Two Nucleic Acid-Containing Components of Tomato Ringspot Virus

I. R. SCHNEIDER; R. M. WHITE, AND E. L. CIVEROLO

Purified tomato ringspot virus (TmRSV) has two nucleic acid-containing components (instead of only one), with estimated sedimentation coefficients of 119 S and 127 S. These two components were present in four different isolates, including a peach yellow bud mosaic isolate. Evidence is presented that the two components are not different conformations of a single component. The relative proportions of the two components were dependent upon whether the virus was increased in cucumber or in tobacco plants. The faster sedimenting component appeared relatively more infectious than the slower sedimenting one. The difference in infectivity may reflect a difference in the degree of separation of the two components rather than an inherent difference between their biological activities. Both components appeared to migrate electrophoretically at the same rate and were not distinguishable by electron microscopy. The 119 S component had a buoyant density in cesium chloride of 1.495, and the 127 S component had a buoyant density of 1.51. Two major nucleic acids were extracted, one, presumably, from each nucleoprotein. They sedimented with estimated values of 30.9 and 32.6 S. After formylation, only one nucleic acid was resolved, with an estimated S value of 18.2 and a molecular weight of about $2.3 \times 10^6$ daltons.

INTRODUCTION

Tomato ringspot virus (TmRSV) and tobacco ringspot virus (TbRSV), although unrelated serologically (Tall et al., 1949), share many common biological and physical properties (State-Smith, 1970A, 1970B). Both are nematode-transmitted members of the Nepo virus group (Teliz et al., 1966; Harrison et al., 1971). Both viruses are endemic in North America (State-Smith, 1966) and infect a wide range of herbaceous and perennial hosts, frequently causing similar symptoms (Kahn and Latterell, 1955; McLean, 1962; Gooding, Jr., 1963; Uyemoto, 1970; Rush and Gooding, Jr., 1970). Both viruses are isometric and indistinguishable in size (Steere, 1956; State-Smith et al., 1965; State-Smith, 1966).

Purified preparations of TmRSV contain top and bottom components not significantly different in size or sedimentation rates from the corresponding top and bottom components of TbRSV (State-Smith, 1966). One difference between the two viruses is that no component corresponding to the middle component of TbRSV is present in TmRSV (State-Smith, 1966). As presently characterized (State-Smith, 1970B), TmRSV is described as a multicomponent virus with one nucleic acid-containing component. However, our examination of several preparations of TmRSV convinces us that there are actually two nucleic acid-containing components: the two components sediment at slightly different rates, and each component contains a different nucleic acid. The evidence for the presence of these two components in TmRSV and an examination of some of their properties are the subjects of this communication.
MATERIALS AND METHODS

Viruses. The isolate of TmRSV (TmRSV-A) used for the bulk of this study was originally isolated by one of us (E. I. Civerolo) from apricot (Prunus armeniaca L.) in Beltsville, Maryland. The other isolates of TmRSV we used are: (1) peach yellow bud mosaic virus (PYBMV-GN), supplied by Dr. George Nyland (University of California, Davis, California); (2) ATCC PV-78; and (3) Isolate A67 (A-67) obtained from Dr. William B. Raymer (Campbell Soup Research Center, Riverton, New Jersey), originally isolated from birdsfoot trefoil (Lotus corniculatus L.) by Dr. S. A. Ostazeski (USDA, Beltsville).

Cucumber mosaic virus (CMV) strain used as a sedimentation marker was obtained from Dr. J. M. Kaper (USDA, Beltsville). TbRSV (ATCC PV-98), and the satellite of TbRSV, as well as their nucleic acids (Schneider, 1971), were also used as sedimentation markers.

Hosts and purification. TmRSV was routinely propagated in cucumber plants: Cucumis sativus L. (cv. “National Pickling”). One cotyledon of each plant (previously dusted with 69D-mesh Carborundum) was inoculated by rubbing it with a pestle dipped into inoculum. Seedlings were harvested 10–14 days after inoculation.

Partial purification of TmRSV was essentially by the method of State-Smith (1966) except that 0.05 M potassium phosphate buffer containing 1% L-ascorbic acid as antioxidant, adjusted to pH 7.0, was used for the extraction of infected tissue. The extract was heated in a water bath (Gooding, Jr., 1963) at 50 C for 15 min before freezing. After partial purification by ammonium sulfate precipitation and differential centrifugation, further purification was effected by one or more cycles of sucrose density-gradient centrifugation.

Density-gradient centrifugation. Linear density gradients were prepared with a Beckman Density Gradient Former equipped with a triple mixing chamber into 9 1/16 × 3 1/2 in. tubes to give a range of 0.2–0.8 M sucrose in phosphate buffer (0.02 M, pH 7.0) (Schwarz/Mann ribonuclease-free gradient grade sucrose). When nucleic acids instead of virus were to be centrifuged, sodium bentonite prepared according to Dunn and Hitchborn (1965) was incorporated with the sucrose density gradient (Kado and Knight, 1966), final concentration 0.004%. One- to two-tenths of a milliliter of an appropriately diluted virus (or nucleic acid) preparation was layered onto each gradient and the tubes were centrifuged in an SW 41 rotor in a Spinco model L3-50 at 40,000 rpm in the case of virus (for 85–90 min) and at 39,000 rpm in the case of nucleic acids (for 16 hr). The gradients were fractionated and analyzed with an ISCO Density Gradient Fractionator and UV Analyzer (Instrumentation Specialities Co., Lincoln, Nebraska).

For the estimation of S values and for separation of components in a second cycle of density-gradient centrifugation, the linear-log gradients described by Brakke and Van Pelt (1970) were used. Centrifugation of virus was for 137 min at 40,000 rpm and centrifugation of nucleic acids was for 16 hr at 33,500.

To concentrate the virus components from each cycle and to remove most of the sucrose before the next cycle of density-gradient centrifugation, the same fractions from many individual tubes were combined, and the components were sedimented into a pellet, either in the SW 65 rotor at 48,000 rpm for 2 hr or in the SW 41 rotor at 40,000 rpm for 3 hr. Each pellet was resuspended in 0.1–0.3 ml of phosphate buffer (0.02 M, pH 7.0) and dialyzed to remove sucrose.

Nucleic acid preparation. Nucleic acids were prepared by the single-phase phenol-SDS method of Diener and Schneider (1968), except that the final concentration of phenol was increased to 5.5% (w/v).

Ultraviolet spectrophotometry. Spectra of virus and nucleic acid were determined by measuring the uv-absorption at 220–300

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nm in a Cary model 14 recording spectrophotometer.

**Infectivity assays.** Infectivity assays were made by comparison of the numbers of lesions in opposite half-leaves of cowpea (Vigna unguiculata (L.) Walp. cv. "Early Ramshorn"). The plants were shaded 4-24 hr before inoculation. Plants were maintained at 21-25 C, 12-hr day, light intensity ca. 1000 lm. One free-falling drop from a Pasteur pipette was rubbed onto each half-leaf with the base of a small glass vial or the base of a 10-ml syringe barrel.

**Buoyant density determination.** Equilibrium centrifugation in CsCl gradients was at 44,770 for 19 hr or longer at 25 C in a Spinco model E ultracentrifuge. The density of CsCl was calculated by measuring its refractive index in an Abbé refractometer and using the formula ρ = 10.8601 + 13.4974 ρ^2 = 1.34974 ρ/cm^3 (Lifl et al., 1961). The buoyant densities were calculated by using the method described by Dirkx (Undated). Components were located in the cell of the Spinco model E ultracentrifuge by use of the high-intensity light source, monochromator, photoelectric scanning system.

RNA samples were formylated according to Boedtker (1968). Nucleic acids were centrifuged in linear-log gradients (Brakke and Van Pelt, 1970) containing 0.1 M sodium phosphate buffer containing 1.1 M formaldehyde (Boedtker, 1968).

**Electrophoresis of virus** was through 5-20% linear density gradient columns of the ISCO model 210 apparatus for 16 to 36 hr at 4 mA (45-50 V). Buffer system was 0.0375 M KCl, 0.0125 M Tris-HCl, 10-5 M MgCl₂ (pH 8.0 (Brakke et al., 1968)), or 0.002 M potassium phosphate and 0.05 M NaCl (adjusted to pH 8.0). The gradients were fractionated and analyzed with an ISCO Density Gradient Fractionator and UV Analyzer.

**RESULTS**

**Sedimentation characteristics of two nucleoproteins.** Yields of purified virus were low and averaged approximately 8 A260 units per 100 g of cucumber tissue. Purified preparations of TmRSV, when centrifuged in sucrose density-gradient columns, consisted of two nucleic acid-containing components (B1 and B2) (Fig. 1A) rather than one bottom component (Stace-Smith, 1966). "T" component (Fig. 1A) probably corresponds to the previously described top component (or empty protein shell) of Stace-Smith, (1966), sedimenting at approximately 53 S. Using top component (TbRSV) and cucumber mosaic virus (CMV) as markers (of 53 S and 98 S, respectively), as well as middle and bottom components of TbRSV (of 95 S and 126 S, respectively) (not shown), in linear-log sucrose density gradients (Brakke and Van Pelt, 1970), we estimate the sedimentation coefficients of components B1 and B2 (TmRSV) to be 119 S and 127 S, respectively (Fig. 1).

To eliminate the possibility that the two components might be characteristic of only the one isolate (TmRSV-A), we examined purified preparations of three other TmRSV isolates: ATCC PV-78; a PYBM isolate (GN); and an isolate from Dr. William B. Raymer (A-67). Each isolate, which reacted in Ouchterlony double diffusion tests with antiserum specific for TmRSV, was also composed of two nucleic acid-containing components sedimenting to a similar position in sucrose density-gradient columns as the B1 and B2 components of TmRSV-A.

To explore the possibility that the two components might be two conformations of a single component, we investigated the influence of three pH values (pH 6, 7, and 8) of phosphate buffer at two concentrations (0.02 and 0.2 M) on the relative proportions, homogeneity, and their sedimentation position relative to each other. After 24-hr dialysis against one of the above six solutions, the virus from each treatment was centrifuged in a sucrose density-gradient column made up with the same buffer used in the 24-hr treatment. No significant difference was observed between the proportions of B1 and B2 components, their homogeneity, or their relative sedimentation positions.

When TmRSV-A was increased in cucumber plants, the quantity of B2 component was always less than the quantity of B1...
component. When tobacco plants (*Nicotiana tabacum* L. cv. "Samsun") were infected with the same isolate, however, the quantity of the B2 component was greater than the B1 component. To investigate this possible "host effect" in a controlled experiment, we used inoculum obtained either from cucumber or from tobacco and inoculated both cucumber and tobacco plants on the same day with each inoculum. Plants were harvested 10 days later and the virus purified from each batch was analyzed by sucrose density-gradient centrifugation. The results obtained from cucumber, as before, contained a larger amount of component B1 and the products obtained from tobacco contained a larger amount of component B2, irrespective of the source of the inoculum. Differences in the relative amounts of B1 and B2 were in the same direction when a different isolate (A-67) was used, but the "host effect" was not as marked.

**Characteristics of partly separated components.** For comparison of the components with respect to UV-absorption spectra and relative infectivity and for examination by electron microscopy, components B1 and B2 were partly separated from each other by one cycle of sucrose density-gradient centrifugation. To improve a very difficult separation, B1 included only that part of the fraction up to 0.05 ml before the peak (B1), and B2 included only that part of the fraction at least 0.05 ml after the B2 peak. A comparison of the degree of enrichment of each component achieved by this procedure shows the relative proportion of B1 and B2 in each fraction in a second cycle of density gradient centrifugation as compared with the composition of the original sample (Fig. 2A).

A summary of the absorption maximum/minimum (260/240 nm) for different lots of separated B1 and B2 is shown in Table 1. Although there was considerable variation between individual lots, presumably reflecting a variation in the degree of purity of each preparation, the maximum/minimum of B1 was consistently higher than the corresponding value for B2. Each sample pair was adjusted to equal A$_{260}$ values at a value of 0.7 A$_{260}$ or greater.

The relative infectivity of partly separated (one-cycle density-gradient centrifugation) B1 and B2 components was compared on opposite half-leaves. At various concentrations tested and based upon equal A$_{260}$ values for the two components, the number of lesions induced by B2 was consistently more than the corresponding number induced by B1. The number of lesions induced by B2 varied from 1.6 to 5.5 times the number of lesions induced by B1 (Table 2).

After a second and third cycle of density-gradient centrifugation, the component B2 induced 2.8 to 16 times as many lesions in cowpea leaves as component B1 (Table 2).

An enhancement of infectivity by a mixture of the two components (after a third cycle of density-gradient centrifugation) compared with the infectivity of the same concentration of either component alone was indicated in one experiment (Table 3), but
Fig. 2. Ultraviolet absorbance profiles of centrifuged density-gradient columns containing purified TmRSV. (A) Unfractionated. (B) B1 enriched fraction. (C) B2 enriched fraction.

TABLE 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>B1 enriched</th>
<th>B2 enriched</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.47</td>
<td>1.29</td>
</tr>
<tr>
<td>2</td>
<td>1.51</td>
<td>1.38</td>
</tr>
<tr>
<td>3</td>
<td>1.40</td>
<td>1.22</td>
</tr>
<tr>
<td>4</td>
<td>1.46</td>
<td>1.35</td>
</tr>
<tr>
<td>5</td>
<td>1.50</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Average 1.47 1.32

the low yields and the technical difficulty in obtaining each component free of the other have not yet permitted us to validate these results.

Electrophoresis of virus. Electrophoresis of virus resulted typically in two migrating components. In agreement with Uyemoto (1970), only the slower of the two migrating components was infectious (and contained both B1 and B2 components) and the faster of the two components appeared to be a mixture of impurities.

Electron microscopy. No difference in size or appearance of particles could be detected when B1- and B2-enriched fractions were compared.

Buoyant density in cesium chloride. TmRSV was stable for 24 hr or longer in 1.468 g/ml of CsCl at 25 C because after dialysis to remove the CsCl, the homogeneity and position of both B1 and B2

TABLE 2

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Concentration</th>
<th>Average number of local lesions</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$A_{260}$</td>
<td>B1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B2/B1</td>
</tr>
<tr>
<td>1</td>
<td>0.013, c</td>
<td>28</td>
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<tr>
<td></td>
<td>0.026</td>
<td>115</td>
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<tr>
<td></td>
<td>0.052</td>
<td>3.4</td>
</tr>
<tr>
<td>2</td>
<td>0.049, c</td>
<td>16</td>
</tr>
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<td></td>
<td>0.099</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>0.198</td>
<td>5.6</td>
</tr>
<tr>
<td>3</td>
<td>0.010, c</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>0.020</td>
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<tr>
<td></td>
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<td></td>
<td>0.080</td>
<td>6.3</td>
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<td>4</td>
<td>0.025, d, f</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>0.100</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>0.200</td>
<td>19.0</td>
</tr>
</tbody>
</table>

$^a$ Average number from 10 half-leaves or 10 leaves.

$^b$ Comparison on opposite half-leaves.

$^c$ After one cycle of sucrose density-gradient separation.

$^d$ Comparison on opposite leaves.

$^e$ After two cycles of sucrose density-gradient separation.

$^f$ After three cycles of sucrose density-gradient separation.
TABLE 3

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Concen $A_{260}$</th>
<th>Total local lesions 10 half-leaves</th>
<th>$B1 + B2$ $B1^a$ or $B2^b$</th>
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<tr>
<td>$B1$</td>
<td>.01</td>
<td>32</td>
<td>10.7$^a$</td>
</tr>
<tr>
<td>$B1 + B2$</td>
<td>.01$^c$</td>
<td>343</td>
<td></td>
</tr>
<tr>
<td>$B1$</td>
<td>.02</td>
<td>151</td>
<td>1.9$^a$</td>
</tr>
<tr>
<td>$B1 + B2$</td>
<td>.01$^c$</td>
<td>287</td>
<td></td>
</tr>
<tr>
<td>$B2$</td>
<td>.01</td>
<td>57</td>
<td>7.4$^b$</td>
</tr>
<tr>
<td>$B1 + B2$</td>
<td>.01$^c$</td>
<td>424</td>
<td></td>
</tr>
<tr>
<td>$B2$</td>
<td>.02</td>
<td>273</td>
<td>1.03$^b$</td>
</tr>
<tr>
<td>$B1 + B2$</td>
<td>.01$^c$</td>
<td>282</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ B1 in denominator.
$^b$ B2 in denominator.
$^c$ Equal volume mixture of $B1$ and $B2$ components, each at $A_{260}$ of 0.02.

components in uv-absorption profiles of sucrose density-gradient columns remained unchanged.

The buoyant density of TmRSV was therefore estimated in CsCl gradients in the Spinco model E ultracentrifuge (Ifft et al., 1961). Components of two buoyant densities [1.49 p and 1.51 p] were found in unfractionated TmRSV (Fig. 3). Components of the same two densities were found in a $B1$-enriched fraction and in a $B2$-enriched fraction from the same purified preparation. By comparing the relative proportion of the two components in sucrose density-gradient columns in each preparation with the relative proportion of the two components in the CsCl gradients, we identify the denser of the two components as $B2$ and the lighter as $B1$.

Nucleic acids extracted from different purified preparations of TmRSV and centrifuged in sucrose density gradient columns consisted of two major nucleic acid components (Fig. 4A). From a comparison of the relative proportion of the slower and faster sedimenting nucleic acid components with the relative proportion of $B1$ and $B2$ in the virus preparation that served as the source, it is likely that the slowly sedimenting nucleic acid was extracted from $B1$ and the faster sedimenting nucleic acid was extracted from $B2$.

Using a 24 S nucleic acid from TbRSV and the 7.3 S nucleic acid from the satellite of TbRSV (S-TRSV) (Schneider, 1971) as markers in linear-log sucrose density gradients (Brakke and Van Pelt, 1970), we estimate that the sedimentation coefficients of the two nucleic acids from TmRSV are 30.9 S and 32.6 S, respectively (Fig. 4). When RNA was formylated (Boedtker,
COMPONENTS OF TOMATO RINGSPOT VIRUS

FIG. 4. Ultraviolet absorbance profiles of sucrose density gradients containing nucleic acids extracted from TmRSV and sedimentation markers. (A) Nucleic acids from TmRSV. (B) 7.3 S and 24 S nucleic acids from the satellite of tobacco ringspot virus and from tobacco ringspot virus, respectively. (C) Mixture of (A) and (B) samples. Centrifugation in linear-log gradients with Spinco SW 11 at 33,500 rpm for 16 hr.

1968), only a single peak with no shoulder indicating the second component remained. The peak sedimented with an estimated S value of 18.2 relative to formylated nucleic acids of TbRSV and its satellite (Kaper and Waterworth, 1973; Kaper, unpublished). Use of an empirical equation (relating sedimentation and molecular weight) that is based upon S values for formylated RNAs according to Boedtker (1968), the molecular weight of both RNAs is about \(2.3 \times 10^6\) daltons.

**DISCUSSION**

The presence of two, rather than one, nucleic acid-containing components in purified preparations of TmRSV does not appear to be an artifact.

We are unable to explain why component B1 consistently had a higher ultraviolet absorption maximum/minimum than component B2 because B1 presumably contains a slightly smaller nucleic acid than B2. The two components appeared equal in size. Electrophoresis of B1 and B2 particles provided no evidence for a difference in electrophoretic mobility between the two components, supporting the probability that the capsid surrounding both components is the same. The anomaly in the ultraviolet absorption maximum/minimum values for the two components may be explained by a difference in the degree of contamination of each component with nonviral impurities.

Although component B2 of partly separated components is relatively much more infectious than component B1, we cannot state that B1 is noninfectious and that B2 is the infectious component because of the incomplete separation of the two components. Recent evidence indicates that both raspberry ringspot and tobacco ringspot viruses are multipartite genome viruses (Murant et al., 1973; Harrison et al., 1972A, 1972B) and both components may be necessary for infection, even though their partly separated components appear to differ considerably in relative infectivity.

Enhancement of the infectivity of a partly separated component by the addition of the second component fits with the possibility of a multipartite genome in TmRSV. With viruses of this type, it has usually been found that the smaller RNA component is considerably less than \(2 \times 10^6\) daltons (Van Kamen, 1972). In contrast, the smaller RNA component of TmRSV appears to be larger than \(2 \times 10^6\) daltons and almost the same size as the larger RNA component. In this respect, the smaller N.A. of TmRSV is similar to that of the cherry leaf roll virus (Jones and Mayo, 1972; Walkey et al., 1973).

If TmRSV should also turn out to be a multipartite genome virus, it would then be more like other well-characterized members of the Néo virus group (Harrison et al., 1971) in this regard as well as in the possession of two different nucleoproteins.

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REFERENCES


