Satellite RNA of cucumber mosaic virus forms a secondary structure with partial 3'-terminal homology to genomal RNAs

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ABSTRACT

Sat-RNA is one of several replicating satellite RNAs which have been isolated from RNA encapsidated in cucumber mosaic virus (CMV) and which are totally dependent on CMV for replication. The 336 residue sequence of Sat-RNA obtained using the dideoxynucleotide chain termination and partial enzymic digestion procedures shows only a few short stretches (up to 11 residues) of sequence homology with one of the three CMV genomal RNAs so far sequenced. Sat-RNA has 88% sequence homology with another, previously sequenced, satellite RNA of CMV, CARNA 5. Analysis of partial digests of 5'- or 3'-^{32}p-Sat-RNA with nuclease S_1 or RNase T_1 under non-denaturing conditions showed that only about 10% of the residues in Sat-RNA were cleaved. Further data on base-paired segments of Sat-RNA were obtained using digestion with RNase T_1 followed by electrophoretic fractionation of the resulting fragments under both non-denaturing and denaturing conditions. On the basis of this data, a complete secondary structure model is proposed for Sat-RNA with 52% of its residues involved in base pairs. A prominent hairpin at the 3'-terminus of Sat-RNA shows considerable sequence and structural homology with parts of the 3'-terminal tRNA-like structure of the CMV genomal RNAs.

INTRODUCTION

Several satellite RNAs have been found encapsidated with viral RNA in some, but not all, isolates of cucumber mosaic virus (CMV) (1-3). These satellite RNAs are small (about 335 residues) linear molecules and are much smaller than the three CMV genomal RNAs of approximately 4,000, 3,400 and 2,200 residues (RNAs 1 to 3, respectively; ref. 4) with which they have essentially no sequence homology detectable by hybridization analysis with complementary DNA (cDNA) (2,5). They are totally dependent on the helper virus for their replication, encapsidation and transmission.

Sequence and structural studies of such satellite RNAs are important for several reasons. They may provide information about their possible origins and, when compared to known sequence and structural features of the helper virus RNAs, about the minimum requirements for viral RNA

replication. Also of interest are possible gene products since the CMV satellite RNAs do not encode a satellite-specific coat protein as do some other plant virus satellite RNAs (3). Further, the satellite RNA can modify the disease symptoms of the parent virus (6,7) which indicates specific interactions with the viral and/or host genomes.

The CMV satellite RNA (Sat-RNA) isolated in Adelaide (2,7) has about 70% sequence homology with another satellite RNA, CMV associated RNA 5 or CARNA 5, isolated in the U.S.A. (1), as determined by hybridization analysis (2,7). CARNA 5 is the only CMV satellite RNA whose sequence has been published (8); it is 335 residues long, has a m^7G cap and two non-overlapping open reading frames encoding possible proteins, and has different biological properties to Sat-RNA (6,7). Other isolates of satellite RNAs have been reported for CMV (9) and for the related peanut stunt virus (10).

In this paper we present the nucleotide sequence of Sat-RNA and a secondary structure model of it which is supported by digestion studies with specific nucleases under non-denaturing conditions. The same model can be applied to CARNA 5. In addition, we consider the striking sequence and structure homology between the 3'-terminal structure of Sat-RNA and that of the genomal RNAs of four strains of CMV which can support Sat-RNA in plant hosts (2,7).

MATERIALS AND METHODS

Materials

Cucumber seedlings (Cucumis sativus cv. Polaris) were infected with CMV (Q strain) and Sat-RNA, virions purified and total RNA extracted (2,4). Sat-RNA was purified by electrophoresis on one 5% polyacrylamide slab gel (11). γ-^{32}P-ATP was prepared from carrier-free ^{32}Pi (12). Phage T4 polynucleotide kinase and nuclease S_1 were from Boehringer and T4 RNA ligase from P-L Biochemicals. Ribonucleases T_1, U_2, PhyM and the extracellular RNase of Bacillus cereus were obtained as in (13).

Sequencing of Sat-RNA

The nucleotide sequence of the first 280 residues from the 3'-end was obtained using the dideoxynucleotide chain termination technique (14,15) with Sat-RNA after in vitro polyadenylation with E. coli poly(A) polymerase (2). Prior to 5'-end labelling using γ-^{32}P-ATP and T4 polynucleotide kinase (13), the m^7G cap was removed by periodate oxidation followed by β-elimination in the presence of aniline, essentially as in (16). The

5'-end sequence was then obtained using the specific partial enzymic cleavage method (13).

Analysis of Secondary Structure

Mapping of nuclease sensitive bonds under non-denaturing conditions. Sat-RNA was either 5'-end labelled as described above or 3'-end labelled using 5'-^{32}p-pCp and T4 RNA ligase (17). The end-labelled RNA was purified by electrophoresis on 40 x 20 x 0.05 cm 3% polyacrylamide, 7 M urea gels followed by elution at room temperature in 0.5 M ammonium acetate, 0.1% SDS, 1 mM EDTA, and ethanol precipitation (18). For digestion with nuclease S_1, end-labelled Sat-RNA was preincubated for 10 min at 37°C in 40 mM sodium acetate, pH 5.0, 1.0 mM $ZnSO_4$, 0.2 M NaCl, 330 µg/ml carrier E. coli. tRNA, and then for 10 min at 37°C in the presence of varying concentrations of nuclease S_1 (see figure legends); final reaction volume was 6 µl for each assay. Digestions were stopped by addition of 2 µl 1% SDS, 50 mM EDTA. For digestions with RNase T_1, end-labelled Sat-RNA was incubated for 10 min at 37°C with either 0.01 or 0.1 units RNase T_1 in 6 µl 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.2 M NaCl, 330 µg/ml carrier E. coli tRNA and terminated as for nuclease S_1 digestions.

After addition of 5 µl loading buffer (95% deionised formamide, 10 mM EDTA, 0.03% xylene cyanol FF, 0.03% bromophenol blue) to each reaction mix, all samples were denatured by heating at 80°C for 1 min followed by snap cooling prior to analysis by electrophoresis on 40 x 20 x 0.05 cm 20% acrylamide gels or 80 x 20 x 0.05 cm 8% acrylamide gels in 90 mM Tris-borate, pH 8.3, 1 mM EDTA, 7 M urea. To enable the precise location of nuclease sensitive bonds, partial RNase T_1 and RNase U_2 digests of RNA plus random alkali cleaved RNA were prepared as for the sequencing procedure using specific partial enzymic cleavage (13) and electrophoresed as reference tracks.

Generation of base-paired fragments with RNase T_1. Unlabelled Sat-RNA (10 µg) in 100 µl 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 M NaCl, was digested with RNase T_1 at 1,000 units/ml at 0°C for 1.0 h. After phenol-chloroform extraction and ethanol precipitation, the RNA fragments were 5'-end labelled with γ-^{32}P-ATP (200 µCi) and polynucleotide kinase (13). After another phenol-chloroform extraction and ethanol precipitation, the labelled fragments were dissolved in 20 µl of 10% (v/v) glycerol, 1 mM EDTA, and fractionated by electrophoresis on a 40 x 20 x 0.05 cm non-denaturing 12% polyacrylamide gel in 90 mM Tris-borate, pH 8.3, 1 mM EDTA, for 6 h at 12 mA. After detection by autoradiography, labelled

bands were excised and recovered by elution as above, and fractionated into individual, end-labelled fragments on a denaturing 12% polyacrylamide gel as above but in the presence of 7 M urea. After elution, each band was identified using partial enzyme digestion with RNases T_1 and U_2 and random alkali cleavage followed by gel electrophoresis (13).

RESULTS

Nucleotide Sequence of Sat-RNA and Potential Polypeptide Products

The sequence of Sat-RNA is given in Fig. 1 and is compared with the sequence of CARNA 5 (8). It contains an m^7G cap, is 336 residues long (excluding the cap residue) and has 88% sequence homology with the 335 residues of CARNA 5. The AUG initiation codon at residues 11-13 of CARNA 5 is not present in Sat-RNA although the in-phase UGA stop codon at residues 92-94 is conserved. There is a common AUG codon at residue 134 of Sat-RNA (residue 135 of CARNA 5) but a single residue deletion after residue 138 in Sat-RNA gives an in-phase UAG termination codon at residue 185 in Sat-RNA compared with a UGA stop codon at residue 261 in CARNA 5. The two RNAs

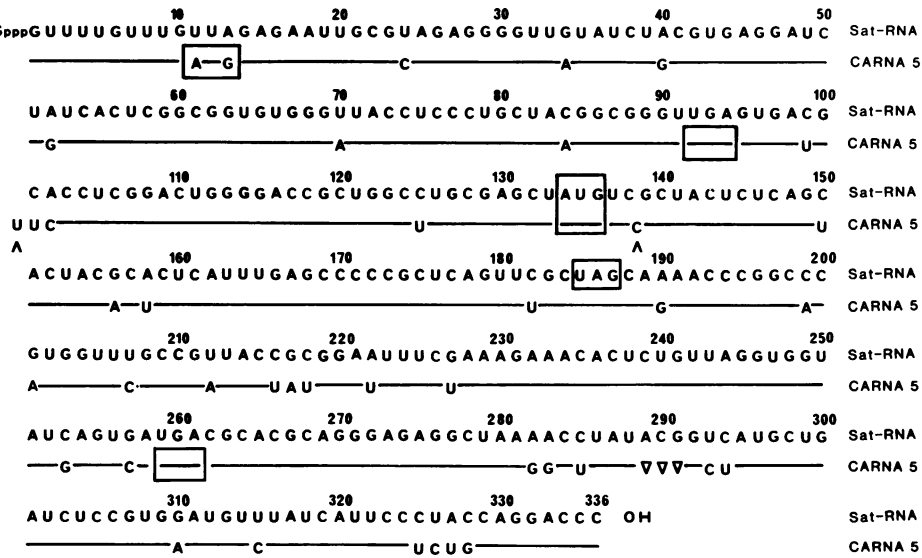


Figure 1. Nucleotide sequence of Sat-RNA and comparison with that of CARNA 5 (from ref. 8). Only CARNA 5 residues differing from those in Sat-RNA are shown while insertions and deletions in CARNA 5 relative to Sat-RNA are represented by Λ and ∇, respectively. Possible initiation codons and their corresponding in-phase termination codons are boxed.

therefore code completely different polypeptides; one 17 residue polypeptide for Sat-RNA and two polypeptides of 27 and 42 residues for CARNA 5.

Secondary Structure Analysis of Sat-RNA

Possible secondary structure models for the Sat-RNA sequence were constructed using the matrix procedure of Tinoco et al. (19). In view of the many possible base-paired structures found, experimental studies were undertaken to determine which structures actually occur in solution. The approach used was to determine the residues present in single-strand and double-strand regions by digestion of Sat-RNA with specific nucleases.

When Sat-RNA was digested under non-denaturing conditions with the single-strand specific nuclease S_1, only about 10% of the residues were cleaved under the conditions used (Fig. 2). Thus, digestion of 5'-^{32}p-Sat-RNA for 10 min at 37°C with two levels of nuclease S_1 showed cleavage at residues 3 to 9 and 30 to 33 only when the digests were analysed on 40 cm long 20% polyacrylamide gels in 7 M urea (Fig. 2A). However, analysis on longer 80 cm 8% polyacrylamide gels disclosed further cleavage, predominantly at sites between residues 133 and 162 (not shown). Similarly, cleavage of 3'-^{32}p-Sat-RNA by nuclease S_1 was observed at residues 314 to 316, and at residue 297 (Fig. 2C). Again, analysis of digests on the longer 80 cm 8% acrylamide gels disclosed further cleavage at sites between residues 220 and 236.

RNase T_1 was found to cleave Sat-RNA at the same regions as nuclease S_1 under the same conditions as shown using both 5'- and 3'-^{32}p-Sat-RNA (Figs. 2B and 2D, respectively). All the bonds cleaved by both enzymes are indicated on the secondary structure model in Fig. 4.

These nuclease cleavage data indicated extensive base-pairing in Sat-RNA but it was necessary to obtain positive information on these base-paired regions in order to derive an overall secondary structure model. Hence, Sat-RNA was digested with a high concentration of RNase T_1 (1,000 units/ml) under conditions designed to maximize base-pairing (0.5 M NaCl, 0°C), followed by 5'-^{32}p-labelling, without prior denaturation, of the double-strand RNA fragments which were then fractionated on a non-denaturing 12% polyacrylamide gel (Fig. 3). Each of the bands A to L was eluted and rerun under denaturing conditions on a 12% polyacrylamide, 7 M urea gel to give from 1 to 9 single-strand fragments (not shown); 25 of the 41 fragments were pure enough to be sequenced and the residue numbers of each fragment are given in Fig. 3.

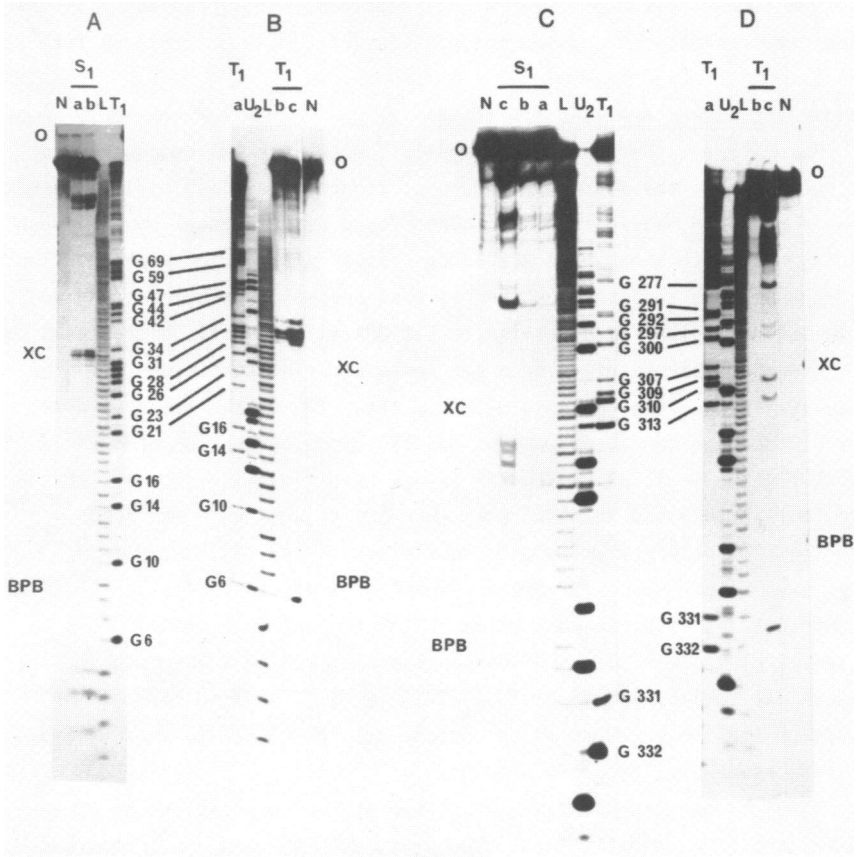
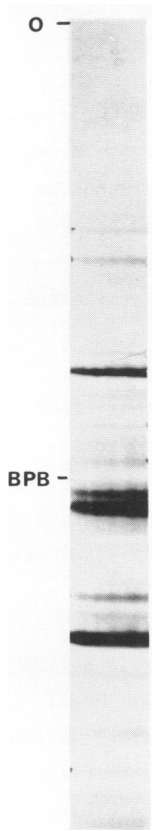


Figure 2. Polyacrylamide gel analysis of partial nuclease digests of 5'- or 3'-^{32}P-Sat-RNA. Sat-RNA was end-labelled and digested under non-denaturing and denaturing conditions as described in Materials and Methods. N, no digestion (control); L, random alkali digestion to produce ladder; U_2, partial RNase U_2 digestion under denaturing conditions used for sequencing. Marker dyes: XC, xylene cyanol FF; BPB, bromophenol blue. 5'-^{32}P-Sat-RNA: Gel A. Nuclease S_1 cleavage under non-denaturing conditions at (a) 70 units/ml and (b) 120 units/ml. T_1, partial RNase T_1 digestion under denaturing conditions used for sequencing. Gel B. RNase T_1 cleavage under non-denaturing conditions at (b) 1.7 units/ml and (c) 17 units/ml. (a), partial RNase T_1 digest under denaturing conditions used for sequencing.
3'-^{32}P-Sat-RNA: Gel C. Nuclease S_1 cleavage under non-denaturing conditions at (a) 40 units/ml, (b) 120 units/ml, and (c) 170 units/ml. T_1, partial RNase T_1 digestion under denaturing conditions used for sequencing. Gel D. RNase T_1 cleavage under non-denaturing conditions at (b) 1.7 units/ml and (c) 17 units/ml. (a), partial RNase T_1 digest under denaturing conditions used for sequencing. All digests were analysed by electrophoresis on 40 x 20 x 0.05 cm 20% polyacrylamide gels in the presence of 7 M urea.



Band	Number of Denatured Fragments	Sequences Present (Residue numbers)
A	1	232 - 332*
B	2	98 - 136; 232 - 332*
C	3	98 - 131; 242 - 332*; 250 - 332*
D	2	47 - 97; 258 - 307
E	3	48 - 90; 258 - 307; 1 n.d.
F	3	3 n.d.
G	4	7 - 28; 35 - 59; 35 - 62; 35 - 63
H	9	35 - 59; 35 - 60; 70 - 85; 69 - 97; 70 - 97; 4 n.d.
I	6	70 - 85; 70 - 93 (2); 70 - 95; 70 - 97; 1 n.d.
J	3	212 - 227; 2 n.d.
K	3	3 n.d.
L	2	2 n.d.

Figure 3. Polyacrylamide gel electrophoresis of base-paired fragments generated by RNase T_1 digestion of Sat-RNA under non-denaturing conditions followed by 5'-^{32}P-labelling as described in Materials and Methods. Bands A to L on the non-denaturing 12% polyacrylamide gel were excised, the RNA eluted and fractionated on a 12% polyacrylamide denaturing (7 M urea) gel. The number of labelled denatured fragments recovered from each band is given together with their residue numbers as determined by sequencing. n.d.; Sequence not determined due to cross contamination. Residues with asterisk; exact termination of fragment could not be accurately determined. Marker dye: BPB, bromophenol blue.

This sequence data showed that the bands on the native gel were of three types. The first type, represented by band A, contained only a single, large 5'-labelled fragment which either could have been present as a single piece of RNA with extensive internal base-pairing or had other RNA fragments base-paired to it that were not 5'-labelled with γ-^{32}P-ATP and polynucleotide kinase under the conditions used. Each of the second type

of band (bands B, C, D, E, G and H) contained two or more fragments derived from two separate regions of Sat-RNA and presumably base-paired over at least some of their sequences.

The third type is represented by band I which contained 6 fragments. Five of these each covered all or most of the sequence between residues 70 and 97 while the sequence of the remaining one could not be determined. These results indicated a prominent hairpin formed between residues 70 and 98. All the RNA molecules containing this sequence (band I, Fig. 3) migrated together in the non-denaturing gel despite the variation in lengths.

Secondary Structure Model of Sat-RNA

The secondary structure model for Sat-RNA in Fig. 4 is consistent with the nuclease cleavage data described above. It contains 87 base-pairs, which involve 52% of the 336 residues, in five major hairpin structures and two other base-paired regions. Within the central third of the molecule, from residues 123 to 234, many nuclease sensitive sites were found; apart from the hairpin structures V and VI (Fig. 4), little secondary structure is possible in this region. There are limited alternative base-pairing structures for those shown between residues 136 to 154 and residues 185 to 198, but these do not affect the overall model significantly. The sites of digestion by nuclease S_1 are in single-strand regions in Fig. 4; these regions are presumably exposed in the tertiary structure of Sat-RNA in solution since nuclease S_1 is unable to cleave single base mismatches (20) and cleaves short single-strand regions poorly (21,22). The large internal loop in region VII of Fig. 4 contained no nuclease S_1 sensitive bonds which indicates that the single strands are protected in some way in the native molecule. Of a total of 11 single-strand stretches longer than 5 residues, only 4 are not cleaved by nuclease S_1.

The overall free energy of the structure is approximately -340 kJ/mol (25°C, 1 M NaCl) calculated according to the rules of G. Steger, H. Gross, J.W. Randles, H. Sänger, and D. Riesner (personal communication). The presence of several branch junctions and most likely of tertiary interactions means that this value is not very accurate. However, it does indicate that the Sat-RNA structure proposed is considerably less stable than the structures proposed for viroids of similar size; e.g., the structure of the 356 residues of chrysanthemum stunt viroid gives a ΔG of -540 kJ/mol (13). The viroid-like RNAs of velvet tobacco mottle virus (365

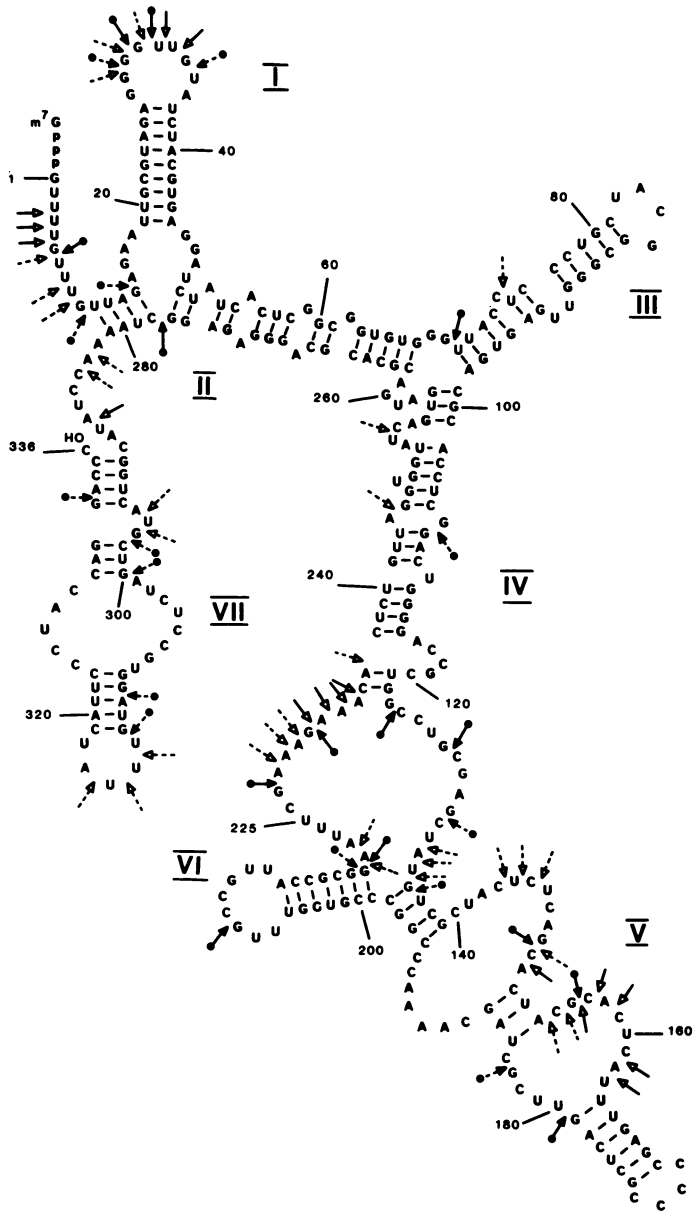


Figure 4. Secondary structure model of Sat-RNA. Residues are numbered from 5'-end as in Fig. 1. Major cleavage sites for nuclease S_1 and RNase T_1 are shown by ⇨ and ➡, respectively. Minor cleavage sites are indicated by dashed arrows. Stems and other base-paired regions are identified by Roman numerals.

residues) and of solanum nodiflorum mottle virus (377 residues), however, have proposed secondary structures with ΔG values more similar to that of Sat-RNA, namely -350 kJ/mol and -450 kJ/mol, respectively (23).

DISCUSSION

Nucleotide Sequence and Secondary Structure of Sat-RNA and CARNA 5

The secondary structure model proposed for Sat-RNA (Fig. 4) provides a basis for further studies on its structure and function and for the comparison of different strains of satellite RNAs of CMV. The model can be applied to CARNA 5 since most of the sequence differences in CARNA 5, relative to Sat-RNA, occur either in single-strand regions or maintain base-pairing. This is especially so in stems I and IV, although the base-paired region of IV requires a slightly rearranged structure with a small decrease in overall stability. It will be of interest to determine if the other satellite RNAs of CMV (9) or of the related peanut stunt virus (PSV) (10) can form a similar secondary structure to the one proposed here for Sat-RNA.

Sat-RNA and CARNA 5 differ greatly in their modification of CMV symptoms in certain host plants (3,6,7). Given the extensive homology in nucleotide sequence and possibly secondary structure, it is feasible that the completely different potential translation products are responsible for these effects. Sat-RNA lacks detectable messenger activity in rabbit reticulocyte lysates but stimulates synthesis of a protein of M_r about 3,000 in the wheat germ system (P. Palukaitis and K.H.J. Gordon, unpublished) which could correspond to the 17 amino acid open reading frame (Fig. 1).

Sequence and Structural Homology betwen Sat-RNA and CMV Genomal RNAs

There are only short scattered stretches of direct nucleotide sequence homology between Sat-RNA and the known sequences of Q-CMV genomal RNAs; i.e., the complete sequence of RNA 3 (24) and the 3'-terminal sequences of RNAs 1 and 2 (15). Ten of the first 12 5'-terminal residues of Sat-RNA are homologous to 10 of the 5'-terminal 11 residues of the subgenomic Q-CMV RNA 4 (24). This homology in a G and U-rich stretch is unlikely to be important for translation of Sat-RNA in view of its poor mRNA activity and, since these G and U-rich stretches are characteristic of the 5'-terminal sequences of some subgenomic mRNAs of plant viruses (25,26), they may represent a signal for the generation of such RNAs from genomal RNAs during replication. Computer analysis has shown other short

stretches of sequence homology between Q-CMV RNA 3 and Sat-RNA; the longest is 11 residues corresponding to residues 63 to 73 in Sat-RNA and residues 1577 to 1587 in RNA 3 (24).

The most prominent structural feature hitherto identified on CMV genomal RNAs is the tRNA-like structure formed by the 3'-terminal 125 residues of the four RNAs (15), the biological significance of which is considered below. In Fig. 5, the 3'-terminal hairpin of Sat-RNA (Fig. 4) is compared to the tRNA-like structure formed by Q-CMV RNA 3 (15) which has been drawn to indicate the possible tertiary structure in a similar way to that employed for tRNA (27). About one-half of the 125 residues of Q-CMV forming this structure are conserved in the genomal RNAs of four strains of CMV studied so far; the Q, P, T and M strains (P.A. Wilson, J.M. Barker and R.H. Symons, unpublished). These conserved residues (shown in dashed plus solid boxes in Fig. 5) are heavily concentrated in that part of the structure which corresponds to the aminoacyl and TψC stems of canonical tRNAs.

The sequences homologous between Sat-RNA and the CMV RNAs are shown in solid boxes. It is striking that the proposed 3'-terminal structure of Sat-RNA shows extensive sequence and structural homology resulting in Sat-RNA possessing a truncated tRNA-like structure. In addition, most of the homologous residues in the CMV structure are also present in the 3'-terminal structure of the genomal RNAs of tomato aspermy virus (28), another cucumovirus which can also support Sat-RNA replication (2,7). The hairpin formed between the 3'-terminal residues 14 to 28 of Sat-RNA represents a shortened version of the CMV loop formed by residues 15 to 36 plus 97 to 100 (Fig. 5), with conserved sequences in the loop and at the stem of the hairpin. A conserved double-strand region is also found near the 3'-terminus of both Sat-RNA and CMV RNAs while there is an intriguing conserved sequence of 8 residues between 3'-terminal residues 54 to 61 of Sat-RNA and 178 to 185 of CMV RNAs.

Only 10 of the 44 residues in the hairpins formed by CMV RNAs residues 53 to 96 (Fig. 5) are conserved in the four strains of CMV (see above). Of these 10, only 3 (UGC) show homology within the 3'-terminal sequence of Sat-RNA but they are not in the same order as the other homologous sequences between the two RNAs. In spite of this, tertiary folding of the CMV RNAs may bring this UGC sequence nearer to residue 100 and hence in an equivalent position to that of Sat-RNA.

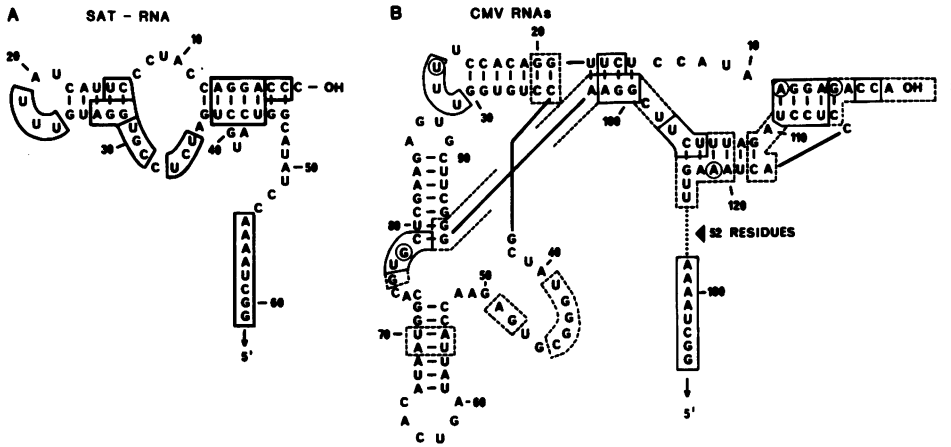


Figure 5. Sequence and structural homology at the 3'-termini of Sat-RNA and CMV genomal RNAs. Residues are numbered from the 3'-terminus in both structures. The Sat-RNA structure (A) is the 3'-terminal hairpin VII in Fig. 4. In B, the model proposed for CMV RNA 3 (15) has been rearranged to show the possible derivation of the three-dimensional tRNA-like structure (27). Residues within dashed and solid boxes in B are conserved in all genomal RNAs of CMV strains Q, P, T and M (ref. 15, Wilson, Barker and Symons, unpublished); of these, the circled residues vary in one or two of the 12 genomal RNAs studied. The solid boxed sequences in the Sat-RNA structure (A) and the CMV RNAs structure (B) are homologous.

Possible Function of 3'-terminal Structure in Replication of Sat-RNA

Although the four RNAs of CMV can be aminoacylated *in vitro* with tyrosine (29,30), Sat-RNA cannot be aminoacylated (P. Palukaitis, personal communication) which is consistent with the lack of a complete tRNA-like structure. It has been suggested that the 3'-terminal aminoacylation of CMV and some other viral RNAs is important in the initiation of viral RNA replication, possibly through facilitating RNA-protein interactions (30-32). However, Sat-RNA would be incapable of replicating in exactly the same way but may parasitize a replication complex which has been assembled using aminoacylated viral RNAs and viral gene products. Some feature of Sat-RNA replication must explain the rapidity with which a very low level of Sat-RNA in virus used for inoculating plants can increase to up to 50% of the total encapsidated RNA (2); for example, the more compact shape of Sat-RNA's truncated tRNA-like structure may play a role. The complete dependence of Sat-RNA on CMV RNAs for replication (2) plus the observation that Sat-RNA is replicated in the same particulate extract of infected

plants in which CMV RNAs are replicated (D.S. Gill and K.H.J. Gordon, unpublished) suggest that Sat-RNA must possess sufficient sequence and structural homology with CMV RNAs to allow it to use the CMV-induced replication machinery.

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