The Structure of Tobacco Rattle Virus Ribonucleic Acids: 
Nature of the 3'-Terminal Nucleosides

By G. DARBY AND A. C. MINSON

Department of Virology, The Medical School, University of Birmingham, 
Birmingham, England

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SUMMARY

The two species of RNA have been purified from the long and short particles 
of the multicomponent plant virus, tobacco rattle isolate CAM. 3'-End-group 
analysis, using the technique of periodate oxidation followed by reduction with 
[3H]-borohydride, showed that both species of RNA had an unphosphorylated 
cytidine residue at the 3' end.

INTRODUCTION

Tobacco rattle virus (TRV) is a multicomponent RNA plant virus which consists of two 
or more types of helical particle. All strains possess a long particle about 190 nm. long and 
one, or sometimes more, short particles whose lengths differ in different strains (Harrison & 

Long particles of the virus are infectious in the absence of short but the product of the 
infection is long particle RNA and no mature particles are made (Frost, Harrison & Woods, 
1967). In contrast, short particles alone are non-infectious. The coat proteins of the different 
particles of any particular strain are identical (Harrison & Nixon, 1959; Lister & Bracker, 
1969) and, in addition, Sanger (1968) has shown that, following infection with long and short 
particles of different strains, the virus produced has the serotype of the added short particles.

These observations suggest that the genome of the short particle has the information for 
the coat protein of the virus whereas the information for an RNA replicase system which 
replicates all viral RNA species may be encoded in the genome of the long particle. If the 
various RNA species share a common replicase, they might be expected to have similar 
nucleotide sequences at their 3'-ends which act as binding sites for the enzyme.

The CAM isolate of TRV consists of two particles, the shorter 52 nm. long and the longer 
197 nm. long (Harrison & Woods, 1966). This strain is particularly convenient because its 
short particle is smaller than that of other strains in which there are only two size classes of 
particles. This simplifies the purification of the particles and the isolation of their RNA species.

In this paper, we describe the purification of the particles from the CAM isolate of TRV 
and the isolation of their RNA species. We have shown that the RNA molecules from both 
particles are terminated at their 3' ends by unphosphorylated cytidine residues.

METHODS

Biological materials. TRV isolate CAM (originally isolated from Bidens pilosa by A. S. 
Costa, Campinas, Brazil) was a gift from Dr B. D. Harrison and was propagated in Nicotiana 
clevelandii. Tobacco mosaic virus (TMV) – type strain – was a gift from Dr A. J. Gibbs. 
Preparation of [14C]-ribosomal RNA from E. coli. Escherichia coli C600 was grown in
TPG medium (Sinsheimer et al. 1962) (10 ml.) in the presence of [2-14C]-uridine (50 μC) (the Radiochemical Centre, Amersham). The cells were harvested after 20 hr and the RNA extracted by the procedure of Godson & Sinsheimer (1967). The RNA was freed of ribonuclease activity by treatment with diethyl pyrocarbonate (Solymosy et al. 1968).

Isolation of TRV long and short particles. Systemically infected leaves of Nicotiana clevelandii were harvested 10 days after inoculation with the CAM isolate of TRV. The tissue was homogenized in a Waring Blender and the sap, after filtration through muslin, was stored for at least 1 week, frozen at −20 °C. The sap was thawed and centrifuged at 16,000 g for 20 min. and the virus was then purified from the supernatant by two cycles of differential centrifugation (Frost et al. 1967). The virus pellet was resuspended in 0.02 M-boric acid, 4 mM-EDTA pH 7.5 (5 ml.) and stored at 4 °C for 48 hr. The long and short particles of TRV were then separated by centrifugation at 60,000 g through a linear sucrose gradient (10 to 40 %, w/v) in 0.05 M-borate, 4 mM-EDTA pH 7.5 (30 ml.). Centrifugation was for 2.5 hr at 4 °C. The gradients were harvested and the two distinct bands of virus particles were collected. Particles were sedimented from sucrose by centrifugation at 100,000 g for 2 hr at 4 °C, resuspended in 0.05 M-tris buffer, 5 mM-EDTA, 0.4 M-NaCl pH 7.5 and resedimented. The final pellets were resuspended in 0.05 M-tris, 5 mM-EDTA, 0.4 M-NaCl pH 7.5.

The particle preparations were examined by electron microscopy to determine the extent of cross-contamination between the particle types.

Purification of TMV. TMV was purified using the method described by Fraenkel-Conrat (1967).

Extraction of RNA. All reagents used in the extraction and purification of RNA were freed from ribonuclease activity by treatment with diethyl pyrocarbonate. Purified virus suspended in 0.05 M-tris, 5 mM-EDTA, 0.4 M-NaCl pH 7.5 (10 to 20 mg./ml.) was mixed with an equal volume of water-saturated phenol and warmed at 55 °C for 10 min. The aqueous layer was removed and extracted twice with equal volumes of phenol at 4 °C. It was then extracted five times with three vol. water-saturated ether and the RNA was precipitated from the aqueous solution by the addition of 2 vol. of ethanol. The mixture was left at −20 °C overnight and the RNA was then pelleted by low-speed centrifugation.

The RNA was further purified by sucrose-gradient centrifugation through a linear (5 to 20 %, w/v) gradient (5 ml.) in 0.05 M-tris, 5 mM-EDTA, 0.4 M-NaCl pH 7.5. The sample was centrifuged at 38,000 rev./min. in the MSE SW 40 rotor for an appropriate time at 4 °C and an internal marker of [14C]-ribosomal RNA from E. coli was included. The required fractions of RNA were pooled and precipitated with 2 vol. of ethanol. The pellet was redissolved in 0.1 M-sodium acetate, 1 mM-EDTA pH 5.4, precipitated once more with ethanol and dried in vacuo.

Exonuclease treatment of TMV RNA. TMV RNA was dissolved in 0.05 M-tris, 0.2 mM-MgCl₂ pH 8.8 (0.5 mg./ml.) and incubated with snake venom phosphodiesterase (20 μg./ml.) (Worthington Biochemical Corp.) for 15 min. at 37 °C. The RNA was then subjected to sucrose density-gradient centrifugation as described in the previous section.

[3H]-labelling of 3’ termini. The methods used for the [3H]-labelling of the 3’ terminal nucleosides were those described by Weber & Weissmann (1970), involving periodate oxidation and reduction with [3H]-sodium borohydride, followed by piperidine hydrolysis of the RNA.

Preparation of nucleoside derivatives. Adenine hydroxymethylglycol and the corresponding analogous derivatives of guanosine, cytidine and uridine were prepared by the method of De Wachter & Fiers (1967) and purified by paper chromatography on Whatman paper 3 MM in propan-2-ol:ammonia (ρ = 0.88):water (7:1:2, v/v).

Identification of labelled nucleosides. RNA containing a labelled 3’-terminal nucleoside
was hydrolysed with piperidine (Weber & Weissmann, 1970), and a mixture of four nucleo-
side derivatives, prepared as described above, was added to act as carrier. The hydrolysate
was divided into two parts (1:2) and these were chromatographed in parallel on Whatman
paper 3 MM in 2-methylpropan-2-ol:butan-2-one:ammonia ($\rho = 0.88$):water (4:3:1:2,
v/v) at 25°C for 15 hr. The papers were dried and the area of the chromatogram containing
the smaller sample was cut into strips (width 0.5 cm.) perpendicular to the direction of
chromatography. Each strip was eluted overnight with water (1.5 ml.) at 37°C and both the
$E_{260}$ of the eluate and its tritium content were determined. Tritium was measured by mixing
an aqueous sample of the eluate (0.5 ml.) with triton/toluene scintillator (1:3.5) (Patterson &
Greene, 1965) and counting by liquid scintillation. The counting efficiency, determined
using an external standard, was usually about 25%.

The area of the chromatogram containing the larger sample was viewed under an ultra-
violet lamp and the nucleoside derivatives located. As a further purification step these were
cut out, eluted with water and rechromatographed on Whatman paper 3 MM in 2-methyl-
propan-2-ol:butan-2-one:formic acid:water (44:44:0.25:11, v/v) for 12 hr at 25°C. The
dried papers were cut into 1 cm. strips which were eluted with water and analysed for
optical density and radioactivity as described above.

**RESULTS**

**Purification of particle and RNA species**

The particles of TRV were purified and then separated by sucrose-gradient centrifugation.
Electron-microscopic examination of the preparations indicated less than 5% contamination
of long particles by short and a similar low contamination of short by long.

The RNA species from each of these were extracted with phenol and further purified by
sucrose-gradient centrifugation. The gradient profiles are shown in Fig. 1 and it can be seen,
that under the conditions used, the RNA from the short particle had a sedimentation
coefficient of 16 s relative to the internal marker of [$^{14}$C]-ribosomal RNA species of *E. coli*
whereas the RNA from the long particle had a relative sedimentation coefficient of 32 s. As
no mol. wt data were available for the RNA species of the CAM isolate of TRV, approximate
values of $0.55 \times 10^6$ and $2.5 \times 10^6$ for the short and long particle RNAs, respectively, were
calculated from the sedimentation data. These values were used in calculating yields in the
labelling experiments. The peak fractions from the gradients (Fig. 1a, fractions 9 to 13
inclusive, and Fig. 1b, fractions 4 to 8 inclusive) were pooled and precipitated. These
samples were used in the end-group determinations.

TMV RNA was similarly purified and material sedimenting in sucrose gradients at about
30 s (usually 60 to 65% of the total RNA) was used in later experiments. Centrifugation of
TMV RNA on sucrose gradients after partial degradation with venom phosphodiesterase
showed that some endonucleolytic as well as exonucleolytic degradation had taken place.
Material sedimenting in a broad band between 16 and 30 s was pooled and used for end-
group determination.

**Determination of 3' terminal nucleosides**

TRV 16 s RNA (35 µg.) and TRV 32 s RNA (100 µg.) were oxidized with periodate and
reduced with [$^{3}$H]-sodium borohydride and the [$^{3}$H]-labelled nucleoside derivatives were
identified. TMV RNA (100 µg.) was treated in parallel as a control, as previous workers
(Whitfield, 1965; Glitz, Bradley & Fraenkel-Conrat, 1968) had shown that TMV RNA is
terminated at its 3' end by an unphosphorylated adenosine residue.
The first-dimension chromatographic profiles of the hydrolysed RNA species are shown in Fig. 2. Approximately 90% of the labelled material in each case remained at the origin. The nature of this material was not investigated further except that it was relatively insoluble in both polar and non-polar solvents and also appeared to be non-u.v. absorbing. It did not therefore appear to have been derived from the nucleic acid. Although the background levels were high (100 to 200 counts/min.) in the nucleoside regions of the chromatograms, there were, in each case two major peaks of [3H] activity. One of the peaks (Fig. 2, peak I) appeared to be common to all three samples but it did not coincide with any of the four nucleoside derivative markers. In the case of TMV the second major peak (Fig. 2a, peak II) coincided with the adenosine derivative marker, whereas the second major peak from each of the TRV species coincided with the cytidine derivative. There was in addition to the two major peaks in the TRV 32 s RNA profile, a third (Fig. 2c, peak III) which was slightly ahead of the adenosine derivative. This component appeared to be present to a lesser extent in the TRV 16 s RNA hydrolysate (Fig. 2b) and may have been present in the TMV hydrolysate, accounting for the asymmetry of the adenosine derivative peak in the [3H] profile (Fig. 2a).
Fig. 2. First-dimension chromatography of RNA hydrolysates. Terminally labelled RNA was hydrolysed and chromatographed in 2-methylpropan-2-ol:butan-2-one:ammonia (ρ = 0.88):water 4:3:1:2, v/v). Fractions were assayed for [H] activity (●) and E_{260} (○). (a) TMV, RNA; (b) TRV 16 s RNA; (c) TRV 32 s RNA. G, U, C and A (Fig. 2a) identify the nucleoside derivative markers.
Fig. 3. Second-dimension chromatography of RNA hydrolysates. After first-dimension chromatography of the RNA hydrolysates the nucleoside derivative markers were identified, eluted and chromatographed in 2-methylpropan-2-ol:butan-2-one:formic acid:water (44:44:0.25:11, v/v). Fractions were assayed for $^3$H activity (●—●) and $E_{260}$ (○—○). (a) TMV RNA; (b) TRV 16 s RNA; (c) TRV 32 s RNA.

Each derivative was re-chromatographed. The profiles are shown in Fig. 3. In this dimension the background levels of radioactivity were reduced to about 50 counts/min., which was close to the background for the counting system. The results confirmed those obtained in the first dimension. The total radioactivity in each nucleoside derivative peak in this dimension was determined and calculated as a percentage of the total radioactivity in nucleoside derivatives. The results are shown in Table 1. With TMV more than 90% of the

<table>
<thead>
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<th>RNA species</th>
<th>Nucleoside derivative</th>
<th>Proportion of total nucleoside label (%)</th>
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<tr>
<td>TMV</td>
<td>Guanosine</td>
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</tr>
<tr>
<td></td>
<td>Uridine</td>
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</tr>
<tr>
<td></td>
<td>Cytidine</td>
<td>4.2</td>
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<td></td>
<td>Adenosine</td>
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<td>TRV (16 s)</td>
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<td></td>
<td>Uridine</td>
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<td></td>
<td>Cytidine</td>
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</tr>
<tr>
<td></td>
<td>Adenosine</td>
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<tr>
<td></td>
<td>Adenosine</td>
<td>0.8</td>
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* Determined from the total activity in each nucleoside derivative peak on the second chromatographic run in solvent 3.
nucleoside derivative label was present in the adenosine derivative. In contrast, for both species of TRV RNA approximately 90% of the activity was present in the cytidine derivative fractions.

The overall yields of radioactivity in the derivatives were calculated using the mol. wts of $0.55 \times 10^6$ and $2.5 \times 10^6$ for the TRV RNA species and $2.05 \times 10^6$ for TMV RNA (Caspar, 1963) and also assuming that the oxidation and reduction reactions were 100% efficient. The results indicated overall yields of 12, 13 and 25% for TMV, TRV 16 s and TRV 32 s RNA species, respectively.

One further control reaction was performed using TMV RNA which had been partially degraded with venom phosphodiesterase to remove nucleotides from the 3' ends of the molecules, thus exposing new termini. The results of end-group determination on this material showed approximately equal labelling of all four nucleosides. There was in addition, on the first chromatographic run, a fifth major peak in the nucleoside region of the chromatogram running in the position of peak I, Fig. 2. The adenosine peak also appeared to contain a second component as it showed asymmetry similar to that of peak II, Fig. 2a.

**DISCUSSION**

In both species of TRV RNA the 3' terminal nucleoside was cytidine. The possibility that the 3' termini were partially phosphorylated was not investigated directly but the efficiency of labelling of the terminal nucleosides was similar to the efficiency of labelling of the terminal adenosine residue of TMV RNA. Because this adenosine residue was known from previous work to be unphosphorylated, the results suggested that the 3' termini of the TRV species were also unphosphorylated. The low overall yields of tritiated derivatives could probably be explained by the low efficiency of reduction of the 3' termini of polynucleotides (Glitz et al. 1968). The difference in yields between the 16 s and 32 s RNA species were probably not significant because in several experiments with TMV RNA, yields of the adenosine derivative varied between 12 and 27%.

In each of the first chromatographic runs there appeared to be two components running in the nucleoside derivative region of the chromatogram which did not coincide with any of the derivative markers. Their presence in the hydrolysate of the exonuclease treated TMV RNA suggested that they were not derived from unusual nucleosides at the 3' termini of the RNA species. It would appear likely that these components were either impurities in the [3H]-sodium borohydride or arose from low level internal labelling of the RNA molecules.

The results obtained are in contrast to those obtained with most other virus RNA species examined (Gilham, 1970), where, in general, the 3' terminal nucleoside appears to be adenosine. However, one other plant virus RNA species, that of tobacco necrosis satellite virus, has a 3' terminal cytidine (Wimmer & Reichmann, 1969). This virus is unable to multiply alone in plants, possibly because it lacks the information to specify a replicase, but it will multiply in plants co-infected with tobacco necrosis virus (Kassanis, 1968). This situation is analogous to the one encountered in the TRV system and it would therefore be of interest to know the 3' terminus of tobacco necrosis virus RNA.

It is probable that the binding site for an RNA replicase would consist of a specific sequence of nucleotides at the 3' end of an RNA molecule. Work is now in progress to determine whether such a sequence occurs in the two distinct species of RNA from the CAM isolate of TRV.
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REFERENCES


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