*REPLICATION OF VIRAL RNA, VI. NUCLEOTIDE COMPOSITION OF THE REPLICATIVE FORM OF TOBACCO MOSAIC VIRUS RNA AND OF ITS COMPONENT STRANDS**

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Multiplication of plant viruses containing single-stranded RNA[1] such as TYMV[2] and TMV[3, 4] is accompanied by the formation of a virus-specific double-stranded RNA designated as the replicative form. Similar findings have been made in the case of animal viruses[5, 6] and bacteriophages.[7–10] We have previously reported on the properties of the partially purified replicative form of TMV-RNA.[4] Because of the small amounts available, the base composition of this material could not be determined at that time. We have now purified the P^{32}-labeled replicative form by a modified procedure and obtained analyses both of the duplex and of its component strands.

Materials and Methods.—Materials were obtained as described previously.[4] In addition, RNAase T_1 was obtained from Sankyo Co., Tokyo. Bentonite was prepared according to Fraenkel-Conrat *et al.*,[11] and suspended in 0.1 *M* glycine buffer, pH 9.5. The general methods employed were the same as in the preceding papers.[4, 7, 12] All P^{32} radioactivities were corrected for decay and are directly comparable.

Replicative form of TMV uniformly labeled with P^{32}: Small Turkish tobacco plants were inoculated with TMV at 0.5 mg per ml. The next day the plants were cut off just above the soil level and placed in about 0.5 ml of radioactive sodium phosphate (1.5–2.1 mc per plant). After the radioactive solution was taken up, the plants were removed and cut in sections containing a piece of stem and one or two leaves. These pieces were floated in Petri dishes containing water, the Petri dishes being placed in a larger covered glass dish also containing water. Ten days after the addition of the isotope the leaves were removed, divided into 30-gm batches, placed in a plastic bag, and frozen in dry ice. Thirty gm of the plant tissue were homogenized in an Omnimixer at low speed and 0° for 20 min, with 60 ml of glycine buffer, pH 9.5, containing 0.1 *M* NaCl and 0.01 *M* EDTA, 120 ml of water-saturated phenol, and 4 ml of 6% bentonite. After centrifugation, the aqueous layer was decanted and re-extracted with 120 ml of phenol and 2 ml of 6% bentonite. This procedure was repeated until the aqueous phase was water-clear. After extracting three times with ether, the ether was removed by bubbling nitrogen through the solution, and the RNA was precipitated by adding 2 vol of 95% ethanol, keeping the mixture in an ice bath for a few hours. After centrifugation, the precipitate was redissolved in a few ml of the glycine buffer and reprecipitated with ethanol. After repeating this procedure once more, the precipitate was taken up in 0.02 *M* Tris-HCl buffer, pH 7.2.

A 15-ml aliquot of the P^{32}-labeled RNA (608 A_{260} units and 2×10^8 cpm) was incubated with 750 μg of DNAase and 2.2 μmoles of $MgCl_2$ for 10 min at 37°. Three ml of 10 × SSC and 1.6 mg of RNAase T_1 were then added. The mixture was diluted to 30 ml and kept at 25° for 15 min. Sixty ml of 1 × SSC and 4.5 mg of pancreatic RNAase A were then added and the incubation at 25° was continued for another 30 min. The digestion was stopped with 0.02 ml of 25% sodium dodecylsulfate and 2 vol of ethanol. After 90 min at −15°, the precipitate was collected by centrifugation, dissolved in 10 ml of 1 × SSC and extracted four times with 2 vol of 80% aqueous phenol (equilibrated with 1 × SSC). The phenol phases were washed with 10 ml of 1 × SSC and the combined aqueous layers extracted three times with 2 vol of ether. After blowing off the ether in a stream of nitrogen, the RNA was precipitated with 2 vol of ethanol and, following standing for 60 min at −15°, centrifuged down at 27,000 × *g* for 30 min. The supernatant was drawn off as completely as possible and the pellet dissolved in 1 ml of 1 × SSC. Most of the

radioactivity of this solution (4.5×10^6 cpm) was contributed by alcohol-precipitable oligonucleotides. A column (0.9-cm diameter) was filled to a height of 25 cm with Sephadex G-200, topped off with a 5-cm layer of Sephadex G-25 (coarse), and equilibrated with 1 × SSC. The P^{32}-labeled RNA preparation was chromatographed on this column at a flow-rate of 3–5 ml/hr. Fractions (0.5 ml) were collected; a small aliquot of each fraction was plated, and the radioactivity determined. A small front-running peak had a total radioactivity of 590,000 cpm; the rest of the material was eluted in a second, larger peak. The fractions containing the RNAase-resistant RNA (first peak) were pooled and dialyzed against 0.1 *M* NaCl, 0.01 *M* Tris-HCl, pH 7.3. An aliquot of this material (443,000 cpm) was diluted to 11 ml with addition of NaCl and potassium phosphate buffer, pH 6.8, to give a final concentration of 0.45 *M* and 0.02 *M*, respectively. This material was chromatographed on a methylated albumin-silicic acid column (0.9 × 7 cm) prepared according to Okamoto and Kawade.[13] Elution was carried out with a linear NaCl gradient from 0.5 to 2.0 *M* (total volume 90 ml, all solutions containing 0.02 *M* potassium phosphate buffer, pH 6.8). A radioactive peak was eluted between 13 and 23 ml of eluant (corresponding to about 0.75 *M* NaCl). Some material, trailing after the peak, was discarded. The peak fractions were pooled, concentrated to a small volume, and dialyzed exhaustively against 0.005 *M* Tris-HCl buffer, pH 7.2. The final preparation (3.5 ml) had a radioactivity of 290,000 cpm (0.2% over-all yield of radioactivity) and contained 0.82 A_{260} units of nucleic acid (0.18% over-all yield of absorbing material).

Denaturation of P^{32}-labeled replicative form and reannealing in the presence of excess unlabeled TMV-RNA: This was done in order to determine separately the nucleotide composition of each of the complementary strands of the P^{32}-labeled duplex. Either of two procedures was followed. (*a*) A sample of P^{32}-labeled replicative form (0.03 A_{260} unit) was mixed with 3.2 A_{260} units of TMV-RNA (freed of heavy metal ions by filtration through Chelex 100, Na^+ form), evaporated to dryness in a glass tube (9-mm diameter), and dissolved in 0.02 ml of 2.5 × SSC. The glass tube was sealed, heated to 120° for 3 min, and transferred to an oil bath at 80° for 1 hr. The contents of the tube were diluted to 1.0 ml to give a final concentration of 1 × SSC. After reannealing, the RNAase-resistant radioactivity, determined on a small aliquot, was 52%. The sample was digested for 15 min at 25° with 50 μg of RNAase T_1; 40 μg of pancreatic RNAase A were then added and the incubation continued for another 15 min. Fifty μg of bovine serum albumin and 0.05 ml of 60% trichloroacetic acid were added and the precipitate was collected on a Millipore HA filter. The filtrate was extracted three times with ether, adjusted to pH 7 with NH_3, and taken to dryness. The precipitate was eluted from the filter with 1.5 *N* NH_3 and the solution was evaporated to dryness. The precipitate fraction, corresponding to the RNAase-resistant, double-stranded RNA was expected to contain the P^{32} predominantly in the "minus" strand. The acid-soluble fraction resulting from the RNAase digestion was expected to contain the radioactive isotope in fragments derived mainly from "plus" strands (cf. ref. 12). The two fractions were hydrolyzed and the nucleotide composition was determined following electrophoresis as described below. In one experiment the total radioactivity of the four nucleotides recovered from the precipitate fraction after hydrolysis and electrophoresis was 786 cpm, that of the filtrate fraction 1230 cpm. (*b*) P^{32}-labeled replicative form (0.234 A_{260} unit) was mixed with 130 A_{260} units of unlabeled TMV-RNA. The mixture was filtered first through a short Chelex-100 (Na^+) column and then through a column of Sephadex G-50 fine (bead form) previously equilibrated with water. The desalted solution (12.5 ml) was heated for 10 min at 100°. A rapidly cooled aliquot was tested for RNAase resistance and was found to be completely sensitive; 1.45 ml of 10 × SSC were added to the solution, and the volume was reduced to 6 ml by lyophilization. After 2 hr at 65° annealing had not occurred. The volume was thereupon reduced to about 0.5 ml and the material was heated for 1 hr at 80°. An aliquot tested at this point was 45% resistant to pancreatic RNAase A. The solution was diluted to 7 ml with water, digested with RNAase T_1 and pancreatic RNAase A, and processed further as described above. In this method, care must be taken to avoid any traces of RNAase during the period when the material is denatured; this requires careful cleaning and flaming of glassware.

Determination of nucleotide composition: Aliquots were taken to dryness in a stream of nitrogen and 0.3–0.5 ml of 1.0 *N* NaOH was added. The samples were routinely kept for 16–18 hr at 30° (unless noted otherwise), neutralized with 6 *N* HCl, and concentrated to a small volume. Aliquots

(500–2,000 cpm) were mixed with 100 μg each of 2′ (3′-)-UMP, 2′(3′)-AMP, 2′(3′-)-CMP, and 2′(3′-)-GMP as carrier and streaked onto strips (4 × 100 cm) of Whatman 3 MM filter paper. Electrophoresis was carried out on a Savant high voltage electrophoresis unit in acetate-pyridine buffer, pH 3.5 (acetic acid:pyridine:H_2O, 10:1:89) for 3 hr at 4,000 V. The strips were scanned on a Nuclear-Chicago Actigraph II radiochromatogram scanning system. The portions of the paper corresponding to individual radioactive peaks were cut out, folded, and placed into scintillation vials. Care was taken to have equal areas of paper in each vial, although little quenching by the paper could be detected. Liquifluor diluted 1:25 with toluene was added and the samples were counted in a Packard Tricarb liquid scintillation spectrometer. About 97% of the radioactivity was recovered in the four peaks.

Results and Discussion.—The procedure used successfully for the purification of the replicative form of phage MS2,[14–16] involving digestion with pancreatic RNAase A of the total cell RNA isolated by the phenol extraction method and chromatography on Sephadex G-200, was first applied to TMV-infected tobacco leaves. However, the TMV replicative form obtained by this procedure (Sephadex step) was only about 55 per cent pure in terms of nucleotide material,[4] as determined by the annealing assay.[7] The base composition of the P^{32}-labeled replicative form of TMV, purified by this method (Sephadex *or* Cs_2SO_4 step), was C, 17.1 ± 0.15 per cent; A, 25.2 ± 0.4 per cent; G, 34.2 ± 0.2 per cent; U, 23.5 ± 0.2 per cent, and suggested an accumulation of guanine-rich, RNAase-resistant "core" (cf. Shipp and Haselkorn[3]). The purification procedure was therefore modified to include a digestion with RNAase T_1 which, as found previously, did not degrade double-stranded MS2 RNA, as well as chromatography on a methylated albumin-silicic acid column. The P^{32}-labeled replicative form of TMV-RNA obtained by this method had a nucleotide composition (Table 1) in good agreement with the values calculated for a double-helical RNA consisting of a viral type RNA ("plus") strand and its complement ("minus" strand). The value of the ratio $G + A/U + C$ was 0.99.

In order to obtain an analysis of the "minus" strand alone, a sample of P^{32}-labeled replicative form was heat-denatured and reannealed in the presence of a large excess of unlabeled TMV-RNA. By this procedure, the P^{32}-labeled "plus" strand is displaced from the duplex[12] and can then be degraded by RNAase. The RNAase-resistant fraction, isolated by acid preci-

TABLE 1

NUCLEOTIDE COMPOSITION OF REPLICATIVE FORM OF TMV-RNA AND ITS COMPONENT STRANDS*

Nucleotide	Total RNA of tobacco leaf (Reddi[17])	TMV-RNA (Knight[18])	P^{32}-TMV-RNA (This work)	P^{32}-Labeled Replicative Form of TMV† Found	Calc.	P^{32}-Labeled "Minus" Strand in Replicative Form‡ Found	Calc.	P^{32}-Labeled "Plus" Strand from Replicative Form§
C	22.0	18.3	19.2	22.3 ± 0.2	21.7	24.8 ± 0.2	24.3	20.2 ± 0.5
A	23.9	28.8	28.2	27.8 ± 0.15	28.3	26.7 ± 0.1	27.7	29.9 ± 0.3
G	31.8	25.1	24.9	21.8 ± 0.1	21.7	20.2 ± 0.3	19.1	22.7 ± 0.1
U	22.3	27.8	27.7	28.0 ± 0.3	28.3	28.3 ± 0.4	28.9	27.2 ± 0.9

* The values given are mean values ± standard error of the mean ($S\bar{x} = \sigma/\sqrt{N-1}$). All analyses were carried out as described in the *Methods* section.
† Purified as described under *Methods*. Mean of six analyses (two samples, hydrolyzed for 24 hr, showed similar mean values and were included in this group).
‡ P^{32}-labeled replicative form after *displacement* of the P^{32}-labeled "plus" strand by unlabeled TMV-RNA. Mean of three analyses of material prepared by method (*b*) (see *Methods*) and one analysis each of two samples prepared by method (*a*).
§ P^{32}-labeled RNA displaced from the P^{32} replicative form by nonradioactive TMV-RNA. Mean of one analysis each of two samples.

pitation, should contain the labeled "minus" strands, whereas the acid-soluble material should contain fragments of the degraded, P^{32}-labeled "plus" strands. Separate analyses of the two fractions indeed indicate that the base composition of the RNAase-resistant fraction is close to that expected of a "minus" strand, whereas the RNAase-sensitive radioactive material has a composition similar to that of TMV-RNA itself.

These experiments thus confirm the conclusion that the TMV-specific, RNAase-resistant RNA found in TMV-infected tobacco leaves consists of a parental type viral strand and its complement. The same conclusion as regards the replicative form of TYMV and MS2 phage is supported by their respective nucleotide compositions.[2, 14] In the case of MS2, the double-helical structure of the replicative form has been demonstrated by X-ray diffraction studies.[14]

Summary.—The nucleotide composition of the replicative form of TMV-RNA and its two component strands was determined and found to be in agreement with the values expected for a double helix consisting of a parental type viral RNA strand and its complement.

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[1] Abbreviations: RNA, ribonucleic acid; TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus; "plus" strand, viral RNA strand of the parental type; "minus" strand, RNA strand with base sequence complementary to that of the "plus" strand; 1 × SSC, 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7; RNAase, ribonuclease; DNAase, pancreatic deoxyribonuclease; cpm, counts per minute; Tris, Tris (hydroxymethyl) aminomethane; A, G, U, C—adenylic, guanylic, uridylic, and cytidylic acid, respectively.

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