Biological Activities of Hybrid RNAs Generated by 3'-End Exchanges between Tobacco Mosaic and Brome Mosaic Viruses

MASAYUKI ISHIKAWA,^{1†*} PHILIP KRONER,^{2,3‡} PAUL AHLQUIST,^{2,3} AND TETSUO MESHI¹

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan,¹ and Institute for Molecular Virology² and Department of Plant Pathology,³ University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 22 January 1991/Accepted 21 March 1991

Sequences within the conserved, aminoacylatable 3' noncoding regions of brome mosaic virus (BMV) genomic RNAs 1, 2, and 3 direct initiation of negative-strand synthesis by BMV polymerase extracts and, like sequences at the structurally divergent but aminoacylatable 3' end of tobacco mosaic virus (TMV) RNA, are required *in cis* for RNA replication *in vivo*. A series of chimeric RNAs in which selected 3' segments were exchanged between the tyrosine-accepting BMV and histidine-accepting TMV RNAs were constructed and their amplification was examined in protoplasts inoculated with or without other BMV and TMV RNAs. TMV derivatives whose 3' noncoding region was replaced by sequences from BMV RNA3 were independently replication competent when the genes for the TMV 130,000-*M_r* and 180,000-*M_r* RNA replication factors remained intact. TMV replicase can thus utilize the BMV-derived 3' end, though at lower efficiency than the wild-type (wt) TMV 3' end. Providing functional BMV RNA replicase by coinoculation with BMV genomic RNAs 1 and 2 did not improve the amplification of these hybrid genomic RNAs. By contrast, BMV RNA3 derivatives carrying the 3' noncoding region of TMV were not amplified when coinoculated with wt BMV RNA1 and RNA2, wt TMV RNA, or all three. Thus, BMV replicase appeared to be unable to utilize the TMV 3' end, and there was no evidence of intervirus complementation in the replication of any of the hybrid RNAs. In protoplasts coinoculated with BMV RNA1 and RNA2, the nonamplifiable RNA3 derivatives bearing TMV 3' sequences gave rise to diverse new rearranged or recombined RNA species that were amplifiable.

Tobacco mosaic virus (TMV) and brome mosaic virus (BMV) are positive-strand RNA viruses of plants. TMV is a rod-shaped virus with a single 6.4-kb genomic RNA (51). BMV is an isometric, tripartite virus with three separately encapsidated genomic RNAs, RNA1 (B1, 3.2 kb), RNA2 (B2, 2.9 kb), and RNA3 (B3, 2.1 kb) (2, 29). Despite their divergent particle multiplicity and genome organization, TMV and BMV share some important basic properties. The genomic RNAs of both viruses have 5' m⁷GpppG caps and 3' aminoacylatable, tRNA-like structures, although each genome is charged with a different amino acid (18, 19). Each virus encodes four known proteins, including one structural and three nonstructural proteins (2, 5, 16, 40). The two largest nonstructural proteins, the 130,000-*M_r* (130k protein) and the 180k protein of TMV and the 1a and 2a proteins of BMV, are involved in viral RNA replication (15, 21, 25, 26, 27, 50; unpublished results). Remarkable amino acid sequence similarities exist among these BMV and TMV replication factors and proteins encoded by the animal alphaviruses and many other positive-strand RNA viruses (6, 20). These and other common features suggest that BMV and TMV have fundamental similarities in their replication strategies.

For BMV, the 3'-terminal 200 bases of all three genomic RNAs are strongly conserved and highly structured (1), and portions of this region play a crucial role in template recognition by BMV replicase. The 3'-terminal 134-base RNA fragment is both aminoacylatable and sufficient to direct

initiation of negative-strand RNA synthesis by an RNA-dependent RNA polymerase extract from BMV-infected barley leaves, which synthesizes RNAs complementary to exogenously input BMV RNAs but not other viral or cellular RNAs (37, 38). Mutational analyses with biologically active viral cDNA clones have confirmed the importance of these 3'-terminal sequences for replication of BMV RNA *in vivo* (10, 13) and the corresponding importance of 3' noncoding sequences for TMV RNA replication *in vivo* (48). 5' and in some cases internal sequences are also required *in cis* for bromovirus RNA replication *in vivo* (13, 42) and similarly contribute to template specificity and activity in the full RNA replication cycle (41a).

To examine the strictness of the relationship between TMV replicase and the 3'-terminal sequence of TMV RNA (23), three chimeric viruses were recently constructed by replacing the 3' noncoding sequence of the TMV-L strain (40) with the corresponding regions from TMV-OM (a common strain [39, 47]), TMV-Cc (a cowpea strain [34, 52]), or cucumber green mottle mosaic virus, another tobamovirus (33, 41). Even though the Val-accepting 3' end of TMV-Cc RNA is remarkably divergent in structure from the His-accepting ends of these other tobamovirus RNAs (34, 44), all of the chimeric viruses were able to replicate, indicating a degree of permissiveness in the interaction between the TMV replicase and the 3' end. Similar permissiveness has also been demonstrated for 3'-terminal exchanges between BMV and cowpea chlorotic mottle virus, another bromovirus (7, 50; unpublished results).

To explore the functional limits of such 3' exchanges, we have now constructed several hybrid genomes transferring the highly divergent 3' noncoding regions between TMV and BMV RNAs. Since TMV and BMV both replicate in tobacco or barley protoplasts, these experiments were also designed

* Corresponding author.

† Present address: Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan.

‡ Present address: The Blood Center of Southeastern Wisconsin, Milwaukee, WI 53233.

to test the possibility of creating chimeric RNA templates for which positive- and negative-strand synthesis *in vivo* would be catalyzed by different replicases from two coinfecting viruses. Such a system would be useful for dissociating and analyzing specific steps in RNA replication. Our results show that TMV replicase was able, at low efficiency, to amplify templates bearing BMV 3' sequences, while BMV replicase was unable to amplify any of the hybrid templates. Simultaneous coinoculation with both BMV and TMV replication helper viruses did not enhance genomic RNA synthesis for any of the hybrid templates.

MATERIALS AND METHODS

Plasmids. pLFW3 is the standard plasmid used to obtain infectious TMV (L strain) RNA by *in vitro* transcription with *Escherichia coli* RNA polymerase (32). pLQDN, a derivative of pLFW3, lacks the 30k protein and coat protein genes (35). pB1TP3, pB2TP5, and pB3TP8 are cDNA clones of BMV RNA1 (B1), RNA2 (B2), and RNA3 (B3), respectively, from which infectious transcripts can be synthesized with T7 RNA polymerase (24). pB3C97 is a pB3TP8 derivative that contains a deletion in the 3a gene (13). pB3BX1 (13), a pB3PM1 derivative (4), has a deletion in the coat protein gene of a B3 cDNA copy linked to an *E. coli* RNA polymerase promoter (4). pB2SB8 is a frameshift derivative of pB2TP5 that produces a truncated, nonfunctional 2a protein (50). pB1SB1 is a frameshift derivative of pB1TP3 that contains the 10-base insertion GGATCGATCC between bases 875 and 876 of the B1 cDNA sequence, leading to production of a truncated, nonfunctional 1a protein (25a).

Plasmid constructions. (i) **pLB1 and pLB2.** The 0.2-kb filled-in *Bst*NI-*Hind*III fragment of pB3C97 was inserted between the filled-in *Mlu*I and *Hind*III sites of pUCG91 (32). The resulting plasmid, pB3M1, has an *Mlu*I site at the junction between the B3-derived sequence and the pUCG91 polylinker sequence. The BMV-derived sequence of pB3M1 was confirmed by sequencing. pB3C97M was constructed by ligating the 3.5-kb *Bam*HI-*Eco*RI and 0.9-kb *Bam*HI-*Hind*III fragments of pB3C97 and the 0.2-kb *Eco*RI-*Hind*III fragment of pB3M1. pB3C97 and pB3C97M are identical except that an *Mlu*I site is introduced just downstream of the BMV sequence in pB3C97M. pB3D1 was constructed by deleting the sequence between the *Bgl*II and *Xba*I sites of pB3C97. pLB1 was constructed by replacing the sequence between the *Ssp*I and *Mlu*I sites of pLFW3, containing the 30k and coat protein genes and the 3' noncoding sequence, with two fragments, the 0.4-kb filled-in *Bam*HI-*Hind*III fragment of pB3D1 and the 0.2-kb *Mlu*I-*Hind*III fragment of pB3M1. pLB2 was constructed by the same method except that the 0.9-kb filled-in *Bam*HI-*Hind*III fragment derived from pB3C97 was used in place of pB3D1.

(ii) **pLB3, pLB4, and pLB6.** pLB1 was digested with *Xba*I, filled in, and religated to create pLB4. The resulting 4-base frameshifting insertion was confirmed by sequencing. pLB6 was constructed by replacing the 1.6-kb *Bgl*II fragment of pLB1 with the corresponding fragment from pLFR3 (21), resulting in a frameshift in the 180k protein gene just downstream of the 130k protein gene. pLB3 was created by ligating three fragments: the 4-kb *Eco*RV-*Mlu*I fragment of pLFW3, the 0.9-kb filled-in *Bam*HI-*Hind*III fragment of pB3C97, and the 0.2-kb *Mlu*I-*Hind*III fragment of pB3M1.

(iii) **pB3PK series.** pOL1, derived from pLFW3, has a TMV-OM-derived 3' noncoding sequence between the *Nsi*I and *Mlu*I sites in place of the authentic L-derived sequence (23). The *Nsi*I-*Mlu*I fragment of pB3C97M containing the 3'

noncoding sequence and the C-terminal portion of the coat protein gene of B3 was replaced with the 0.2-kb *Nsi*I-*Mlu*I fragments of pOL1 and pLFW3, creating pB3PK1 and pB3PK2, respectively. The 1.8-kb *Sph*I-*Nsi*I fragment of pB3TP8 was introduced into the corresponding regions of pB3PK1 and pB3PK2 to create pB3PK3 and pB3PK4, respectively.

(iv) **Plasmids for RNA probe production.** pT732 was constructed by inserting the *Nsi*I-filled-in *Mlu*I fragment of pLFW3, the 3' noncoding sequence of TMV-L, between the *Pst*I and *Sma*I sites of pT7T3-19U (Pharmacia). pBCPSN1 was constructed by inserting the *Sac*I-*Nsi*I fragment of pB3TP8, derived from the internal portion of the BMV coat protein gene, between the *Pst*I and *Sac*I sites of pT7T3-19U. pB3HE1 carries the sequence derived from the 3' noncoding region of B3 (13).

In vitro transcription. Plasmids were linearized with *Mlu*I (TMV-based constructs, pB3C97M, and pB3PK series constructs) or *Eco*RI (BMV-based constructs). According to the promoter linked to the viral cDNA, linearized plasmids were then transcribed *in vitro* with *E. coli* RNA polymerase or T7 RNA polymerase as described previously (3, 13, 35). When used alone as the inoculum for tobacco protoplasts, *in vitro* transcripts produced by *E. coli* RNA polymerase were purified by phenol extraction and ethanol precipitation. Template DNAs were not removed because their presence did not cause a serious decrease in infection efficiency, because digestion of templates with DNase I occasionally resulted in inefficient infection, and because RNA at low concentrations is recovered inefficiently by LiCl precipitation. In experiments requiring the coinoculation of transcripts produced by T7 and *E. coli* RNA polymerases, the transcripts were mixed and purified by LiCl precipitation since the presence of BMV cDNA templates in inocula causes inefficient infection of barley cells (15). TMV transcripts were efficiently recovered along with BMV transcripts by this method.

Protoplast inoculation. Tobacco protoplasts prepared from suspension-cultured cells (56) were inoculated with *in vitro* transcripts essentially as described previously (54). Briefly, 6×10^5 protoplasts were inoculated by electroporation with *in vitro* transcripts produced from 7.5 μ g of linearized plasmid for *E. coli* RNA polymerase and/or 2 to 2.5 μ g of plasmid for T7 RNA polymerase. For some experiments, 2 μ g of TMV-L virion RNA, extracted from tobacco plants inoculated with LFW3 transcripts, was used as a TMV-L inoculum in place of pLFW3 transcripts (see Fig. 5). Inoculated protoplasts were divided into four parts, and each aliquot (1.5×10^5 cells) was cultured in the dark in the presence of dactinomycin (25 μ g/ml). Total nucleic acids were extracted as described before (53). For Northern (RNA blot) analysis, DNA was removed with DNase I (22) and total RNAs were glyoxalated and separated by electrophoresis on 1% agarose gels (49). Infection in each experiment was concurrently checked by pulse-labeling with [3 H]uridine from 21 to 23 h postinoculation (p.i.), followed by gel electrophoresis of total nucleic acids that were extracted and fluorographed as described before (53). Immunofluorescence with anti-BMV antibodies showed that typically 20 to 30% of inoculated tobacco cells were infected by the procedures used here.

Before undertaking coinoculation experiments with B1 and B2, we confirmed that the amount of *E. coli* RNA polymerase transcript used as the inoculum for the LB series hybrid RNAs was sufficient in principle for detectable replication by B1 and B2. This was done by coinoculating B1 and B2 transcripts together with a suitable range of dilutions of

FIG. 1. Schematic diagram of TMV-BMV hybrid RNAs LB1, LB2, LB3, LB4, and LB6, with comparisons to wt TMV-L and wt BMV RNA3 (B3). LB1, LB2, and LB3 are diagrammed individually, and the positions of the LB4 and LB6 frameshift mutations (see text) are indicated below the LB1 structure. LQDN, B3BX1, and B3C97 are highly replicating deletion mutants of TMV-L and B3 that were used as models and intermediates for construction of the hybrids (see Materials and Methods) and as controls for some later experiments. TMV- and BMV-derived sequences are shown by thin and bold lines, respectively. Open circles indicate 5' cap structures. Coding regions for the TMV 130k, 180k, 30k, and coat (CP) proteins and the BMV 3a and coat (CP) proteins are shown by open boxes. The B3 intercistronic oligo(A) tract is denoted by a solid rectangle. The starting positions of BMV (14) and TMV subgenomic mRNAs are shown by bent arrows. The starting positions indicated for the TMV-L 30k and CP subgenomic mRNAs are based on the corresponding start sites in the closely related TMV-OM and TMV-U1 strains (17, 55). Thick bars below the wt TMV and B3 diagrams identify the regions subcloned to make the TMV 3', BMV 3', and BMV CP in vitro transcript probes, which were used for Northern blot analysis of progeny RNA. nt, nucleotides.

E. coli RNA polymerase transcripts of B3BX1, a replication-competent B3 deletion derivative (13). To confirm that the extra 3' nucleotides provided by the *Mlu*I termination site of LB series transcripts did not inhibit amplification by BMV replicase, we introduced an equivalent *Mlu*I site into the 3' end of the B3 derivative B3C97 and demonstrated that the resultant RNA is replicated in protoplasts coinoculated with B1 and B2 (13) (see plasmid pB3C97M in this section).

Barley protoplasts were isolated and inoculated as previously described (26). Protoplasts (10^5) were inoculated with in vitro transcripts produced from 1 μ g of each appropriate linearized plasmid and, when appropriate, with 2 μ g of TMV virion RNA. Following inoculation, 10^5 protoplasts were incubated at 30°C for 5 to 24 h. Total nucleic acids were extracted and separated by electrophoresis on agarose gels following denaturation in the presence of glyoxal (26).

Northern blotting. Four different probes were prepared to specifically detect BMV RNA, TMV RNA, and the different hybrid transcripts. Nick-translated pLFW3 or pLQDN was used for detection of TMV-derived sequences. 32 P-labeled transcripts derived from pT732, pB3HE1, and pBCPSN1 were used to specifically detect the 3' end of TMV, the four BMV RNAs, and derivatives with the BMV coat protein gene sequence, respectively. Transcript probes were pre-

pared essentially as described previously (31). Hybridization with GeneScreen membrane (Du Pont) was performed according to the supplier's protocol with slight modifications. Hybridization with ZetaProbe membrane (Bio-Rad) was performed as described previously (26).

RESULTS

Independent replication of TMV derivatives bearing 3'-terminal sequences from BMV RNA3. To assess whether the tRNA-like 3' end of a BMV genomic RNA could functionally replace the 3' end of TMV RNA and support RNA replication by TMV replicase, hybrids LB1 and LB2 were constructed in cloned viral cDNA (Fig. 1). LB2 contains the 5' 4.9 kb of wild-type (wt) TMV-L RNA, including the 5' noncoding sequence and complete 130k and 180k protein genes, followed by the 3' 1.1 kb of BMV RNA3 (B3), including the intercistronic noncoding region, the coat protein gene, and the 3' noncoding region. LB1 differs from LB2 in lacking the BMV coat protein gene and some of its 5'-flanking sequence, including the core promoter for synthesis of the BMV subgenomic coat protein mRNA (14, 30).

In vitro transcripts from the LB1 and LB2 cDNA clones were inoculated separately into tobacco protoplasts, and

FIG. 2. Accumulation of 5' TMV-3' BMV hybrid RNAs in tobacco protoplasts. The transcripts used for inoculation are indicated above each lane. Total nucleic acids were extracted at 5 or 24 h p.i., treated with DNase I, denatured with glyoxal, separated by 1% agarose gel electrophoresis, and blotted to GeneScreen membranes (Du Pont). Membranes were probed with (A) nick-translated pLQDN (TMV probe) and with (B) a transcript probe complementary to the conserved 3'-terminal 200 bases of all three BMV genomic RNAs (BMV 3' probe; see Fig. 1). No cross-hybridization was observed with either probe (see lanes B1 + B2 + B3 in panel A and LQDN in panel B). One-fifteenth of the total RNA from 1.5×10^5 inoculated protoplasts was used for each lane, except for lanes LQDN (A) and B1 + B2 + B3 (B), for which 1/15,000th of the total RNA from 1.5×10^5 protoplasts was loaded. The positions of LQDN, wt BMV RNAs 1 (B1), 2 (B2), and 3 (B3), and subgenomic RNA4 (B4) are indicated to the right of the panels, with a bracket (LB) encompassing the positions of the LB1, LB2, LB4, and LB6 hybrid RNAs. Arrowheads in panel B indicate bands discussed in the text.

their fate was examined by Northern blot analysis of total RNA extracted at 5 and 24 h p.i. Bands corresponding to the LB1 and LB2 genomic RNAs were detected with probes specific to either the TMV or the BMV sequences that they contain (Fig. 2). At 5 h p.i., only faint genomic RNA-sized bands were visible and most of the hybridization signal was found in a lower-molecular-weight (MW) RNA smear that presumably represents degraded inoculum. By 24 h p.i., most of this low-MW material had disappeared, but there was a substantial increase in the intensity of the LB1 and LB2 genomic RNA bands, showing that these hybrid RNAs were replicated in vivo. The levels of LB1 and LB2 accumulation, however, were 100- to 1,000-fold lower than that of LQDN (Fig. 2A), an internal deletion mutant of TMV that shares one deletion boundary with LB1 and LB2 (Fig. 1) but has wt RNA replication activity (35).

In addition to genomic RNA, some discrete lower-MW RNAs were also seen at 24 h p.i. in LB1 and LB2 infections (Fig. 2). Most of these RNAs were of low intensity relative to genomic RNA, and many varied in level between different experiments. Similar low-intensity bands often accompany wt TMV and BMV replication. These bands may include degradation products of the genomic RNA. For both LB1 and LB2, the BMV 3' probe also consistently revealed one lower-MW RNA species with strong intensity relative to the genomic RNA (bands marked by arrowheads in Fig. 2B). Hybridization to the BMV 3' probe (Fig. 2B) indicates that these RNAs are coterminal or nearly coterminal with the 3' end, while lack of a detectable signal with the LQDN TMV probe (Fig. 2A) shows that they contain little or no TMV sequence. These facts and the observed electrophoretic

FIG. 3. Accumulation of 5' TMV-3' BMV hybrid RNAs in tobacco protoplasts coinoculated with B1 and B2. The transcripts used for inoculation are indicated above each lane, and RNAs were analyzed as described in the legend to Fig. 2. One-fifteenth of the total RNAs from 1.5×10^5 inoculated protoplasts was used for each lane, except for lanes LQDN and LQDN + B1 + B2 in panel A and lane B1 + B2 + B3 in panel C, for which 1/15,000th of the total RNA from 1.5×10^5 protoplasts was loaded. Nick-translated pLQDN was used as a TMV-specific probe in panel A. Panel B shows hybridization of the BMV 3' probe with the same samples as in the first 15 lanes of panel A. The BMV 3' and the CP (coat protein) probes used in panels B and C are mapped in Fig. 1. The BMV CP probe does not cross-hybridize to wt TMV RNA (see Fig. 5). The positions of relevant RNA bands are shown in the margins, and an arrowhead in panel C marks a band discussed in the text.

mobilities suggest that the marked bands may represent subgenomic RNAs synthesized from the TMV 30k subgenomic mRNA promoter, whose start site is present in LB1 and LB2 shortly 5' to the junction with BMV sequences (Fig. 1).

Hybrids LB3, LB4, and LB6 were made to confirm the dependence of LB1 and LB2 replication on the TMV replication genes. LB3 is an LB2 derivative with a large deletion in the 130k and 180k protein-coding sequences (Fig. 1). LB4 and LB6 are LB1 derivatives with 4-base frameshifting insertions in the 130k and 180k protein gene, respectively (Fig. 1). LB3 and LB4 do not produce either the 130k or 180k protein, and only the 130k protein is produced by LB6. From 5 to 24 h p.i., the intensity of the genome-sized LB3, LB4, and LB6 RNA bands from the input inoculum decreased rather than increased (Fig. 2A for LB4 and LB6 and Fig. 3C

for LB3). Thus, just as for wt TMV (21), at least the 180k protein is required for detectable replication of LB1 and LB2.

Coinoculation of 5' TMV-3' BMV hybrids with BMV RNAs 1 and 2. To determine whether BMV replicase could further enhance the amplification of LB1 and LB2, these hybrid RNAs were coinoculated with BMV RNA1 (B1) and RNA2 (B2), which encode BMV RNA replication proteins 1a and 2a, respectively. Since LB1 and LB2 (Fig. 1) contain all BMV 3' sequences required for initiation of negative-strand synthesis *in vitro* by BMV polymerase extracts (12, 37), BMV replicase may recognize the LB series RNAs as templates for negative-strand synthesis *in vivo*. If the resultant BMV replicase-derived LB negative-strand RNAs could then serve as templates for positive-strand synthesis by TMV (or possibly BMV) replicase, greater hybrid RNA accumulation might occur than in cells inoculated with the LB series hybrids alone.

In protoplasts coinoculated with B1 and B2, the accumulation of LB1 (Fig. 3A and B) and LB2 (Fig. 3C) genomic RNA was slightly enhanced over that after inoculation of LB1 or LB2 alone. However, equivalent enhancement of LB1 and LB2 accumulation was also observed after coinoculation with the nonreplicating B1 and B2 mutants B1SB1 and B2SB8, which bear lethal frameshift mutations in the 1a and 2a genes, respectively (Fig. 3A and B for LB1, and Fig. 3C for LB2). The increase in LB1 and LB2 accumulation in such coinoculations thus appears to be due to relatively nonspecific effects, such as the higher total RNA level in the inoculum or the use of LiCl precipitation in the final purification of inoculum transcript mixtures containing BMV components (see Materials and Methods) rather than the replicative action of the BMV 1a and 2a proteins. LB3, LB4, and LB6, which were incapable of independent replication, still showed no amplification when coinoculated with B1 and B2 (Fig. 3A for LB4 and LB6, Fig. 3C for LB3). Hybridization of strand-specific probes to blots of glyoxal-denatured protoplast RNA also revealed no detectable accumulation of LB3, LB4, and LB6 negative-strand RNA after coinoculation with B1 and B2 (results not shown). Such probing reliably and specifically detects negative-strand RNA in infections with wt BMV, wt TMV, and any of their derivatives that replicate to a significant level (26, 27; unpublished results).

As shown in Fig. 1, LB2 contains the complete sequence of the BMV subgenomic coat protein mRNA, RNA4, and all of the upstream subgenomic promoter sequences required *in vivo* for RNA4 synthesis by BMV replicase (14). Upon coinoculation of LB2 with B1 and B2, a new RNA band appeared that hybridized to the BMV coat gene probe and exactly comigrated with BMV subgenomic RNA4 (Fig. 3C, indicated by arrowhead). This RNA did not appear when LB2 was coinoculated with B1SB1 and B2SB8 (Fig. 3C).

Behavior of BMV RNA3 derivatives bearing 3'-terminal sequences from TMV RNA. To explore the consequences of reciprocal exchanges, BMV RNA3 (B3) derivatives carrying TMV 3' noncoding sequences were constructed. Figure 4 shows the structure of hybrid B3PK4, in which a 3' portion of the BMV coat gene and 3' noncoding region were replaced with the 3' noncoding region of TMV-L. This change did not significantly alter B3 stability *in vivo*, since, in the absence of B1 and B2, the decrease in input B3PK4 transcripts from 5 to 24 h p.i. was similar to that observed for wt B3 (long exposures of Fig. 5 and similar experiments).

Coinoculation with B1 and B2 or with TMV-L RNA, or all three, did not alter the decline in the B3PK4 RNA band

FIG. 4. Schematic diagram of BMV-TMV hybrid RNA B3PK4 and comparison with wt B3 and wt TMV-L. Abbreviations and conventions are the same as in Fig. 1.

between 5 and 24 h p.i. (Fig. 5), indicating that B1, B2, and TMV-L were unable to direct detectable replication of B3PK4. Nevertheless, whenever B3PK4 was coinoculated with B1 and B2, new RNA bands appeared that did not comigrate with B3PK4 (arrowheads in Fig. 5, lanes B1 + B2 + B3PK4 and B1 + B2 + B3PK4 + L). The appearance of such novel RNA bands was observed in a large number of samples derived from many independent coinoculations of B3PK4, B1, and B2, in the presence or absence of TMV-L. The novel RNAs were visible at 24 h p.i. but not at 5 h p.i., indicating that they were amplified progeny RNAs. Since these RNAs hybridized to the B3-specific BMV CP probe, they must be derived from B3PK4, while their altered mobility on denaturing gels shows that the RNA3 sequences

FIG. 5. Analysis of progeny RNA in tobacco protoplasts inoculated with BMV-TMV hybrid B3PK4 in the presence or absence of B1, B2, and TMV-L RNA (L). The transcripts used for inoculation are indicated above each lane, and RNAs were analyzed as described in the legend to Fig. 2. One-fifteenth of the total RNA from 1.5×10^5 inoculated protoplasts was used for each lane, except for lane B1 + B2 + B3, for which 1/15,000th of the total RNA from 1.5×10^5 protoplasts was loaded. The TMV-L inoculum for this experiment was TMV-L virion RNA extracted from tobacco inoculated with LFW3 transcripts (see Materials and Methods). Arrowheads mark the position of novel bands discussed in the text. The positions of wt BMV RNAs 3 and 4 are indicated at the right, and the position of the input B3PK4 RNA is seen in the 5-h p.i. lanes. RNA bands were visualized with the BMV CP probe (see Fig. 4).

FIG. 6. Diversity of putative recombinants detected in tobacco protoplasts inoculated with B3PK4 plus B1 and B2. In each of three independent experiments (denoted 1, 2, and 3), a pool of protoplasts was inoculated with B1, B2, and B3PK4 and then divided into several aliquots (1.5×10^5 cells each) that were cultured separately. Individual aliquots were harvested at 5, 22, and 24 h p.i. as shown, and total RNA was extracted from each and analyzed by Northern blotting as described for Fig. 2. (A) Hybridization with the BMV CP probe. (B) Analysis of the same samples from lanes 5 to 8 of panel A with the TMV 3' probe. Arrowheads mark bands in lanes 5 and 6 of panel A for comparison with the corresponding positions in panel B (see text). Lanes 1 to 8 each contain 1/15th of the total RNA isolated from the full 1.5×10^5 cell population of the relevant aliquot. Thus, the signal in each lane is produced by 1/15th of the viral RNA present in each infected cell of the original population. For signal strength comparisons (see Discussion), total RNA from cells inoculated with wt B1, B2, and B3 was diluted so that lanes 9 and 10 contain the equivalent of 1/15th of the total RNA from 1 and 10 cells, respectively, inoculated with B1, B2, and B3. RNA from mock inoculations was included so that the total RNA concentration was unchanged during dilution. The position of the input B3PK4 transcript, seen in lane 2, is indicated at the left. The BMV CP and TMV 3' probes, used in panels A and B, respectively, are mapped in Fig. 1. A host RNA that weakly cross-hybridized with the BMV CP probe is indicated by an arrow at the left. The TMV 3' probe did not hybridize to wt BMV RNAs (panel B).

must be covalently associated with new sequences or sequence rearrangements; i.e., these bands must have arisen from recombination events. This conclusion is considered further in the Discussion.

The variability exhibited by the novel B3PK4-derived RNAs is further demonstrated in Fig. 6. In this experiment, several pools of tobacco protoplasts (1, 2, and 3) were coinoculated with B1, B2, and B3PK4. Each pool was then divided into additional separate aliquots prior to incubation. Individual aliquots were harvested at 5, 22, or 24 h p.i., and total RNA was analyzed as shown. As in Fig. 5, only the input B3PK4 transcript was visible in cells harvested at 5 h p.i., while new RNAs appeared in all cell populations harvested at 22 or 24 h p.i. (Fig. 6A). The number and size distribution of bands were different in each of the aliquots, which constitute independent populations of inoculated cells. This appearance of distinct patterns of bands in aliquots separated after inoculation clearly demonstrates that the novel RNAs did not arise from any contamination of the initial inoculum. Furthermore, the variety of different bands that emerged in separate aliquots (each containing 1.5×10^5 cells) implies that most individual bands arose from

events that were relatively infrequent even in cell populations of this size. Comparison of the bands marked by arrowheads in Fig. 6A with the equivalent lanes and positions in Fig. 6B shows that some of the novel bands revealed by the BMV coat gene probe did not hybridize to the TMV 3' probe and have thus lost the TMV 3' sequences originally present in B3PK4. All of the novel bands were of extremely low intensity compared with B3 signals from wt BMV infections (Fig. 5 and 6A).

In addition to tobacco cell experiments such as those shown in Fig. 5 and 6, equivalent results were obtained after inoculating barley cells with B3PK4 in the presence and absence of B1 and B2. Other hybrids were also made by replacing the 3' B3 region deleted in B3PK4 with the 3' noncoding region of TMV-OM (hybrid B3PK3) and by replacing the corresponding 3' region of B3 deletion mutant B3C97 (Fig. 1) with the 3' noncoding regions of TMV-OM (hybrid B3PK1) or TMV-L (hybrid B3PK2). As with B3PK4, coinoculation of these hybrids with B1 and B2, with or without TMV-L, did not amplify the starting hybrid but did lead to the appearance of diverse, novel RNAs late in infection.

DISCUSSION

Amplification of 5' TMV-3' BMV hybrid genomes. The results presented above for hybrids LB1 and LB2 demonstrate that TMV replicase can utilize 3'-terminal sequences from BMV in place of essential sequences at the 3' end of TMV RNA. This strengthens the view that the RNA replication mechanisms of TMV and BMV RNA are related, as was first indicated by the similarity of their RNA replication genes (6, 20). Because prior mutational analyses (48) imply specific interactions between TMV replicase and 3'-proximal sequences in its templates, it was not apparent before this that the His-accepting 3' end of TMV-L RNA could be functionally, if inefficiently, replaced by the Tyr-accepting 3' end from a virus as divergent as BMV. The primary sequences of the TMV and BMV 3' sequences are not detectably similar. Moreover, while both 3' noncoding regions are highly structured and contain potential pseudoknots, their higher-order structures appear to differ in many fundamental ways (1, 44, 48). This suggests that successful interaction with TMV replicase may involve some common subset of structural features in the TMV and BMV 3' noncoding regions, or that distinct but related cellular factors might mediate the interaction of these differently aminoacylatable ends with the virus-coded components of TMV replicase.

In contrast to the above results, reciprocal exchanges revealed that BMV replicase was unable to detectably amplify templates bearing the 3' noncoding regions of TMV-L or TMV-OM RNAs. Since even the successful replication of LB1 and LB2 was extremely low compared with that of wt TMV, this may not reflect basic differences in BMV and TMV RNA replication. Alternatively, BMV replicase might be intrinsically less tolerant of variant 3' structures than TMV. For example, the high specificity of BMV negative-strand synthesis appears to be derived solely from recognition of 3'-proximal sequences (12, 37), while template recognition by TMV replicase might also be stabilized by interaction with internal TMV RNA sequences retained in LB1 and LB2. Internal sequences do contribute to replicase-RNA interactions for bacteriophage Q β (36) and possibly also for BMV RNA3 positive-strand synthesis (13). Alternatively, as suggested for alphaviruses (45, 46) and poliovirus (9, 28), a translation-coupled or otherwise *cis*-acting mech-

anism might exist for template selection by the TMV-coded replication components. The inherent template selectivity of a *cis*-acting replicase could allow more relaxed requirements for 3' template structure while retaining the necessary initiation specificity. Unlike these single-component viruses, the BMV replicase must act *in trans* to initiate negative-strand synthesis on each of its three genomic RNAs.

Lack of intervirus complementation in replication of hybrid genomic RNAs. The coinoculation experiments of Fig. 3 and 5 show that providing functional BMV and TMV replicase in combination did not enhance the replication of any TMV-BMV or BMV-TMV RNA hybrids in this study. This observation is most significant for TMV-BMV hybrids LB1 and LB2, whose independent replication was severely limited due to 3' modifications that presumably interfered with negative-strand synthesis by TMV replicase and which contained all BMV 3' sequences required for replication *in vivo* (13) and initiation of negative-strand synthesis by BMV polymerase *in vitro* (12, 37). If BMV replicase synthesized negative strands from the LB1 and LB2 templates *in vivo*, these negative strands were neither utilized as templates for positive-strand synthesis by BMV replicase nor accessible to TMV replicase. The only potential complementation effect seen was the possible BMV-directed synthesis of subgenomic BMV RNA4 after coinoculation of LB2, B1, and B2 (Fig. 3C). However, BMV-directed RNA4 synthesis might have occurred from BMV-synthesized LB2 negative strands, and so may not represent actual complementation between the BMV and TMV replication machinery.

Emergence of new, replicatable RNAs in cells inoculated with nonreplicatable 5' BMV-3' TMV hybrids. Although BMV replicase did not amplify BMV RNA3 derivatives bearing TMV 3' sequences, coinoculation of B3PK4 and similar hybrids with B1 and B2 gave rise to diverse new RNAs that were amplified during infection (Fig. 5 and 6). These RNAs hybridized to RNA3 probes and, in most cases, also to a TMV 3' probe, showing that they were derived from B3PK4. However, the electrophoretic mobilities of these bands showed that the B3PK4-derived sequences had become linked to additional sequences or had undergone sequence rearrangements. The novel RNAs thus must represent amplifiable recombinants arising from B3PK4. Since B3PK4 contained a full BMV subgenomic promoter, some of the faster-migrating novel bands may be subgenomic RNAs derived from higher-MW recombinants.

Bujarski and Kaesberg found that plants infected with a B3 variant bearing a partially debilitating 3' noncoding mutation gave rise to more actively replicating B3 derivatives that had acquired conserved 3'-terminal sequences from B1 or B2 by homologous and nonhomologous recombination (11). Similar recombination between B3PK4 and the 3' ends of B1 or B2 would restore B3 amplification, as observed in Fig. 5 and 6, and is one potential explanation for the B3PK4 results. At least some of the recombinants have undergone 3' modifications since they have lost the 3'-terminal TMV sequences originally present in B3PK4 (Fig. 6). Because of the extremely low intensity of the recombinant bands (see below), lower-MW background from wt B1 and B2 (e.g., Fig. 3B) prevented meaningful probing of Northern blots to determine whether the recombinants contain B1 or B2 3' sequences. This question as well as the nature of the presumed recombination junctions will be addressed by more sensitive approaches in future experiments.

The diverse sizes of novel RNAs that emerged in separate experiments imply that a variety of recombination events

generated amplifiable B3PK4 derivatives. This is consistent with possible recombination between B3PK4 and the B1 or B2 3' ends, which would necessarily involve nonhomologous crossovers due to the extent of B3 3' sequence deleted from B3PK4 (Fig. 4). An origin by diverse, individually rare recombination events of any kind would imply that few cells gave rise to any single detectably amplified recombinant, and the weak intensity of the novel RNA bands is in keeping with this. Even when total RNA from 1.5×10^5 protoplasts inoculated with B3PK4, B1, and B2 was assayed, the intensity of individual recombinant bands was intermediate between the wt B3 signals produced by extracts equivalent to 1 and 10 protoplasts inoculated with B1, B2, and B3 (Fig. 6A, compare lanes 3 to 8 with lanes 9 and 10). Since typically 20 to 30% of inoculated protoplasts were infected (see Materials and Methods), the wt B3 signals in lanes 9 and 10 of Fig. 6 should be equivalent to about 0.2 and 2 infected cells, respectively. The relative band intensities are thus consistent with each of the more strongly visualized recombinants being generated early in infection in a single cell of the relevant 1.5×10^5 cell population and having amplification efficiencies similar to that of wt B3. Weaker recombinant bands may have arisen later in infection or may have lower amplification efficiencies than wt B3.

Intermolecular recombination during bromovirus replication has been documented previously in whole-plant experiments with the related bromovirus cowpea chlorotic mottle virus (8) as well as BMV (11). In these *in planta* experiments, recombinants were only visualized 4 to 10 days p.i., after selective amplification by multiple passages through secondarily infected cells. The results reported here indicate that recombinants can be visualized within a virus population even when amplification is limited to the single infection cycle within which the recombinants were generated.

While this manuscript was in preparation, Rao and Hall reported similar results for a nonamplifiable 3' deletion mutant of B2, from which amplifiable variants also arose in protoplasts coinoculated with wt B1 and B3 (43).

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