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Bean Mild Mosaic Virus: Genome and its Translation Products

V. HARI, I.P.S. GADH, D.BAUNOCH, AND P. DAS
Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA

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Requests for reprints should be addressed to V. Hari, Dept. of Biological Sciences, Wayne State University, Detroit, Michigan 48202, USA.

Abstract

Viral RNA from purified preparations of bean mild mosaic virus (BMMV) was resolved into two RNAs of mol. wt 1.5×10^6 daltons (R1) and 0.76×10^6 daltons (R2). Complementary DNA hybridizations, infectivity tests, and in-vitro translation studies established that R2 is a subgenomic RNA derived from R1. In-vitro translation studies showed that the genomic RNA (R1) coded for two major proteins of mol. wt. 40.5 kD and 25.7 kD. Of these, the 40.5 kD protein was shown to be the capsid protein that is translated in vitro from both the R1 and R2 RNAs. Time-course studies on the appearance of in-vitro translation products showed that the 25.7 kD product is the earliest product made when the genomic R1 RNA is translated. This is not one of the products of R2 RNA. Several of the characteristics of BMMV described in this paper show similarities with turnip crinkle virus (TCV) and carnation mottle virus (CarMV).

Introduction

Isometric plant viruses that sediment as a single component and that contain an RNA of mol. wt. 1.4×10^6 daltons have been classified in the tombus group, sobemo group, or under the unclassified virus group (1,2). Bean mild mosaic virus (BMMV) is presently placed in the unclassified group (2). BMMV particles are reported to be approximately 28 mm in diameter, sedimenting at 127 S and contain a single plus polarity RNA of mol. wt 1.27×10^6 (3,4). The virus is serologically unrelated to several isometric viruses, including viruses of the como, tombus,

sobemo, and carnation mottle virus-related groups. The virus is transmitted mechanically and by beetles (3,4). This paper deals with further characterization of this virus with respect to its genome, its capsid proteins, and its in-vitro translation products.

Materials and methods

Virus, virus purification, and viral RNA extraction

An inoculum of BMMV was obtained in the form of dried dessicated tissue from the USDA plant introduction station at Glen Dale, MD. A piece of the dessicated tissue was ground in 0.02 M phosphate buffer (pH 7.5) and inoculated onto young *Phaseolus vulgaris* var. pinto plants. About 2 weeks after inoculation, the young trifoliate leaves of these plants showed mild mosaic symptoms. These leaves served as source inoculum for future infections.

The virus was purified by a procedure modified from that reported earlier (3). Approximately 300 g of fresh leaves from infected bean plants were blended in 3 volumes of 0.02 M phosphate buffer (pH 7.5) containing 0.02 M mercaptoethanol. Cold choloroform, at a rate of 1 ml/15 ml, was added to the grinding buffer. The homogenate was then filtered through two layers of cheesecloth. After filtering, the slurry was centrifuged at 10,000 g for 10 min. The virus from the supernatant was precipitated by the addition of polyethylene glycol (PEG) at a final concentration of 10%. The pellets collected by centrifugation at 10,000 g were resuspended in a small volume of grinding buffer and clarified by low-speed centrifugation at 5000 g for 10 min. The supernatant was centrifuged at 100,000 g for 90 min. the resultant pellets containing the virus were resuspended in 12 ml of grinding buffer and centrifuged at 5000 g for 10 min to remove insoluble material. The supernatant was made 35% w/vol with respect to cesium chloride and centrifuged for 22 hr at 100,000 g. The virus that banded at a density of 1.336 was collected, diluted with 5 volumes of grinding buffer, and pelleted at 100,000 g for 90 min. The pelleted virus was dissolved in a small volume of 0.02 M Tris-HCl (pH 7.0) buffer, centrifuged at 5000 g for 10 min to remove impurities, and stored at 4°C. The virus concentration was estimated spectrophotometrically using an extinction coefficient of 6.0 at a wavelength of 260 nm for a 1 mg/ml solution.

RNA was extracted from purified virus preparations after proteinase K treatment (5,6). Alternately, RNA was extracted by treatment with phenol:choloroform: isoamyl alcohol (25:24:1) in an extraction buffer of 0.1 M Tris (pH 9.0) containing 5% $NaDodSO_4$ and 5% mercaptoethanol. Nucleic acids from leaf and tissue RNA was isolated from powdered tissue after treatment with liquid nitrogen (7).

Agarose gel electrophoresis and purification of RNA

Nucleic acids were electrophoresed in 0.7% or 0.8% agarose gels in borate:EDTA (BE) buffer pH 8.2 (8), except that methyl mercuric hydroxide at a concentration of

10 mM was added to the sample alone. The RNA samples were visualized after staining with ethidium bromide. Molecular weight estimations were made with reference to healthy bean or tobacco-leaf RNA containing 25S RNA (1.3×10^6 daltons), 23S RNA (1.05×10^6 daltons), 18S RNA (0.7×10^6 daltons), and 16S RNA (0.56×10^6 daltons), as well as tobacco mosaic virus (TMV) RNA (2×10^6 daltons) and tobacco etch virus RNA (3×10^6 daltons).

RNA from purified virus was fractionated by sucrose gradient centrifugation in 5-20% sucrose gradients made in 0.1 M Tris (pH 7.6), 0.1 M EDTA, 0.1% NaDod-SO_4, and 0.1% mercaptoethanol (6). Centrifugations were carried out at 24000 rpm in a Sorvall OTD-2 ultracentrifuge using a SW 27.1 rotor.

Genomic and subgenomic RNAs present in viral RNA preparations were also purified from agarose gels after electrophoresis onto NA 45 paper (Schleicher and Schuell). Briefly, the method consisted of inserting a small piece of NA 45 paper into a gel slot just ahead of the nucleic acid of interest and electrophoresing the RNA onto the gel, followed by eluting the RNA with a high-salt buffer (9,10).

cDNA synthesis and northern blot analysis

Complementary DNA (cDNA) to BMMV-RNA was prepared by the random primer method (11). After agarose gel electrophoresis, the RNAs were transferred to diazotrized paper (12) and subjected to northern hybridization (7,13).

Immunoblots, immunoprecipitation, and serological tests

Virus capsid proteins or in-vitro viral RNA translation products were analyzed after transblotting the electrophoretically separated proteins onto cellulose nitrate paper. The blots were probed with BMMV antiserum, followed by exposure to alkaline-phosphatase-conjugated goat anti-rabbit antibodies (GAR-AP) and AP-color development reagent, according to a protocol supplied by Bio-Rad Laboratories, Richmond, CA 94804.

For immunoprecipitation, the translation products obtained after in-vitro translation of viral RNA were dissolved in 2 vol. of Laemmli sample buffer without merceptoethanol (14) heated at 70°C for 15 min, and centrifuged at 10 K/10 min to remove insoluble material. An equal volume of antiserum to BMMV diluted in 50 mM Tris (pH 7.4), 5 mM EDTA, 180 mM NaCl, 0.1% Tween-20, and 10% BSA was added to the supernatant and incubated at 37°C for 1 hr. Immunoprecipitates of the antigen-antibody complexes were obtained by the addition of staphylococcus A protein (15). The immunoprecipitates were washed and dissolved in Laemmli (14) buffer and subjected to gel electrophoresis and fluorography (16).

BMMV was tested against antiserum to BMMV, red clover necrotic mosaic virus (RCNMV), sweet clover necrotic mosaic virus (SCNMV), and cowpea mosaic virus (CpMV) by Ouchterlony double-diffusion analysis and by rocket immunoelectrophoresis.

In-vitro translations

RNA samples were translated in vitro in the presence of 5 μCi of ^{35}S-methionine per reaction using commercially available rabbit reticulocyte lysate (Bethesda Research Labs or Promega-Biotek) as per the protocol supplied by the manufacturer. The translations were carried out at 30°C for 1 hr. At the end of the translations, the samples were mixed with an equal volume of 2x Laemmli buffer (14), boiled for 5 min, and subjected to electrophoresis in $NaDodSO_4$-polyacrylamide gels. The gels were soaked after electrophoresis in Enhance (New England Nuclear) and processed for fluorography (16). In all cases ^{14}C-labeled protein molecular weight standards were run during electrophoresis of proteins. The mol. wt. standards used were myosin-H (200 kD), phosphorylase b (97.4 kD), bovine serum albumin (68 kD), ovalbumin (43 kD) α-chymotrypsinogen (25.7 kD), β-lactoglobin (18.4 kD), and lysozyme (14.3 kD).

RNA dot blots

One leaf disc of 0.8 cm diameter was punched out of healthy or infected plant leaves and ground in 50 μl of 4% $NaDodSO_4$, in Tris (0.01 M) EDTA (2 mM) pH 7.6 buffer. Ten microliters of the grindate was used for dot-blot hybridization using ^{32}P-labeled random cDNA probes to BMMV-RNA. The hybridizations were conducted at 50°C in 50% formamide (13).

Results

Viral RNA

Two RNAs of mol. wt. 1.5×10^6 daltons and 0.76×10^6 daltons were resolved on 0.8% agarose gels after ethidium bromide staining when total RNA extracted from BMMV was subjected to gel electrophoresis along with various marker RNAs of known molecular weights (Fig. 1A). It was important to establish that neither of these RNAs were host RNA contaminants, since both viral RNAs had molecular weights close to those of 25S and 18S ribosomal RNAs. The viral origin of these RNAs was established when randomly primed cDNA probes to BMMV RNA hybridized to both RNAs from BMMV, whereas a similar probe prepared using bean leaf RNA as template failed to hybridize to these RNAs (Fig. 1B, 1C). A third RNA of mol. wt. 0.65×10^6 daltons also hybridized to the cDNA to BMMV RNA probe, although this RNA was not discernible in ethidium-bromide-stained gels. Subsequent experiments have shown that the 0.65×10^6 dalton RNA does not show up in all viral RNA preparations.

R1 (1.5×10^6 daltons) and R2 (0.76×10^6 daltons) RNAs were gel purified by the NA 45 paper method (9,10). Northern blots of bean leaf RNA, BMMV-infected

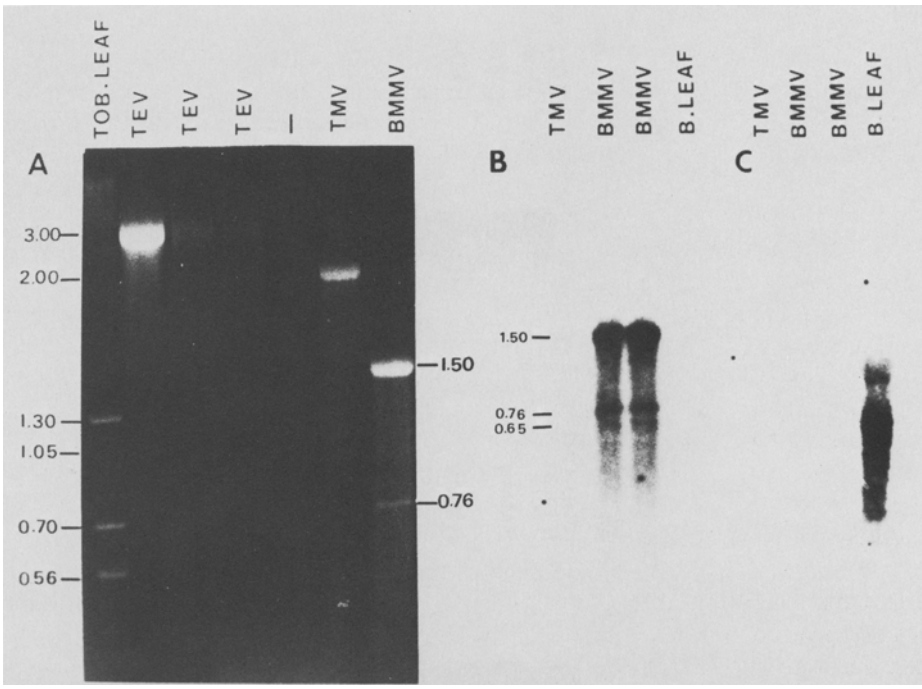

Fig. 1. Size analysis and hybridization of BMMV RNA probed with ^{32}P-labeled cDNA to BMMV RNA or bean leaf RNA. *A:* BMMV RNA and other RNAs of known molecular weights were separated by electrophoresis on 0.8% agarose gels under denaturing conditions and stained with ethidium bromide. Starting left to right the gels contained, respectively, tobacco leaf RNA (Tob. leaf) and three separate samples of tobacco etch virus RNA (TEV), tobacco mosaic virus RNA (TMV), and BMMV RNA. The molecular weights of these RNAs are marked on the left and right of the figure. *B:* Northern blot of RNAs isolated from TMV and two separate samples of BMMV RNA and bean leaf RNA probed with randomly primed ^{32}P-labeled BMMV cDNA probe. The molecular weights are marked on the left. *C:* Northern blot of RNAs as in Fig. 1B but probed with a randomly primed ^{32}P-labeled cDNA to bean leaf RNA.

leaf RNA, total virus RNA, and gel-purified R1 and R2 RNAs were probed with ^{32}P-labeled cDNA probes prepared using R1 or R2 RNAs as templates. Both R1 and R2 cDNA probes hybridized to R1 as well as to R2 RNAs, which showed that both RNAs shared sequence homologies (Fig. 2A,2B).

Dot blot and northern blot analysis of RNA, and northern blot analysis of proteins, from bean plants infected with gel-purified R1 or R2 RNA showed that R1 alone is infectious. No viral RNA or proteins could be recovered from plants infected with R2 alone. Further, plants infected with R1 alone showed the presence of R2 RNA. Thus, R2 appears to be a subgenomic RNA derived from R1.

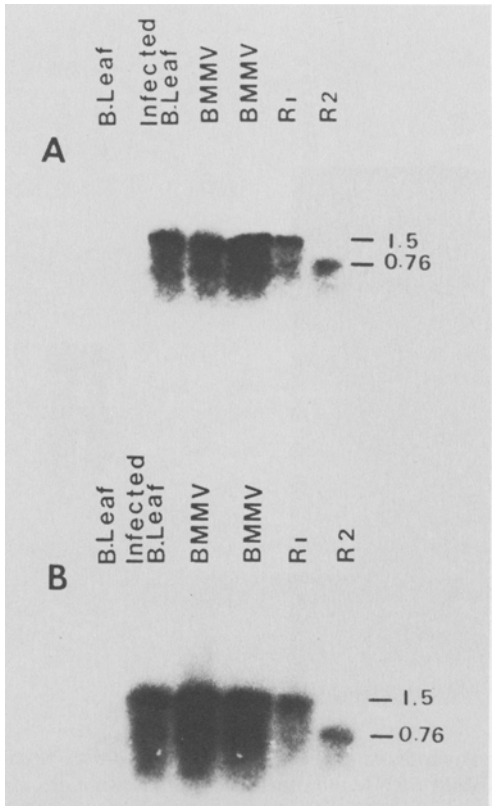

Fig. 2. Analysis of gel-purified BMMV RNA of mol. wt. 1.5×10^6 daltons (R1) and 0.76×10^6 daltons (R2) by northern blot analysis using cDNA probes to R1 (A) or R2 RNA (B). *A:* RNA samples from bean leaf (B. leaf), BMMV-infected bean leaf (infected B. leaf), total BMMV-RNA, gel-purified R1 RNA, and gel-purified R2-RNA were separated by electrophoresis on 0.8% agarose gels and subjected to northern blot analysis using cDNA to R1 RNA as a probe. *B:* Northern blots of the various RNAs identified in Fig. 2A probed with ^{32}P-labeled cDNA to gel-purified R2 RNA.

The viral capsid protein(s)

Viral capsid proteins from BMMV were extracted either by treatment with 2 volumes of glacial acetic acid (17) or by boiling the virus solutions in Laemmli dissociation buffer (14), and were analyzed by Coomassie blue staining after polyacrylamide gel electrophoresis (Fig. 3). Alternately, immunoblots of the electrophoretically separated proteins were treated with primary antibodies (anti-BMMV) followed by secondary antibodies (goat anti-rabbit conjugated with alkaline phosphatase) and AP-color reagent. In both cases, a single major protein of mol. wt. 40.5 kD was found along with a barely visible minor protein of 81 kD. We conclude that the major protein is the capsid protein monomer, and we infer

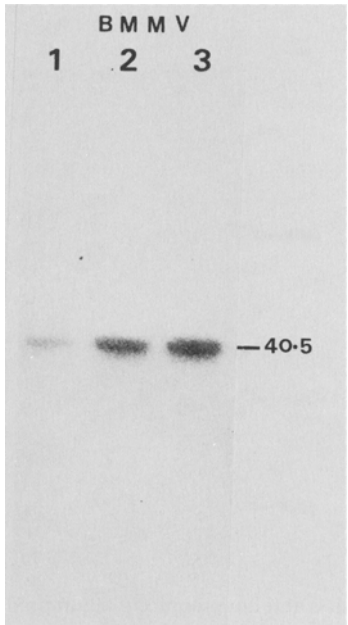

Fig. 3. Polyacrylamide gel electrophoresis of purified capsid protein from BMMV. Lanes 1, 2, and 3 contain 1, 2, and 5 μg, respectively, of purified capsid protein prepared from BMMV. The molecular weight capsid protein monomer (40.5) is shown. The gel was stained with Coomassie blue. An 81.0 kD component is not visible in the print but is seen in the gel.

that the minor protein is a dimer of the capsid protein. Other isometric viruses have been shown to produce coat protein dimers (18).

Cell-free translation

Purified BMMV-RNA was translated in an in-vitro translation system prepared from rabbit reticulocyte lysate or from wheat-germ extracts. The major translation products had molecular weights of 40.5 kD and 25.7 kD. Other translation products found had molecular weights of 87 kD, 57 kD, and 49 kD (Fig. 4A).

Sucrose-gradient-purified or NA45-paper-purified R1 and R2 RNAs were also translated in the reticulocyte system. The R1 translation products were similar to those of total BMMV RNA translation, whereas, the R2 RNA was translated into a 40.5 kD product only (Fig. 4A).

Immunoprecipitation studies using anti-serum to BMMV to precipitate the in-vitro products showed that all three RNAs (total BMMV RNA, and R1 and R2 RNAs) could be translated to produce the 40.5 kD capsid protein (Fig. 4B). Im-

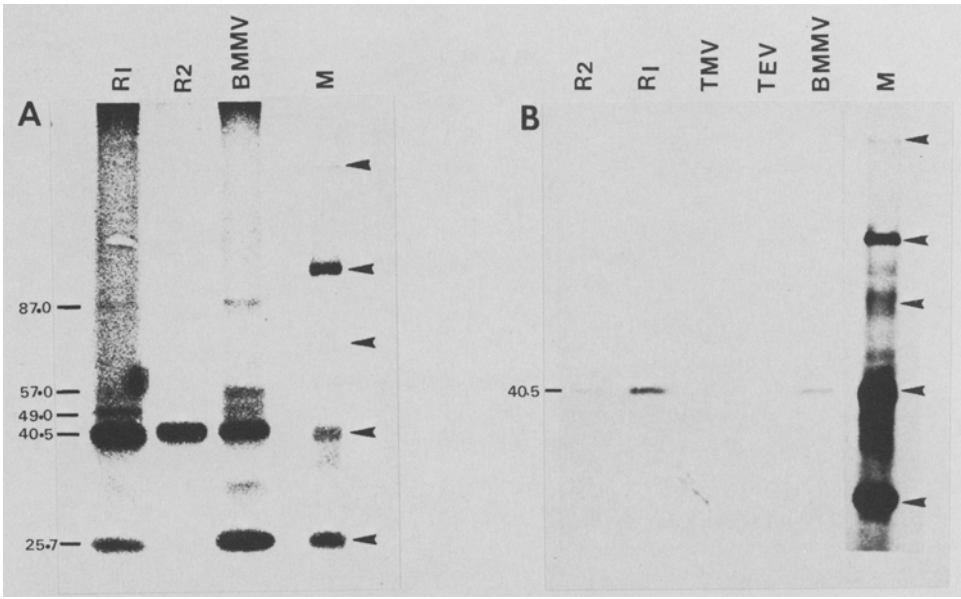

Fig. 4. Total translation products and immunoprecipitation products generated after in-vitro translation of BMMV, R1, and R2 RNAs. *A:* ^{35}S-labeled translation products after translation of R1, R2, and BMMV RNAs. Translation products of mol. wt. 87×10^3, 57×10^3, 49×10^3, 40.5×10^3, and 25.7×10^3 are shown. The lane marked "M" contains ^{14}C mol. wt. marker proteins shown by arrows of mol. wt. 200 kD, 97.4 kD, 68 kD, 43 kD, and 25.7 kD. *B:* ^{35}S-labeled in-vitro translation products obtained after translation of RNAs from BMMV, TEV, TMV, R1, R2, and endogenous RNAs were immunoprecipitated with antiserum to BMMV capsid protein. The precipitates were subjected to electrophoresis on 14% polyacrylamide gels and fluorographed. The lane on the right marked "M" contains ^{14}C-labeled marker proteins of known molecular weights, as in Fig. 4A. The capsid protein (40.5) is indicated.

munoblots of dissociated virus proteins and in-vitro translation products of BMMV RNA, and R1 and R2 RNAs probed with anti-BMMV serum and GAR-AP secondary antibodies also showed that the 40.5 kD capsid protein is a product of both R1 and R2 RNAs.

Time-course studies on the appearance of various translation products programmed by purified R1 RNA as a function of time of translation showed that the earliest product to appear is the 25.7 kD protein. No conclusions could be drawn with respect to appearance of the other proteins (Fig. 5).

Serological tests

Serological tests using homologous and heterologous antisera showed that BMMV is unrelated to RCNMV, SCNMV, and CpMV. Previous tests by other investigators had shown that BMMV is serologically unrelated to several other viruses (3,4).

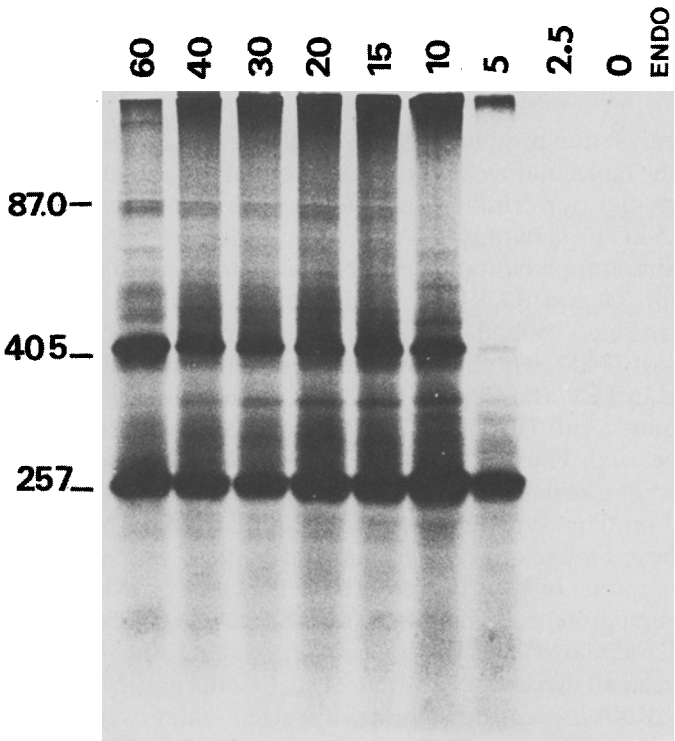

Fig. 5. Fluorogram of ^{35}S-labeled products obtained after various times of translation of R1-BMMV RNA. R1 RNA was translated in the rabbit reticulocyte lysate system, and starting from time 0 the translations were stopped at various time periods and the products generated at each time period were separated by polyacrylamide gel electrophoresis and subjected to fluorographic analysis. The 25.7, 40.5, and 87.0 $\times$ 10^3 dalton products are identified. The time of translation in minutes is indicated.

Discussion

Previous reports on BMMV have dealt with its pathology, transmission, and serology. Only limited information on the characteristics of the viral genome was available (3,4). Thus the early reports described the virus as containing a single RNA of mol. wt. 1.27 $\times$ 10^6 daltons based on electrophoresis of RNA under nondenaturing conditions. However, the experiments reported here where electrophoresis of RNA was carried out under denaturing conditions showed that at least two RNAs of mol. wt. 1.5 $\times$ 10^6 daltons and 0.76 $\times$ 20^6 daltons are associated with viral preparations. Of these, this study has shown that the larger RNA (R1) is the genomic RNA, whereas the shorter one is subgenomic in nature. Both RNAs are efficient messengers in vitro with the R2 RNA coding for the capsid protein. R1 RNA is also translatable into capsid protein in vitro, and thus the need for generation of subgenomic RNA for capsid protein synthesis is not clear. A similar situation also

prevails in the case of TCV and CarMV (18,19). It is possible that in vivo in these viruses, the initiation site for capsid protein synthesis is unavailable for initiation, necessitating the synthesis of a subgenomic RNA for efficient synthesis of the capsid protein.

The major translation products of BMMV RNA are proteins of mol. wt. 40.5 kD and 25.7 kD. The molecular weights of these proteins are similar to the major translation products of turnip crinkle virus (TCV) and carnation mottle virus (CarMV) RNAs. The 40.5 kD protein of BMMV has been shown in this study to be the capsid protein by immunoprecipitation experiments. This protein is also a translation product of both R1 and R2 RNAs. Similarly, the capsid proteins of TCV and CarMV are translated by both genomic and subgenomic RNAs and have molecular weights of 38 kD in both cases. The capsid protein gene has been placed at the 3′ most end in TCV and Car MV (18-20). A further similarity between BMMV translation products and TCV, and CarMV is in regard to the size of the first product to be synthesized. Thus in BMMV, translation time-course studies show that the first product of translation has a mol. wt. of 25.7 kD, which is similar to the 25 kD and 27 kD proteins synthesized by TCV and Car MV RNAs during in-vitro translation. These proteins have been mapped at the 5′ end of the RNA. Lastly, like the 87 kD product of BMMV translation, TCV and CarMV translations produce read-through proteins of mol. wt. 80 kD and 86 kD respectively (18-20). Thus, although TCV and CarMV are serologically unrelated to BMMV, there is strong similarity between all these viruses with respect to virus particle size, genome size, subgenomic RNAs, capsid protein size, and types of major translation products. These studies lend support to the already suggested inclusion of BMMV as a prospective member of the newly proposed carmovirus group (18).

Acknowledgments

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