

Identification of Domains in Brome Mosaic Virus RNA-1 and Coat Protein Necessary for Specific Interaction and Encapsidation

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Received 16 April 1993/Accepted 26 July 1993

Even though many single-stranded RNAs are present in the cytoplasm of infected cells, encapsidation by brome mosaic virus (BMV) coat protein is specific for BMV RNA. Although the highly conserved 3' region of each of the three BMV genomic RNAs is an attractive candidate for the site of recognition by the coat protein, band shift and UV cross-linking assays in the presence of specific and nonspecific competitors revealed only nonspecific interactions. However, BMV RNA-1 formed a retarded complex (complex I) with the coat protein in the absence of competitors, and two domains of RNA-1 that specifically bound coat protein in a small complex (complex II), presumably early in the encapsidation process, were identified. Strong nonspecific, cooperative binding was observed in the presence of high concentrations of coat protein, suggesting that this provides the mechanism leading to rapid encapsidation seen in vivo. In contrast, no binding to a coat protein mutant lacking the N-terminal 25 amino acids that has been shown to be incapable of encapsidation in vivo (R. Sacher and P. Ahlquist, *J. Virol.* 63:4545–4552, 1989) was detected in vitro. The use of deletion mutants of RNA-1 revealed the presence of domains within the coding region of protein 1a that formed complexes with purified coat protein. One deletion mutant (B1SX) lacking these domains was only slightly more effective in dissociating RNA-1-coat protein complexes than were nonspecific competitors, further suggesting that regions other than the 3' end can participate in the selective encapsidation of BMV RNAs.

Encapsidation is an important event in the virus life cycle. In addition to providing the virus nucleic acid genome with a protective protein coat against nuclease degradation, it regulates many facets of viral biosynthesis. For example, virion formation provides a sink that effectively removes free viral nucleic acids from the host cytoplasm. In the case of multipartite RNA viruses such as brome mosaic virus (BMV), preferential encapsidation of specific RNAs could probably cause a transition from early to late functions. For example, removal of RNA-1 and RNA-2, which encode replicase subunits, increases the proportion of RNA-3 and RNA-4, which dramatically increase the ratio of plus to minus strands synthesized (16), undoubtedly favoring cell-to-cell movement through the production of large quantities of protein 3a (thought to be the BMV movement protein).

Since BMV virions form in the cytoplasm of the host cell (15), the opportunity for coencapsidation of cellular tRNAs, mRNAs, or rRNA fragments exists. However, Cuillel et al. (7) found the interaction between BMV genomic RNAs and coat protein to be specific in vivo and in vitro. A localization of viral RNA and coat protein subunits in the same compartment within the cell (18) and a requirement for sequence- and/or structure-dependent interaction should ensure that the majority of the virions contain viral RNAs.

The noncoding 3' 200 nucleotides (nt) of each of the three genomic and single subgenomic RNAs of BMV share a highly conserved sequence (1). Although this region appears to be a likely candidate for sequences involved in encapsidation, no evidence exists to support this possibility, and the major encapsidation sequence for tobacco mosaic virus, whose genome exhibits several similarities with that of BMV (13), lies in

the open reading frame (ORF) of the coat protein or the movement protein rather than in the noncoding 3' end. Recent studies on Sindbis virus (28) and turnip crinkle virus (TCV) (27) have identified RNA domains that contribute to specific interactions with viral coat protein. In the latter report, it was also demonstrated that nonspecific interactions can readily occur between the viral coat protein and nonviral RNAs.

We have developed band shift and UV cross-linking procedures to investigate encapsidation processes for BMV RNA-1. The experiments described reveal both specific and nonspecific interactions with the virus coat protein and also show the ability of the coat protein to associate cooperatively with viral and nonviral RNAs. Additionally, we used these techniques to further explore the observation of Sacher and Ahlquist (21) that deletion of the N-terminal 25 amino acids of BMV coat protein eliminates encapsidation.

MATERIALS AND METHODS

Plasmid constructions. An 852-bp *Xba*I fragment from pT7B1 (which contains a full-length cDNA sequence corresponding to RNA-1 [8]) was cloned into the *Xba*I site in the polylinker of pBluescript (pBS; Stratagene), yielding pB1X, from which the transcript B1X is produced (see Fig. 5). The deletion derivative B1SX (see Fig. 5) was obtained by cutting the cDNA of RNA-1 to completion with *Xba*I and then partially cutting with *Sph*I for 10 min and subsequent treatment with mung bean nuclease and ligation of the blunt ends. The 3' 200 nt of RNA-3 was cloned into the polylinker of the transcriptional vector 19U (Pharmacia), by using the *Bam*HI and *Hind*III sites in the vector and the insert, to construct p19UB3200wt (20a). The construct psub047, kindly supplied by David Frisch, contains a 231-bp *Eco*RV fragment from the β -glucuronidase (GUS) gene cloned into pBS. Another GUS

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construct, pGEM4.2-GUS (4), was a kind gift of Jim Carington.

In vitro transcription. Transcription reactions for the synthesis of ^{32}P -labeled transcripts were performed for 2 h at 37°C. The reaction mixture contained 4 μl of $5\times$ transcription buffer (Bethesda Research Laboratories); 2 μl of 0.1 M dithiothreitol; 1 μl of RNA guard (Pharmacia); 4 μl of 5 mM ATP, CTP, and GTP and 3 mM UTP; 2 μg of linearized template; 2 μl of 60 U of T7/T3 RNA polymerase (Pharmacia) per μl ; and 5 μl of [α - ^{32}P]UTP (Du Pont-NEN). For unlabeled transcripts, the MEGAscript transcription kit (Ambion) was used. The DNA templates were prepared by linearization at various restriction sites. *Tth*1111 was used for linearizing p19UB3200wt, which was then transcribed with T7 RNA polymerase. *Hind*III was used for linearizing psub047, and the 305-nt fragment of GUS was transcribed by using T7 RNA polymerase. The vector 19U was linearized with *Pvu*I and was transcribed with T7 RNA polymerase. The cDNA of RNA-1 and its truncations were transcribed with T7 RNA polymerase. Full-length and B1SX transcripts were synthesized by linearization at the *Bam*HI site, while the truncations were transcribed from templates that were linearized at the sites indicated by the names of the truncations. T3 RNA polymerase was used for transcribing Bluescript (BS), after pBS was linearized at the *Afl*III site. After linearization with *Eco*RI, pGEM4.2-GUS was transcribed with T7 RNA polymerase to produce GUS RNA. T3 RNA polymerase was used for transcribing B1X by linearizing pB1X at the *Sac*I site in the polylinker of pBS.

Preparation of protein extracts from barley plants infected with BMV. Secondary BMV-inoculated leaves (2 g) of barley plants, harvested 5 days postinoculation, were ground with glass in 15 ml of buffer A (50 mM Tris-HCl [pH 7.4], 10 mM KCl, 1 mM EDTA, 10 mM magnesium acetate, 15% [vol/vol] glycerol, 10 mM dithiothreitol). The paste was centrifuged in a Beckman JA20 rotor for 10 min at $1,088\times g$. The supernatant was then centrifuged for 20 min at $362,000\times g$ in a Beckman Ti60 rotor. One milliliter of 1% (wt/vol) dodecyl- β -D-maltoside in buffer A per g of leaf material was used for resuspending the pellet by stirring for 90 min at 4°C. The suspension was then centrifuged for 30 min at $145,000\times g$ in a Beckman Ti40 rotor. The pellet obtained was resuspended in 0.1% (wt/vol) dodecyl- β -D-maltoside in buffer A (1 ml/2 g of leaf material). The suspension was then layered over 30 ml of buffer A containing 40% (wt/vol) sucrose and 0.1% dodecyl- β -D-maltoside and centrifuged for 2 h at $46,162\times g$ in a Beckman SW27 rotor. The pellet obtained was resuspended in 1 ml of buffer B (50 mM Tris-HCl [pH 8], 0.1% [wt/vol] dodecyl- β -D-maltoside, 10 mM dithiothreitol, 0.5 mM magnesium acetate) per 2 g of leaf material, and aliquots were stored at -80°C . These extracts were then subjected to $(\text{NH}_4)_2\text{SO}_4$ fractionation and were then dialyzed against buffer B. The pellet of the 60% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction was found to contain the majority of the coat protein (data not shown), and a 200- μl aliquot of this fraction was treated with 2.5 μl of 50 mM calcium acetate and then with 8.5 μl of a 15-U/ μl suspension of micrococcal nuclease for 30 min at 30°C to remove the endogenous RNAs. Micrococcal nuclease activity was then terminated by the addition of 18 μl of 0.2 M ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and total protein was determined by the bicinchoninic acid method (Pierce).

Overexpression of coat protein and deletion mutant of coat protein in *Escherichia coli*. *Nde*I and *Bam*HI sites were generated at the 5' and 3' ends of the coat protein ORF by polymerase chain reaction. The *Bam*HI site was created at the 3' end of the 3' noncoding region of RNA-3. The same sites

were generated for the coat protein deletion mutant ($\Delta\text{N}25\text{CP}$), except that the *Nde*I site was introduced 25 codons downstream of the coat protein start codon. These fragments were cloned into the overexpression vector pET-9a (Novagen) by using the *Nde*I and *Bam*HI sites to produce the constructs pETCP and pET $\Delta\text{N}25\text{CP}$. These constructs were used for transforming *E. coli* BL21(DE3)pLysE, and the protein synthesis induction and protein purification were carried out by the method of Citovsky et al. (5). The purification and solubilization scheme was modified slightly for optimal recovery of the proteins. Briefly, the cells were harvested by centrifugation for 10 min at $7,000\times g$ at 4°C and resuspended in 2 ml of buffer L (5) containing 100 μg of lysozyme per ml, and then 200 μl of 1% (vol/vol) Triton X-100 was added to the cells, which were then incubated for 15 min at 30°C. By using a Sonifier (cell disruptor 350; Branson Sonic Power Co.) the cells were then sonicated at the maximum microtip output setting four times for 10 s (each) with a 1-min interval between each pulse. The cell lysate was spun for 5 min at $12,000\times g$ at 4°C, the pellet was resuspended in 1 ml of buffer L containing 1 M NaCl and pelleted again by centrifugation for 5 min at $12,000\times g$ at 4°C, and the pellet was resuspended in 1.5 ml of buffer L containing 10 M urea and 1 M NaCl and then incubated for 15 min at 72°C. After being spun in an Eppendorf microcentrifuge for 10 min, the supernatant was dialyzed against buffer L for 30 h, and the amount of protein was estimated as described above.

Purification of coat protein from BMV virions. The coat protein was purified from isolated BMV virions by using a protocol based on that described by Verduin (24). A 10-mg/ml BMV virion suspension (pH 4.5), in 50 mM sodium acetate and 8 mM magnesium acetate, was dialyzed against 0.5 M CaCl_2 -1 mM dithiothreitol in 50 mM Tris-HCl (pH 7.5). Overnight dialysis (at 4°C) at a slightly alkaline pH disrupted the virion structure, facilitating removal of RNA by spinning for 20 min in a microcentrifuge. The supernatant, containing most of the coat protein, was then centrifuged for 30 min in a microcentrifuge to remove residual undissociated virus. This protein solution was dialyzed for 6 h against 1 M NaCl in 50 mM Tris-HCl (pH 7.5). The coat protein was then precipitated by saturating the solution with 60% $(\text{NH}_4)_2\text{SO}_4$. The pellet was resuspended in buffer B and dialyzed overnight against buffer B. Micrococcal nuclease treatment and protein estimation were done as described above.

Band shift assays. The gel-purified, ^{32}P -labeled 3' 200 nt of RNA-3 (20 fmol) was incubated for 15 min at 30°C with 5 μg of protein contained in the pellet of the 60% $(\text{NH}_4)_2\text{SO}_4$ fraction of the infected plant extract in a final volume of 15 μl , made up with buffer B. Before incubation with the probe, the $(\text{NH}_4)_2\text{SO}_4$ fraction was made free of contaminating RNases by incubation for 10 min at 30°C with 1 U of Inhibit-ACE (5'→3' Inc.), as described by Nakhasi et al. (20). In order to demonstrate cooperative binding by the coat protein, various amounts of coat protein were incubated with the probe. For band shift competition experiments, the ^{32}P -labeled probe and unlabeled competitors were added together to the protein extracts, to give both of the RNAs an equal chance of binding to the coat protein. After adding 1.5 μl of 75% (vol/vol) glycerol, the samples were loaded onto a nondenaturing 4% (wt/vol) polyacrylamide gel with $0.8\times$ Tris-borate-EDTA as the running buffer. Electrophoresis was carried out at 150 V for 2.5 h, after which the gel was dried and exposed to X-ray film. For the binding reactions involving RNA-1 and its truncations and purified coat protein, 20 ng of gel-purified ^{32}P -labeled probe was incubated for 8.5 min at 30°C with 5 μg of coat protein in the presence of 0.4 M NaCl. Because of the

greater length of these probes, the complexes were resolved on a 0.7% (wt/vol) agarose gel by electrophoresis at 80 V. The gel was then dried and exposed to X-ray film.

UV cross-linking assays. The binding reaction was identical to that of the band shift assays, except that 100 ng of gel-purified, ^{32}P -labeled probe was incubated with 10 μg of virion-purified coat protein or overexpressed proteins. The RNA and protein were cross-linked in the lids of 1.7-ml Eppendorf tubes in a Stratalinker 1800 (Stratagene) with 1.8 J of UV light (5). The cross-linked extract was then mixed with RNase A (6 μg) and RNase T₁ (6 U). Following incubation for 30 min at 37°C, the samples were boiled in Laemmli sample buffer for 5 min, and RNA-protein complexes were resolved by electrophoresis in sodium dodecyl sulfate (SDS)-12% (wt/vol) polyacrylamide gels. The gels were then dried and autoradiographed.

RESULTS

Coat protein from infected barley leaves binds nonspecifically to the 3' 200 nt of RNA-3. In preliminary experiments to investigate the binding of proteins to the 3' 200 nt of RNA-3, replicase extracts (3, 12, 19) from barley plants infected with BMV were used. It was known that the viral coat protein was a major component of these extracts (3). Band shift and UV cross-linking assays, together with the use of coat protein antibodies, confirmed that the 20-kDa coat protein present in the fraction of the replicase extract precipitated by treatment with 60% $(\text{NH}_4)_2\text{SO}_4$ was capable of binding to the 3' 200 nt of RNA-3 (data not shown).

To determine whether the interaction between the coat protein and the 3' 200 nt was specific, competition band shift experiments were carried out. Radiolabeled sequences corresponding to the 3' 200 nt of RNA-3 were incubated with coat protein in the presence of three unlabeled competitors: the 3' 200 nt of RNA-3, a 224-nt transcript from a T7/T3 vector (19U), and a 305-nt transcript corresponding to a fragment from the coding region of GUS (Fig. 1). The interaction between the coat protein and the 3' 200 nt of RNA-3 resulted in the formation of a retarded ribonucleoprotein (RNP) complex (Fig. 1, arrowhead) that persisted upon the addition of equimolar amounts of the three unlabeled competitors (Fig. 1, lanes 2, 6, and 10) but was inhibited by a twofold molar excess (Fig. 1, lanes 3, 7, and 11). Since it was anticipated that the 3' region common to each genomic RNA would contain a specific encapsidation signal, these results were unexpected, as they indicate that the interaction between the coat protein and the 3' 200 nt of RNA-3 is nonspecific.

BMV coat protein binds cooperatively to nonspecific and specific RNAs. A purified preparation of coat protein was obtained from isolated BMV virions (24), and the pattern of binding to various RNAs was examined. The band shift experiments shown in Fig. 2A reveal cooperative binding between the coat protein and a 305-nt ^{32}P -labeled transcript from the coding region of GUS. At low protein concentrations, both free probe and RNP complexes were seen (Fig. 2A, lanes 3 and 4). This result is similar to the cooperative binding observed for the movement protein of tobacco mosaic virus to RNA (5). In the presence of moderate levels of coat protein, intermediate-size and fully shifted RNP complexes were detected (Fig. 2A, lanes 5 and 6), but with 5 μg of coat protein only fully shifted complexes were detected. At this coat protein concentration, the RNP complex was massive and barely entered the gel.

A similar pattern of cooperative binding was observed when ^{32}P -labeled full-length RNA-1 was used in band shift assays

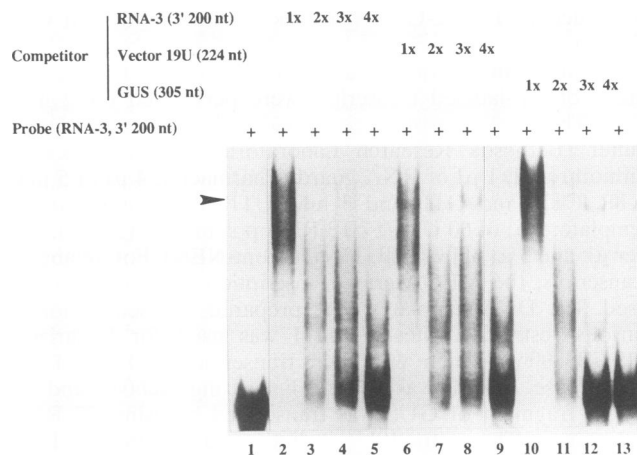


FIG. 1. Lack of specificity in the interaction between the coat protein in the pellet of the 60% $(\text{NH}_4)_2\text{SO}_4$ fraction of BMV-infected barley plant extract and the 3' 200 nt of BMV RNA-3. An autoradiograph of a native polyacrylamide gel shows band shift assays demonstrating the binding activity of coat protein to the 3' 200 nt of RNA-3 in the presence of various competitors. A one- to fourfold molar excess of three unlabeled competitors was included with the ^{32}P -labeled 3' 200 nt of RNA-3 and the coat protein in the binding reactions: the 3' 200 nt of RNA-3 (lanes 2 to 5), 224 nt of 19U (lanes 6 to 9), and 305 nt of GUS (lanes 10 to 13). Lane 1, free probe. The arrowhead indicates the RNP complex that persists in the presence of equimolar amounts of the three competitors.

with increasing concentrations of the coat protein. At 5 μg of coat protein (Fig. 2B, lane 7), most of probe was a part of a highly retarded RNP complex (possibly virions) and the rest of the probe was in a complex having a mobility slightly less than that of the free probe, suggesting that this complex contained relatively few coat protein molecules. In the presence of 10 μg of coat protein (Fig. 2B, lane 8), because of the rapid cooperative interaction of the coat protein with the RNA, only the highly retarded complex was seen.

The N-terminal 25 amino acids of coat protein contain specific and nonspecific determinants for BMV RNA binding. Sgro et al. (22) found that residues 11 to 19, 26 to 40, and 44 to 80 of BMV coat protein cross-linked to viral RNA, and Sacher and Ahlquist (21) found that a coat protein mutant lacking the first 25 amino acids was incapable of directing RNA encapsidation *in vivo* or producing systemic infection. Eight of the first 25 amino acids of BMV coat protein are basic, and the data in Fig. 1, together with the findings of Sacher and Ahlquist (21), indicate that the positively charged N terminus is required for nonspecific RNA binding or compaction of the RNA essential for the encapsidation process. However, these data do not address the possibility that the BMV RNAs interact specifically with a region of the coat protein other than the first 25 amino acids. In order to test this possibility, purified wild-type (CP) and $\Delta\text{N}25\text{CP}$ (the coat protein mutant lacking the first 25 amino acids) proteins were obtained by overexpression in *E. coli* (see Materials and Methods) and used in UV cross-linking experiments with ^{32}P -labeled full-length RNA-1.

The wild-type coat protein formed a specific complex with ^{32}P -labeled RNA-1 (Fig. 3, lane 1, arrowhead) that could be completely inhibited by a 30-fold excess of unlabeled RNA-1 (Fig. 3, lane 2). The other RNP complex seen in Fig. 3 (lanes 1 to 3) with a higher molecular weight probably resulted from the interaction of ^{32}P -labeled RNA-1 and dimers of the coat protein. The CP complex persisted, though at a slightly re-

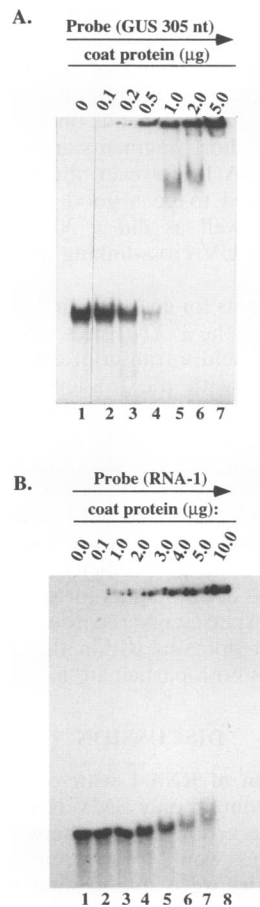


FIG. 2. Cooperative binding of virion-purified BMV coat protein to nonspecific and specific RNAs. (A) Autoradiograph of a native polyacrylamide gel showing band shift assays demonstrating the cooperative binding of coat protein to a ^{32}P -labeled 305-nt transcript of a fragment from the GUS gene at increasing concentrations of purified coat protein (lanes 2 to 7). Lane 1, free probe. (B) Autoradiograph of a native agarose gel showing the cooperative binding of coat protein to full-length ^{32}P -labeled RNA-1 of BMV. Lane 1, free probe; lanes 2 to 8, increasing concentrations of the coat protein.

duced level, in the presence of a 30-fold excess of nonspecific competitor (Fig. 3, lane 3, BS RNA). The slight decrease in the intensity of the RNP complex band was due to the nonspecific interaction of the coat protein with BS RNA. In the case of ΔN25CP , no complex with RNA-1 was detected in the absence or presence of competitors (Fig. 3, lanes 4 to 6). Taken together with the absence of any RNP complex formation with the ΔN25CP mutant protein, these results provide direct evidence that the major determinants (specific and nonspecific) for binding to BMV RNA lie in the N-terminal 25 amino acids of BMV coat protein.

Specific binding of virion-purified coat protein to BMV RNA-1. Despite the nonspecificity of RNA binding by BMV coat protein found under certain circumstances *in vitro* (Fig. 1) (2), it is evident that a high degree of specificity is exhibited *in vivo*. For example, Cuillel et al. (7) failed to detect any nonviral RNAs in BMV virions isolated from infected cells. They also found specific encapsidation of BMV RNA-4 by BMV coat protein in reassembly assays in the presence of alfalfa mosaic virus RNA-4 and yeast tRNA. These considerations led us to extend our RNA-coat protein binding studies to include ^{32}P -labeled full-length transcripts of RNA-1.

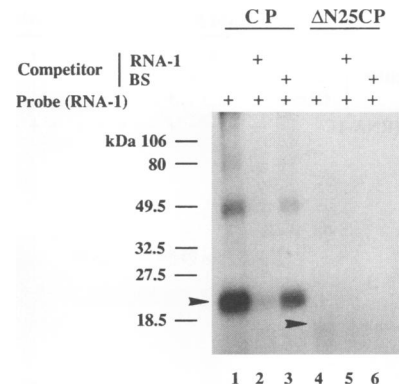


FIG. 3. The 25 N-terminal amino acids of BMV coat protein are essential for binding to RNA. An autoradiograph of an SDS-polyacrylamide gel containing RNP complexes formed after UV cross-linking ^{32}P -labeled RNA-1 to coat protein (CP; lanes 1 to 3) and an N-terminal deletion mutant of the coat protein (ΔN25CP ; lanes 4 to 6), both overexpressed in *E. coli*. Lanes 1 and 4 contained the RNP complexes formed between radiolabeled RNA-1 and either CP or ΔN25CP , respectively, in the absence of competitors. Lanes 2 and 5 contained a 30-fold excess of unlabeled RNA-1 included in the cross-linking reaction mixture with radiolabeled RNA-1 and either CP or ΔN25CP , respectively. Lanes 3 and 6 contained a 30-fold excess of unlabeled BS RNA (a 2,602-nt transcript from the vector pBS) included in the cross-linking reaction mixture with radiolabeled RNA-1 and either CP or ΔN25CP , respectively. The arrowheads indicate the positions of CP and ΔN25CP after staining with Coomassie brilliant blue (R-250). In the case of CP, the arrowhead also indicates the position of the RNP complex. The positions of protein molecular mass markers are indicated on the left.

In band shift assays, radiolabeled RNA-1 formed an RNP complex with virion-purified coat protein under three different salt conditions (Fig. 4, complex I). The addition of a 20-fold excess of nonspecific (BS RNA or GUS RNA) unlabeled competitor converted complex I into complex II (Fig. 4, lanes 4, 8, and 12 and 5, 9, and 13, respectively), which had a greater mobility than complex I but was still retarded in comparison with the migration of supplied free probe or probe released by a specific competitor (Fig. 4, lanes 1, 3, 7, and 11, F). Evidence that these nonspecific competitors (e.g., BS RNA) were less effective in disrupting the RNA-viral coat protein complex than was specific competitor (BMV RNA-1) can be derived from the experiments shown in Fig. 3 (lanes 2 and 3). The findings with virion-purified coat protein (Fig. 4) indicate that complex II is specific, nonspecifically bound coat protein present in complex I, having been removed by the excess of nonspecific RNA competitor. In the presence of low concentrations of the specific competitor, unlabeled RNA-1, complex II is formed; higher concentrations of this competitor liberate the probe from the complex (data not shown). In contrast, when high concentrations of nonspecific competitors (e.g., BS RNA) are included in the binding reaction, dissociation of radiolabeled RNA-1 from complex II was not observed (data not shown). A similar specific complex having a mobility only slightly reduced from that of the free probe was described by Wei and Morris (27) for the interaction of TCV RNA with its coat protein. Additionally, the persistence of the BMV coat protein-RNA-1 complex at a high salt concentration (0.8 M NaCl) indicates the existence of a strong association between the coat protein and RNA-1.

Identification of domains of RNA-1 responsible for specificity in the interaction between RNA-1 and BMV coat protein. To identify the region in RNA-1 conferring specificity to the

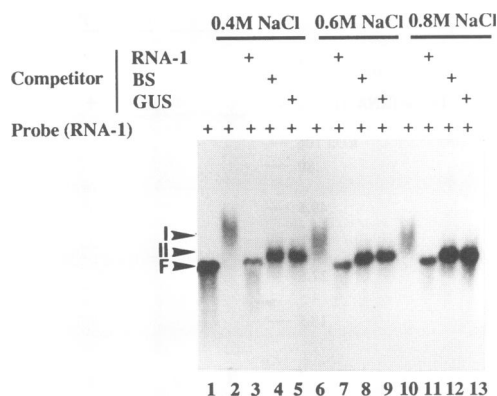


FIG. 4. Strong and specific binding of virion-purified BMV coat protein to RNA-1. An autoradiograph of a native agarose gel showing band shift assays demonstrating binding of virion-purified coat protein to ^{32}P -labeled RNA-1 in the presence of three competitors, in three salt concentrations: 0.4 M (lanes 1 to 5), 0.6 M (lanes 6 to 9), and 0.8 M (lanes 10 to 13). Lane 1, free probe (F). Lanes 2, 6, and 10, RNP complex I formation in the absence of any competitors; lanes 3, 7, and 11, displacement of radiolabeled RNA-1 from RNP complex I in the presence of a 20-fold mass excess of unlabeled RNA-1; lanes 4, 8, and 12, conversion of complex I into complex II when binding reactions were carried out in the presence of a 20-fold mass excess of unlabeled BS RNA. The same is also seen in the case of lanes 5, 9, and 13, in which binding reactions were performed in the presence of a 20-fold mass excess of unlabeled GUS RNA (a 2,023-nt transcript from pGEM4.2-GUS). The arrowheads on the left indicate the positions of free probe (F) and the two RNP complexes (I and II).

interaction between RNA-1 and coat protein, a series of ^{32}P -labeled transcripts (Fig. 5A) were made corresponding to 3' truncations of RNA-1 (see Materials and Methods). The longer transcripts resulting from linearization of RNA-1 cDNA downstream of the *Xba*I site at position 1764 did not lead to a loss of specificity in band shift assays (data not shown). As in the experiments described above (Fig. 4), radiolabeled BMV RNA-1 formed complex I with virion-purified coat protein in the absence of competitor (Fig. 5B, lane 1) and complex II was obtained in the presence of a 10-fold excess of GUS RNA as nonspecific competitor (Fig. 5B, lane 4). Also as in Fig. 4, addition of unlabeled RNA-1 as a specific competitor resulted in the release of free probe (Fig. 5B, lane 3). The formation of complexes analogous to complex II and the release of free probe were also observed for the *Xba*I and *Hind*III RNA transcripts in the presence of a 10-fold excess of unlabeled GUS RNA or BMV RNA-1, respectively (Fig. 5B, lanes 7 and 10 and 6 and 9, respectively). In the case of the *Afl*III RNA transcript, all of the RNA probe was released from the RNP complex in the presence of a 10-fold excess of unlabeled RNA-1 (Fig. 5B, lane 12). However, in the presence of a 10-fold excess of unlabeled GUS RNA, most of the radioactivity comigrated with the ^{32}P -labeled *Afl*III RNA transcript (FA), and very little RNP complex (IIA) analogous to complex II was detected (Fig. 5B, lane 13).

The substantial loss in specificity for coat protein seen for the *Afl*III RNA transcript suggested that a domain which participates in the formation of a specific complex exists downstream from the *Afl*III site. To explore this possibility, *Afl*III-*Hind*III, *Sph*I-*Hind*III, and *Xba*I-*Xba*I fragments (Fig. 5A) were cloned into pBS, and their unlabeled transcripts were used as competitors in band shift and UV cross-linking experiments involving coat protein and radiolabeled RNA-1.

Disappointingly, the *Afl*III-*Hind*III and *Sph*I-*Hind*III tran-

scripts did not compete specifically for coat protein (data not shown). Possibly, these domains cannot function independently because they are a part of a larger region that is responsible for sequence-specific interaction. It is also possible that the specific interaction depends on a tertiary folding that is absent when these short fragments are removed from their normal context in RNA-1. However, the *Xba*I-*Xba*I transcript (Fig. 5A, B1X) proved to be a good competitor: a 30-fold excess competed as well as did a 30-fold mass excess of full-length RNA-1 in UV cross-linking assays (Fig. 5C, compare lanes 3 and 5).

If all the determinants for coat protein binding were present between the *Afl*III and the 3' *Xba*I sites, deletion of this region from RNA-1 should yield a transcript exhibiting no ability to compete for binding with the full-length RNA. Since the *Afl*III-truncated transcript retained a small ability to form a complex II-like RNP complex (Fig. 5B, lane 13, IIA), a site (*Sph*I) slightly upstream was chosen and a deletion mutant of RNA-1 (*Sph*I-3' *Xba*I [Fig. 5A, B1SX]) was constructed. Unlabeled B1SX RNA competed only slightly better than the nonspecific competitor BS RNA or GUS RNA (Fig. 5C, lanes 6 to 11), showing that, as predicted, the deleted region probably contains the determinants for specific binding to the coat protein. The experiments recorded in Fig. 5B and C further indicate that domains within the *Afl*III-*Hind*III and *Xba*I-*Xba*I fragments both participate in specific binding.

DISCUSSION

Specific interaction of RNA-1 with coat protein of BMV. Since BMV virions contain only BMV RNAs (7), it is evident that specificity of interaction exists; otherwise, nonviral RNAs and nonviral proteins would be present. In these studies, focusing on RNA-1, we were able to show that in addition to specific binding, nonspecific binding occurred. Specific complexes were readily dissociated by competition with RNA-1 and were unaffected by the presence of nonviral RNA competitors. Additionally, coat protein and RNA-1 appear to have a strong affinity for each other, since complexes between them are resistant to dissociation by a high salt (0.8 M NaCl) concentration (Fig. 4). The formation of a small, specific complex (Fig. 4 and 5, complex II) in the presence of nonspecific competitors suggests that the specific interaction between the coat protein and genomic RNAs occurs at an early stage of assembly, when only a few coat protein molecules are associated with the RNA.

Interestingly, a marked decrease in specific binding was only evident upon extensive 3' sequence deletions (to the *Afl*III site [Fig. 5B, lanes 11 to 13]). Within the region deleted, we identified two domains that appeared to contribute to binding specificity, those contained within the *Afl*III-*Hind*III and *Xba*I-*Xba*I fragments (Fig. 5B and C). Our experiments indicate that both domains are independently capable of specific binding: complex II formation was seen with RNA containing the *Afl*III-*Hind*III fragment but lacking the *Xba*I-*Xba*I fragment (Fig. 5B, lane 7), and the *Xba*I-*Xba*I fragment competed strongly for RNP formation between RNA-1 and coat protein (Fig. 5C, lane 5). It is also possible that the region upstream of the *Afl*III site makes a small contribution to binding specificity, since B1SX RNA was marginally more effective than nonspecific RNA (BS RNA or GUS RNA) in dissociating RNA-1-coat protein complexes (Fig. 5C). The existence of multiple regions exhibiting binding suggests that the secondary or quaternary structure of the viral RNA contributes to specificity. Such considerations may explain why it is difficult to demonstrate specific binding *in vitro* by using individual short

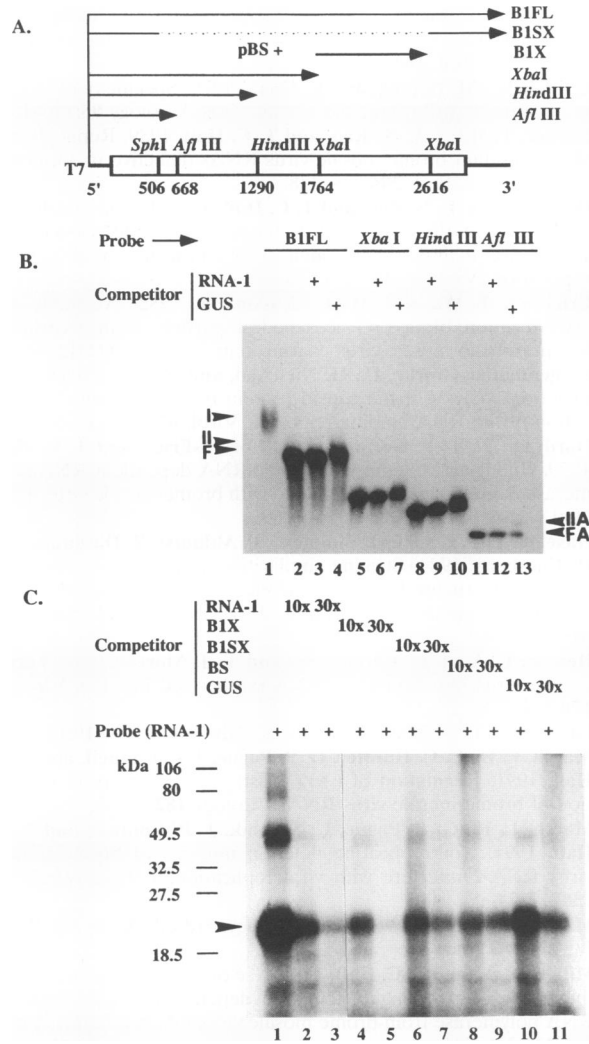


FIG. 5. Identification of regions of RNA-1 responsible for specific binding to BMV coat protein. (A) Schematic diagram of the cDNA of RNA-1 downstream of the T7 promoter. The open rectangle represents the ORF of protein 1a, and the solid lines at the 5' and 3' ends of the ORF represent the 5' and 3' noncoding regions, respectively. The restriction sites used for making truncations and deletions of RNA-1 are indicated. The arrows originating from the T7 promoter represent the various transcripts shown on the right. The dotted line indicates the extent of the deletion in B1SX. In case of B1X, the transcript is derived from pBS containing the XbaI fragment of RNA-1. (B) Autoradiograph of a native agarose gel showing a band shift competition assay involving virion-purified coat protein and full-length ³²P-labeled RNA-1 (B1FL) and its truncations. Lane 1, RNP complex I, formed between radiolabeled RNA-1 and the coat protein; lanes 2, 5, 8, and 11, free radiolabeled RNA-1 (F) or the XbaI-, HindIII-, and AflIII-truncated transcripts, respectively; lanes 3, 6, 9, and 12, effects of inclusion of a 10-fold mass excess of unlabeled RNA-1 in the binding reaction mixture which contained coat protein and radiolabeled RNA-1 and XbaI-, HindIII-, and AflIII-truncated RNAs, respectively; lanes 4, 7, 10, and 13, effects of a 10-fold mass excess of unlabeled GUS RNA on the RNP complex formation between virion-purified coat protein and radiolabeled RNA-1 (complex II) and XbaI-, HindIII-, and AflIII-truncated RNAs, respectively. The arrowheads on the left indicate the positions of free radiolabeled RNA-1 (F) and the two RNP complexes containing radiolabeled RNA-1 (I and II). The arrowheads on the right show the position of the RNP complex between the coat protein and the

fragments, even though these fragments may function as specific competitors. For example, the fragments may or may not assume the conformation necessary for binding when removed from the context of the genomic RNA, and consequently, they may (as in the case of the XbaI-XbaI fragment) or may not (as in the case of the AflIII-HindIII fragment) be able to compete with specific binding determinants to destabilize or disrupt the RNP complex. Evidence for important intramolecular interactions has been adduced for other functions of BMV. Examples include the debilitation of replication by exchange of the similar but not identical 3' termini of the genomic RNAs (9) or by the deletion of internal sequences (for which there is no evident promoter function) that yield nonreplicating, interfering RNAs (17).

Evidence for the presence of two distinct but distant domains that interact with coat protein, leading to the assembly of virus particles, has also been obtained for the genomic RNA of TCV by Wei et al. (26). Furthermore, defective interfering RNAs of TCV are efficiently encapsidated (14), even though a domain important for their encapsidation was not identified as contributing to interactions with the coat protein when present in the context of the genomic RNA (26). In the case of Sindbis virus, Weiss et al. (28) showed that a domain in the region encoding nonstructural protein nsp1 was responsible for specific binding of coat protein to both genomic and defective interfering RNAs. Interestingly, this domain is analogous in position to the AflIII-HindIII domain of RNA-1 of BMV.

Significance of the nonspecific and cooperative binding nature of BMV coat protein. Nonspecific protein-RNA interactions have been widely found for viral RNAs. In their studies on TCV, Wei and Morris (27) suggested that a lack of specificity may aid in virion assembly by compaction of the RNA through the neutralization of the negative charge on the phosphate backbone. In addition to the specific interactions discussed above, our data (Fig. 1) reveal that BMV coat protein interacts with various RNAs in a nonspecific manner. These findings are in accord with previous in vitro studies describing encapsidation of double-stranded DNA and oligo(U) by BMV coat protein (2).

The cooperative pattern of binding exhibited by BMV coat protein (Fig. 2) probably accelerates encapsidation through protein-protein interactions, after a few molecules of the coat protein bind to a specific region of the RNA. This observation explains the rapid in vitro assembly of BMV empty protein shells by Cuillel et al. (6). Besides assisting in virion assembly, the cooperative mode of interaction of the coat protein may have regulatory functions, such as the sequestration of progeny viral RNAs to prevent their participation in processes such as replication, translation, and movement.

The N terminus of BMV coat protein is responsible for its interaction with RNA. Many plant icosahedral RNA viruses have coat proteins with highly basic amino-terminal regions

AflIII-truncated RNA (complex IIA), analogous to complex II and the position of free AflIII-truncated RNA (FA). (C) An SDS-polyacrylamide gel showing the RNP complexes formed after UV cross-linking ³²P-labeled RNA-1 and virion-purified coat protein in the presence of various unlabeled competitors. Lane 1, RNP complex formed in the absence of any competitors (arrowhead); lanes 2, 4, 6, 8, and 10, effects on RNP complex formation caused by the presence of a 10-fold excess of unlabeled RNA-1, B1X, B1SX, BS, or GUS RNA, respectively; lanes 3, 5, 7, 9, and 11, effects on RNP complex formation by the presence of a 30-fold excess of unlabeled RNA-1, B1X, B1SX, BS, or GUS RNA, respectively. The positions of protein molecular mass markers are shown on the left.

thought to interact with the RNA inside the capsid shell (10, 23, 25). BMV coat protein has 8 amino acid residues with basic side chains in its first 25 amino acids, and three regions in the first 80 amino acids have been found to interact with the viral RNA (22). Also, Sacher and Ahlquist (21) found that a coat protein deletion mutant lacking the first 25 amino acids was unable to package BMV RNA *in vivo*, resulting in a lack of systemic movement of the mutant virus. Here, we have constructed the same mutant as described by Sacher and Ahlquist (21) and overexpressed it in *E. coli*. This mutant failed to bind ³²P-labeled RNA-1 in a UV cross-linking assay (Fig. 3, lanes 4 to 6). Because of the complete abrogation of RNA binding in case of the overexpressed deletion mutant, it seems highly unlikely that a site, other than the one in the first 25 amino acids, exists for specific binding in the first 80 amino acids. However, once inside the capsid shell, the RNA could interact nonspecifically with other regions of the coat protein, as shown by the *in situ* cross-linking experiments of Sgro et al. (22). The apparent existence of a single domain in BMV coat protein that binds to RNA is similar to the situation reported for Sindbis virus (11). These observations for BMV and Sindbis virus differ from the suggestion by Wei and Morris (27) that two sites exist in TCV coat protein, one for specific binding to a genomic fragment and the other for nonspecific binding to poly(U).

The studies described in this paper provide valuable insight into regions within BMV RNA-1 that are responsible for selective binding to the coat protein and show that this RNA also participates in cooperative, nonspecific binding that is probably important in rapid encapsidation. However, they do not address the situation for RNA-2, RNA-3, or RNA-4, each of which is encapsidated. It is possible that differential signals exist for each RNA, although some common mechanism for encapsidation seems likely. The 3' region common to each of the RNAs is attractive for this function, although no persuasive evidence for such function has yet been adduced. Despite the many questions remaining unresolved, this study lays a solid foundation for further investigation of the processes for specific encapsidation of BMV.

ACKNOWLEDGMENTS

We thank Jim Carrington for providing pGEM4.2-GUS; David Frisch for providing psub047; Greg Pogue for the use of p19UB3200wt; Fred Lahser for advice regarding the replicase extract; Jan Anthony, David Frisch, Lonke van der Geest, and Jim Carrington for useful discussions; and Denise Ariola for technical assistance.

This work was supported in part by National Science Foundation grant DMB-8921023.

REFERENCES

- Ahlquist, P., R. Dasgupta, and P. Kaesberg. 1984. Nucleotide sequence of the brome mosaic virus genome and its implications for viral replication. *J. Mol. Biol.* **172**:369–383.
- Bancroft, J. B., E. Hiebert, and C. E. Bracker. 1969. The effect of various polyanions on shell formation of some spherical viruses. *Virology* **39**:924–930.
- Bujarski, J. J., S. F. Hardy, W. A. Miller, and T. C. Hall. 1982. Use of dodecyl- β -D-maltoside in the purification and stabilization of RNA polymerase from brome mosaic virus-infected barley. *Virology* **119**:465–473.
- Carrington, J. C., and D. F. Freed. 1990. Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. *J. Virol.* **64**:1590–1597.
- Citovsky, V., D. Knorr, G. Schuster, and P. Zambryski. 1990. The P30 movement protein of tobacco mosaic virus is a single strand nucleic acid binding protein. *Cell* **60**:637–647.
- Cuillel, M., C. Berthet-Colominas, B. Krop, A. Tardieu, P. Vachette, and B. Jacrot. 1983. Self assembly of brome mosaic virus capsids. Kinetic studies using neutron and X-ray solution scattering. *J. Mol. Biol.* **164**:645–650.
- Cuillel, M., M. Herzog, and L. Hirth. 1979. Specificity of *in vitro* reconstitution of brome grass mosaic virus. *Virology* **95**:146–153.
- Dreher, T. W., A. L. N. Rao, and T. C. Hall. 1989. Replication *in vivo* of mutant brome mosaic virus RNAs defective in aminoacylation. *J. Mol. Biol.* **206**:425–438.
- Duggal, R., A. L. N. Rao, and T. C. Hall. 1992. Unique nucleotide differences in the conserved 3' termini of brome mosaic virus RNAs are maintained through their optimization of genome replication. *Virology* **187**:261–270.
- Erickson, J. W., and M. G. Rossmann. 1982. Assembly and crystallization of a T=1 icosahedral particle from trypsinized southern bean mosaic virus coat protein. *Virology* **116**:128–136.
- Geigenmüller-Gnirke, U., H. Nitschko, and S. Schlesinger. 1993. Deletion analysis of the capsid protein of Sindbis virus: identification of the RNA binding region. *J. Virol.* **67**:1620–1626.
- Hardy, S. F., T. L. German, L. S. Loesch-Fries, and T. C. Hall. 1979. Highly active template-specific RNA-dependent RNA polymerase from barley leaves infected with brome mosaic virus. *Proc. Natl. Acad. Sci. USA* **76**:4956–4960.
- Haseloff, J., P. Golet, D. Zimmern, P. Ahlquist, R. Dasgupta, and P. Kaesberg. 1984. Striking similarities in amino acid sequence among nonstructural proteins encoded by RNA viruses that have dissimilar genomic organization. *Proc. Natl. Acad. Sci. USA* **81**:4358–4362.
- Heaton, L. A., J. C. Carrington, and T. J. Morris. 1989. Turnip crinkle virus infection from RNA synthesized *in vitro*. *Virology* **170**:214–218.
- Lane, L. C. 1974. The bromoviruses. *Adv. Virus Res.* **19**:151–220.
- Marsh, L. E., C. C. Huntley, G. P. Pogue, J. P. Connell, and T. C. Hall. 1991. Regulation of (+):(-)-strand asymmetry in replication of brome mosaic virus RNA. *Virology* **182**:76–83.
- Marsh, L. E., G. P. Pogue, U. Szybiak, J. P. Connell, and T. C. Hall. 1991. Non-replicating deletion mutants of brome mosaic virus RNA-2 interfere with viral replication. *J. Gen. Virol.* **72**:2367–2374.
- Matthews, R. E. F. 1991. *Plant virology*, 3rd ed. Academic Press, Inc., New York.
- Miller, W. A., and T. C. Hall. 1983. Use of micrococcal nuclease in the purification of highly template dependent RNA-dependent RNA polymerase from brome mosaic virus-infected barley. *Virology* **125**:236–241.
- Nakhasi, H. L., T. A. Rouault, D. J. Haile, T.-Y. Liu, and R. D. Klausner. 1990. Specific high-affinity binding of host cell proteins to the 3' region of rubella virus RNA. *New Biol.* **2**:255–264.
- Pogue, G. P., and T. C. Hall. Unpublished data.
- Sacher, R., and P. Ahlquist. 1989. Effects of deletions in the N-terminal basic arm of brome mosaic virus coat protein on RNA packaging and systemic infection. *J. Virol.* **63**:4545–4552.
- Sgro, J.-Y., B. Jacrot, and J. Chroboczek. 1986. Identification of regions of brome mosaic virus coat protein chemically cross-linked *in situ* to viral RNA. *Eur. J. Biochem.* **154**:69–76.
- Sorger, P. K., P. G. Stockley, and S. C. Harrison. 1986. Structure and assembly of turnip crinkle virus. II. Mechanism of reassembly *in vitro*. *J. Mol. Biol.* **191**:639–658.
- Verduin, B. J. M. 1978. Ph.D. thesis. Agricultural University of Wageningen, Wageningen, The Netherlands.
- Vriend, G., M. A. Hemminga, B. J. M. Verduin, J. L. DeWit, and T. J. Schaafsma. 1981. Segmental mobility involved in protein-RNA interaction in cowpea chlorotic mottle virus. *FEBS Lett.* **134**:167–171.
- Wei, N., L. A. Heaton, T. J. Morris, and S. C. Harrison. 1990. Structure and assembly of turnip crinkle virus. VI. Identification of coat protein binding sites on the RNA. *J. Mol. Biol.* **214**:85–95.
- Wei, N., and T. J. Morris. 1991. Interactions between viral coat protein and a specific binding region on turnip crinkle virus RNA. *J. Mol. Biol.* **222**:437–443.
- Weiss, B., H. Nitschko, I. Ghattas, R. Wright, and S. Schlesinger. 1989. Evidence for specificity in the encapsidation of Sindbis virus RNAs. *J. Virol.* **63**:5310–5318.