A Non-capsid Protein Associated with Unencapsidated Virus RNA in Barley Infected with Barley Stripe Mosaic Virus

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SUMMARY

Barley tissue with an acute systemic infection of barley stripe mosaic virus contained a large amount of unencapsidated virus RNA which was stable in extracts made in ribosome isolation buffer. The virus RNA in ribosome preparations sedimented in a broad band at 80S to 100S in sucrose gradients, which is less than the virion sedimentation rate of 180S to 200S. A protein of apparent Mr 60,000, which sedimented with the virus RNA, was present in ribosome extracts from infected plants but absent from those from uninfected plants. The protein is probably a virus protein because its apparent molecular weight varied slightly with the strain of virus. The structure containing the Mr 60,000 protein did not sediment in sucrose gradients in a compact zone as would be expected for a particle of uniform size. The Mr 60,000 protein was present at a concentration equal to or slightly higher (up to 400 μg/g leaf tissue) than the unencapsidated virus RNA (up to 300 μg/g leaf tissue). Sedimentation results support the conclusion that the virus RNA and the Mr 60,000 protein were combined in polydispersed nucleoprotein particles which may or may not have been attached to ribosomes or ribosome subunits. The Mr 60,000 protein was not serologically related to the capsid protein. Gold-labelled antibodies to the Mr 60,000 protein stained the cytoplasm in thin sections of infected cells but not that of uninfected cells. However, no distinct immunogold-labelled particle could be identified as the presumed nucleoprotein.

INTRODUCTION

Barley stripe mosaic virus (BSMV) is a rod-shaped virus with three genomic RNAs. The RNAs are encapsidated separately in three virions that have 4% RNA, capsid protein of Mr 23,000, and sedimentation coefficients between 180S and 200S (for reviews, see Atabekov & Dolja, 1986; Jackson & Lane, 1981). The initial systemic symptoms are acute and are associated with a high concentration of 20S RNA, which was identified as virus RNA by its infectivity and sedimentation rate (Pring, 1971). About 90% of this virus RNA is not encapsidated (Pring, 1971). The amount of virus RNA in these young, acutely infected leaves is about the same as that of one of the ribosomal RNAs, and it is readily detected by sucrose density gradient centrifugation. This contrasts with the low concentrations of non-virion virus RNA associated with polyribosomes, replication complexes, and translation complexes; such RNA usually requires sensitive techniques based on radioisotopes for its detection (Atabekov & Morozov, 1979; Dorokhov et al., 1983, 1984).

The maximum concentration of BSMV virions in young acutely infected leaves is 500 to 1000 μg/g. Leaves appearing after the acute stage have a chronic systemic infection with 200 to 300 μg of virions per g of leaf tissue. The virus replicates in young leaves and the amount of virus per leaf increases as long as the leaf is growing, but remains constant or decreases by up to 20%
after the leaf has stopped growing (Brakke et al., 1987). The amount of unencapsidated virus RNA in young leaves with acute symptoms exceeds that usually associated with replication or translation complexes by at least one order of magnitude.

It is unlikely that a molecule with a high negative charge such as RNA would be free in the cell, and this research was initiated to find out whether a protein(s) was associated with the unencapsidated virus RNA. The results suggest that a virus-coded protein of apparent Mr 60000 forms a polydisperse nucleoprotein with the virus RNA.

METHODS

Virus culture. Most experiments were done with the ND18 strain of BSMV maintained in barley (Hordeum vulgare L., cv. 'Larker') plants grown in a greenhouse at 25 °C to 30 °C. Plants in the early two-leaf stage were inoculated by rubbing leaves with sap (diluted several-fold with water) from infected leaves mixed with Celite (diatomaceous earth). Ten other strains used were Type, White Leaf, Argentine Mild, Canadian Severe, Yellow Leaf, and a strain from an Oklahoma wheat nursery (1945), all received from H. H. McKinney (McKinney & Greeley, 1965), Norwich received from L. T. Lane, ND 159 received from R. G. Timian, and Montana isolates no. 1 and no. 2 received from T. Carroll.

Ribosome and virus RNA assays. The RNA assay was based on previously published results showing that extraction of RNA from leaves in high salt, high pH buffers gives twice the yield obtained by phenol extraction and preserves the infectivity of added virus RNA (Brakke, 1972). Leaves were ground in 9 to 18 volumes (ml/g) of AAE buffer [110 mM-NH₄HCO₃, 330 mM-NH₄Cl, 11 mM-EDTA adjusted to pH 9.3 with NaOH] (Brakke, 1972). The extract was filtered through cheesecloth, added to 1 to 2 volumes of 10% SDS, incubated at 25 °C for 30 min and then in an ice bath for 30 min to precipitate SDS-complexes of proteins. After low speed centrifugation (10 min at 10000 g), 100 to 200 μl of the clarified extract was centrifuged for 3-25 h at 54000 r.p.m. (30000 g mean radial centrifugal force, RCF) at 15 °C through a 75 to 300 mg/ml linear sucrose gradient in NaGPS buffer (50 mM-NaHPO₄, 100 mM-glycine, 300 mM-NaCl, 1 mM-EDTA, pH 9.4; Brakke, 1972). The centrifuged gradient columns were scanned for absorbance at 254 nm and RNA concentrations were determined from areas under peaks on the recorded scanning pattern (Brakke, 1963). Ribosome and virus RNA concentrations of extracts in other buffers were determined by diluting a portion of the extract with an equal volume of 2 x AAE buffer containing 2% SDS and then incubating, centrifuging and scanning as above.

Protein electrophoresis. Samples to be analysed were boiled for 2 min in cracking buffer (50 mM-Tris, 10 mM-EDTA, 2% SDS, 1% 2-mercaptoethanol, pH 8.8) and electrophoresed in 12% polyacrylamide gels with discontinuous buffers (Laemmli, 1970). Gels were stained in Coomassie Brilliant Blue R-250 or by silver (Merril et al., 1981). Concentrations were estimated by scanning the Coomassie Brilliant Blue-stained gels for absorbance at 570 nm. Different amounts of bovine serum albumin or of purified BSMV were electrophoresed, stained and measured to create a standard curve. Molecular weight markers were myosin (M, 205000), β-galactosidase (M, 116000), phosphorylase B (M, 97400), bovine serum albumin (M, 66000), ovalbumin (M, 45000) and carbonic anhydrase (M, 29000).

Isolation and sedimentation analysis of the M, 60000 protein-BSMV RNA complex. Barley leaves with an acute, systemic infection were ground in ribosome isolation buffer (200 mM-Tris-HCl, 60 mM-KCl, 30 mM-MgCl₂, 200 mM-sucrose, 5 mM-iodoacetamide, pH 8.5; modified from Davies et al., 1972) (5 to 10 ml/g) at 2 °C in a mortar and pestle. Triton X-100 was then added to a final concentration of 2% and the extract was clarified by low speed centrifugation. The clarified extract was centrifuged for 90 min at 40000 r.p.m. (106000 g mean RCF) in a Beckman Ti 50 rotor through a 1 cm layer of 30% sucrose in ribosome gradient buffer. Gradients were centrifuged for 5 hr at 26000 r.p.m. (89000 g mean RCF) in a SW28 Beckman rotor at 5 °C, and scanned for absorbance at 254 nm in an Isco density gradient fractionator with a UA 5 monitor. Samples were collected, mixed with 2 volumes of ethanol, incubated at −20 °C, and centrifuged. The precipitate was analysed for protein by boiling in cracking buffer followed by gel electrophoresis, or for RNA by incubation in AAE buffer with 1% SDS followed by density gradient centrifugation as above.

Serology. The M, 60000 protein was purified by preparative slab gel electrophoresis of a ribosome pellet. The gel was stained in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol with 1% acetic acid and destained in 50% methanol with no acetic acid (to limit acid hydrolysis). Stained strips were cut out, chopped into 1 mm cubes, frozen, and ground using a mortar and pestle in 100 mM-Tris-acetate pH 8.3, 0.1% SDS, 0.1% 2-mercaptoethanol. The mixture was stirred at 60 °C for 2 to 4 h, and centrifuged at low speed to pellet the polyacrylamide. The protein was collected by ethanol precipitation and dissolved in 0.1% SDS. Protein, emulsified in Freund's complete
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Unencapsidated virus RNA is stable in ribosome buffer

Acute, systemic symptoms appeared on leaf three at 4 or 5 days post-inoculation. Subsequent leaves were systemically infected and had chronic symptoms (McKinney & Greeley, 1965). Virus RNA was readily detected in gradient patterns of total cellular RNA from young acutely infected tissue (Fig. 1 b), but not from uninfected tissue (Fig. 1 a). The amount of virus RNA in typical experiments was 100 to 300 µg/g of young acutely infected leaf tissue (Table 1). The minimum detectable amount of virus RNA in these total cellular RNA extracts was about 50 µg/g leaf tissue. Detectable virus RNA survived when infected leaves were ground in ribosome isolation buffer (Fig. 1 c), but not when they were ground in other buffers, e.g. in those used to isolate virions, chloroplasts or nuclei. A typical result is shown in Fig. 1 (d), which is the RNA from an extract of leaves ground in a chloroplast isolation buffer (Dean & Leech, 1982). The chloroplast isolation buffer extract was diluted into AAE buffer 1 min after the leaves were ground, but the virus RNA had already been degraded. It is unlikely that RNA was lost by any mechanism except degradation because no fraction of the extract was discarded before incubation in the AAE buffer.
Virus RNA was detected in a sample of acutely infected leaf three from plants at 4 days after inoculation, but not in another sample of leaf three from the same batch of plants harvested 11 days after inoculation (Fig. 2a, 2 and a, 4). The concentration of virions was essentially the same at the two harvest dates as shown by the concentration of capsid protein in gel electrophoresis (Fig. 2). The amount of RNA in virions was not sufficient to detect by density gradient centrifugation of total cellular RNA (Fig. 2a, 4).

Virus RNA was not detectable by density gradient centrifugation of total cellular RNA from systemically infected leaves with chronic symptoms regardless of the age of these leaves when assayed.

Ribosome pellets have a disease-specific M, 60000 protein

Ribosome preparations from infected plants that contained a high concentration of unencapsidated virus RNA also contained an M, 60000 protein and capsid protein (M, 23000) (Fig. 2, lane 2) in addition to proteins detected in similar preparations from uninfected plants (Fig. 2, lanes 1 and 3). The M, 60000 protein was not detected in acutely infected leaf three at 11 days after inoculation (Fig. 2, lane 4), nor within the first 3 days after inoculation (Table 1).
Table 1. *Concentration of viral RNA, capsid protein and M<sub>v</sub> 60000 protein at various times post-inoculation*

<table>
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<tr>
<th>Time post-inoculation (h)</th>
<th>Virus RNA (µg/g)</th>
<th>Capsid protein (µg/g)</th>
<th>M&lt;sub&gt;v&lt;/sub&gt; 60000 protein (µg/g)</th>
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*Values are for a typical experiment. Plants were inoculated in the early two leaf stage and the basal portion of the third leaf was harvested and assayed at various times thereafter. The error (coefficient of variation) in determinations of virus RNA and capsid protein for a given extract was about 5%. Values varied over about a twofold range from experiment to experiment, but the changes with time and relative values were similar.

Symptoms did not appear in all plants at the same time. Virus RNA, capsid protein and M<sub>v</sub> 60000 protein were detected in extracts of symptomless plants harvested when other plants in the inoculated batch first showed symptoms (data not shown).

The M<sub>v</sub> 60000 protein reached its highest concentration (about 400 µg per g leaves; Table 1) in leaf three at 4 to 5 days post-inoculation, then decreased in concentration and by 11 days post-inoculation was not detectable in leaf three (Fig. 2, lane 4). Low concentrations of the M<sub>v</sub> 60000 protein could be detected by Western blots and immunogold staining, but not by Coomassie Brilliant Blue staining, in very young portions (the basal 5 cm, which had not emerged from the whorl, of leaves less than half the length of the next oldest leaf) of leaves four, five and six up to 20 days post-inoculation (data not shown). Later times were not tested. Virus RNA was detectable by density gradient centrifugation of whole cell nucleic acid preparations as soon after inoculation as the capsid and M<sub>v</sub> 60000 proteins were (4 or 5 days post-inoculation). The highest concentration of the unencapsidated virus RNA was about 300 µg per g of leaves (Table 1).

*Sedimentation of virus RNA and the M<sub>v</sub> 60000 protein*

Both ribosomes and the presumed virus nucleoprotein were partly aggregated after pelleting by high speed centrifugation and could not be completely dissolved. Therefore, sedimentation analysis was done with clarified extracts that had not been concentrated by high speed centrifugation. The virus RNA was recovered from the fractions of the extracts of infected tissue that sedimented at 85S to 100S in sucrose gradients (Fig. 3, infected leaf RNA gradients 7 to 10). Analysis of proteins in samples from a companion gradient revealed that the protein of M<sub>v</sub> 60000 was present in all material sedimenting between 60S and 180S (fractions from 60S to 115S shown in gel lanes 1 to 12, Fig. 3c); the highest concentrations were in fractions 4 to 9 (gel lanes 4 to 9, Fig. 3e). Monodisperse particles, such as 70S monosomes, were collected in three fractions.

Chloroplast 23S rRNA was not detected in polysomes (fractions 7 to 12, infected leaf RNA gradients, Fig. 3b) from infected plants, but was present in polysomes from uninfected plants (fractions 8 to 12, Fig. 3b). The 70S ribosome peak was also smaller in the infected than in the uninfected plants. A shoulder (arrow) was present on the heavy side of the 80S monosomes from infected plants that was absent in the extract from uninfected plants. The small shoulder on the heavy side of the 80S monosome peak in the uninfected leaf extract is the dimer of the 70S chloroplast ribosomes as shown by presence of 23S RNA in fractions 8 to 10.
Fig. 3. The absorbance of sucrose gradients with separated polyribosomes from uninfected and infected barley plants is shown in (a). Twelve samples were collected from about 60S to 115S from each of the gradients as indicated. These were analysed for the presence of RNA (using density gradient centrifugation; b) and protein (using SDS-PAGE with silver staining; c). The two main ribosome peaks are 70S (chloroplast monosomes) and 80S (cytoplasmic monosomes). The arrow (a) indicates the position of a shoulder on the heavy side of the 80S monosome peak found only in extracts from infected plants.
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Attempted elution of Mr 60000 protein from ribosomes

The Mr 60000 protein was not eluted from pelleted ribosomes from infected plants by washes with KCl concentrations up to 0.8 M in a variety of buffers from pH 7.0 to 9.4, nor with 10 mM-EDTA in 100 mM-NaHPO₄, pH 7.0, or 2 μg RNase/ml at pH 8.5, or 4 M-urea at pH 8.5.

Serology

Antisera to the Mr 60000 protein reacted in Western blots with the Mr 60000 protein but not with capsid protein (Fig. 4). A protein of similar, but not identical, size was detected in tissue infected with 10 other strains (including Type, Norwich, White Leaf and Argentine Mild) that were tested. These proteins reacted with antisera to the Mr 60000 protein of the ND18 strain. Reactions with proteins of five strains are shown in Fig. 5. The mobility and, therefore, the apparent molecular weight of the detected protein varied with the strain. The reactions with proteins of faster or slower mobility than the Mr 60000 protein or the capsid protein (Mr 23000) are presumably due to aggregates or breakdown products.

No particle of discrete and characteristic morphology that could be correlated with the Mr 60000 protein could be identified by electron microscopy of negatively stained preparations collected from gradient columns. No morphologically unique particles could be identified in thin sections of BSMV-infected tissue stained with gold-labelled antibody. The antibody was distributed rather uniformly over the cytoplasm, a distribution similar to that of ribosomes (data not shown). Thin sections of uninfected leaves did not react with antibody to the Mr 60000 protein.

DISCUSSION

Barley stripe mosaic virus is unusual among plant viruses in causing the production of a large amount of unencapsidated RNA during acute systemic infections (Pring, 1971). Small amounts of unencapsidated virus RNA should theoretically be present in translation, replication and transport complexes in tissue at the appropriate stages of infection. The presence of BSMV RNA in replication and translation complexes is indicated because polysomes from BSMV-infected plants synthesize virus proteins (Gustafson et al., 1981) and dsRNA has been isolated from infected plants (Pring, 1972; Jackson & Brakke, 1973; Dolja & Atabekov, 1987). Unencapsidated virus RNA extracted with high pH, high salt buffers from infected tissue may originate from such complexes (Diener, 1962; Brakke, 1972). Association of virus RNAs with polyribosomes has been reviewed by Atabekov & Morozov (1979). Dorokhov et al. (1983, 1984) have reported a non-virion nucleoprotein containing tobacco mosaic virus RNA, which may be involved in translocation. In contrast to these reports, where the concentration of unencapsidated RNA is small and detected only by use of radioactive labels or other sensitive methods, the concentration of unencapsidated BSMV RNA in acutely infected leaves is large enough for it to be readily detected by density gradient centrifugation of total cellular RNA. In some cases, the concentration of virus RNA in these leaves exceeded the concentration of any one of the ribosomal RNAs (Pring, 1971). Such a high concentration of virus RNA is rare among the viruses with which we have worked.

BSMV replicates in young systemically infected leaves in the chronic stage of infection, reaching a final concentration half that of acutely infected leaves. The young chronically infected leaves should have about half the total amount of virus RNA in polyribosomes and RNA replication complexes as that in the acutely infected leaves. However, the chronically infected leaves do not contain half as much unencapsidated virus RNA as the acutely infected leaves. BSMV RNA is not even detectable in the chronically infected leaves by density gradient centrifugation of total cellular RNA. Unencapsidated BSMV RNA from replication and translation complexes would undoubtedly be detected in chronically infected leaves by more sensitive assays. Density gradient centrifugation of total cellular RNA extracts is a convenient method to detect the high concentrations of unencapsidated BSMV RNA in acutely infected leaves because it does not detect the low concentrations of RNA in replication and translation complexes, or even the higher concentrations of RNA encapsidated in virions.
We have shown that a protein of $M_r\ 60000$ is present in leaves that contain unencapsidated BSMV RNA. This protein is probably the same as the $M_r\ 67000$ protein reported by Jackson et al. (1983) to be associated with the acute phase of BSMV infection. An $M_r\ 67000$ protein is not a major product of translation in vitro of encapsidated BSMV RNA, but is synthesized during in vitro translation of polyribosomes from infected plants and in vivo by infected, but not uninfected, leaves (Gustafson et al., 1981; Dolja et al., 1983). The difference in molecular weight between this and previous reports is not as large as it first appears because Gustafson et al. (1981) used values of 69000 and 46000 for the molecular weights of the marker proteins bovine serum albumin and ovalbumin, whereas we used values of 66000 and 45000. The report by Jackson et al. (1983) does not identify marker proteins, but indicates one marker at $M_r\ 68000$, presumably bovine serum albumin. They also found a slight variation in electrophoretic mobility of the protein with different strains of virus, which supports the conclusion that this protein is coded by the virus. Gustafson et al. (1987) suggest that this protein may be a post-translational cleavage product of open reading frame a of RNA-γ. Alternatively, it may be the protein of $M_r\ 58098$ coded by open reading frame 2 of RNA-β and translated from a subgenomic RNA (Gustafson & Armour, 1986).
Pring (1971) concluded from the relative concentrations of RNA and virions that the majority of virus RNA in plants with an acute infection was unencapsidated. We have confirmed his conclusion by showing that the virus RNA is in a particle sedimenting more slowly than virions (80S to 100S compared to 180S to 200S for virions), and which is stable only in magnesium-containing buffers whereas virions are stable in a variety of buffers (Brakke, 1962).

The time of appearance of the Mr 60000 protein, its concentration and sedimentation properties are all consistent with the hypothesis that it is bound to the unencapsidated virus RNA to form a polydisperse nucleoprotein. This nucleoprotein is similar to ribosomes in its stability and sedimentation and may or may not be attached to the ribosomes. If bound to ribosomes, there must be several copies of the Mr 60000 protein per ribosome. The function of the protein is unknown, but it could be part of a replication, transport or translation complex. The high concentration of the Mr 60000 protein and virus RNA in the acute stage is probably an aberration resulting from a lack of regulation leading to over-production of these components and extreme damage to the host cell. The low concentration of the protein in young leaves with a
chronic infection, where the protein can be detected by serology but not by Coomassie Brilliant Blue stain, may be a better indication of the amount of this protein needed for perpetuation of the virus infection than is the high concentration during the acute phase.

Previous research has shown a decrease in chlorophyll, chloroplast ribosomal RNA and chloroplast-associated proteins in BSMV-infected plants (White & Brakke, 1983; Brakke et al., 1987). The present results add a decrease or absence of chloroplast polyribosomes to this list of physiological changes found in the early stages of virus infection.

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REFERENCES


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