The EMBO Journal vol.7 no.9 pp.2645–2651, 1988

Identification of regions affecting virulence, RNA processing and infectivity in the virulent satellite of turnip crinkle virus

Anne E.Simon[1], Henk Engel[2], Richard P.Johnson and Stephen H.Howell[3]

Department of Biology, C-016, University of California San Diego, La Jolla, CA 92093, USA
[1]Present address: Department of Plant Pathology, University of Massachusetts, Amherst, MA 01003, USA
[2]Present address: Department of Biochemistry, University of Groningen, Nijenborgh 16, 9747 AG Groningen, The Netherlands
[3]Present address: Boyce Thompson Institute, Cornell University, Tower Road, Ithaca, NY 14853–1801, USA

Communicated by A.-L.Haenni

Turnip crinkle virus (TCV) supports a small family of satellite RNAs (RNAs C, D and F). RNA C is a virulent satellite, producing severe symptoms in host plants, while RNAs D and F are avirulent satellites. The virulent satellite (RNA C) has two major domains—a 5′-domain similar to the avirulent satellites and a 3′-domain similar to the 3′-end of the TCV genome. To demonstrate that the 3′-domain of RNA C determines virulence, a chimeric satellite was constructed composed mostly of the 5′-domain of the avirulent satellite (RNA F) and the 3′-domain of the virulent satellite (RNA C). To locate other functional regions, small DNA fragments were inserted or deleted at various sites in the cDNA of virulent satellite (RNA C). Most small internal deletions and insertions in the midsection of the molecule had no detectable effects while those near the 3′-end of RNA C destroyed infectivity. Modifications in a small region centering on an AGCAGC repeat in the domain of satellite homology blocked the accumulation of monomers and presumably the processing of RNA C. Other modifications in this region produced more intense symptoms. Hence, these experiments reveal regions of the satellite which determine virulence, are essential for infectivity, affect monomer accumulation (RNA processing) and modulate symptom expression.

Key words: plant virus/satellite RNAs/RNA processing/*in vitro* RNA synthesis

Introduction

Satellite RNAs are small, infectious RNAs which require a helper virus to replicate in host plants (Murant and Mayoi, 1982; Francki, 1985). An interesting property of satellite RNAs, which is poorly understood, is their ability to alter symptom expression in infected plants. Turnip crinkle virus (TCV) supports a family of satellite RNAs (Altenbach and Howell, 1981; Simon and Howell, 1986), one of which, the virulent satellite (RNA C), intensifies TCV symptoms in turnip. Two other TCV satellites, the avirulent satellites (RNAs F and D), have no apparent effect on symptoms. Members of the TCV satellite family are related by sequence similarity (Simon and Howell, 1986). The virulent satellite RNA C (355 bases) is an unusual, hybrid molecule composed of two domains, a 3′-domain (166 bases) similar to two regions at the 3′-end of the helper virus genome and a 5′-domain (189 bases) similar to the entire sequence of the avirulent satellite (RNA D). Hence, the virulent TCV satellite is intermediate between a true satellite which has no appreciable similarity to the helper virus genome (Murant and Mayo, 1982) and a defective interfering RNA, such as that associated with tomato bushy stunt virus (Hillman *et al.*, 1987) which is similar to discontinuous regions of the helper virus genome. The avirulent TCV satellite (RNA F) which has no appreciable similarity to the helper virus genome differs from another avirulent satellite (RNA D) mainly by a 36-base insert near the 3′-end of RNA F (Simon and Howell, 1986). Based on the relationship between satellites, we proposed that the determinants of virulence might be vested in the 3′-domain of RNA C, which is unique to the virulent satellite and similar to the 3′-end of the viral genome (Simon and Howell, 1986).

Virulence determinants in other small pathogenic RNAs, such as viroids and other satellite RNAs, have been determined by comparing the sequences of mild and severe isolates. By such means, two 'virulence modulating regions' in potato spindle tuber viroid (PSTV) (Schnölzer *et al.*, 1985) and one in citrus exocortis viroid (CEV) (Visvader and Symons, 1986) have been located. However, sequence comparisons of various cucumber mosaic virus (CMV) satellites, some of which can cause severe necrosis in tomato, have not pinpointed virulence determinants because there are many single base differences among various isolates (Collmer *et al.*, 1983; Garcia-Arenal *et al.*, 1987).

Recently, infectious RNA copies of viroids (Tabler and Sänger, 1985; Visvader and Symons, 1985) and satellites (Collmer and Kaper, 1986; Gerlach *et al.*, 1986; Kurath and Palukaitis, 1987; Simon and Howell, 1987) have been synthesized *in vitro*. This development has made it possible to generate site-specific mutations in *in vitro* synthesized transcripts of viroids and satellites and, subsequently, to assay for infectivity and symptom production in plants. However, most single-base alterations destroy the infectivity of viroid transcripts (Ishikawa *et al.*, 1985; Owens *et al.*, 1986) except for one which bears a compensatory mutation that maintains base pairing (Hammond and Owens, 1987).

In this paper a major domain responsible for virulence has been located in RNA C as well as other regions which are essential for infectivity, affect monomer accumulation (and presumably, affect RNA processing) and modulate symptom expression.

Results

Construction of a chimeric satellite

The virulent satellite (RNA C) is composed of two major domains—a 5′-domain similar to the other avirulent satellites and a 3′-domain similar to the 3′-end of the helper virus

©IRL Press Limited, Oxford, England

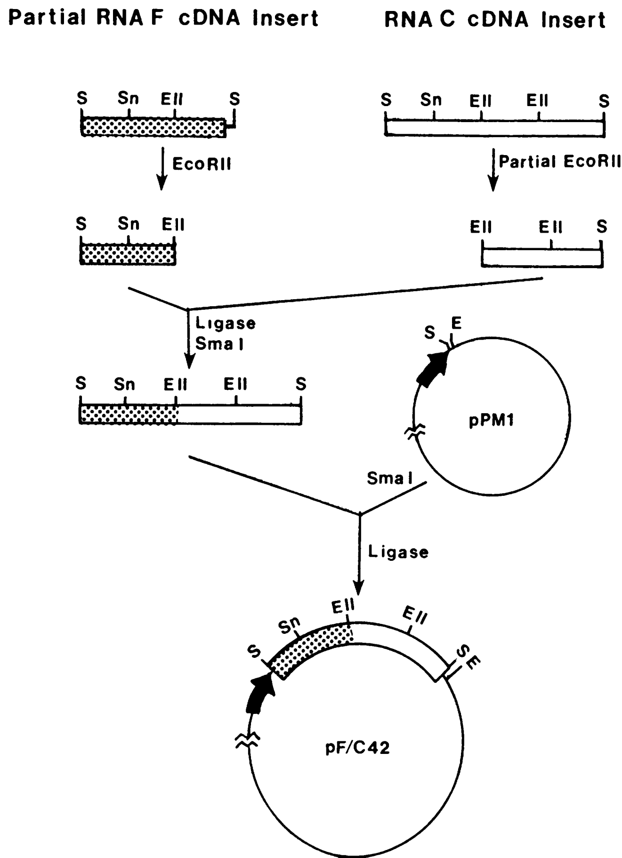

Fig. 1. Construction of the F/C chimeric satellite. pFfl23, a nearly full-length RNA F cDNA clone lacking two cytosine residues at the 3′-end, and pPM1-2-47M+, a full-length RNA C clone, were grown in the dcm- *E.coli* strain dcm 6 in order to facilitate subsequent digestion with *Eco*RII. The partial RNA F cDNA insert (shaded bar) and the full-length RNA C cDNA insert (open bar) were purified after digesting pFfl23 and pPM1-2-47M+, respectively, with *Sma*I. The RNA F and RNA C cDNA fragments were subjected to either full or partial digestion with *Eco*RII, followed by ligation of the 5′ *Sma*I–*Eco*RII fragment of RNA F to the 3′ *Eco*RII–*Sma*I fragment of RNA C. The F/C cDNA was digested with *Sma*I and ligated into the *Sma*I site of pPMI1 creating plasmid pF/C42. Restriction site abbreviations are S, *Sma*I; Sn, *Sna*BI; EI!, *Eco*RII; E, *Eco*RI. Black arrow indicates λ Pr promoter.

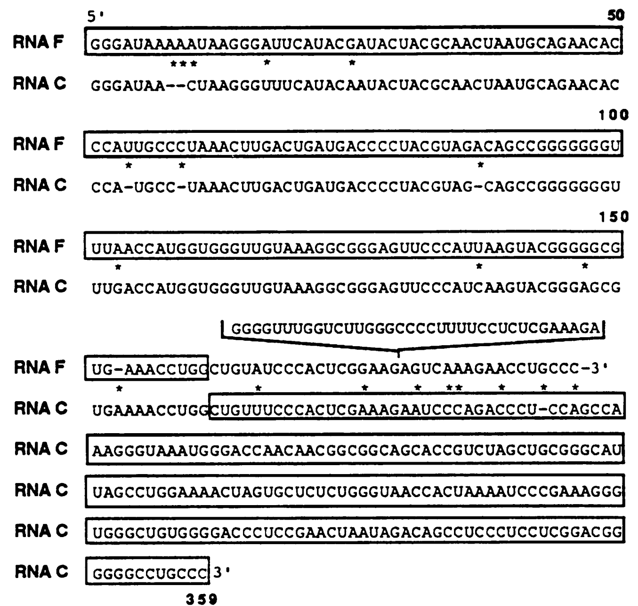

Fig. 2. Sequence of the RNA F/RNA C chimeric satellite. Sequences from RNA F and RNA C which make up the chimeric satellite are boxed. Asterisks (*) indicate base differences between RNA F and RNA C. RNA F also contains a 36-base insert at position 179. The RNA F sequence shown here is from a cloned sequence and differs from a previously published RNA F sequence (Simon and Howell, 1986) by a G → A change at position 9 and by the addition of an adenine residue at position 10.

genome. To determine which of these two domains was responsible for the virulence of RNA C, a chimeric satellite was constructed composed of 155 bases at the 5′-end of the non-virulent satellite (RNA F) joined to 200 bases from the 3′-end of the RNA C (Figure 1). The 155 bases at the 5′-end falls just short of the full 194 bases in the domain of satellite homology; however, a convenient *Eco*RII restriction site, commonly shared between the two satellites, permitted us to make an exchange at this site. This segment at the 5′-end of RNA F differs from the corresponding sequence of RNA C by 12 nucleotides including five inserted bases and one deleted base (Figure 2). When plants were inoculated with the chimeric satellite RNA along with helper virus inoculum (HVI described in Simon and Howell, 1987), several plants at 3 weeks post-inoculation (at a frequency comparable to inoculations with normal satellite) showed severe (RNA C-type) symptoms, typical of those produced by normal RNA C and clearly distinguishable from the symptoms produced by HVI alone (Figure 3). In plants that showed symptoms, a satellite RNA of the size expected for a hybrid F/C satellite was recovered (lanes D and E, Figure 3; the double band observed in lane D may be the result of incomplete RNA denaturation—we have no evidence for RNA heterogeneity in the hybrid F/C satellite). In order to demonstrate that plants were infected with the chimeric satellite and not with a wild-type RNA C revertant or contaminant, the accumulated satellite RNA was sequenced by extending the primer oligonucleotide (oligo-2), which was complementary to nucleotides 174–199. The sequence of RNA in the region analyzed (from oligo-2 to the 5′-end) corresponded exactly to the original chimeric transcript which was used to inoculate plants (data not shown). Therefore, we concluded that the severe symptoms produced by plants infected with the *in vitro* synthesized RNA F/C chimeric satellite resulted from the 200 bases at the 3′-end of RNA C, which is largely composed of the domain of homology to the 3′-end of the helper virus genome (Figure 4).

In vitro mutagenesis of RNA C

In an effort to locate determinants in the virulent satellite (RNA C) which affect other satellite functions, we introduced small internal deletions and insertions at various restriction sites in RNA C. Mutations were created mainly by filling in 5′ overlaps at convenient restriction sites with *Escherichia coli* DNA polymerase large fragment (Klenow) or by inserting linkers or polylinkers at these restriction sites.

Transcripts containing the various mutations along with the HVI were used to inoculate turnips. A total of 13 different insertions and deletions were tested for infectivity and symptom production spanning nearly the entire length of RNA C (Figure 4). Mutant satellites containing alterations in the midsection of the satellite, covering ~50% of the molecule and ranging from single-base to 60-base insertions, were infectious (Table I and Figure 4). Mutants altered in the 3′-region of the molecule were not infectious, i.e.

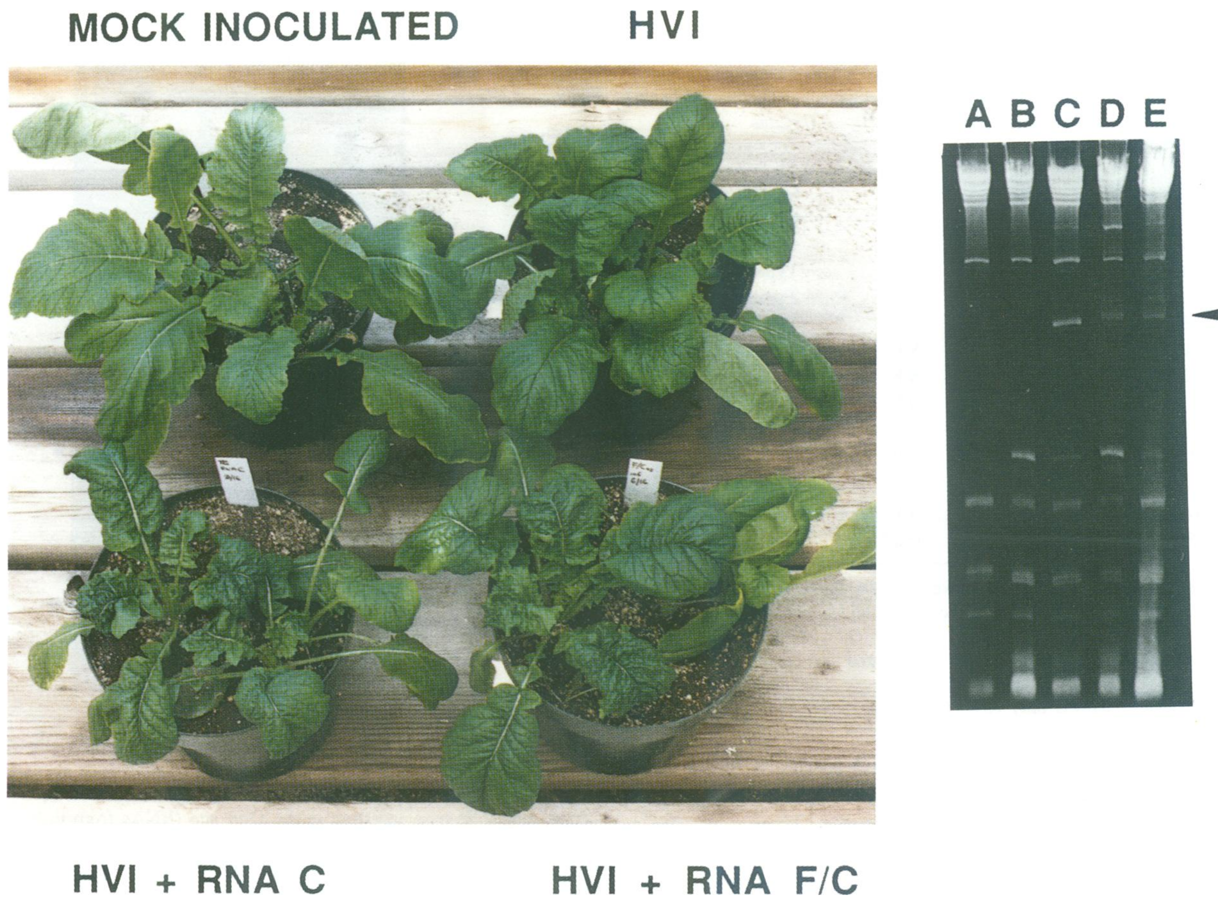

Fig. 3. Symptoms produced in turnip plants (**left**) 3 weeks following inoculation with a mock inoculum; HVI, HVI plus *in vitro* synthesized normal RNA C; HVI plus *in vitro* synthesized F/C chimeric satellite. Gel pattern (**right**) of total RNA isolated from turnip plants inoculated with (A) mock inoculum; (B) HVI; (C) HVI plus *in vitro* synthesized normal RNA C; (D and E) HVI plus *in vitro* synthesized F/C chimeric satellite (RNAs from two different inoculated plants).

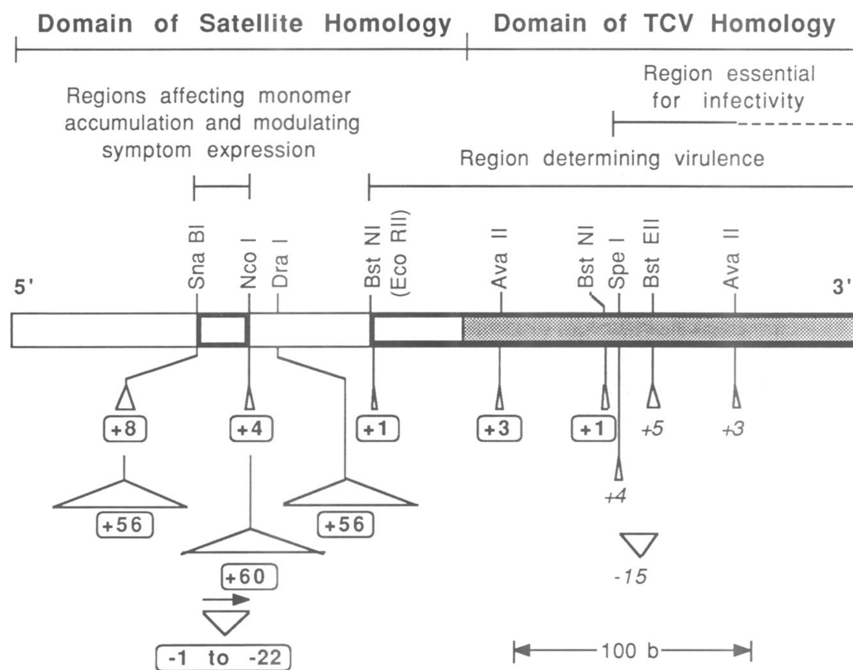

Fig. 4. Map of mutations and modifications in RNA C. Full-length RNA C *Sma*I insert from pPM1-2-47M+ is shown. Sequences were inserted (+ number of bases) and deleted (− number of bases) at various restriction sites as described in Materials and methods. RNA C mutants which were infectious and produced symptoms are boxed (see Table I.)

Table I. Infectivity of RNA C mutants

Restriction site (nt position)	No. of inserted or deleted bases	No. of RNA C-positive plants[a]	Symptoms in RNA C-positive plants
Insertions			
*Sna*BI (79)	8	8/8	Greater severity
*Sna*BI (79)	56	7/9	Greater severity
*Nco*I (105)	4	3/4	Typical severity[b]
*Nco*I (105)	60	4/9	Typical severity
*Dra*I (115)	56	4/9	Typical severity
*Bst*NI (156)	1	8/9	Typical severity
*Ava*II (211)	3	8/8	Typical severity
*Bst*NI (252)	1	8/8	Typical severity
*Spe*I (262)	4	0/5	—
*Bst*EII (277)	5	0/5	—
*Ava*II (310)	3	0/9	—
Deletions			
*Sna*BI–*Nco*I (79–100)	22	6/8[c]	Greater severity
*Bst*EII (265–279)	15	0/27	—

[a]Plants containing the mutant derivative of RNA C. Negative plants contained neither the mutant derivative nor a wild-type revertant of RNA C.
[b]Typical symptoms are severe. Plants are stunted and leaves heavily wrinkled.
[c]Positive plants contained only RNA C dimers.

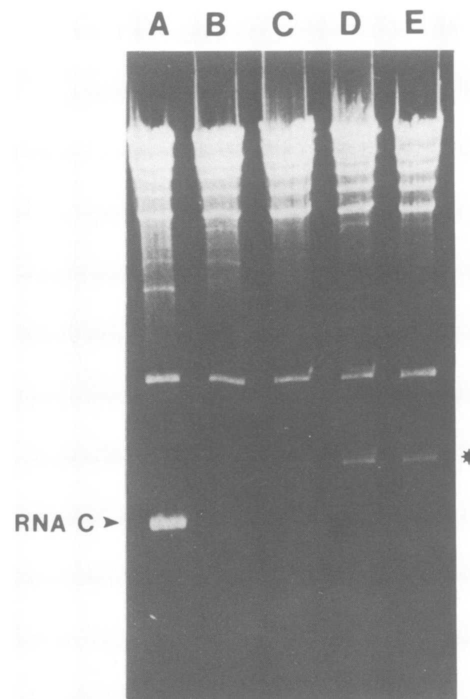

Fig. 5. Gel pattern of total RNA isolated from turnip plants inoculated 3 weeks previously with (**A**) HVI plus *in vitro* synthesized normal RNA C; (**B**) HVI; (**C–E**) HVI plus *in vitro* synthesized RNA C containing a 56-base insertion in the *Dra*I site (RNAs from three different inoculated plants). Asterisk (*) indicates the gel migration position of the *in vitro* synthesized RNA C satellite with the 56-base insertion.

produced neither symptoms nor altered forms of RNA C. In no case did we find a mutation that retained infectivity but lost the ability to produce symptoms. However, the mutants with insertions at the *Sna*BI site showed more intense symptoms (Table I). Symptoms in these plants appeared earlier (~1 week) and were even more intense (greater stunting) than the symptoms produced by the normal, unmodified satellite.

To demonstrate that the modified RNA can be recovered in its altered form, RNA was extracted from leaves of infected plants 3 weeks post-inoculation, subjected to electrophoresis on denaturing polyacrylamide gels and the presence or absence of RNA C was determined. An example of the resulting RNA C-type satellite derived from symptomatic plants inoculated with a 56-base insertion at the *Dra*I site is shown in Figure 5. A larger RNA C-type satellite which nearly co-migrated with the inoculated insertion mutant was recovered (lanes C–E) from infected plants. In this situation, some reduction was observed in the yield of the larger satellite however, this was not always the case. [The inoculated RNA (not shown) was slightly larger than the recovered progeny RNAs because the inoculated satellite has up to seven extra bases on the 3′-end due to the location of restriction sites at the corresponding end of the template. None the less, the extra bases are not found in the progeny satellite RNAs (Simon and Howell, 1987). This finding indicated that the satellite retained its insertion during propagation in the infected plant. However, long-term studies on the stability of the insert-bearing satellites upon serial passage have not been undertaken.

In the case where the insertion or deletion was too small to detect by a shift on polyacrylamide gels, the sequence of the resulting satellite RNA was determined in the region of the modification by sequencing primer-extended cDNAs. Two different oligonucleotide primers which specifically hybridize to RNA C were used to synthesize the cDNAs: oligo-7, complementary to terminal 19 bases at the 3′-end of RNA C was used to synthesize cDNA corresponding to the 3′-domain of RNA C, and oligo-2, described above, was used to confirm the presence of mutations in the 5′-domain of RNA C. In all cases, the variant RNA C recovered from infected plants was identical to the *in vitro* synthesized transcripts which were originally used in the inoculum (except for the extra bases at the 3′-end). An example is shown in Figure 6 where the satellite RNA recovered from plants still retained the 3-base insertion at the *Ava*II site (nucleotide position 211).

Deletions affecting monomer RNA C accumulation

The consequence of having a 22-base deletion mutant between the *Sna*BI and *Nco*I sites was most unusual. The deletion resulted in the accumulation of dimers, not monomers, and like the insertions at the *Sna*BI site, symptoms produced in the infected plants were more intense. By sequencing the RNA accumulated in infected plants, we demonstrated that the dimers were tandem dimers and that the 22-base deletion was still present and found in both arms of the dimer (data not shown). Sequencing was performed as before by extending on oligo-2-; however, two cDNAs were produced (by extension from primer bound to either binding site on the dimers) which were separated on denaturing polyacrylamide gels.

In order to locate more precisely the sequence between the *Sna*BI and *Nco*I sites which affected monomer accumulation, a series of *Bal*31 deletions were created in satellite template (pPM2-247M+) from the *Sna*BI site (nucleotide position 79) toward the *Nco*I site (nucleotide position 100). The deletions ranged from 1 to 11 bases. RNAs from respresentative plants in the deletion series (Figure 7) demonstrated that deletions >4–5 bases resulted

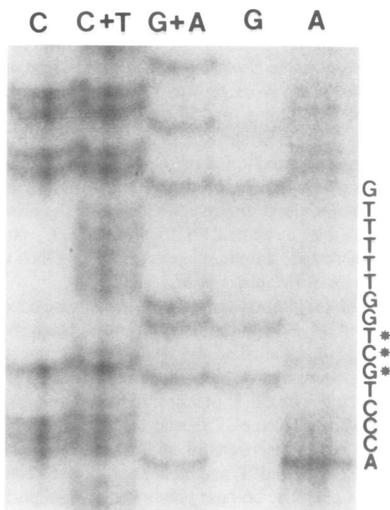

Fig. 6. Sequence analysis of RNA from plants inoculated with RNA C transcripts containing a three-base insertion at the 5'-most *Ava*II site (GGACC, see Figure 4). The sequence was generated by hydridizing ^{32}P-labeled oligo-7 to the 3'-end of RNA C in an extract from infected turnip leaves, synthesizing cDNA with MMLV reverse transcriptase, then sequencing the cDNA by chemical degradation. Asterisks (*) indicate the three inserted bases. Since the cDNA has been sequenced, the sequence is the complement of the published sequence (Simon and Howell, 1986).

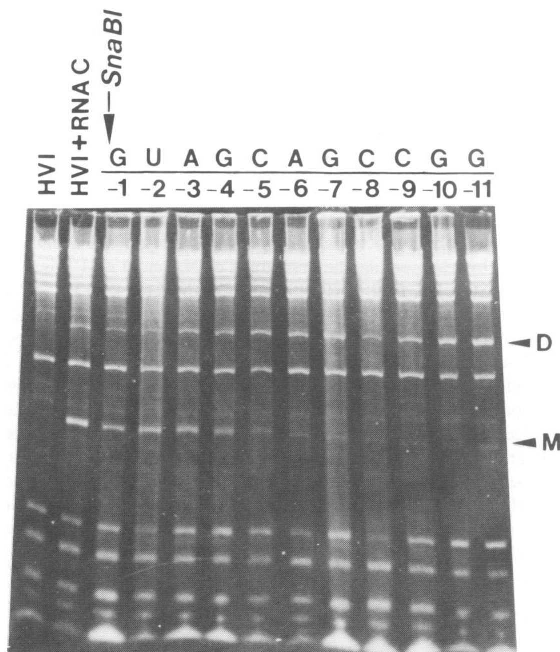

Fig. 7. Gel pattern of total RNA isolated from turnip plants inoculated with the helper virus inoculum (HVI); HVI plus RNA C; HVI plus *in vitro* synthesized RNA Cs with stepwise single-base deletions (designated −1 to −11) from the *Sna*BI site toward the 3'-end of RNA C. The sequence of RNA C corresponding to the single-base deletions is shown above the line. The general gel migration position of monomers (M) and dimers (D) of RNA C is shown on the right.

in a progressive loss of monomers and a preferential accumulation of dimers. Thus, it appears that removing a small block of bases adjacent to the *Sna*BI site and extending into the AGC of an AGCAGC repeat alters the accumulation pattern of monomeric forms of RNA C.

Discussion

The virulent satellite, RNA C, is composed of two domains, a 5'-domain of satellite homology and a 3'-domain of viral genome homology (Simon and Howell, 1986 and Figure 4). Since the 3'-domain is unique to the virulent satellite, we have suggested that this domain or elements within it determine virulence. We have shown here that a chimeric satellite composed largely of the 5'-domain of satellite homology from the avirulent satellite (RNA F) and the 3'-domain of virus homology from the virulent satellite (RNA C) was infectious and produced severe RNA C-type symptoms. This result argues that the terminal 200 bases of RNA C, which is composed primarily of the 166-base TCV 3'-domain of virus homology, is a region determining virulence (Figure 4).

The integrity of the 3'-end of the satellite appeared to be essential for infectivity, because small insertions in the 3'-end destroyed infectivity (Figure 4). It is curious that a region in the domain of virus homology is essential for infectivity since this domain is absent in the infectious, avirulent satellites. The avirulent and virulent satellites share only 7 bases in common at the 3'-end (Simon and Howell, 1986). Only two insertions toward the 5'-end of the domain of virus homology, a 3-base insertion at the *Ava*II site and a single-base insertion at the *Bst*NI site, yielded infectious satellite (Figure 4), and both produced severe RNA C-type symptoms. So far, we have not been able to produce small mutations in the region determining virulence that alter symptom expression without destroying infectivity. We cannot eliminate the possibility that the simple accumulation of RNA C in any replicating form will produce symptoms. However, we are currently constructing single-base mutations at the 3'-end of RNA C in an effort to separate virulence-determining from infectivity-determining elements.

Viroids have extensive secondary structure and are more vulnerable to modifications brought about by *in vitro* mutagenesis (Ishikawa *et al.*, 1985; Owens *et al.*, 1986; Visvader and Symons, 1986; Hammond and Owens, 1987). This is not the case for RNA C. We have demonstrated that RNA C tolerates as much as 60 additional bases inserted at various sites in the midsection of the satellite (covering ~50% of the molecule). Because this large region of the molecule can tolerate small insertions and deletions, we asked whether the entire midsection of the molecule could be deleted. In preliminary experiments where stepwise deletions from the *Nco*I toward the 3'-end of molecule were analyzed, deletions >18 bases were not infectious. Therefore, although small fragments of DNA can be inserted or deleted from a large region of the midsection of the satellite, that region is not entirely dispensable for infectivity. The 3'-end of satellite is quite intolerant to small insertions or deletions, and the sensitivity of this region may be due to structural requirements of a replicase. It has been shown that for the unrelated virus, brome mosaic virus, deletions 130 bases from the 3'-end affect the level of virus replication *in vitro* (Bujarski *et al.*, 1985, 1986). Other reasons for the non-infectivity of RNA C altered in the 3'-end could be the loss of the ability to be encapsidated or the loss of some other component that regulates satellite replication.

The most intriguing mutations in RNA C were deletions which block the accumulation of RNA C monomers and which presumably affect satellite RNA processing. In a stepwise series of *Bal*31 deletions it was demonstrated that the region affecting monomer accumulation encompasses an

AGCAGC repeat (Figure 7). We presume that the sequence itself and not some aspect of the change in secondary structure of RNA C may be important to RNA processing or monomer accumulation, because substantial insertions at nearby sites (56 bases at the *Sna*BI site and 60 bases at the *Nco*I site) do not affect monomer accumulation. Whether these mutations reveal anything about the normal mode of replication or processing of the satellite is not known. It is curious that the mutant satellite accumulates as a dimer—a possible intermediate in a satellite RNA maturation process. Alternatively, the mutant monomer may be unstable and lost from the stready-state population.

Recent evidence has demonstrated the ability of some viroids (Hutchins *et al.*, 1986) and satellites (Prody *et al.*, 1986; Buzayan *et al.*, 1986; Forster and Symons, 1987a,b) to self-process. The self-cleavage reaction involves a hammerhead structure composed of conserved sequences (Forster and Symons, 1987a,b). We have not observed self-processing by *in vitro* synthesized RNA C dimers under a variety of conditions (A.E.Simon and K.Klucher, unpublished results). Since only circular satellites (virusoids), satellites which circularize inside the plant (satellite of tobacco ringspot virus) or viroids undergo self-cleavage reactions, a different mechanism may be involved in processing linear satellite RNAs such as RNA C.

Finally, some of the mutations in the region which affect monomer accumulation also modulate symptom expression. For example, the *Sna*BI−*Nco*I deletion and the small insertions at the *Sna*BI site intensify symptom expression. This was unexpected since these mutations lie in the domain of satellite homology, in common with the avirulent satellites and outside the region determining virulence (Figure 4). However, we regard these mutations as having an indirect effect on virulence, perhaps by altering the rate of accumulation of the satellite. Plants inoculated with these mutants express symptoms earlier than with the normal, virulent satellite and it appears that the mutant blocks leaf growth at an earlier stage. We presume that because some, but not all, mutations which affect monomer accumulation also produce intensified symptoms, the region affecting monomer accumulation and the region modulating symptom expression overlap, but do not exactly coincide.

This analysis has generated a map of the virulent satellite in which various functions can be roughly located (Figure 4). Thus, by generating a chimera between a virulent and avirulent satellite, we have demonstrated that the region which determines virulence is composed largely of the domain of TCV homology. The 3′-half of this region is essential for infectivity, i.e. small insertions or deletions in this region are lethal mutations. Two small and apparently overlapping regions in the domain of satellite homology affect monomer accumulation and modulate symptom expression. As further modifications of the satellite are analyzed the functional regions described here can be carefully delimited and new functional regions will undoubtedly come to light.

Materials and methods

In vitro mutagenesis of RNA C

The plasmid used to construct mutations in the virulent satellite (RNA C) was pPM1-2-47M+ (Simon and Howell, 1987), which contains a full-length monomer cDNA of the RNA C satellite cloned into the *Sma*I site of pPM1, a vector designed for the synthesis of RNA *in vitro* using an *E.coli* RNA polymerase promoter (Ahlquist and Janda, 1984) or pPM2-2-47M+, a plasmid identical to pPM1-2-47M+ with the exception of an *Xho*I linker directly downstream from the *Eco*RI site at the 3′-end of the satellite template.

To insert 4 bases at the *Nco*I site (nt 105), 4 bases at the *Spe*I site (nt 262), 1 base at the *Bst*NI sites (nt 156 or 252), 3 bases at the *Ava*II sites (nt 211 or 310) or 5 bases at the *Bst*EII site (nt 277) in RNA C, 1 μg of pPM1-2-47M+ was subjected to full or partial digestion with the appropriate enzymes, and the overlapping ends were blunted by the action of 1 U of *E.coli* DNA polymerase large fragment (Klenow) in the presence of all four dXTPs. The DNA was incubated for 15 min at 25°C, phenol extracted, ethanol precipitated and the termini religated using T4 DNA ligase according to standard procedures (Maniatis *et al.*, 1982).

An 8-base *Xho*I linker inserted at the *Sna*BI site was introduced by digesting 1 μg of pPM1-2-47M+ with *Sna*BI, followed by removing terminal phosphates with 1 U of calf alkaline phosphatase for 15 min at 37°C. Kinased *Xho*I linkers (5 μg) were ligated to the linearized pPM1-2-47M+ plasmid, followed by digestion with 50 U of *Xho*I and subsequent religation of the plasmid. The polylinker from pUC19 was inserted in the RNA C template in order to create larger insertions of 56−60 bases. pUC19 (10 μg) was digested with *Eco*RI and *Hin*dIII, and blunt ends formed using Klenow DNA polymerase and dXTPs. The 56-base polylinker was gel purified and ligated into calf-alkaline-phosphatase-treated pPM1-2-47M+ digested with either *Sna*BI, partial *Dra*I (followed by gel purification of linear plasmid) or *Nco*I (after filling in the 5′ overhanging sequence with Klenow and dXTPs).

Nucleotides 79−100 were deleted by digesting 1 μg of pPM1-2-47M+ with *Sna*BI and *Nco*I, filling in the ends with Klenow and dXTPs, gel purifying the large linear plasmid fragment, and religating the ends. Nucleotides 265−279 were deleted by digesting 1 μg of pPM1-2-47M+ with *Bst*EII, followed by treatment with 7 U of mung bean nuclease (Pro Mega Biotech) for 15 min at 37°C according to the manufacturer's instructions.

Cloning of near-full-length RNA F

We described previously the cloning of pF2, a plasmid containing a partial RNA F cDNA insert composed of nucleotides 21−228 cloned into the *Kpn*I site of pUC 19 (Simon and Howell, 1986). In order to lengthen the sequence of this clone to the 5′-end of RNAF, pF2 was linked to another partial RNA F cDNA clone which extended to the 5′-end. The partial RNA F cDNA clone extending to the 5′-end was generated as follows: 0.5 μg of oligo-3, a 15-base oligomer complementary to nucleotides 96−110 was hybridized as previously described (Simon and Howell, 1986) to 12.5 μg of total RNA from plants infected only with RNA F and the helper virus. cDNA was synthesized using 200 U of MMLV reverse transcriptase (BRL) according to the manufacturer's instructions. Second-strand cDNA was synthesized by resuspending the first-strand cDNA in 50 mM Tris, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 40 μM of each of the dXTPs, 20 μCi of [α^{32}P]dATP, 50 μg/ml BSA, 1 U of Klenow DNA polymerase, and 4 μg of oligo-6, a 15-base oligomer homologous to the 15 terminal nucleotides at the 5′-end of RNA F. The reaction was terminated after 1 h at 23°C by the addition of 1 μl of 10% SDS, followed by phenol extraction and ethanol precipitation. The double-stranded cDNA was blunt-end ligated into *Sma*I-digested, calf-alkaline-phosphatase-treated pPM1 and transformed into *E.coli* strain dH5α. The insert from one positive clone pF5′, which contained the complete 5′ sequence of RNA F, was combined with pF2 to create the nearly complete (minus 2 bases at the 3′-end) RNA F cDNA clone pFfl23. Briefly, the large *Sna*BI−*Sca*I fragment from pF2 (containing nucleotides 82−228 of RNA F) was ligated to the small *Sna*BI−*Sca*I fragment of pF5′ (containing nucleotides 1−81 of RNA F).

Bal31 deletions

Stepwise deletions between the *Sna*BI and *Nco*I sites were created in pPM2-2-47M+. *Sna*BI-digested pPM2-2-47M+ ((3 μg) was treated with 1.5 U of the slow-acting form of *Bal*31 (BRL) for 1−9 min according to the manufacturer's directions. DNAs from the various time points were mixed, treated with Klenow DNA polymerase and dXTPs, then digested with *Pst*I. The 1-kb fragment containing the RNA C cDNA deletions was gel purified and ligated to the 3-kb *Pst*I−*Sna*I fragment from pPM2-2-47M+. Plasmids containing deletions of different sizes were identified by visualization of *Sma*I−*Nco*I fragments on 6% polyacrylamide gels as well as by sequencing the DNA in the region of the deletion by chemical degradation. Infectivity assays, primer extensions and DNA sequencing have been previously described (Simon and Howell, 1987).

Acknowledgements

This work was supported by the NSF (DM84-17352) and a NIH postdoctoral fellowship to A.E.S.

References

Ahlquist,P. and Janda,M. (1984) *Mol Cell Biol.*, **12**, 2876–2882.
Altenbach,S.B. and Howell,S.H. (1981) *Virology*, **112**, 128–135.
Bujarski,J.J., Dreher,T.W. and Hall,T.C. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5636–5640.
Bujarski,J.J., Ahlquist,P., Hall,T.C., Dreher,T.W. and Kaesberg,P. (1986) *EMBO J.*, **5**, 1769–1774.
Buzayan,J.M., Gerlach,W.L. and Bruening,G. (1986) *Nature*, **323**, 349–353.
Collmer,C.W. and Kaper,J.M. (1986) *Biochem. Biophys. Res. Commun.*, **135**, 290–296.
Collmer,C.W., Tousignant,M.E. and Kaper,J.M. (1983) *Virology*, **127**, 230–234.
Forster,A.C. and Symons,R.H. (1987a) *Cell*, **49**, 211–220.
Forster,A.C. and Symons,R.H. (1987b) *Cell*, **50**, 9–16.
Francki,R.I.B. (1985) *Annu. Rev. Microbiol.*, **39**, 151–174.
Garcia-Arenal,F., Zaitlin,M. and Palukaitis,P. (1987) *Virology*, **158**, 339–347.
Gerlach,W.L., Buzayan,J.M., Schneider,I.R. and Bruening,G. (1986) *Virology*, **151**, 172–185.
Hammond,R.W. and Owens,R.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3967–3971.
Hillman,B.I., Carrington,J.C. and Morris,T.J. (1987) *Cell*, **51**, 427–433.
Hutchins,C.J., Rathjen,P.D., Forster,A.C. and Symons,R.H. (1986) *Nucleic Acids Res.*, **14**, 3627–3640.
Ishikawa,M., Meshi,T., Okada,Y., Sano,T. and Shikata,E. (1985) *J. Biochem.*, **98**, 1615–1620.
Kurath,G. and Palukaitis,P. (1987) *Virology*, **159**, 199–208.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Clonong. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Murant,A.F. and Mayo,M.A. (1982) *Annu. Rev. Phytophathol.*, **20**, 49–70.
Owends,R.A., Hammond,R.W., Gardener,R.C., Kiefer,M.C., Thompson,S.M. and Cress,D.E. (1986) *Plant Mol. Biol.*, **6**, 179–192.
Prody,G.A., Bakos,J.T., Buzayan,J.M., Schneider,I.R. and Bruening,G. (1986) *Science*, **231**, 1577–1580.
Schnölzer,M., Haas,B., Ramm,K., Hofmann,H. and Sanger,H.L. (1985) *EMBO J.*, **4**, 2181–2190.
Simon,A.E. and Howell,S.H. (1986) *EMBO J.*, **5**, 3423–3428.
Simon,A.E. and Howell,S.H. (1987) *Virology*, **156**, 146–152.
Tabler,M. and Sänger,H.L. (1985) *EMBO J.*, **4**, 2191–2199.
Visvader,J.E. and Symons,R.H. (1985) *Nucleic Acids Res.*, **13**, 2907–2920.
Visvader,J.E. and Symons,R.H. (1986) *EMBO J.*, **5**, 2051–2055.

Received on April 5, 1988; revised on June 6, 1988