Identification of Single- and Double-Stranded RNAs Associated with Maize Stripe Virus

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ABSTRACT

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Purified nucleoprotein from maize stripe virus (MStpV)-infected plants formed a single band with a density of 1.27 g/cc in cesium sulfate equilibrium density gradients, but sedimented in rate-zonal sucrose density gradients as four major nucleoprotein components with sedimentation coefficients between ~70 and 187 S. The purified nucleoproteins from both the cesium sulfate and sucrose gradients were infectious. A complex mixture of both single- and double-stranded (ss- and ds-, respectively) RNAs was identified in all purified nucleoprotein preparations of MStpV when RNAs were extracted with SDS and phenol and analyzed by nondenaturing gel electrophoresis. However, electrophoresis of denatured RNAs showed five RNAs of ~3.01, 1.18, 0.81, 0.78, and 0.52×10^6 relative mass (M_r). The ds-RNA species detected in purified nucleoprotein preparations coelectrophoresed with ds-RNAs extracted from MStpV-infected tissues, except that one small ds-RNA ($\sim 0.66 \times 10^6$ M_r) was found in tissue extracts and not in nucleoprotein samples. The nucleoprotein ds-RNAs were estimated to be ~4.9, 2.58, 1.73, 1.67, and 0.87×10^6 M_r under nondenaturing conditions. Denatured ss- and ds-RNAs separated by 2M LiCl fractionation had identical mobilities, suggesting that the ds-RNAs are complementary to the respective ss-RNAs. The sizes of the ss- and ds-RNAs in individual nucleoprotein components were proportional to the sedimentation coefficients. The nucleoprotein 0.52×10^6 ss- and 0.87×10^6 ds-RNAs were isolated from the slowest-sedimenting component (70S), while the 3.01×10^6 ss- and 4.9×10^6 ds-RNAs were isolated from the fastest-sedimenting component. Nondenaturing electrophoresis of RNAs isolated and electrophoresed immediately from purified individual nucleoprotein components showed mostly ss-RNAs, whereas SDS and phenol extraction yielded both ss- and ds-RNAs. The possibility that only ss-RNAs are encapsidated during virion assembly, and that ds-RNAs arise during RNA extraction is discussed.

Maize stripe virus (MStpV) is serologically related to rice stripe virus (RSV); these two viruses may represent a new group of plant viruses (6,20). Characteristics of this group include planthopper vectors, production of large amounts of a 16,000 relative mass (M_r) noncapsid protein in infected plants, the presence of flexuous threadlike particles in purified preparations, a capsid protein of 32,000 M_r, and possibly single-stranded (ss-) RNA genomes (6,20). Until recently (6,20), these viruses were uncharacterized and the virion nucleic acids had been identified for only RSV. MStpV also is thought to have an ss-RNA genome (6); however, no specific nucleic acids have been identified.

Our objectives were to identify the nucleic acids associated with MStpV and to compare these findings with those reported for RSV.

MATERIALS AND METHODS

Viruses. The planthopper vector (*Peregrinus maidis* Ashmead) of MStpV was reared on healthy maize plants (*Zea mays* L. 'Guardian') in a growth room at 24 C and 16 hr of light (21). MStpV was propagated by giving adult, nonviruliferous *P. maidis* a 72-hr acquisition access period on MStpV-infected plants. Groups of *P. maidis* were then transferred to maize seedlings and kept in growth chambers at 24 C and 16 hr of light for 10 days. *P. maidis* were transferred weekly to new seedlings. This was continued for 3–4 wk. After the removal of *P. maidis*, the maize seedlings were sprayed with malathion and kept in the greenhouse for 4–6 wk. Viruses used as standards were the brome mosaic virus (BMV) and tobacco mosaic virus (TMV) isolates used previously (3), and turnip yellow mosaic virus (TYMV) which was obtained from P. R. Desjardins, University of California, Riverside. Cucumber mosaic virus (CMV) was isolated from naturally infected celery (*Apium graveolens* L.) in Belle Glade.

Purification of the MStpV nucleoprotein. The MStpV nucleoprotein was purified by using a modification of the method used previously for MStpV (6). Leaves from healthy and MStpV-infected maize plants were separately diced with a razor blade; mixed with three to five volumes (w/v) of 0.1 M potassium phosphate buffer containing 0.5% 2-mercaptoethanol, 200 μg of bentonite per milliliter, and 0.01 M disodium ethylenediamine tetraacetic acid (EDTA), pH 7.0, and thoroughly ground with a chilled mortar and pestle. The homogenate was squeezed through cheesecloth and clarified by stirring for 1 min with 1/3 volume of chloroform. The emulsion was broken by centrifugation at 5,500 *g* (max.) for 10 min in the Beckman JA 14 rotor. The aqueous phase was removed, layered onto 2 ml of 20% sucrose (w/v) in 1 × SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) and centrifuged at 106,000 *g* (max.) for 3.5 hr in the Beckman Type 30 rotor. Pellets were resuspended overnight in 1 × SSC. The pellets were ground thoroughly in a tissue grinder and Triton X-100 was added to 1% (v/v) and the sample was stirred on ice for 30 min. Samples were centrifuged at 7,700 *g* (max.) for 10 min and the supernatant, containing the partially purified nucleoprotein, was then further purified by cesium sulfate equilibrium density gradient centrifugation and/or rate-zonal sucrose density gradient centrifugation.

Partially purified MStpV nucleoprotein was separated by equilibrium density gradient centrifugation in cesium sulfate gradients with mean densities of 1.20 or 1.27 g/cc. Gradients were centrifuged for 18 hr at 150,000 *g* (max.) at 20 C in the SW 50.1 rotor. Gradients were scanned at 254 nm and fractionated into 0.6-ml fractions which were dialyzed overnight against SSC.

Rate-zonal sucrose density gradient centrifugation of the partially purified nucleoprotein of MStpV was done in linear 5 to 30% sucrose gradients (using SSC buffer) in the SW 28 rotor for 3 hr at 141,000 *g* (max.), or linear 10 to 40% sucrose gradients in the SW 50.1 rotor at 243,000 *g* (max.) for 1.5 hr. Gradients were

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fractionated by using an ISCO (Lincoln, NE) model 640 density gradient fractionator.

Infectivity of the partially purified MStpV nucleoprotein was monitored by microinjection into adult *P. maidis* by using needles prepared from microcapillary glass tubes.

Polyacrylamide gel electrophoresis of proteins. Protein samples were prepared for electrophoresis by reduction and alkylation with dithiothreitol and iodoacetamide, respectively (9), and then labeled with MDPF (2-methoxy-2,4-diphenyl-3 [2*H*]-furanone, kindly supplied by W. E. Scott) (10). Samples were adjusted to pH 10 and 0.2 volume of MDPF (1 mg/ml) was added. After 10 min at room temperature, samples were readjusted to pH 7.0 with HCl, heated at 50 C for 5 min, and electrophoresed. Proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in mini slab (IDEA Scientific, Corvallis, OR) gels composed of a 12% resolving and 5% stacking gel (acrylamide/bisacrylamide = 30/0.8) and the system of Laemmli (18). Gels were 0.8 mm thick and had resolving and spacer gels that were 5.5 and 1.0 cm high, respectively. Electrophoresis was for 1 hr at room temperature at 200 V, constant voltage. Gels were photographed with ultraviolet radiation (302 nm) by using a Wratten 9 filter and Polaroid Type 55 positive/negative film.

Analysis of MStpV nucleic acids. Double-stranded RNA was extracted and purified from MStpV-infected maize plants by using a modification of the method of Morris and Dodds (17). Samples (40 g) of freshly harvested leaf tissue were blended in a Waring blender containing 80 ml of STE buffer (0.1 M NaCl, 0.05 M tris-HCl, 0.001 M EDTA, pH 6.9), 16 ml of 10% SDS, and 80 ml of freshly prepared water-saturated phenol (pH 7.0) containing 0.1% 8-hydroxyquinoline. The extract was centrifuged for 10 min at 5,500 *g* (max.) in the JA 14 rotor and the aqueous phase was removed and adjusted to 15% ethanol. Three grams of Cellex N-1 cellulose powder (BioRad Laboratories, Richmond, CA 94804) was added and the solution was stirred for 5 min. The Cellex mixture was centrifuged for 5 min at 3,000 *g* (max.) in the JA 20 rotor, and the Cellex pellet was resuspended in STE buffer that was adjusted to 15% ethanol (v/v) (15% ethanol-STE) and poured into 1-cm-diameter glass columns. Single-stranded RNA and most of the DNA was eluted from the Cellex by using 15% ethanol-STE. The eluant was monitored at 254 nm and when the absorbance returned to zero, STE containing no ethanol was added to elute ds-RNAs. The ds-RNA fraction was then incubated in 0.01 M $MgCl_2$, 0.2 M tris-HCl, 0.2 M NaCl, pH 7.3, containing 2 μg/ml DNase I (Sigma Chemical Co., St. Louis, MO 63178) for 30 min at room temperature to remove residual DNA. Double-stranded RNAs were then precipitated with 2.5 volumes of ethanol and 0.15 M sodium acetate, pH 5.5, at −20 C overnight.

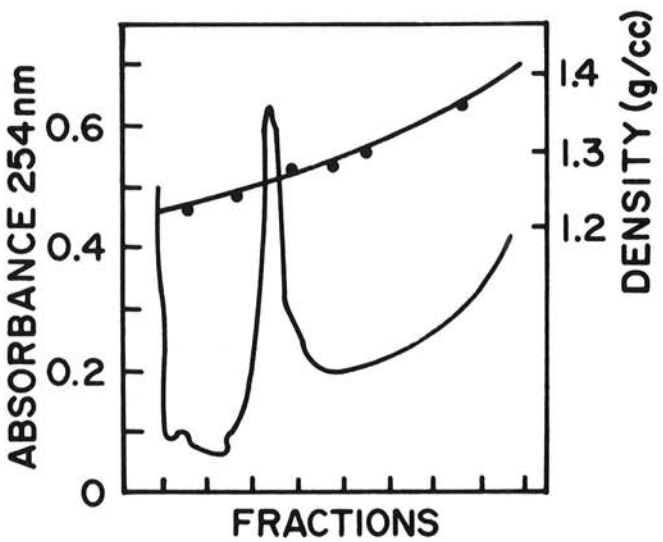

Fig. 1. Absorbance profile of a fractionated cesium sulfate equilibrium density gradient containing purified MStpV nucleoprotein. Centrifugation was at 20 C for 18 hr at 150,000 *g* (max.) in the Beckman SW 50.1 rotor.

Nucleic acids were extracted from purified MStpV nucleoprotein preparations by mixing 1 vol of the nucleoprotein with 1 vol of 0.2 M tris-HCl, 0.02 M EDTA, 2.5% SDS, 2.5% *n*-lauroyl sarcosine (pH 8.9) and 0.1 vol of purified bentonite (4). After 20 min, 2 vol of phenol (pH 7.0) containing 0.1% 8-hydroxyquinoline were added, the sample was shaken at room temperature for 10 min and centrifuged at 3,000 *g* (max.). The aqueous phase was reextracted with phenol and the nucleic acids were precipitated with ethanol. RNAs also were released from the purified nucleoproteins by heating for 5 min at 55 C in 1% SDS, 0.5% 2-mercaptoethanol, 0.05 M sodium phosphate, 2 mM EDTA, and 10% sucrose (pH 7.4) just prior to electrophoresis.

Nondenaturing electrophoresis was done in the buffer of Loening (17). Nucleic acids (in sterile distilled water) were diluted with 1 vol of 1.0% SDS, 0.5% 2-mercaptoethanol, 0.05 M sodium phosphate, 2 mM EDTA, and 10% sucrose (pH 7.4). Nucleic acids were electrophoresed in 0.8-mm-thick × 6.5-cm-high × 9.5-cm-wide mini slabs of 5% acrylamide (29:1 acrylamide:bisacrylamide) gels for 3 hr at 75 V at room temperature or composite gels of 1.0% agarose/2.5% acrylamide for 1.5 hr and 75 V at room temperature. Nucleic acids were stained with ethidium bromide (40 ng/ml in 1 mM EDTA), visualized by exposing the gel to ultraviolet radiation, and photographed by using Wratten 9 and 23A filters and Polaroid Type 55 positive/negative film.

Electrophoresis of nucleic acids was performed under nondenaturing and denaturing conditions in 1% agarose horizontal gels as described (16), except that buffers and the gel contained 20 mM sodium phosphate buffer (pH 7.0), and 1 mM EDTA. Electrophoresis was at room temperature in 20-cm-long × 15-cm-wide × 3-mm-thick horizontal slab gels of 1.0% agarose for 2 hr at 100 V. Gels were submerged 3 mm below the buffer during electrophoresis. Nucleic acids were stained with acridine orange or ethidium bromide. Acridine orange staining was done by soaking gels for 20–30 min in acridine orange (30 μg/ml) followed by extensive destaining in 20 mM phosphate (pH 7.0) for 2–3 hr. Ethidium bromide staining of nondenatured nucleic acids in agarose gels was as described for acrylamide gels. Denatured nucleic acids were stained after first soaking the gel in 50 mM NaOH for 20 min, 0.5 M ammonium acetate for 20 min, and 0.1 M ammonium acetate for 1 hr (15). Nucleic acids were stained for 1 hr with ethidium bromide (400 μg/ml) and visualized by using ultraviolet radiation.

Ribonuclease A treatment utilizing high- and low-salt buffers was used to differentiate ss- and ds-RNAs (23). Nucleic acids in water or 0.2 M NaCl, 0.1 M tris, 0.01 M $MgCl_2$ (pH 7.3), were treated with ribonuclease A (Sigma) 10 μg/ml, or DNase I (Sigma) 10 μg/ml, for 30 min at 25 C, followed by proteinase K (Sigma) 4 μg/ml, in 2 × SSC containing 0.05% SDS for 30 min at room temperature. Nucleic acids were then analyzed by electrophoresis.

Single- and ds-RNAs were separated by 2 M LiCl fractionation (1). One volume of 4 M LiCl was added to the nucleic acid solution and kept overnight at 3 C. The ss-RNAs were precipitated by centrifugation at 12,000 *g* (max.) for 10 min. The supernatant fraction was mixed with 2.5 vol of ethanol to precipitate the ds-RNAs. Nucleic acids were then analyzed by denaturing or nondenaturing electrophoresis.

RESULTS

Purification of the MStpV nucleoprotein. Purified nucleoproteins formed a single UV-absorbing band at a density of 1.27 g/cc in cesium sulfate gradients (Fig. 1). When the purified nucleoprotein was centrifuged in rate-zonal sucrose density gradients, four major UV-absorbing bands (labeled 1–4) were detected (Fig. 2). An identical pattern was found when the purified nucleoprotein from cesium sulfate gradients was subsequently centrifuged in rate-zonal sucrose density gradients. Occasionally a minor zone also was detected that sedimented slightly slower than peak 1. No bands were detected in cesium or sucrose gradients in preparations from healthy maize.

The MStpV nucleoprotein components separated by rate-zonal sucrose density gradient centrifugation in the SW 28 rotor were

kept separate, pelleted by high-speed centrifugation (Type 40, 2 hr at 145,000 *g* [max.]), and separately recentrifuged in sucrose gradients in the Beckman SW 50.1 rotor. Two cycles of sucrose centrifugation were necessary to purify the individual components, as minor contaminants were present after a single cycle (Fig. 3). Estimates of the relative sedimentation coefficients for the four MStpV nucleoproteins were obtained by comparison to TYMV top and bottom components (53 and 116 S [14]), BMV (78 S [2]), and TMV (189 S) markers in SW 50.1 linear sucrose gradients. Components 1, 2, and 3 were between the 70 and 116 S markers while component 4 sedimented slightly behind purified virions of TMV.

The purified nucleoproteins from both cesium sulfate and sucrose gradients were infectious when assayed by injecting *P. maidis*. However, it was necessary to add bentonite (200 μg/ml) to the purified preparations before injection which also was found by Gingery et al (6). The association of infectivity with any specific nucleoprotein component has not yet been determined. No attempts were made to test the efficiency of transmission by individually injected *P. maidis*. However, 80–100% of the test plants were infected when three to four *P. maidis*, injected with nucleoprotein from cesium sulfate gradients, were placed on individually caged maize seedlings.

Polyacrylamide gel electrophoresis of proteins. When the four nucleoprotein components from the sucrose gradients and the single nucleoprotein fraction from cesium sulfate gradients were analyzed for purity by SDS-PAGE, only a 32,000 M_r (32 K) protein was detected, indicating that these samples were highly purified (Fig. 4). Also, this 32 K protein corresponds to the estimated size of the MStpV capsid protein given previously (6).

Analysis of MStpV nucleic acids. A complex pattern of RNAs was obtained in nondenaturing gels when the nucleic acids extracted from MStpV nucleoproteins using SDS and phenol were

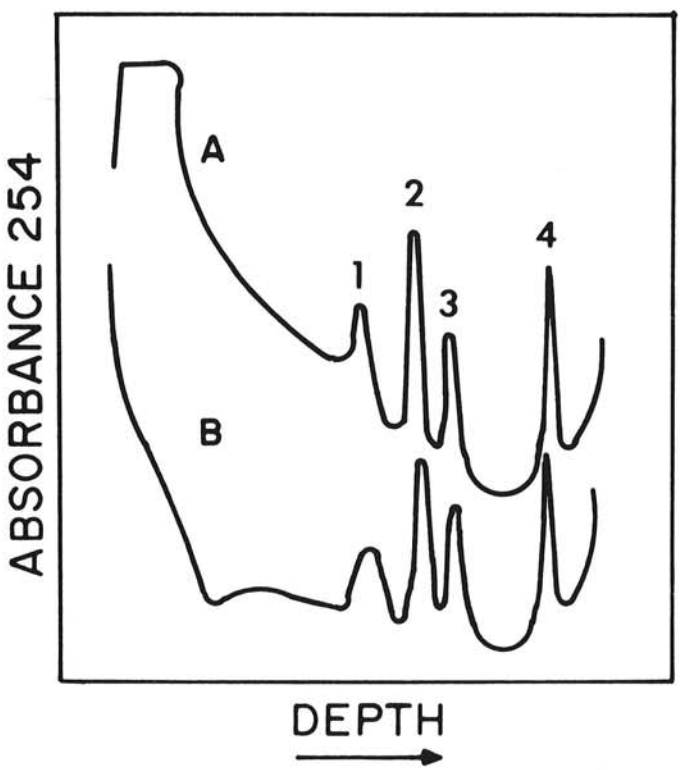

Fig. 2. Absorbance profile of fractionated MStpV nucleoproteins in linear 10–40% sucrose gradients. Centrifugation was at 3 C for 1.5 hr at 243,000 *g* (max.) in the Beckman SW 50.1 rotor. Pattern A shows the MStpV nucleoproteins purified only by differential centrifugation, followed by sucrose gradient centrifugation. Pattern B shows MStpV nucleoproteins purified by differential centrifugation and cesium sulfate equilibrium density gradient centrifugation followed by sucrose gradient centrifugation. The individual nucleoproteins are numbered 1 to 4 in order of increasing sedimentation.

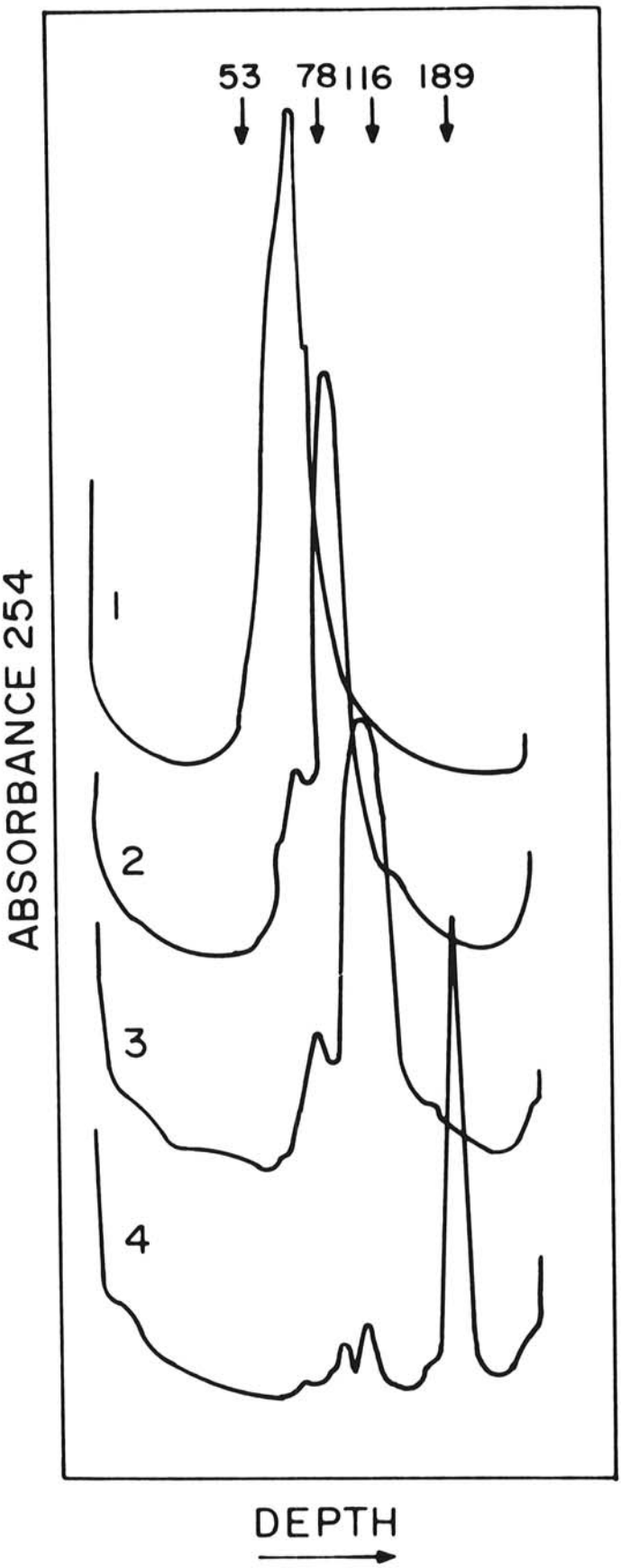

Fig. 3. Absorbance profiles of fractionated rate-zonal sucrose density gradients. The four MStpV nucleoprotein components were separated by one cycle of rate-zonal sucrose density gradient centrifugation in the SW 28 rotor. Each component was then pelleted by ultracentrifugation and recentrifuged on rate-zonal sucrose gradients in the Beckman SW 50.1 rotor for 1.5 hr at 243,000 *g* (max.). Markers are TYMV top and bottom components (53 and 116 S, respectively), BMV (78 S), and TMV (189 S).

examined (Fig. 5A). The individual RNAs were not all well resolved, but appeared as two separate clusters in the gel. The RNAs in the upper region of the gel consisted of a cluster of at least four incompletely resolved RNAs which were designated as group 1. Group 2 RNAs were four to five poorly resolved RNAs in the lower part of the gel. Nucleic acids were not detected in similarly processed preparations from healthy maize. The nucleic acids were identified as RNA by their susceptibility to digestion by RNase A and resistance to digestion by DNase I (*unpublished*). Denaturing electrophoresis was used in attempts to more clearly resolve the MStpV RNAs. Analysis of the same nucleoprotein RNA preparations on 1% agarose gels after denaturation with glyoxal and DMSO showed five distinct RNAs (Fig. 5B). The sizes of the MStpV RNAs (labeled 1 to 5 in order of decreasing M_r) were determined to be 3.01, 1.18, 0.81, 0.78, and 0.52×10^6 M_r for RNAs 1 to 5, respectively, when compared to denatured TMV and BMV RNAs in the same gels.

The complexity of the RNAs associated with the MStpV nucleoprotein was not completely unexpected as the related RSV has been shown to have four RNAs (19,20). However, only five RNAs were found when the MStpV RNAs were analyzed by denaturing electrophoresis while more than five bands were seen in our nondenaturing gels. Also the slower electrophoretic mobilities for more than one MStpV RNA relative to the TMV ss-RNA marker in nondenaturing gels while only one MStpV RNA was slower in denaturing gels suggested the possibility that structurally different MStpV RNA species might be present in the nondenatured RNA preparations. This prompted an evaluation of the ss- or ds-RNA nature of these RNAs.

TMV virion ss-RNA, TYMV ds-RNAs, and MStpV nucleoprotein RNAs isolated by SDS and phenol were incubated overnight in 2 M LiCl. The 2 M LiCl-soluble (ds-RNA) and 2 M LiCl-insoluble (ss-RNA) fractions were separated by centrifugation and analyzed by nondenaturing electrophoresis in agarose/acrylamide composite gels and by denaturing electrophoresis in horizontal agarose gels. The majority of the TMV and TYMV control ss- and ds-RNAs were found in the 2 M LiCl-insoluble and 2 M LiCl-soluble fractions, respectively, while MStpV RNAs were found in both fractions. Under nondenaturing conditions it was found that most of the slower-migrating MStpV RNAs (group 1) were in the 2 M LiCl-soluble fraction (ds-) and could be resolved as five RNAs (Fig. 6). The faster-migrating group 2 RNAs were primarily in the insoluble fraction (ss-) and were not as well resolved. Under denaturing conditions, the MStpV 2 M LiCl-soluble and 2 M LiCl-insoluble RNAs also were seen to have identical mobilities (Fig. 7), indicating that the ds-RNAs most likely are the ds-RNA counterparts to the ss-RNAs. The RNAs were completely denatured after glyoxal and DMSO treatment; when stained with acridine orange they all fluoresced pink. Nondenatured RNAs in gels stained with acridine orange showed group 2 ds-RNAs that fluoresced green and group 1 ss-RNAs that fluoresced pink. The ss- and ds-RNA nature of the group 1 and 2 RNAs also was confirmed by RNase treatment in high and low salt. DNase had no effects, but group 1 and 2 RNAs were digested in RNase and low salt, whereas only group 2 RNAs were digested in RNase plus high salt (*unpublished*). The results confirmed the structural heterogeneity of the MStpV nucleoprotein RNAs by indicating the presence of ss- and ds-RNAs in the SDS and phenol extracted MStpV nucleoprotein RNAs.

We next proceeded to isolate and analyze the replicative ds-RNAs from MStpV-infected plant tissues to compare with the MStpV nucleoprotein ds-RNAs. The ds-RNA fraction isolated from MStpV-infected maize contained six major ds-RNAs with estimated sizes (relative to TYMV and CMV ds-RNA markers) of ~4.9, 2.58, 1.73, 1.67, 0.87, and 0.66×10^6 M_r (Fig. 8) for ds- RNAs

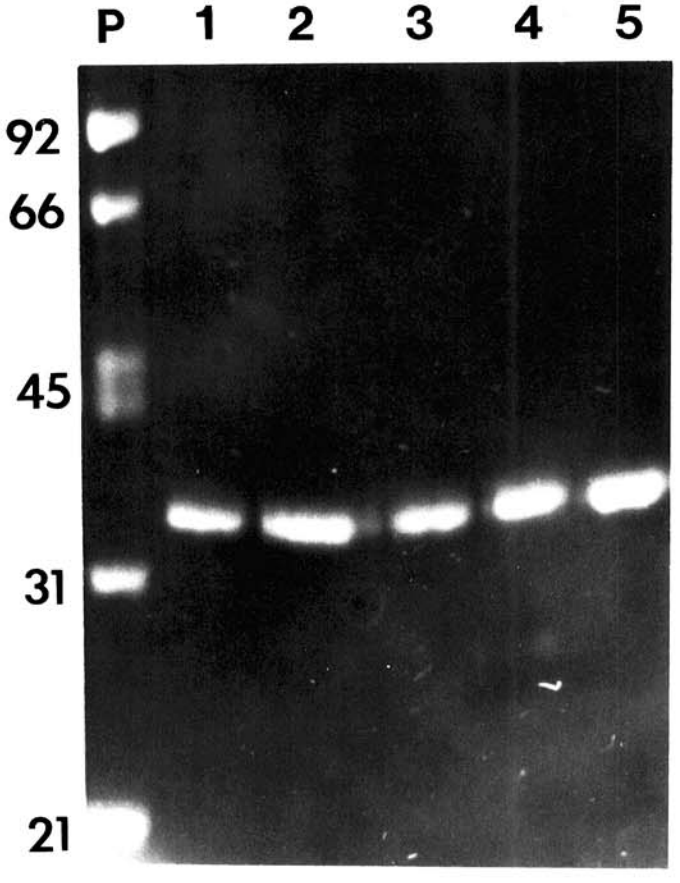

Fig. 4. SDS-polyacrylamide gel showing proteins associated with the purified MStpV nucleoproteins. Lane P shows protein standards. Values at left are relative masses (M_r) $\times 10^{-3}$ of standards. Lanes 1 to 4 are the proteins of the MStpV nucleoprotein components 1 to 4 from sucrose density gradients, and lane 5 is the protein from MStpV nucleoprotein purified by equilibrium density gradient centrifugation in cesium sulfate gradients.

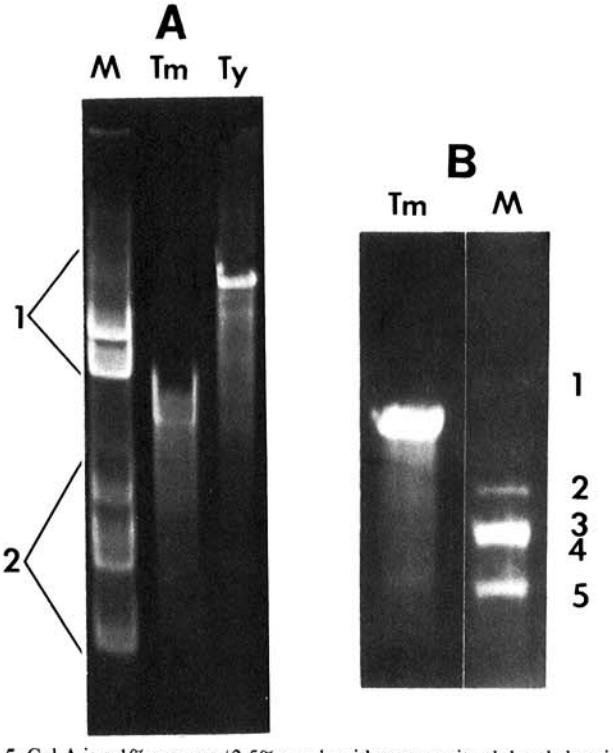

Fig. 5. Gel A is a 1% agarose/2.5% acrylamide composite slab gel showing the MStpV nucleoprotein RNAs isolated by SDS and phenol extraction. Lane M shows MStpV RNAs, lane Tm shows TMV ss-RNA and lane Ty shows TYMV ds-RNA after nondenaturing electrophoresis. Groups 1 and 2 MStpV RNAs are designated at left. Gel B is a 1% agarose gel showing MStpV (M) and TMV (T) RNAs after denaturation with glyoxal and DMSO and subsequent electrophoresis. MStpV RNAs are numbered 1 to 5 in order of decreasing M_r.

1 to 6, respectively. When the MStpV nucleoprotein RNAs were analyzed on the same gel, the five group 1 ds-RNAs coelectrophoresed with the tissue extracted ds-RNAs 1 to 5. There was no nucleoprotein ds-RNA that corresponded to ds-RNA 6. Larger ss-RNAs will not enter the 5% gels used here and only the MStpV nucleoprotein ds-RNAs can be seen.

It was somewhat surprising to find both ss- and ds-RNAs in the MStpV nucleoprotein RNA fraction. Thus, we attempted to determine if both ss- and ds-RNAs might be virion-encapsidated, and the distribution of the RNAs in the four MStpV nucleoprotein components. The separated nucleoproteins from two cycles of density gradient centrifugation were then used for nucleic acid analysis. Under denaturing conditions it was demonstrated that the sizes of the RNAs in each nucleoprotein component increased with the sedimentation coefficients of the respective nucleoproteins. The smaller RNAs (1 to 4) were detected in extracts from the slower-sedimenting components with the majority of RNA 5 in component 1, the majority of RNAs 3 and 4 in component 2 and RNA 2 in component 3. Only the largest RNA (RNA 1) was in the peak 4 extract (Fig. 9). However, when these RNAs were analyzed by using nondenaturing electrophoresis, more than one type of RNA (both ss- and ds-RNA) was evident in each nucleoprotein extract.

Therefore, to test whether both ss- and ds-RNAs were encapsidated in the MStpV nucleoproteins, or whether one or the other may be produced as a result of the nucleic acid extraction method, the nucleoprotein RNAs isolated by the SDS and phenol extraction were compared with RNAs released directly by heating the nucleoproteins in SDS immediately prior to electrophoresis. When this was done and the RNAs were analyzed by using 2.5% acrylamide/1% agarose gels, ss- and ds-RNAs were seen in the SDS- and phenol-extracted RNA preparations (Fig. 10). The sizes of both ss- and ds-RNAs increased with the sedimentation of coefficients of the nucleoproteins. Both ss- and ds-RNA 1 were detected in extracts from component 4. The majority of both ss- and

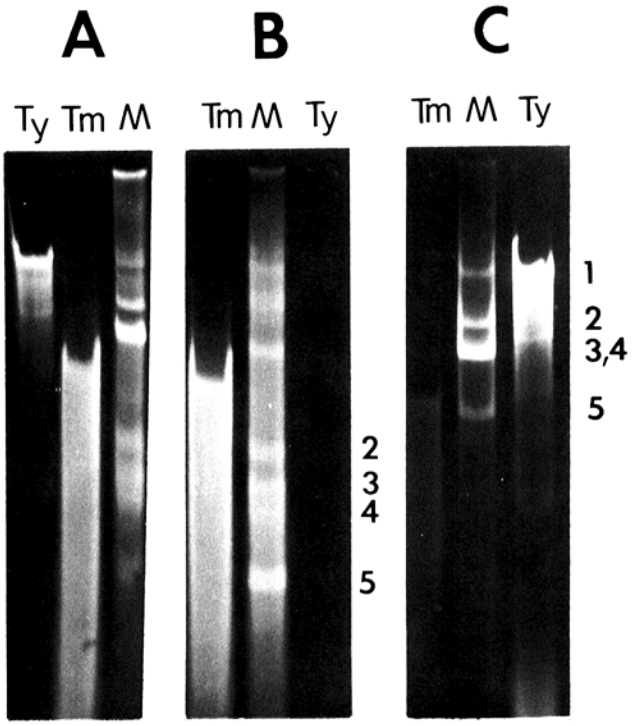

Fig. 6. A 1% agarose/2.5% acrylamide composite slab gel showing nondenaturing electrophoretic separation of TMV ss-RNA (Tm), TYMV ds-RNA (Ty), and MStpV nucleoprotein RNAs (M) before and after 2 M LiCl fractionation. Gel A shows unfractionated RNAs. Gel B shows the 2 M LiCl-insoluble (ss-) RNAs and gel C shows the 2 M LiCl-soluble (ds-) RNAs. Numbers to the right of gel B refer to the positions of MStpV ss-RNAs 2, 3, 4, and 5 (lane M). RNA 1 was not resolved in this system because of residual ds-RNA in the upper region of the gel. Numbers to the right of gel C refer to the MStpV ds-RNAs (lane M) 1, 2, 3, 4, and 5.

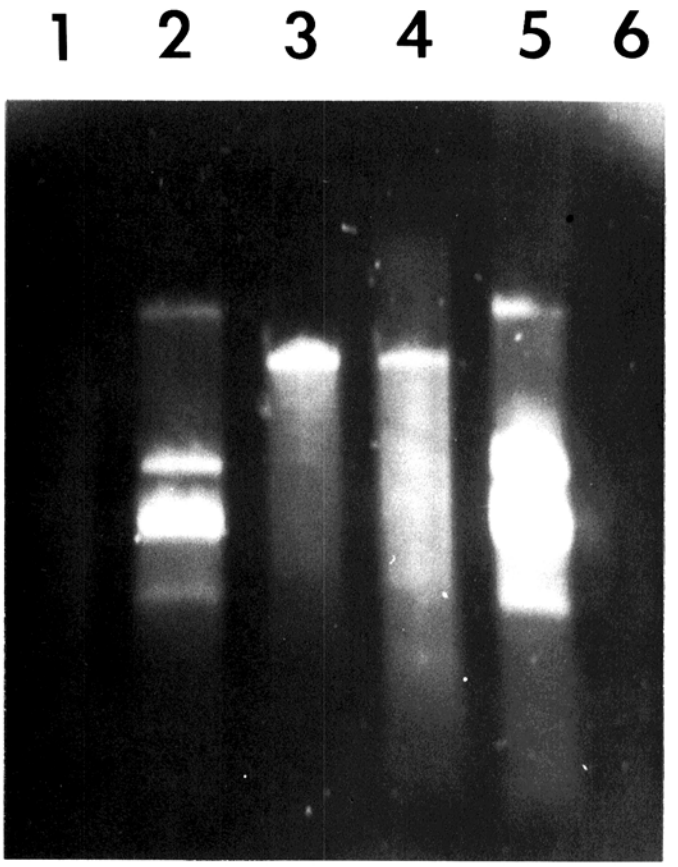

Fig. 7. A 1% agarose gel showing glyoxal and DMSO-denatured RNAs after 2 M LiCl fractionation and subsequent electrophoresis. Lanes 1 to 3 are the 2 M LiCl-soluble (ds-) RNAs and lanes 4 to 6 are the 2 M LiCl-insoluble (ss-) RNAs of TMV ss-RNA, MStpV nucleoprotein RNAs, and TYMV ds-RNA, respectively.

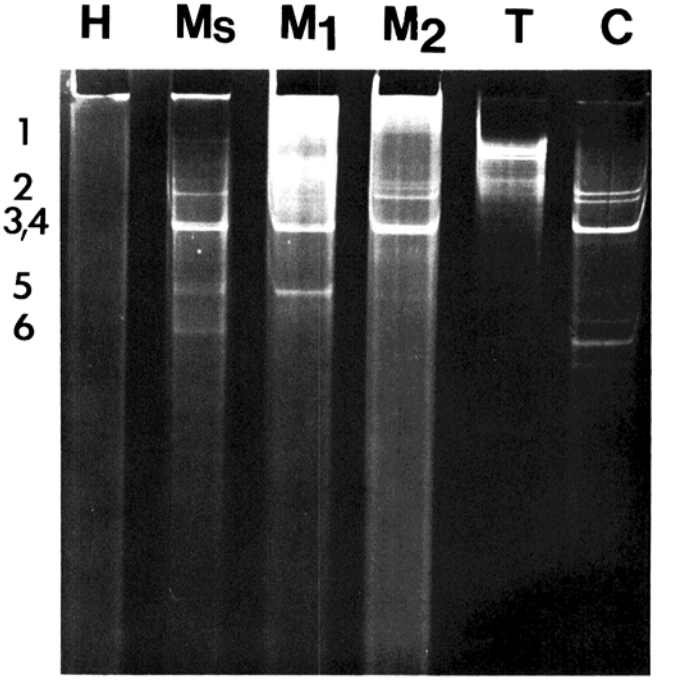

Fig. 8. A 5% polyacrylamide slab gel showing electrophoretic separation of double-stranded (ds-) RNAs. Lane H shows a ds-RNA preparation from healthy maize; Ms shows the ds-RNAs from MStpV-infected maize plants; M_1 and M_2 show ds-RNAs present in SDS and phenol extracts from purified MStpV nucleoprotein; T shows the ds-RNAs extracted from TYMV-infected chinese cabbage, and C shows the four major ds-RNAs extracted from CMV-infected peppers. Numbers to the left of the gel refer to the positions of MStpV ds-RNAs 1 to 6 in order of decreasing M_r.

ds-RNA 2 were associated with component 3, ss- and ds-RNAs 3 and 4 were associated with component 2, and ss- and ds-RNA 5 were both associated with component 1. However, in the SDS-treated nucleoprotein samples the majority of the RNAs were ss-RNAs. The ss- and ds- nature of these RNAs was confirmed by acridine orange staining. Also, when these same samples were examined by electrophoresis in 5% gels, ds-RNAs were detected only in the SDS and phenol extracts (*unpublished*).

DISCUSSION

MStpV and RSV are serologically related viruses that have been suggested to represent a new group of plant viruses (6,20). The properties shown here for MStpV support this idea; however, we also have shown some new and significant properties for MStpV.

We found the purified MStpV nucleoprotein to have a density in cesium sulfate of 1.27 g/cc which is similar to the value reported previously (6). We have found four major nucleoprotein components by rate-zonal sucrose density gradient centrifugation. The sedimentation values for the MStpV nucleoprotein components were between ~70 and 187 S. This conflicts somewhat with previous work on MStpV and the related RSV. MStpV previously was found to have three to six sedimenting components between 51 and 70 S (6) and RSV has only three components of undefined sedimentation coefficients (20).

The most unique characteristic found here for MStpV was the nature of the RNAs detected by gel electrophoresis. Five RNAs were detected by electrophoresis under denaturing conditions. This is slightly different than for RSV in which only four RNAs were found under denaturing conditions (20). Interestingly, the four smaller RNAs of MStpV are similar in size to the RSV RNAs, while no RSV RNA was similar to MStpV RNA 1. Most interesting, however, was finding both ss- and ds-RNAs when the nucleoprotein RNAs were extracted by using SDS and phenol and analyzed by nondenaturing electrophoresis. The MStpV ds-RNAs isolated from the nucleoprotein preparations are identical in electrophoretic mobility to the ds-RNAs 1 to 5 extracted from infected tissues. However, a small ds-RNA (ds-RNA 6) consistently was detected in tissue ds-RNA extracts and not in nucleoprotein extracts. It seems likely that the nucleoprotein ds-RNAs represent ds-RNA complements to the nucleoprotein ss-RNAs. When the nucleoprotein ss- and ds-RNAs were fractionated using 2 M LiCl and then analyzed by denaturing electrophoresis, they had identical mobilities. The control RNAs in this experiment, TMV ss-RNA (2×10^6 M_r) and TYMV ds-RNA (4×10^6 M_r), also coelectrophoresed when denatured. The TYMV ds-RNA is composed of two complementary RNAs each of 2×10^6 M_r which migrate as 2×10^6 M_r when denatured and therefore coelectrophorese with TMV ss-RNA when denatured, but not when nondenatured. Thus, the co-electrophoresis of the denatured MStpV ss- and ds-RNAs suggests that the nucleoprotein ds-RNAs are twice the size of the nucleoprotein ss-RNAs and most likely the ds-RNA complements to the ss-RNAs. The estimated sizes given here for the nondenatured ds-RNAs from electrophoresis in 5% gels are not exactly twice the ss-RNA values, but are close to twice the size estimates for the denatured RNAs of MStpV. Also, the denatured size estimates are probably more accurate than the nondenaturing estimates, for nondenaturing estimates of ds-RNAs have been shown to underestimate the corresponding ss-RNA sizes when determined under denaturing conditions (5).

We also found that different size RNAs are associated with the various nucleoprotein components, with the slowest-sedimenting nucleoprotein (component 1) having the smallest RNAs (mostly RNA 5, but we always detected some RNA 3 and 4). The fastest-sedimenting nucleoprotein (component 4) contains the largest RNA (RNA 1). Thus, MStpV may be somewhat analogous to

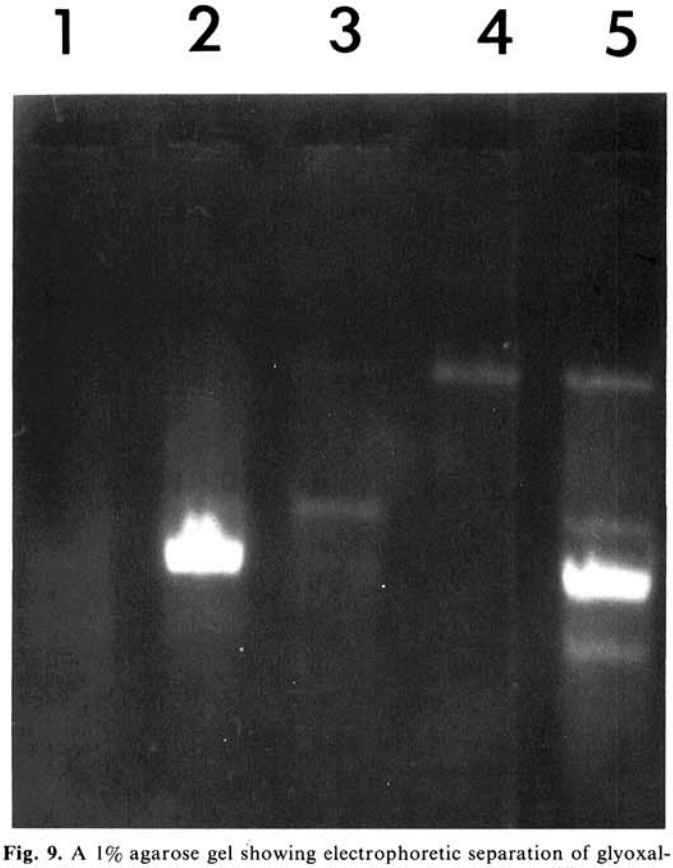

Fig. 9. A 1% agarose gel showing electrophoretic separation of glyoxal- and DMSO-denatured RNAs extracted from individual-purified MStpV nucleoprotein components 1, 2, 3, and 4. Lanes 1 to 4 show the RNAs from components 1 to 4, respectively, and lane 5 shows the RNAs extracted from a mixture of all four MStpV nucleoproteins.

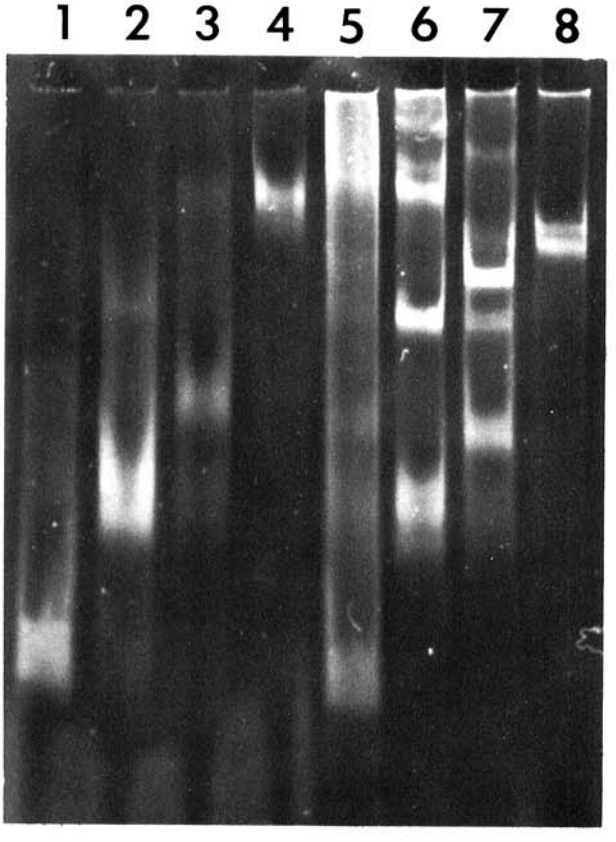

Fig. 10. Nondenaturing electrophoretic separation of RNAs in 2.5% acrylamide/1% agarose composite slab gels. Lanes 1 to 4 show the MStpV RNAs from nucleoproteins 1 to 4, respectively, isolated by SDS and heating just prior to electrophoresis. Lanes 5 to 8 show the MStpV RNAs from nucleoproteins 1 to 4, respectively, isolated by using SDS and phenol. Arrows at left show the respective positions of TYMV ds-RNA (upper arrow) and TMV ss-RNA (lower arrow) in the same gel.

many other viruses such as alfalfa mosaic virus (22) or tobacco rattle virus (11), where the sizes of the encapsidated individual RNAs are proportional to the sedimentation coefficients of the nucleoprotein components. However, additional infectivity data and analysis of the individual nucleic acids for any relationships is necessary to confirm a possible multicomponent nature. The related RSV, however, also has been shown to have its largest RNA associated with the fastest sedimenting nucleoprotein, but it appears that the fastest sedimenting nucleoprotein component of RSV contains all of the RSV RNAs (20).

Both ss- and ds-RNAs always were detected in the RNAs extracted by using SDS and phenol, even from highly purified MStpV nucleoprotein isolated by cesium sulfate followed by sucrose density gradient centrifugations, or by two cycles of sucrose density gradient centrifugation. The only protein detected in these preparations was the MStpV 32 K capsid protein. Because these were highly purified preparations, the likelihood that ds-RNAs resulted from contaminated nucleoprotein preparations or that replicative structures containing ds-RNAs (3) copurified with the MStpV nucleoproteins is highly unlikely. Also the lack of ds-RNAs detected in the nucleoprotein RNA preparations that were prepared by disrupting the nucleoproteins in SDS immediately prior to nondenaturing electrophoresis, rules out this possibility. Control ds-RNAs were not denatured to ss-RNAs when heated in SDS and thus, this tends to rule out the unlikely possibility that heating in SDS does not allow detection of ds-RNAs because they are denatured. It also seems improbable that some nucleoprotein particles might encapsidate ss-RNAs and some might encapsidate ds-RNAs, because only the ss-RNAs are obtained upon heating nucleoproteins in SDS just before electrophoresis.

One possible explanation for the presence of both ss- and ds-RNAs in the MStpV RNA samples prepared by SDS and phenol extraction is that only ss-RNAs are encapsidated during assembly, but that "plus" and "minus" polarity RNAs are separately encapsidated and then anneal under the conditions of extraction in SDS and phenol, but not when the RNAs are rapidly isolated by heating the nucleoproteins in SDS just prior to electrophoresis. This is not without precedent as some adeno-associated ss-DNA viruses contain "plus" and "minus" DNA strands that are separately encapsidated, but which anneal to ds-DNA under extraction conditions similar to those used here (15,18). If this were the case for the MStpV RNA samples prepared by SDS and phenol extraction, then either the annealing reaction must not go to completion or an unequal amount of "plus" and "minus" RNAs are encapsidated and thus, both ss- and ds-RNAs are detected by nondenaturing electrophoresis. Unequal encapsidation of ss- and ds-RNAs also would explain why previous workers using base ratio analysis suggested a single-stranded nature for MStpV RNA (6). Experiments to test these possibilities by extracting RNAs under various conditions that either promote or prevent annealing and to determine the ss- and ds-RNA relationships by hybridization reactions may help to answer these questions. These results also further support the contention that MStpV is representative of a new and unique group of RNA viruses.

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