Deletion Analysis of Brome Mosaic Virus 2a Protein: Effects on RNA Replication and Systemic Spread

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Brome mosaic virus (BMV) genomic RNA2 encodes the 94-kDa 2a protein, which is one of two BMV nonstructural proteins required for RNA replication and subgenomic mRNA transcription. 2a contains a central polymeraselike region, which has extensive sequence similarity with the Sindbis virus nsP4 and tobacco mosaic virus (TMV) 183-kDa replication proteins, and also contains N- and C-terminal flanking segments without counterparts in the Sindbis virus and TMV nonstructural proteins. To further investigate the roles of the central and flanking segments in 2a, we have constructed a series of deletion and frameshift mutants in a biologically active BMV RNA2 cDNA clone and tested their ability to support viral RNA replication in barley protoplasts and systemic infection in whole barley plants. The entire 125-amino-acid C-terminal segment following the polymeraselike region was dispensable for RNA replication and transcription. Within the 200-amino-acid N-terminal flanking segment, deletion of the first 50 residues dramatically reduced genomic and subgenomic RNA accumulation, and deletion of 100 or more residues abolished detectable RNA synthesis. All mutations removing residues from the central polymeraselike domain also blocked RNA replication in *trans*. Sequences required in *cis* for RNA2 replication or stability were found to occur within the first 300 nucleotides of the 2a coding region. In whole barley plants, systemic infection was inhibited even by 2a deletions that supported strong RNA replication in protoplasts. Some replication-competent 2a variants failed to spread to uninoculated leaves, while others showed 10- to 500-fold-reduced virus yield in both inoculated and uninoculated leaves. These reductions were not due to any defects in RNA2 encapsidation.

Positive-strand RNA viruses include many significant human and animal pathogens and the majority of plant viruses. Within this class, many outwardly dissimilar viruses encode related nonstructural proteins involved in viral RNA replication (5, 13, 21, 25). The plant bromoviruses, for example, encode RNA replication factors that have extensive similarities with nonstructural proteins of the animal alphaviruses, tobacco mosaic virus (TMV), alfalfa mosaic virus, and many other plant viruses.

The type member of the bromoviruses is brome mosaic virus (BMV). Like other bromoviruses, BMV is an icosahedral virus whose genome is divided among RNA1 (3.2 kb), RNA2 (2.9 kb), and RNA3 (2.1 kb) (2, 4). RNA3 encodes nonstructural protein 3a, which is required for systemic spread of infection (8), and the subgenomic coat protein mRNA, RNA4. RNAs 1 and 2 encode proteins 1a (109 kDa) and 2a (94 kDa), respectively, which together are sufficient to direct viral RNA replication in protoplasts (19, 31). A variety of mutations in BMV 1a or 2a perturb or abolish RNA replication (33, 34, 53). 1a is related to the TMV 126-kDa protein and to Sindbis virus proteins nsP1 and nsP2 (5), which have also been implicated in RNA replication (23, 24, 28, 52). Recent results suggest that the conserved domains in 1a and its homologs may encode a helicase (22, 26) and possibly methyltransferase (38) and guanylyltransferase (16) activities involved in viral RNA capping.

BMV protein 2a has an extended region of even higher

sequence similarity with the TMV 183-kDa protein, Sindbis virus nsP4, and several known RNA-dependent RNA polymerases (9, 25, 30, 43). This nearly 500-amino-acid, polymeraselike conserved domain constitutes the C-terminal 85% of the Sindbis virus nsP4 readthrough protein and the entire readthrough domain of the TMV 183-kDa protein, which is translated by readthrough of the leaky termination codon following the 1a-like 126-kDa open reading frame. The corresponding BMV sequences are located in the central portion of the 822-residue 2a protein, beginning near amino acid 200 and ending near amino acids 683 to 689, which align with the C termini of the TMV 183-kDa and Sindbis virus nsP4 proteins (25). Thus, 2a contains substantial N- and C-terminal segments that lack apparent counterparts in the corresponding polymeraselike proteins of TMV and Sindbis virus.

To investigate the roles of the N- and C-terminal segments flanking the central polymeraselike core of BMV 2a protein, we have constructed a series of 2a frameshift and deletion mutants and tested these mutants for replication in protoplasts and systemic infection of whole barley plants. The results show that essentially the entire C-terminal segment but only a small portion of the N-terminal segment can be deleted from 2a without blocking RNA replication. Moreover, although some N- and C-terminal 2a deletion mutants are capable of supporting systemic infection of barley plants, the in planta yield of virus bearing these mutations was substantially reduced compared with the wild type (wt) and did not parallel the relative levels of RNA accumulation in protoplasts. These results complement those of an earlier RNA replication study that exchanged selected 2a gene segments between BMV and the related bromovirus cowpea chlorotic mottle virus (CCMV) (53).

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MATERIALS AND METHODS

Plasmid constructions. Full-length cDNA copies of wt BMV RNA1, RNA2, and RNA3 are contained in plasmids pB1TP3, pB2TP5, and pB3TP8, respectively; infectious transcripts can be synthesized from these templates by T7 RNA polymerase (29). Deletion and frameshift mutations were introduced into pB2TP5 as described below, by standard recombinant DNA techniques (10, 36). RNA2 sequence numbering below is as given in reference 2.

Oligonucleotide-directed, in-frame deletions in the N-terminal region of the BMV 2a gene were performed according to the method of Kunkel et al. (35). The deletions in the initially mutagenized clones were verified by restriction fragment analysis and nucleotide sequencing. To avoid possible inclusion of spurious mutations that might conceivably have been introduced elsewhere in the cloned cDNA, small restriction fragments encompassing the desired changes were then subcloned back into wt plasmid pB2TP5. Clone pB2PT14 carries a deletion of nucleotides (nt) 109 to 174 (codons 3 to 24); pB2PT16 has a deletion of nt 111 to 257 (codons 3 to 51); pB2PT18 has a deletion of nt 110 to 406 (codons 3 to 101); and pB2PT24 has a deletion of nt 109 to 708 (codons 3 to 202). In pB2PT20, oligonucleotide-directed mutagenesis was used to change base 97 from C to G and base 99 from C to T, creating a *Bcl*I site and a new AUG codon nine bases 5' to the wt initiation codon of the 2a reading frame. Translation initiation at this position would give Met-Ile-Lys-Met, where the underlined Met corresponds to the normal translational start of the 2a protein.

Internal deletions in the 2a open reading frame were constructed by excising selected restriction fragments and religating the resulting ends. Noncompatible ends were made blunt with T4 DNA polymerase prior to ligation. Deletions were confirmed by restriction fragment analysis. pB2PA11 has an in-frame deletion of the *Nco*I-*Kpn*I fragment, nt 887 to 1252 (codons 262 to 383). pB2PA12 has an in-frame deletion of the *Kpn*I-*Mlu*I fragment, nt 1249 to 1680 (codons 383 to 526). pB2PA13 has a frameshifting deletion of the *Mlu*I-*Sac*I fragment, nt 1680 to 2373 (codons 528 to 757); at the ligated junction, the reading frame shifts and terminates after nine codons. pB2PT60 has a frameshifting deletion of nt 113 to 442, which was constructed by ligating *Bam*HI-bounded fragments from pB2DR18 and pB2DR19 (provided by D. Richards), which are pB2TP5 mutants in which 6-base *Bam*HI linkers were inserted after nt 111 and 442, respectively. pB2PT70 has an in-frame deletion of nt 444 to 880 (codons 114 to 259) made by deletion of the *Bam*HI-*Nco*I fragment from pB2DR19. pB2PT80 has a frameshifting deletion of nt 113 to 880 (codons 4 to 259) made by deletion of the *Bam*HI-*Nco*I fragment from pB2DR18.

Frameshift mutations were created by DNA cleavage at unique restriction sites previously introduced by linker insertion mutagenesis, followed by blunting and religation. pB2SB8 and pB2SB9, which bear frameshifts after 599 and 672 codons, respectively, have been described previously (53). pB2PT10, which has a frameshift after wt codon 697, was made by cleavage at a 6-base *Apa*I linker inserted after nt 2194 (pB2DR25; provided by D. Richards), followed by T4 DNA polymerase treatment and religation. pB2PT11, which has a frameshift after wt codon 790, was made by religating mung bean nuclease-treated, *Sal*I-digested wt pB2TP5 DNA.

pB2PT50 has a deletion of the *Apa*I-*Stu*I fragment in pB2DR25, nt 2195 to 2502 (codons 698 to 800); the reading frame terminates after one alternate-frame codon. pBBC2

has been described previously (53); this hybrid RNA2 encodes a 2a protein in which the C-terminal 231 amino acids and 3' noncoding region have been replaced by the analogous segment from the related virus CCMV.

Double deletion mutants were constructed by digesting individual RNA2 plasmids at their unique sites for *Eco*RV (within the center of the 2a open reading frame) and *Eco*RI (3' to the BMV cDNA insert) and ligating appropriate fragments recovered from low-melting-point agarose gels. pB2PT64 has a combination of the respective N- and C-terminal deletions of mutants pB2PT14 (codons 3 to 24) and pB2PT50 (codons 698 to 822); pB2PT66 combines the respective deletions of mutants pB2PT16 (codons 3 to 51) and pB2PT50.

In vitro transcription, protoplast inoculation, and RNA analysis. Preparation of infectious transcripts, isolation and inoculation of barley protoplasts, RNA extraction, glyoxalation, and Northern (RNA) blot analysis were performed as described before (33, 53). Encapsulation of viral RNAs was assayed by nuclease treatment of polyethylene glycol (PEG)-precipitable virus particles as follows. Approximately 10^5 inoculated protoplasts were incubated for 40 h and then sonicated for 45 s at 40 W with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc.; courtesy of P. Wolk). Cell debris was pelleted at 10,000 rpm for 5 min. The supernatant was made 9% PEG and 0.185 M NaCl, and 12 μ g of carrier TMV virions was added. After being rocked on ice for 1 h, precipitated virus was pelleted at 10,000 rpm for 15 min and the virions were resuspended in 100 μ l of 0.5 M sodium acetate (pH 4.8)–80 mM MgCl₂. Samples were adjusted to 1 mM CaCl₂, and 300 U of micrococcal nuclease (Pharmacia) was added for digestion at 37°C for 15 min. The reaction was stopped by addition of EGTA (ethylene glycol tetraacetic acid) to a final concentration of 5 mM. PEG-precipitable, nuclease-protected viral RNAs were extracted with phenol-chloroform (1:1) in the presence of 1% sodium dodecyl sulfate (SDS)–0.1 M glycine–0.1 M NaCl–1 mM EDTA (pH 9.5)–2.4 mg of bentonite per ml. Recovered RNAs were denatured with glyoxal and subjected to Northern blot analysis as described before (33). RNA blots were probed with SP6 or T7 polymerase transcripts complementary to the conserved 3'-proximal 200 nt of BMV (18) or CCMV (6). The probe specific to RNA2 (used in Fig. 4) was a T7 polymerase transcript of a cDNA clone containing nt 30 to 2479 of BMV RNA2.

Barley inoculation and virion RNA recovery. Seven-day-old barley plants (*Hordeum vulgare* L. cv. Morex) were inoculated with 0.2 μ g each of wt RNA1 and RNA3 transcripts and the appropriate mutant or wt RNA2 transcript in 5 mg of bentonite per ml–0.25 M NaCl–25 mM Tris-Cl–25 mM NaH₂PO₄–5 mM EDTA, pH 8.0 (3). All mutants were tested in four or more separate experiments. For each experiment with a given mutant, 10 or 12 plants were inoculated. At 10 to 14 days postinoculation (p.i.), the inoculated leaves from all 10 or 12 plants were harvested and pooled, and small end portions from all such leaves were trimmed and discarded as needed to yield a total 1-g sample. Uninoculated leaves were similarly pooled and trimmed to yield a separate 1-g sample. These leaf samples were homogenized with a Brinkman Polytron PT3000 (courtesy of R. Allison). The homogenates were clarified, and virions were precipitated with PEG and NaCl by the methods of Verduin (54). Viral RNA was ethanol precipitated from phenol-chloroform extracts of 1% SDS-lysed virus, electrophoresed in 0.8% agarose gels, transferred to nylon membrane, and hybridized with the BMV 3'-specific probe. The CCMV

3'-specific probe was also included in experiments with BBC2. Virus yields were estimated by quantitation of recovered viral RNA by absorbance, by inspection of ethidium bromide-stained gels and autoradiograms, and, for some experiments, by quantitation of probed filters with an AM-BIS MkII radioanalytic scanner.

RESULTS

To examine the possible contributions of the central polymeraselike domain and the flanking N- and C-terminal domains of the BMV 2a protein to RNA replication and systemic spread of BMV infection, a series of frameshift and deletion mutations were introduced into a biologically active wt BMV RNA2 cDNA clone (Fig. 1 and Table 1). The design of these mutations was aided by inspection of 2a sequence alignments with similar proteins from alfalfa mosaic virus, TMV, and Sindbis virus (25). Deletion borders and frameshift sites were verified by restriction analysis and nucleotide sequencing as described in Materials and Methods. In vitro translation of transcripts from each construct yielded products of the expected sizes (results not shown). Most of the RNA2 mutants were from a plasmid series designated PT n , where n is the identification number of each mutant. To simplify labeling, these mutants are referred to in the figures by identification number alone (e.g., 16 = PT16). RNA2 mutants derived from other series of plasmid constructions are referred to with their identifying prefixes (e.g., SB8, PA11, BBC2). All RNA2 mutants used in this study are diagrammed and labeled in Fig. 1.

The ability of the altered 2a proteins to support viral RNA replication was tested by inoculating barley protoplasts with in vitro transcripts of each RNA2 derivative plus transcripts from wt RNA1 and RNA3 cDNA clones. Each mutant was tested in at least six independent protoplast experiments and gave consistent results. In preliminary experiments, all mutations that removed residues from the central, polymeraselike domain of 2a prevented detectable RNA replication (results not shown). These mutations include contiguous deletions PT70, PA11, PA12, and PA13, which together span the entire polymeraselike region (Fig. 1). Frameshift mutations SB8 and SB9, affecting only C-terminal portions of this region (Fig. 1), also block RNA replication (53). Based on these results, the N- and C-terminal 2a segments were targeted for more detailed deletion analysis as described below.

N-terminal deletions show a gradient of RNA replication activity. Northern blot analysis showed that 2a proteins with increasingly larger amino-terminal deletions supported decreasing levels of viral RNA accumulation in protoplasts (Fig. 2). Deletion of amino acids 3 to 24 in mutant PT14 caused a slight decrease in activity compared with the wt. Deletion of amino acids 3 to 51 in mutant PT16 substantially reduced but did not abolish RNA synthesis, as evidenced by the production of visible bands for genomic RNAs 1 to 3 and subgenomic RNA4, which is not present in the inoculum. Deletion of amino acids 3 to 101 and 3 to 202, in mutants PT18 and PT24, respectively, blocked detectable RNA replication. In mutant PT20, base substitutions creating a new AUG codon three codons upstream of the wt 2a initiation site had no apparent effect on 2a activity (Fig. 2); however, we have not determined the translation start site(s) actually used in this mutant.

C-terminal domain flanking the polymeraselike 2a core is dispensable for RNA replication. A series of frameshift mutations were used to demarcate the C-terminal boundary of

FIG. 1. Schematic diagram of BMV RNA2 frameshift and deletion mutants. The central diagram labeled wt depicts the structure of wt RNA2, with the 2a open reading frame boxed and the flanking noncoding regions shown as single lines. The shaded portion of the 2a open reading frame, bounded by vertical dotted lines, encodes the polymeraselike region whose amino acid sequence shows similarity with proteins encoded by TMV, Sindbis virus, and certain other viruses (25). Above the wt RNA2 map, asterisks mark the positions of frameshifts in mutants SB8, SB9, PT10, and PT11, and the subsequent untranslated portions of the 2a gene are marked by dashed lines. (For brevity, PT n series mutants are denoted in this and later figures by identification number alone.) Mutant PT20, also shown above the wt map, contains two base substitutions that create a new AUG positioned three codons 5' to the normal 2a start site (see text). Below the wt RNA2 map, the bold lines show the extent of sequences deleted in mutants PT14, -16, -18, -24, -50, -60, -64, -66, -70, and -80, PA11, PA12, and PA13. Mutants PT64 and PT66 contain deletions at both ends of the reading frame, as indicated. All deletions preserve the 2a reading frame except for PT60, PT80, and PA13, which introduce frameshifts following the deletion (see asterisks); these mutants were constructed primarily for mapping *cis*-acting sequences (Fig. 4). PA13, which expresses over half of the 2a sequence, was also tested for *trans*-acting activity (see Results). BBC2, diagrammed at the bottom, is an RNA2 hybrid in which the last 231 codons of the 2a gene and the 3' noncoding region have been replaced by the analogous segment (hatched region) from the related virus CCMV (53). Exact descriptions of all alterations are given in Materials and Methods, and the codon positions of the N- and C-proximal alterations are listed in Table 1. Scales at the top give kilobases from the start of RNA2 and the number of encoded amino acids from the start of the 2a gene.

2a protein sequence required for RNA replication (Fig. 1). The frameshift in mutant PT11, which prevented translation of the last 32 amino acids, had no detectable effect on viral RNA accumulation (Fig. 3). Mutant PT10 contained a frameshift only eight codons after the end of the polymeraselike region, preventing translation of the last 125 wt codons. This truncated 2a protein was also able to direct RNA replication, though viral RNA accumulation was greatly reduced (Fig. 3). Frameshifts located 150 codons (mutant SB9) and 223 codons (mutant SB8) before the end of the 2a open reading frame truncated the 2a protein within the conserved polymeraselike domain. Each of these alterations abolished detectable RNA replication (Fig. 3) (53).

TABLE 1. Summary of amino acids present in 2a proteins with N- or C-terminal alterations

RNA2 variant	Type of mutation ^a	wt residues present	wt residues missing ^b	Alternative-frame codons added ^c
wt		1-822		
N-terminal extension PT20	E	1-822		N 3
N-terminal truncations				
PT14	D	1-2, 25-822	N 22	
PT16	D	1-2, 52-822	N 49	
PT18	D	1-2, 102-822	N 99	
PT24	D	1-2, 203-822	N 200	
C-terminal truncations				
PA13	D, F	1-527	C 295	C 9
SB8	F	1-599	C 223	C 5
SB9	F	1-672	C 150	C 6
PT10	F	1-697	C 125	C 9
PT50	D	1-697	C 125	C 1
PT11	F	1-790	C 32	C 9
N- and C-terminal truncations				
PT64	DD	1-2, 25-697	N 22, C 125	C 1
PT66	DD	1-2, 52-697	N 49, C 125	C 1

^a D, deletion; E, extension; F, frameshift; DD, double deletion.

^b Number of wt residues eliminated from either end of the proteins by frameshift or deletion. N, N-terminal residues; C, C-terminal residues.

^c Number of additional, alternative-frame residues added to either end of the protein by frameshift or deletion. N, N-terminal residues; C, C-terminal residues.

To confirm the ability of frameshift mutant PT10 to support RNA replication and to rule out the possibility of reversion or leaky termination, deletion mutant PT50 was constructed (Fig. 1 and Table 1). The proteins encoded by mutants PT10 and PT50 each contain the same wt amino acids, but while the reading frame in mutant PT10 extends nine codons after the introduced frameshift, that in mutant PT50 terminates after a single alternative-frame codon downstream from the deleted segment. Unlike the relatively low level of mutant PT10 activity, mutant PT50 directed nearly wt levels of RNA accumulation (Fig. 3). As was found previously (53), RNA2 hybrid BBC2 also directed replication at levels only slightly below wt (Fig. 3). In BBC2 the BMV 2a segment following amino acid 591 has been replaced with the corresponding 2a segment from a distinct bromovirus, CCMV (see reference 7 for comparison of the BMV and CCMV 2a proteins).

FIG. 2. Effects of N-terminal 2a deletions on BMV RNA replication. The autoradiogram shows total protoplast nucleic acids extracted at 20 h p.i., denatured with glyoxal, electrophoresed, transferred to a nylon membrane, and probed to detect progeny BMV RNAs. Inocula consisted of in vitro transcripts from cDNA clones corresponding to wt RNAs 1 and 3 plus the wt or PTn series RNA2 derivative indicated above each lane. The structure of the RNA2 mutants is given in Fig. 1. The positions of the progeny RNAs are shown on the left.

Mutant PT64 combines the N- and C-terminal deletions of mutants PT14 and PT50, encoding a 2a protein of 676 amino acids. The level of RNA accumulation directed in protoplasts by this double deletion mutant (Fig. 3) was similar to that of mutant PT14 (Fig. 2). Mutant PT66, which bears the N- and C-terminal deletions of mutants PT16 and PT50, encodes a 2a protein of 649 amino acids. In repeated experiments and direct comparisons, the RNA replication activity of this double truncated protein (Fig. 3) was greater

FIG. 3. Effects of C-terminal and combined N- and C-terminal 2a alterations on BMV RNA replication. Northern blot analysis of viral RNA replication in protoplasts was conducted as described in the legend to Fig. 2. Inocula consisted of in vitro transcripts from cDNA clones corresponding to wt RNAs 1 and 3 plus the BBC2 derivative indicated above each lane. Unprefixed numbers denote PTn series mutants, and the structure of all RNA2 mutants is given in Fig. 1. As shown previously (53) and in Fig. 5 below, BBC2 RNA2 accumulates to a significant level in such infections. The BBC2 RNA2 band was not visualized in this experiment because this blot was only probed to detect RNAs containing the conserved 3'-terminal sequences common to wt BMV virion RNAs, while in BBC2 the 3'-terminal noncoding sequences and a C-terminal section of the 2a gene have been replaced with the corresponding sequences from CCMV RNA2 (Fig. 1).

FIG. 4. Amplification of deleted RNA2 templates after coinoculation with the complete wt BMV genome. The autoradiogram shows a Northern blot of protoplast nucleic acids from four-component inoculations, in which the inoculum consisted of *in vitro* transcripts from cDNA clones of wt RNAs 1, 2, and 3 plus the additional deleted form of RNA2 indicated above each lane. Unprefixed numbers denote PTn series RNA2 mutants. The lane marked -- contains RNA from protoplasts inoculated only with transcripts of wt RNAs 1, 2, and 3. The blot was hybridized with a probe specific for RNA2. Positions of wt and deleted RNA2s are indicated on the left. All deletion derivatives are electrophoretically distinct from wt RNA2 and migrate within the bracketed region.

than that of mutant PT16, which has only the relevant N-terminal deletion (Fig. 2).

For all frameshift and deletion mutants that supported RNA replication, negative-strand RNA accumulation was determined by probing Northern blots of glyoxal-denatured protoplast RNA with a negative-strand-specific BMV probe, as in previous experiments (33, 34). In all cases, the ratio of negative- to positive-strand RNA accumulation was similar to that in the wt (results not shown). To explore the possible existence of multiple independent 2a functions or domains, protoplasts were inoculated with selected combinations of nonviable N- and C-terminal RNA2 mutants plus wt RNA1 and RNA3. No replication was detected when mutant PT18 or PT24 was coinoculated with either mutant SB8 or SB9 (results not shown). Similarly, coinoculation of weakly active mutants PT16 and PT10 plus wt RNA1 and RNA3 transcripts did not enhance the low level of RNA replication directed by either of these mutants individually.

Some deletions show *cis*-acting effects on RNA2 accumulation. Sequences required in *cis* for normal accumulation of bromovirus RNAs include but are not always restricted to terminal regions (1, 11, 17, 39, 42, 44, 47). For example, sequences in the central intercistronic region, as well as 5'- and 3'-proximal regions, are necessary for efficient BMV RNA3 accumulation *in vivo* (17). Experiments with BMV-CCMV RNA2 hybrids also suggested that 5'-proximal sequences extending into the 2a reading frame influence the accumulation of BMV RNA2 and the differential *in vivo* recognition of BMV and CCMV RNA2 by their respective replicases (53).

To further localize internal sequences with *cis*-acting effects on RNA2 accumulation, the activity of the deletion mutants shown in Fig. 1 as templates for *in vivo* replication was examined. Collectively these deletions span all but the extreme 3' end of the 2a open reading frame (Fig. 1). Viable N-terminal 2a deletion mutants PT14 and PT16 have no obvious *cis*-acting defect, since the amplification of these RNA2 derivatives in normal three-component inoculations is comparable to that of the wt RNA1 level in the same infections (Fig. 2). A high level of RNA2 template activity

FIG. 5. Encapsidation of altered RNA2 molecules. Protoplasts were inoculated with transcripts from cDNA clones of wt RNAs 1 and 3 and the RNA2 derivative indicated above each lane. Unprefixed numbers denote PTn series RNA2 mutants. After 40 h of incubation, protoplasts were harvested and sonicated. Virus was precipitated from the clarified sonicate with PEG. After nuclease treatment of the virus pellet, the encapsidated RNAs were extracted with phenol, electrophoresed, transferred to a nylon membrane, hybridized with two distinct 32 P-labeled probes complementary to the 3' noncoding regions of BMV and CCMV RNAs (to detect BBC2), and autoradiographed.

was similarly apparent in inoculations with C-terminal RNA2 deletion mutant PT50 (Fig. 3).

All other deletions in this collection blocked the ability of 2a protein to support RNA replication *in trans* (Fig. 2 and results not shown). To determine the *cis*-acting effects of these remaining deletions, each mutant was coinoculated into protoplasts along with *in vitro* transcripts from wt RNA1, RNA2, and RNA3 cDNA clones, thus providing all virus functions including wt 2a protein *in trans* (53). Differences in electrophoretic mobility allowed the wt and deleted forms of RNA2 to be clearly separated in agarose gels and the level of their respective accumulation to be compared by hybridization to an RNA2-specific probe. Mutants PA11, PA12, and PA13 were amplified in four-component inoculations, although they accumulated to lower levels than wt RNA2 (Fig. 4). These mutants have large nonoverlapping deletions within the central conserved region of 2a (Fig. 1). By contrast, there was no detectable accumulation of N-terminal deletion mutants PT18, PT24, PT60, and PT80 and internal deletion mutant PT70 when wt BMV proteins were supplied *in trans* (Fig. 4).

Viable RNA2 derivatives are encapsidated. RNA sequences that direct specific interactions with viral capsid protein have been described for several viruses, including TMV (20) and Sindbis virus (55). Accordingly, as preparation for whole-plant tests, the BMV RNA2 derivatives that supported significant RNA replication *in trans* were assayed for their ability to be encapsidated into virus particles in barley protoplasts. These RNA2 derivatives (mutants PT11, -14, -50, -64, and -66 and BBC2) were all recovered from infected protoplasts in a PEG-precipitable, nuclease-resistant form (Fig. 5). Previous results confirm that this encapsidation assay is completely dependent on the expression of BMV coat protein and its ability to interact with viral RNA (6, 50). The similar ratios of progeny genomic and subgenomic

FIG. 6. Effects of BMV RNA2 mutations on virus accumulation in barley plants. The autoradiogram shows a Northern blot of virion RNAs recovered at 14 days p.i. from inoculated (i) and uninoculated (u) leaves of barley plants inoculated with *in vitro* transcripts from cDNA clones of wt RNAs 1 and 3 plus the indicated wt or mutant RNA2. Unprefixed numbers denote PTn series mutants. Samples from equivalent amounts of tissue were used for all lanes except for the samples from wt-infected plants, for which equivalent volumes of 20-fold-diluted sample (1/20) were loaded. BMV RNA positions are shown on the left. Both BMV- and CCMV-specific probes were used as in Fig. 5.

RNAs in the encapsidated (Fig. 5) and total RNA (Fig. 2 and 3) extracts show that the introduced deletions did not detectably reduce the efficiency of RNA2 encapsidation.

RNA2 mutations affect BMV systemic infection of barley. Omission of any single BMV genomic RNA transcript from the inoculum prevents systemic infection of barley plants (3). Inactivation of either the 3a or the coat gene on RNA3 also abolishes systemic spread of bromoviruses in host plants (8, 50). To examine the possible effects of deletions in RNA2 and the 2a protein on infection of barley, transcripts of RNA2 mutants PT11, -14, -50, -64, and -66 and BBC2 were inoculated onto barley plants together with wt RNA1 and RNA3 transcripts. These mutants were chosen because they exhibited wt or only moderately reduced RNA replication in protoplasts (Fig. 2 and 3). Each mutant was tested in four or more separate experiments. At 7 to 10 days p.i., 80% or more of wt BMV-inoculated plants showed typical mosaic symptoms, stunting, and chlorosis in both inoculated and uninoculated leaves. Plants inoculated with any of the above mutants remained symptomless as long as they were observed (4 weeks p.i.).

At 2 weeks p.i., virus was separately purified from inoculated and uninoculated leaves of the above plants, and viral RNAs were recovered and subjected to Northern blot analysis (Fig. 6). In all cases, much less virion RNA was recovered from the leaves of mutant-infected plants than from wt-infected plants. Despite the nearly wt level of viral RNAs in protoplasts inoculated with mutant PT14, similarly inoculated plants accumulated approximately 100-fold less virus than wt-infected plants. Plants inoculated with mutant PT11, which exhibited wt RNA replication levels in protoplasts, accumulated 10- to 20-fold less virus than wt-inoculated plants. Mutant PT50-infected plants contained 20- to 30-fold less virus than wt-infected plants. Double deletion mutants PT64 and PT66, which replicated to intermediate levels in protoplasts, accumulated to levels approximately 500-fold below the wt in inoculated leaves; trace amounts of mutant PT64 but not of PT66 were recovered from uninoculated leaves. Similarly, RNA2 hybrid BBC2 accumulated to a very low level in inoculated leaves but was never detected in uninoculated leaves (Fig. 6).

DISCUSSION

The sequence of BMV 2a protein can be divided into three segments: a large central domain with extensive similarity to polymeraselike proteins from many other RNA viruses and N- and C-terminal flanking domains without obvious counterparts in the nonstructural proteins of these other viruses (25). We have used a series of BMV RNA2 mutants encoding truncated 2a proteins to examine the role of the nonconserved flanking domains in directing RNA replication in protoplasts and systemic infection in barley. These experiments have identified terminal regions of the 2a protein that are dispensable for RNA replication and defined a functional core that provides all 2a functions required for the synthesis of positive- and negative-strand genomic RNAs and subgenomic RNA. The results show some parallel with mutational analysis of the bacteriophage-encoded Q β replicase protein, which identified a central polymeraselike domain intolerant of amino acid changes, flanked by terminal domains more permissive of insertions and substitutions (40). Our experiments also demonstrate that alterations in 2a protein can have differential effects on RNA replication in protoplasts and virus accumulation in whole plants.

trans-acting effects of N- and C-terminal 2a deletions on RNA replication. No clear-cut boundary between required and dispensable 2a sequences in the N-terminal flanking segment was evident. Small and moderate-sized deletions were associated with corresponding reductions in overall RNA accumulation; larger N-terminal deletions abolished RNA replication (Fig. 2). This gradient of diminishing activity extends earlier evidence that the N-proximal sequences in 2a contribute an important function or functions to RNA replication. By exchanging selected 2a gene segments between BMV and the related bromovirus CCMV, we previously found that sequences within the N-terminal 358 amino acids of 2a are a major determinant of the successful interaction of 2a with 1a protein, RNA1, or both (53). Recent results suggest that 1a, 2a and several host proteins exist as an immunoprecipitable complex in BMV polymerase extracts (45). A role for the nonconserved N-terminal 2a segment in forming a noncovalent complex between 2a and 1a would explain the absence of corresponding sequences in TMV, since the TMV protein domains analogous to 1a and to the polymeraselike core of 2a are covalently linked by translational readthrough in the TMV 183-kDa RNA replication protein (see the introduction).

In contrast to the relatively gradual decline in activity exhibited by N-terminal deletions, the C-terminal boundary of BMV 2a sequences required for RNA replication was more clearly demarcated. Proteins lacking the last 125 amino acids were active; those lacking the last 150 residues were completely inactive (Fig. 3). As shown in Fig. 1, these endpoints closely flank the C-terminal boundary of the polymeraselike region in 2a, which has similarity with Sindbis virus nsP4, TMV 183-kDa protein, and other viral proteins. Significantly, the region of similarity defining this boundary extends to within a few residues of the ends of the Sindbis virus and TMV proteins (25). The deletion results may partially explain the previous finding that antibodies against a peptide containing the last 26 amino acids of BMV 2a failed to inhibit RNA synthesis by a 2a-containing extract from BMV-infected cells (46). In fact, while deleting the last 125 amino acids of 2a caused a very slight reduction in RNA accumulation, deleting the last 32 amino acids had no detectable effect on RNA replication (Fig. 3, mutants PT50 and PT11). The high replicative activity of mutant PT11 is

also consistent with the observation that the C-terminal 19 amino acids of BMV 2a are missing from the closely related but shorter CCMV 2a protein (7).

By combining N- and C-terminal deletions into one doubly truncated protein, mutant PT66 defines a core region of the 2a protein that is sufficient for all requisite RNA replication and transcription functions (Fig. 1 and 3). Presently it remains unclear why the loss of 2a activity due to the N-terminal deletion of mutant PT16 was partially restored in mutant PT66 by subsequent deletion of C-terminal sequences (Fig. 2 and 3). This might reflect protein folding and/or stability differences, and the dissimilar replication activities of mutants PT10 and PT50, which differed only in the number of alternative-frame codons translated after the introduced frameshift or deletion, may have a similar basis.

BMV RNA replication entails multiple functions, including template recognition and binding for positive- and negative-strand synthesis, subgenomic promoter binding, initiation, elongation, strand separation, and capping. The large sizes (103 and 94 kDa) of the BMV 1a and 2a proteins and the presumptive multiplicity of virus-specific functions they must fulfill suggest that one or both may be multifunctional. The phenotypes of previously characterized site-specific 2a substitution mutants are also consistent with this possibility (33). None of the mutants in this study, however, exhibited a defect demonstrably specific to positive-strand, negative-strand, or subgenomic RNA synthesis, and lethal or weakly active N- and C-terminal deletions failed to complement each other. Thus, the required N-terminal region and central polymeraselike core of 2a may carry out a single function or linked functions, or some physical constraints may prevent complementation between these regions when carried on separate proteins.

***cis*-acting effects of deletions on RNA2 accumulation.** Viral RNAs necessarily contain sequences or structures that designate them as templates for viral replicase. Negative-strand promoter functions reside in the highly structured, tRNA-like end conserved among the 3' noncoding regions of all three BMV genomic RNAs (1, 15, 17, 47). 5' nontranslated sequences of BMV and CCMV RNAs are also required in *cis* for RNA replication, apparently for promoting positive-strand synthesis (17, 42, 44), and intercistronic noncoding sequences strongly influence BMV RNA3 amplification in *cis* (17). Results presented here show that *cis*-acting sequences regulating BMV RNA accumulation are not always restricted to noncoding regions. Deletion analysis defined a region within the 2a open reading frame, less than 300 nt long, that contains sequences required for detectable accumulation of RNA2 in BMV-infected cells (Fig. 1 and 4). Deletions in this region either may remove some *cis*-acting primary sequence or may influence the higher-order structure of RNA2. These data are consistent with earlier results that suggested that sequences important for RNA2 replication template activity reside within a large 5'-proximal domain extending into the coding region (53). The Sindbis virus nsP1 coding region similarly contains a *cis*-acting RNA encapsidation signal (55) and a 51-base conserved sequence suspected but not proven to have a *cis*-acting role in positive-strand initiation (41).

Differences in RNA stability as well as synthesis may contribute to differences in accumulation within inoculated cells. The mutant RNA2s that failed to accumulate after coinoculation with the complete wt genome (mutants PT18, -24, -60, -70, and -80; Fig. 4) appeared no more labile than wt RNA2 when inoculated singly into protoplasts and examined at 20 h p.i. (data not shown). This suggests that the effective

in vivo stabilities of the mutant RNAs were equivalent to that of wt RNA2, though it does not prove this, since the transfected RNA population monitored in such assays might not have the same intracellular distribution as actively replicating viral RNA. If the mutants are not substantially reduced in RNA2 stability, their failure to support RNA replication in *trans* could not result solely from a *cis*-acting defect in RNA2 amplification. RNA2 made nonreplicable by removal of the 3' noncoding region, when inoculated together with wt RNAs 1 and 3, supports easily detected amplification of RNAs 1, 3, and 4 (32, 49). The absence of detectable *trans*-acting replication activity in mutants PT18, -24, -60, -70, and -80 (Fig. 2 and results not shown) therefore suggests that these deletions may inactivate the encoded 2a protein as well as disrupting RNA2 accumulation.

Influence of RNA2 and 2a protein changes on systemic infection. When viable RNA2 mutants with N-terminal, C-terminal, or combined changes in the 2a open reading frame were tested in barley plants, virus accumulation in inoculated and uninoculated leaves did not parallel the level of viral RNA accumulation in protoplasts (Fig. 6). Rather, 2a mutants showing wt or nearly wt RNA replication in protoplasts all displayed much greater overall disadvantages (10-fold to over 500-fold) in plants relative to wt virus. All detectable spread of virus from inoculated to uninoculated leaves was blocked for deletion mutant PT66 and hybrid BBC2, in which a C-terminal 2a portion was replaced by the equivalent gene segment from CCMV, a bromovirus adapted to systemically infect legumes rather than grasses (53). These effects were not due to defects in encapsidation of the RNA2 derivatives (Fig. 5). Because the mechanisms of symptom induction in bromovirus-infected plants are unknown, it is unclear whether the lack of symptoms in the mutant-infected plants was due to the reduced virus concentration alone or to more specific effects.

The disparity between the replication levels of these mutants on an individual protoplast basis and the relative accumulation of virus in whole plants suggests that the observed yield reductions in plants may result from defects in the spread of infection or conceivably from alterations in host responses to infection (27). Plant virus infection entails both short-range (cell-to-cell) and long-range (systemic) spread of virions or viral components in host plant tissues. Despite important recent advances (12, 56), the transport mechanism(s) underlying such infection spread remains uncertain. For TMV and BMV, normal spread of infection throughout host plants requires a nonstructural virus protein dispensable for RNA replication (TMV 30-kDa protein and BMV 3a protein [8, 14, 37]) and coat protein (8, 50, 51). Previous reassortment studies with the genomic RNAs of BMV, CCMV, and related viruses have also shown that RNA1, RNA2, or both play a role in systemic spread and/or host specificity (6, 48, 57). The possible role of 2a protein in systemic spread or in the induction of host responses that limit virus infection (27) is currently unclear. Overall, the effects of the RNA2 mutations on whole-plant infection suggest either that RNA2 encodes functions other than its recognized role in RNA replication or that transport of progeny genomes is linked in some way to their synthesis.

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