Size Variation Among Proteins Induced by Sugarcane Mosaic Viruses in Plant Tissue

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ABSTRACT

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Virus-induced proteins, infectivity on johnsongrass (*Sorghum halepense*), and symptom expression on sorghum (*S. bicolor*) were compared for 12 isolates of maize dwarf mosaic virus (MDMV) and four isolates of sugarcane mosaic virus (SCMV). Two virus-specific proteins were identified. The capsid protein was 37 kDa and varied in size among isolates from 34.4 to 39.7 kDa. A 66-kDa protein found in infected plants of three species—sorghum, maize (*Zea mays*), and pearl millet (*Pennisetum americanum*)—was not serologically related to the capsid protein and was probably a cytoplasmic cylindric inclusion protein. This protein also varied in size (from 64.2 to 67.5 kDa) among the 16 isolates, which fell into five groups. Although host range and symptom expression on the indicator plants tended to correlate, there were enough exceptions to preclude definitive correlation of these characteristics with the size of induced proteins. The 16 isolates showed many variations in inclusion protein size, capsid protein size, host range, and symptom expression. SCMV variations may be greater than generally supposed.

Maize dwarf mosaic virus (MDMV) strains, which constitute a subdivision of sugarcane mosaic virus (SCMV) (17), are usually differentiated by host range, symptoms, or serology (1,4,6,11,15, 17,19,21,24). These criteria often give variable results, and diagnosis of specific strains is not always easy or accurate. Accurate, reproducible identification is important in breeding for host plant resistance, in studying epidemiology, and for a number of other concerns.

Potyviruses produce proteinaceous inclusions (5,8,9,18). Alper et al (2) have shown that these proteins can be diagnostic for different viruses in the potyvirus group. We wanted to know if such proteins could be identified in sorghum infected with MDMV or SCMV and, if found, if such proteins could be used to identify virus strains and be correlated with host range and symptom expression.

MATERIALS AND METHODS

The grain sorghum (*Sorghum bicolor* (L.) Moench) hybrid Bugoff (Asgrow Seed Co.) was the test plant except where otherwise specified. Plants were grown in 10-cm-diameter clay pots containing a greenhouse soil mixture. A dilute balanced fertilizer was added weekly. About 7 days after planting, when in the two-leaf stage, the plants were dusted lightly with Carborundum and mechanically inoculated with infected plant sap diluted 1:1 with tap water. For 5–6 days, or until symptoms appeared, the plants were held in a greenhouse at 27 ± 5 C with supplemental light to give 16 hr of light per day. The plants were then moved to a controlled environment chamber at 27 ± 1 C and 16 hr of illumination (500 μE sec^{-1} M^{-2}) per day for the remainder of the experiment. Johnsongrass (*S. halepense* (L.) Pers.) was tested for susceptibility to the virus isolates in the greenhouse. Symptom expressions were determined on Bugoff and on DeKalb E59+ hybrid sorghums maintained in a growth chamber at 27 ± 1 C for 7–10 days after inoculation. After notes were taken on symptoms, the plants were subjected to a temperature of 11 ± 1 C for 3 days, then returned to the warm temperature. After 5–7 days, notes were taken on red leaf symptoms, expressed as various patterns of necrosis.

The preliminary studies on protein extraction and characterization were conducted with isolates MDMV-A and MDMV-B. For extraction of total plant proteins, 200 mg of young leaf tissue harvested 2–3 wk after inoculation was ground in a Waring Blendor in 14 ml of cold buffer, pH 8.4, containing 50 mM Tris, 30 mM HCl, 0.1 M KCl, 2 mM dithioerythritol (DTE) (or 2-mercaptoethanol), 5 mM disodium ethylenediaminetetraacetic acid (EDTA), 10 mM disodium diethyldithiocarbamate (DIECA), and 0.7 M sucrose. The sap was filtered through Miracloth and blended with an equal volume of water-saturated phenol. The emulsion was broken by centrifugation for 10 min at 5,900 *g* in a Sorvall SS34 rotor. The proteins were precipitated from the phenol by adding five volumes of 0.1 M ammonium acetate in methanol, incubated for 24 hr at −20 C, and collected by centrifugation for 10 min at 6,000 *g*. The pellet was washed once with methanol and pelleted again. This pellet was resuspended in a minimum volume (0.1 ml) of dissociation buffer, pH 8.8, containing 175 mM Tris, 10 mM DTE, 5% sucrose, 2% (w/w) sodium dodecyl sulfate, and 0.001% crystal violet. The sample was boiled for 1 min, and 2- to 5-μl aliquots were used for electrophoresis.

To assay the virus coat proteins, we used the minipurification procedure of Lane (13). Young leaf tissue (2 g) was ground in 15 ml of 0.1 M ammonium citrate grinding buffer, pH 6.5, containing 0.25% mercaptoethanol, 0.1% Na DIECA, and 1% polyvinylpyrrolidone, in a small Waring Blendor cup. Samples were expressed through Miracloth, and large fragments were removed by centrifuging for 15 min at 5,900 *g*. The supernatant was mixed with 4 ml of 33% Triton X-100, and the virus was centrifuged through a 5-ml pad of 20% sucrose in ammonium citrate buffer, pH 6.5, for 2 hr at 85,000 *g*. The pellet was suspended in 200 μl of one-half concentration citrate grinding buffer, and the proteins were reduced and alkylated with DTE and iodoacetamide (14). Sucrose and crystal violet were added before the sample was loaded on the gel.

The 66-kDa proteins that were seen in the whole-plant protein extracts in low concentration were assumed to be inclusion body proteins. The method of Brakke et al (3), a purification procedure for wheat streak mosaic virus inclusions, was effective for concentrating MDMV inclusions. Tissue (3–5 g) was ground in a Waring Blendor in five volumes (w/v) of an inclusion extraction buffer consisting of 5 mM Tris, 1 mM KCl, 0.1 mM Na EDTA, 1

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mM MgCl, 10% glycerol, 1% Triton X-100, and 0.1% 2-mercaptoethanol. The sap was expressed through Miracloth and centrifuged for 2 min at 2,000 *g* to remove particulates. The supernatant was then centrifuged over a 5-ml pad of 20% sucrose in inclusion extraction buffer for 25 min at 17,000 *g*. Pellets were suspended in the previously described dissociation buffer (0.25 ml/g of tissue) for electrophoresis.

Pellets from MDMV-A and MDMV-B were also fixed, embedded, sectioned, stained, and examined in an electron microscope.

Electrophoresis was in a discontinuous buffer system with 10–20% polyacrylamide gradient gels, 0.75 mm thick and 15 cm long, containing 2% bis-acrylamide (12). Electrophoresis at approximately 90 V was carried out at room temperature for 16 hr, or until the dye front reached the bottom of the gel.

The proteins were visualized using Morrissey's silver nitrate stain (16). Processing time was reduced by one-half because gels were thin. The gel was photographed and the resulting slide projected to a standard enlargement to record the migration of each band. The relative molecular weights (rmw) of the proteins were computed from the regression of the migration distance of five standard proteins plotted against the log of their rmw according to the method of Weber and Osborn (23). Each gel had at least two lanes of standards. A best-fit regression line with very high precision was computed by combining several sets of standards, but accuracy was only as good as the estimates of the rmw of the protein standards. If the regression coefficient did not exceed 0.99 or if the extracted proteins did not give sharp, well-resolved bands, the experiment was discarded. Precision was such that migration distances of proteins were visually and statistically different ($P<0.05$) when the best estimates of protein rmw differed by 200 Da or more. At least three extractions and six measurements were made on each protein from each virus isolate. The number of observations was usually much greater, however, because proteins similar in size were electrophoresed in various juxtapositions to assure that differences or similarities were visual as well as computed.

Potential relationships between 66-kDa proteins and capsid proteins were examined by western blotting. After electrophoresis, a gel with healthy proteins, proteins from plants infected with MDMV-A, and purified MDMV-A was divided, with each half having lanes of each preparation. One half was stained with silver, and the proteins of the other half were transferred electrophoretically to a cellulose nitrate membrane (22). Optimum conditions for the test were developed according to Hawkes et al (7). Sites were blocked with 3% BSA and the blot was treated with anti-MDMV-A antiserum. Excess antiserum was removed by five washings for 10 min each in 10 mM Tris-Cl buffer, pH 7.4, to which 0.9% NaCl was added. The membrane was then incubated with goat-antirabbit antiserum conjugated with peroxidase. Treating the membrane with *o*-dianisidine revealed spots that had reacted with antibody to virus.

That the 66-kDa proteins were host proteins induced by viral infection was also possible. To test this hypothesis, MDMV-A was inoculated to four cultivars or lines of grain sorghum giving different symptom responses to the virus and also to maize (*Zea mays* L.) and pearl millet (*Pennisetum americanum* (L.) Leeke) (10). All of the hosts developed mosaic symptoms 5–7 days after inoculation. Total protein extracts from healthy and virus-infected plants for all of the hosts were compared after electrophoresis.

TABLE 1. Sources and characteristics of 16 virus strains or isolates

Strain or isolate	Supplier[a]	Origin	Source	Differentiation plant
I-188	JH	Iowa	Maize	Type culture of MDMV-B
Minn-5	PB	Minnesota	Maize	No infection on johnsongrass
Minn-B	PB	Minnesota	Maize	No infection on johnsongrass
SCMV-A	PB	Unknown	Sugarcane	Varietal reaction
SCMV-B	PB	Unknown	Sugarcane	Varietal reaction
MDMV-B	JH	Iowa	Maize	No infection on johnsongrass
MDMV-B?	PB	Texas	Unknown	No infection on johnsongrass
SCMV-H	PB	Louisiana	Sugarcane	Varietal reaction
SCMV-M	PB	Louisiana	Sugarcane	Varietal reaction
MDMV-A	JH	Iowa	Maize	Type culture of MDMV-A
Minn-11	PB	Minnesota	Maize	Infection on johnsongrass
Minn-6	PB	Minnesota	Maize	Infection on johnsongrass
Ohio-D	PB	Ohio	Maize	Maize inbred reaction
Ohio-E	PB	Ohio	Maize	Maize inbred reaction
Ohio-F	PB	Ohio	Maize	Maize inbred reaction
KS-1	JM	Kansas	Sorghum	Unique symptoms on sorghum

[a]JH = John Hill, Iowa State University, Ames; PB = Phil Burger, Texas A&M University, College Station; JM = T. J. Martin, Kansas State University, Hays.

RESULTS

Gels of total plant proteins showed two proteins of about 37 and 66 kDa that were specific to virus-infected plants. The 37-kDa protein comigrated with capsid protein from purified virus of the strain used to infect the plants. When the proteins were electrophoretically transferred to a cellulose nitrate membrane, only the 37-kDa proteins from purified virus or from the total proteins of infected plants reacted with virus antiserum. The 66-kDa proteins are, by this criterion, unrelated to virus coat protein.

In a test of total proteins from three susceptible species—sorghum, maize, and pearl millet—the healthy plant proteins were nearly identical for the sorghums but greatly different between species. The 37- and 66-kDa proteins that were specific to infected plants, however, were identical from all hosts. This supports the assumption that both the 37-kDa protein, which is capsid protein, and the 66-kDa protein are coded for by the virus genome, not the host.

Capsid proteins from purified MDMV-A and purified MDMV-B migrated to slightly different positions (37.5 vs. 37.3 kDa). The 66-kDa proteins from these two strains also migrated to significantly different positions. The 66-kDa proteins in total protein extracts from MDMV-A were always distinctive, but the proteins from the B strain were sometimes faint or obscured by host proteins. When the 66-kDa proteins were partially purified and concentrated, however, the host proteins no longer interfered.

Because both the 37-kDa and the 66-kDa proteins of MDMV-A and MDMV-B differed distinctly, proteins of additional strains or isolates of MDMV and SCMV were compared (Table 1). Sharpness and resolution using total protein extracts were poor, so protein rmw in Table 2 were compared after partial purification and concentration using the minipurification technique of Lane (13) for capsid protein and the method of Brakke et al (3) for the inclusion protein. The virus-induced proteins of different strains always occurred in the same position relative to each other. Figure 1 shows differences among capsid proteins of seven strains, and Figure 2 shows differences among inclusion proteins of six strains.

Table 2 summarizes rmw of the virus-induced proteins and host reactions of the 16 isolates. The first criterion for ordering isolates in Table 2 was infectivity or noninfectivity on johnsongrass, and the second criterion was size of inclusion protein. The inclusion proteins of the 16 isolates fit into five size categories. Two exceptions disrupt a perfect ordering of the isolates on both criteria. Isolate KS-1 had the largest inclusion protein (67.5 kDa) but differed from others with large inclusion proteins by infecting johnsongrass. Some of the group with 64.2-kDa inclusion proteins could infect johnsongrass and some could not.

Table 2 does not show relative concentrations of 66-kDa proteins in the extracts. Isolate I-188, which is the type culture of MDMV-B, produced so little 66-kDa protein that it was often hardly visible in the silver-stained gels of total proteins and was not found at all after

the concentration procedure. Size of this protein was calculated from total protein gels. Since I-188 produces inclusion bodies that can be seen in the cell in the electron microscope (W. G. Langenberg, *personal communication*), our failure to detect them after concentration suggests that the 66-kDa protein is probably lost during this procedure.

When pellets of 66-kDa proteins were fixed and embedded for electron microscopy, structures were observed resembling those of potyvirus cytoplasmic cylindric inclusions. Only strains A and B were examined, but both had the same appearance.

The capsid proteins of the 16 isolates could not be grouped, and their size did not correlate with other properties. The largest and smallest capsid proteins were both in the 64.2-kDa inclusion protein group. KS-1 had the largest inclusion protein but an intermediate-sized capsid protein. The average capsid protein of johnsongrass-infecting isolates was smaller than the average capsid protein of isolates not infecting johnsongrass, but the size ranges overlapped.

Isolates not infecting johnsongrass also produced the temperature-sensitive necrotic red leaf reaction on the sorghum hybrid Bugoff and, with few exceptions, caused some necrosis on the hybrid E59+. One exception, isolate KS-1, infected johnsongrass and also caused some necrosis on E59+. The other significant exception was I-188, which produced only faint symptoms on E59+ after long incubation.

DISCUSSION

In the past, members of the SCMV subdivisions have been differentiated mainly on the basis of host range or symptom characteristics. We studied the properties of virus-induced proteins to examine this group further, and our work confirms, in principle, the work of Alper et al (2), who used biophysical properties to differentiate the proteins induced by distinctly different potyviruses. We found, however, that strains or even isolates of very similar viruses can also be differentiated by these methods.

TABLE 2. Comparison of proteins[a] and host reactions of 16 strains or isolates of MDMV and SCMV

Strain or isolate	Inclusion protein (kDa)	Capsid protein (kDa)	Infectivity on johnsongrass[b]	Symptom[c] expression on: Bugoff Warm[d]	Bugoff Cool	E59+ Warm	E59+ Cool
I-188	67.2	35.5	−	M	N	NS	NS
Minn-5	67.2	37.6	−	M	N++	M	N
Minn-B	67.2	37.2	−	M	N++	M	N−
SCMV-A	66.5	36.9	−	M	N++	M	N++
SCMV-B	66.4	36.7	−	M	N++	M	N+
MDMV-B	65.8	37.3	−	M	N++	N−	N−
MDMV-B?	64.2	39.7	−	M	N++	M	N++
SCMV-H	64.2	39.1	−	M	N++	M	N++
SCMV-M	64.2	37.4	−	M	N+	M	N+
MDMV-A	64.2	37.5	+	M	M	M	M
Minn-11	64.2	36.4	+	M	M	M	M,L
Minn-6	64.2	35.3	+	M	M,L,N−	M	M
Ohio-D	64.2	37.0	+	M	M,L	M	M,L
Ohio-E	64.2	35.1	+	M	M	M	M
Ohio-F	64.2	34.4	+	M	M	M	M
KS-1	67.5	35.6	+	M	M	M	N−

[a] Differences of 0.2 kDa gave visually and statistically different migration distances. No size differences were observed among members of an inclusion protein group.

[b] Results of two experiments with identical results; − = no infection, + = infection.

[c] M = mosaic, N = necrosis, L = local necrosis of inoculated leaf only, NS = no symptoms; − = small necrotic flecks, + = severe, ++ = plants dead.

[d] Warm = 27 C for 7 days; cool = 11 C for 3 days, then 27 C for 5 days.

Understanding variability of this group is important when studying epidemiology and when considering biological properties, such as host range and host reaction, and control procedures in breeding for host plant resistance.

Serology is another biochemical property used to differentiate the SCM viruses. Using polyclonal antibodies, we found that among our isolates, MDMV-A and MDMV-B, with capsid proteins differing in size by only 200 Da, were not serologically related by direct ELISA. Conversely, I-188 (capsid protein rmw, 35.5 kDa) reacted with MDMV-B (capsid protein rmw, 37.3 kDa) antiserum. Comparisons of isolates in this collection by others have shown that finding serological differences or similarities is relatively easy (6,15,21,24). It remains to be seen if the inclusion proteins described here are serologically related, but differences would not be surprising. Aphid transmission "helper proteins" of other potyviruses, even though similar in size and function, can be serologically unrelated to one another (20).

Differences in protein properties were not related to geographic origin. Minn-5 and Minn-B normally would be identified as isolates of MDMV-B, but their coat proteins were different sizes. Isolates of MDMV-A, Minn-11, and Minn-6 could also be resolved by the sizes of their coat proteins. The Ohio isolates, all similar to MDMV-A but separable on the basis of symptoms on maize (15), could also be differentiated by coat protein sizes. Our attempts to repeat the separation on maize according to Louie and Knoke (15) were not successful, probably because of environmental differences, but we did see other differences in symptoms in the Ohio group. All of these protein differences were seen despite the short time the disease has been observed in these geographic areas,

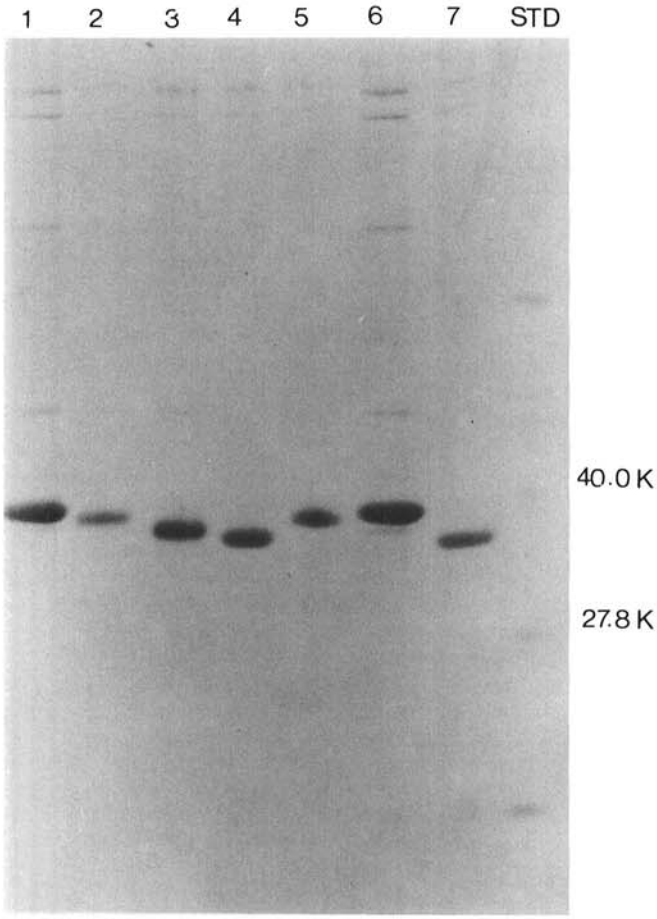

Fig. 1. Virus particles concentrated by minipurification, converted to sodium dodecyl sulfate derivatives, separated by polyacrylamide gel electrophoresis, and silver-stained. Lane 1, MDMV-A; lane 2, Ohio-D; lane 3, Minn-6; lane 4, Ohio-E; lane 5, Minn-11; lane 6, MDMV-A; and lane 7, Ohio-F. Standards: BSA (67.9 kDa), glutamate dehydrogenase (52.2 kDa), aldolase (40 kDa), and chymotrypsin (27.8 kDa).

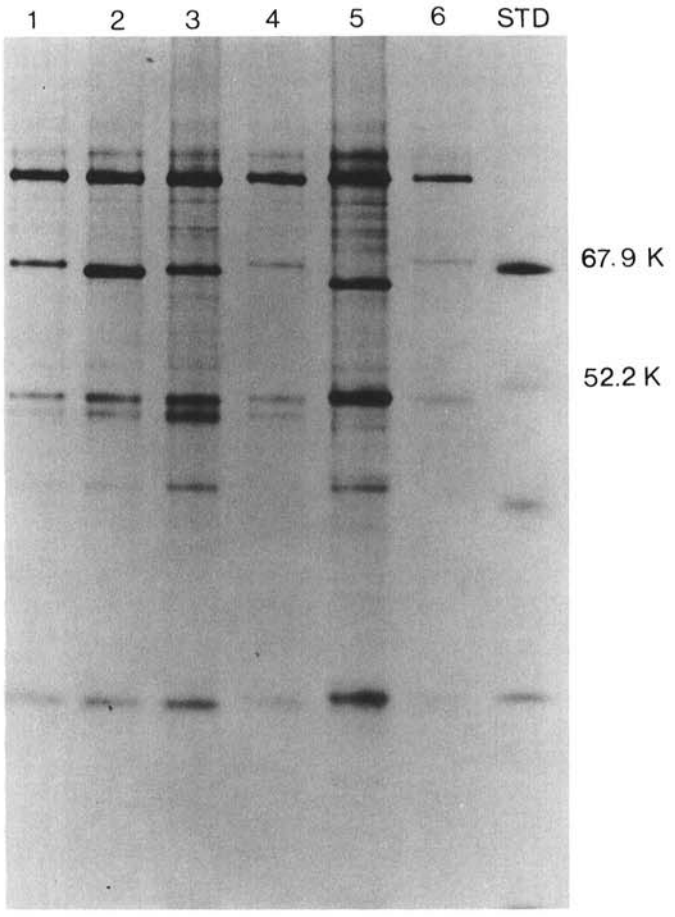

Fig. 2. Inclusion proteins concentrated, converted to sodium dodecyl sulfate derivatives, separated by polyacrylamide gel electrophoresis, and silver-stained. Lane 1, Minn-B; lane 2, MDMV-B; lane 3, SCMV-A; lane 4, Minn-5; lane 5, MDMV-A; and lane 6, KS-1. Standards: BSA (67.9 kDa), glutamate dehydrogenase (52.2 kDa), aldolase (40 kDa), and chymotrypsin (27.8 kDa).

suggesting a greater potential for variability than is commonly acknowledged.

Biological properties of these isolates were stable, and we found no recombination of properties in several hundred plants with mixed MDMV-A and MDMV-B infections. None of the isolates recovered from these plants could infect johnsongrass, typical of strain A, and also produce necrotic symptoms on Bugoff, typical of strain B. Assuming that protein size properties are equally stable and not subject to recombination during mixed infection, an independent evolution of each isolate is indicated.

In greenhouse experiments using mixed infections with two isolates, the inclusion proteins appeared as two bands whose sizes corresponded with those of the virus isolates that had gone into the mixture. Thus, the dual infection did not alter the size of the inclusion protein. This test also served as an internal control demonstrating that the isolates reported in these studies were not mixtures.

In a preliminary trial in which we isolated inclusion proteins of MDMV-A and MDMV-B at intervals ranging from 5 to 21 days after inoculation, we found variation in the amount of protein over time but no variation in the size. Thus, the size seems stable, at least on the short term.

All of this suggests that the concepts of host range, host reaction, serology, etc., that have been used in classifying isolates and strains of the SCMV subdivisions have not identified nearly all of the diversity. The addition of two concepts—the size of the capsid protein and the size of the inclusion protein—demonstrates that considerable variability exists within groups of isolates that appear homogeneous on the basis of other concepts. As a case in point, a naturally infected sorghum plant collected in the field appeared, by host reaction and serological typing, to be carrying a MDMV-B-like strain. When the inclusion protein was extracted and separated by electrophoresis, two distinct bands were seen, suggesting that the plant was infected with two virus strains similar in biological properties. Another plant in the same collection was infected with a virus with MDMV-B-like biological properties, but only one inclusion band was seen.

Knowledge of the variability of these proteins may be useful in further characterizing isolates to improve identification, in following mixed infections with similar isolates, in using unique isolates to follow epidemiology, in developing or following mutants, and in a number of other applications. This variability must also be taken into account when considering replicative strategy of the virus or the potential for variation in structures, such as virions or inclusions, that are made up of these proteins.

The meaning of the variability in size of these proteins is unknown, particularly for the 66-kDa protein. Assuming this is an inclusion protein, no function has been identified for these structures and differences in size cannot be related to any properties reported here. In the long term, meaningful grouping or classification of these virus isolates and strains on the basis of the size of induced proteins may have to wait for a better understanding of the viral genomic function and the host interaction.

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