Homogenization-Resistant and -Susceptible Components of Tobacco Mosaic Virus Replicative Form RNA

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SUMMARY

When prepared from tissue frozen with liquid nitrogen, tobacco mosaic virus replicative form RNA (TMV RF) was uniform in size but when prepared by high-speed homogenization, or when TMV RF prepared with liquid nitrogen was homogenized, 80 to 90% of the RF broke into relatively discrete pieces. The unbroken RF was not fragmented by additional homogenization. The TMV RF components susceptible and resistant to breakage, respectively, were synthesized with similar kinetics in relation to length of labelling period, but the slightly more resistant component was synthesized during the early infection period. Both components were produced by different strains of TMV but leaves infected with cowpea chlorotic mottle or southern bean mosaic viruses yielded only RF resistant to breakage. TMV replicative intermediate RNA was also broken by homogenization. The occurrence of the two RF components may be of significance in the replication of RNA viruses.

INTRODUCTION

Replicative form RNA (RF) and replicative intermediate RNA (RI) have been found in plants infected by RNA viruses (Ralph, 1969). Initial characterizations described TMV RF as a heterogeneous population of molecules with mol. wt. considerably lower than would be expected of a structure consisting of a TMV-RNA molecule and its complementary strand. Efforts to reduce the amount of polydisperse material obtained, such as using gentle extraction procedures and eliminating RNase from the purification scheme, have met with varying degrees of success. Jackson, Mitchell & Siegel (1971) demonstrated that a molecule with the expected characteristics of TMV RF could be obtained by powdering tissue frozen with liquid nitrogen in a mortar, extracting with phenol, and purifying it by CF-11 chromatography. However, similar procedures have been less successful with some other viruses, resulting in RF molecules that are totally (tobacco ringspot virus: Rezaian & Francki, 1973) or partially (pea enation mosaic virus: German & deZoeten, 1975) heterodisperse in size.

This paper reports the isolation and characterization of two classes of tobacco mosaic virus (TMV) RF from infected tissue; one that is resistant to breakage on homogenization and retains the properties expected of TMV RF (Jackson et al. 1971) and another component that breaks down to give a population of smaller molecules.
METHODS

Viruses and hosts. Strain U1 of TMV, the tobacco form of the cowpea strain of TMV (Cp TMV-TF), and a transition strain of TMV (supplied by Dr J. G. Bald; Bald, Gumpf & Heick, 1974) were examined in tobacco (Nicotiana tabacum L. cv. Xanthi). The cowpea form of the cowpea strain of TMV (Cp TMV-CF), cowpea chlorotic mottle virus (CCMV), and the cowpea strain of southern bean mosaic virus (SBMV) were examined in cowpea (Vigna sinensis (Torner) Savi cv. California Blackeye). In most experiments with TMV, young tobacco leaves were systemically inoculated at 3 °C using the differential temperature procedure to synchronize multiplication (Dawson, Schlegel & Lung, 1975). Plants were maintained in a plant growth chamber at 25 °C with a 14 h photoperiod at 20000 lux.

Labelling procedure. Detached leaves were submerged and vacuum infiltrated with either 3H-uridine (40 μCi/ml; 8 Ci/mmol) or H235PO4 (60 to 100 μCi/ml; carrier free), and placed in Petri dishes and incubated at 25 °C at 20000 lux for the designated labelling period. In most experiments 65 μg/ml actinomycin D was included in the labelling solution.

RNA extraction procedures

Liquid nitrogen procedure. Tissue (5 g) was frozen with liquid nitrogen and powdered using a mortar and pestle. The fine powder was transferred to a beaker containing 12 ml of cold 0.01 M-tris-HCl, pH 7.6 containing 0.05 M-NaCl and 1 % SDS, and 12 ml water-saturated phenol containing 10 % m-cresol and 0.1 % 8-hydroxyquinoline and stirred at room temperature for 15 min. The aqueous phase was recovered after centrifugation, NaCl added to 0.5 M, and stirred 15 min at room temperature after addition of 12 ml of the above phenol solution. The aqueous phase was again collected after centrifugation and 2 vol. ethanol was added. The resulting precipitate was dissolved in 2 ml of 0.05 M-tris-HCl, pH 6.85, containing 0.1 M-NaCl, 0.001 M-EDTA, and 0.1 % N-lauroyl sarcosine, and dialysed against that buffer overnight. The nucleic acids were precipitated with 2 vol. ethanol, dissolved in 1.0 ml of 0.01 M-MgCl2 and incubated 1 h at 37 °C with 50 μg/ml DNase (RNase free). LiCl was then added to 2 N, the mixture stored overnight at 5 °C, and the precipitate collected by centrifugation at 10000 g for 10 min. The 2 N-LiCl supernatant was mixed with 2 vol. of ethanol and the precipitate dissolved in E buffer (0.04 M-tris-acetate, 0.02 M-sodium acetate, 0.002 M-EDTA, pH 7.8) containing 0.2 % SDS and 20 % sucrose.

Homogenization procedure. This was identical to the above process except that tissue was homogenized in a Virtis homogenizer in ice water for about 1 min at high speed in the presence of 12 ml of the extraction buffer and 12 ml of the phenol solution.

RNase treatment. The 2 N-LiCl supernatant fraction, or the 2 N-LiCl precipitate, was dissolved in 0.5 ml of 0.2 M-NaCl, 0.01 M-MgCl2, 0.01 M-tris-HCl, pH 7.6, and incubated with 5 μg/ml RNase A and 0.5 μg/ml RNase T1 for 30 min at 37 °C. Ethanol (2 vol.) was then added to samples and the precipitate dissolved in E buffer plus 0.2 % SDS and 20 % sucrose for electrophoresis.

CF-11 chromatography. Two cycles of chromatography on CF-11 cellulose were made according to the procedures of Jackson et al. (1971) and Franklin (1966).

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed essentially by the procedure of Loening (1967) with the addition of 0.2 % SDS to the electrophoresis buffer. After electrophoresis, the gels were scanned at 260 nm with a Beckman Acta II spectrophotometer equipped with a gel scanner.

Determination of radioactivity. Polyacrylamide gels were sliced into 2 mm slices. Gel slices labelled with 32P were put into 10 ml of 2.5 mm-7-amino-1,3-naphthalene disulphonic
acid and radioactivity was counted in a liquid scintillation counter by Cerenkov radiation (Läuchli, 1969). Gel slices labelled with \(^{3}H\) were dissolved in \(\text{H}_{2}\text{O}_{4}\) and counted in PPO-dimethyl-POPOP as described previously (Dawson & Schlegel, 1976).

**Isopycnic centrifugation.** Samples purified by cellulose chromatography were analysed by isopycnic centrifugation in Cs\(_{2}\)SO\(_{4}\) gradients according to Szybalski & Szybalski (1971).

**RNA hybridization.** \(^{3}H\)-labelled TMV-RNA was extracted from purified virus using phenol. Double-stranded RNA labelled with \(^{32}P\) was eluted from polyacrylamide gels by the procedure of Young & Young (1974). Hybridization with \(^{3}H\)-TMV RNA and \(^{32}P\)-double-stranded RNA was by the method of German & deZoeten (1975).

**RESULTS**

**Comparison of RF extracted using liquid nitrogen or homogenization**

Fig. 1 (c) shows that homogeneous TMV RF was prepared by powdering tissue frozen with liquid nitrogen in a mortar and extracting with phenol (first extraction method). However, when TMV RF was extracted from infected tissue by a similar procedure, differing in that the tissue was homogenized in a Virtis homogenizer (second method) a different profile resulted (Fig. 1 a). There was a sharp peak at the expected position for TMV RF, but also a series of polydisperse peaks that migrated faster than RF during electrophoresis. Approximately the same amount of radioactivity was found in the RF plus polydisperse RNA extracted by homogenization as in the RF of liquid nitrogen extracted RNA. In other experiments RNA from tissue homogenized with a Waring blender gave identical results. Neither the sharp RF peak nor the polydisperse peaks occurred with preparations from healthy tissue.

The different extraction procedures affected only double-stranded TMV RNA. Single-stranded TMV-RNA, ribosomal RNA, transfer RNA and 5S RNA exhibited identical electrophoresis profiles when isolated by either extraction procedure.

**Re-extraction of RF by homogenization**

To test whether RF was progressively broken down by forces acting during homogenization, different samples of tissue were blended for periods from 2 to 10 min. Regardless of the duration of homogenization, the same proportion of RF to polydisperse RNA resulted, 10 to 20 \% RF and 80 to 90 \% polydisperse RNA.

Another way to examine the effect of homogenization upon RF was to re-extract by the homogenization procedure TMV RF that was prepared by the liquid nitrogen procedure. RF prepared by the two methods was electrophoresed (Fig. 1 a, c) and the gel fractions containing RF were cut out and added to healthy tissue, re-extracted by the homogenization procedure, and analysed by polyacrylamide gel electrophoresis. The RF previously extracted by homogenization did not break down (Fig. 1 b). However, most of the RF extracted by the liquid nitrogen procedure broke down into polydisperse RNA (Fig. 1 d). Thus TMV RF consists of two components, one component that is resistant to breakage during homogenization and one that breaks down into smaller double-stranded molecules.

**Hybridization of polydisperse RNA with TMV RNA**

The polydisperse RNA that was obtained by homogenization was found only in infected tissue, but there was no evidence that this RNA was TMV specific. To answer this question, the polydisperse RNA was hybridized with TMV RNA. Leaves infected with TMV were labelled with \(^{32}P\) and the RNA was extracted by the homogenization procedure and
Fig. 1. Effect of homogenization upon TMV RF. Infected tissue was labelled with \(^{32}\)P for 4 h and RNA was extracted from (a) tissue homogenized in a Virtis homogenizer or from (c) tissue frozen with liquid nitrogen and powdered with a mortar and pestle. The RNA soluble in 2 N-LiCl was analysed by electrophoresis on 2.5 % polyacrylamide gels run 5 h at 7 mA/gel. After polyacrylamide gel electrophoresis, the portion of the gel containing RF (within dashed lines) was cut out, added to healthy tissue not labelled with a radioisotope and RNA was re-extracted by the homogenization procedure and again analysed by polyacrylamide gel electrophoresis. (b) and (d) show the profiles obtained after re-extraction of the material from (a) and (c) respectively. The tops of the gels are on the left.
TMV replicative form RNA

Fig. 2. Hybridization of virion $^3$H-TMV RNA to $^{32}$P-polydisperse double-stranded RNA and RF. The $2 \text{~N-LiCl}$ soluble RNA extracted by the homogenization procedure was electrophoresed on 2.5% polyacrylamide gels for 6 h at 7 mA/gel. The gels were sliced and RNA was eluted and divided into two equal fractions. One fraction melted, self-annealed, and treated with RNase (RNase resistant counts). The other fraction was melted, annealed in the presence of excess $^3$H-TMV RNA, treated with RNase, and the amount of RNase-resistant $^3$H-TMV RNA determined.

Fractionation of the polydisperse RNA with $2 \text{~N-LiCl}$. The $2 \text{~N-LiCl}$-soluble RNA was electrophoresed on polyacrylamide gels as in Fig. 1(a) and the RNA from each fraction was eluted from gel slices and divided into two fractions. One fraction was melted and re-annealed in the absence of added TMV-RNA and then treated with RNase. The other fraction was melted and re-annealed in the presence of excess $^3$H-labelled virion TMV RNA. Fig. 2 shows that the polydisperse RNA specifically hybridized with TMV RNA. Approx. 80% of the polydisperse RNA was resistant to RNase after melting and self-annealing, demonstrating its double-stranded nature. When the polydisperse RNA was annealed in the presence of excess $^3$H-TMV RNA, slightly more than one-half of the $^{32}$P counts were displaced.

**RNase treatment of RF**

When the $2 \text{~N-LiCl}$ soluble RNA extracted by the homogenization procedure was treated with RNase prior to analysis by polyacrylamide gel electrophoresis, a profile identical to that in Fig. 1(a) resulted, demonstrating that the polydisperse RNA (fractions 14 to 45 of Fig. 1a) was double-stranded.

To test whether in the homogenization-susceptible RF the sites susceptible to homogenization might also be susceptible to RNase, homogenous RF prepared by the liquid nitrogen procedure was treated with RNase and analysed by polyacrylamide gel electrophoresis. A
profile almost identical to that of Fig. 1(c) resulted, showing that TMV RF was not susceptible to RNase in the manner that it was susceptible to homogenization.

Characterization of the polydisperse RNA

RNA was extracted from TMV infected leaves by the homogenization procedure and the 2 N-LiCl soluble fraction was chromatographed on CF-11 cellulose. The RNA that eluted with 100 % buffer (double-stranded RNA) gave a profile identical to that in Fig. 1(a) when analysed by polyacrylamide gel electrophoresis; the polydisperse RNA eluted from CF-11 cellulose with RF.

Centrifugation of RF plus polydisperse RNA obtained from CF-11 chromatography to equilibrium in Cs$_2$SO$_4$ gradients resulted in a homogeneous band with a density of 1.625 g/ml.

The mol. wt. of the various species of polydisperse RNA were estimated by comparing their mobilities on electrophoresis in 2.5 %, 3.5 % and 5.0 % polyacrylamide gels to that of double-stranded RNA from bacteriophage $\phi 6$ (kindly supplied by Dr J. S. Semancik). Under the conditions of electrophoresis, TMV RF co-migrated with the $4.5 \times 10^6$ dalton component of $\phi 6$ RNA in each of the gels (Fig. 3). Although the polydisperse RNA did not migrate as discrete components, reproducible peaks were resolved. Similar mol. wt. were estimated for the RNA in these peaks in the different gels; the approximate values were 3.0, 2.0 to 2.4, 1.0 to 1.2, 0.6 to 0.7 and $0.2 \times 10^6$.

This suggests that on homogenization there were specific sites of the molecule that were more susceptible to breakage resulting in relatively specific breakdown products. Most of the polydisperse RNA was 2.0 to 3.0 $\times 10^6$ daltons. A large proportion of the polydisperse RNA resulted from only one or two breaks of RF molecules.

Effect of labelling period on ratio of RF components labelled

To determine whether a precursor–product relationship existed between the two components and whether their rates of synthesis or breakdown differed, infected leaves were labelled with $^{32}$P for periods of 5 min to 24 h and the relative amounts of the two RF components determined. From 13 to 21 % of the RF was resistant to breakage in the different samples, but there was no trend with increasing labelling period, demonstrating that both components were synthesized with similar kinetics.

Relation of ratio of RF components to the infection cycle

With the development of a system that results in synchronous synthesis of TMV in intact tobacco leaves (Dawson et al. 1975), we have previously examined the kinetics of TMV RNA synthesis in relation to the infection cycle (Dawson & Schlegel, 1976). The synthesis of single-stranded TMV RNA, RF and RI were each detected first at 6 to 8 h after infection, each increased exponentially until 18 to 24 h after which each increased linearly. To examine the ratio of the two RF components in relation to time of the infection cycle, leaves were labelled for 2 h with $^{32}$P at different periods between 6 and 96 h after infection and RF was extracted by the homogenization procedure. The results are shown in Table 1. The percentage of homogenization-resistant RF was higher during the early infection period; however, both RF components were synthesized during all stages of the infection cycle.

Sensitivity of RFs of different viruses and virus strains to homogenization

To determine whether the degree of sensitivity of RF to homogenization is strain-specific the RFs of strain U1, the tobacco form of the cowpea strain of TMV (Cp TMV-TF), the cowpea form of the cowpea strain of TMV (Cp TMV-CF), and a transition strain of TMV
Fig. 3. Estimation of the mol. wt. of the polydisperse RNA. Infected tissue was labelled 4 h with 3H-
uridine and RNA was extracted by the homogenization procedure. The 2 M-LiCl-soluble RNA was
compared to that of double-stranded RNA from the bacteriophage 66 and to single-stranded r-RNA.
The RNA was electrophoresed at 7 mA/gel for 5 h on 2.5% gels, 10 h on 3.5% gels, and 20 h
on 5.0% gels. Solid lines represent ct/min and dashed lines represent optical density at 260 nm.
The peaks are labelled with mol. wt. in millions.

were extracted by homogenization and analysed by polyacrylamide gel electrophoresis. All
the TMV RFs had similar degrees of susceptibility to breakage, each possessing homogeniz-
ation-resistant and homogenization-susceptible components. Each electrophoresis profile
was similar to that in Fig. 1(a).
Table 1. Incorporation of $^{32}P$ into the components of TMV RF at different times after infection

<table>
<thead>
<tr>
<th>Hours after infection</th>
<th>Resistant RF</th>
<th>Polydisperse RNA</th>
</tr>
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<tbody>
<tr>
<td>6-8</td>
<td>42</td>
<td>58</td>
</tr>
<tr>
<td>8-10</td>
<td>31</td>
<td>69</td>
</tr>
<tr>
<td>10-12</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td>14-16</td>
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<td>76</td>
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<td>24-26</td>
<td>19</td>
<td>81</td>
</tr>
<tr>
<td>72-7</td>
<td>18</td>
<td>82</td>
</tr>
<tr>
<td>96-98</td>
<td>9</td>
<td>91</td>
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</table>

To compare different viruses, RFs were isolated by the homogenization procedure from cowpea leaves infected with Cp TMV-CF, the cowpea strain of southern bean mosaic virus (SBMV) or cowpea chlorotic mottle virus (CCMV), and analysed by polyacrylamide gel electrophoresis (Fig. 4). Although the RF of Cp TMV-CF consisted of homogenization-resistant and homogenization-susceptible components, the RFs of SBMV and CCMV were not susceptible to breakage on homogenization. The electrophoretic profile of CCMV RF consisted of 3 sharp peaks similar to that reported for brome mosaic virus (Philipps, Gigot & Hirth, 1974), a close relative of CCMV. The RF of SBMV consisted of one homogeneous species. The RFs extracted by the liquid nitrogen procedure from tissue infected with each of these viruses and virus strains gave the expected sharp peaks.

**Effect of homogenization upon RI**

Nilsson-Tillgren (1970) and Jackson et al. (1971) demonstrated that RNase treatment of TMV RI converted it into a totally double-stranded molecule similar to RF. To examine the effect of homogenization upon RI, RNA was extracted from infected leaves by the liquid nitrogen procedure and by the homogenization procedure, precipitated with 2 N-LiCl, treated with RNase and analysed by polyacrylamide gel electrophoresis. The RNase-resistant core of RI extracted by the liquid nitrogen procedure was relatively homogeneous (Fig. 5a), whereas that extracted by homogenization consisted almost entirely of molecules smaller than RF (Fig. 5b). The entire population of RI was susceptible to breakage during homogenization.

**DISCUSSION**

We have shown that TMV RF consists of two components; one comprising about 10 to 20% of the RF that is resistant to breakage on homogenization and one that fragments into polydisperse double-stranded RNA. This is in contrast to the homogeneous molecule that is extracted in the absence of shear.

In relation to that of other viruses, TMV RF is of intermediate stability. For instance, the RF of tobacco ringspot virus (TRSV) was not isolated as a full length structure, even when extracted by the liquid nitrogen procedure (Rezaian & Francki, 1973). Schneider, White & Thompson (1974) isolated TRSV RF that was larger but still polydisperse. However, the RFs of CCMV and SBMV remained intact when extracted by either procedure. This difference in stability is not due simply to the size of these molecules. Although TMV and TRSV are viruses with larger expected RFs (TMV, $4.1 \times 10^6$ daltons; TRSV, $4.4$ and $2.4 \times 10^6$ daltons), the small species of TRSV RF is smaller than that of SBMV ($2.8 \times 10^6$ daltons). Also, the component of TMV RF that breaks down is the same size as that which does not break down.
TMV replicative form RNA

Fig. 4. Effect of homogenization on the RFs of different viruses. Cowpea leaves infected with (a) Cp TMV-CF, (b) SBMV or (c) CCMV were labelled with $^{32}$P for 2 h and RNA was extracted by the homogenization procedure. The RNA that was soluble in 2 n-LiCl was analysed by polyacrylamide gel electrophoresis on 2.5% acrylamide gels run 7 h at 7 mA/gel.

TMV RI also breaks down upon homogenization, but without a resistant component. Although the labile component of RF breaks down in a manner similar to RI, this component does not result from breakdown of RI. Exposure of tissue infected with TMV or temperature-sensitive mutants of TMV to restrictive temperatures results in the synthesis of RF but not RI (unpublished data). Under these conditions, both RF components occur in proportions similar to those reported here.
The function of RF in virus synthesis is not understood. However, the conditions that determine resistance or susceptibility to homogenization may represent different functional states of the molecule. Small TMV-specific RNA species other than full length virion RNA have been reported in infected tissue (Siegel, Zaitlin & Duda, 1973) and in association with polysomes (Beachy & Zaitlin, 1975), and can be translated in vivo (Knowland et al. 1975). These data are consistent with the idea that TMV RNA is processed into small pieces for translation. It is interesting to note that the new proteins that are found after infection by TMV are of sizes, 165 to 195, 135 to 155, 37 and 17.5 x 10^{3} daltons (Zaitlin & Hariharasubramanian, 1972; Sakai & Takebe, 1974; Paterson & Knight, 1975), that would require messenger RNAs of approx. one half the mol. wt. of the peaks of polydisperse RF that resulted from homogenization.
REFERENCES


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