A Study of Sequence Homology between Tobacco Rattle Virus Ribonucleic Acids

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(Accepted 2 January 1973)

SUMMARY

A comparison was made of the two RNA species of the CAM isolate of tobacco rattle virus. The RNAs were found to have significantly different base compositions. Double-stranded virus-specific RNA was extracted from virus infected tissue and was used with [3H]-labelled virus RNA in hybridization experiments. Hybridization competition experiments showed that most sequences in each RNA species were unique to that species, but that a sequence of about 500 nucleotides may be common to both.

INTRODUCTION

The CAM isolate of tobacco rattle virus (TRV), a multicomponent plant virus, consists of two types of helical particles; a 197 nm long rod and a 52 nm short rod (Harrison & Woods, 1966). The two particles have an identical capsid protein and each contains 5% RNA (Harrison & Nixon, 1959; Lister & Bracker, 1969; Miki & Okada, 1970).

Long particles of the virus are infectious but the product of the infection is long particle RNA only: no virus particles are made (Frost, Harrison & Woods, 1967; Lister, 1968). The short particles alone do not appear to be infectious, but in mixed infections with long particles, the short particle genome provides the information for the synthesis of capsid protein (Sänger, 1968, 1969).

It is apparent that information in both parts of the virus genome must be utilized to achieve a successful infection resulting in virus particles but it is not known whether there is duplication of information in the two parts. It is probable that both RNA species depend on a single replicase system, and it has been suggested that there might be a common enzyme recognition site at the 3' end of each RNA (Darby & Minson, 1972). Experiments are now in progress to investigate this possibility, but such experiments cannot be interpreted if there is extensive sequence homology between the two RNA species.

These considerations led us to compare the two RNA species. In this paper we describe experiments to determine the base composition of the two RNAs, and the extent of sequence homology using the technique of RNA–RNA competition hybridization.

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. It was propagated in Nicotiana clevelandii. Virus was grown under controlled conditions in the non-systemic host Nicotiana
tabacum var. White Burley. Purified tobacco necrosis virus, strain A, was a gift from Dr B. Kassanis. Bacteriophage T2 was grown in *Escherichia coli* strain b.

**Purification of unlabelled TRV RNA species.** Leaves of *N. clevelandii* systemically infected with TRV were harvested 10 days after inoculation. The tissue was homogenized in a Waring Blender and after filtration through muslin the sap was stored at $-20\, ^\circ \text{C}$ for at least 1 week. The sap was thawed and clarified by centrifuging at 16 000 g for 20 min at 4 °C. The virus was purified by two cycles of differential sedimentation (Frost et al. 1967), and the final pellet was resuspended in 0.1 M-phosphate buffer, pH 7.2 (PB). After standing at 4 °C for 5 h the particles were separated by centrifuging through a linear 10 to 40 % (w/v) sucrose gradient in PB at 60 000 g for 2.5 h at 4 °C. Two distinct light-scattering bands of virus were visible and these were harvested by piercing the tube below each band and collecting each separately. The particles were then sedimented from the sucrose by centrifuging at 200 000 g, resuspended in PB and re-banded as described above. The final pellets were suspended in 0.05 M-tris, 0.1 M-NaCl, 0.005 M-EDTA, pH 7.5 (TES).

The RNA species were extracted and purified by centrifuging in a sucrose density gradient (Darby & Minson, 1972), and were dissolved in 0.3 M-NaCl, 0.03 M-sodium citrate (2 × SSC) at a concentration of 10 μg/ml. Molar concentrations of RNA were calculated assuming a mol. wt. of $2.5 \times 10^6$ for long RNA and $0.70 \times 10^6$ for short RNA (Tollin & Wilson, 1971; Cooper & Mayo, 1972).

**Preparation of bacteriophage T2.** A culture of *E. coli* containing approximately $3 \times 10^8$ cells/ml was inoculated with bacteriophage T2 at an input multiplicity of 5 p.f.u./cell. The culture (2 l) was incubated for 4 h or until the culture cleared. Phage was concentrated from the culture by precipitation with polyethylene glycol and purified by differential and sucrose gradient sedimentation.

**Virus growth curves.** Leaves of White Burley tobacco were inoculated with TRV using an inoculum containing $5 \times 10^{11}$ particles/ml (ratio of long particles to short, 1:4) and carborundum powder. The plants were then kept in a controlled environment (22 to 23 °C, 90 % relative humidity and 16 h day-length at about 4000 lux), and the inoculated leaves of six plants were removed at various times. The sap was extracted and particle counts were made using the electron microscope according to the method of Watson, Russell & Wildy (1963).

**Preparation of [3H]-TRV RNA of high specific activity.** White Burley tobacco leaves were inoculated with TRV and the plants were placed in a controlled environment. Sixteen h after infection the inoculated leaves were detached from the plants and strips (15 × 2 mm) were cut from the central portion of each leaf. The strips were washed, dried on filter paper and placed in 5 cm plastic Petri dishes (1 g tissue per dish); 10 ml 0.02 M-KH$_2$PO$_4$ was added to each dish and the solution was infiltrated into the tissue strips under vacuum (Zaitlin & Hariharasubramanian, 1970); 2.5 mCi [5-3H]-uridine (30 Ci/m-mol, Radio-chemical Centre, Amersham) were added to each dish, and the dishes were returned to the constant environment chamber. The strips were harvested 70 h after infection.

The strips were frozen at $-20\, ^\circ \text{C}$ overnight, thawed and then mixed with 2 ml of sap from uninfected plants and ground with a pestle and mortar. This homogenate was frozen at $-20\, ^\circ \text{C}$ for 48 h. It was then thawed, filtered through muslin and diluted to 10 ml with PB. The purification procedure for the labelled particles was then similar to that for unlabelled particles except that 0.5 mg purified bacteriophage T2 was added whenever the TRV particles were to be sedimented into a pellet. After separation of the particles by sucrose gradient sedimentation in TES, 100 μg yeast RNA was added as carrier (RNA, purchased from Sigma, was further purified by phenol extraction and was freed of RNase
activity by treatment with diethyl pyrocarbonate; Solymosy et al. 1968). The RNAs were extracted as above. The final yield of each RNA species in experiments using 5 mCi [3H]-uridine was 2 to $10 \times 10^5$ ct/min.

**Preparation of [32P]-TRV RNA.** [32P]-labelled virus was prepared using leaf strips from inoculated leaves of White Burley tobacco as described above except that the strips were infiltrated with water, and 2 mCi carrier-free [32P]-orthophosphate (Radiochemical Centre, Amersham) was added to each gram of tissue. Purified, unlabelled TRV (5 mg) was added to act as carrier during the purification of virus and extraction of RNA. The efficiency of incorporation of [32P] into the virus RNA was similar to that obtained with [3H]-uridine.

**Preparation of double-stranded RNA.** Leaves of White Burley tobacco were inoculated with TRV and the plants were maintained in a constant environment, as described above, until they were harvested 40 h after infection. The infected tissue (50 g) was homogenized in a Waring Blender with 100 ml ice-cold TES (made 0.1 % with respect to sodium dodecysulfate) and 100 ml water-saturated phenol. The homogenate was filtered through muslin and, after separation by centrifuging, the aqueous phase was extracted twice with 100 ml phenol. The RNA was then precipitated from the aqueous phase by the addition of two vol. of ethanol. After 2 h at 0 °C the precipitate was collected by centrifuging and dissolved in 8 ml 0.01 m-tris, 0.01 m-NaCl, 0.001 m-EDTA, pH 7.5. Polysaccharide was removed by the addition of 8 ml 2.5 m-K$_2$HPO$_4$ and 8 ml 2-methoxyethanol (Kirby, 1956). The reagents were mixed thoroughly and, after separation, the aqueous phase was collected. RNA was precipitated from the aqueous phase by adding 60 ml ice-cold 0.01 m-tris, 0.01 m-NaCl, 0.001 m-EDTA containing 0.1 % cetyltrimethylammonium bromide (Ralph & Bellamy, 1964), and after 15 min at 0 °C the precipitate was collected by centrifuging. It was washed with 50 ml of the above solution to remove residual K$_2$HPO$_4$, dissolved in 8 ml 0.05 m-tris, 0.5 m-NaCl, 0.005 m-EDTA, and precipitated with 2 vol. ethanol. The RNA was dissolved in 3 ml TES, extracted twice with 3 ml phenol, precipitated with ethanol and dissolved in TES. The total yield of RNA was then determined spectrophotometrically. An equal vol. of 4 M-LiCl was added to the RNA solution and after 18 h at 0 °C the precipitate of high mol. wt. single-stranded RNA was removed by centrifuging (Baltimore & Girard, 1966). The RNA in the supernatant fluid was precipitated with 2 vol. ethanol and finally dissolved in 2 × SSC at a concentration of 10 mg equivalents/ml (1 mg equivalent is the quantity of double-stranded RNA in 1 mg of total RNA extracted from the tissue). This fraction of RNA is the double-stranded RNA fraction (DS-RNA).

**Base composition analysis.** Approximately 20000 ct/min of [32P]-labelled RNA was hydrolysed with 10 % piperidine in a sealed tube at 100 °C for 90 min. The nucleotides were separated by electrophoresis on Whatman paper No. 2 in 0.05 m-formate, pH 3.6, for 1 h at 60 V/cm. The nucleotide spots were eluted with 10 ml water and the total [32P] in each nucleotide was measured directly by Cerenkov counting (Braunsberg & Guyver, 1965).

**Hybridization.** Hybridization techniques were modified from those described by Weissman & Ochoa (1967). All experiments were performed in 2 × SSC in a total vol. of 0.2 ml. DS-RNA and [3H]-TRV RNA (usually 2500 to 5000 ct/min), and in competition experiments unlabelled RNA, were mixed and sealed in Pyrex tubes. The samples were heated at 115 °C for 15 min in an oil bath and then quickly transferred to a water bath at 70 °C where annealing took place for a suitable length of time (usually 20 h). At the end of the annealing period the tubes were rapidly cooled to 0 °C. Pancreatic ribonuclease (50 μg, purchased from Sigma) and T1 ribonuclease (50 units, purchased from Worthington) were added in a vol. of 0.2 ml and the mixture was incubated for 20 min at 25 °C. The undigested RNA was precipitated by the addition of 100 μg denatured calf thymus DNA.
Fig. 1. Multiplication of TRV CAM in inoculated leaves of White Burley tobacco. Attached leaves (●—●); infiltrated leaf strips prepared 16 h after inoculation (○—○).

Fig. 2. Sucrose gradient sedimentation of [32P]-labelled TRV RNA. Sedimentation is from right to left. The fractions indicated by shading were pooled for base composition analyses.

RESULTS

Virus growth

Fig. 1 shows the accumulation of TRV in inoculated leaves of White Burley tobacco. The results are similar to those described by Frost & Harrison (1967). Maximum concentration of virus was achieved by about 50 h after infection and this concentration was maintained until at least 96 h after infection. Samples taken less than 16 h after infection contained less than $5 \times 10^8$ particles/g of tissue and this was not significantly higher than that due to the residual inoculum. The same result was obtained when leaf strips were infiltrated under phosphate buffer 16 h after infection, showing that this treatment had no adverse effect on virus growth.

Since the infection of White Burley tobacco with TRV-CAM is symptomless, the growth curves shown in Fig. 1 were used to determine suitable times after infection for isotope labelling and harvesting of tissue. Thus labelled virus was obtained by adding the isotope
Sequence homology between TRV RNAs

Table I. Base composition of TRV-CAM RNA

<table>
<thead>
<tr>
<th>Base</th>
<th>Molar contribution (%)</th>
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<tbody>
<tr>
<td></td>
<td>Short RNA</td>
</tr>
<tr>
<td>Adenine</td>
<td>26.1 ± 0.1</td>
</tr>
<tr>
<td>Guanine</td>
<td>24.3 ± 0.2</td>
</tr>
<tr>
<td>Cytosine</td>
<td>17.6 ± 0.3</td>
</tr>
<tr>
<td>Uracil</td>
<td>32.0 ± 0.3</td>
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Fig. 3. Sucrose gradient sedimentation of [3H]-uridine labelled TRV. Sedimentation is from right to left. Fractions indicated by shading were pooled and RNA extracted for hybridization experiments.

16 h after infection and harvesting the tissue at 72 h, whereas double-stranded RNA was extracted from tissue harvested at 40 h when the concentration of the virus in the tissue was increasing rapidly.

Base composition

[32P]-labelled TRV RNA was fractionated into 16 and 32 S components by sucrose gradient sedimentation. The profile is shown in Fig. 2. It was considered that this single fractionation provided an adequate separation of the species. After precipitation of the RNA species their base compositions were determined. Table I shows the mean results of four independent determinations together with the standard errors. The base compositions of the two RNA species were found to be significantly different. The results are in contrast to those of Semančík & Kajiyama (1967), who found that RNA components of TRV-B had very similar base compositions.

Hybridization studies

Purification of RNA species

High specific activity [3H]-TRV was prepared as described in Methods, and long and short particles were separated by sucrose gradient sedimentation. The very low virus concentration resulted in a good particle separation (Fig. 3) and re-running of the bands gave no apparent improvement in purity. RNA was therefore extracted from the particles following a single sucrose gradient fractionation.

Unlabelled TRV RNA for use as competitor was required in optically detectable quantities...
and at the higher concentrations necessary the separations of particles or of RNA species on sucrose gradients proved less effective. Since the purity of the competitor RNA was critical to the interpretation of the competition experiments, the particles were separated by two sucrose gradients and, following extraction, the RNAs were further purified by a single sucrose gradient sedimentation.

**Demonstration of virus-specific complementary strands in infected tissue**

To determine whether virus-specific complementary strands were present in double-stranded RNA fractions from infected tissue, hybridization experiments were done using a constant concentration of [\textsuperscript{3}H]-TRV long or short RNA with increasing quantities of double-stranded RNA up to a maximum of 750 \mu g equivalents. The results in Fig. 4 show that increasing the amount of double-stranded RNA in the hybridization mixture increased the amount of [\textsuperscript{3}H]-RNA fixed in acid-precipitable material after annealing.

In control experiments using similar fractions from uninfected tissue, there was no detectable fixing of the labelled RNA. The experiments clearly demonstrated the presence of virus-specific complementary RNA strands, presumably in double-stranded structures, in the tissue of infected leaves. It also appeared that double-stranded structures derived from both long and short TRV RNA species were present.

Virus-specific complementary RNA could also be detected in the total RNA extracted from infected leaves. However, the efficiency of the total RNA in fixing [\textsuperscript{3}H]-TRV RNA was much lower than that of the LiCl supernatant fraction. Thus the total RNA extracted from leaves 40 h after infection presumably contained a large excess of virus RNA strands relative to complementary strands.

**Optimum time for annealing**

In competition hybridization experiments it is essential that the annealing process is allowed to reach an equilibrium state. In order to determine the time required for this an experiment was performed using samples of 250 \mu g equivalents of double-stranded RNA and mixed [\textsuperscript{3}H]-labelled TRV RNAs (5000 ct/min per sample). Hybridization was done under the standard conditions except that samples were assayed after various times of annealing to determine the quantity of [\textsuperscript{3}H] fixed. It can be seen from the results in Fig. 5 that an equilibrium state had been reached after about 15 h at 70 °C.
Sequence homology between TRV RNAs

Fig. 5. Hybridization of [H]-TRV RNA with DS-RNA for different times.

Fig. 6. Hybridization of [H]-short RNA with DS-RNA in the presence of increasing amounts of unlabelled short RNA (○—○) or unlabelled long RNA (●).

Competition hybridization

To determine whether there is sequence homology between long and short TRV RNA species competition hybridization experiments were performed. Initially [H]-TRV short RNA was hybridized to complementary RNA in the presence of various concentrations of either unlabelled long or unlabelled short RNA as competitor. The results of such an experiment are shown in Fig. 6(a) and (b). The theoretical relationship between the concentration of competitor and the fraction of [H]-TRV RNA fixed in the homologous competition experiment is given by the following equation:

\[ \frac{1}{F} = 1 + \frac{C}{A} \]

where \( F \) = fraction [H] fixed relative to that fixed in the absence of competitor, \( C \) = concentration of competitor, \( A \) is a function of the concentrations of [H]-RNA and double-stranded virus RNA. In competition experiments this function is constant. Thus \( 1/F \) is linearly related to \( C \), and when \( C = 0 \), \( 1/F = 1 \).

Fig. 6(b) shows that the homologous competition data fit the theoretical relationship. Fig. 6(a) shows that the long RNA preparation also behaved as a competitor in this experiment. Line (1) is the theoretical line which would be obtained if 25 % of the sequences present in pure short RNA are also present in the long. Line (2), however, is that obtained if the long RNA contains no common sequences, but contains an 8 % molar contamination of...
short RNA. Since long RNA is four times the length of short this would represent a 2\% contamination by weight. It is apparent from Fig. 6(a) that our data do not distinguish between these possibilities.

Competition experiments were also performed using [\(^3\)H]-TRV long RNA. The results are shown in Fig. 7. The homologous competition data again fit the theoretical relationship but the heterologous RNA apparently did not compete. However, this is not inconsistent with the previous experiment because if 25\% of the short sequences are present in the long, these sequences would only represent 6\% of the long RNA. Hence short RNA could not compete with more than 6\% of the long sequences and it is doubtful if this would be detectable.

Finally, in order to show that the competition observed in the experiments was specific, [\(^3\)H]-TRV RNA was hybridized with TRV double-stranded RNA in the presence of TNV RNA. TNV RNA had no effect on the hybridization even at concentrations tenfold higher than those of the TRV competitors used in the competition experiments.

**DISCUSSION**

Both RNA species of the multi-component virus TRV-CAM are required for complete infection. It has been established that the RNAs contain different genetic information (Sänger, 1968; Lister, 1969), but it is not known whether all the information in each RNA species is unique, or whether there is some genetic redundancy. Furthermore both RNAs presumably contain sequences which are not translated, but are recognition sites for replication and translation. Such sequences might be similar in both RNAs.

We attempted to investigate these possibilities by competition hybridization experiments. The results are consistent with the conclusion that 25\% of the short RNA sequences are found in the long RNA. This would represent a sequence of 500 nucleotides, sufficient for a polypeptide of about 20000 mol. wt. However, we have shown that the results could also be explained by a 2\% weight contamination of long competitor RNA by short RNA, and we have been unable to eliminate the possibility that this level of contamination was present. Nevertheless the experiments demonstrate that most of the sequences in each RNA are specific to that species. At least 94\% of the long RNA sequences are not found in the short RNA, and at least 75\% of the short RNA sequences are not found in the long.
Sequence homology between TRV RNAs

Van Kammen (1971) working with cowpea mosaic virus, found that the RNAs from the ‘bottom’ and ‘middle’ components of the virus behaved independently in hybridization experiments with double stranded virus RNA. He concluded that the two RNAs had no sequences in common.

Our results with tobacco rattle virus do not allow us to draw such a clear conclusion. It should be possible to eliminate the present ambiguity with respect to the remaining 500 nucleotides by using, as competitor, long RNA completely free of short RNA or by using double stranded RNA composed entirely of long sequences. A defective isolate of the virus, consisting only of infectious long RNA (Lister, 1966, 1968; Frost et al. 1967) would provide a good source of these reagents. Preparation of such an isolate is now in progress.

We wish to thank Miss Vicky Maxwell for skilful technical assistance, and Dr R. A. C. Jones and Mr D. R. Smith for assistance in propagating TRV.

This work was supported by a grant from the Agricultural Research Council.

REFERENCES


(Received 9 October 1972)