PCR Amplification, and Southern Analysis of DNA. Total leaf DNA from carnation (*Dianthus caryophyllus* L.) was extracted as described (15) and purified by CsCl equilibrium sedimentation. When indicated, DNA samples (4 μg) were subjected, prior to PCR amplification, to the following alternative treatments: (*i*) incubation with 0.5 M NaOH at 65°C for 1 hr and neutralization or (*ii*) digestion at 37°C for 1 hr with either 1 μg of RNase A (DNase-free) per μl or with 1 unit of DNase I (RNase-free) per μl, heating at 70°C for 15 min, and extraction with phenol/chloroform. Aliquots (1/40) of these preparations and of untreated controls were PCR-amplified (16) using a CarSV-specific pair of adjacent primers, PI and PII, complementary and identical to nt 257–14 and nt 15–39 of CarSV RNA, respectively, *Taq* DNA polymerase in 1.5 mM $MgCl_2$, and 30 cycles of 40 s at 94°C, 30 s at 60°C, and 2 min at 72°C, followed by a final extension of 10 min at 72°C. Aliquots were analyzed by PAGE in 5% gels and Southern blot hybridization. Membranes were hybridized (55°C, 50% formamide) and washed as reported (17). The probe was a radioactive full-length RNA complementary to CarSV RNA obtained by transcription with T3 RNA polymerase of the recombinant plasmid pCA15 that contains a complete monomer of CarSV DNA in the *Sma* I site of pBluescript II KS+ (10).

Fractionation of DNA. Samples (10 μg) of total DNA (cv. "Indios"), purified by CsCl equilibrium sedimentation and then digested with either RNase plus *Sau*3A or with RNase only, were applied on 10–40% sucrose density gradients. After centrifugation in a Beckman SW40 rotor at 26,000 rpm for 18 hr at 20°C, the DNA from 1-ml fractions was recovered by ethanol precipitation. Aliquots were electrophoresed in 0.8% agarose gels with TBE buffer (89 mM Tris/89 mM boric acid/2.5 mM EDTA) and stained with ethidium bromide. Other aliquots containing equal volumes of the DNA preparations from contiguous fractions were assayed by PCR amplification using primers PII and PIII (identical and complementary to nt 15–39 and 143–174 of CarSV RNA, respectively) and the products were analyzed as indicated above. The *Sau*3A site in CarSV DNA encompasses nt 209–212 (the same numbers are used in the DNA as in the RNA) and is located outside the fragment amplified with primers PII and PIII.

Nested PCR Amplification. Fractions 1 and 2 from the sucrose gradient containing the DNA treated only with RNase were pooled and the DNA was digested with *Hae* III that generates blunt ends and does not cut within the CarSV DNA. The fragments were ligated to a double-stranded oligonucle-

Abbreviations: CarSV RNA, carnation small viroid-like RNA (see † footnote); CarMV, carnation mottle virus; CERV, carnation etched ring caulimovirus.

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†CarSAV was initially proposed as an acronym for carnation stunt-associated viroid (9). Since this RNA is not infectious, it does not seem to be associated with a stunting syndrome (10), and because it also exists as a DNA, we prefer to name it carnation small viroid-like RNA and to use the abbreviations CarSV RNA/DNA for the corresponding forms.

otide with blunt and protruding ends obtained by annealing PIV, TATCAGGATCCAGTCGAATTCACGG, and PV, CCGTGAATTCGACTGGATCCTGAT. Regions containing CarSV and non-CarSV sequences were amplified by two consecutive PCRs using first primers PIV and PVI (complementary to nt 83–102 of CarSV RNA) and then PIV and PI (complementary to nt 257–14 of CarSV RNA). PCR products were examined by PAGE and Southern blot hybridization using a CarSV-specific RNA probe, and the DNA generating the strongest signal was eluted, cloned, and sequenced following established protocols (18).

Northern Blot Hybridization. Nucleic acid preparations enriched in CarSV RNA sequences were obtained as described (10). RNAs were examined in urea/TBE gels (89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.3, for the electrode buffer and the same plus 8 M urea for the gel buffer). Before loading, the nucleic acids were dissolved in the gel buffer, denatured at 100°C for 1.5 min, and snap-cooled on ice. After ethidium bromide staining the RNAs were electroblotted to nylon membranes and fixed by UV irradiation. The membranes were then hybridized as indicated above with the exceptions that the hybridization temperature was 70°C and that plus and minus radioactive CarSV RNAs of full-length, synthesized by transcription of plasmid pCA15 with T7 and T3 RNA polymerases, respectively (10), were used as probes.

Sequence Comparisons. The fragments of highest similarity scores were obtained with the BESTFIT program (gap weight, 5; length weight, 0.3) of the Genetics Computer Group package (19). The statistical significance of the alignment was tested by a procedure (20) that estimates the probability, as a function of the sequence length, that a given alignment, including mismatches or not, is due to randomness.

RESULTS AND DISCUSSION

Transmission Assays. In preliminary experiments using purified circular CarSV RNA, no infectivity was observed in carnation plants (10). Repeated attempts using this inoculum or a dimeric CarSV RNA obtained by *in vitro* transcription were also negative (data not shown). These results suggested that CarSV RNA could be a satellite RNA depending on a helper virus for its replication and/or transmission, instead of an autonomously replicating viroid. The best candidate for such a virus was carnation mottle virus (CarMV), since at least part of the CarSV RNA appears to be physically associated with the CarMV particles.‡ To test this hypothesis, the following inocula were prepared from plants positive for CarSV RNA and CarMV by dot-blot hybridization: crude homogenates, purified CarMV virions, RNAs from virions (supplemented with purified CarSV RNA), and total plant nucleic acids (also supplemented with a cloned dimeric CarSV DNA, or the corresponding sense and antisense dimeric RNAs). Inoculations were made on carborundum-dusted carnation plants negative for CarSV RNA and either positive or negative for CarMV. While CarMV or its RNA was transmissible in all instances, CarSV RNA was not. Grafting pieces of tissue from carnation plants positive for CarMV and CarSV RNA onto plants negative for both of them also failed in transmitting CarSV RNA, whereas CarMV was detected shortly after inoculation. On the other hand, we observed that some carnation plants contained naturally CarSV RNA but not CarMV. The absence of CarMV was confirmed by bioassay in *Chenopodium quinoa* and, moreover, some of these plants were seedlings and no seed transmission of CarMV has been found (21). Therefore, CarSV RNA was not horizontally transmissible and its propagation in the plant was not dependent on CarMV. However, CarSV RNA was transmissible through ovules and pollen since it was detected in 30 (F_1) seedlings analyzed from a carrier plant pollinated by a noncarrier one, whereas the reciprocal cross (fertilization of a noncarrier plant with pollen from a carrier one) led to 25 carrier and 5 noncarrier seedlings. Attempts to eliminate CarSV RNA by meristem propagation have been so far negative.

Detection of a CarSV DNA. These results led us to consider the existence of a CarSV DNA form in some carnation plants. Another hint in this direction was our previous finding that the hammerhead structure of the plus CarSV RNA is more related to that of the 330-nt transcript of a tandemly repeated sequence scattered throughout the newt chromosomes, the only known animal RNA with a hammerhead structure (22), than to the remaining 15 hammerhead structures of plant origin (10). Fig. 1*A* shows that the hammerhead structures of the plus CarSV RNA and the newt transcript are identical in having helix III of 2 bp closed by a loop of only two C and G residues and in having helix II, but not helix I, closed by a short loop. Moreover, a search for the fragments of highest similarity between CarSV and newt RNAs revealed an observed length higher than the minimum expected significant one (Fig. 1*B*) and also higher than the lengths observed previously between the CarSV RNA and some viroid and viroid-like satellite RNAs (10).

To determine whether a DNA form of CarSV exists, total carnation DNA was electrophoresed in agarose and polyacrylamide gels and analyzed by Southern blot hybridization using CarSV-specific DNA and RNA probes. No hybridizations were observed with undigested DNA or DNA digested with several enzymes, including *Asp* 700, *Hin*dII, and *Sau*3A, each of which cuts inside the CarSV sequence and would be expected to produce unit-length CarSV DNA (data not shown). Under these conditions 10 pg of cloned CarSV DNA was clearly detectable, indicating that, if present, only low amounts of CarSV DNA accumulate in carnation.

As a more sensitive approach to the question of CarSV DNA, carnation DNA was assayed by PCR amplification using primers PI and PII (complementary and identical, respectively, to two adjacent regions of the CarSV RNA sequence) and then

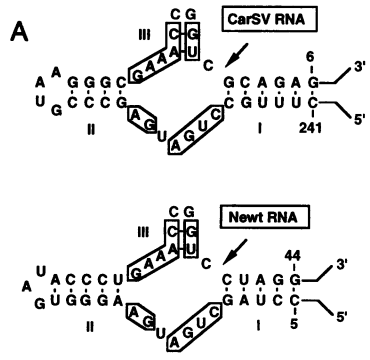

FIG. 1. Structural and sequence similarities shared by the CarSV RNA and the transcript of the newt. (*A*) Hammerhead structures of the plus CarSV RNA (10) and of the newt transcript (22). The structures are represented using the adopted convention (23) with the 13 conserved nucleotides present in most hammerhead structures boxed and the self-cleavage sites indicated by arrows. (*B*) Fragments of highest similarity between the CarSV and the newt RNAs. The percent similarity was 71.8%, the observed length was 41, and the minimum expected significant length (at the 0.05 level) was 31. The sequence of the newt transcript was obtained from the EMBL data base (accession no. X04478).

‡Darós, J. A. & Flores, R., Ninth International Congress on Virology, Aug. 8–13, 1993, Glasgow, Scotland, abstr. P67-12, p. 353.

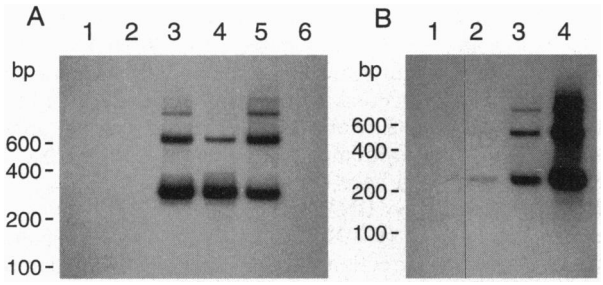

FIG. 2. PAGE and Southern blot hybridizations of the PCR-amplified products from different carnation DNA samples. (*A*) Lane 1, complete reaction mixture without added DNA; lane 2, DNA from the cultivar "Sarinah" (negative for the CarSV RNA); lanes 3–6, DNA from the cultivar "Indios" (positive for CarSV RNA) without pretreatment (lane 3) or treated prior to PCR amplification with NaOH (lane 4), RNase (lane 5), and DNase (lane 6). (*B*) Lane 1, complete reaction mixture without added DNA; lanes 2–4, DNA from cultivars "Solar," "Virginie," and "Killer," respectively (each is positive for CarSV RNA). Sizes of some DNA markers are indicated in bp.

examined by PAGE and Southern blot hybridization. Bands with the mobilities expected for mono- (275 bp) and oligomeric CarSV DNAs were observed only when the DNA was from a plant positive for CarSV RNA (Fig. 2*A*, lane 3) but neither in the reaction without DNA nor in that with DNA from a CarSV RNA-free plant (Fig. 2*A*, lanes 1 and 2). To rule out that the amplified products could have resulted from the reverse transcriptase activity of the *Taq* DNA polymerase (24) acting on residual CarSV RNA, the DNA was preincubated with NaOH or RNase. The amplified products were not affected by these pretreatments (Fig. 2*A*, lanes 4 and 5), but they disappeared when prior to PCR amplification the DNA was incubated with DNase (Fig. 2*A*, lane 6). These results indicate the existence of a CarSV DNA that it is organized as a series of head-to-tail multimers, since the alternative orientation would have generated a different pattern of PCR products.

We observed a correlation between the presence of CarSV circular RNA and that of CarSV DNA (as revealed by Northern blot hybridization and PCR amplification, respectively) in the 14 carnation varieties studied: both CarSV forms were present in all of the cuttings of 7 varieties and absent in those of the other 7 varieties. The intensities of the CarSV-specific PCR products were dependent on the carnation DNA source (Fig. 2*B*), indicating a variable content in CarSV DNA. However, the levels of the CarSV RNA did not reflect the variations observed in the CarSV DNA (data not shown). Sequencing of four complete CarSV DNA clones showed an identical sequence, except for minor changes (G67 → A; C78 → T; A137 → G; G108, deleted), to that of the CarSV RNA reported (10) and to some other ones recently obtained (unpublished data).

Localization of CarSV DNA. To establish whether CarSV DNA is integrated into the carnation genome, total carnation DNA was fractionated by sucrose density gradient centrifugation. The DNA from paired fractions was then amplified by PCR using the nonadjacent primers PII and PIII (see *Materials and Methods*) and analyzed by PAGE and Southern blot hybridization. A DNA with the size expected (160 bp) for the main CarSV-specific PCR product (Fig. 3*C*) was observed in fractions 1 and 2 containing low molecular size DNA (Fig. 3*A*). In fractions 3–12, which contain DNA of higher molecular size (Fig. 3*A*), other additional CarSV-specific PCR products of 160 + 275 bp, 160 + 550 bp, and 160 + 825 bp were detected (Fig. 3*C*). These results confirm the organization of CarSV DNA as a series of multimers and indicate that these multimers do not appear to be integrated into the chromosomal DNA (a major part of CarSV DNA was detected in the upper half of

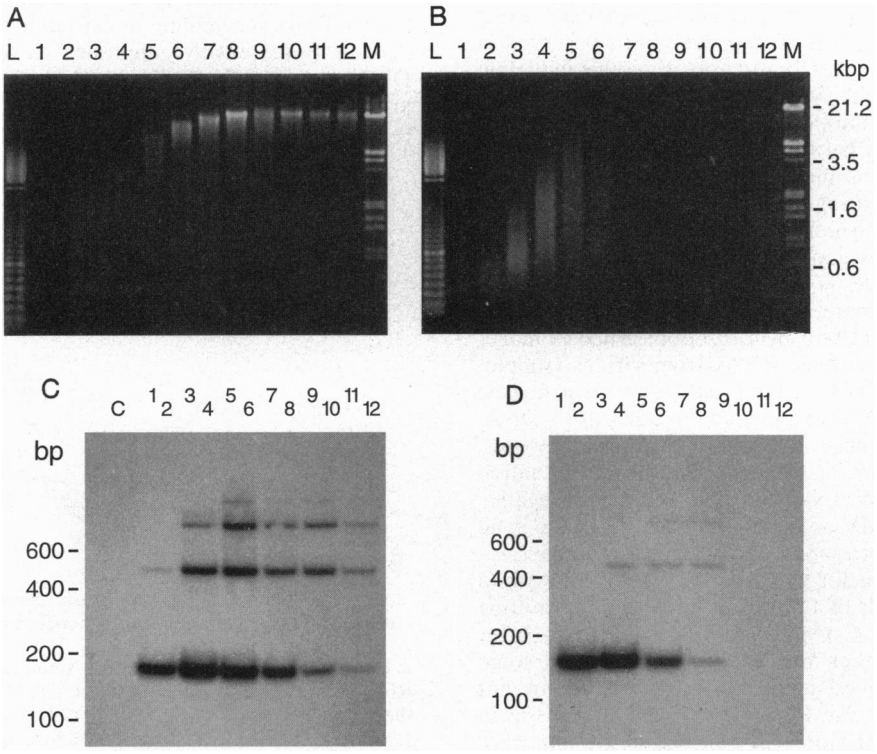

FIG. 3. Distribution of CarSV DNA sequences after sucrose gradient centrifugation of carnation DNA. (*A* and *B*) Agarose gel electrophoresis of the DNAs from the fractions of two sucrose density gradients containing undigested carnation DNA or DNA digested with *Sau*3A, respectively. Gels were stained with ethidium bromide. Lanes L, DNA ladder of 100-bp multimers; lanes 1–12, fraction number of the gradients from top to bottom; lanes M, DNA markers of 21.2, 5.1, 5.0, 4.3, 3.5, 2.0, 1.9, 1.6, 1.4, 0.9, 0.8, 0.6, and 0.1 kbp. (*C* and *D*) PAGE and Southern blot hybridizations of the PCR-amplified products from the DNAs present in fractions analyzed in *A* and *B*, respectively. Lanes C, complete reaction mixture without added DNA; lanes 1 and 2 to 11 and 12, fraction number of the gradients (pooled in pairs). Sizes of some DNA markers are indicated in bp.

the gradient, whereas the bulk of cellular DNA was found in the bottom half of the gradient and, moreover, CarSV DNA multimers were fractionated in the gradient according to their sizes). More likely, CarSV DNA exists in a smaller extrachromosomal element consisting of a variable number of CarSV DNA repeats that could also contain additional non-CarSV sequences. The non-Mendelian inheritance of the CarSV system through ovules and pollen (see above) also supports an extrachromosomal location for CarSV DNA, although we cannot exclude that a minor fraction of it could be integrated into the plant genome. Susceptibility of CarSV DNA to digestion with *Sau*3A demonstrated that it is double-stranded (Fig. 3*D*).

Analysis of the Non-CarSV DNA Sequences of the Extrachromosomal Element. Digestion of total carnation DNA with *Hae* III, which does not cut within the CarSV DNA sequence, displaced CarSV DNA signals to fractions of the sucrose gradient of lower molecular sizes (data not shown), indicating the presence of non-CarSV DNA sequences in the extrachromosomal element. Sequence analysis of the products obtained following nested PCR amplification revealed that these non-CarSV sequences are homologous to regions of the DNA of carnation etched ring caulimovirus (CERV), a plant pararetrovirus (25) (Fig. 4*A*). Direct PCR amplification of total carnation DNA with CarSV- and CERV-specific primers and sequencing of the products confirmed this observation and showed additional junctions (Fig. 4*B*). These junctions were characterized by 4–6 nt shared by the CarSV and CERV sequences. Interestingly, CarSV sequence starts in two of them at nt 1, which is the position immediately following the self-cleavage site of plus CarSV RNA (10). With regard to CERV sequences flanking the junctions, one of them rich in G residues (Fig. 4*A*) is located around the presumed gap 2 of CERV (25), one of the two regions where synthesis of the second strand of the DNA is initiated. The identification of CarSV DNA fused with genuine pararetroviral sequences is consistent with its extrachromosomal location (since the DNA of pararetroviruses does not become integrated into their host chromosomes) and, on the other hand, supports the contention that the CarSV RNA and DNA constitute a retroviroid-like system.

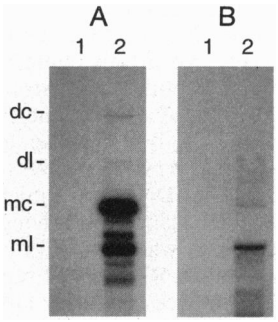

FIG. 5. Northern blot hybridization of carnation RNAs separated by PAGE in a 5% denaturing gel. Autoradiograms obtained using RNA probes for plus (*A*) and minus (*B*) CarSV sequences are shown. Lanes 1 and 2, nucleic acid extracts from the carnation cultivars "Sarinah" and "Indios" (negative and positive for CarSV RNA, respectively). The positions of monomeric circular (mc) and linear (ml) and of the apparent dimeric circular (dc) and linear (dl) CarSV RNAs are indicated. Other prominent bands moving faster than the 275-nt mc and ml CarSV forms (*A*, lane 2) correspond to circular and linear CarSV RNAs that are smaller than unit length as a result of containing deletions (unpublished data). The hybridization solutions had the same acid-precipitable cpm of either plus or minus probes and the films were exposed for the same time.

Relationships of the CarSV RNA/DNA System with Other Small Circular RNAs. The existence of DNA tandem repeats of the CarSV RNA has no precedent in the small infectious circular RNAs either from plants, such as viroid and viroid-like satellite RNAs (1, 2), or from animal origin, such as the human δ virus RNA (3). The closest correlate to CarSV RNA is the small newt transcript (22), although this RNA is linear and has a hammerhead structure only in the plus strand. Some parallel also exists with the VS DNA and RNA found in the mitochondria of an isolate of *Neurospora* (26). The VS RNA is a single-stranded circular molecule complementary to one strand of a low-copy double-stranded circular VS DNA population organized as a series of head-to-tail multimers. The VS RNA is able to self-cleave *in vitro* but lacks a hammerhead structure and is unlikely to replicate by a rolling-circle mechanism, because no minus VS RNA could be detected (26).

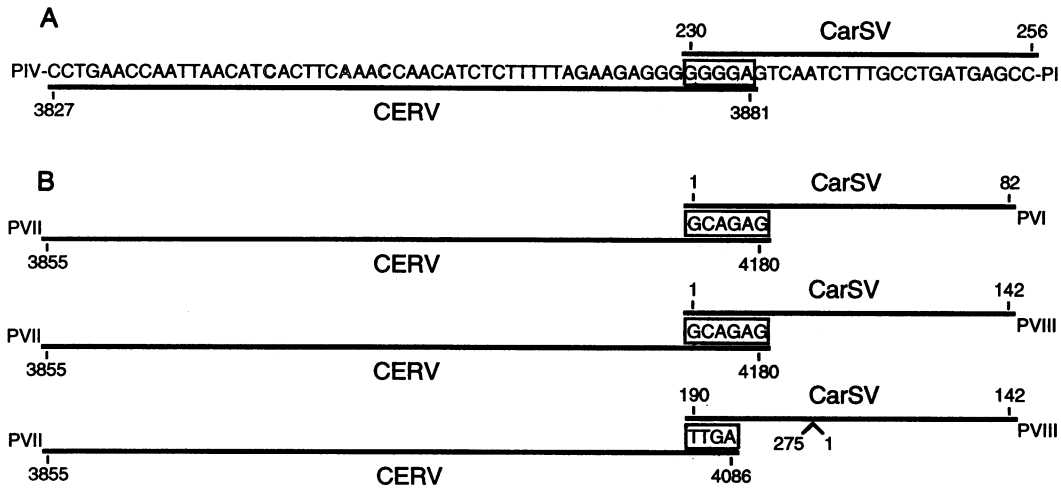

FIG. 4. Primary structure of the extrachromosomal element around several junctions (boxed) between CarSV and non-CarSV DNA sequences. (*A*) Product formed by CERV nt 3827–3881 and CarSV nt 230–256 resulting from two consecutive PCR amplifications, the second one with primers PI (complementary to nt 257–14 of CarSV RNA) and PIV (see text). Outlined fonts indicate substitutions with respect to CERV DNA reported (25). CERV sequence starts with the dinucleotide CC as expected from the initial digestion with *Hae* III. (*B*) Other products from direct PCR amplifications with primers PVI (complementary to nt 83–102 of CarSV RNA) and PVII (identical to nt 3827–3854 of CERV with the three substitutions indicated above) or with PVIII (complementary to nt 143–174 of CarSV RNA) and PVII. With the last pair of primers, two fragments of similar size but with different junctions were amplified. Heavy lines indicate CarSV and CERV sequences, which in the first case were identical to CarSV RNA reported (10) and in the second one showed 91–92% similarity with CERV DNA reported (25). CERV sequences in *A* and *B* were identical within the shared interval.

Conversely, the CarSV RNA could replicate through such a mechanism, since longer-than-unit plus and minus RNAs are found in carnation (Fig. 5). In this context, the presence of hammerhead structures in both CarSV strands provides indirect evidence in support of the symmetric variant of the rolling-circle model (4, 7, 8). However, unlike viroid and viroid-like satellite RNAs, the CarSV RNA lacks an infectious extracellular phase and its DNA form might be involved in some replicative step, for example, by giving rise to an initial input of CarSV transcripts, which, after processing and circularization, would assume an RNA-based replication pathway. The almost identical sequences of CarSV DNA and RNA also suggest a functional link between the two CarSV forms.

Since the newt transcript resembles the small infectious plant RNAs in size, in the occurrence of DNA multimers, and in its ability to self-cleave through hammerhead structures, it has been speculated that a similar infectious agent could have incorporated its RNA by retroposition via a cDNA intermediate (22). The CarSV DNA could have emerged by a similar mechanism in plants coinfected by CarSV RNA and CERV, probably making use of the reverse transcriptase activity that this virus needs for its replication. The structure of the junctions between CarSV and CERV sequences in the extrachromosomal element (Fig. 4) suggests that they could result from template switchings of the polymerase. Although the plants in which these junctions were characterized gave a positive reaction with a CERV antiserum, the extrachromosomal element, once generated, seems to replicate autonomously, because it exists also in plants negative for CERV by immunoassay (data not shown). Since the CarSV RNA/DNA system is vertically transmissible (see above), whereas seed transmission has been reported neither for CERV (27) nor for the other 13 caulimoviruses where it has been studied (28), this is probably the way by which some carnation plants have acquired only the former of the two systems. Going one step further, the sequence similarities shared by CarSV and newt RNAs, and specially the striking parallelism of their hammerhead structures, might reflect common ancestry. Nevertheless, the alternative possibility that these similarities could have resulted from convergent evolution cannot be dismissed.

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1. Diener, T. O. (1991) *FASEB J.* **5,** 2808–2813.
2. Bruening, G., Feldstein, P. A., Buzayan, J. M., van Tol, H., deBear, J., Gough, G. R., Gilham, P. T. & Eckstein, F. (1991) in *Viroids and Satellites: Molecular Parasites at the Frontier of Life*, ed. Maramorosch, K. (CRC, Boca Raton, FL), pp. 141–158.
3. Taylor, J. (1990) *Semin. Virol.* **1,** 135–141.
4. Branch, A. D. & Robertson, H. D. (1984) *Science* **223,** 450–455.
5. Branch, A. D., Benenfeld, B. J. & Robertson, H. D. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9128–9132.
6. Sänger, H. L. (1987) in *The Viroids*, ed. Diener, T. O. (Plenum, New York), pp. 117–166.
7. Hutchins, C. J., Keese, P., Visvader, J. E., Rathjen, P. D., McInnes, J. L. & Symons, R. H. (1985) *Plant Mol. Biol.* **4,** 293–304.
8. Daròs, J. A., Marcos, J. F., Hernández, C. & Flores, R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12813–12817.
9. Boccardo, G., d'Aquilio, M., Palukaitis, P., Marzachi, C. & Lisa, V. (1988) *Acta Hortic.* **234,** 109–114.
10. Hernández, C., Daròs, J. A., Elena, S. F., Moya, A. & Flores, R. (1992) *Nucleic Acids Res.* **20,** 6323–6329.
11. Branch, A. D., Lee, S. E., Neel, O. D. & Robertson, H. D. (1993) *Nucleic Acids Res.* **21,** 3529–3535.
12. Bruening, G. (1989) *Methods Enzymol.* **180,** 546–558.
13. Symons, R. H. (1989) *Trends Biochem. Sci.* **14,** 445–450.
14. Hernández, C. & Flores, R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3711–3715.
15. Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) *Plant Mol. Biol. Rep.* **1,** 19–21.
16. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–494.
17. Flores, R. (1986) *J. Virol. Methods* **13,** 161–169.
18. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
19. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12,** 387–395.
20. Karlin, S., Ost, F. & Blaisdell, B. E. (1989) in *Mathematical Methods for DNA Sequences*, ed. Waterman, M. S. (CRC, Boca Raton, FL), pp. 133–157.
21. Hollings, M. & Stone, O. M. (1970) *CMI/AAB Descriptions of Plant Viruses*, Commonwealth Mycological Institute/Association of Applied Biologists (Culross & Son, Coupar Argus, Perthshire, Scotland), no. 7.
22. Epstein, L. M. & Gall, J. G. (1987) *Cell* **48,** 535–543.
23. Hertel, K. J., Pardi, A., Uhlenbeck, O. K., Koizumi, M., Ohtsuka, E., Uesugi, S., Cedergren, R., Eckstein, F., Gerlach, W. L., Hodgson, R. & Symons, R. H. (1992) *Nucleic Acids Res.* **20,** 3252.
24. Jones, M. D. & Foulkes, N. S. (1989) *Nucleic Acids Res.* **17,** 8387–8388.
25. Hull, R., Sadler, J. & Longstaff, M. (1986) *EMBO J.* **5,** 3083–3090.
26. Saville, B. J. & Collins, R. A. (1990) *Cell* **61,** 685–696.
27. Lawson, R. H., Hearon, S. S. & Civerolo, E. D. (1977) *CMI/AAB Descriptions of Plant Viruses*, Commonwealth Mycological Institute/Association of Applied Biologists (Culross & Son, Coupar Argus, Perthshire, Scotland), no. 182.
28. Matthews, R. E. F. (1991) *Plant Virology* (Academic, San Diego), 3rd Ed., pp. 339–343.