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# *cis*- and *trans*-Acting Functions of Brome Mosaic Virus Protein 1a in Genomic RNA1 Replication▽

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**RNA viruses employ a combination of mechanisms to regulate their gene expression and replication. *Brome mosaic virus* (BMV) is a tripartite positive-strand RNA virus used to study the requirements for virus infection. BMV genomic RNA1 encodes protein 1a, which contains a methyltransferase (MT) domain and a helicase domain that are required for replication. 1a forms a complex with the 2a RNA-dependent RNA polymerase for the replication and transcription of all BMV RNAs. RNA1 expressed with 2a from *Agrobacterium*-based vectors can result in RNA1 replication in *Nicotiana benthamiana*. A mutation in the 1a translation initiation codon significantly decreased RNA1 accumulation even when wild-type (WT) 1a and 2a were provided in *trans*. Therefore, efficient RNA1 replication requires 1a translation from RNA1 in *cis*, indicating a linkage between replication and translation. Mutation analyses showed that the full-length 1a protein was required for efficient RNA1 replication, not just the process of translation. Three RNA1s with mutations in the 1a MT domain could be partially rescued by WT 1a expressed in *trans*, indicating that the *cis*-acting function of 1a was retained. Furthermore, an RNA motif in the 5′-untranslated region of RNA1, named the B box, was required for 1a to function in *cis* and in *trans* for BMV RNA accumulation. The B box is required for the formation of the replication factory (M. Schwartz, J. Chen, M. Janda, M. Sullivan, J. den Boon, and P. Ahlquist, Mol. Cell 9:505–514, 2002). Results in this work demonstrate a linkage between BMV RNA1 translation and replication.**

For all positive-strand RNA viruses, the genomic RNA(s) serves first as the mRNA to translate the replication proteins and then as the template for RNA replication. The proper timing of the two processes prevents the translating ribosomes, which move from 5′ to 3′ on the RNA, from colliding with the replicase that reads the RNA from 3′ to 5′ during negative-strand RNA synthesis. For poliovirus, ribosomes on the RNA template can actively prevent negative-strand RNA synthesis by providing a timing switch for the two processes. Replication can take place only after the clearing of ribosomes (6). Similar coupling of translation and replication/transcription has been reported for several animal and plant RNA viruses and with defective interfering RNAs (3, 7, 8, 24, 25, 29, 30, 34, 36, 40, 47, 50, 52, 53, 57, 58).

*Brome mosaic virus* (BMV), a member of the alphavirus-like superfamily of RNA viruses, is a model segmented positive-strand RNA virus for studying viral gene expression and replication, RNA encapsidation, and recombination (23, 35, 44, 51). The BMV genome consists of three capped, messenger-sense genomic RNAs that have a tRNA-like structure within the 3′-untranslated region (3′UTR). Genomic RNA1 and RNA2 encode nonstructural proteins 1a and 2a, respectively, which direct RNA replication. Genomic RNA3 is a bicistronic RNA encoding the cell-to-cell movement protein (MP) and coat protein (CP). The MP is translated from RNA3, whereas the 3′-proximal CP is translated from subgenomic RNA4 (33, 35). In addition to replication in various plant hosts, BMV can replicate its genome in the surrogate host *Saccharomyces cerevisiae* (21).

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The BMV 1a protein contains two domains separated by a proline-rich sequence. The N-terminal 516 residues contain activities for $m^7G$ methyltransferase and the binding of GTP through a covalent intermediate that is required for viral RNA capping (1, 26). The C-terminal 436 residues of 1a contain all of the motifs found in RNA helicases, although only ATPase, but not helicase, activity has been documented (56). 1a is the primary viral protein determinant for the localization of the replicase to perinuclear and endoplasmic reticulum membranes (12, 45, 46). In yeast, 1a could recruit viral RNA2 and RNA3 to the replication complexes in a reaction that involves an RNA sequence named the B box that is within the intercistronic region of RNA3 and the 5′UTR of RNA2 (9, 21, 49).

The 2a protein is an RNA-dependent RNA polymerase (RdRp) (59). 2a has a large central domain that contains all of the hallmark motifs of an RdRp, and it is flanked by less conserved N and C termini (37). RNA1 and RNA2 can replicate in transfected protoplasts and *Nicotiana benthamiana* plants in the absence of RNA3 or RNA4 (4, 14, 18, 41, 42). Thus, the movement and the capsid proteins encoded by BMV RNA3 are not essential parts of the BMV replicase, although they could have regulatory roles in the accumulation of molar excesses of BMV positive-strand RNAs (31).

The BMV 1a and 2a proteins function together as a complex for BMV RNA replication. 1a and 2a can interact when expressed in rabbit reticulocytes and in yeast as components of the two-hybrid assay. The C-terminal portion of 1a can interact with the N-terminal portion of 2a (10, 22, 38, 48). The interaction of 1a and 2a also is found in enzymatically active BMV replicase (43) and in the endoplasmic reticulum of plant cells, the site of BMV RNA synthesis (45, 46). The interaction between 1a and 2a is species specific, as determined by examining

TABLE 1. Plasmids made in this study[a]

| Construct | Primer | Sequence[b] (5′–3′) | Amplification or cloning strategy | Primer pair for amplification or cloning |
|---|---|---|---|---|
| R1KO | (+)BglII | GAAGATCTCGTGTGGTTGGCATGCACA | Fusion PCR; PCR products were cloned into pBR1 cut with BglII and MluI | |
| | (−)R1KO | GTTCTTTGTTTTTCACCAACAAATTGTCAAGT TCTATCGATTTGCTG | | 1. (+)BglII and (−)R1KO |
| | (+)R1KO | CAGCAAATCGATAGAACTTGAGAATTTGTTG GTGAAAAACAAAGAAC | | 2. (−)R1KO and (+)R1/ MluI |
| | (−)R1/MluI | GTACCACGTAGTACGCGTACTCCATGCGG ATGC | | 3. (+)BglII and (−)R1/ MluI |
| R2KO | (+)BglII | See above | Fusion PCR; PCR products were ligated into pBR2 cut with BglII and MluI | |
| | (−)R2KO | GGCGAACGAAATCAGCAGCCCAGGTTTTCGA AGACAACTTGGTGATAGTAGAAAGAAC | | 1. (+)BglII and (−)R2KO |
| | (+)R2KO | GTTCTTTCTACTATCACCAAGTTGTCTTCGAA AACCTGGGCTGCTGATTTCGTTCGCC | | 2. (−)R2KO and (+)R2/ MluI |
| | (−)R2/MluI | GTAAACGCGTCACCAGTTCTGCGTTGG | | 3. (+)BglII and (−)R2/ MluI |
| R1ΔN2-50 | (+)BglII | See above | Ligation; PCR products were cut with restriction enzymes and ligated into pRB1 cut with BglII and SnaBI | |
| | (+)SalI | GTCGACCACGGTTCTGCTACTTGTTCTTTGTT TTTCACCAACAAAATGAAGCTCTCTATTGA GGAGGCTGAC | | 1. (+)BglII and (−)SalI |
| | (−)SalI | GTCGACAAGGGATTGAACCTCGTTC | | 2. (+)SalI and (−)SnaBI |
| | (−)SnaBI | TACGTACTTAGATATGTCTTCAAACCATAC | | |
| R1ΔC50 | (+)AatII | GACGTCAGGATGGCTTTATTCCCTGAC | Ligation; PCR products were cut with the corresponding enzyme and cloned into pRB1 cut with (+)AatII and (−)ApaI | |
| | (−)XhoI | CTCGAGCAGAGGTCTCACACAGAGACAAGC GCATCAGTCAACAGAGATCCCTTGCGCTTC | | 1. (+)AatII and (−)XhoI |
| | (+)XhoI | CTCGAGGAGAGCCCTGTTCCAGGTAGGAAC | | 2. (+)XhoI and (−)ApaI |
| | (−)ApaI | GGGCCCCCCGTCGAGGTCGACGGTATC | | |
| R1PM5 | (+)PM5 | GCGAATCGTAAATCGGCC**T**AGGACGTCAGG ATGGCTTTATTC | Site-directed mutagenesis of pBR1 | |
| | (−)PM5 | GAATAAAGCCATCCTGACGTCCT**A**GGCCGAT TTACGATTCGC | | |
| R1PM6 | (+)PM6 | AACGGCGAGCTCGCTGGTGATT**AA**ATCTTTA ATTGTGTTAAGTGA | Site-directed mutagenesis of pBR1 | |
| | (−)PM6 | TCACTTAACACAATTAAAGAT**TT**AATCACCA GCGAGCTCGCCGTT | | |
| R1-PK17 | (+)AatII | See above | Fusion PCR; PCR products were ligated into pBR1 cut with AatII and ApaI | |
| R1-PK18 | (−)ApaI | See above | | 1. (+)AatII and (−)XbaI |
| | (+)XbaI | GAGTCCAAAGTTTCTAGAAGTCTCACGAAGC | | 2. (+)XbaI and (−)ApaI |
| | (−)XbaI | GCTTCGTGAGACTTCTAGAAACTTTGGACTC | | 3. (+)AatII and (−)ApaI |
| H80A | (+)H80 | CAGCAGTATCATGCGCCC**GC**TAGCCTGGCTG GTGCTC | Site-directed mutagenesis of pBR1 | |
| | (−)H80 | GAGCACCAGCCAGGCTA**GC**GGGCGCATGAT ACTGCTG | | |
| D106A | (+)D106 | GAAGACCCCGTTATAG**CG**TTCGGAGGGTC TTGG | Site-directed mutagenesis of pBR1 | |
| | (−)D106 | CCAAGACCCTCCGAA**CG**CTATAACGGGGT CTTC | | |
| R136A | (+)R136 | GTTAGAGACGCTGCC**GC**ACATGAGGAGAGG ATGTGC | Site-directed mutagenesis of pBR1 | |
| | (−)R136 | GCACATCCTCTCCTCATGT**GC**GGCAGCGTCTC TAAC | | |
| C168A | (+)C168 | TAACCGAGCTCAAGAT**GC**TGATGTCCAAGCT GATTGG | Site-directed mutagenesis of pBR1 | |
| | (−)C168 | CCAATCAGCTTGGACATCA**GC**ATCTTGAGCT CGGTTA | | |
| Y248A | (+)Y248 | GAAAGCACATTATCT**GC**CATCCACGGATGG CAAG | Site-directed mutagenesis of pBR1 | |
| | (−)Y248 | CTTGCCATCCGTGGATG**GC**AGATAATGTGC TTTC | | |
| 2(1a)1 | (+)BglII | See above | Fusion PCR; PCR products were cloned into pBR1 cut with BglII and SnaBI | |
| | (−)SnaBI | See above | | 1. (+)BglII and (−)R25′U |
| | (−)R25′U | CTTCAGCAAATCGATAGAACTTGACATCTTG GTGATAGTAGAAAGAAC | | 2. (+)R25′U and (−)SnaBI |
| | (+)R25′U | GTTCTTTCTACTATCACCAAGATGTCAAGTTC TATCGATTTGCTGAAG | | |
| 3(1a)1 | (+)BglII | See above | Fusion PCR; PCR products were cloned into pBR1 cut with BglII and SnaBI | |
| | (−)SnaBI | See above | | 1. (+)BglII and (−)R35′U |
| | (−)R35′U | CTTCAGCAAATCGATAGAACTTGACATCTTG GTGATAGTAGAAAGAAC | | 2. (+)R35′U and (−)SnaBI |

*Continued on facing page*

TABLE 1—*Continued*

| Construct | Primer | Sequence[b] (5′–3′) | Amplification or cloning strategy | Primer pair for amplification or cloning |
|---|---|---|---|---|
| | (+)R35′U | GTGATACTGTTTTTGTTCCCGATGTCAAGTTC TATCGATTTGCTGAAG | | |
| Bb/M | (−)MS2/Bb | GCAGAACCGTGGTCGACAACATGGGTAATA CTCATGTCGTTCCGTGGTCTACTCC | Site-directed mutagenesis of pBR1 | |
| | (+)MS2/Bb | GGAGTAGACCACGGAACGACATGAGGATTA CCCATGTTGTCGACCACGGTTCTGC | | |
| ΔBb | (+)ΔBbox | GAGAGGAGTAGACCACGGAACGAGGTCCCT TGTCGACCACGGTTCTGC | Site-directed mutagenesis of pBR1 | |
| | (−)ΔBbox | GCAGAACCGTGGTCGACAAGGGACCTCGTTC CGTGGTCTACTCCTCTC | | |

[a] (+) denotes a forward primer; (−) denotes a reverse primer.
[b] Restriction sites are underlined, and mutated residues are in boldface.

the replication of homologous and heterologous combinations of BMV 1a and 2a and the orthologs from the related bromovirus cowpea chlorotic mottle virus (13, 38). The extracted replicase from barley plants or transiently expressed replicase in *Nicotiana benthamiana* plants or yeast can support in *trans* genomic RNA3 replication and subgenomic RNA4 transcription (18, 21). In addition, 1a and 2a expressed in yeast can replicate genomic RNA2 and RNA3 (9). Using various hosts or in vitro systems, a number of *cis*-acting elements has been identified for genomic replication and subgenomic transcription.

While developing a minimal replicon system using the *Agrobacterium*-based transient expression system, we found that efficient RNA1 replication requires the translation of 1a protein in *cis*. Furthermore, the B box in the 5′UTR of RNA1 is required for 1a to function in *trans* for both negative- and positive-strand RNA1 replication. Based on these and previous results, RNA1 replication is dependent on the ability to translate the 1a protein, which is required to bridge the timing of translation and RNA replication.

## MATERIALS AND METHODS

**Plasmid constructs.** Mutant plasmids generally were made using overlapping templates and three primer pairs. First, two PCR products that overlapped by ca. 20 bp were independently generated using the first two primer pairs. The products were purified from agarose gels and then used as templates for amplification with the third primer pairs, which anneal to the termini of the first two templates. The fusion PCR product was digested with enzyme and inserted into the corresponding site of pBR1 or pBR2, replacing the wild-type (WT) sequence. These constructs are designated as being generated by a fusion PCR method (Table 1). A second cloning strategy uses the DNA fragments generated by restriction enzyme digestion and ligation into the appropriate plasmid using a subcloning strategy (Table 1). A third method to make constructs uses site-directed mutagenesis in which two complementary oligonucleotides containing the desired sequence are used to extend the desired sequence from the target plasmid. The products were digested with the modification-sensitive restriction enzyme DpnI prior to transformation into *Escherichia coli* DH5α. Plasmids then were purified, and their sequences were determined to identify the desired mutants.

**Agroinfiltration.** The *Agrobacterium*-mediated delivery of recombinant tDNAs was used to express BMV genomic RNAs for replication or the BMV-encoded replication proteins 1a and 2a (18). Plasmids were introduced into *Agrobacterium tumefaciens* C58C1 by electroporation using an electroporator 2510 set at 2,000 V (Eppendorf). Cultures were grown in Luria-Bertani (LB) medium amended with 50 μg/ml kanamycin for 24 h at 30°C with vigorous shaking. A 0.5-ml aliquot of the culture was transferred to 25 ml LB medium containing 10 mM morpholineethanesulfonic acid (MES) and 40 μM acetosyringone and was incubated at 30°C overnight with vigorous shaking. The bacteria were pelleted by centrifugation at 3,000 × *g* for 10 min and resuspended in 5 ml of infiltration buffer containing 10 mM MES buffer, 10 mM $MgCl_2$, and 1.25 mM of acetosyringone. The mixture was kept at room temperature for at least 3 h without shaking and then was adjusted to the desired $A_{595}$.

Plants used for agroinfiltration were grown in an environmentally regulated growth chamber set to 27°C with a 16-h light/8-h dark regimen and a humidity of 70%. Plants that had six leaves were used for agroinfiltration, and usually only the fully expanded leaves were used. The leaves were infiltrated by gently pressing the end of a 3-ml syringe to the leaf surface and flooding the interstitial areas of the leaf. For each sample tested, at least two independently infiltrated samples were analyzed.

**RNA extraction and Northern blotting.** Total RNA was extracted from 50 mg of leaf tissue by maceration of the tissue with disposable pestles in the presence of a lysis buffer (0.1 M glycine, pH 9.0, 40 mM EDTA, 100 mM NaCl, 2% sodium dodecyl sulfate, and 0.05% bentonite), and then extraction with an equal volume of phenol and chloroform and precipitation with an equal volume of isopropanol. Glyoxylated RNA (usually 7.5 μg denatured at 60°C for 1 h) was loaded into a 1.2% agarose gel. After transferring the RNA to the nylon membrane, Northern blotting was performed with α-$^{32}$P-labeled strand-specific riboprobes, as described by Hema and Kao (20). Briefly, to detect negative-strand RNAs, the EcoRI-linearized plasmid, which contains 200 nucleotides of the tRNA-like sequence of the BMV 3′UTR, was used as the template for in vitro transcription with a T7 megascript transcription kit (Epicenter Inc.). The HindII-linearized plasmid was used as the template for in vitro transcription with SP6 polymerase to detect positive-strand RNA. All Northern blottings were repeated at least three times. All signals in the Northern blottings were quantified using ImageQuant software.

**RT-PCR.** Total RNA was extracted using the Mini RNA Easy kit using the manufacturer's protocol (Qiagen Corp.) and then was treated with RNase-free DNase I at 37°C for 20 min. After DNase I was inactivated, 1 μg of total RNA was used for the reverse transcription (RT) with the reverse primer 5′-TACGT ACTTAGATATGTCTTCAAACCATAC-3′ and SuperScript II reverse transcriptase as described by the manufacturer (Invitrogen Inc.). PCR was performed with both the reverse and forward primer (5′-GTCGACCACGGTTCTGCTAC TTGTTC-3′) for 40 cycles, with 54°C as the annealing temperature. The PCR products were separated in a 1% agarose gel and stained with ethidium bromide. The PCR product was cloned into pGEM-T Easy vector (Promega, Inc.), and 10 random clones were selected for sequencing using the BigDye sequencing system.

**Protein analysis and Western blotting.** Leaf tissues were macerated with a pestle in TB buffer (50 mM Tris-acetate, pH 7.4, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and the Complete proteinase inhibitor cocktail) to generate a lysate for Western blotting. The lysate was kept on ice for 20 min and clarified by a 5,000 × *g* centrifugation for 5 min at 4°C to remove the plant cell debris. The supernatant was centrifuged at 16,000 × *g* for 30 min at 4°C to separate the pellet and the supernatant. The pellet was dissolved in 150 μl of TB buffer, and an equal amount of protein was loaded into a 4 to 20% denaturing polyacrylamide gel. The proteins were transferred to a polyvinyl difluoride membrane and blocked with 3% nonfat milk. Rabbit anti-1a polyclonal antibody (1:2,000) was used as the primary antibody. The primary antibody was detected with a goat anti-rabbit polyclonal antibody linked to horseradish peroxidase. The signal was detected with an enhanced chemiluminescence kit (Amersham Inc.).

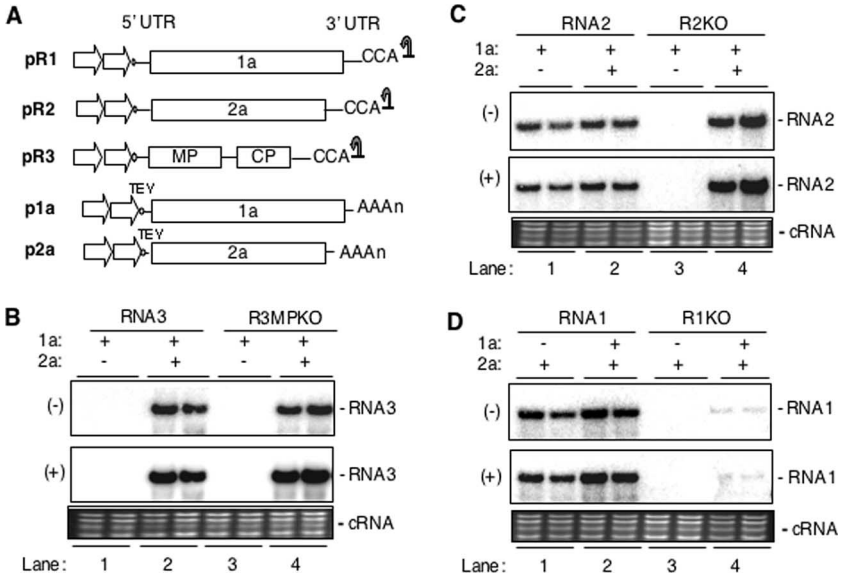


FIG. 1. *cis*-acting function of BMV 1a protein in genomic RNA1 replication. (A) Schematic representations of BMV constructs used to express BMV viral RNAs or BMV-encoded replication proteins in a binary vector (18). The two copies of the cauliflower mosaic virus 35S promoter are shown as double arrows, and the ribozyme used to generate the 3′ termini of RNAs by *cis* cleavage by the ribozyme derived from tobacco ring spot virus satellite RNA is represented by a curved arrow. The tobacco etch virus translational enhancer at the 5′ end is denoted by the letters TEV, and the polyadenylation signal is represented by AAAn. (B) Transiently expressed BMV 1a and 2a proteins can replicate RNA3 and R3MPKO. *Agrobacterium* cultures that express 1a or both 1a and 2a (at an $A_{595}$ of 0.2) and RNA3 (at an $A_{595}$ of 0.5) were coinfiltrated into *N. benthamiana* leaves. Total RNA was extracted at 48 hpi, and negative- and positive-strand RNAs were detected with strand-specific probes by Northern blotting. Gel images labeled cRNA denote cellular RNA detected in the ethidium bromide-stained agarose gel used for the Northern blots. The autoradiographs of negative-strand RNA were exposed overnight, while the autoradiographs of positive-strand RNA were exposed for 2 h. (C) *Agrobacterium* expressing 1a and 2a can support RNA2 and R2KO replication. (D) Efficient accumulation of RNA1 required the translation of 1a protein in *cis*.

## RESULTS

**Efficient BMV RNA1 replication requires the translation of the 1a protein in *cis*.** The agroinoculation system has the advantage of delivering hundreds of tDNA copies into the nuclei of cells of an *N. benthamiana* plant (17). This feature can be used to mix and match the expression of the three BMV genomic RNAs either individually or in combination with one or more of the BMV-encoded proteins. The three genomic cDNAs were cloned into a binary tDNA vector that contained the cauliflower mosaic virus 35S promoter and a *cis*-acting ribozyme to ensure a proper 3′ terminus for the BMV RNAs. The p1a and p2a plasmids that express BMV replication proteins were constructed by cloning the coding sequences of RNA1 and RNA2 in the binary vector with the tobacco etch virus translational enhancer at the 5′ end and a poly(A) signal at the 3′ end (Fig. 1A).

To study the *cis*- and *trans*-acting requirements of BMV RNAs, *Agrobacterium* that can express genomic RNA1 and 2a or RNA2 and 1a was infiltrated into *N. benthamiana* leaves. Total RNA was extracted at 48 h postinfiltration (hpi), and the negative- and positive-strand RNAs were detected with strand-specific probes by Northern blot assays (Fig. 1C and D). Both negative- and positive-strand RNA1 and RNA2 were observed at 48 hpi. In these reactions, the RNAs serve as the template for replication and provide one of the two BMV-encoded proteins of the BMV replicase, while the other protein is provided by a replication-incompetent mRNA. When RNA1 was expressed with both replication proteins 1a and 2a, RNA accumulation was even higher (Fig. 1D, lane 2), demonstrating that RNA silencing was not a significant factor on RNA1 accumulation under these conditions. Similar results were obtained with RNA2 (Fig. 1C, lane 2). The replication of BMV RNA3 also can take place with 1a and 2a expressed in *trans* (Fig. 1B, lane 2). This minimal replicon system can be used to study *cis*- and *trans*-acting functions of BMV-encoded proteins during genomic RNA replication.

To examine whether the BMV genomic RNA replication requires the translation of encoded protein in *cis*, we mutated the first translation initiation codons of BMV RNA1, RNA2, or RNA3 to UUG, resulting in constructs named R1KO, R2KO, and R3MPKO, respectively. To demonstrate that the mutations did not result in sufficient leaky initiation or alternative translation initiation for RNA replication, a combination of R2KO and 1a or R1KO and 2a was introduced into plants. Neither of these combinations replicated the mutated RNA efficiently, indicating that the backgrounds for these reactions were suitable for further analysis (Fig. 1C and D, lanes 3).

R2KO and R3MPKO were found to accumulate to levels comparable to those of WT RNA2 and RNA3, respectively, when both 1a and 2a were expressed in *trans* (Fig. 1B and C, lanes 4). These results demonstrate that RNA2 and RNA3 can efficiently use the replicase produced in *trans*. In contrast, the accumulation of R1KO was decreased to ~10 to 20% of that of WT RNA1 even when 1a and 2a were provided in *trans* with any of several concentrations of inoculum that can produce 1a (Fig. 1D, lane 4, and data not shown). These results lead us to

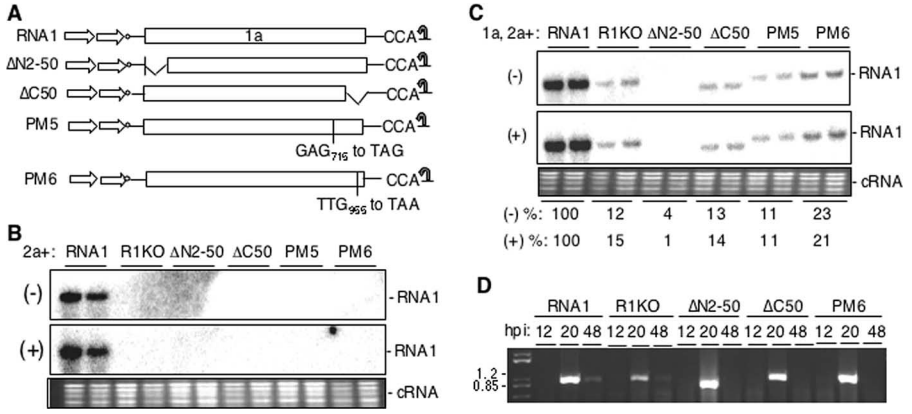


FIG. 2. Translation of full-length 1a is required for RNA1 replication. (A) The schematic represents the binary constructs used in this experiment. ΔN2-50 and ΔC50 represent deletions of N-terminal codons 2 to 50 and the last C-terminal 50 codons, respectively, from the 1a coding sequence of RNA1. PM5 and PM6 have Glu715 (GAG) and Leu955 (TTG) codons changed to termination codons. (B) The constructs described in the legend to panel A cannot replicate in *N. benthamiana* leaves in the presence of WT 2a protein. The autoradiographs of negative-strand RNA were exposed overnight, while the autoradiographs of positive-strand RNA were exposed for 2 h. (C) The process of translation is not responsible for the lack of efficient RNA1 replication. *Agrobacterium* expressing 1a and 2a proteins and one of the cultures expressing R1KO, ΔN50, ΔC50, PM5, and PM6 were coinfiltrated. (D) RT-PCR was used to detect the transcribed RNA from a binary vector. *Agrobacterium* that can express each of the mutant RNAs was infiltrated into plants at a final $A_{595}$ of 0.5. Total RNA was extracted at 12, 20, and 48 hpi and treated with RNase-free DNase I. After RNA purification, approximately equal amounts of RNA were subjected to RT-PCR. The PCR products were separated in 1% agarose gels and stained with ethidium bromide. The DNA marker is shown in the left-most lane.

hypothesize that efficient RNA1 replication requires the translation of the encoded 1a protein in *cis*.

**Translation of full-length 1a is required for efficient RNA1 amplification.** A requirement for 1a translation in *cis* for RNA1 replication could result from a need for the act of translation, perhaps to reconfigure the structure of RNA1. To test this, we made several constructs with mutations within the 1a-coding sequence of RNA1, all of which should retain the ability to direct translation without producing full-length 1a. First, we deleted codons 2 to 50 in a construct named ΔN2-50 as well as the C-terminal 50 codons of the 1a coding sequence in a construct named ΔC50 (Fig. 2A). Both mutants can efficiently translate the truncated protein in rabbit reticulocyte extracts (data not shown), but they are defective for RNA replication (Fig. 2B). When WT 1a was provided in *trans*, the accumulation of constructs ΔN2-50 and ΔC50 remained low (Fig. 2C), indicating that the act of translation is not sufficient for RNA accumulation. To confirm these results and to minimize the potential effects of changes in the RNA sequence, we made two constructs with premature translation codons in the C-terminal portions of the 1a coding regions, named PM5 and PM6 (changing the E715 codon to TAG and the L955 codon to TAA) (Fig. 2A). PM5 and PM6 accumulated to levels that were 11 and 21%, respectively, of that of WT RNA1 in the presence of 1a in *trans* (Fig. 2C). These results support our hypothesis that the production of the functional 1a protein from RNA1 is needed for efficient genomic RNA1 replication.

A possible explanation for the poor accumulation of RNA1 mutants is that the mutant RNAs are unstable in plants. To rule out this possibility, total RNAs were extracted from *N. benthamiana* plants inoculated with WT and mutant variants of RNA1 at 12, 20, and 48 hpi. After treatment with RNase-free DNase I followed by phenol-chloroform purification, equal amounts of total RNA were subjected to RT-PCR amplification. Different concentrations of input cDNAs were used to ensure that the product synthesis was in the linear range. As shown in Fig. 2D, the relative amounts of amplified DNA from ΔN2-50, ΔC50, and PM6 were similar to those of WT RNA1 at all three time points assayed. We note that there is no replication in this assay, but the detection of similar levels of RNA transcribed from tDNA constructs indicates that RNA stability is not responsible for the low accumulation of RNA1 when full-length 1a was not produced.

**Some 1a mutants can be partially complemented in *trans*.** Since 1a is required in *trans* for the replication of RNA2 and RNA3 and is required both in *cis* and in *trans* for RNA1, it is possible that some defects in 1a would affect only the *cis*- or *trans*-acting function of 1a. Furthermore, it should be possible to complement the *cis*-acting defect by 1a expressed in *trans*. To test this possibility, we tested several mutants, named the PK mutants, that have 2- or 3-amino-acid in-frame insertions in the 1a coding region of RNA1 (27). The ones selected for characterization can produce an amount of protein approximately equal to that of WT 1a in yeast, but they are severely defective for RNA replication in barley (27, 38). Among these mutants, PK7, PK11, PK12, and PK13 have amino acid insertions in the methyltransferase (MT) domain, while PK15, PK17, and PK18 have insertions in the helicase domain. To test whether these mutants are defective for RNA replication in *N. benthamiana*, *Agrobacterium* expressing each of the PK mutants was coinfiltrated with cultures expressing 2a and RNA3. No RNA replication was observed for any of the PK mutants in the absence of 1a (Fig. 3B), indicating that all of the PK mutants are defective for the *trans*-acting function for RNA3 replication in *N. benthamiana* as well as in barley protoplasts.

To examine whether PK mutants can be rescued by WT 1a in *trans*, *Agrobacterium* that can express a PK mutant was coinfiltrated with transiently expressed 1a and 2a. In four independent experiments containing eight independent samples, all positive-strand RNA from PK7 and PK12 accumulated to at least 50% of the WT RNA1 level, while the other PK mutants were not efficiently rescued. In two of the four experiments,

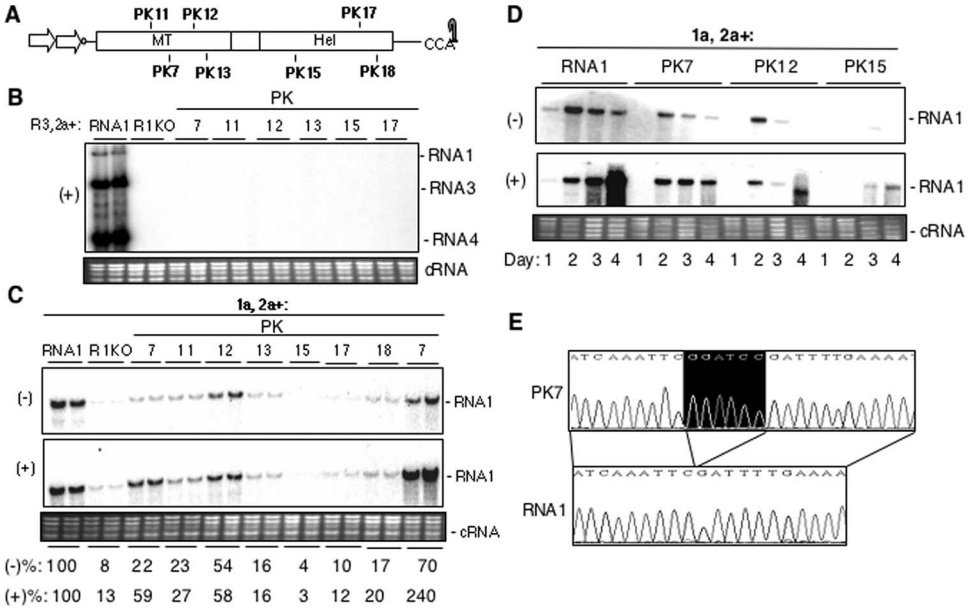


FIG. 3. Complementation of PK mutants with WT 1a provided in *trans*. (A) A schematic showing the location of PK mutants with 2- or 3-codon in-frame insertions in the 1a coding sequence. PK7, PK11, PK12, and PK13 have mutations in the MT domain, while PK15, PK17, and PK18 have insertions in the helicase domain. (B) Results of Northern blotting demonstrating that all of the PK mutants analyzed are defective for RNA replication in *N. benthamiana*. The constructs expressed by infiltrated *Agrobacterium* cultures are listed above the gel image of the Northern blot detecting positive-strand BMV RNAs. The autoradiographs of negative-strand RNA were exposed overnight, while the autoradiographs of positive-strand RNA were exposed for 2 h. (C) Northern blotting for the results for the *trans*-complementation of PK mutants by the WT 1a protein. (D) A time course experiment to confirm and extend the *trans*-complementation results. Total RNA was extracted on days 1, 2, 3, and 4, and viral RNA was detected by Northern blotting. (E) The accumulation of PK7 mutant was due to *trans*-complementation but not to repair or recombination. Total RNA was extracted and treated with DNase I and then subjected to RT-PCR. The sequence from a representative of the 10 clones of the PCR fragment derived from PK7 RNA is shown. All 10 clones retained the same 6-nucleotide insertion in the 1a coding region.

PK7 RNA accumulated to levels comparable to or much higher than that of the WT (Fig. 3C, far right lane). In a time course experiment that spanned 4 days, we found that the levels of negative-strand WT RNA1 efficiently accumulated by day 1 and then gradually decreased, while positive-strand RNA1 accumulated until day 4. For the PK7 mutant, the positive-strand RNA efficiently accumulated from day 1 to day 4, while PK12 accumulated only up to day 2 (Fig. 3D). The partial complementation by WT 1a demonstrates that PK7 and PK12 likely retain at least some of the *cis*-acting activity of 1a.

Although it is unlikely that the increased accumulation of PK7 is due to the repair of the RNA, we wanted to rule out this possibility. Total RNA from plants infiltrated to express the PK7 mutant was digested with DNase I. After purification, the RNA was reverse transcribed, and the cDNA was amplified with primers flanking the insertion in PK7. The amplified DNA fragment was cloned into the pGEM-T Easy vector, and 10 randomly selected clones were sequenced. All 10 clones derived from the PK7 mutant retained the insertion (Fig. 3E and data not shown), indicating that the accumulation of PK7 was not due to repair or recombination. This result supports the hypothesis that PK7, and likely PK12, can be complemented in *trans* by WT 1a.

**Examination of additional mutations in the MT domain.** Both PK7 and PK12 have amino acid insertions in the MT domain. We hypothesize that constructs with mutations in the 1a MT domain could retain *cis*-acting functions. To produce 1a proteins that are likely to be inactive (and hence decrease the background level of RNA accumulation), an alignment of comparable proteins from several members of the alphavirus-like family was used to identify five conserved motifs in the MT domain (Fig. 4A) (2). Conserved residues H80, D102, R136, C168, and Y248 in the five motifs then were mutated to alanines. All mutant RNAs lost the ability to replicate in *N. benthamiana* (Fig. 4B). In the presence of 1a provided in *trans*, the accumulation of D106A, R136A, C168A, and Y248A RNA levels remained low, but mutant H80A accumulated to nearly WT levels (Fig. 4C). Therefore, H80A, along with PK7 and PK12, in the MT domain retains the *cis*-acting function and can be *trans*-complemented by WT 1a.

***cis*-acting element in the 5′UTR of RNA1 is required for 1a to function in *trans*.** It is important to identify the *cis*-acting RNA element required for 1a to function in *cis* for RNA1 replication. Since BMV's three genomic RNAs have nearly identical RNA sequences and structures in the 3′UTR, we suspect that the *cis*-acting RNA element is located in the 5′UTR of RNA1. To test this, chimeric RNAs that swap the 5′UTR of RNA1 with that of RNA2 or RNA3, named 2(1a)1 and 3(1a)1, respectively, were made and coinfiltrated with *Agrobacterium* expressing the 2a protein into *N. benthamiana*. Construct 2(1a)1 accumulated to ~40% of the level of WT RNA1, while 3(1a)1 accumulated to only 2% of the level of WT RNA1 (Fig. 5B). The 5′UTRs of RNA1 and RNA2 differ in length (74 and 102 nucleotides, respectively) and in the number of predicted RNA secondary structures, but they share

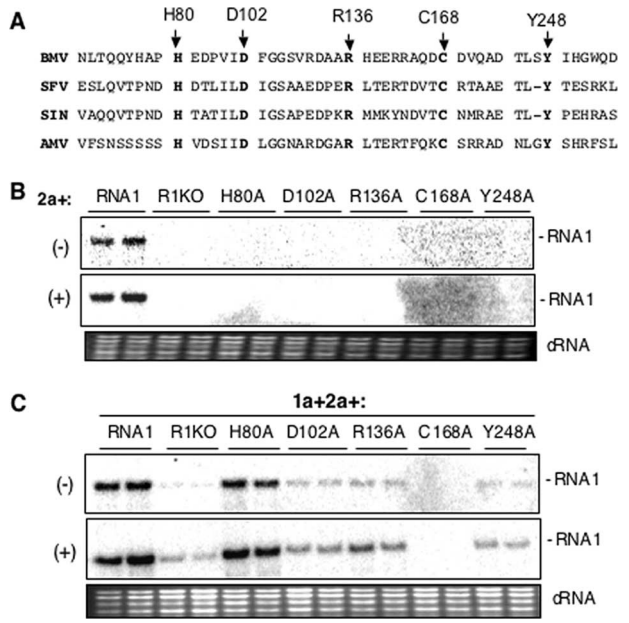


FIG. 4. *trans*-complementation of RNA1 mutants in the conserved MT domain. (A) Amino acid alignment of MT domains from different viruses in the alphavirus family, including alfalfa mosaic virus (AMV), Semliki Forest virus (SFV), and SIN. Conserved residues H80, D102, R136, C168, and Y248 in the five motifs were mutated to alanines. (B) A construct with a mutation in the MT domain of RNA1 cannot function in *trans* for RNA replication. *Agrobacterium* cultures expressing 2a protein were coinfiltrated with each of the RNA1 mutants. Total RNA was extracted at 48 hpi and detected by a Northern blot assay. The autoradiographs of negative-strand RNA were exposed overnight, while the autoradiographs of positive-strand RNA were exposed for 2 h. (C) Complementation of defective RNA1 by WT 1a protein provided in *trans*. *Agrobacterium* expressing each of the mutants was coinfiltrated with a culture expressing replication proteins 1a and 2a.

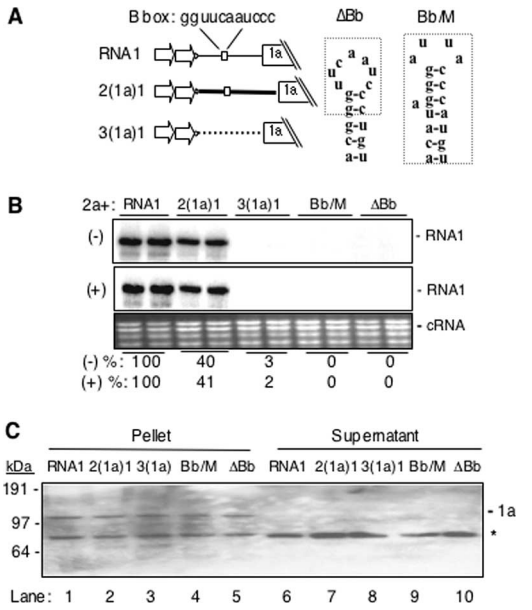


FIG. 5. *cis*-acting element in the 5′UTR of RNA1 required for 1a to function in *trans* for RNA1 replication. (A) Schematic representation of the chimeric constructs and the deletion mutation in the 5′UTR of RNA1. 2(1a)1 and 3(1a)1 are chimeric constructs containing the 5′UTR of RNA1 swapped with that of RNA2 and RNA3, respectively. Bb/M denotes a construct that has the B box replaced by the phage MS2 capsid binding RNA motif. ΔBb has a deletion of the B box motif. The secondary structures of the relevant sequences are shown to the right of the schematics of the 5′UTRs in the sequence. The deleted portion of the B box and the MS2 replacement sequence are shown in dashed boxes. (B) The WT B box in the 5′UTR of RNA1 is required for 1a to function in *cis*. The RNAs examined were extracted at 48 hpi. The autoradiographs of negative-strand RNA were exposed overnight, while the autoradiographs of positive-strand RNA were exposed for 2 h. (C) Western blot analysis detecting the 1a protein expressed from plants infiltrated with WT RNA1 or the chimeric constructs. *Agrobacterium* expressing each of the constructs was infiltrated at an $A_{595}$ of 0.5. Total protein was extracted at 22 hpi. The asterisk denotes an *N. benthamiana* protein that cross-reacts with the antiserum to the 1a protein.

an identical B box motif, while the 5′UTR of RNA3 lacks this element.

To examine whether the B box is the *cis*-acting element that works in concert with 1a for the *cis* replication of RNA1, we made two mutants: Bb/M, which replaced the B box with phage MS2 RNA binding sequence, and ΔBb, which deleted the B box from the 5′UTR. Both Bb/M and ΔBb were defective for RNA replication and could not be rescued by the 1a protein expressed in *trans* (Fig. 5B), demonstrating that the B box in the 5′UTR of RNA1 is required for 1a to function in RNA replication, likely by interacting with the *cis*-translated 1a protein.

In order for the B box to be a *cis*-acting element for 1a recognition, it should not affect the level of 1a protein produced. To examine this, *N. benthamiana* plants infiltrated to express all of the constructs tested and shown in Fig. 5B were examined for 1a production by Western blotting. All of the chimeric BMV RNAs and Bb/M and ΔBb produced similar levels of 1a in the membrane pellet fraction of *N. benthamiana* leaf lysate (Fig. 5C), the expected location of 1a (45, 46). Furthermore, the detection of comparable amounts of translation products from the chimeric RNAs suggests that they are able to localize to the endoplasmic reticulum. These results indicate that the mutation in the B box, but not the translation of WT 1a, was responsible for the lack of RNA accumulation.

## DISCUSSION

BMV RNA1 has a requirement for replication that is not shared by RNA2 and RNA3. RNA1 can be replicated in *N. benthamiana* only if it encodes and translates a functional 1a protein. All five of the mutants in the helicase-like domain of 1a prevented complementation in *trans*, but three of nine constructs with mutations in the MT domain partially retained the *cis*-acting function, suggesting that the helicase-like domain must be kept intact for *cis* function. The B box in the 5′UTR of RNA1 is required for the *cis* replication of RNA1, since mutations in the B box prevented replication of RNA1 even when the RNA is producing a functional 1a protein. Furthermore, the 5′UTR of RNA2 that contains a B box can partially restore RNA1 replication.

Based on these results, we propose that the 1a protein translated from RNA1 marks RNA1 for competence in replication, likely through a *cis*-specific recognition of the B box in RNA1.

Based on our results and those of the Ahlquist laboratory (9, 49, 60), there are at least three identified functions required for BMV infection due to the proposed 1a-B box interaction. First, the translation of 1a from RNA1 leads to the assembly of a complex on RNA1 as well as the formation of 1a complexes that act on the B boxes of RNA2 and RNA3. It is possible that RNA1 is translated from an RNA1 that is circularized due to proteins that interact at the 5′- and 3′UTRs so that the termination of 1a can result in the ribosome being close to the B box at the 5′ terminus of the RNA, as the 5′ cap and 3′UTRs of BMV RNAs both stimulate translation and RNA stability (15). Second, the 1a protein could recruit 2a during the assembly process, as 1a can physically interact with 2a, even 2a protein that is still in the process of translation (11, 22, 38). A build-up of sufficient numbers of subunits of 1a and/or 1a complexed with 2a then can result in the repression of translation. Third, the oligomerization of 1a on RNA containing the B box redirects the RNA from use as the template for translation to use in the formation of the replicase to becoming membrane-associated replication factories. The net effect of the three steps can result in an elegant timing switch from BMV translation to replication.

The B box is involved in 1a's role in translation, replicase formation, and the *cis*-specific recognition of RNA1. The 1a protein also can regulate translation from RNA1 and RNA2 through the B box (18, 60) as well as the targeting of RNA2 and RNA3 to the membrane-associated replication complexes (9, 49). Direct binding of the B box by 1a has not been demonstrated by biochemical methods. Indeed, this activity has proved to be difficult because of 1a's association with the plant membranes. However, the effects of mutations in the B box for RNA1 accumulation without affecting 1a levels are consistent with the idea that an interaction between the B box and 1a is central to the regulatory events. If cellular proteins are involved, a number of yeast proteins identified and characterized by Mas and colleagues are promising candidates (32).

The partial *trans*-complementation of three constructs with mutations in the 1a MT domain (PK7, PK12, and H80) is consistent with the model that 1a must form a complex, perhaps in association with the B box. A similar ability to be *trans*-complemented was reported for mutations in the MT domain of comparable proteins in the *Alfalfa mosaic virus* and *Sindbis virus* (SIN) members of the alphavirus superfamily (28, 54). For SIN, it was shown that cells expressing WT Nsp1 protein, a homologue to BMV 1a, could rescue the replication of a SIN strain that was conditionally defective in methyltransferase activity (28). We interpret these results to mean that the 1a protein may oligomerize through the helicase-like domain. The oligomerization that involves cellular membranes may eventually provide the framework to form the BMV replication factory, perhaps providing an explanation for the presence of one BMV RNA per replication spherule (48).

There is precedent for viral helicases to form oligomers, as demonstrated for the large T antigen of the DNA tumor virus simian virus 40 (55). Goregaoker and Culver (19) demonstrated that the helicase domain of the tobacco mosaic virus P183 protein could form hexamers. Should a similar situation take place with the BMV 1a protein, we hypothesize that mutants PK7, PK12, and H80 retain binding to RNA while being able to form oligomers with the *trans*-complementing 1a molecules.

The interaction within 1a subunits likely is more complex than the simple oligomerization of only the helicase-like domains. Significant conformational changes in 1a may be a part of the regulatory mechanism. O'Reilly et al. (39) previously used yeast two-hybrid analyses of BMV mutants, including PK7, to demonstrate that a full-length 1a protein can interact with another 1a protein through the MT domain. Also, the MT domain can interact with the helicase-like domain of 1a (38, 39). Thus, it is not clear whether the mutations in PK7, PK12, and H80A affect MT-MT interactions and/or another activity of the MT domain, such as RNA binding (perhaps binding to the B box). If some or all three mutations affect 1a-1a interactions, then the helicase-helicase interaction may be able to restore a partial replication-competent state. If some or all three of the mutations affect RNA binding, then it is possible that the *trans*-complementing WT 1a is acting by binding the B box in RNA1 while the mutant subunits oligomerize with the WT 1a. We have not observed the rescue of any of the replication-defective RNA1 mutants by 1a in *trans*, an observation incompatible with this model. However, it is currently difficult to rule out that high levels of 1a expression have other regulatory effects, as has been demonstrated for translation (60). A biochemical analysis of the 1a protein is needed to distinguish between these two models.

In summary, we have identified a previously unreported *cis* preference in the replication of BMV RNA1 that is likely an early event in efficient genomic RNA replication. This process might play an important role in the molecular switch from translation to RNA replication in BMV. All positive-strand RNA viruses likely contain a similar timing switch operated by distinguishable mechanisms to allow optimal infection (5, 6, 16).

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