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Analysis of terminal structures of RNA from potato virus X

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ABSTRACT

The 5'-end structure of potato virus X RNA was determined following enzymatic methylation in vitro. A single ^{3}H-methyl group was introduced into the 2'-position of the 5'-penultimate residue and the end structure was determined as $m^{7}GpppG^{(m)}pAp(Xp)_{3}G$. This part of the RNA apparently is involved in binding to ribosomes since it can be partially protected against RNase digestion by wheat germ 40S ribosomes. PVX RNA was not retained by poly(U)-sepharose, indicating that it does not contain a 3'-terminal poly(A) tract.

INTRODUCTION

Most animal cell and virus mRNAs are modified by addition of a poly(A) tract at the 3'-end and a blocked methylated "cap", $m^{7}GpppX$ at the 5'-end (for reviews see refs. 1 and 2). The same kind of 5'-terminal cap is present in several plant virus genome RNAs (3-9) that also function as messenger for virus-specific protein synthesis (9-14). However, the 3'-ends of most plant virus RNAs are not polyadenylated but resemble transfer RNA both in structure (15,16) and the ability to be charged with a specific amino acid in a reaction catalyzed by aminoacyl-tRNA-synthetase (17-19). Exceptions to these general properties of eukaryotic messengers have been described. For example, certain virus RNAs of animal [polio (20,21), EMC (22)] and plant [STNV (23,24), CPMV (25)] origin do not contain a 5'-cap; furthermore, the 3'-end of the plant virus RNA, CPMV is polyadenylated (26) while human reovirus mRNA is not (27).

Potato virus X is a flexuous rod-shaped virus consisting of multiple copies of a single coat protein and a single-stranded infectious RNA of molecular weight 2×10^{6} daltons (28). PVX RNA is translated efficiently in cell-free extracts prepared from wheat germ (29) but its terminal structures have not been characterized previously. In this report we present evidence that PVX RNA has $m^{7}GpppGpA$ at the 5'-end and a 3'-terminus that is not polyadenylated.

MATERIALS AND METHODS

Preparation of RNAs.

PVX RNA was prepared by the hot SDS-phenol method as previously described (29). ^{32}P-Labeled PVX RNA was obtained by incubating 40 eight-day old infected tobacco leaves in water containing ^{32}P-orthophosphoric acid (0.02 mCi, Amersham-Searle) for 24 hr and isolating the virus according to Goodman (30). Rabbit globin mRNA and STNV RNA were kindly provided by B. Kemper and J.M. Clark, Jr. of the University of Illinois.

Preparation of guanylyltransferase and methyltransferase--methylation of RNA.

A preparation containing guanylyltransferase and methyltransferase activities, free of DNA, was prepared from purified vaccinia virus according to Ensinger et al. (31). Methylation of RNA was carried out essentially as described by Moss (32). Reaction mixtures in a total volume of 50 µl contained 3 µg of PVX RNA, 50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 1 mM DTT, 0.82 µM of ^{3}H-S-adenosylmethionine (67 Ci/mmole, NEN), and the indicated volume of the preparation of vaccinia proteins (10 mg/ml). After incubation at 37°C for 25 min, incorporation of radioactivity into PVX RNA was assayed by precipitating the RNA with 5% TCA at 4°C, collecting the insoluble material on Millipore filters and counting in toluene-based scintillant.

For preparative methylation of PVX RNA, the reaction mixture was scaled up to 0.5 ml including 75 µl of enzyme. Following incubation at 37°C for 25 min, RNA was extracted with an equal volume of phenol containing 0.2% SDS, passed through a column of Sephadex G-50 equilibrated with 20 mM Tris-HCl (pH 7.5) and 0.2% SDS, and precipitated with 2 volumes of ethanol in the presence of 0.2 M NaCl. The specific activity of ^{3}H-methyl PVX RNA obtained was usually 12,000 to 15,000 cpm/µg.

Digestion of ^{3}H-methyl-PVX RNA with RNases and analysis of products.

Digestions of PVX RNA with Penicillium nuclease (P_1, Yamasa Ltd., Tokyo), venom nucleotide pyrophosphatase (Sigma) and bacterial alkaline phosphatase (Worthington) were done as described by Furuichi et al. (33). PVX RNA digested with P_1 plus alkaline phosphatase was analyzed by descending chromatography on 3 MM Whatman paper in isobutyric acid/0.5 N NH_4OH, 10:6 (v/v). Radioactive material was eluted from the paper with water, digested with nucleotide pyrophosphatase plus alkaline phosphatase and analyzed by paper electrophoresis on Whatman 3 MM at 2600 V for 40 min in pyridine acetate buffer (pH 3.5). Authentic compounds were located under ultraviolet light; the paper was dried and cut into 1 cm strips for

counting in scintillation fluid.

Digestion with purified tobacco phosphodiesterase (kindly provided by Dr. M. Miwa, Natl. Cancer Center Research Institute, Tokyo, Japan) was essentially as described (34). PVX RNA (1 µg) in a total volume of 50 µl containing 50 mM NaOAc pH 6.0 and 10 mM β-mercaptoethanol were incubated with 1.8×10^{-3} units of enzyme for 30 min at 37°C. The pH was then adjusted to 4.5 with HOAc, 2.5 units RNase T_2 were added and the sample of 100 µl incubated at 37° for 4 hr. The pH was again adjusted to 7.5 with Trizma base and incubation with alkaline phosphatase (5 µg) was for 1 hr at 37°. An aliquot (60%) of the digested sample was mixed with a RNase A digest of yeast tRNA as marker and analyzed by chromatography on a column (27 x 0.7 cm) of DEAE-cellulose equilibrated with 50 mM Tris-HCl (pH 7.5) containing 7 M urea and 25 mM NaCl (35). The oligonucleotides were eluted with a linear gradient of NaCl (100 ml each of 25 or 50 mM NaCl and 0.3 M NaCl) in the same buffer. Fractions of 1 ml were collected and alternate fractions counted in 15 ml of xylene-based scintillator. Another aliquot (20%) was analyzed by high voltage paper electrophoresis as described above except that the 1 cm strips were eluted with H_2O before adding scintillation fluid. T_1 RNase (Sankyo) digestion was done on 0.8 µg of PVX RNA with 1.5 units of enzyme (∿0.2 µg) in 0.1 ml of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA for 15 hr at 37°C. Alkaline phosphatase (5 µg) was added following RNase digestion and incubation continued for 1 hr at 37°C. The digest was chromatographed on DEAE-cellulose.

Ribosome binding and protection.

Binding of PVX RNA to ribosomes in wheat germ extracts followed by analysis in glycerol gradients was as described previously (36,37). For the protection experiments, binding mixtures were treated with 2.5 µg/ml of RNase A for 10 min at 0°C or with 250 units/ml of RNase T_1 for 15 min at 25°C before glycerol gradient analysis.

RESULTS

PVX RNA can be translated in the wheat germ system (29) which for most mRNAs is cap-dependent (2). During these studies we found that m^7GMP, which has been shown to inhibit the translation of capped mRNAs (38-40), also inhibits the translation of PVX RNA (Fig. 1). Globin mRNA (41) and STNV RNA (24) served as comparative controls for capped and uncapped messengers, respectively. Translation of globin RNA was completely inhibited by 0.5 mM m^7GMP whereas translation of STNV RNA was decreased by only 20%. These results suggested that PVX RNA contains a 5'-cap.

In order to determine the 5'-end structure of the PVX viral RNA, we

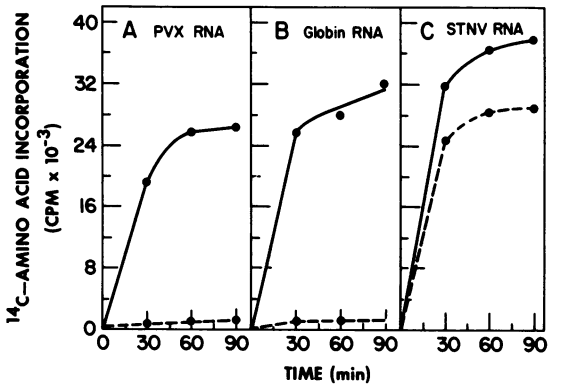

Figure 1: Effect of m^7GMP on the translation of PVX, globin and STNV RNAs in the wheat germ system. Translation was carried out as described (29) in 50 µl reaction mixtures containing 3 mM $Mg(OAc)_2$, 0.1 M KCl and the following RNAs: 1 µg PVX RNA (A); 0.5 µg globin RNA (B); 6 µg STNV RNA (C). Mixtures were incubated with (---) or without (——) 0.5 mM m^7GMP, and 10 µl portions removed at the indicated times were precipitated with 10% TCA, filtered, washed, dried and counted.

took advantage of the fact that the caps of all plant viral RNAs so far analyzed contain an unmethylated residue rather than a 2'-O-methylated nucleotide next to the m^7G. Therefore, we checked if ^3H-methyl groups from radioactive S-adenosylmethionine could be incorporated into PVX RNA by incubation with guanylyltransferase and methyltransferase activities prepared from solubilized vaccinia virus (32). Table I shows that PVX RNA is methylated by the vaccinia virus enzymes. The site of incorporation of the ^3H-methyl group is probably the 2'-OH of X in preformed cap structures of the type m^7GpppX and not due to guanylylation followed by methylation since GTP did not significantly stimulate ^3H-methyl incorporation into RNA (Table I). Maximal incorporation of radioactivity yielded about one mole of ^3H-methyl groups/mole of RNA. In order to confirm that methylation occurred in the 2'-position of X in the cap structure, methylated RNA was digested with P_1 RNase followed by bacterial alkaline phosphatase, and analyzed by paper chromatography. As shown in Fig. 2A, all the radioactivity migrated in a position identical to authentic m^7GpppG^m, indicative of labeling in a cap structure. To establish definitively the site of methylation in the PVX RNA cap, the radioactive oligonucleotide was eluted from the paper, digested with nucleotide pyrophosphatase and subjected to paper electrophoresis. All the radioactivity moved to a position identical to marker pG (Fig. 2B). The results indicate that the 5'-terminal structure of PVX RNA is m^7GpppG which is converted to m^7GpppG^m by methyltransferase in solubilized vaccinia virus.

To obtain information about the cap-proximal nucleotides, ^3H-methyl-labeled PVX RNA was incubated with purified tobacco phosphodiesterase, to

Table I

Methylation of PVX RNA

µl Enzyme	^{3}H-CPM TCA insoluble
0	1,185
2	15,828
4	35,966
4 + 2 mM GTP	40,151
10	60,734

Incubation mixtures and conditions were described in Materials and Methods. A reaction mixture containing 10 µl enzyme but no PVX RNA yielded 1597 TCA-insoluble CPM.

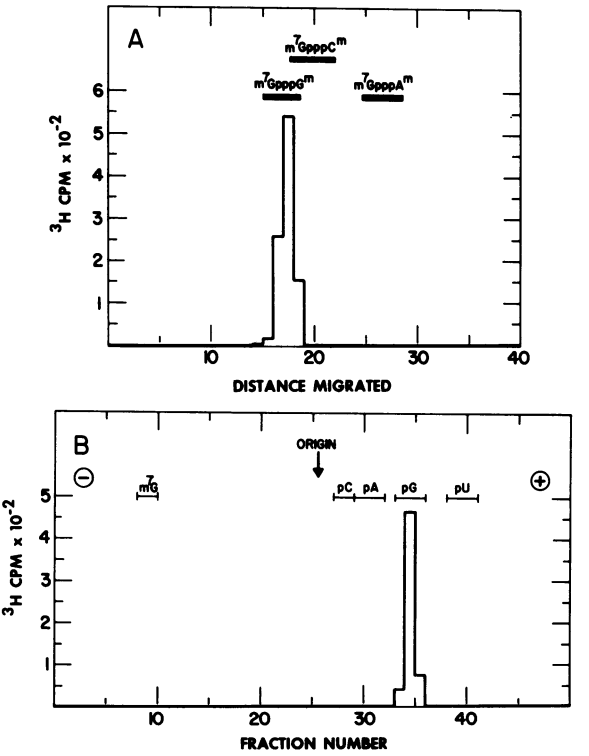

Figure 2: Chromatographic and electrophoretic analyses of enzymatically digested ^{3}H-methyl-labeled PVX RNA. A: RNA (15,000 cpm, ∿1 µg) was digested with Penicillium nuclease and bacterial alkaline phosphatase and chromatographed as described in Materials and Methods. The paper was dried and cut into 1 cm strips which were counted in toluene-based scintillant. B: Radioactive material was eluted from the paper, digested with nucleotide pyrophosphatase and analyzed by high voltage paper electrophoresis at pH 3.5.

remove the m^7G portion of the molecule, followed by T_2 RNase and bacterial alkaline phosphatase. This treatment yields a ^3H-methyl-labeled dinucleotide at net charge -1 (Fig. 3A). It migrates by paper electrophoresis with marker GpA (Fig. 3B), consistent with the 5'-sequence $m^7GpppG^{(m)}pA$. The same results were obtained after sequential digestion with tobacco phosphodiesterase, KOH (0.3 M, 18 hr, 37°) and phosphatase. Similarly, treatment with RNase U_2 (1.25 units/ml, 1 hr, 37°) and phosphatase yielded an oligonucleotide that eluted from DEAE-cellulose with a net charge between -3 and -4 as expected for m^7GpppG^mpA.

Figure 4 is the pattern of labeled nucleotides obtained after RNase T_1 digestion. Most of the radioactivity eluted with a net charge of -7.5, implying that a guanosine residue is located in PVX RNA about seven nucleotides from the 5'-end, i.e. $m^7GpppG^{(m)}pAp(Xp)_3G$.

Messenger RNAs of eukaryotes apparently are functionally monocistronic and initiate protein synthesis near the 5'-end. Consistent with ribosome binding close to the 5'-end, the caps of reovirus mRNAs (37) and some VSV mRNA species (42) in initiation complexes are protected against digestion by RNases T_1 and A. The same experimental approach was applied to PVX RNA. ^3H-

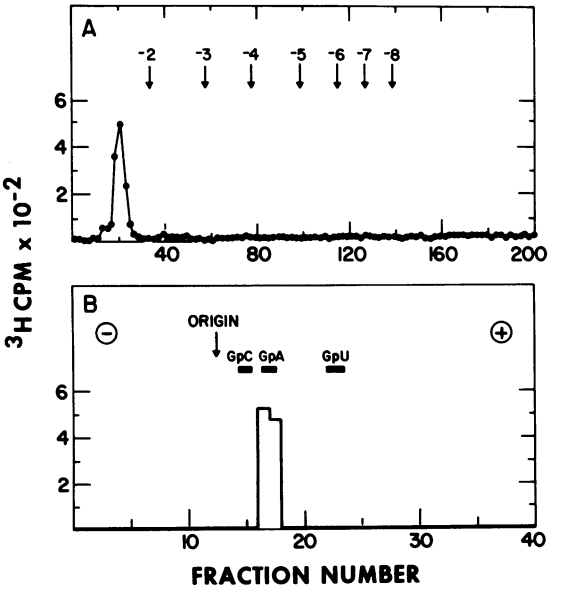

Figure 3: Analysis of ^3H-methyl-labeled 5'-terminal fragments from PVX RNA. A: RNA was digested with tobacco phosphodiesterase, RNase T_2 and alkaline phosphatase and an aliquot was chromatographed on DEAE-cellulose as described in Materials and Methods. B: A second aliquot was analyzed by high voltage paper electrophoresis with marker compounds.

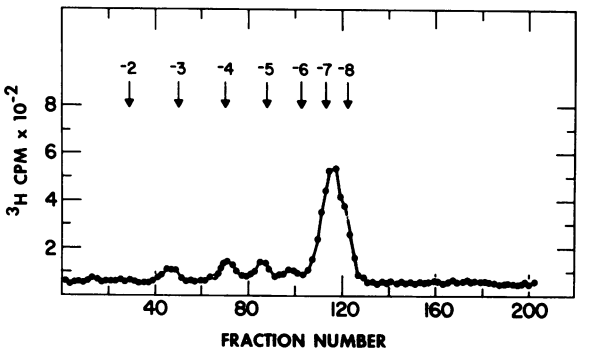

Figure 4: Chromatography of RNase T_1 digested ^{3}H-methyl-PVX RNA. After enzyme treatment the digest was chromatographed on DEAE-cellulose as in Fig. 3.

methylated PVX RNA was tested for the ability to form initiation complexes in cell-free extracts of wheat germ. Complex formation was carried out in the presence of GMPPCP, a non-hydrolyzable analogue of GTP that partially inhibits conversion of 40S to 80S complexes (37). As shown in Fig. 5A, ^{3}H-methyl-labeled PVX RNA can efficiently form 40S and 80S initiation complexes. In order to determine if the 5'-end of messenger is protected against RNase digestion, complexes were treated with RNase A or T_1 before glycerol gradient analysis. The results indicate that the cap structure is partially protected against the RNases in 40S initiation complexes but not in 80S complexes. When RNase A was used, 41% of the ^{3}H-methyl-labeled m^7GpppG^m in mRNA in 40S initiation complexes was protected against digestion (Fig. 5B); the degree of protection against RNase T_1 digestion was lower (28%) (Fig. 5C). The same experiments were repeated with a 3-fold lower level of PVX RNA to eliminate the possibility that mRNA was in excess and consequently more accessible to RNase digestion because of a limiting amount of some protecting component (43). This was not the case since similar results were obtained, i.e. 49% and 19% protection of mRNA in 40S complexes, when these complexes were treated with RNase A and T_1, respectively.

Affinity chromatography on poly(U)-Sepharose has been used to isolate and characterize mRNAs containing 3'-terminal poly(A) (44,45). Unlike poly(A) which bound quantitatively to poly(U)-Sepharose, PVX RNA labeled with ^{32}P during infection of tobacco plants, did not bind (Fig. 6). The ^{32}P-labeled PVX RNA eluted in the load buffer indicating that it does not contain a poly(A) tract of size sufficient to bind poly(U). CPMV RNA under the same conditions bound to the same poly(U)-Sepharose to the extent of 50% (not shown).

DISCUSSION

Cap structures in viral RNAs are often identified by radiolabeling the

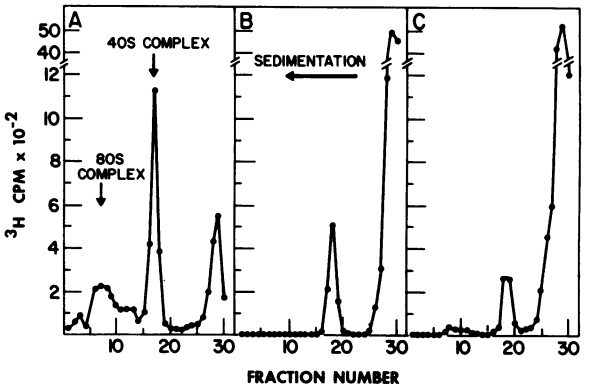

Figure 5: Binding of ^{3}H-methyl PVX RNA to wheat germ ribosomes and protection of cap structure against RNase digestion. ^{3}H-methyl PVX RNA (15,000 cpm, ∿1 µg) was bound to wheat germ ribosomes (A), digested with RNase A (B) or T_1 (C) and analyzed on glycerol gradients as described in Materials and Methods. The total radioactivity in fractions collected from RNase-digested samples was greater than in the undigested control sample because RNA that pelleted in the control sample was released to the top of the gradient by RNase digestion.

RNA with ^{32}P *in vivo*, followed by extraction and analysis of the 5'-terminal sequence. This approach is limited because the cap comprises less than 0.1% of the radioactivity in most RNAs, the low proportion of radioactivity making cap analysis difficult. To obviate the use of ^{32}P for studying the terminal structures of PVX RNA, we used a previously described technique (32) which depends upon the ability of capping and methylating enzymes from vaccinia virus to use heterologous polynucleotides as substrates (47). Previous studies of the 5'-end structure of several plant viral RNAs including TMV, CMV, BMV, TRV, AMV and TYMV (3-9) demonstrated that the penultimate nucleotide following the 5'-m^{7}G nucleotide is not methylated, and it seemed possible that PVX RNA caps would have a similar structure. Based on that premise we isolated PVX viral RNA and showed that it could be methylated by vaccinia methyltransferase. Methylation occurs specifically on the 2'-position of the guanosine following the 7-methylguanosine in the structure, m^{7}GpppG. These results place PVX RNA among the majority of plant viral RNAs [TMV, CMV, BMV, TYMV and AMV (3-7,9)] which contain m^{7}GpppG at their 5'-terminus. One plant viral RNA (short species of TRV) was found to contain a m^{7}GpppA cap (8), and two plant viral RNAs [STNV (23,24) and CPMV (25)] do not contain any cap structure at their 5'-terminus. The absence of a 2'-O-methyl in the 5'-penultimate nucleotide in plant viral RNAs is similar to the situation in slime mold (48) and yeast mRNAs (49) and is in marked con-

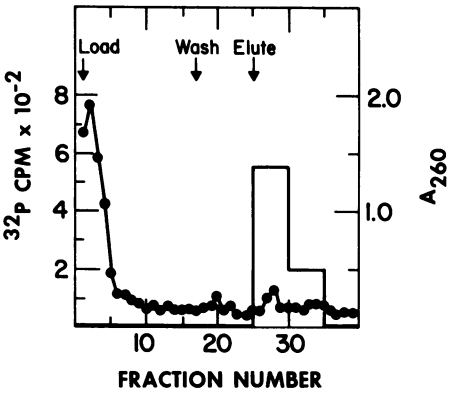

Figure 6: Absence of PVX RNA binding to poly(U) Sepharose. ^{32}P-labeled PVX RNA (80 µg) was applied to a column of 1 ml poly(U) Sepharose (Pharmacia) in loading buffer consisting of 0.4 M NaCl, 10 mM Hepes buffer (pH 7.4), 5 mM EDTA and 0.5% SDS (46). The column was washed with 10 mM Hepes buffer (pH 7.4) containing 2 mM EDTA and 0.5% SDS, and elution was with 70% formamide in 1 mM Hepes buffer (pH 7.4) containing 2 mM EDTA. Fractions of 1 ml were collected and 0.1 ml aliquots were counted in 10 ml Bray's solution. As a control, poly(A) (2 A_{260} units, P-L Biochemicals) was applied to the same poly(U) Sepharose column and eluted as described for PVX RNA. Fractions were pooled, precipitated with ethanol, dissolved in H_2O and monitored for absorbance (solid line).

trast to all mammalian cellular mRNAs and most animal viral mRNAs (2). This may indicate that the methylation following the m^7G in the cap structure may have arisen late in the evolutionary process and might have some advantage in animal mRNA function. The physiological significance of the 2'-O-methylation has not been elucidated, although Muthukrishnan et al. found that when vaccinia mRNA was tested in wheat germ extracts or reticulocyte lysates at concentrations above saturation, the ribosome-bound fraction was enriched for messengers with 5'-terminal m^7GpppA^m (50).

^{3}H-methyl-labeled PVX RNA binds well to ribosomes in wheat germ extract. In 40S initiation complexes, the cap was partially protected against digestion by RNase A or T_1, suggesting that the 5'-end of PVX RNA is involved in ribosome binding during initiation of protein synthesis. Partial protection by 40S subunits of the cap in TMV RNA was also obtained (20% resistance to 2.5 µg/ml of RNase A at 0° for 10 min). At higher levels of enzyme (10 µg/ml) the ^{3}H-methyl-labeled caps of PVX and TMV RNA were completely sensitive (N.S., unpublished results). Incomplete protection of the cap was observed when saturating amounts of reovirus mRNA were used in wheat germ extracts (43) and reticulocyte lysates (51). However, the studies with PVX RNA were done at subsaturating concentrations of RNA.

The degree of protection may be affected by the length and structure of the region between the cap and the initiation codon and by the stability of 40S initiation complexes, or may reflect the affinity of the cap for a component which confers protection against RNase digestion (52). It should be noted that the protection assay provides a way to select the 5'-terminal fragment of PVX and other RNAs for sequence determination (37,42).

Concerning the 3'-end of PVX RNA, the results demonstrate the absence of a poly(A) tract. Absence of a poly(A) tract at the 3'-end of plant viral RNAs has been reported for all plant viruses studied with the exception of CPMV which contains a poly(A) tract of about 200 nucleotides (26). Many plant viral RNAs can be charged by addition of a specific amino acid to the 3'-end, and for some RNAs like BMV (16) and TYMV (15), it was shown that the 3'-end sequence is structurally similar to tRNA. It is not known if PVX RNA can be aminoacylated. Finally, the method used here for labeling specifically the 5'-end of PVX RNA might be a useful tool for determining if the 5'-region of the RNA is involved in the association of coat protein and genome RNA during virion assembly.

ABBREVIATIONS

PVX, potato virus X; EMC, encephalomyocarditis virus; STNV, satellite tobacco necrosis virus; CPMV, cowpea mosaic virus; TMV, tobacco mosaic virus; BMV, brome mosaic virus; TRV, tobacco rattle virus; AMV, alfalfa mosaic virus; CMV, cucumber mosaic virus; TYMV, turnip yellow mosaic virus; P_1, Penicillium nuclease; m^7GMP, 7-methylguanosine-5'-monophosphate.

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