
Physical and functional heterogeneity in TYMV RNA : evidence for the existence of an independent messenger coding for coat protein.

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Received 9 August 1976

ABSTRACT

Turnip yellow mosaic virus RNA can be separated into two distinct components of 2×10^6 and 300 000 daltons molecular weight after moderate heat treatment in the presence of SDS or EDTA. The two species cannot have arisen by accidental *in vitro* degradation of a larger RNA, as they both possess capped 5' ends. Analysis of the newly synthesized proteins resulting from translation of each RNA by a wheat germ extract shows that the 300 000 molecular weight RNA can be translated very efficiently into coat protein. When translated *in vitro* the longer RNA gave a series of high molecular weight polypeptides but only very small amounts of a polypeptide having about the same mass as the coat protein. Thus our results suggest that the small RNA is the functional messenger for coat protein synthesis in infected cells.

INTRODUCTION

Within recent years an ever increasing number of plant viruses have been shown to have divided genomes. Members of the tobnavirus family have bipartite genomes with the information for the coat protein carried by the shorter genome pieces. Alfalfa mosaic virus and members of the bromovirus family have a tripartite genome; as in the tobnaviruses the coat protein cistron is on the smallest of the genome pieces although the actual messenger for coat protein synthesis appears to be a still smaller RNA derived from the small RNA of the genome in the course of infection¹. In the case of tobacco mosaic virus (TMV) it has been shown that the functional message for coat protein synthesis is a small RNA (the LMC) which is produced *in vivo* from the full-length RNA of the virion by either specific cleavage or partial transcription². Although the full-length RNA carries the coat protein cistron, it is not expressed, at least not *in vitro* in protein synthesizing systems. The biological significance of the divided genome of the multicomponent viruses or the processed genome of TMV is not yet clear but it may be an adaptation to the protein synthetic machinery of the eucaryotic cell which seems to

translate monocistronic mRNAs more readily than polycistronic ones³.

Up until now the existing evidence has indicated that the turnip yellow mosaic virus (TYMV) does not have a multicomponent genome. Centrifugation of TYMV in gradients of caesium chloride resolves a number of minor nucleoprotein bands in addition to the principal nucleoprotein component and empty protein shells, but biological tests show that infectivity is associated only with the major nucleoprotein species and is not enhanced by addition of the minor components^{4,5}. RNA extracted from the virus is often heterogeneous and the heterogeneity increases markedly upon heating or treatment with EDTA^{6,4}, but generally this has been attributed to accidental breakage of the infectious RNA molecule rather than taken as evidence for a specific cleavage.

In this paper we shall show that moderate heat treatment in the presence of SDS or EDTA causes TYMV RNA to separate into distinct heavy and light components with molecular weights about 2×10^6 and 300 000 daltons respectively. The lightest component carries the coat protein cistron and is easily translated in a cell-free system. Translation of the heavy RNA can give rise to polypeptides of mass superior to 165 000 but the coat protein cistron, if it is present, is translated only poorly or not at all. Thus, our findings suggest that TYMV should be added to the growing list of viruses with multicomponent or processed genomes, in which the genome is divided or must undergo division in the infected cell in order to express all of its functions.

MATERIALS AND METHODS

1 - Virus purification

Turnip yellow mosaic virus was propagated on chinese cabbages var. Pé-Tsai and purified by established procedures⁷. ³²P TYMV was prepared by the following method : six days after infection, about 15 cabbages were removed from their pots and, after having been carefully washed with water, the roots were dipped into distilled water containing ³²P phosphate (100 mCi, 1mCi/ml). After absorption of the ³²P phosphate each plant was made to imbibe 5 ml 10^{-5} M nonradioactive phosphate and then distilled water. Seven days later the virus was purified from the leaves by means of the same procedure used for nonradioactive virus.

2 - RNA and protein purification

RNA was extracted with water-saturated phenol in the presence of bentonite (0.5 mg per mg of virus) to inhibit traces of ribonucleases. The RNA in the aqueous phase was precipitated with ethanol after residual phenol had been eliminated by ether. RNA solutions were prepared in sterile distilled water and stored frozen. ^{32}P RNA had a specific radioactivity of 0.03 Ci/g.

TYMV protein was prepared by heating purified TYMV to 37°C for 5 min in the presence of 8 M urea. The mixture was then dialyzed at 4°C against 0.01 M borate buffer, pH 9, and ultracentrifuged (2 hr at 105,000 g) to remove undegraded virus. The protein present in the supernatant was precipitated twice in 20% ammonium sulphate, resuspended in 0.01 M borate buffer, pH 9, dialyzed extensively against water, and lyophilized (G. Jonard, unpublished observations).

3 - Wheat germ extracts

Commercial wheat germ (General Mills Inc., Vallejo, California, or Kenosha variety) was extracted by the procedure of Marcu and Dudock⁸. Incubation mixtures were prepared as described by Mayo *et al.*⁹ with some modifications described below. The optimum concentration of Mg^{++} was about 2.75 mM, and that of spermidine was 0.60-0.70 mM; other components were 20 mM HEPES (N-2-hydroxyethyl piperazine N'-2-ethane sulfonate), 5 mM Tris, 100 mM potassium acetate, 2.5 mM ATP, 0.375 mM GTP, 5 mM phosphoenolpyruvate, 1.8 mM dithiothreitol, and 0.025 mM in each amino acid. Maximum incorporation was obtained with RNA concentrations of 80-100 µg/ml after about 2.5 hr incubation.

4. Analysis of incorporation

Incubation mixtures contained either ^3H leucine (54 Ci/mmol, 2.5 nmol/ml) or ^{35}S methionine (320 Ci/mmol, 2.5 nmol/ml) or a mixture of the following: ^{14}C arginine (318 mCi/mmol, 6 nmol/ml), ^{14}C leucine (236 mCi/mmol, 25 nmol/ml), ^{14}C lysine (348 mCi/mmol, 10 nmol/ml), ^{14}C phenylalanine (522 mCi/mmol, 7 nmol/ml), ^{14}C proline (290 mCi/mmol, 5 nmol/ml), ^{14}C serine (174 mCi/mmol, 27 nmol/ml), and ^{14}C threonine (232 mCi/mmol, 9.7 nmol/ml). The extent of incorporation of amino acids into protein was determined by measuring the radioactivity retained on Whatmann 3MM paper discs after washing with hot and cold trichloroacetic acid, ethanol, and ether.

5 - Analysis in polyacrylamide-SDS gel

Incubation samples were prepared for slab gel electrophoresis as described by Mayo *et al.*⁹. Electrophoresis and subsequent autoradiography were performed following previously described procedures^{10,11,12}.

Autoradiography was on Kodirex film (Kodak) at room temperature. Protein used as size markers were: *E. coli* polymerase (molecular weights 165 000, 155 000, 95 000 and 39 000), bovine serum albumin (66 000), pyruvate kinase (57 000), ovalbumin (43 000), carbonic anhydrase (29 000), chymotrypsinogen (25 000), TYMV protein (20 000), and TMV protein (17 500).

6 - Analysis of tryptic peptides

Bands of newly synthesized protein were localized by autoradiography and excised. Protein was eluted from the piece of gel by incubation in 66% (v/v) formic acid for three days at 37°C. TYMV coat protein (400 to 500 µg) was added after the first day. After low-speed centrifugation, the supernatant containing the protein solution was dialyzed extensively against water, then against 0.1 M ammonium bicarbonate, pH 8, and lyophilized. The residue was resuspended in 1 ml of either 0.1 M ammonium bicarbonate or distilled water adjusted to pH 8 with 1% NH₄OH. Trypsin-TPCK (Merck) 2% (v/v) was added and the solution was incubated for 18 hr at 37°C. The resulting peptide solution was lyophilized, resuspended in 5 to 10 µl of electrophoresis buffer (pyridine, acetic acid, water 10/0.4/90, v/v/v, pH 6.5) or in the same volume of 15% acetic acid and applied to a cellulose thin layer (Macherey Nagel Polygram Cel 400). Electrophoresis was at 8 mA (about 400 V) for 60 min. Subsequent chromatography was in pyridine, acetic acid, butanol, water (10/3/25/12, v/v/v/v). The peptides of the carrier TYMV coat protein were visualized by staining with ninhydrin. Radioactive peptides were located by autoradiography on Kodirex film at room temperature.

7 - Serology

Coat protein antiserum was prepared by giving a rabbit three injections of purified TYMV protein (10 mg/ml) at three-week intervals. For the first injection, incomplete Freund's adjuvant was used. Serum was collected about two months after the final injection. In gel plate tests¹³ the titer against protein (0.1 mg/ml) was estimated to be 1/8.

8 - Isolation and characterization of the 5' terminal group of TYMV RNAs

³²P-labeled light and heavy TYMV RNAs in 0.05 M sodium acetate, 10 mM

EDTA, pH 4.7, were digested to completion with a mixture of T_1 RNase (100 units enzyme/ml), T_2 RNase (100 units enzyme/ml), and pancreatic RNase (100 μ g/ml). Digestion was for 15 hr at 37°C with about 5 μ l of the enzyme mixture per 50 μ g RNA. The products were fractionated by electrophoresis on DEAE paper at pH 3.5 (5% acetic acid plus 0.5% pyridine) until the xylene cyanol blue marker had migrated about 10-12 cm. The slowly migrating 5' terminal structure was eluted from the paper with 2 M triethylamine bicarbonate (pH 8) and further characterized by methods that have been described elsewhere^{14,15}. 32 P-labeled m⁷G^{5'}ppp^{5'}Gp prepared from TMV RNA as described by Zimmern¹⁴ was used as a reference material.

9 - Isolation of the 3'terminal U_2 RNase oligonucleotide of TYMV RNAs
 32 P-labeled light and heavy TYMV RNAs in 0.05 M sodium acetate, 10 mM EDTA, pH 4.7, were treated at 37°C overnight with U_2 RNase (0.5 units enzyme/mg RNA). The positively charged 3' terminal oligonucleotide was separated from the other products of digestion by electrophoresis on Whatman 3 MM paper at pH 2.6¹⁶.

RESULTS

1 - In vitro translation of unfractionated TYMV RNA

When unfractionated TYMV RNA is incubated in a cell-free protein synthesizing system derived from wheat germ, there is a 20- to 40- fold stimulation of radioactive amino acid incorporation into trichloroacetic acid insoluble material. Electrophoresis of the translation products in a 10% polyacrylamide gel reveals three main bands of protein having molecular weights of 37 000, 35 000, and 20 000 daltons (Fig. 1a). The fastest of the above three bands comigrates with coat protein and has been identified as the viral coat protein by tryptic peptide mapping. Coat protein has also been identified among the translation products of TYMV by Benicourt and Haenni¹⁷. The heaviest translation product which could be detected has a molecular weight of 100 000, but is present only in minor amounts.

One possible explanation for the relative scarcity of long translation products in the above experiment is that the secondary structure of the RNA hinders movement of ribosomes along the polynucleotide chain. In the case of TYMV RNA, such secondary structure may be especially stable since the RNA extracted from the virion is known to be complexed with spermidine¹⁸. Accordingly, we attempted to alter the secondary structure as

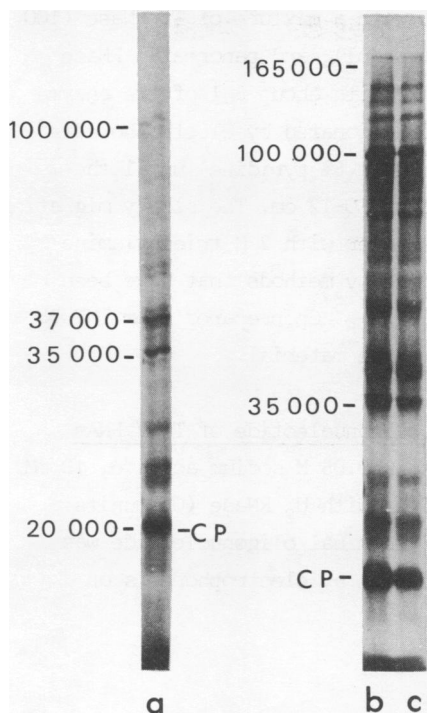


Figure 1. Analysis of TYMV RNA translation products on 10% polyacrylamide gels.

- a - Total TYMV RNA
- b - TYMV RNA heated in the presence of EDTA
- c - TYMV RNA dialyzed against 1 M NaCl (see text)

CP = coat protein

much as possible by heat treatment or by treatment to remove polyamines. Heat treatment was for 10 min at 60°C in 0.01 M Tris, 1 mM EDTA, pH 7.4. The solution was rapidly cooled in ice and the RNA precipitated with ethanol. RNA-spermidine complexes are known to be unstable in high salt concentrations¹⁹. Consequently we dialyzed TYMV RNA overnight at 4°C against 1 M NaCl, 0.01 M Mg acetate followed by dialysis against 1 mM EDTA and finally against water. The RNA was then precipitated with ethanol.

Fig. 1 b,c shows a polyacrylamide gel of the translation products of TYMV RNA subjected to the above two treatments. Both treatments result in the appearance of longer polypeptides with molecular weights of at least 165 000, of 150 000, and of 120 000 daltons, and augmented synthesis of coat protein. Thus under certain circumstances TYMV RNA can be made to express all or almost all of its genetic information. Indeed the sum of the molecular weights of the principal translation products greatly exceeds the theoretical coding capacity of the RNA, suggesting that the sequence of the 150 000 and 120 000 dalton polypeptides largely overlaps that of the 165 000+ chain.

2 - Fractionation of TYMV RNA

The above findings show that the properties of TYMV RNA as a messenger can be dramatically altered by heating or by dialysis against high concentrations of salt. This led us to examine the effect of one such treatment (heating) upon the integrity of the RNA preparation. 10 to 20 μg of TYMV RNA in 50 μl of water was heated to 60°C for 10 min before being loaded on a composite polyacrylamide-agarose (2.4% and 0.5%) gel²⁰. In this case and for most of the other experiments described below, 1% SDS was also included in the incubation mixture, but identical results were obtained when the SDS was omitted or replaced with 1 mM EDTA.

After staining the gel with o-toluidine blue, two major bands were visible (Fig. 2). The faster band comigrates with BMV RNA 4 and must therefore have a molecular weight of about 300 000 daltons. The slower band migrates to about the same depth in the gel as TMV RNA and hence must have

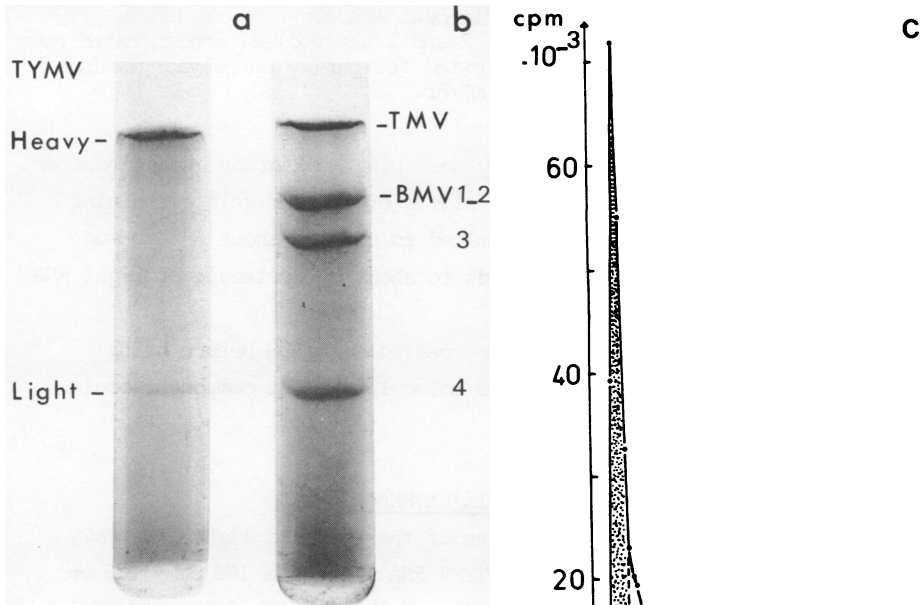


Figure 2.
 a - ³²P-labeled TYMV RNA on polyacrylamide-agarose (2.4%-0.5%)gels, stained with o-toluidine blue.
 b - Position of marker RNAs run in a parallel gel.
 c - Profile of radioactivity in 1.5 mm slices of gel a.

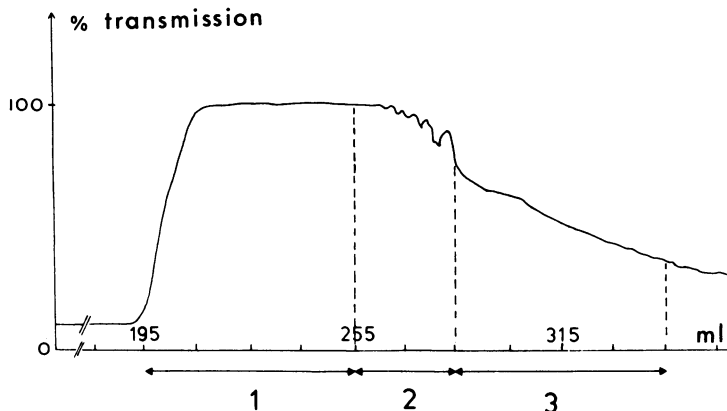


Figure 3. Purification of light and heavy RNAs of TYMV by gel filtration in a column (2.5 x 100 cm) of Ultrogel Aca22. The material in fractions 1, 2 and 3 was pooled, concentrated by alcohol precipitation, and tested for purity on polyacrylamide-agarose gels. Outflow was 9 ml/hr.

a molecular weight of about 2×10^6 . By measuring peak areas in densitometer tracing of stained gels or by counting slices of similar gels containing ^{32}P -RNA, the faster band has been estimated to contain about 6% as much RNA as the slower band. This corresponds to about one molecule of light RNA for every two molecules of heavy RNA.

When the TYMV RNA was not heated or treated with SDS before being loaded on the gel, little or no 300 000 molecular weight component could be detected.

3 - Preparation of purified heavy and light RNAs

In order to prepare large quantities of the purified light and heavy RNAs we passed heat- and SDS- treated TYMV RNA through a 100 cm x 2.5 cm column of Ultrogel Aca22(LKB). The profile of the outflow from a typical column is shown in Fig. 3. The indicated three cuts of RNA were concentrated by ethanol precipitation and analyzed on polyacrylamide-agarose gels as before. Fraction 1 contained heavy RNA (Fig. 4b), while fraction 3 was greatly enriched in the 300 000 molecular weight component. Fraction 2 contained a mixture of heavy and light RNAs, plus intermediate RNAs (molecular weights ranging from 400 000 to 700 000) which have not yet been characterized as they are in very small quantities.

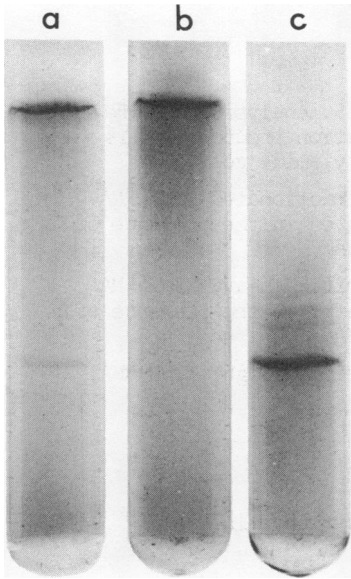


Figure 4. Analysis of column-purified TYMV RNAs on polyacrylamide-agarose (2.4% and 0.5%) gels.

- a - Total TYMV RNA (nonfractionated)
- b - Purified heavy TYMV RNA (fraction 1)
- c - Purified light TYMV RNA (fraction 3 after further purification).

It was sometimes necessary to pass fraction 3 through a second Ultrogel column (0.8 cm x 60 cm) to eliminate traces of heavy RNA, but no light RNA contamination of fraction 1 could be detected even if 80 µg of RNA was loaded on the gel. Hence, fraction 1 was generally used without further purification. Although fraction 3 could be easily freed of heavy RNA by passage through the second column, it always contained traces of the lighter of the intermediate RNA species (Fig. 4).

4 - Translation of purified light and heavy RNAs in wheat germ extracts

The proteins synthesized by a wheat germ extract primed with heavy and light RNAs as messengers are shown in Fig. 5. The pattern of polypeptides coded by the purified heavy RNA (not shown in the figure) resembles that obtained with unfractionated TYMV RNA with one important exception, the band of coat protein has greatly diminished. Treatment of purified heavy RNA with EDTA augments production of the same heavier polypeptides which appear upon EDTA treatment of unfractionated TYMV RNA (Fig. 5a and b) but synthesis of the 20 000 dalton protein is not stimulated.

Translation of purified light RNA produces one major protein band which comigrates with the coat protein (Fig. 5c). Identical results were obtained with gel-purified light RNA after extraction of the RNA from the gel²¹. Hence the possibility can be eliminated that one of the minor components

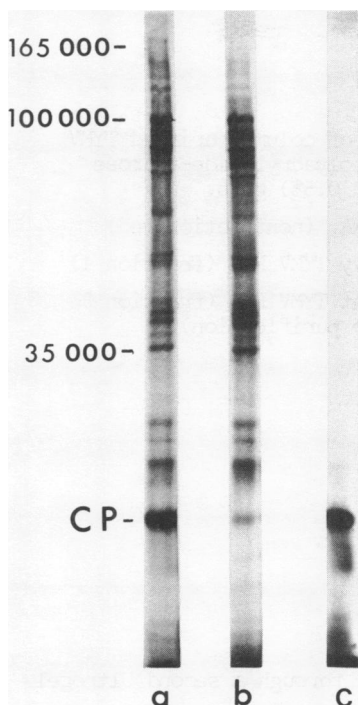


Figure 5. Analysis of TYMV RNA translation products on 11% polyacrylamide gel.

- a - Unfractionated TYMV RNA heated in the presence of EDTA
- b - Purified heavy TYMV RNA heated in the presence of EDTA
- c - Purified light TYMV RNA.

CP = coat protein

present in the column-purified light RNA rather than the light RNA itself codes for the 20 000 dalton protein.

5 - Nature of the translation products of light RNA

Serological reactivity. The 20 000 dalton polypeptide resulting from translation of the light RNA was tested to see if it was serologically identical to coat protein. After in vitro protein synthesis in the presence of ^3H leucine, the incubation medium was sedimented for 1 hr at 105 000 g (in order to eliminate polysomes) and the supernatant was dialyzed against phosphate buffer (PBS), pH 7 (0.85% NaCl, 0.01 M phosphate, 0.02% NaN_3). Samples for serological tests consisted of 50 μl of the dialyzed supernatant (containing the translation products) plus 60 μl of TYMV protein antiserum plus 10 μl of TYMV protein (10 mg/ml). Samples were incubated for 30 min at 30°C and for 15 hr at 4°C. The resulting precipitates were sedimented at low speed and the pellets were washed with 200 μl of PBS containing 2% Triton X-100, sedimented again, and resuspended in 50 μl 6M urea, 0.05 M Tris, pH 6.8, 2.5% SDS, 2.5% β -mercaptoethanol.

Table 1. Amounts of precipitation from three RNA translation products reacting with TYMV coat protein antiserum.

	300 000 Mol. Wt		
	Total TYMV RNA	Component of TYMV RNA	TMV RNA
Initial counts/min	12 140	18 450	26 510
Precipitated counts/min	1 016	6 702	356
Percentage counts/min precipitated	8.4	36	1.3

Table 1 shows that 36% of the protein synthesized with light RNA as a messenger is precipitated. A similar efficiency of precipitation has been observed for the translation products of alfalfa mosaic virus (AMV) 12S RNA reacted with AMV coat protein antiserum²² and for the reaction between tobacco rattle virus (TRV) coat protein antiserum and the translation products of short TRV RNA⁹.

When the newly synthesized proteins which are coded for by unfractionated TYMV RNA were mixed with the coat protein antiserum, only about 8.4% of the counts precipitated. This result is in line with expectations, however, since we estimate that no more than 20% of the synthesized protein has the molecular weight of 20 000 daltons excepted for coat protein. Nonspecific precipitation (determined by testing the TYMV coat protein antiserum against the translation products of TMV RNA) was only 1.3%.

Tryptic fingerprint. The above observations show that translation of 300 000 dalton TYMV RNA gives material serologically similar to coat protein. In order to find out if the translation product is chemically identical to coat protein, we characterized its tryptic fingerprint. Using column-purified 300 000 dalton RNA, the putative coat protein was synthesized in the standard incubation medium in which seven of the amino acids were ¹⁴C-labelled. The newly synthesized 20 000 dalton protein was purified by gel electrophoresis (see Material and Methods).

Fig. 6 shows an autoradiogram of a tryptic fingerprint of such protein. The spots detectable by ninhydrin staining come from coat protein which was added as a carrier. From the amino acids sequence of the coat protein a maximum of 10 stained tryptic peptides are predicted whereas we regularly obser-

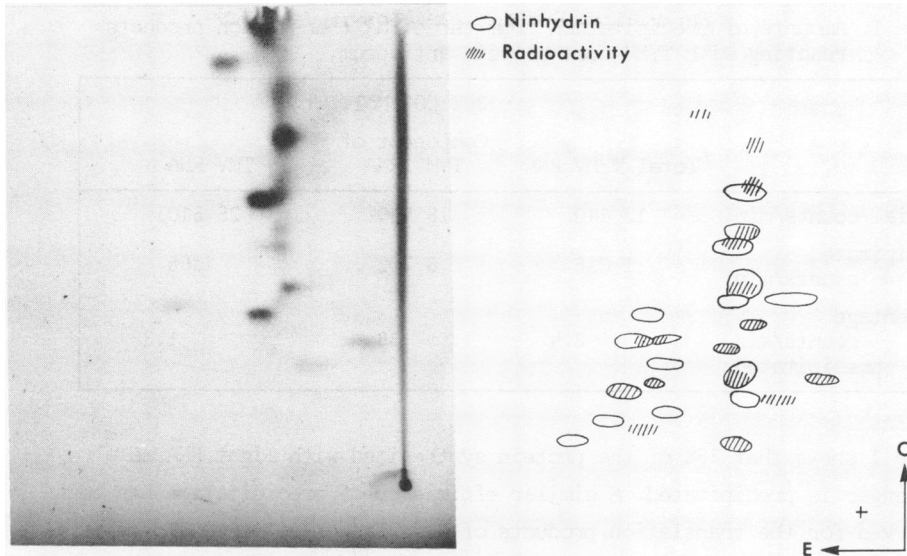


Figure 6. Fingerprint analysis of radioactive 20 000 dalton mol. wt. protein synthesized in wheat germ extract under the direction of 300 000 dalton mol. wt. TYMV RNA.

- a - Autoradiogram of the tryptic digest of the 20 000 dalton mol. wt. protein
- b - Composite drawing of the ninhydrin spots corresponding to purified TYMV coat protein peptides. The shadowed spots correspond to the labelled spots shown in a.

E = electrophoresis ; C = chromatography.

ved about 20 ninhydrin spots on the plate. The supplementary spots may be due to a contaminating chymotryptic activity.

Eleven of the 15 radioactive spots on the plate, including most of the darker spots, correspond to ninhydrin-stained peptides, leaving little doubt that coat protein has been synthesized. The sequence of coat protein²³ is such that 70% of the ¹⁴C-amino acids will be concentrated in four large tryptic peptides, two with 21-22 radioactive amino acids per peptide and two with 11-12. These four peptides probably account for the four darkest coat protein spots of the autoradiogram. The ninhydrin-stained but apparently unlabelled products may well be authentic tryptic peptides (or chymotryptic breakdown products of such peptides) which do not contain enough ¹⁴C-amino acids to be detectable.

More puzzling are those spots on the autoradiogram which do not correspond to ninhydrin spots. These peptides of unknown origin may signal that,

in addition to coat protein, significant amounts of other protein(s) of about the same size have been synthesized. These proteins could well arise from translation of minor RNA species contaminating the column-purified light RNA.

6 - Characterization of the 5' extremity of light and heavy TYMV RNAs

The most straightforward interpretation of the above findings is that the light RNA is the functional messenger for coat protein in the infected cell. A possible objection to such a conclusion is that the heterogeneity of TYMV RNA may be an artifact, that is, that the light and heavy species may have arisen from specific degradation of a longer RNA during virus purification or subsequent manipulations. In order to rule out this possibility, we have undertaken a study of the 5' extremities of both heavy and light species. A number of plant viral RNAs have been shown to be "capped" at their 5' ends with a group of the type $m^7G^{5'}ppp^{5'}Xp$ 14, 24-26. Evidently if light and heavy TYMV RNA have extremities of this type they cannot be products of in vitro degradation.

^{32}P -labelled TYMV RNA was separated into light and heavy components by heat-SDS treatment and gel filtration as before. The purified heavy and light RNAs were treated with a mixture of T_1 , T_2 , and pancreatic ribonucleases which cleaves the phosphodiester linkage provided that the 2' position of the ribose is not methylated. ^{32}P -labelled TMV RNA which is known to have the group $m^7G^{5'}ppp^{5'}Gp$ at its 5' terminus, was similarly hydrolyzed to provide markers. After hydrolysis, the mononucleotides were separated from products containing two or more nuclease-resistant phosphates by electrophoresis on DEAE paper at pH 3.5. Both the light and the heavy TYMV RNA give rise to products which migrate with about one fourth the mobility of the blue marker on the DEAE paper (fig. 7). The mobility of the nuclease-resistant product coming from the heavy RNA corresponded to that of $m^7G^{5'}ppp^{5'}Gp$ from TYMV RNA. The hydrolysate of the light RNA contained two nuclease resistant products: spot a, which migrated like $m^7G^{5'}ppp^{5'}Gp$, and spot b, which migrated slightly faster. The relative proportion of radioactivity found in products a and b was shown to be 1:1 and 2:1 in two separate experiments. That both a and b derive from the 300,000 molecular weight species and not from minor components of the column-purified light RNA was confirmed by performing the same analysis upon light RNA further purified by gel electrophoresis. Assuming that each nuclease-resistant oligonucleotide contains four phosphates (an assump-

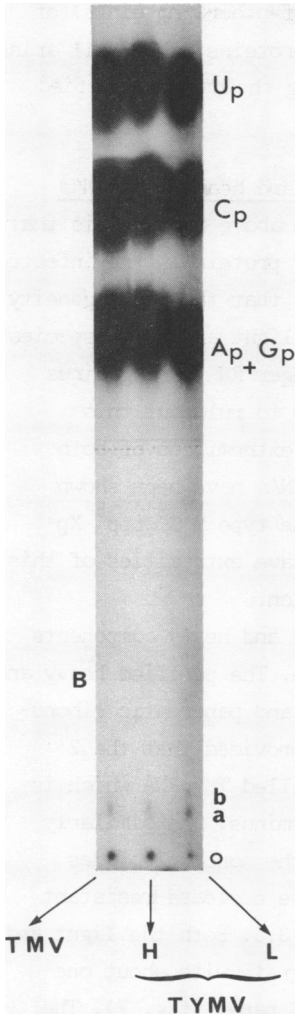


Figure 7. Electrophoresis of a total T₁ RNase + T₂ RNase + pancreatic RNase hydrolysate of ³²P-labeled TMV RNA (lane 1), purified TYMV heavy RNA (lane 2), and purified TYMV light RNA (lane 3) on DEAE paper at pH 3.5. B marks the position of the blue marker, and O indicates the origin of migration.

tion that will be proven below), the fraction of RNA chains possessing such a group may be readily determined from the ratio of radioactivity in the nuclease-resistant product to that in the mononucleotides. If we take the chain length of heavy RNA to be 6600 nucleotides, we can calculate that at least 90% of the heavy RNA molecules must contain the modified group. Taking a chain length of 1000 for the 300 000 molecular weight RNA and summing the radioactivity in spots a and b, we arrive at a similar molarity (90%) for the RNase-resistant group in the light RNA.

Phosphatase treatment of the nuclease-resistant products from both

light and heavy RNAs caused a four-fold increase in mobility, which is expected for the loss of a single phosphate¹⁵. The dephosphorylated nuclease-resistant species from heavy RNA and the dephosphorylated product a of the light RNA comigrated with $m^7G^{5'}ppp^{5'}G_{OH}$ from TMV RNA on the DEAE paper at pH 3.5. The dephosphorylated product b migrated about 30% faster.

The behavior of the nuclease resistant products before and after phosphatase treatment is consistent with a cap structure of the type $m^7G^{5'}ppp^{5'}Xp$. In order to confirm this identification, we have digested all three dephosphorylated RNase-resistant oligonucleotides with venom phosphodiesterase and separated the digestion products by one-dimensional chromatography on thin layers of cellulose (G1440 Schleicher and Schüll) in isobutyric acid : 0.5 M NH_3 (5/3 v/v). The RNase-resistant structure from heavy RNA and product a from light RNA gave three well-defined spots after venom phosphodiesterase which comigrated with the three products released from m^7GpppG_{OH} from TMV RNA by identical treatment (Fig. 8).

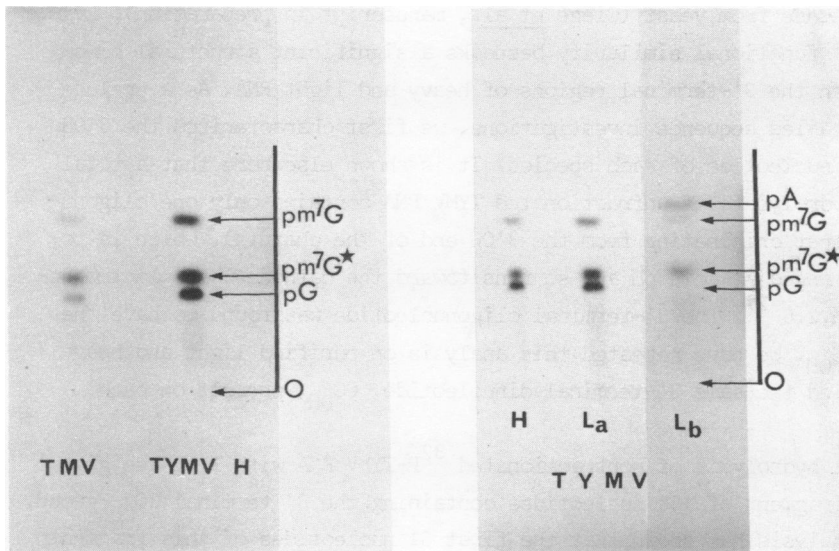


Figure 8. Total venom phosphodiesterase digestion of dephosphorylated RNase-resistant oligonucleotides derived from ³²P-labelled TMV RNA (lane 1), purified heavy TYMV RNA (lanes 2 and 3) spot a (lane 4) and spot b (lane 5) from purified light RNA. Chromatography was on thin layers of cellulose in isobutyric acid : 0.5 M NH_3 (5/3 v/v). The origin of migration is indicated by O.

From their mobilities and by comparison with the markers, we have identified these spots as pm^7G , pm^7G^* , (2-amino-4-hydroxy-5-N-methyl formamido 6-ribosyl-amino pyridine¹⁵, an alkaline degradation product of pm^7G which is produced, presumably, in the course of elution of the RNase-resistant oligonucleotides from DEAE paper with triethylamine bicarbonate), and pG. The fourth expected product, inorganic phosphate, migrates as an indistinct spot in the region of pm^7G^* . Chromatography of the products of venom phosphodiesterase digestion of the nondephosphorylated nucleotides in two dimensions gave spots migrating like pGp, or pAp, pm^7G and inorganic phosphate (Data not shown). The RNase-resistant moiety from the heavy RNA and the light RNA spot must therefore have the structure $\text{m}^7\text{G}^5' \text{ppp}^5' \text{Gp}$ while the sequence of the product b from light RNA must be $\text{m}^7\text{G}^5' \text{ppp}^5' \text{Ap}$.

7 - Preliminary studies on 3'OH terminus of light and heavy RNA

We shall report elsewhere that both heavy and light RNA species can accept an adenosine at the 3' terminus and, after adenylation, both can serve as substrates for aminoacylation with valine by purified valyl-tRNA synthetase from yeast (Giégé *et al.*, manuscript in preparation). Evidently this functional similarity bespeaks a significant structural homology between the 3'-terminal regions of heavy and light RNA. As a prelude to more detailed sequence investigations, we first characterized the 3'OH terminal dinucleotide of each species. It is shown elsewhere that a total U₂ RNase hydrolysate of unfractionated TYMV RNA contains only one oligonucleotide, that originating from the 3'OH end of the chain(s), which is positively charged at acid pH and so runs toward the cathode upon electrophoresis at pH 2.6²⁷. The 3'-terminal oligonucleotide was found to have the sequence CC_{OH} . We have repeated this analysis on purified light and heavy RNAs and find the same 3'-terminal dinucleotide, CC_{OH} , present on each chain.

Partial hydrolysis of nonfractionated ³²P-TYMV RNA with T₁ RNase gives rise to a fragment of 158 nucleotides containing the 3' terminal CC_{OH} group. Sequence analysis has shown that the first 51 nucleotides of this fragment extend into the 3' terminal portion of the coat protein cistron²⁷. When ³²P-labelled column purified light TYMV RNA was subjected to a similar partial T₁ RNase hydrolysis and the products of digestion separated by gel electrophoresis, a large 3' terminal fragment could be detected. The T₁ RNase fingerprint of this fragment is identical to that of the 3' terminal fragment obtained from unfractionated TYMV RNA; in particular the oligonucleotides shown by Briand *et al.*²⁷ to arise from the 51 last nucleotides

of the coat protein are present. This observation provides yet another proof that the genetic information for the coat protein is on the light RNA.

DISCUSSION

We have demonstrated in this paper that the TYMV virion contains significant amounts of two distinct RNA species, one of which (light RNA, mol. wt. 300 000) is an efficient messenger for coat protein in in vitro protein synthesis while the other (heavy RNA, mol. wt. 2×10^6) is not. Both RNAs can be aminoacylated with comparable efficiency by valyl-tRNA synthetase (Giégé et al., to be published) and both have the same 3' terminal séquence, PuCC_{OH}. Thus the evidence suggests that the 3' terminal tRNA-like portions of heavy and light RNAs are closely similar if not identical in sequence.

The origin of light RNA is not known. The only firmly established point is that it does not arise from scission of a larger RNA molecule during virus purification or subsequent manipulations. The presence of caps on both the heavy and light RNA components shows that each exists as a separate and presumably functional entity in vivo: The heterogeneity at the 5' terminus of light RNA is an unexpected finding. It will be interesting to discover if this heterogeneity is limited to the 5' terminal position.

An important unanswered question is whether the light RNA is necessary for infection or if the heavy RNA contains all the genetic information (including the cistron for the coat protein) necessary for pathogenicity. Among the translation products of column-purified heavy RNA (and of heavy RNA extracted from polyacrylamide gels) we have always observed a faint band which comigrates electrophoretically with coat protein. This finding, however, does not by itself constitute proof that the coat protein cistron is present on the heavy RNA since (1) it is not known if the 20 000 dalton product is coat protein (there is too little of it synthesized for tryptic peptide or serological analysis) and (2) even if the polypeptide is indeed coat protein it is difficult to eliminate the possibility that its synthesis is primed by traces of light RNA remaining associated with the heavy RNA.

An indirect line of evidence suggesting that the coat protein cistron of TYMV is carried by the heavy RNA has come from studies with the RNA of eggplant mosaic virus (EMV), another member of the tymovirus group²⁸. The RNA of the infectious bottom component of EMV does not give rise to its coat protein when translated in the wheat germ system, and all efforts to release a light RNA similar to that observed with TYMV RNA have failed (C. Klein and C. Fritsch, unpublished observations). We suggest that, in

infected cells, the messenger for EMV coat protein is a "light" RNA derived in vivo from the virion RNA by partial replication or specific cleavage. Thus EMV would resemble TMV in that a small, transient (nonencapsidated) coat protein messenger is generated in the course of infection². It has recently been shown that the coat protein messenger of the cowpea strain of TMV is encapsidated²⁹. We expect that the relationship between EMV and TYMV will prove to be parallel to that between TMV and its cowpea strain: the heavy RNAs of both tymoviruses contain the coat protein information and in both cases a smaller functional messenger can be generated from the longer RNA in vivo processing. In the case of TYMV, however, the coat protein messenger (or some of it) becomes encapsidated while apparently for EMV it is not.

It has long been known that TYMV RNA can serve as a substrate for valyl-tRNA synthetase³⁰. Briand et al.²⁷ have recently sequenced a 3'OH terminal fragment of 158 nucleotides isolated from a partial T₁ RNase digest of unfractionated TYMV RNA. The 5' terminal 51 nucleotides of this fragment extend into the last part of the coat protein cistron and the stretch of 107 untranslated nucleotides which follows the cistron can be folded into a cloverleaf secondary structure having some points in common with known species of tRNA^{Val}. The principal finding reported in this paper, that TYMV RNA is composed of not one but two major components, raises the question of which component has the reported sequence at its 3' extremity. The fact that the sequence overlaps the coat protein cistron suggests strongly that it represents the 3' end of the light RNA, a conclusion confirmed by direct nucleotide sequence analysis. If, however, the coat protein cistron proves to be redundant, an identical sequence should be found at the 3' end of the heavy RNA.

Evidently, if the coat protein information is present on the heavy RNA one would expect purified heavy RNA to be infectious. Because of the lack of a high-sensitivity local lesion assay for TYMV, however, interpretation of infectivity tests will inevitably be difficult or impossible. Hence, we will probably have to await studies of sequence homology between the heavy and the light RNAs or hybridization experiments to determine the degree of redundancy. Such studies should allow us to establish whether TYMV is a multicomponent virus or whether the TYMV genome, like that of TMV, is a single RNA chain which is processed in the infected cell.

ACKNOWLEDGEMENTS

We thank Mrs Odile Hemmer for her skilful technical assistance.

This work was supported by grants from the "Délégation à la Recherche Scientifique et Technique" and the "Commissariat à l'Energie Atomique".

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