Journal of General Virology (1991), **72**, 205–208. *Printed in Great Britain*

VPg-mediated aggregation of potyviral RNA

Carl S. Luciano,[1] John F. Murphy,[1] Robert E. Rhoads[2] and John G. Shaw[1]*

Departments of [1]Plant Pathology and [2]Biochemistry, University of Kentucky, Lexington, Kentucky 40546, U.S.A.

RNA prepared from the potyvirus tobacco vein mottling virus contained aggregates of the 9·5 kb genomic RNA with electrophoretic mobilities corresponding to 20 and 41 kb species. Similar aggregates were present in preparations of the RNAs of two other potyviruses. Aggregation occurred during or after purification of the RNA by sucrose gradient centrifugation and alcohol precipitation and was dependent upon the presence of a protein apparently bound covalently to a region at or near the 5′ terminus of the viral RNA. This protein is probably the VPg. The RNAs of tobacco mosaic virus and cowpea mosaic virus did not form aggregates when isolated from purified virus by similar procedures.

Potyviruses are filamentous plant viruses whose genomes consist of a single 9·5 kb molecule of single-stranded, positive-sense RNA. Potyviral RNA contains a covalently linked protein (VPg) at the 5′ terminus (Siaw *et al.*, 1985; Riechmann *et al.*, 1989; Murphy *et al.*, 1990) and is polyadenylated at the 3′ terminus (Hari *et al.*, 1979). During electrophoretic analysis in agarose gels of the RNA of the potyvirus tobacco vein mottling virus (TVMV), some RNA migrated more slowly than the genomic RNA. In this communication we report that the more slowly migrating RNA consisted of VPg-dependent aggregates of the viral RNA.

TVMV was propagated in *Nicotiana tabacum* cv. Burley 21 and purified by procedures described by Calvert & Ghabrial (1983) and Murphy *et al.* (1990). Other potyviruses, tobacco etch virus (TEV) and potato virus Y (PVY), were purified from infected *N. tabacum* using Method 1 of Moghal & Francki (1976). Potyviral RNA was isolated by disruption of purified virus in 25 mM-Tris–HCl pH 9·0 (at 20 °C), 1 mM-EDTA, 1% SDS and 250 μg/ml bentonite followed by sucrose gradient centrifugation (Hellmann *et al.*, 1980). Tobacco mosaic virus (TMV) and cowpea mosaic virus (CPMV) were purified by methods similar to those of Boedtker & Simmons (1958) and van Kammen & De Jager (1978), respectively. Tobacco leaf RNA was isolated from leaves of uninoculated plants by the method of Loening & Ingle (1967).

After electrophoresis in 1% agarose gels of glyoxal-treated TVMV, PVY or TEV RNA (McMaster & Carmichael, 1977), we consistently observed at least two bands in addition to the genomic RNA (Fig. 1*a*). The amounts of the slowly migrating RNAs relative to the 10 kb RNA varied somewhat between different preparations. By comparison with *Hin*dIII-cut bacteriophage lambda DNA (Fig. 1*a*) and with bacteriophage L-47.1 DNA (not shown; provided by S. Bingham, Martek Corporation), the larger species had sizes of approximately 20 and 41 kb. The high M_r bands were also observed in gels loaded with non-denatured RNA but none was observed after treatment of TVMV RNA with RNase A (50 μg/ml) or 10 mM-NaOH for 1 h at 37 °C (data not shown).

To verify that the high M_r bands contained viral RNA, samples were denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% agarose gel containing formaldehyde and, after limited alkaline hydrolysis, transferred to nitrocellulose for Northern blot analysis (Maniatis *et al.*, 1982). The hybridization probe consisted of a mixture of ^{32}P-labelled DNA obtained from cloned cDNAs that corresponded to 80% of the TVMV genome (Hellmann *et al.*, 1983); hybridization and washing conditions were those described by Thomas (1983). The probe hybridized to the 9·5 kb viral RNA and to the high M_r RNAs, but not to RNA isolated from leaves of uninoculated tobacco plants (Fig. 1*b*). This result identified the slowly migrating RNAs as aggregates of TVMV RNA.

The possible involvement of an RNA-associated protein in the aggregation phenomenon was investigated. Incubation of TVMV RNA with proteinase K (100 μg/ml in 10 mM-sodium phosphate pH 7·0, 1% Sarkosyl, 10 mM-EDTA) for 2 h at 37 °C removed the aggregates but had no effect on the 9·5 kb RNA (Fig. 2*a*) indicating that the formation or stability of the aggregated forms was protein-dependent. The high M_r RNAs

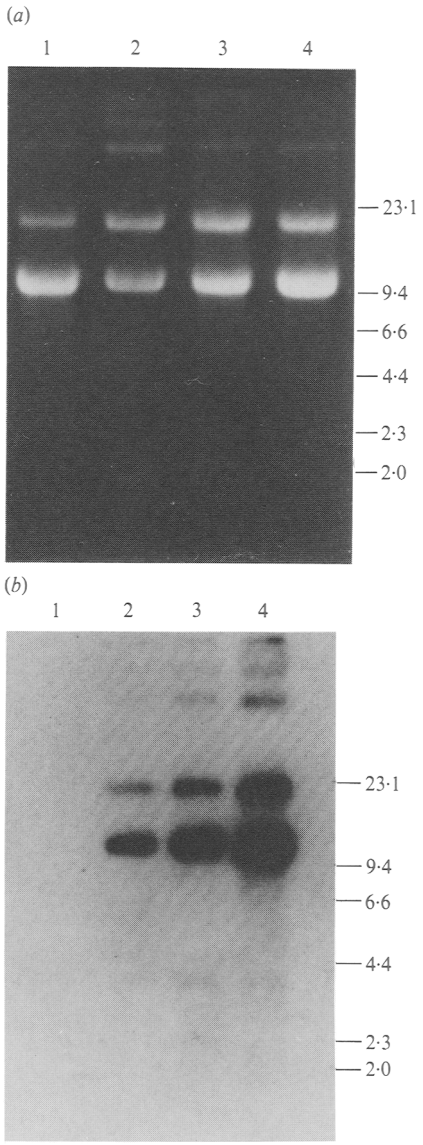

Fig. 1. Electrophoretic (*a*) and Northern hybridization (*b*) analysis of aggregates of potyviral RNAs. (*a*) TVMV (lanes 1 and 2), PVY (lane 3) and TEV (lane 4) RNAs were treated with glyoxal and analysed by electrophoresis in 1% agarose gels and ethidium bromide staining. Each lane contains 2 μg of RNA and samples of RNA from two different preparations of TVMV are shown. (*b*) Northern blot analysis of TVMV RNA. Lane 1, 2·5 μg tobacco leaf RNA (control); lane 2, 100 ng RNA; lane 3, 200 ng RNA; lane 4, 400 ng RNA. The hybridization probe was a mixture of ^{32}P-labelled TVMV cDNAs. The positions of glyoxal-treated *Hin*dIII lambda phage DNA fragments of known size (kb) are indicated at the right of each panel.

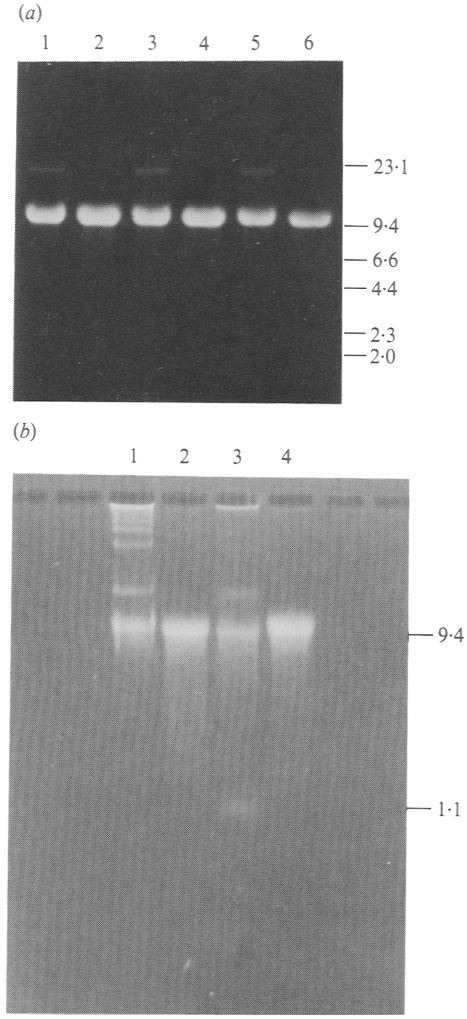

Fig. 2. Involvement of protein in aggregation of TVMV RNA. (*a*) Effects of proteinase K treatment and protein denaturants on aggregates. TVMV RNA (2·5 μg per lane) was treated as follows: lane 1, no treatment; lane 2, digestion with proteinase K; lane 3, extraction with phenol/chloroform in the presence of 1% SDS; lane 4, phenol/chloroform extraction followed by proteinase K digestion; lane 5, extraction with $NaClO_4$; lane 6, $NaClO_4$ extraction followed by proteinase K digestion. The RNA was then denatured with glyoxal and analysed by agarose gel electrophoresis with ethidium bromide staining. (*b*) Effects of RNase H digestion of TVMV RNA–oligodeoxyribonucleotide hybrids on aggregates. Samples of TVMV RNA were hybridized with oligodeoxyribonucleotides complementary to TVMV RNA nucleotide residues 408 to 420 (lane 2), 8374 to 8388 (lane 3) or 55 to 75 (lane 4), treated with RNase H, denatured and analysed by agarose gel electrophoresis. Gels were stained with ethidium bromide. Lane 1, RNA was not hybridized to an oligodeoxyribonucleotide or treated with RNase H.

were not eliminated by treatment of viral RNA with phenol/chloroform (1 : 1) or $NaClO_4$ (75% w/v) but they remained sensitive to proteinase K after treatment with either of these reagents (Fig. 2*a*). Viral RNA treated by heating in 5% SDS and 5% 2-mercaptoethanol for 5 min at 100 °C, in 8 M-urea and 1% SDS for 10 min at 65 °C, or in 10 mM-EDTA pH 7·0 containing 1% Sarkosyl for 2 h at 37 °C still contained aggregates (data not shown). These results strongly suggest that a covalently linked

protein, such as the VPg, is responsible for the aggregation phenomenon.

Evidence that the VPg is involved in the aggregation of potyviral RNA was obtained by annealing TVMV RNA to specific oligodeoxyribonucleotides and treating the hybrids with RNase H. The TVMV RNA used in this experiment had been purified by four cycles of sucrose gradient centrifugation in a medium containing 0·5% SDS, a treatment that removed all viral coat protein detected by Western blot analysis (J. F. Murphy, unpublished data) but that did not eliminate the aggregated forms of the RNA (Fig. 2*b*, lane 1). Samples containing 2·5 μg of this preparation of viral RNA were hybridized (Kunkel *et al.*, 1987) with a 10-fold molar excess of oligonucleotides complementary to nucleotide residues 55 to 75, 408 to 420 or 8374 to 8388. Hybrids were treated with 2 units of RNase H at 37 °C for 1 h, denatured with formamide and formaldehyde and analysed by electrophoresis in 1% agarose gels containing formaldehyde. When the RNA was annealed to the oligonucleotide complementary to a region near the 3′ terminus of the RNA (Domier *et al.*, 1986) and then treated with RNase H, the aggregates were not eliminated (Fig. 2*b*, lane 3). The appearance of a more rapidly migrating band of approximately 1100 bases demonstrated that the TVMV RNA–cDNA hybrid had been cleaved by the RNase H. However, the aggregates were eliminated by RNase H treatment after hybridization of the RNA to oligonucleotides complementary to sequences located near the 5′ terminus (Fig. 2*b*, lanes 2 and 4). Samples which were either hybridized with the above oligodeoxyribonucleotides or were treated with RNase H contained aggregates of RNA similar in size to those in the untreated sample (data not shown). These data indicate that a protein linked to (or near) the 5′ terminus of TVMV RNA, such as the VPg, is involved in the aggregation phenomenon. Furthermore, they suggest that the orientation of the aggregated RNA molecules, at least in the case of dimers, is 5′ to 5′ ('head-to-head').

We analysed by agarose gel electrophoresis the RNAs of CPMV and TMV that had been prepared from purified virus by the method used to prepare RNA from TVMV. TVMV RNA contained the familiar set of aggregates, but no aggregation of CPMV or TMV RNA was observed (Fig. 3*a*). Furthermore, when CPMV [the genomic RNAs of which have VPgs (Stanley *et al.*, 1978)] and TVMV particles were mixed and their RNAs copurified, there was no evidence of aggregates of CPMV RNA after ethidium bromide staining (Fig. 3*a*) or Northern blot analysis using ^{32}P-labelled CPMV cDNA probes (kindly provided by L. L. Domier; data not shown). The aggregation of viral RNA therefore does not seem to be a general phenomenon among plant viruses.

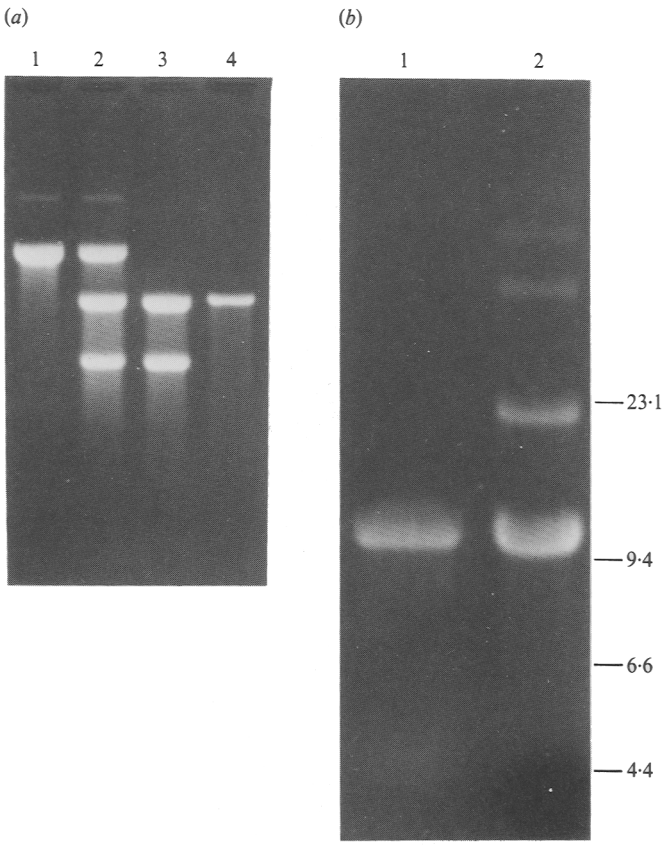

Fig. 3. (*a*) Electrophoresis of TVMV, CPMV and TMV RNAs. RNAs were denatured with glyoxal and analysed by agarose gel electrophoresis with ethidium bromide staining. Lane 1, 1·5 μg TVMV RNA; lane 2, 2·5 μg RNA isolated from a mixture of 10 mg TVMV and 3 mg CPMV; lane 3, 1·25 μg CPMV RNA; lane 4, 0·5 μg TMV RNA. (*b*) Electrophoresis of TVMV RNA before and after purification in sucrose gradients. Glyoxal-treated RNAs were analysed by electrophoresis in a 1% agarose gel and ethidium bromide staining. Lane 1, 2 μg of TVMV RNA immediately after disruption of virus; lane 2, 2 μg of TVMV RNA from the same preparation of virus after sucrose gradient centrifugation and alcohol precipitation.

The aggregates of potyviral RNA seemed to be generated during the later stages of the purification procedure. The RNA released from TVMV particles by treatment with the disruption mixture (25 mM-Tris–HCl pH 9·0, 1 mM–EDTA, 1% SDS and 250 μg/ml bentonite) did not aggregate (Fig. 3*b*, lane 1), whereas RNA that had been centrifuged in sucrose gradients and then precipitated with ethanol did aggregate (Fig. 3*b*, lane 2).

The experiments described here indicate that the aggregation of RNA isolated from potyvirus particles is dependent upon the VPg. Aggregation of the 3K to 4K VPg of CPMV has been reported to occur (Stanley & van Kammen, 1979), but this apparently does not cause the formation of stable aggregates of the RNA. The stability of the potyviral RNA aggregates may be due to the unusually large size of the VPgs. Some potyviral VPgs

have been reported to be 22K to 24K proteins (Siaw *et al.*, 1985; Riechmann *et al.*, 1989) and it has been suggested that the entire 49K proteinase of TEV may be a VPg (Murphy *et al.*, 1990). We do not know whether the aggregation of potyviral RNA molecules occurs *in vivo* and might therefore have some functional significance or if it is merely a phenomenological curiosity, the result perhaps of the molecular 'stickiness' of potyviral VPgs.

We thank Gary Hellmann, Susan Ballard and Paula Podhasky for their assistance. This work was supported by Kentucky Tobacco and Health Research Institute Grant 4E021 and USDA Grant 85-CRCR-1-1536.

References

BOEDTKER, H. & SIMMONS, N. S. (1958). The preparation and characterization of essentially uniform tobacco mosaic virus particles. *Journal of the American Chemical Society* **80**, 2550–2557.

CALVERT, L. A. & GHABRIAL, S. A. (1983). Enhancement by soybean mosaic virus of bean pod mottle virus titer in doubly infected soybean. *Phytopathology* **73**, 992–997.

DOMIER, L. L., FRANKLIN, K. M., SHAHABUDDIN, M., HELLMANN, G. M., OVERMYER, J., HIREMATH, S. T., LOMONOSSOFF, G. P., SHAW, J. G. & RHOADS, R. E. (1986). The nucleotide sequence of tobacco vein mottling virus RNA. *Nucleic Acids Research* **14**, 5417–5430.

HARI, V., SIEGEL, A., ROZEK, C. & TIMBERLAKE, W. E. (1979). The RNA of tobacco etch virus contains poly(A). *Virology* **92**, 568–571.

HELLMANN, G. M., SHAW, J. G., LESNAW, J. A., CHU, L.-Y, PIRONE, T. P. & RHOADS, R. E. (1980). Cell-free translation of tobacco vein mottling virus RNA. *Virology* **106**, 207–216.

HELLMANN, G. M., SHAHABUDDIN, M., SHAW, J. G. & RHOADS, R. E. (1983). Molecular cloning of DNA complementary to tobacco vein mottling virus RNA. *Virology* **128**, 210–220.

KUNKEL, T. A., ROBERTS, J. D. & ZAKOUR, R. A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods in Enzymology* **154**, 367–382.

LOENING, U. E. & INGLE, J. (1967). Diversity of RNA components in green plant tissue. *Nature, London* **213**, 363–367.

MCMASTER, G. K & CARMICHAEL, G. G. (1977). Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels using glyoxal and acridine orange. *Proceedings of the National Academy of Sciences, U.S.A.* **74**, 4835–4838.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory.

MOGHAL, S. M. & FRANCKI, R. I. B. (1976). Towards a system for the identification of potyviruses. I. Serology and amino acid composition of six distinct viruses. *Virology* **73**, 350–362.

MURPHY, J. F., RHOADS, R. E., HUNT, A. G. & SHAW, J. G. (1990). The VPg of tobacco etch virus RNA is the 49 kDa proteinase or the N-terminal 24 kDa part of the proteinase. *Virology* **178**, 285–288.

RIECHMANN, J. L., LAÍN, S. & GARCÍA, J. A. (1989). The genome-linked protein and 5′ end RNA sequence of plum pox potyvirus. *Journal of General Virology* **70**, 2785–2789.

SIAW, M., SHAHABUDDIN, M., BALLARD, S., SHAW, J. G. & RHOADS, R. E. (1985). Identification of a protein covalently linked to the 5′ terminus of tobacco vein mottling virus RNA. *Virology* **142**, 134–143.

STANLEY, J. & VAN KAMMEN, A. (1979). Nucleotide sequences adjacent to the proteins covalently linked to the cowpea mosaic virus genome. *European Journal of Biochemistry* **101**, 45–49.

STANLEY, J., ROTTIER, P., DAVIES, J. W., ZABEL, P. & VAN KAMMEN, A. (1978). A protein covalently linked to the 5′ termini of both RNA components of the cowpea mosaic virus genome. *Nucleic Acids Research* **12**, 4505–4522.

THOMAS, P. S. (1983). Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. *Methods in Enzymology* **100**, 255–266.

VAN KAMMEN, A. & DE JAGER, C. P. (1978). Cowpea mosaic virus. *CMI/AAB Descriptions of Plant Viruses*, no. 197.

(*Received 11 July 1990; Accepted 16 October 1990*)