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The virulent satellite RNA of turnip crinkle virus has a major domain homologous to the 3′ end of the helper virus genome

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RNA C (355 bases), RNA D (194 bases) and RNA F (230 bases) are small, linear satellite RNAs of turnip crinkle virus (TCV) which have been cloned as cDNAs and sequenced in this study. These RNAs produce dramatically different disease symptoms in infected plants. RNA C is a virulent satellite that intensifies virus symptoms when co-inoculated with its helper virus in turnip plants, while RNA D and RNA F are avirulent. RNA D and RNA F, the avirulent satellites, are closely related to each other except that RNA F has a 36-base insert near its 3′ end, not found in RNA D. The 189 bases at the 5′ end of RNA C, the virulent satellite, are homologous to the entire sequence of RNA D. However, the 3′ half of RNA C, is composed of 166 bases which are nearly identical to two regions at the 3′ end of the TCV helper virus genome. Hence, the virulent satellite is a composite molecule with one domain at its 5′ end homologous to the other avirulent satellites and another domain at its 3′ end homologous to the helper virus genome. All four TCV RNAs, RNAs C, D and F and the helper virus genome have identical 7 bases at their 3′ ends. The secondary structure of RNA C deduced from the sequence can be folded into two separate domains – the domain of helper virus genome homology and the domain homologous to other TCV satellite RNAs. Comparative sequences of several different RNA C clones reveal that this satellite is a population of molecules with sequence and length heterogeneity.

Key words: plant virus/satellite RNAs/turnip crinkle virus/small RNAs

Introduction

Turnip crinkle virus (TCV) encapsidates and supports the replication of a family of small RNAs. These RNAs are called satellite RNAs because they require the TCV genome for replication, yet TCV can replicate in their absence. One prominent TCV satellite, called RNA C, is highly virulent, intensifying the symptoms of TCV infection (Altenbach and Howell, 1981). The effect of RNA C on symptom expression is not understood because RNA C, like viroids and some other small satellite RNAs, does not appear to encode any proteins (Altenbach and Howell, 1982). Furthermore, RNA C does not behave like several other plant virus satellites, such as the CARNA-5 satellite of cucumber mosaic virus, which tend to reduce, not intensify, virus symptoms in certain plant hosts by competing with the helper virus genome for replication and accumulation in infected plants (Waterworth *et al.*, 1979).

Viruses in a number of different plant RNA virus groups support satellites, but there is little in common among these satellites. Most satellites are encapsidated in helper virus particles and have little homology with the helper virus genome (see Murant and Mayo, 1982). However, there are exceptions. For example, the satellite of tobacco necrosis virus (STNV) is encapsidated in a separate virus particle which the satellite itself encodes (Leung *et al.*, 1976; Salvato and Fraenkel-Conrat, 1977). Satellites vary in size (from ~200 to 1700 bases), coding capacity and form. The satellites of several Australian plant viruses are circular and resemble viroids (Haseloff and Symons, 1982), while most others are linear.

In this paper, we have cloned and compared the sequence of the virulent TCV satellite, RNA C, with two avirulent TCV satellites, RNAs D and F. We have found that RNA C is a complex molecule composed of two domains, one homologous to the 3′ end of the helper virus genome and the other common to the avirulent satellites, RNAs D and F.

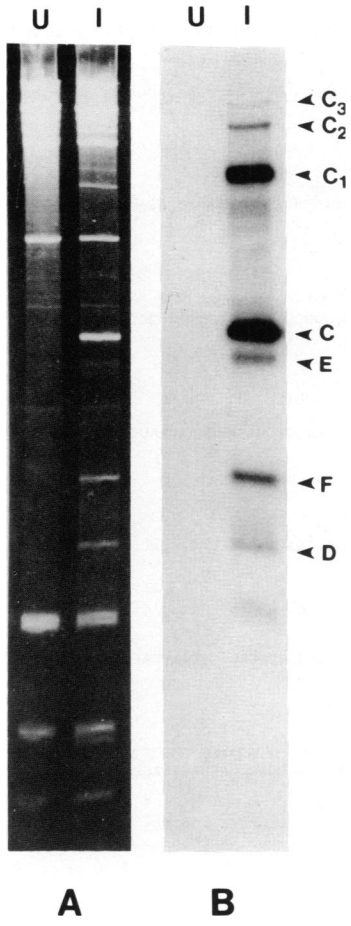

Fig. 1. Family of satellite RNAs associated with TCV infection. 10 μg of RNA extracted from (U) uninfected or (I) infected leaves were (**A**) visualized with ethidium bromide after electrophoresis on 4% polyacrylamide gels containing 50% urea or (**B**) transferred to nitrocellulose filters and hybridized to ^{32}P-labeled pSTCV-1, a partial cDNA clone of RNA C which hybridizes to all TCV satellite RNAs. The nomenclature for identifying satellite RNA forms and species is described in Altenbach and Howell (1981, 1984).

Results

Cloning and sequencing RNA C, the virulent TCV satellite

The sequence was determined for pSTCV-1, a partial cDNA clone (135 bases) of RNA C, produced in an earlier study (Altenbach and Howell, 1984) by polyadenylation of RNA C and reverse transcription using oligo(dT) as a primer. On blots of infected plant leaf RNA, pSTCV-1 hybridized to all small RNAs associated with a standard isolate of TCV (John Innes isolate). This included RNA C and the more rapidly migrating species RNAs D, E and F as well as the multimers of RNA C − RNA C1, C2, etc. (Figure 1). To clone the remainder of RNA C an oligodeoxynucleotide primer (oligo-1) complementary to the sequence 5′-GGGUUUCAUAAUACUACGCAAC-3′ near the 5′ end of pSTCV-1 was synthesized. However, using this primer and gel-purified RNA C (monomer) as template, we were unable to extend more than a few bases past the primer. We surmised that sequences complementary to oligo-1 were either near the 5′ end of RNA C or near a 'hard stop', i.e. a block to reverse transcription.

Therefore, we attempted to extend the oligo-1 primer on multimers of RNA C found in total infected leaf RNA (Figure 1). RNA blots showed that oligo-1 hybridized exclusively, under stringent conditions, to RNA C and its multimers, and not to the other satellite RNAs nor to the TCV genome (data not shown). Thus, we expected the primer extension products from oligo-1 would be derived only from RNA C and its multimers even though a complex mixture of RNAs was used as template in the reverse transcription reaction. Extension products, the anticipated size of full-length RNA C (~350 bp), were double-stranded, cloned and sequenced as described in Materials and methods.

The sequences of six clones shown in Figure 2 were circularly permuted with respect to pSTCV-1, overlapping both the 5′ and 3′ ends of pSTCV-1. This would be expected if the cDNAs were derived from tandem multimers of RNA C. (The sequences were aligned according to the ends of RNA C described below.) Two cDNA clones (p1-23 and p2-13) were judged to be full-length, because their 5′ ends terminated at a base immediately downstream from the 3′ end of the sequence complementary to oligo-1 in pSTCV-1 (base 35 in Figure 2). The insert in p1-23 is 355 bases in length while the insert in p2-13 is 356 bases long.

The clones showed both length and sequence heterogeneity.

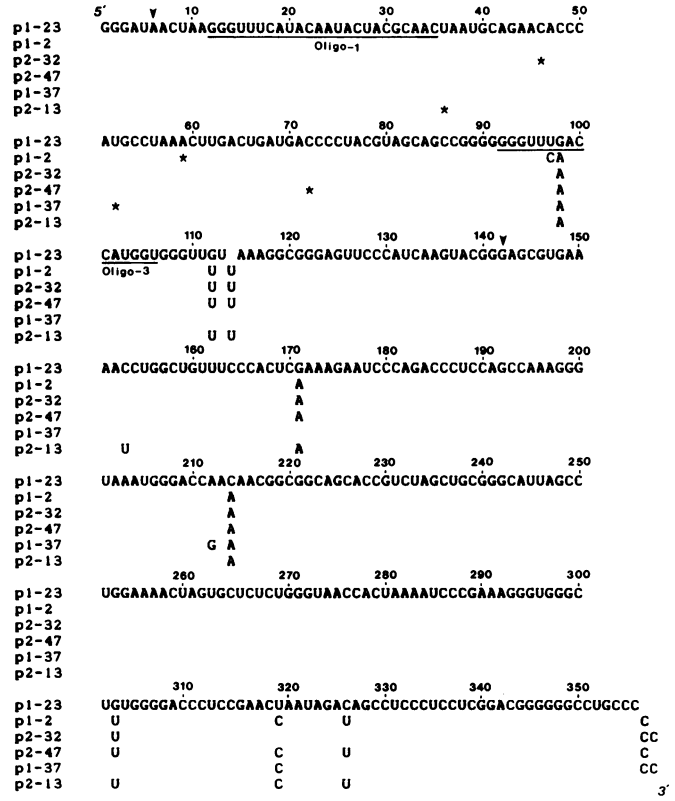

Fig. 2. Sequence of RNA C as determined from cDNA clones. Sequences are aligned from the 5′ and 3′ ends of RNA C as determined from primer extension analysis and direct RNA sequencing, respectively. Sequences complementary to the synthetic oligodeoxynucleotides oligo-1 and -3 are indicated. All clones shown here were produced by extending on the oligo-1 primer and only p1-23 and p2-13 are full-length. Asterisks (*) indicate the positions of prematurely truncated ends of the partial cDNA clones. Arrows (↓) indicate the ends of the partial cDNA clone, pSTCV-1, which was identical in sequence to p1-37. Bases which differ between p1-23 and the other cDNA clones are shown.

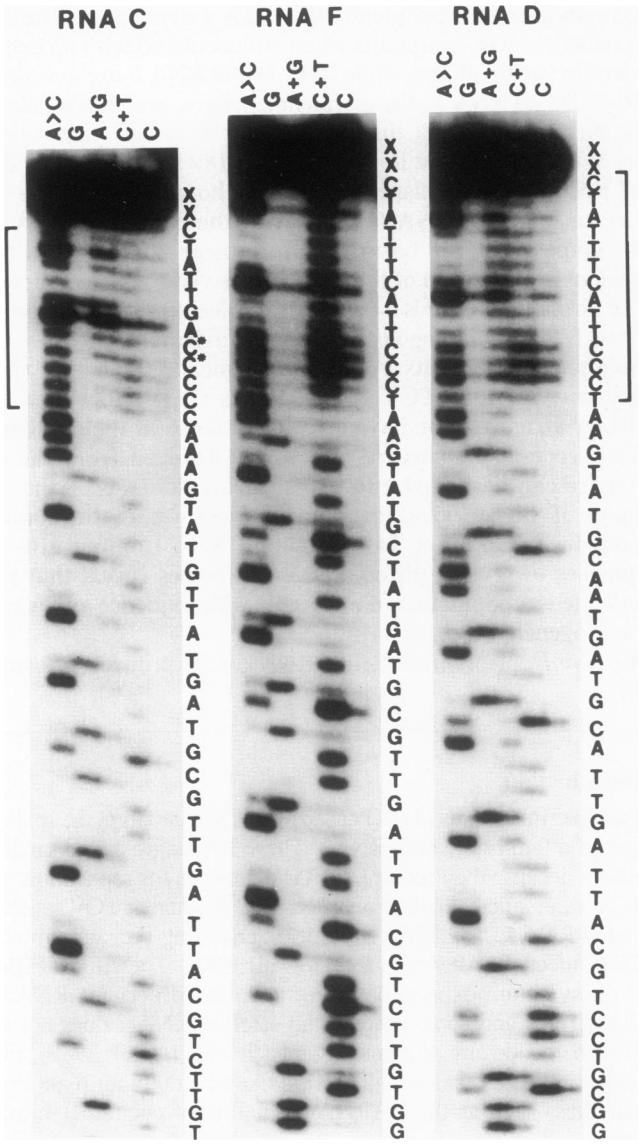

Fig. 3. Primer extension on RNAs C, F and D. (**A**) The sequence of the products extended from 5′ end-labeled oligo-3 on RNAs C, F and D was determined on an 8% polyacrylamide gel containing 50% urea (Maxam and Gilbert, 1980). Brackets indicate region of 5′ end heterogeneity. In this region, the sequence represented by the most prominent band was read. Starred (*) bases indicate a sequence difference from the cloned cDNA sequence in Figure 2, base positions 10 and 11.

Base substitutions among the six sequences were found at nine positions while insertions and deletions were localized at two positions, 114 and beyond 356 (Figure 2). We have reason to believe that the heterogeneity represents actual variants in the satellite RNA population and not artifacts in cloning. In recent experiments, we have observed that at least two of these clones (p1-23 and p2-47) are biologically active in that they can be transcribed *in vitro* to yield infectious satellite cRNA (Simon and Howell, 1987).

The 3′ and 5′ ends of RNA C

In order to locate the 3′ end of the monomeric RNA C, the 3′ end of gel-purified RNA C (monomer) was labeled with [^{32}P]-pCp and RNA ligase and sequenced by direct RNA sequencing using the chemical modification procedure of Peattie (1979). The 3′ end sequence of the monomer was clear and homogenous (data not shown), composed of three C residues while the number of Cs at the junction between the 3′ and 5′ ends of the multimer varied between three and five in the different clones (Figure 2). Possibly, extra C residues are gained or lost in the formation or processing of multimers.

To confirm the assumed location of the 5′ end of the RNA C monomer with respect to the sequence of the multimer, we carried out primer extension with reverse transcriptase using another oligodeoxynucleotide primer (oligo-3) complementary to the sequence 5′-GGGUUUAACCAUGGU-3′ (Figure 2). Oligo-3 hybridized to RNAs C, D and F (data not shown), unlike oligo-1 which only hybridized to RNA C.

Using gel-purified RNA C as a template, we found that reverse transcriptase could extend oligo-3 by an additional 91 bases (Figure 3). Although the position of the 5′ end could be accurately determined by primer extension, the nucleotide sequence of the first two nucleotides, found at the top of the sequencing ladder, could not be read. However, it was assumed that these two bases correspond to the two Gs in the multimer sequence. The sequence of the extension product, from the oligonucleotide primer to base 14 in RNA C matched the cloned sequence exactly. However, at base 14 the sequence became somewhat heterogenous and more difficult to read. Despite the heterogeneity, the predominating sequence at the 5′ end in the primer-extended product matched the cloned sequence except at positions 10−11 where two G residues (read as two Cs in the primer-extended product, Figure 3) were found instead of two As in all the cDNA clones. The difference could be due to the possibility that the As at these two positions are found in multimers from which the clones were derived,but not in the monomers which were used in primer extensions. Another possibility is that different RNA preparations were used in the two experiments and the RNA C sequence may have 'drifted' during serial propagation of the virus in infected plants.

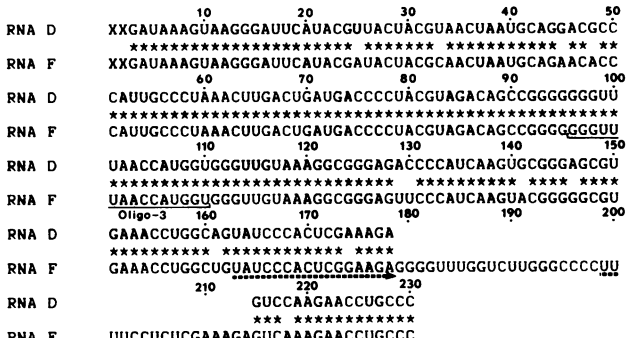

Fig. 4. Comparison of the sequence of the non-virulent satellite RNAs D and F. The 3′ ends of the RNAs were sequenced by direct RNA sequencing. Partial cDNA clones, pD22 and pF2 were cloned by reverse transcriptase-mediated extension on gel purified RNAs D and F, respectively, using oligo-4 as a primer. The insert in pD22 extended upstream to base 27, and the insert in pF2 extended upstream to base 21. The position and sequence of the 5′ end was determined by sequencing reverse transcripts extended on gel-purified RNAs D and F from the primer, oligo-3. The 5′-terminal two bases at the top of the sequencing ladder could not be determined. Stars (*) indicate sequence identity. Hatched underlined sequences indicate a direct repeat in RNA F.

Cloning and sequencing RNAs D and F, two avirulent TCV satellites

RNAs D and F are smaller satellite RNAs found along with RNA C in the John Innes isolate of TCV. These RNAs are independent satellite RNAs by the criteria that they replicate in plants only in the presence of the helper virus, but RNAs D and F are not required for helper virus replication (Simon and Howell, unpublished observations). However, unlike RNA C, RNAs D and F do not intensify viral symptoms (Simon and Howell, unpublished observations).

The sequences of gel-purified RNAs D and F were determined by a combination of direct RNA sequencing from the 3′ end, sequencing of cloned cDNA and primer extension sequencing of the 5′ end (Figure 4). From the 3′ end sequence an 11-base oligodeoxynucleotide, oligo-4 complementary to the sequence 5′-AAGAACCUGCC-3′) was constructed to serve as a pimer for reverse transcriptional synthesis of cDNA from the 3′ end of RNAs D and F. The sequence of a nearly full length cDNA clone of RNA F, pF2 (Figure 4), matched the 122 bases of sequence obtained by direct RNA sequencing of RNA F. Likewise, the sequence of the nearly full-length clone of RNA D, pD22, also matched the sequence of RNA D obtained by direct RNA sequencing. The 5′ ends of RNAs D and F were determined by sequencing the reverse transcriptase extension products primed by oligo-3 (Figure 3). Oligo-3, previously constructed for primer extension on RNA C, was also found to hybridize to the two smaller satellites and the sequence of oligo-3 was the exact complement of the region between nucleotides 96 and 110 in pD22 and pF2. The sequence of the product extended from oligo-3 up to a position equivalent to the end of pD22 or pF2 matched exactly the cloned cDNA sequences. Heterogeneity present at the 5′ end of RNA C was also observed toward the 5′ end of RNAs D and F (Figure 3). Again, the first two bases at the 5′ end could not be determined because they were at the top of the sequencing ladder.

Alignment of the sequences of RNAs D and F demonstrates the close relationship between the two avirulent satellite RNAs (Figure 4). RNA D differs from RNA F by only 11 single base changes (94% homology) and by a 36-base insert in RNA F. The insert, which accounts for the size difference between the two avirulent satellites (RNA D, 194 bases; RNA F, 230 bases), is composed of a 20-base segment of unknown origin and a 16-base segment which is a direct repeat (12 bases/16 bases) of the sequence directly upstream from the insert.

Discussion

A striking homology was found between the 3′ end of the virulent satellite, RNA C and two segments at the 3′ end of the TCV genome (Figure 5, Carrington *et al.*, 1986). The longest homologous segment (155 bases) lies at the very 3′ end of the TCV genome and the other shorter segment (15 bases) lies 116 bases upstream. The 166 bases at the 3′ end of RNA C

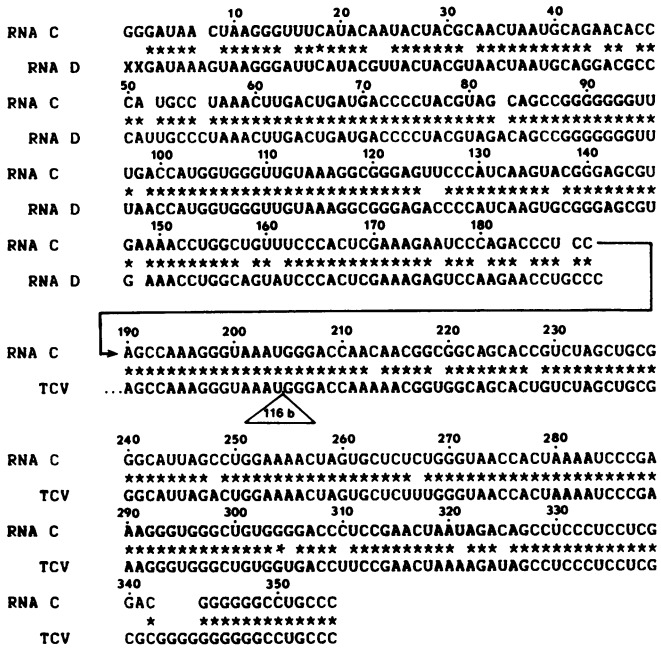

Fig. 5. (A) Homology between the sequence at the 5′ end of RNA C (clone p1-23) and RNA D and the 3′ ends of RNA C and TCV RNA (Carrington *et al.*, 1986). Stars indicate sequence identity.

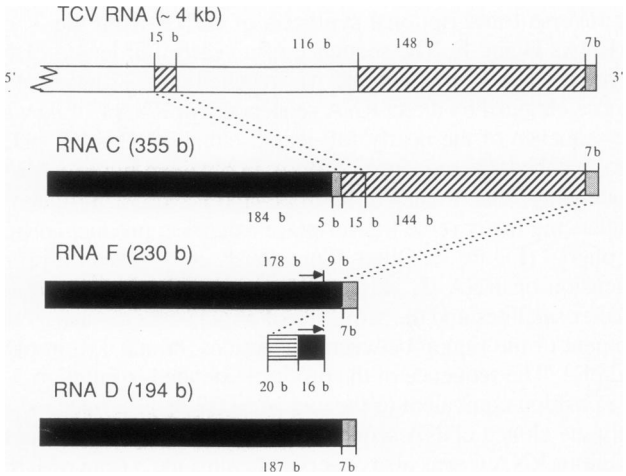

Fig. 6. Schematic representation of the homology among the satellites and the 3′ end of the helper virus genome.

(represented in clone p1-23, bases 190−355) corresponding to the 3′ end segment of the TCV genome differs by only 11 base changes (93% homology) and an insertion of four G residues at position 343 in the sequence of RNA C.

Not only is the 3′ end of RNA C homologous with the TCV genome, but the remainder, the 5′ half of RNA C is closely related to the avirulent TCV satellites, RNAs D and F (Figure 5). Among the 189 bases at the 5′ end of RNA C, only 22 bases differ between RNA C and the same region in RNA D (equivalent to 88% homology). In the same region RNA C and RNA F are 92% homologous (differing by only 16 base changes and the 36-base insert found in RNA F, but not in the other satellites). Interestingly, the sequence, 5′....CCUGCCC-OH occurs at the 3′ ends of all three RNAs, the TCV genome, RNA C, D and F.

Given these relationships, RNA C can be divided into two homology domains − roughly the 3′ half with homology to the TCV genome and the 5′ half with homology to RNAs D and F (Figure 6). The homology between RNA C and the TCV genome was not observed in previous hybridization studies (Altenbach and Howell, 1984) because the hybridization probe, a partial cDNA clone (pSTCV-1), did not contain the region homologous to the helper virus genome. Nonetheless, comparative T1 RNase fingerprinting of RNA C and TCV showed that five out of 12, or roughly half, of the characteristic oligonucleotides of RNA C were identical to oligonucleotides in the TCV genome.

The most probable secondary structure (lowest free energy form) of RNA C, determined by computer analysis (using the sequence of the full length clone p1-23), is a structure in which the two regions of homology are folded into separate structural domains (Figure 7). The folded structure is not highly base-paired on itself (55% base pairing), and the molecule can be folded into other forms by substituting the base changes found in different clones. Clearly, the structure(s) of RNA C will have to be determined by physical studies. Nonetheless, neither this structure nor the other forms are reminiscent of any other small, infectious RNA.

RNA C is unique among TCV satellites in intensifying viral symptoms, the crinkling and stunting of the infected turnip leaves (Hollings and Stone, 1972; Altenbach and Howell, 1981). When either RNA D or F is co-inoculated with the helper virus genome, the symptoms produced are no different from those with TCV alone (Simon and Howell, in preparation). It is an interesting, but open question whether the symptoms produced by RNA C are due to some property of the 3′ domain in RNA C which distinguishes RNA C from RNAs D and F. RNA C differs from the two smaller satellites in other aspects including several base differences. If the region of TCV homology in RNA C is responsible for intensifying symptom expression, then the mechanism by which it does so is difficult to understand. The domain of TCV homology represents mainly the 3′-untranslated region of the TCV genome (Carrington *et al.*, 1986), and in the context of RNA C this domain has no greater coding capacity than in the TCV genome. It is possible that the 3′-untranslated region of TCV itself produces mild symptoms in infected plants and that RNA C intensifies symptoms simply by accumulating greater numbers of copies of the viral 3′ end in infected plants. RNA C outnumbers TCV RNA molecules in infected plants by ~10:1, while in virions RNA C and TCV RNA are present in about equal numbers (Altenbach and Howell, 1981). RNA C does not stimulate TCV RNA accumulation, in fact, it seems to do just the opposite. RNA C appears to be a weak competitor of TCV RNA replication or accumulation (Simon, unpublished observations).

It is not known whether the base sequence heterogenity observed in different RNA C clones endows RNA C types with different phenotypic properties. However, this question may be approached using infectious clones of the different types. Recently, we have been able to produce *in vitro* infectious RNA from cloned cDNAs of RNA C (Simon and Howell, 1987), so such experiments are feasible. Furthermore, it will now be possible to alter RNA C copies *in vitro* in order to study such properties as virulence of the molecule. It will also be interesting to determine how long cloned RNA C remains homogeneous in serially infected plants. This should give some indication as to whether the heterogeneity observed in these experiments is acquired rapidly by mutation or whether it results from the continuous propagation of a collection of different forms which have a long history in association with the virus.

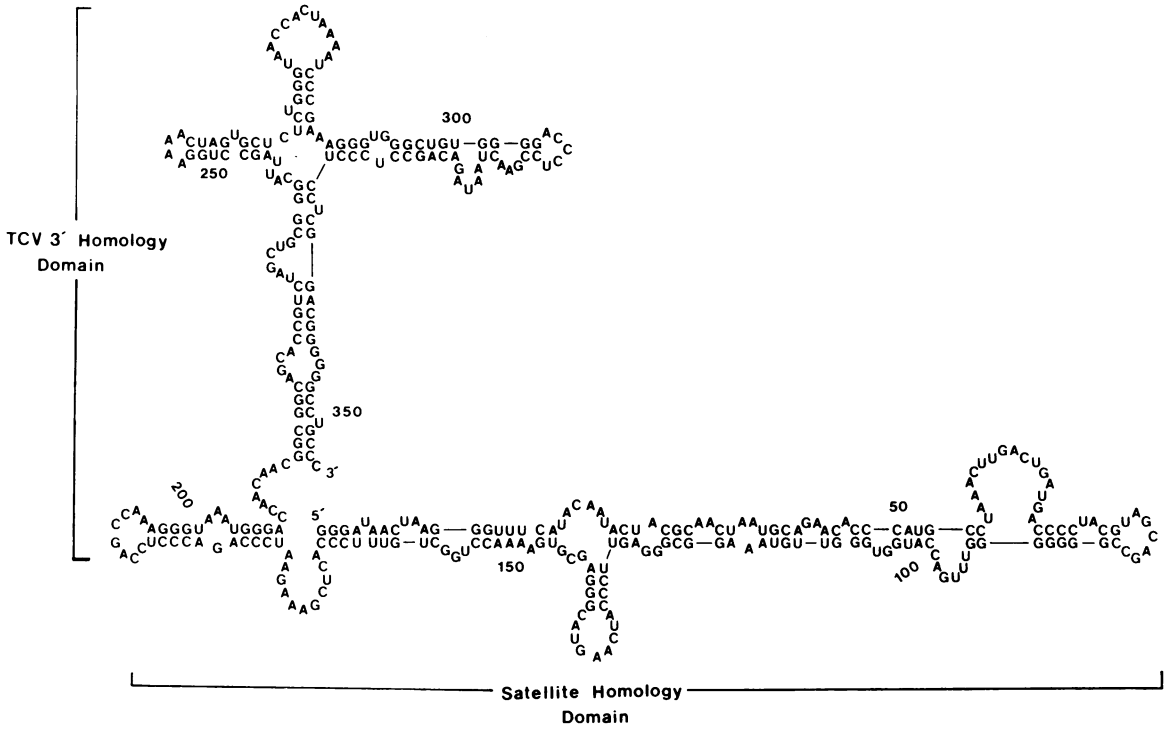

Fig. 7. Folded structure of RNA C (clone p1-23) generated from the computer algorithm of Zuker and Stiegler (1981).

The close relationship between RNAs C, D and F may indicate that the satellite RNAs have a common origin. Of the three satellites, RNA D is the smallest and simplest. RNA F is somewhat more complex. It has the basic structure of RNA D, but contains, in addition, a 36-base insertion, part of which (16 bases) is a repeat of the adjacent sequence in RNA F (Figure 6). At first glance, RNA C appears to be a simple recombinant between RNA D and the 3′ end of TCV RNA. Even at the junction between the two domains of RNA C, five out of the seven bases characterisic of the 3′ end of the satellite RNAs have been conserved. However, while the regions of homology are similar to each other in the three satellites, they are not identical, suggesting that the common parts of these satellites do not undergo continual exchange. Furthermore, the virulent satellite, RNA C, has not appeared spontaneously in infections of RNAs D or F with TCV RNA. These observations are consistent with the idea that the various satellite RNAs may have arisen by internal rearrangements and/or intermolecular recombination, but the sequences have drifted somewhat away from each other and the individual satellites have established themselves as rather stable populations of RNA molecules.

One property usually included in the definition of satellite RNAs is that satellites have no appreciable homology to their helper virus genome (see, for example, Murant and Mayo, 1982). However, sequence analysis of various satellites has revealed subtle or limited homologies, such as that between the 3′ end of the satellite RNA of cucumber mosaic virus (CMV) and the 3′ ends of the helper virus RNAs (Gordon and Symons, 1983) and that between the 5′ end of the same satellite and the complement of the 5′ end of CMV RNA 1 (Rezaian *et al.*, 1985). Nonetheless, the absence of appreciable homology is one property that distinguishes plant satellites from the RNAs of defective interfering (DI) particles associated with certain animal viruses (see, for example Holland, 1985). However, DI particle RNAs are totally derived from their helper virus genome and usually represent a series of internal deletions in the viral genome. RNA C is different. It is an apparently composite molecule related to both the TCV genome and to other TCV satellite RNA species. Also, RNA C is just one member of a family of TCV satellite RNAs in which the others seem to be more conventional satellites.

Materials and methods

RNA extraction and analysis

RNA was extracted from leaves of infected turnip plants, *Brassica campestris* var. *rapa* cultivar Just Right turnip, 3–6 weeks after inoculation by procedures modified from Altenbach and Howell (1981). Briefly, 3–10 g of turnip leaves were ground in liquid nitrogen and the powder was resuspended in extraction buffer (200 mM Tris–HCl, pH 9.0, 400 mM LiCl, 25 mM EDTA and 1% SDS). The mixture was phenol extracted three times, and RNA was ethanol precipitated. The RNA pellet was washed twice with 2 M LiCl and ethanol precipitated again. RNA was dissolved in water and stored frozen at −80°C. Low mol. wt RNAs were analyzed on 4% polyacrylamide–50% urea gels and visualized by staining with ethidium bromide. For RNA blots, gels were treated with 6% formaldehyde for 30 min before transferring RNA to nitrocellulose filters. Hybridizations were carried out as previously described (Altenbach and Howell, 1984).

Cloning RNA C

To clone cDNA copies of RNA C, 220 μg of total leaf RNA from plants infected with TCV (plus RNA C) was hybridized to 5 μg of the oligodeoxynucleotide primer, oligo-1. Hybridization was carried out at 70°C for 10 min in 100 μl of HB buffer, 0.4 M NaCl and 10 mM Pipes (pH 6.4). The reaction was then cooled slowly to 25°C by placing the reaction tube in a 400-ml beaker of water at 70°C and allowing the water to cool to room temperature. RNA and primer were ethanol precipitated, and the first DNA strand was synthesized using 20 units of AMV reverse transcriptase (New England Biolabs) after Maniatis *et al.* (1982). The first strand product was precipitated twice with 2 M ammonium acetate, and the second DNA strand was synthesized using 20 units of *Escherichia coli* DNA polymerase I (New England Biolabs) and 100 μCi [α-^{32}P]dCTP in the presence of 1 unit of RNase H (PL Biochemicals) as described by Gubler and Hoffman (1983). 1 μg of kinased *Xho*I linkers was ligated onto the cDNA overnight at 15°C. After heat inactivation of the ligase and ethanol precipitation of the cDNA, the linkers were digested with 100 units of *Xho*I (BRL) for 3 h at 37°C. To determine the size of the cDNAs, the cDNA preparation was loaded directly onto a 4% non-denaturing acrylamide gel and subjected to electrophoresis. The band corresponding to full-length RNA C was electroeluted from the gel, purified over DEAE Sephacel (Pharmacia) and ligated into the *Sal*I site of pUC19. Six cDNA clones containing the largest inserts were selected for further analysis.

Cloning cDNAs for RNAs D and F

To clone cDNAs for RNAs D and F, 1 μg of gel-purified RNA D or F was hybridized to 0.5 μg of the oligodeoxynucleotide primer, oligo-4, in 40 μl of HB buffer. The hybridization reaction was slowly cooled to 25°C after heating for 10 min at 70°C. RNA and primer were ethanol precipitated and the first strand DNA was synthesized using 20 units of MMLV reverse transcriptase (Bethesda Research Laboratories) according to manufacturer's instructions with the following modifications: KCl as eliminated from the reaction mix to reduce the total salt concentration, and synthesis was carried out at 25°C for 45 min. Twenty additional units of enzyme were added, incubation was carried out for an additional 45 min at 37°C. Second strand synthesis and cloning of the double-stranded cDNA was carried out as above except that *Kpn*I linkers were ligated onto the cDNA and the cDNA was inserted into the *Kpn*I site of pUC19.

5′ End determination

Oligodeoxynucleotide 3, oligo-3 (see Figure 2) was used as a primer in primer extension reactions to determine the 5′ ends of the three satellite RNAs. Oligo-3 was kinased using [γ-^{32}P]ATP and purified by gel electrophoresis. For primer extensions on RNA C, 0.5 μg of oligo-3 was hybridized in HB buffer to 1 μg of gel-purified RNA C. For primer extensions on RNAs D and F, 0.5 μg of oligo-3 was hybridized to 12.5 μg of total RNA from plants infected only with the respective satellites and the helper virus genome, i.e. no RNA C. Hybridizations of primers to satellite RNAs were carried out in 40 μl of HB buffer by heating for 10 min at 70°C and slow cooling to 25°C as described above. Primer extension was carried out using 20 units of MMLV reverse transcriptase (Bethesda Research Laboratories) according to the manufacturer's instructions. DNA:RNA hybrids were disassociated by boiling for 3 min in 100% formamide and full-length cDNAs were size-selected on 4% polyacrylamide−50% urea gels and subjected to DNA sequencing.

DNA and RNA sequencing

DNA sequencing was carried out by the chemical degradation method of Maxam and Gilbert (1980) as modified by Bencini *et al.* (1984). For direct RNA sequencing, the 3′ ends of gel-purified RNAs C, D and F were labeled with 200 μCi of [^{32}P]pCp (Amersham Corp.) using 10 units of RNA ligase (Bethesda Research Laboratories) according to the manufacturer's instructions and sequenced according to Peattie (1979). The 3′-terminal C residues on RNAs C, D and F were identified based on their comigration with pCp.

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