Extensive sequence homology at the 3'-termini of the four RNAs of cucumber mosaic virus

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Received 23 August 1979

ABSTRACT

The sequences of 270 residues from the 3'-termini of the four RNAs of cucumber mosaic virus have been determined by copying the in vitro polyadenylated RNAs with reverse transcriptase using d(pTpG) as primer and the 2',3'-dideoxynucleoside 5'-triphosphates as specific chain terminators. The terminal sequences of RNAs 3 and 4 were identical; this was expected since hybridization data has shown that the sequence of RNA 4 was present at the 3'-end of RNA 3 (Gould and Symons (1978) Eur. J. Biochem. 91, 269-278). The first 138 residues of RNAs 1 and 2 were identical to those of RNAs 3 and 4 except for one residue in RNA 1 and three residues in RNA 2. From residue 139 to 270 from the 3'-terminus, RNAs 1 and 2 showed, relative to RNAs 3 and 4, a non-homologous region of 33 residues, a homologous region of 40 residues, a partially homologous region of 46 residues and a homologous region of 14 residues which probably extended to about residue 300. There were 11 residues different between RNAs 1 and 2.

INTRODUCTION

Purified virions of cucumber mosaic virus (CMV) contain four major single-stranded RNA species, designated RNAs 1 to 4, with molecular weights of 1.35×10^6, 1.16×10^6, 0.85×10^6, and 0.35×10^6, respectively (1). The genome is functionally divided since the three largest of the four RNA species are required for infectivity (1-3). We have shown previously (4,5) by hybridization analysis using labelled complementary DNA (cDNA) that the sequence of RNA 4, which contains the coat protein gene, is completely contained at the 3'-end of RNA 3 and that there is a homologous region of about 200 residues at the 3'-end of RNAs 1, 2 and 3. Apart from this terminal homology, there was no other sequence homology between the three largest RNAs.

This work has now been extended to the determination of the sequence of about 270 residues at the 3'-ends of the four CMV RNAs using the Sanger et al. (6) dideoxynucleotide chain termination technique as applied to RNA (7-10).

MATERIALS AND METHODS

The four viral RNAs of CMV (Q strain) were purified as described (11) and polyadenylated at their 3'-termini with Escherichia coli ATP:RNA adenyltransferase (poly(A) polymerase) essentially as given by Gould et al. (12). 2',3'-Dideoxynucleoside 5'-triphosphates (ddNTPs) and $d(pT_8G)$ were obtained from P.L. Biochemicals. Reverse transcriptase from avian myeloblastosis virus was generously provided by the Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Maryland. α-^{32}P-dCTP and α-^{32}P-dGTP at specific activities of 200 - 350 Ci/mmol were prepared by the method of Symons (13) and contained unlabelled ATP which did not interfere with the sequencing reactions. γ-^{32}P-ATP of high specific activity (14) was a gift of R. Richards or was prepared by an unpublished modification of the method of Schendel and Wells (15).

Preparation of 5'-^{32}P-$d(pT_8G)$

About 2 µg of purified $d(pT_8G)$ (see below) was incubated with 0.001 unit of purified calf intestinal phosphatase (16) in 15 µl of 30 mM Tris-HCl, pH 9.0, at 37°C for one hour and was then phosphorylated with γ-^{32}P-ATP (300 µCi, 200 pmol) in 25 µl of 50 mM Tris-HCl, pH 9.0, 10 mM $MgCl_2$, 20 mM sodium acetate, 5 mM dithiothreitol, 15 mM sodium phosphate (to inhibit the phosphatase; ref. 16), and 4 units of T4-phage polynucleotide kinase (Boehringer) at 37°C for 45 min. The reaction mixture was extracted with 25 µl of phenol-chloroform (1:1 by vol.) and the organic phase back-extracted with 25 µl of water. The pooled aqueous phases were extracted three times with three volumes of ether, lyophilized and the residue dissolved in 25 µl of water. The 5'-^{32}P-$d(pT_8G)$ solution was used without further purification for sequencing reactions.

Purification of $d(pT_8G)$

In order to remove small amounts of oligonucleotide contaminants, about 50 µg of $d(pT_8G)$ plus 1 - 2 µCi of 5'-^{32}P-$d(pT_8G)$ was electrophoresed as a band 10 cm wide on a thin 20% polyacrylamide slab gel (17) at 15 mA until the bromophenol blue marker was nearly to the bottom of the 34 cm gel. The main band of 5'-^{32}P-$d(pT_8G)$, which ran just slower than the bromophenol blue, was located by autoradiography, cut out and eluted overnight in 0.5 M ammonium acetate, 0.1% sodium dodecylsulphate, 1 mM EDTA. This solution was diluted 5-fold with water and applied to a 0.6 cm x 2.0 cm column of DEAE-cellulose (HCO_3^-). The column was extensively washed with freshly prepared 0.1 M NH_4HCO_3 and 5 ml of 0.2 M NH_4HCO_3 before the 5'-^{32}P-$d(pT_8G)$ was eluted with about 3 ml of 1.0 M NH_4HCO_3. Triethylamine (0.3 ml) and 0.3 ml of ethanol

were added and the solution lyophilized in a siliconized flask. The residue was dissolved in 100 µl of water and complete removal of sodium dodecylsulphate ensured by passage over a 1.0 x 25 cm column of Sephadex G50 and elution with water. Peak fractions were pooled, lyophilized and the purified $d(pT_8G)$ was dissolved in 100 µl of 0.1 mM EDTA, pH 7.

Sequencing procedure

Using polyadenylated RNA as template and purified $d(pT_8G)$ as primer, cDNA was transcribed using reverse transcriptase in four reactions, each containing a different ddNTP. Reaction mixtures were prepared in siliconized capillary tubes and contained in a final volume of 4 µl; 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, about 0.1 µg of polyadenylated RNA and 0.05 to 0.1 µg of $d(pT_8G)$, each of the four dNTPs at 20 µM for short sequences up to about 50 residues and at 50 µM for longer sequences, 2.5 units of reverse transcriptase, and various concentrations of ddNTPs. The concentration of each ddNTP to give long, medium or short sequences was determined empirically and varied from 33 to 250 µM for ddATP, 3 to 50 µM for ddCTP, 5 to 50 µM for ddGTP, and 8 to 80 µM for ddTTP. Each reaction mixture was then used to dissolve a freshly dried down mixture of about 5 µCi each of α-^{32}P-dCTP and α-^{32}P-dGTP in a small siliconized glass tube. Incubation was at room temperature for 5 min, and then at 37°C for 30 min. The reaction was stopped by the addition of 20 µl of formamide-dye mix (95% deionised formamide, 10 mM EDTA, 0.03% bromophenol blue, 0.03% xylene cyanol F.F.) and heating at 100° for two minutes.

In order to obtain the first 10 - 15 residues, it was necessary to replace the α-^{32}P-dCTP and α-^{32}P-dGTP by 5'-^{32}P-$d(pT_8G)$; thus, each reaction mixture for short sequences contained 0.5 µl of the solution of 5'-^{32}P-$d(pT_8G)$ prepared as described above.

Labelled nucleotides in each reaction mixture were separated on the basis of chain length by electrophoresis on thin polyacrylamide gels (17). Sequences up to about 50 residues long were determined on 17 cm x 34 cm x 0.4 mm 20% acrylamide, 0.67% methylene bisacrylamide gels run at 15 mA for 2 to 4 hours. All longer sequences were determined on 17 cm x 77 cm x 0.5 mm 6% acrylamide, 0.4% methylene bisacrylamide gels run at 15 to 24 mA for 7 to 24 hours. Autoradiography was carried out at -70°C using pre-flashed Fuji RX medical X-ray film and Ilford fast tungstate intensifying screens (18). Usually only the bottom 40 cm of long gels was autoradiographed after transfer of the gel from the glass plate to a piece of developed X-ray film.

RESULTS AND DISCUSSION

Sequencing approach and procedures

In view of the successful application by others (7-10) of the Sanger et al. (6) dideoxynucleotide chain termination sequencing technique to polyadenylated RNA in the presence of a specific primer, the same approach was adopted here to determine the sequence of about 270 residues at the 3'-terminus of each of the four CMV RNAs. The 3'-terminal residue of these RNAs was shown to be either A or C (19) and the terminal sequence was assumed to be C-C-A since the four CMV RNAs can be aminoacylated by tyrosine in the presence of aminoacyl tRNA synthetases (20). Hence, the four purified RNAs (11) were polyadenylated with E. coli poly(A) polymerase and $d(pT_8G)$ was used as a primer for the reverse transcriptase catalysed synthesis of cDNA.

By empirically altering the concentration of each ddNTP in the sequencing reaction mixtures, it was possible to obtain sequences of at least 270 residues from the 3'-terminus of all four CMV RNAs. However, this required the routine use of thin sequencing gels nearly 80 cm long (21) rather than the standard short 40 cm thin gels of Sanger and Coulson (17). Such gels are easy to prepare and run and can provide autoradiograms in which the successive nucleotide bands are usually well separated and the sequence can be rapidly read off.

The quality of sequencing gels has shown frustrating variation. Many gels were easily read (e.g., Fig. 1 and 2) while others were difficult to read because of a high background banding level in each of the four ddNTP tracks. It was difficult to pinpoint the causes of this variability but several factors were considered important; the purity of each RNA, the quality of the poly(A) polymerase used for polyadenylation of RNA, and the quality and age of the high specific activity α-^{32}P-dNTPs. Further, it has been found more reliable to prepare sequencing reaction mixtures with the four unlabelled dNTPs at the same concentration and to add one or two α-^{32}P-dNTPs to each reaction mixture. This is different to the usual practice (7-9) of preparing reaction mixtures which contain three unlabelled dNTPs plus one α-^{32}P-dNTP at a much lower concentration.

Sequences adjacent to the 3'-termini of the four CMV RNAs

It was found impossible to read sequences adjacent to the unlabelled $d(pT_8G)$ primer when a mixture of α-^{32}P-dCTP and α-^{32}P-dGTP was used as the source of label in the sequencing reaction mixtures. However, this problem was overcome by the use of 5'-^{32}P-$d(pT_8G)$ in the presence of unlabelled dNTPs; an example of a sequencing gel obtained for CMV RNAs 3 and 4 is given

in Fig. 1. The numbering of residues starts at the A residue of the terminal C-C-A so that the very dark band of ^{32}P-d(pT$_8$G) represents residue 2. The first residue incorporated was a G (residue 3), the band of which was usually obscured by the broad band of ^{32}P-d(pT$_8$G) unless brief exposure of the film was taken. The next residue always appeared as a band (X) in all four ddNTP tracks and with the four CMV RNAs. In most gels of this type it was possible to read at least 50 residues from the 3'-terminus. In the case

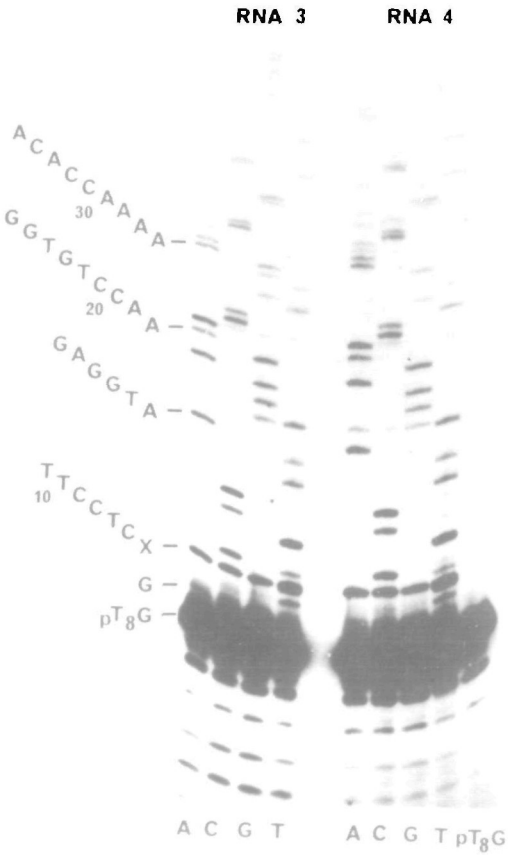

Figure 1. Sequence at the 3'-ends of CMV RNAs 3 and 4. Reaction mixtures were prepared using ^{32}P-d(pT$_8$G) as described in Materials and Methods and electrophoresed on a short 20% acrylamide gel at 15 mA for 3.0 hours. Only the bottom 28 cm of the gel is shown. The very dark band is unreacted ^{32}P-d(pT$_8$G) which is taken as residue number 2 (see text). The marker pT$_8$G was a sample of the ^{32}P-d(pT$_8$G) used in this experiment. A,C,G and T and the tracks of reaction mixtures containing ddATP, ddCTP, ddGTP and ddTTP, respectively. Residue 3 is not known and is given as X.

of Fig. 1, the identical sequence of the first 34 residues of RNAs 3 and 4 is given.

The several weak bands in the ddTTP track just above the ^{32}P-d(pT$_8$G) were taken as background; they possibly arose from priming by oligo(dT) contaminants which were present in the original ^{32}P-d(pT$_8$G) or which originated by partial hydrolysis of the primer during the sequencing reaction. That such hydrolysis occurred was shown by the increased appearance of labelled bands shorter than the primer in Fig. 1 and in other gels. There was significant cross-banding at residues 12 and 18 but the strongest band in each case was taken as correct.

The cross-band X (Fig. 1) cannot be due to compression of nucleotide bands (6,8,22,23) since the product at this stage was only 11 residues long. Confirmation of this was obtained by the substitution of dGTP by dITP in the sequencing reaction mixtures; identical gel patterns were found with either dGTP or dITP. It has been shown recently that nucleotide band compression in sequencing gels of the RNA product of the phage Qβ RNA replicase can be eliminated by substitution of GTP by ITP in the RNA replicase reaction mixtures (23). It is considered that the cross-banding at this second residue (X in Fig. 1) was due to inhibition of movement of the reverse transcriptase prior to the start of a postulated base-paired structure (Fig. 5). Such inhibition of movement would be expected to give a band in each of the ddNTP tracks. The marked cross-banding may also be related to the small size of the elongated d(pT$_8$G) primer and the low stability of the primer-template complex.

Sequences up to 270 residues from the 3'-termini of CMV RNAs

An example of a long sequencing gel for CMV RNA 1 is shown in Fig. 2. The autoradiogram shows the bottom 40 cm of the 77 cm long gel with three separate loadings of the reaction mixture. In this case, it was possible to read the sequence of about 135 residues, from residue 135 to residue 270. From this and many other similar gels, it was possible to determine the sequence of 270 residues from the 3'-end of each of the four CMV RNAs (Fig. 3). Data for each RNA were obtained from gels of several different sequencing reaction mixtures while regions which were difficult to read in one gel could usually be resolved by reference to one or more other gels. A background banding pattern was always found in all four ddNTP tracks (Fig. 2) and this often proved useful in lining up gel bands and in ensuring that no bands were omitted.

A prominent cross-banding occurred at residue 174 in the four RNAs

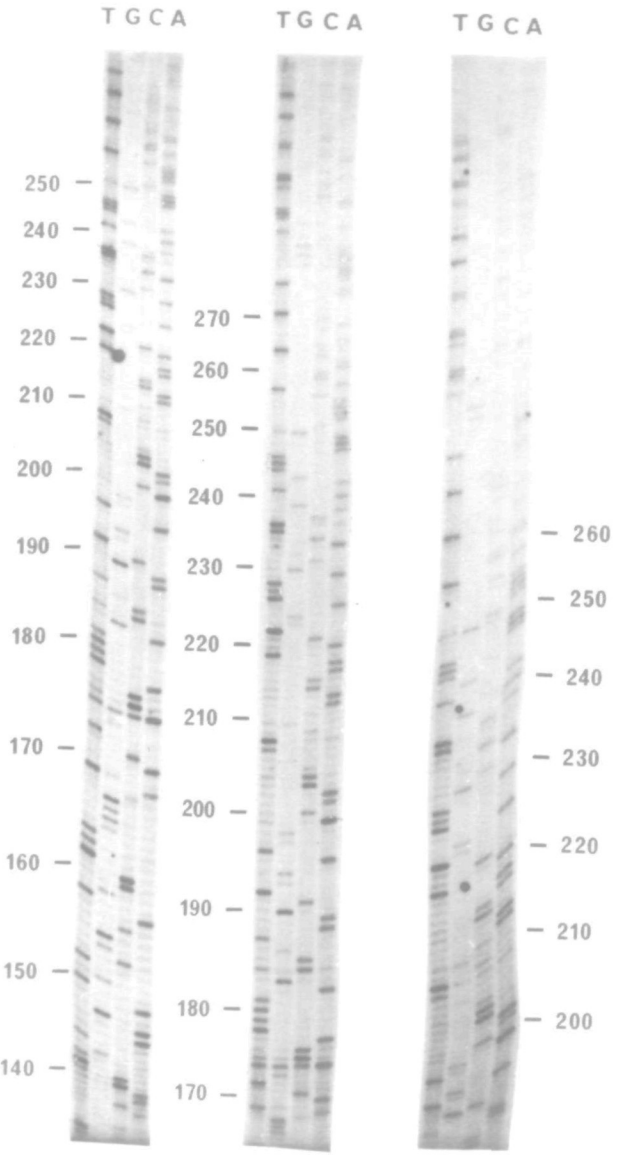

Figure 2. Sequence determination from residues 135 to 270 for CMV RNA 1. Reaction mixtures were prepared as described in Materials and Methods and electrophoresed on a long 6% acrylamide gel at 15 - 18 mA for 23 hours. Three loadings were made at 0, 4 and 8 hours. Only the bottom 40 cm of the gel is shown. The numbers refer to residues from the 3'-end as given in Fig. 3. A,C,G and T are the tracks of reaction mixtures containing ddATP, ddCTP, ddGTP and ddTTP, respectively.

(Fig. 2 and 3). Although this residue was most likely A, it is given in brackets in Fig. 3 to indicate uncertainty. To remove the possibility that this cross-banding was due to nucleotide compression, two sequencing reaction mixtures were prepared with RNA 3, one as usual and another in which dGTP was

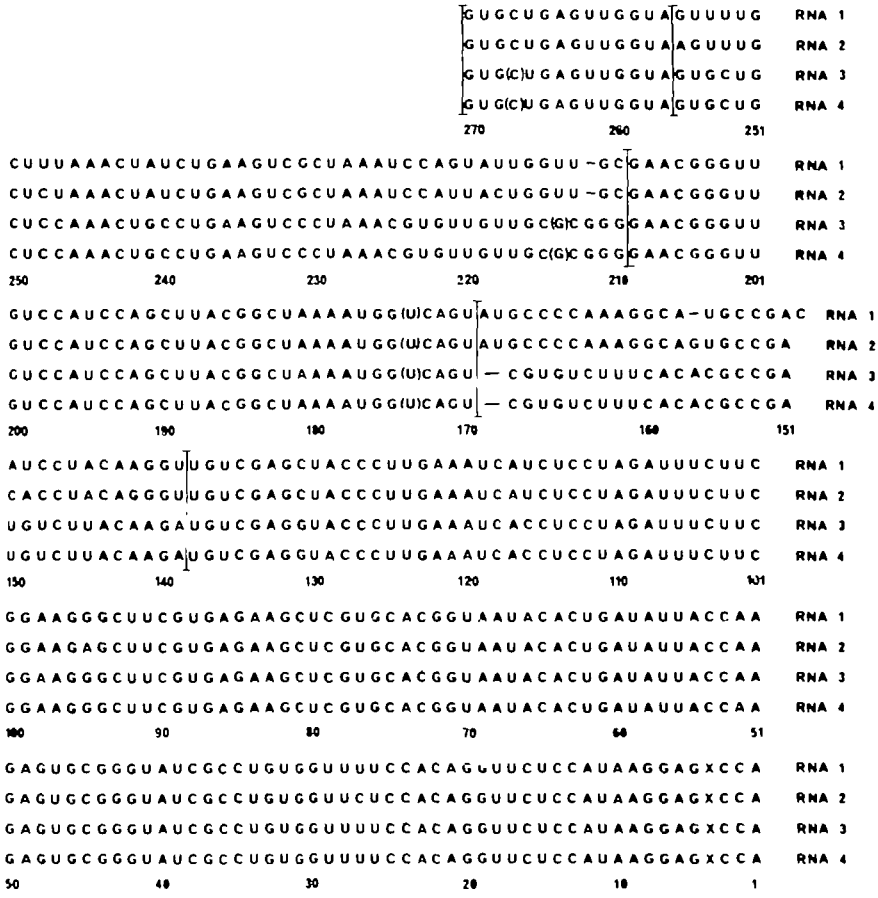

Figure 3. Sequence of the 3'-terminal region of the four CMV RNAs. Residues are numbered from the 3'-terminal A for all RNAs and are continuous from residues 1 to 270 for RNAs 3 and 4. In the case of RNAs 1 and 2, sequences are arranged vertically with those of RNAs 3 and 4 to show maximum homology. Thus, there is an additional residue between residues 150 and 151 in RNA 1 and an additional two residues between residues 169 and 170 in RNAs 1 and 2. The absence of a residue is indicated by a dash (-). Where there is ambiguity, the most likely residue is given in parenthesis. Residue 3 is unknown and is given as X. The vertical lines correspond with the vertical divisions in Fig. 4.

replaced by dITP (23). The only labelled nucleotide present was α-^{32}P-dCTP. Gel analysis of the reaction mixtures showed identical electrophoretic mobility for all bands up to the top of the autoradiogram (about residue 190). Hence, other possibilities must be considered for the origin of this cross-band.

There are a number of important features of the sequence data of Fig. 3 which are summarized diagrammatically in Fig. 4.

1. The sequence of the first 138 residues is identical for the four RNAs except for residues 27 and 95 of RNA 2, and residue 116 of RNAs 1 and 2. These differences between RNAs 1 and 2 together with those considered below eliminate the possibility that the sequences of RNA 1 were actually derived from contaminating RNA 2 molecules and vice-versa.

2. The sequence of RNA 3 is identical to RNA 4 as expected since

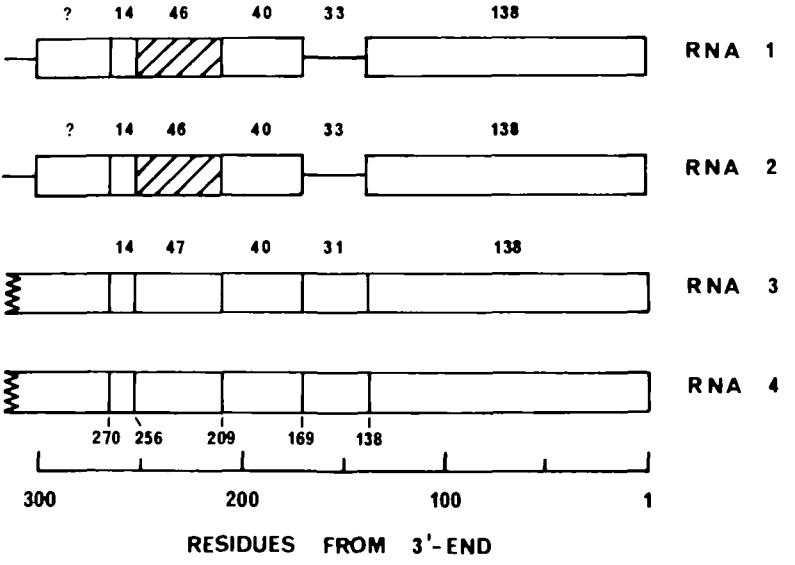

Figure 4. Diagrammatic summary of the regions of sequence homology between the four CMV RNAs as given in the data in Fig. 3. The open box areas correspond to regions of complete or almost complete homology while the cross-hatched areas represent partial homology of RNAs 1 and 2 with RNAs 3 and 4. The single horizontal lines represent no homology of RNAs 1 and 2 with RNAs 3 and 4. The numbers above each region give the number of residues in that region. The question mark represents a region of homology of about 30 residues. The scale at the bottom gives the number of residues from the 3'-end of the RNAs.

hybridization data showed that the sequence of RNA 4 is at the 3'-end of RNA 3 (5).

3. The 3'-terminal homology is interrupted after residue 138 by a stretch of 33 residues in RNAs 1 and 2, the sequence of which is different to the corresponding stretch of 31 residues in RNAs 3 and 4. There are differences in the 33 residue sequence between RNAs 1 and 2.

4. Sequence homology for the four RNAs is restored at residue 170 of RNAs 3 and 4 (actually residue 172 of RNAs 1 and 2) with an identical stretch of 40 residues.

5. The next 47 residues of RNAs 3 and 4 (residues 210 to 256) form a region of partial sequence homology with a 46 residue stretch of RNAs 1 and 2 (the cross-hatched area in Fig. 4). There are 18 residues different (includes one base deletion) between RNA 1 and RNAs 3 and 4 and 19 residues different (one base deletion) for RNA 2. There are 5 differences in sequence between RNAs 1 and 2 in this region.

6. Complete sequence homology between the four RNAs is present between residues 256 and 270. Sequences between residues 270 and 310 could be mostly read but not with sufficient accuracy to be reported here. However, there was extensive sequence homology in this region for the four CMV RNAs (shown by a question mark in Fig. 4) and this appeared to terminate between residues 300 and 305 in RNAs 1 and 2. Whether or not sequence homology returned at longer sequences could not be determined.

Given that the sequence homology terminates at residue 300, there are approximately 222 residues (138 + 40 + 14 + 30) homologous between the four RNAs which is to be compared with the 200 residue homology shown by our earlier hybridization data (5). However, the approach used in this hybridization work did not give any indication of the two regions of no homology and partial homology shown by the sequence data.

The coat protein of CMV contains approximately 238 amino acids (3) which corresponds to 714 residues out of a total of about 1,000 residues in RNA 4 (1). On the basis of the statement of Hidaka *et al.* (24) that the initiation codon of RNA 4 of the yellow strain of CMV starts at residue 30 from the 5'-end, the termination codon should occur about 260 residues from the 3'-end of RNA 4. Although UGA and UAG codons occur between residues 250 and 270 (Fig. 3) it is not possible to locate the termination codon in the absence of C-terminal amino acid sequence data for the coat protein and accurate base sequences in excess of 300 residues from the 3'-end. It would seem most likely that coat protein termination would occur outside any region

of homology between the four RNAs.

Accuracy of sequence data

The sequence data of Fig. 3 was derived from numerous sequencing gels run over a period of about six months. Most sequences were unambiguous and encouragingly reproducible. The region which presented most difficulty was from residue 150 to 180 for RNA 2 and it is possible that one or more errors may be present. The major difficulty in the sequencing approach used is that there is no confirmatory evidence available, such as sequencing of the complementary strand, so that a low error frequency is possible.

Possible base-paired regions in 3'-terminal sequences

Since CMV RNAs can be aminoacylated with tyrosine (20), possible base-paired regions were searched for with the aid of the computer programme of Staden (25) to see if a tRNA-like structure existed. Maximum base-pairing for the 3'-terminal 138 residues of the four RNAs was obtained with the structure of Fig. 5 in which 84 residues (61%) were base-paired. It is of

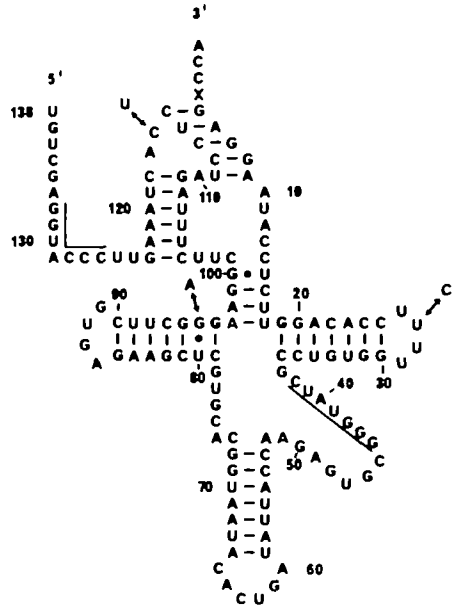

Figure 5. One possible base-paired structure for the 3'-terminal 138 residues of the four CMV RNAs. The primary sequence is for RNAs 3 and 4. The base changes shown at residues 27 and 95 are for RNA 2 and at residue 116 for both RNAs 1 and 2. X is an unknown residue. For the sake of clarity, the base-pairing between residues 126 to 132 and residues 38 to 44 (shown by lines) has not been drawn.

interest that the single base changes at residue 27 of RNA 2 and at residue 116 for RNAs 1 and 2 occur in single-strand regions while the base change at residue 95 of RNA 2 converts a G-U base-pair to a more stable A-U base-pair.

The structure of Fig. 5 bears some resemblance to the two-dimensional clover leaf pattern in which the sequences of tRNA are usually drawn. The base-pairing between residues 126 to 132 and residues 38 to 44 (shown by lines) could presumably occur with appropriate twisting of the molecule.

Significance of 3'-terminal sequence homology

The extensive and complicated arrangement of the various regions of complete homology, partial homology and no homology at the 3'-end of the four CMV RNAs is intriguing, especially in the absence of any information to indicate a biological role for these terminal sequences or for the ability of the RNAs to be aminoacylated with tyrosine *in vitro* (20). It is of obvious interest to see to what extent the actual sequences and sequence arrangement are conserved in other strains of CMV and in other members of the Cucumovirus family, e.g., tomato aspermy virus. At least some of the terminal sequence must be involved in the recognition site of the RNA replicase responsible for the replication of the CMV RNAs; the presumptive enzyme has been extensively purified (26) but no data is available on this aspect.

Brome mosaic virus (BMV), a member of the Bromoviruses which are closely related to the Cucumoviruses, contains four RNAs which have many properties similar to the corresponding CMV RNAs (27). Like the CMV RNAs, BMV RNAs can be aminoacylated by tyrosine (20). Dasgupta and Kaesberg (28) have determined the sequence of 161 residues from the 3'-end of BMV RNA 4 and have provided data that the corresponding sequences of the other three RNAs are nearly the same. Any comparison of the arrangement of regions of complete, partial or non-homology between CMV (Fig. 4) and BMV RNAs must await more extensive sequence data for the BMV RNAs.

ACKNOWLEDGMENTS

This work was initiated while on Study Leave in 1978 with Dr. F. Sanger, M.R.C. Laboratory of Molecular Biology, Cambridge. The author is most grateful to Dr. Sanger for his generous hospitality, encouragement and advice and also for numerous helpful discussions with many of his colleagues. The reverse transcriptase was kindly provided by the Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Maryland. The author thanks Miss J.L. Rosey for assistance and Dr. J.C. Wallace and Miss M. Fraser

for help with the computer programme. This work was supported by the Australian Research Grants Committee.

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