The Structure of the 5'-Terminus of Papaya Mosaic Virus RNA

(Accepted 11 January 1978)

SUMMARY

The RNA of the sinuous papaya mosaic virus particles was labelled in vivo with $^{32}$P or in vitro after oxidation by periodate and reduction by KB$_3$H$_4$. After digestion with RNases T$_1$, T$_2$ and A, the oligonucleotides were fractionated by electrophoresis or by chromatography on a DEAE-cellulose column. After such fractionation, an oligonucleotide resistant to the RNases and phosphatase with a structure of m$_7$G$_3$ppp$^v$Gp was obtained from the 5'-terminus of the RNA.

It has recently been shown that the RNA species of brome mosaic virus (BMV) (Dasgupta et al. 1975), tobacco mosaic virus (Zimmern, 1975), alfalfa mosaic virus (Pinck, 1975) and tobacco rattle virus (AbouHaidar & Hirth, 1977) contain 7-methyl guanosine triphosphate, m$_7$G$_3$ppp$^v$N, at their 5' ends. Such identification, beyond being of comparative interest in respect to other messenger RNA species from a variety of cells and from both DNA and RNA viruses (see Cory & Adams, 1975 and Griffin, 1976, for comprehensive lists), is of considerable use in the specification of the direction of virus assembly. Accordingly, we have undertaken to identify the 5' end of the RNA of a sinuous plant virus named papaya mosaic virus (PMV) (Purcifull & Hiebert, 1971), which we are employing as a model reconstitution system. We demonstrate that PMV-RNA is also terminated by methylated guanosine and that the penultimate base is guanosine.

Labelled PMV was grown in young (about 25 cm tall) papaya plants (Carica papaya), already infected for 5 days. The stems were cut and dipped in water containing 1 mCi/ml of $^{32}$P-labelled phosphoric acid (1 mCi/plant). After 5 days of incubation, the virus was purified by a method similar to that described by Purcifull & Hiebert (1971). PMV-RNA was prepared by adding one volume of cold 5 M-guanidine at pH 8.3 containing 10$^{-3}$ M-EDTA to one volume of virus (10 mg/ml in 0.05 M-tris-HCl, 10$^{-3}$ M-EDTA, pH 7.5) (Reichmann & Stace-Smith, 1959). The precipitated RNA was dissolved in water before precipitation with 2 volumes of cold 95% ethanol. $^{32}$P-labelled tobacco mosaic virus (TMV) was grown in Nicotiana tabacum var. White Burley labelled with $^{32}$P-phosphoric acid for 5 days (1 mCi/plant), and was purified by the method of Von Wechmar & van Regenmortel (1970). $^{32}$P-labelled TMV-RNA was extracted with phenol.

To label PMV-RNA with $^3$H in vitro we used the following methods based on the sequential oxidation and reduction of the two free hydroxyl groups on the ribose at the 3'-OH end of the RNA and at the 5'-terminus when the latter is terminated by a 5'-5' linkage. PMV-RNA was dissolved in 0.05 M-Na-acetate buffer, pH 5.5, at 5 mg/ml and oxidized by the addition of fresh NaIO$_4$ at a final concentration of 0.01 M for 45 to 60 min at 4 °C in the dark. The oxidized RNA was then precipitated and dissolved in 0.05 M-phosphate buffer, pH 7.5, before reduction by solid KB$_3$H$_4$ (Amersham/Searle, 12.4 Ci/mmol) at a final concentration of 10$^{-3}$ M. The reduction was carried out for 1 h at 20 °C followed...
Short communications

by 15 h at 0 to 4 °C in the dark. Excess borohydride was removed by dialysis against H₂O at 4 °C for 24 h.

Purified ³²P-labelled PMV-RNA and ³²P-labelled TMV-RNA (used as a control) were digested by RNases T₁, T₂ and A as before (AbouHaidar & Hirth, 1977). These enzymes cleave phosphodiester linkages of the RNA to yield 3' nucleotides. Any RNA containing 2'-o-methyl nucleosides yields dinucleotides (NmNp) since a 2'-o-methyl group blocks the enzyme action (Uchida & Egami, 1971). The digest was fractionated by electrophoresis on DEAE-cellulose paper (Whatman DE-81) at pH 3.5. Fig. 1(A) shows that the digest of PMV-RNA contains a product migrating with a mobility of 0.22 relative to the blue marker xylene cyanol FF. This product is resistant to further digestion with RNases T₁, T₂ and A. TMV-RNA shows a similar product (Fig. 1A) which is m⁷GpppGp (Zimmern, 1975). The slowly migrating species originating from the digestion of PMV-RNA and the known m⁷GpppGp from TMV-RNA were eluted from the DEAE-cellulose paper by triethylamine bicarbonate (TEABC) and treated with the alkaline phosphatase from Escherichia coli. The products were analysed by electrophoresis on DEAE-cellulose paper at pH 3.5. After dephosphorylation, the PMV and TMV products migrate with an electrophoretic mobility of 0.71 relative to the xylene cyanol FF marker (Fig. 1B). The shift in electrophoretic mobility is expected for the loss of one phosphate residue (Cory & Adams, 1975). Moreover, the ratio of radioactivity in the phosphate released to that in the dephosphorylated product was about 1 to 3, signifying the loss of one phosphate residue.

These results suggest that the oligonucleotides from both PMV and TMV contain three 'internal' phosphates. The nuclease-resistant phosphorylated oligonucleotide originating from PMV-RNA and the m⁷GpppGp from TMV-RNA were treated with venom phosphodiesterase (3'-exonuclease liberating the nucleoside 5'-phosphate). The digestion products were analysed by electrophoresis on Whatman No. 1 paper at pH 3.5 in the presence of markers. Fig. 1(C) shows that the products obtained from the cleavage of the oligonucleotide originating from PMV-RNA were pm⁷G, pG and Pi with electrophoretic mobilities similar to the products obtained from m⁷GpppG from TMV. The molarities are pm⁷G (m = 0.27), pG (m = 0.29) and Pi (m = 0.44) from PMV-RNA and 0.21, 0.30 and 0.49, respectively, from TMV. The molarity of Pi was a little higher than the 0.33 expected for the release of one phosphate residue and it is not inconceivable that the snake venom phosphodiesterase has some phosphatase action on the oligonucleotide from both viruses. Nevertheless, the results show clearly that the 5'-terminus of PMV-RNA is that of TMV-RNA and the structure is therefore m⁷G₅'pp⁵'Gp----.

Assuming that the mol. wt. of PMV-RNA is 2 x 10⁶ (approx. 7000 nucleotides) and knowing that the cap contains four phosphates, we were able to calculate the ratio of radioactivities present in the cap to that in the rest of the RNA. We found that at least 90 to 95 % of the molecules have their 5'-terminal ends blocked.

Purified ³H-labelled PMV was applied to a DEAE-cellulose column (0.9 x 40 cm) after digestion with RNases T₁, T₂ and A, and the product was compared with a RNase A digest of cold PMV-RNA which served as the optical marker. The oligonucleotides were eluted with a linear 0.005 to 0.4 M-NaCl gradient containing 0.02 M-tris-HCl, pH 7.6, and 7 M-urea. After elution, the radioactivity resolved into three major peaks labelled I, II and III (Fig. 2a). Peak I, which is eluted from the DEAE-cellulose column with 0.05 M-NaCl, corresponds to a nucleoside which is probably the 3'-OH end nucleoside of PMV-RNA. Peak II was eluted from the DEAE-cellulose column as mononucleotides. After electrophoresis at pH 3.5 on Whatman 3 MM paper, it separated into Cp, Ap, Gp and Up (result not shown). This material probably originates from nonspecific labelling of the
Fig. 1. Determination of the structure of the 5'-terminus of PMV-RNA. (A) An electrophoretogram of RNases T₁, T₂ and A digest of ³²P-labelled TMV-RNA (a) and PMV-RNA (b). The digests were fractionated by electrophoresis on DEAE-cellulose paper (Whatman DE-81) in 0.5% pyridine, 5% acetic acid buffer, pH 3.5. (B) The RNase-resistant material shown in Fig. 1(A) was eluted and digested with the alkaline phosphatase of E. coli. The digest was fractionated by electrophoresis on DEAE-cellulose paper at pH 3.5 as described in section A above. (C) Material from TMV-RNA. (d) Material from PMV-RNA. (C) Characterization of products after treatment with snake venom phosphodiesterase. The oligonucleotides originating from TMV-RNA (e) and PMV-RNA (f) after treatment with alkaline phosphatase, were treated with snake venom phosphodiesterase and the digests were analysed by electrophoresis on Whatman No. 1 paper at pH 3.5. O = Origin; B = blue marker xylene cyanol FF.
Fig. 2. Column chromatography on DEAE-cellulose. (a) Ribonucleases T₁, T₂ and A digest of \(^{3}H\)-labelled PMV-RNA was fractionated on a DEAE-cellulose column. The positions of the oligonucleotide markers with net charges of \(-2\) (mono) to \(-5\) (tetra) were determined by absorbance at 254 nm and are indicated by the arrows. Fractions corresponding to peak III were used for subsequent analysis. (b) Determination of the number of phosphate residues in the 5'-terminal oligonucleotide resistant to the ribonucleases. The resistant oligonucleotide from peak III (a) was desalted, treated with alkaline phosphatase, mixed with the RNase A digest of cold PMV-RNA and analysed by DEAE-cellulose chromatography. ---, Elution profile of marker oligonucleotides monitored at 254 nm; ----, elution profile of \(^{3}H\)-labelled oligonucleotide from PMV-RNA.

four bases along the RNA molecules. Peak III was eluted from the DEAE-cellulose column between the tri- and tetrancleotides (Fig. 2a). This oligonucleotide probably contains four phosphates. The material from this peak was treated with venom phosphodiesterase and the products of digestion were analysed by electrophoresis. The radioactivity was present in a single peak corresponding to pm\(^{2}\)G. After treatment with alkaline phosphatase, the products migrated towards the cathode at the position of the m\(^{2}\)G marker (result not shown). These results show that material from peak III corresponds to m\(^{2}\)GpppGp and that the first nucleotide is pm\(^{2}\)G. To determine the number of internal phosphates, the material from peak III was eluted from the column, re-treated with ribonucleases T₁, T₂ and A and alkaline phosphatase, and re-run on a DEAE-cellulose column. Fig. 2(b) shows that the dephosphorylated oligonucleotide is now eluted between the mono and dinucleotides with a net charge of \(-2\frac{1}{2}\). Since each internal phosphate residue must contribute one negative charge and the m\(^{2}\)G half a positive charge (Sripati et al. 1976), this oligonucleotide must have three internal phosphate residues.

We have presented evidence obtained from two approaches that PMV-RNA is terminated by m\(^{2}\)GpppGp. In doing so, we have shown that experiments designed to determine the polarity of reconstitution may indeed be pursued profitably and that the RNA of at least one member of the large potato virus X family is capped at the 5'-terminus.
We wish to thank Dr D. B. Smith for use of his high voltage electrophoresis equipment and Mr J. W. Erickson for discussion.

This work was supported in part by the National Research Council of Canada.

Department of Plant Sciences
The University of Western Ontario
London, Ontario N6A 5B7
Canada

MOUNIR ABOUHAIDAR
J. B. BANCROFT

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(Received 16 November 1977)