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The Barley Stripe Mosaic Virus 58-Kilodalton βb Protein Is a Multifunctional RNA Binding Protein

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The barley stripe mosaic virus (BSMV) βb gene product is the major viral nonstructural protein synthesized during early stages of the infection cycle and is required for systemic movement of the virus. To examine the biochemical properties of βb, a histidine tag was engineered at the amino terminus and the protein was purified from BSMV-infected barley tissue by metal affinity chromatography. The βb protein bound ATPs in vitro, with a preference for ATP over dATP, and also exhibited ATPase activity. In addition, βb bound RNA without detectable sequence specificity. However, binding was selective, as the βb protein had a strong affinity for both single-stranded (ss) and double-stranded (ds) RNAs but not for tRNA or DNA substrates. Mutational analyses of βb purified from *Escherichia coli* indicated that the protein has multiple RNA binding sites. These sites appear to contribute differently, because mutants that were altered in their binding affinities for ss and ds RNA substrates were recovered.

The movement of plant viruses from cell to cell is presumed to occur through plasmodesmata, which are intercellular channels between plant cells that permit movement of macromolecules (for reviews, see references 20 and 46). Viruses are thought to move from cell to cell via plasmodesmata by two mechanisms. In the first mechanism, virus particles are believed to move from cell to cell via tubular structures that pass through and extend from plasmodesmata. Several viruses, including tomato ringspot virus (43), cowpea mosaic virus (42), and cauliflower mosaic virus (27), have virus-encoded movement proteins (MPs) associated with these modified plasmodesmata. In the second mechanism, MPs apparently facilitate the cell to cell movement of infectious entities consisting of viral RNA associated with MPs. Studies with several MPs, including those encoded by tobacco mosaic virus (TMV) (39, 44, 45), red clover necrotic mosaic virus (RCNMV) (11), and bean dwarf mosaic virus (24), suggest that these virus-encoded proteins also have the dual function of binding to nucleic acids and increasing the permeability of plasmodesmata.

Barley stripe mosaic virus (BSMV), the type member of the *Hordeiviridae*, is a positive-sense, single-stranded (ss) RNA virus composed of three genome components, designated α, β, and γ, that encode a total of eight proteins. The α and γ RNAs are strictly required for viral replication, while the β RNA is necessary for systemic movement (29, 30, 47). The β RNA contains five open reading frames (ORFs). The 5′-proximal ORF encodes the coat protein, which is dispensable for viral infectivity (31). Following the coat protein and an intergenic region are a series of overlapping ORFs (βb, βd, and βc) termed the triple gene block. A triple gene block is present not only in hordeivirus genomes but also in those of carla- furo-, and potexviruses (15). Recently, we have shown that the triple gene block of BSMV is unique among these viruses in that it encodes a fourth protein, βd′, which results from a translational read-through of the βd amber stop codon (47). The three major proteins encoded by the triple gene block have been shown to be individually required for movement of BSMV (31), white clover mosaic potexvirus (1), and beet necrotic yellow vein furovirus (BNYVV) (12), whereas the BSMV βd′ read-through derivative is not required for systemic movement (18). The βb homologs encoded by the 5′-proximal ORFs of each triple gene block contain a nucleoside triphosphate (NTP) binding-helicase domain that appears to be related to helicase superfamily II (13–15). The two remaining triple gene block ORFs encode hydrophobic proteins that appear to be associated with membranes (9, 23). At present, little is known about the biochemical natures of the proteins encoded by the triple gene block. However, the proteins encoded by the 5′-proximal ORF of the triple gene blocks of foxtail mosaic potexvirus (FMV) (35) and BNYVV (2) have been purified following overexpression in *Escherichia coli*. The 26-kDa FMV protein was shown to bind RNA and NTPs and to exhibit ATPase activity in vitro, while the 42-kDa BNYVV protein was shown to bind both ss and double-stranded (ds) RNA and DNA in vitro.

Variation occurs with respect to the sizes of the proteins involved in the movement of viruses containing the triple gene block. The triple gene block-encoded proteins lack obvious relatedness to the TMV MP. However, complementation experiments using mixed viral inocula have shown similarities in function, since the cell to cell movement of BSMV can be complemented by TMV (21). In addition, replacement of the BSMV triple gene block by the TMV MP gene permits limited viral movement in common hosts but not in selective hosts (38). Although complex interactions can influence systemic movement of BSMV (28), the available evidence suggests that the most obvious candidate for the BSMV homolog of the TMV MP is the βb 58-kDa protein. BSMV βb accumulates early in infection (9) and is associated with viral RNA in vivo (3). To extend these studies, we have engineered a histidine tag at the amino terminus of βb, purified the protein from BSMV-infected barley by metal affinity chromatography, and performed biochemical characterizations of the protein. In addition, we have recovered wild-type and mutagenized forms of βb from *E. coli* and have compared the RNA binding properties of the wild-type and mutant βb derivatives.

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence	Description
βMObM1	GGCTCAGGACGATCAACCATTGTGC	Mutagenesis of K→R in domain I
βMObM2	GGCTCAGGAGCATCAACCATTGTGC	Mutagenesis of K→A in domain I
βMObM3	GATTTATTGATCATAAATCAATATACACTTGCT	Mutagenesis of D and E→N and Q in domain II
βMObM4	GTGTTGTTAGTCGCGAATGTAGCTGAAGGAAAAGC	Mutagenesis of G, D, and Q→A, N, and E in domain III
βMObM5	AGACGACTTATGCTTTGGGACAAGAGAC	Mutagenesis of R→A in domain IV
βMObM6	GCAATTGATGTGGAAGCGAAAGAATTCGAT	Mutagenesis of Q and G→E and A in domain V
βMObM7	GCTTTGAGCGCACATAAGTCGAAGT	Mutagenesis of R→A in domain VI
βMObΔ3	TGCAGCAAGCAGGGTAACAGATCTGCAAAACCGGATGAAGACCAC	Introduction of *Bgl*II site
βMObΔ4	AAGTTGGCAACAACTGTGGAATTCTTTGAAATGATTAAGCTCGCAT	Introduction of *Eco*RI site
βMObΔ5	AAGGAAACTCTAAAAGTTGCCTTGGCAACAACTGTGGAAAAGGAAC	Introduction of *Sty*I site
βMObΔ6	GATTCAGTGAAAGGTGTTTTTAAAGTTGCCGATCAGACTCCATT	Introduction of *Dra*I site
βMObΔ7	AATGTGAGTGAGAACTATACT<>CGACAGTATTTCAGAGCTAGAT	Primer used to delete 58 amino acids in βb
βMObΔ8	GAACCTGAGTTGAAACCCGGGATTTCTAAGGAAGCAGCT	Introduction of *Sma*I site
βMObΔ9	GTTTAGTCGCTTTGAGCAGATCTGCAAAACCGGATGAAGACCAC	Introduction of *Bgl*II site
βMObΔ10	GTGGAAGGGACACAGTGATCGATTGTGCCCTGGCAATTGAT	Introduction of *Cla*I site
βMObΔ12	TGTGAAAGATTGCGGGTAC<>CATAAGTCGAAGTTAATCATC	Primer used to delete 40 amino acids in βb
β-O-Sal	GAAATACTGTCGACCAGTGTAC	Primer used in PCR
βbhis-1	GGCCCATGGGACATCACCATCACCATCACGACATGACGAAAACTGTTG	Introduction of his-tag by PCR
βb-2	GGACAGGAAGTGGGTGGTG	Mutagenesis of βb′ AUG→GUG

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used for expression of recombinant BSMV βb protein have been described previously (9).

Cloning and mutagenesis. Site-directed mutagenesis was performed on β42SpI, a full-length cDNA clone derived from the β RNA of the ND18 strain of BSMV, and B7 (31), a mutant of β42SpI lacking the βa AUG codon. The gene product βb′ is initiated from an AUG located 102 nucleotides (nt) downstream of the βb AUG. To prevent expression of βb′, the AUG used for initiation of βb′ was mutagenized to GUG essentially as described previously (16) to generate B7.β8 from B7. Subsequently, sequences encoding a 6-histidine tag (his-tag) were inserted directly after the βb AUG in B7.β8 by a PCR procedure. For this purpose, oligonucleotides β-O-Sal and βbhis-1 (Table 1) were used to generate a PCR product which was subsequently digested with *Nco*I and *Sal*I and ligated into *Nco*I- and *Sal*I-digested B7.β8 to generate B7.β8His6.

A 10-bp substitution containing an *Xho*I site was engineered adjacent to the βb AUG of β42SpI to generate β42b1. Additional mutagenesis was performed on β42b1 with the oligonucleotides in Table 1. The Δ1 deletion mutant was created by digestion with *Pst*I and *Sal*I, treatment with Klenow fragment, and subsequent ligation, while the Δ2 deletion mutant was generated by digestion with *Bcl*I and subsequent religation. The Δ11 deletion mutant was created by digestion with *Sal*I and *Ssp*I and subsequent ligation. These BSMV βb deletion mutants were subcloned into the expression vector pET3aXL (9) by excision of the mutated βb ORF from the respective β42 deletion derivative with *Xho*I and *Ssp*I and ligation to pET3aXL between the *Xho*I and Klenow fragment-treated *Bam*HI sites. All mutations were confirmed by DNA sequence analysis.

Purification of his-tagged βb protein from infected barley. Prior to transcription reactions, the cDNA clones of the α and γ RNAs were linearized with *Mlu*I while β cDNA clones were linearized with *Spe*I. Transcripts were prepared and inoculated onto 7-day-old barley plants as described previously (30). Systemically infected barley leaves were harvested 6 days postinoculation and extracted in buffer C (50 mM Tris [pH 8.0], 600 mM NaCl, 0.1% Nonidet P-40, 5% glycerol, 0.1% β-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin per ml, and 0.5 μg of leupeptin per ml (1:4, tissue/buffer) at 4°C. All further steps were performed at 4°C. The extract was strained through four layers of cheesecloth and centrifuged at 8,000 rpm (Sorval SS34 rotor) for 10 min. The supernatant was recovered and centrifuged at 21,000 rpm (Beckman Ti70.1 rotor) for 35 min. The supernatant was then applied to a 1-ml Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) column equilibrated in buffer C. The column was washed with 30 ml of buffer C, 10 ml of buffer C containing 25 mM imidazole, and 10 ml of buffer C containing 75 mM imidazole. Proteins were eluted from the column with buffer C containing 150 mM imidazole. Column fractions containing the βb protein were either assayed directly or pooled and dialyzed against buffer D (50 mM Tris [pH 8.0], 40 mM NaCl, 0.1% Nonidet P-40, 5% glycerol, 0.1% β-mercaptoethanol) for 4 to 5 h with two changes of buffer.

Purification of recombinant βb proteins from *E. coli*. Wild-type and mutated forms of the βb protein were purified from *E. coli* [BL21(DE3)pLysE] transformed with pET3βb plasmids. For these experiments, we used at least two independent plasmid transformants with independently verified cDNA clones. Protein was purified essentially as described by Citovsky et al. (4) with modifications for solubilization of recombinant protein from *E. coli* inclusion bodies (22). Purified proteins were examined following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by Coomassie blue staining and Western blot analysis (7). Aliquots of βb which were frozen at −70°C retained full RNA binding activity for as long as a year after preparation. In contrast, some of the deletion mutant preparations, such as that with Δ4, had significant losses of activity within a week after purification. Therefore, to ensure experimental reproducibility, at least two independent preparations were tested in vitro for biochemical activity.

Biochemical assays. (i) RNA binding. Native PAGE was used to resolve protein-RNA complexes from free radiolabeled probe in the mobility shift assay essentially as described previously (8). RNA and DNA probes (8) were diluted to 10 ng/μl in buffer C for βb purified from BSMV-infected barley or in buffer L (10 mM Tris [pH 8.0], 200 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol) for βb purified from *E. coli*, heated at 70°C for 3 min, and cooled rapidly on ice before use. Protein was mixed with 10 ng of probe RNA in a final volume of 20 μl of buffer C or L and incubated at 4°C for 30 min before electrophoresis. In competition experiments, protein was preincubated with unlabeled competitor RNA or DNA for 20 min before the probe was added. After an additional 20 min of incubation at 4°C in the presence of the probe, the samples were analyzed on a 4% native polyacrylamide gel.

(ii) Cross-linking of NTPs or RNA. Purified protein was incubated with [α-^{32}P]NTP or RNA probe as described previously (35). Protein was analyzed by SDS-PAGE followed by Coomassie staining and autoradiography.

(iii) ATPase assays. βb protein preparations were tested for ATPase activity by a charcoal adsorption assay (37). RNA substrates (5 to 10 μg per reaction mixture) were added to reaction mixtures to test their effects on the ATPase activity.

RESULTS

Infectivity of βb mutants. We have previously shown that βb is essential for movement in plants (29, 31). To investigate motifs and/or regions in βb that are important for cell to cell movement, deletion and point mutations were introduced into βb. The amino acid sequence of βb was aligned with other putative helicases (14) (Fig. 1A), and point mutations (M1 to M7) were introduced in the conserved domains I to VI (Fig. 1B and Table 1). The effects of the point and deletion mutations were tested by inoculating barley with wild-type α and γ RNAs and with β RNA containing mutations.

Two of the βb deletion mutants retained infectivity in barley. The first mutant, βbΔNco.7, eliminated expression of the first 35 amino acids by an 8-nt deletion which destroyed the 5′-proximal initiating AUG. As reported earlier (32), this mutation had no pronounced effect on the symptom phenotype. The

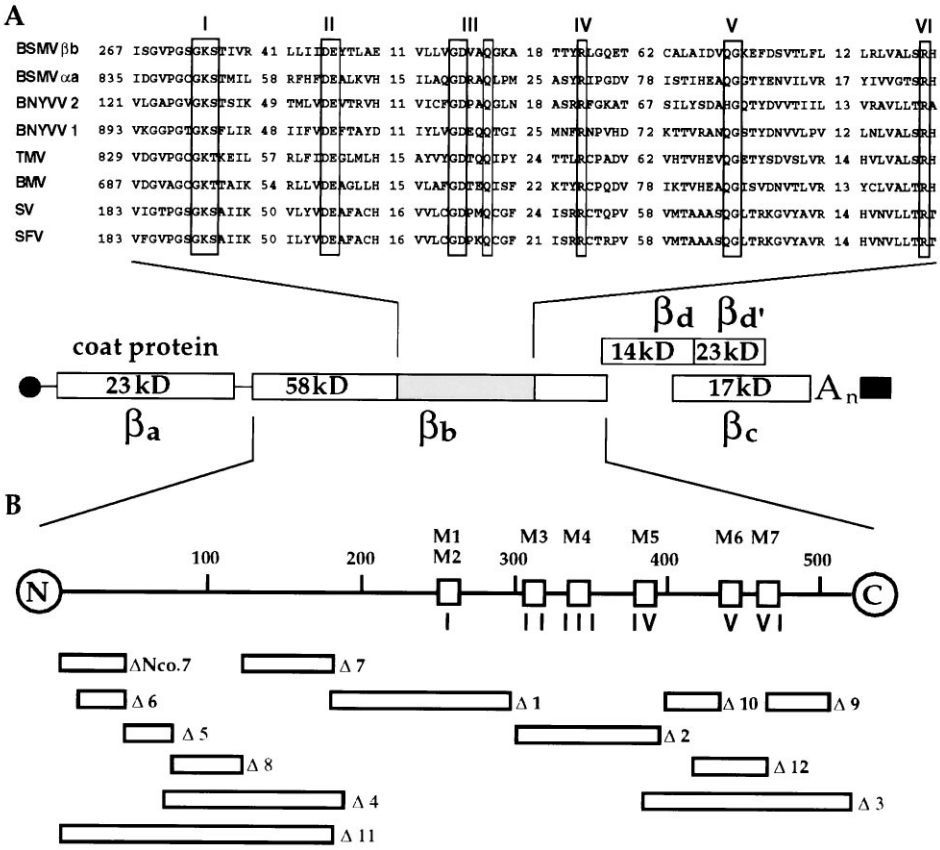

FIG. 1. (A) Amino acid sequences of BSMV, BNYVV, TMV, brome mosaic virus (BMV), Sindbis virus nsP2 (SV), and Semliki Forest virus nsP2 (SFV) were aligned. The boxed regions labeled I to VI indicate the relative positions of the blocks of homology that define an RNA helicase motif; such motifs are common to superfamilies of replicative proteins involved in nucleic acid replication and recombination (15). (B) Schematic illustration of the deletion and missense mutants constructed within the βb protein. The mutant forms of the βb ORF were subcloned into the full-length β cDNA clone or the T7 polymerase expression vector pET3aXL. Invariant amino acids within the blocks of homology were targeted by the introduction of missense mutations (M1 to M7). Deletions in the βb gene are designated as open boxes.

second nonlethal mutant, Δ5, caused an attenuated phenotype in which symptoms were delayed, erratic, and less uniform than in wild-type infections. Levels of the coat protein and the mutant βb protein, which was distinguishable from wild-type βb protein by its mobility, also appeared to be normal (data not shown). The βb deletion mutants Δ11 and Δ2 were not tested for infectivity on barley. However, these two deletion mutants are expected to have null mutations, since smaller deletions spanning the region deleted by Δ11 and point mutations M3, M4, and M5 located within deletion Δ2 eliminated infectivity. Each of the mutations (M1 to M7) targeted amino acid residues that are strictly conserved among the plant viral RNA helicase-like motifs, and each eliminated the ability of BSMV to infect barley systemically. These results indicate that multiple motifs within βb are required for virus movement and that the invariant amino acids within the helicase motif are essential for this process.

Purification of BSMV βb protein from infected barley. To permit purification of βb by metal affinity chromatography, a his-tag was engineered at the N terminus of βb (B7.β8His6). In this cDNA clone (Fig. 2), the AUGs of the coat protein and βb′ were mutagenized. These properties enabled us to conduct experiments with a derivative expressing only a single βb protein analog. More importantly, however, use of the B7 derivative eliminated the complicating effects of the coat protein, which is present in large quantities in wild-type-infected tissue and forms aggregates that potentially could have interfered with the purification and analysis of βb. Plants inoculated with β RNAs containing these mutations developed systemic infections within 5 days postinoculation, but the plants were more stunted than those infected with wild-type virus and, as previously reported (31, 32), the symptoms did not progress to the milder chronic recovery stage characteristic of wild-type infections.

The wild-type α and γ RNAs were coinoculated with the B7.β8His6 RNA (designated his-tagged βb) or B7.β8 RNA (designated wild-type βb because the his-tag was lacking) into barley. The presence of the his-tag at the N terminus of βb appeared not to alter the timing or severity of symptoms compared to those of infections with B7 derivatives unable to express the coat protein (data not shown). The his-tagged βb was purified from systemically infected barley leaves collected 6 days postinoculation, when high levels of βb appear (data not shown). A series of imidazole washes were used to remove nonspecifically bound proteins (Fig. 2). Following the 25 mM imidazole wash, a more stringent 75 mM imidazole wash was used for removal of contaminating proteins, and during these steps a proportion of the βb protein was also eluted from the column. However, in the final elution (150 mM imidazole), a single major protein species that corresponded to βb in size and that cross-reacted with βb antiserum was detected (Fig. 2B, lanes 3 and 7). The βb protein was not detected following

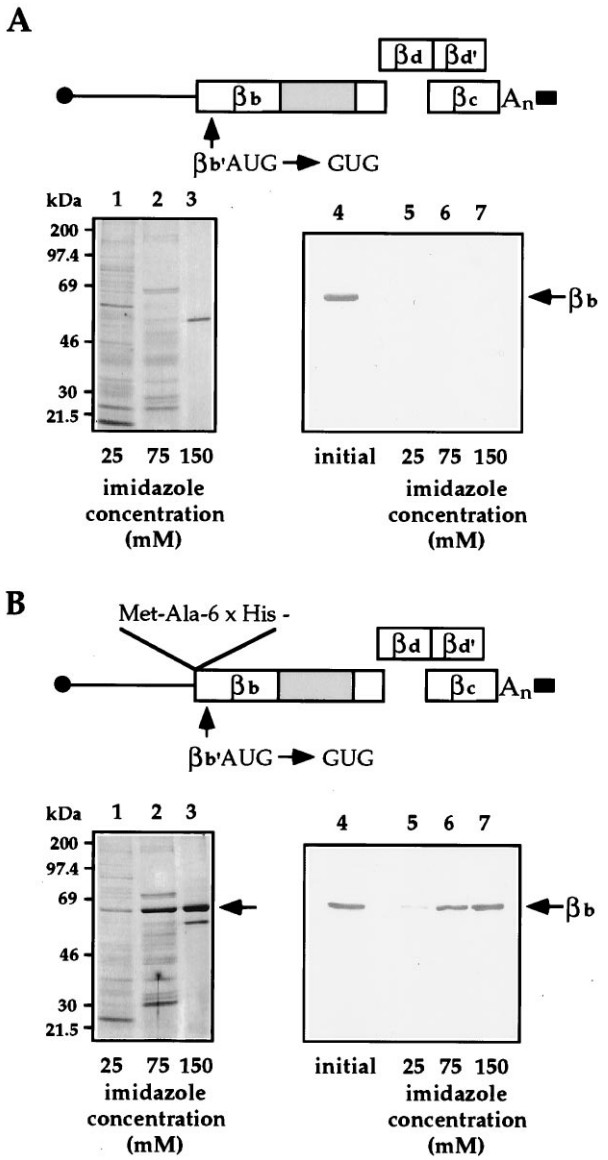

FIG. 2. Analysis of proteins eluted from Ni-NTA affinity columns loaded with either BSMV wild-type (A) or his-tagged (B) βb extracts. The schematic illustrations above each panel show the wild-type or his-tagged βb RNAs used to infect plants. The coat protein gene is absent from these constructs, as it is not expressed. Proteins eluted from affinity columns by washes containing different concentrations of imidazole were separated on a 10% polyacrylamide-SDS gel followed by silver staining (lanes 1 to 3) or immunoblotting with antiserum against βb (lanes 4 to 7). Molecular mass markers are shown at the left.

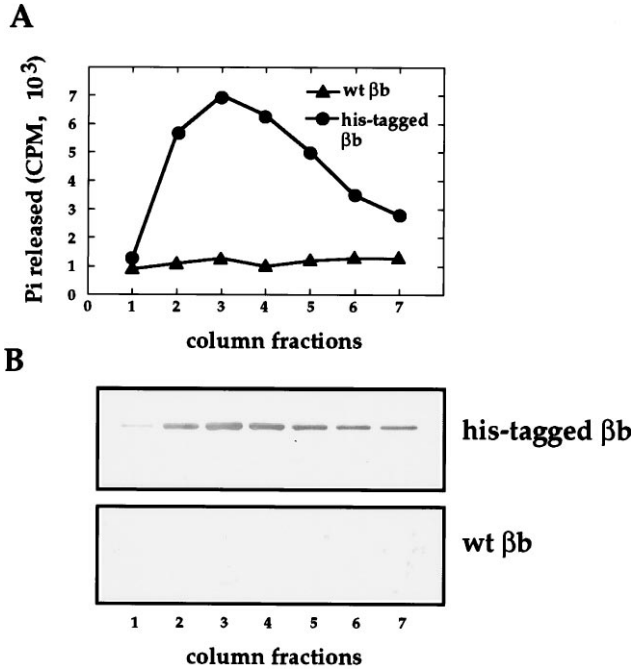

FIG. 3. Analysis of proteins eluted from Ni-NTA-affinity columns with 150 mM imidazole. Fractions from columns loaded with either wild-type or his-tagged βb extracts were analyzed for ATPase activity (A) or on a 10% polyacrylamide-SDS gel followed by immunoblot analysis with antiserum against βb (B). wt, wild type.

150 mM imidazole elution from a column that was loaded with wild-type extracts (Fig. 2A, lanes 3 and 7). However, small amounts of a host protein of approximately 52 kDa were present following the final elution in both the his-tagged and wild-type βb preparations.

βb exhibits ATPase activity and ATP binding. Helicase superfamily II, to which BSMV βb belongs (13, 15), includes putative RNA helicases of positive-strand RNA viruses that contain highly conserved regions thought to be involved in RNA binding, NTP binding, and ATPase activity (Fig. 1). To determine if ATPase activity was associated with his-tagged βb, individual column fractions were analyzed for the amounts of βb detected in immunoblots (Fig. 3B) and assayed for their ability to hydrolyze γ-^{32}P-labeled ATP (Fig. 3A). ATPase activity in the column fractions correlated quantitatively with the presence of his-tagged βb. No substantial activity was detected in the column fractions derived from tissue infected with wild-type virus, suggesting that the minor 52-kDa contaminating protein did not contribute to the ATPase activity. Because βb has a helicase motif, the ability of RNA to stimulate the ATPase activity associated with βb was also tested. However, we were unable to detect stimulated ATPase activity following addition of ss or ds RNA from either a viral or nonviral source, even though RNA was not found associated with the purified protein (data not shown).

βb also contains an NTP binding domain; therefore, nucleotide binding and the specificity of binding were investigated. Purified βb was incubated with α-^{32}P-labeled ATP, CTP, GTP, UTP, or dATP, and protein-nucleotide interactions were examined following UV cross-linking and SDS-PAGE. Coomassie staining for visualization of βb was followed by autoradiography to permit detection of the binding of the radioactive nucleotide species (Fig. 4). ATP was bound by βb, and dATP was bound to a lesser extent, but CTP, GTP, and UTP failed to bind to βb.

βb has RNA binding activity. The ability of his-tagged βb to bind RNA probes in vitro was initially assessed by incubating radiolabeled RNA with purified βb. The protein-RNA complexes were subjected to UV cross-linking followed by digestion with RNase A and analysis by SDS-PAGE (Fig. 5A). A 3.3-kb β RNA probe, a 256-nt probe complementary to the 3′ terminus of all three BSMV RNAs, a 220-nt probe complementary to the γa-γb intergenic region, and a 182-nt *petD* probe that corresponds to the 3′ end of the spinach chloroplast *petD* gene were all bound by βb. In control experiments, RNA-βb complexes were not subjected to UV cross-linking prior to RNase A treatment and SDS-PAGE, and a signifi-

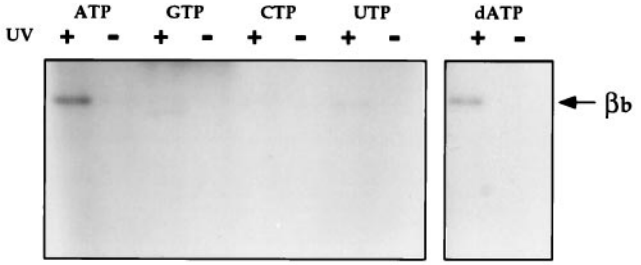

FIG. 4. Nucleotide binding of βb. βb was incubated in the presence of α-^{32}P-labeled NTP or dATP for 15 min at room temperature followed by UV cross-linking. Samples were analyzed on a 10% polyacrylamide-SDS gel followed by Coomassie blue staining and autoradiography.

cantly reduced level of RNA was associated with βb. Therefore, βb is able to bind to RNA in a sequence-nonspecific manner.

To examine the stability of the RNA-βb interactions, RNA binding assays were performed, as described above, in the presence of different concentrations of salt (Fig. 5B). His-tagged βb was able to bind ss RNA in the presence of 40 to 300 mM NaCl. However, if the NaCl concentration was increased to >300 mM, βb failed to bind to the RNA probe. During the βb purification protocol, a concentration of 600 mM NaCl was used. Therefore, it is probable that the βb-RNA interactions observed previously (3) would have been disrupted during our extraction procedure.

The RNA binding activity of the his-tagged βb protein was

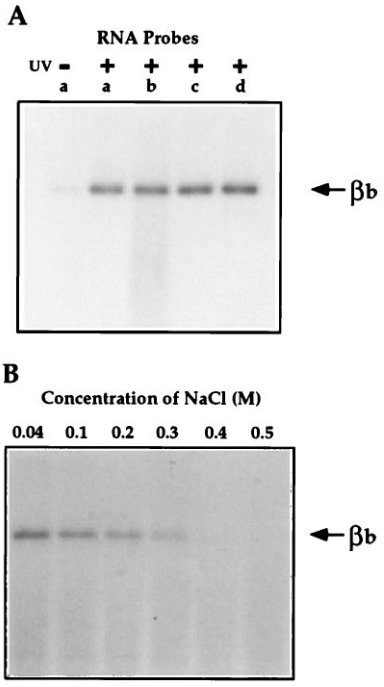

FIG. 5. RNA binding activity of βb. (A) βb was incubated with 10 ng of RNA probe for 15 min at room temperature, UV cross-linked (+) or not UV cross-linked (−), and subjected to RNase A digestion. Samples were analyzed on a 10% polyacrylamide-SDS gel followed by autoradiography. The labeled probes were the 256-nt RNA complementary to the common 3′ termini of BSMV RNAs (lanes a), the 3.3-kb β RNA (lane b), a 220-nt RNA complementary to the γa-γb intergenic region (lane c), and the 182-nt *petD* RNA (lane d). (B) Effect of salt on the ability of βb to bind RNA. βb was incubated with 10 ng of the 3.3-kb β RNA probe in the presence of different concentrations of NaCl for 15 min at room temperature. Samples were analyzed as described for panel A.

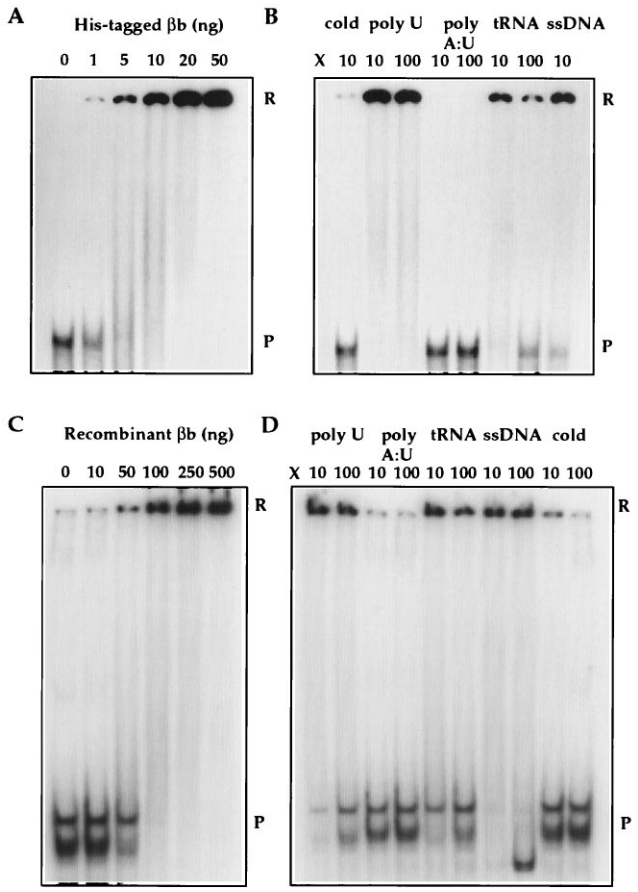

FIG. 6. RNA binding activity of βb isolated from infected plants or *E. coli*. (A) Ten nanograms of a 256-nt radiolabeled RNA probe corresponding to the common 3′ termini of BSMV RNAs was incubated with different amounts of his-tagged βb (plant derived) for 20 min at 4°C. The βb-RNA complexes (R) were separated from free probe (P) on a 4% native polyacrylamide gel and analyzed following autoradiography. (B) Competition experiments were performed by incubating unlabeled nucleic acids with 20 ng of his-tagged βb (the concentration for probe saturation) for 20 min at 4°C prior to the addition of the labeled RNA probe. The competitors (10:1 and 100:1 mass ratios relative to that of the probe) used were polyuridine (poly U), ds polyadenine-polyuridine (poly A:U), yeast tRNA (tRNA), ss DNA, and unlabeled probe (cold). (C) Ten nanograms of a 220-nt, radiolabeled RNA probe complementary to the γa-γb intergenic region was incubated with recombinant βb (*E. coli* derived) at 4°C for 20 min. βb-RNA complexes were analyzed as described for panel A. (D) Competition experiments were performed with 100 ng of recombinant βb (the concentration for probe saturation) and unlabeled nucleic acid molecules, all as described for panel B.

also investigated by gel mobility shift assays to resolve RNA-βb complexes formed after incubation of purified protein with radiolabeled RNA (Fig. 6A). Shifted RNA-βb complexes were observed as the amount of βb in the assays was increased. The specificities of the RNA-βb interactions were further assessed from the abilities of unlabeled RNA or DNA substrates to compete for binding with radiolabeled probe that had been preincubated with βb. Figure 6B shows the results of an experiment in which a 256-nt BSMV RNA probe corresponding to the 3′ conserved region of each of the BSMV RNAs was used to measure the affinity of βb for various competing substrates. The competitors, used in 10-fold and 100-fold excess (by weight) relative to the ss RNA probe, included polyuridine, ds polyadenine-polyuridine, yeast tRNA, ss DNA, and unlabeled RNA homologous to the probe RNA. The ss DNA, tRNA, and homopolymeric RNA competitors failed to com-

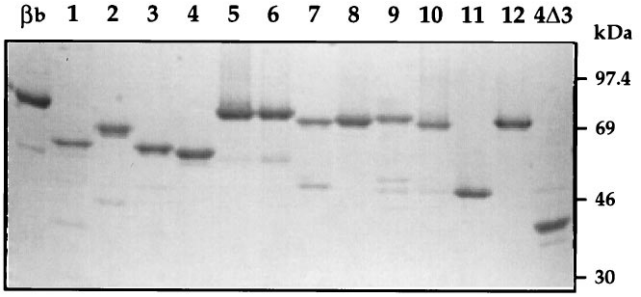

FIG. 7. Wild-type and deletion mutants of βb protein were purified from *E. coli*. Proteins were analyzed on a 10% polyacrylamide-SDS gel and visualized by Coomassie blue staining. Molecular mass markers are noted at the right.

pete efficiently with the ss RNA probe for binding. In contrast, the competition of polymeric synthetic ds RNA for binding was similar to the competition observed with homologous unlabeled RNA. Essentially identical results were obtained in RNA binding experiments with plus- and minus-sense RNA probes (data not shown). Thus, the results reveal that βb has selectivity in substrate binding, with a strong preference for ss or ds RNA. However, βb binding to RNA seems to be relatively sequence nonspecific. In addition, the lack of significant competitive RNA binding activity observed with homopolymeric polyuridine and with yeast tRNA suggests that βb may discriminate between different RNA substrates on the basis of RNA conformation.

Since βb was shown to bind RNA and ATP and to exhibit ATPase activity, additional experiments were conducted to assess the possible helicase activity of the protein. Several different ds RNA templates, including ds RNA regions ranging in size from 20 to 200 bp and containing blunt ends or a 3′ or 5′ overhanging sequence, were used as substrates in helicase assays in the presence of βb. However, helicase activity was not detected in any of these experiments (data not shown).

RNA binding by βb protein deletion mutants resolves multiple binding motifs. As shown earlier, the majority of the mutations introduced into βb rendered the virus noninfectious, so we were unable to recover mutant βb from barley for biochemical assays. Therefore, to map the βb domains involved in RNA binding, mutant βb protein derivatives were purified from *E. coli*. Protein of greater than 95% purity was obtained reproducibly from insoluble inclusion body preparations by solubilization in guanidinium HCl-NaCl buffer followed by dialysis (Fig. 7).

The RNA binding activity of βb produced in *E. coli* was assessed by gel mobility shift assays which showed, in agreement with the his-tagged βb results, that RNA-βb complexes were formed (Fig. 6C). In addition, RNA binding of the *E. coli*-derived βb protein was similar to that of the his-tagged βb protein isolated from BSMV-infected tissue in its selectivity of RNA binding (compare Fig. 6B and D). The *E. coli*-derived βb protein was also able to bind both ss and ds RNA derived from the BSMV RNAs and a *petD* (chloroplast-derived) probe and to protect them from RNase A digestion in vitro (data not shown).

The RNA binding activities of the purified mutant βb protein preparations were also compared by gel mobility shift analyses (Fig. 8). RNA specificity was monitored by the ability of RNA substrates to compete for binding with the 256-nt RNA probe complementary to the 3′ termini of the BSMV RNAs. Unlabeled polyuridine, the ds homopolymer polyade-

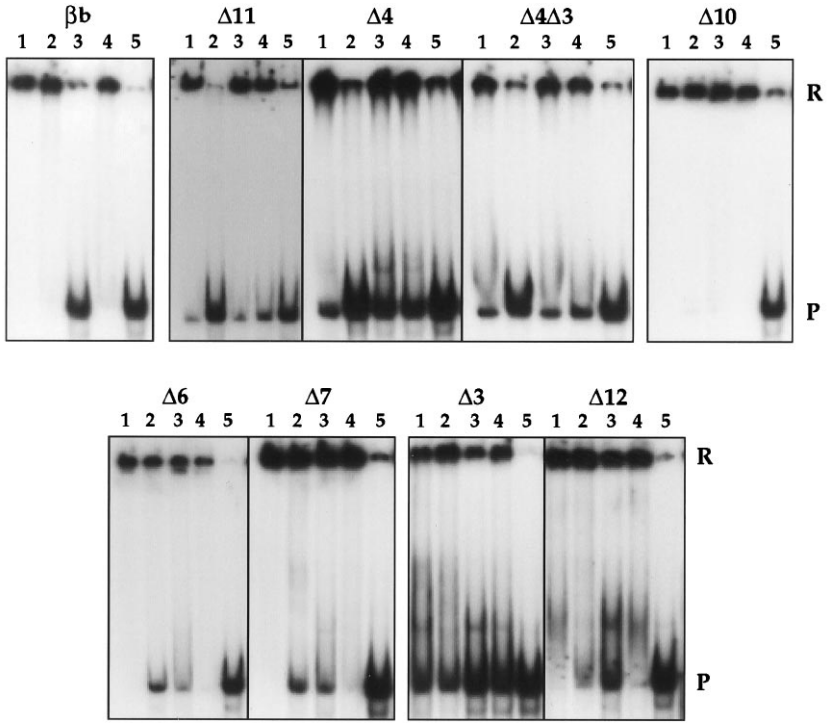

FIG. 8. RNA binding activities of βb deletion mutants. The ability of unlabeled RNA competitors at 10-fold (wt/wt) excess to compete for binding with the labeled, 256-nt RNA probe complementary to the 3′ termini of BSMV RNAs was measured at probe saturation as described in the legend to Fig. 6. The positions of the unbound probe (P) and the RNA-protein complexes (R) are shown at the side. Lanes 1, RNA-protein complex at probe saturation with no competitor; lanes 2, polyuridine; lanes 3, ds polyadenine-polyuridine; lanes 4, yeast tRNA; lanes 5, homologous probe.

nine-polyuridine, yeast tRNA, and RNA homologous to the probe RNA were used as competitors. Surprisingly, none of the deletions within the βb gene completely eliminated the RNA binding activity of the encoded protein. In fact, several deletions, including those of Δ1, Δ2, Δ5, Δ8, and Δ9, had no discernible effect on substrate specificity in competition experiments, as they exhibited the same band shift profiles as those of the full-length βb protein (data not shown). However, other deletions had a substantial effect on the selectivity of binding to the synthetic polymeric ds RNA and ss RNA competitors but not to yeast tRNA or the homologous BSMV RNA competitors. These effects could be separated into four characteristic classes (Fig. 8). The first class, consisting of the effects of the deletion mutants Δ11 and Δ4 and the double mutant Δ4Δ3, was the most dramatic because the binding affinities of these βb derivatives for synthetic homopolymeric RNAs relative to unmutated βb were reversed. The mutant proteins simultaneously gained an affinity for the ss polyuridine substrate in the competition experiments that was not previously detected in βb and lost (Δ11 and Δ4Δ3) or significantly reduced (Δ4) the ability to bind to ds RNA. The second binding affinity class, which was exhibited only by Δ10, was characterized by a complete loss of competitive ds RNA binding. The third and fourth classes consisted of intermediate effects on RNA binding activity with respect to the first two classes. In the third class, the effects of the deletion mutants Δ6 and Δ7 were similar to those of the first class (mutants Δ11 and Δ4Δ3), but they exhibited a moderate reduction in competition for the ds, synthetic RNA substrate, with the concomitant appearance of a small, but detectable, affinity for polyuridine. The fourth class, consisting of the effects of the overlapping-deletion mutants Δ3 and Δ12, exhibited reductions in ds RNA binding activity that were intermediate between those obtained for the third class (mutants Δ6 and Δ7) and the first class (mutants Δ11 and Δ4Δ3), but some residual ds RNA binding could still be detected.

Although none of the deletions completely eliminated binding of βb to radiolabeled RNA probes, four of the deletion mutants, Δ4, Δ11, Δ3, and the double-deletion mutant Δ4Δ3, had noticeable effects on the activity of RNA binding, as determined in separate titration experiments (data not shown). These effects, which are also evident in Fig. 8, included the inability of the mutant protein to bind 100% of the probe, even when increased levels of protein were used.

DISCUSSION

We have purified the 58-kDa βb protein from BSMV-infected barley plants by engineering a his-tag on the N terminus of the protein and using metal affinity chromatography. This approach proved very successful, as we were able to recover nearly homogeneous protein suitable for biochemical characterization. A similar approach was also reported (19) for the cucumber mosaic virus (CMV) MP isolated from CMV-infected zucchini squash, but the yields in these experiments were not sufficient for biochemical characterization.

The subcellular localization of the putative helicases encoded by the triple gene block-containing viruses is variable. We have found that the βb protein is localized in both the soluble and cell wall fractions (9). In contrast, the analogous 26-kDa protein of FMV was present primarily in the soluble fraction (35) whereas the 25-kDa potato virus X protein was found in lamellar inclusions (6) and the 42-kDa BNYVV protein was restricted to the membrane fractions (23). However, the biochemical characteristics of βb, the 26-kDa FMV protein (35), and the 42-kDa BNYVV protein (2) are similar in their nonspecific binding to ss RNA. In addition, βb and the 26-kDa FMV protein (35) are similar in their specificities of nucleotide binding and in their ATPase activities. Both βb and the FMV 26-kDa protein exhibit specificity for ATP, while the CMV and TMV MPs bind GTP very strongly but fail to bind ATP (19). The βb protein is required for systemic movement, and the strict requirement for amino acids residing in the core of the type A and type B ATPase domains (blocks I and II in Fig. 1) strongly suggests that ATPase activity is essential for the function of βb. Interestingly, the ATPase activity associated with βb was not stimulated by the addition of RNA. This characteristic was also observed with the 26-kDa FMV protein (35). The putative NTPase-helicase proteins of positive-sense RNA viruses have been classified into three groups: (i) Sindbis virus-like, (ii) picornavirus-like, and (iii) poty-flavi-pestivirus-like proteins (13). RNA stimulation of ATPase activity of proteins encoded by members of all three groups has been demonstrated (10, 17, 33, 34, 41). This includes the nsP2 protein of Semliki Forest virus (33), which like the βb and 2b-kDa FMV proteins is a member of the Sindbis virus-like group. Therefore, the ATPase activities of the βb and 26-kDa FMV proteins appear to be unique, as their ATPase activities are unaffected by the presence of RNA.

The biochemical properties of βb purified from plant tissue or from *E. coli* appeared to be very similar with respect to their abilities to bind RNA. βb isolated from both sources bound RNA in a sequence-nonspecific manner but was selective for ss RNA and ds RNA molecules. The selectivity displayed by βb for RNA substrates, particularly its affinity for ds RNA, distinguishes the BSMV protein from the MPs encoded by viruses such as TMV (4), CMV (19), and RCNMV (25). These MPs are able to bind RNA in a sequence-nonspecific manner, but in contrast to βb, they exhibit only ss RNA or ss DNA binding affinities. Therefore, the possibility exists that BSMV differs fundamentally from viruses such as TMV, CMV, and RCNMV in that βb may bind replicative-form ds RNA and mediate movement of this complex from cell to cell.

We recognize that results from deletion experiments, such as those described for βb, need to be interpreted cautiously. The pitfalls of this approach were illustrated previously in a deletion analysis of the TMV MP, where preliminary results suggested the presence of two putative RNA binding domains (4). However, more extensive experiments subsequently revealed that the primary affects of the TMV MP deletions were related to protein stability or protein folding rather than to a direct role for the deleted amino acids in RNA binding per se (5). However, despite this caveat, a major conclusion to be drawn from the βb analyses is that the βb protein has multiple binding sites. Our experiments demonstrated that each of the single-deletion mutants, whose mutations together span the length of the protein, retains some RNA binding activity. Even the double-deletion mutant Δ4Δ3, which encodes a protein 45% smaller than the full-length βb protein, retains the ability to form an RNA-protein complex with the majority of the RNA probe. This double mutant was expected to exhibit a substantial reduction in RNA binding activity, because the effects on RNA substrate selectivity observed with the individual βb deletion mutants Δ4 and Δ3 can be mapped to smaller determinants within their boundaries, such as those defined by deletion mutants Δ7 and Δ12. The central portion of the βb protein, which is spanned by the deletions of Δ1 and Δ2 and which constitutes 40% of the protein, seems at first glance to be entirely dispensable for RNA binding. However, this may not necessarily be true because the apparent redundancy of RNA binding sites of other motifs may provide compensatory activities.

A second important conclusion that can be drawn from the

data is that several determinants that map to different regions of βb contribute to ds RNA binding affinity. The N-terminal-deletion mutant Δ11 and the C-terminal-deletion mutant Δ10 abolish ds RNA binding, and the N-terminal-deletion mutants Δ7, Δ6, and Δ4 and the C-terminal-deletion mutants Δ3 and Δ12 exhibit substantially reduced binding to ds RNA. One interpretation suggested by these data is that amino acid sequences within individual deleted regions, such as those encompassed by deletions Δ6, Δ7, Δ10, and Δ12, might interact in the native, folded protein to form a binding pocket with ds RNA binding affinity. However, it is also possible that some of these mutations exert their effects indirectly, by altering the folding of the protein. In this case, the appearance of polyuridine binding activity in deletion mutants Δ6, Δ7, Δ10, and Δ12 could be explained by changes in the folding of the mutated βb protein that result in exposure of a site(s) capable of binding the homopolymer. This hypothesis is supported by similar experiments conducted with the TMV MP (4).

From sequence analyses, a conserved RNA helicase motif was found (14) in the βb protein that places it in the DEXH family of proteins, which are members of helicase superfamily II (13, 15). Members of this family all contain highly conserved amino acid regions (I to VI) (14) that are thought to be involved in RNA binding, NTP binding, and ATPase activity (Fig. 1). Our infectivity experiments have demonstrated that these invariant amino acids individually have critical roles in the function of βb. We hypothesized that the ATPase activity of βb couples nucleotide hydrolysis with melting of duplex RNA in vivo. However, we were unable to demonstrate helicase activity with purified βb in vitro (data not shown). One explanation for the lack of detectable helicase activity is that βb may have a requirement for an ancillary protein or proteins, derived from either the host or the virus, that were eliminated during purification of the βb protein. The interaction of βb and such putative additional proteins may be dependent on the presence of an intact βb-RNA complex, which we presume was also disrupted during extraction. The majority of the helicases studied to date do not require additional proteins for activity in vitro; however, eIF-4A does require a second protein, eIF-4B, for activity (36). In this case, a model in which eIF-4A binding of ATP is followed by RNA binding and subsequently by the binding of eIF-4B was proposed (26). This hypothesis was supported experimentally, because helicase activity was detected only in conjunction with the binding of eIF-4B. Therefore, the presence of an additional protein or proteins may be required to mediate βb helicase activity.

A second explanation for the inability of βb to act as a helicase is that βb behaves in a manner similar to that of the TMV MP by binding to the viral RNA molecules and subsequently altering the secondary structure to produce a linear, βb-coated RNA molecule. Our in vitro experiments suggest that βb has a strong affinity for ds RNA. This affinity, however, may reflect an in vivo propensity of βb to bind to specific structural elements of BSMV RNA (for example, helical stem-loop structures) that provide more opportunities for specific recognition. This putative binding specificity may require additional proteins that are not associated with purified βb. This suggests a model in which βb binds to and melts ds RNA, in cooperation with additional proteins, to produce βb-coated viral RNA molecules that are less highly structured than the uncoated RNA molecules and are more amenable to passage through plasmodesmata. If this hypothesis is correct, helicase activity in vitro may require additional proteins to interact with βb prior to binding to specific regions of ds RNA to facilitate unwinding. In this regard, activities of virus-encoded RNA helicases in vitro have been shown to vary substantially, depending on overhanging or blunt sequences at the 5′ or 3′ termini of the ds RNA templates (36, 40).

It appears that functionally related MPs encoded by several different RNA viruses exhibit varied biochemical properties in vitro. These include differences in the binding specificities of BSMV βb and the TMV MP. However, despite these differences, the TMV MP can replace the BSMV triple gene block to facilitate movement in common hosts (38), and in complementation studies, the TMV MP can be replaced by the BSMV (21) triple gene block. The ability of these viruses to utilize, albeit to a somewhat limited extent, foreign MPs for cell to cell movement suggests that these proteins may have similar functional activities in vivo. These complex processes undoubtedly are dependent on numerous interactions, including protein-protein and RNA-protein associations involving both virus and host proteins. We hope to elucidate some of these interactions in subsequent studies of the roles of the triple gene block proteins in viral movement.

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R. G. K. Donald and D. M. Lawrence contributed equally to this work and share senior authorship.

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