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In vivo transfer of barley stripe mosaic hordeivirus ribonucleotides to the 5′ terminus of maize stripe tenuivirus RNAs

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ABSTRACT **The *Tenuivirus* maize stripe virus (MStV) shares many properties with viruses in the genus *Phlebovirus* of the family *Bunyaviridae*. Besides genome organization and gene expression strategies, one property shared by these plant- and vertebrate-infecting viruses is that transcription gives rise to virus-specific mRNAs containing nonviral 5′-terminal nucleotide sequences. The 5′-terminal nucleotides are believed to be derived from host mRNA sequences as a result of "cap-snatching." We investigated whether specific nucleotide sequences could serve as primer donors for cap-snatching *in vivo*. Barley (*Hordeum vulgare*) plants were singly and doubly infected with MStV and the *Hordeivirus* barley stripe mosaic virus (BSMV). A reverse transcription–PCR assay was used to identify chimeric BSMV/MStV RNAs. Specific reverse transcription–PCR products were detected from doubly infected plants by using one PCR primer corresponding to the 5′ termini of the BSMV RNAs (α, β, and γ) and a second primer complementary to MStV RNA 4. The resulting cDNAs were cloned, and nucleotide sequence analysis showed them to be chimeric, containing BSMV 5′-terminal sequences as well as MStV RNA 4 sequences. All clones contained the BSMV RNA 5′ primer nucleotide sequence, but they also showed characteristics common to *Tenuivirus* mRNAs. More than 80% of the clones contained BSMV RNA nucleotides not present on the PCR primer. Several lacked the exact 5′ terminus of MStV RNA 4, a feature also seen for viruses in the *Bunyaviridae*. These data show that heterologous virus RNAs (BSMV) can serve as primer donors for MStV mRNA capped RNA-primed transcription in doubly infected plants.**

Maize stripe virus (MStV) and other plant-infecting viruses within the genus *Tenuivirus* exhibit many properties characteristic of several vertebrate-infecting viruses, particularly those within the *Phlebovirus* genus of the *Bunyaviridae*. Members of both the *Tenuivirus* and *Phlebovirus* genera have a number of similarities including: multipartite genomes composed of both negative and ambisense segments; the genomic RNAs for viruses in each of these genera have eight identical nucleotides (5′-ACACAAAG) at the 5′ termini of each genome segment while the 3′ termini are conserved and complementary to the 5′ termini; the genomes have similar arrangements of protein coding regions; and some of the encoded proteins for different viruses show significant similarity (1–11). Transcription of the genomic RNAs yields mRNAs with capped 5′ termini (12–21), and between the cap and the viral RNA sequence are short stretches of nucleotides that are not of virus origin (12, 14).

The origin of the 5′-terminal nonviral nucleotides is thought to result from recruiting the cap structure and short stretches of nucleotides from host cellular mRNAs to initiate transcription (2, 12–15, 22). This process, first referred to as capped RNA-primed transcription was identified in the *Orthomyxovirus* influenza virus (23–25). Capped RNA-primed transcription or cap-snatching is facilitated by a virus-encoded cap-specific endonuclease that cleaves the donor RNA (16, 26). After cleavage the viral polymerase extends the RNA primer according to the viral RNA template. Thus, the viral mRNAs appear to be chimeric molecules containing both host mRNA and viral sequences.

The factors for selecting primer donor RNA sequences are presently unknown, but some features of the nonviral sequences are common. The sequences are typically short segments of 10–15 ribonucleotides (12–15, 17, 18, 27, 28). *In vitro* transcription assays with the *Bunyavirus*, La Crosse virus, and a defined donor mRNA resulted in viral mRNAs with 5′ extensions of 12–14 nucleotides (16). The specific nucleotide composition of the 5′ ribonucleotide extensions on *in vivo* generated viral mRNAs is also variable in size and sequence (12, 14, 18, 27), but for some viruses given nucleotides at specific positions may be important (29).

In this article, we present evidence for specific *in vivo* cap-snatching by the *Tenuivirus* MStV. We used a reverse transcription–PCR (RT-PCR) assay and analyzed MStV mRNAs from barley plants infected by both the *Hordeivirus* barley stripe mosaic virus (BSMV) and MStV. We show that nucleotide sequences corresponding to the 5′ termini of the three BSMV RNAs (α, β, and γ; refs. 30–33) could be detected on the 5′ termini of the MStV mRNA 4 from doubly but not singly infected plants.

MATERIALS AND METHODS

Plants and Viruses. Seedlings of barley (*Hordeum vulgare* cv. Briggs) and maize (*Zea mays* L. cv. Challenger) plants were infected with MStV in Florida as described (34). After 1–2 weeks seedlings were mechanically inoculated with BSMV in California. Plants were maintained in a glasshouse for 1–3 weeks, and assayed for MStV and BSMV infection by indirect ELISA using antibodies to the MStV noncapsid protein (NCP) (34) and BSMV virions (35).

RT-PCR Assay. A nested RT-PCR assay was performed using total RNAs isolated (36) from individual healthy plants, individual plants infected singly with MStV or BSMV, or individual plants doubly infected with MStV and BSMV (see Fig. 1). The cDNA primers (Table 1) were designed to be complementary to BSMV RNAs, α, β, and γ, and MStV RNA 4. The 5′ PCR primers (24 nucleotides) each had 14 5′-identical nucleotides to aid in the PCR reaction and subsequent cloning. The 10 3′-terminal nucleotides of these primers were identical to the 5′-terminal 10 nucleotides for each of the

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Abbreviations: MStV, maize stripe tenuivirus; BSMV, barley stripe mosaic hordeivirus; RT-PCR, reverse transcription–PCR; NCP, noncapsid protein.

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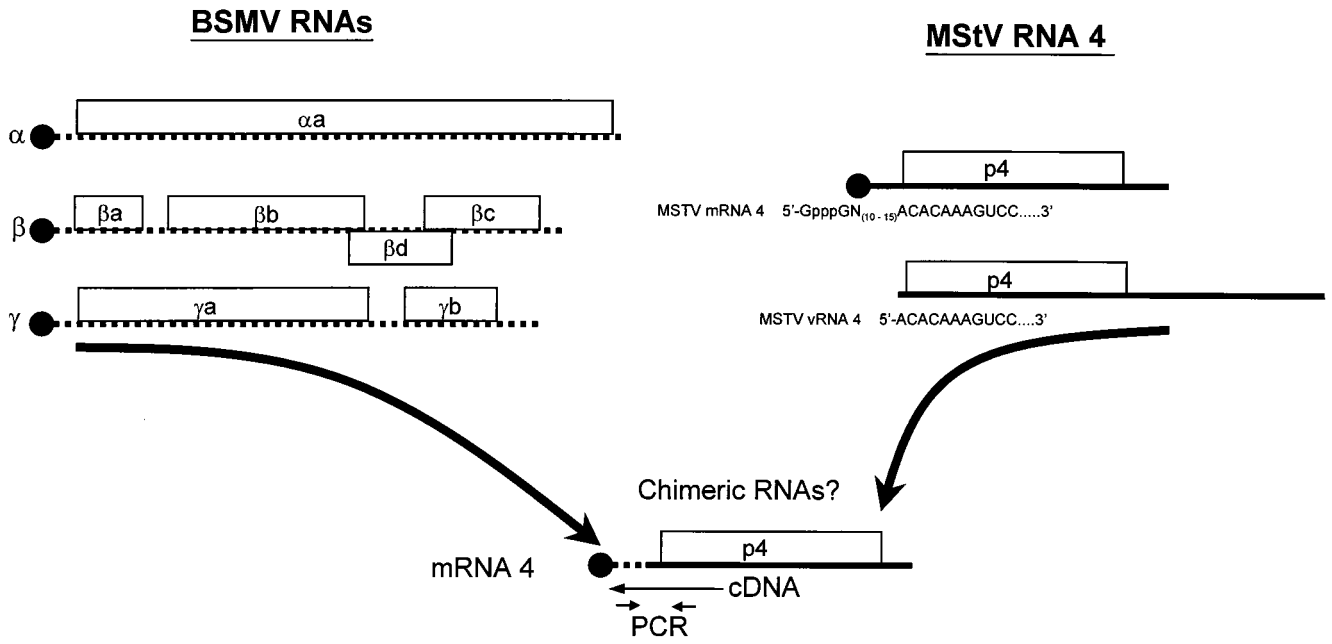

FIG. 1. RT-PCR experimental design. BSMV RNAs (α, β, and γ), and MStV genomic (vRNA 4) and mRNA 4 are indicated. Open boxes indicate ORFs, and letters inside boxes indicate the respective encoded proteins. The 5′-terminal nucleotide sequences for the MStV RNAs are shown below the vRNA 4 and mRNA 4. Arrows show the RT-PCR strategy used to detect BSMV/MStV chimeric RNAs containing BSMV 5′-terminal nucleotides (indicated at bottom). cDNA was synthesized using the MStV primer NCP (see Table 1). PCR was performed using a 5′ BSMV and 3′ MStV primer.

respective virus genomic RNAs. The 3′ PCR primers were designed to be complementary to each of the viral RNAs, but at a site 5′ of the cDNA primers, and in some cases included extra nucleotides to aid in cloning.

For cDNA synthesis, 10 μl of total RNA (250 ng) in 1× 1st strand buffer (50 mM Tris, pH 8.3/75 mM KCl/3 mM $MgCl_2$; BRL) containing 15 ng of cDNA primer was heat denatured at 90°C for 3 min. After slow cooling to at least 42°C, RT was done at 37–42°C for 1 hr after the addition of 10 μl of a reverse transcriptase mixture (1× 1st strand buffer/1.0 mM dNTPs/20 mM DTT/200 units of Moloney murine leukemia virus reverse transcriptase). RNA was hydrolyzed by adding 2.5 μl of 10× PCR buffer (500 mM KCl/100 mM Tris, pH 9.0/1% Triton X-100), 1.25 μl of H_2O, and 1.25 μl of 20 mM NaOH followed by incubation at 60°C for 1 hr (37). After adding 1.25 μl of 20 mM HCl, one-fifth of the cDNA reaction was used for PCR by diluting to 100 μl in 1× PCR buffer, 200 mM dNTPs, 1.625 mM $MgCl_2$, 150 ng of each PCR primer, and 2.5 units of *Taq* polymerase. PCR conditions were as follows: 1 cycle of 94°C, 3 min; 30 cycles of 94°C, 30 sec, 58°C, 30 sec, 72°C, 1 min; and 1 cycle of 72°C, 10 min. RT-PCR products were electrophoretically separated in nondenaturing 15% polyacrylamide (38) or 3.5% Metaphore agarose (FMC) in 1× TAE (0.04 M Tris·acetate/0.001 M EDTA) and visualized by ethidium bromide staining. The RT-PCR products were cloned into pGEM-T and sequenced as described (10).

RESULTS

Experimental Design. The nested RT-PCR was designed to detect chimeric MStV RNA 4 molecules containing 5′-terminal extensions derived from the 5′ termini of the BSMV α, β, or γ RNAs. As outlined in Fig. 1, cDNA was synthesized using the MStV cDNA primer, MStV50 (Table 1). If cap snatching of the BSMV genomic RNA 5′-terminal sequences occurred during infection, BSMV-MStV chimeric RNAs should only be detected only from plants infected by both MStV and BSMV. Furthermore, if chimeric RNAs were present, they should then be detected by amplifying the cDNA with one of the 5′BSMV PCR primers (Table 1) and the 3′ MStV PCR primer, 3′NCP (Table 1).

Table 1. Primers used for RT-PCR

Virus RNA*	cDNA primer†	cDNA product‡	5′ PCR primer†	3′ PCR primer†	PCR product§
BSMVα	BSMV7α	211	5′BSMV1α	3′BSMV4α	180
	T*ACGTGCGAGCACAGTACGT*		TTGGATCCCCCCCC***GTATGTAAGT***	GCGAATTC***GGAAGCTGTGCTAATC***	
BSMVβ	BSMV8β	189	5′BSMV2β	3′BSMV5β	149
	CCCACCAATCGTCAAATACT		TTGGATCCCCCCCC***GTAAAAGAAA***	GCGAATT***CGATGTAGTGTCCTCC***	
BSMVγ	BSMV9γ	441	5′BSMV3γ	3′BSMV6γ	137
	ATGTCCCGGTAAAGACCACG		TTGGATCCCCCCCC***GTATAGCTTG***	GCGAATT***CTGCTACTACAGTCAC***	
MStV RNA 4	MStV50	173	5′MStV-CON	3′NCP	121
	CAGATCTAGAAGAGTGATAT		TTGGATCCCCCCCC***ACACAAAGTC***	***CAGGCCTGTAATAGGGCCAA***	

*Indicates the virus RNA.
†Boldface, italicized letters indicate nucleotides that are complementary (for the cDNA and 3′ primers) or identical (the 5′ primer) to the respective virus RNA sequence. Letters not appearing as boldface and italicized were added to primers to aid cloning.
‡Indicates expected size of cDNA product, in nucleotides.
§Indicates expected size of the RT-PCR product, in nucleotides.

RT-PCR Analysis of Singly and Doubly Infected Plants. When the MStV RNA 4 primers (Table 1) were used in the RT-PCR assay, a product of the predicted size (121 bp) was detected using RNAs extracted from MStV-infected plants (Fig. 2*A*). The same sized RT-PCR products were detected using RNAs isolated from plants infected singly by MStV, from plants doubly infected by both MStV and BSMV, and when RNAs from plants infected singly by BSMV or MStV were mixed *in vitro*. No RT-PCR products were detected using the MStV RT-PCR primers and RNAs from healthy plants or plants infected by only BSMV. Similarly, when primers corresponding to the BSMV α, β, and γ RNAs (Table 1) were used for RT-PCR, DNAs of the expected sizes (180, 149, and 137 bp, respectively) were detected (Fig. 3*A–C*). These products were obtained using RNAs from plants infected by only BSMV, from plants doubly infected by BSMV and MStV, and the mixture made *in vitro* using RNAs from plants infected singly by BSMV and MStV. No RT-PCR products were obtained with the BSMV primers and RNAs from healthy or plants infected by only MStV.

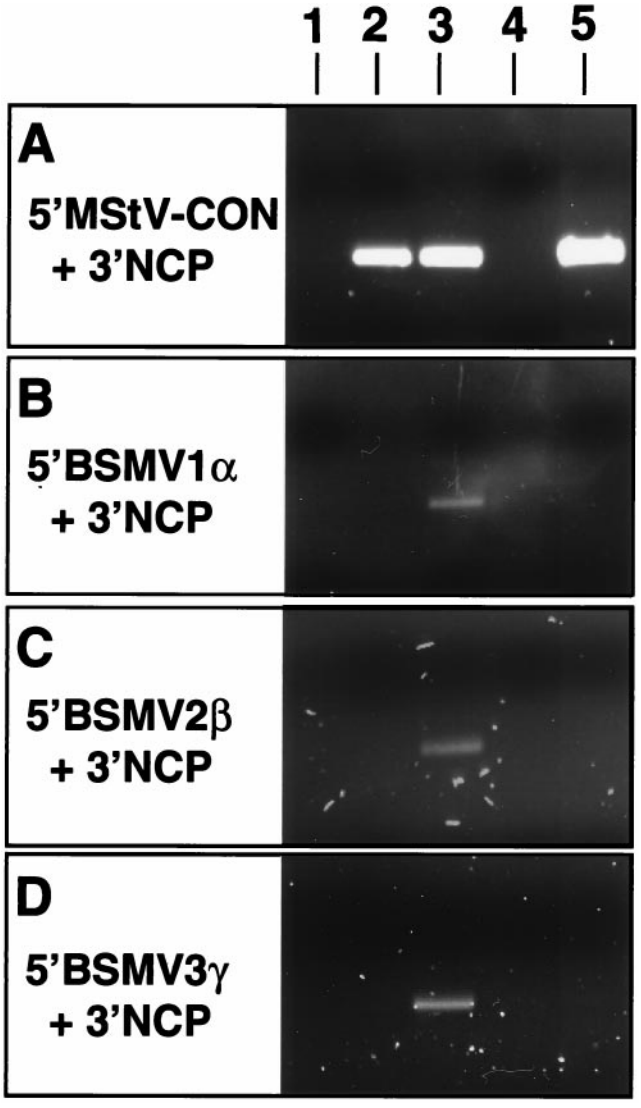

FIG. 2. RT-PCR detection of chimeric MStV mRNA 4 sequences. Total RNA from individual healthy plants (lane 1), MStV-infected plants (lane 2), MStV- + BSMV-infected plants (lane 3), BSMV-infected plants (lane 4), plus mixed RNAs from MStV- and BSMV-infected plants (lane 5) was converted to cDNA using primer MStV50 (see Table 1). The cDNA was amplified using primers 5′MStV-CON and 3′NCP (*A*) to detect MStV RNA 4; the following primer sets: 5′BSMV1 and 3′NCP (*B*), 5′BSMV2 and 3′NCP (*C*), or 5′BSMV3 and 3′NCP (*D*) were used in attempts to detect BSMV/MStV chimeric RNAs. One-tenth of the PCR reaction was electrophoretically separated in 3.5% Metaphore agarose followed by staining with ethidium bromide. Sizes of fragments were estimated by using the BRL 1-kb DNA ladder (not shown).

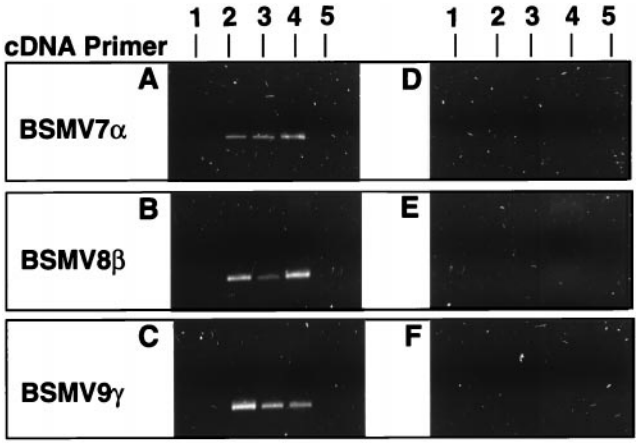

FIG. 3. RT-PCR detection of nonchimeric BSMV RNA sequences. Total RNA from individual healthy plants (lane 1), MStV- + BSMV-doubly infected plants (lane 2), BSMV-infected plants (lane 3), mixed RNAs from plants singly infected by MStV and BSMV (lane 4), and RNA from a MStV-infected plant (lane 5), was converted to cDNA using the BSMV primers BSMV7 (*A*, *D*), BSMV8 (*B*, *E*), or BSMV9 (*C*, *F*, see Table 1). The cDNA was amplified using the following PCR primer pairs: 5′BSMV1 and 3′BSMV4 (*A*), 5′BSMV2 and 3′BSMV5 (*B*), 5′BSMV3 and 3′BSMV6 (*C*), to detect BSMV RNAs α, β, and γ, respectively. Primers 5′MStV-CON and 3′BSMV4 (*D*), 5′MStV-CON and 3′BSMV5 (*E*), or 5′MStV-CON and 3′BSMV6 (*F*) were used in attempts to detect MStV/BSMV chimeric RNAs. One-tenth of the PCR reaction was electrophoretically separated in 3.5% Metaphore agarose followed by staining with ethidium bromide. Sizes of fragments were estimated by using the BRL 1-kb DNA ladder (not shown).

Because of the specificity demonstrated by the nested RT-PCR, it was used in attempts to detect chimeric BSMV/MStV RNAs from plants doubly infected with MStV and BSMV. After cDNA synthesis with MStV50, RT-PCR products were detected using the 3′ NCP PCR primer, and any of the 5′ primers corresponding to the BSMV α, β, or γ genomic RNA 5′ termini (Fig. 2*B–D*). Based on our experimental design, these RT-PCR products should represent BSMV/MStV chimeric RNAs. RT-PCR products of the expected sizes were detected only using RNAs from a plant doubly infected with MStV and BSMV. No RT-PCR products were detected by using these same primers and RNAs from healthy plants, plants singly infected by MStV or BSMV, or from the mixture prepared *in vitro* of MStV and BSMV total RNAs.

To demonstrate that the MStV NCP mRNA uniquely possessed BSMV 5′-terminal sequences, and that this was not an RT-PCR artifact, cDNAs corresponding to the α, β, and γ BSMV RNAs were subjected to PCR using the 5′ primer, MStV-CON, and the corresponding 3′ BSMV PCR primers. If RT-PCR products resulted, this would suggest the presence of chimeric BSMV RNAs containing 5′ sequences of the MStV NCP mRNA. No RT-PCR products corresponding to such MStV/BSMV chimeric RNAs were ever detected irrespective of the source of RNAs used for the RT-PCR (Fig. 3 *D–F*). Thus, Figs. 2 and 3 show that chimeric RNAs containing 5′ BSMV and 3′ MStV sequences were detected only from plants infected by both MStV and BSMV.

The initial RT-PCR detection of BSMV/MStV chimeric RNAs was obtained using RNAs extracted from a single plant doubly infected by BSMV and MStV. Therefore, to assess whether this was an isolated event, an additional 394 barley and 99 maize plants were inoculated with both MStV and BSMV.

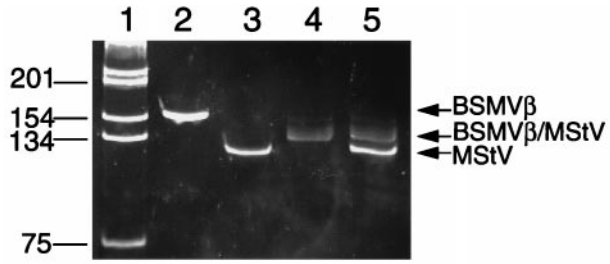

FIG. 4. RT-PCR product size comparisons. Total RNA from a BSMV-infected plant was converted to cDNA using the cDNA primer BSMV8 followed by amplification with primers 5′BSMV2 and 3′BSMV5 (lane 2, see Table 1 for primers). Total RNA from individual plants infected by MStV (lane 3) and doubly infected by MStV plus BSMV (lane 4) was converted to cDNA by primer MStV50 followed by amplification with primers 5′MStV-CON and 3′NCP or 5′BSMV2 and 3′NCP, respectively. The RT-PCR products were electrophoretically separated in 15% nondenaturing polyacrylamide gel followed by ethidium bromide staining. Included on the gel was the BRL 1-kb DNA ladder (lane 1) and in lane 5, a mixture of portions of the RT-PCR products shown in lanes 3 and 4.

ELISA analyses identified 39 barley and two maize plants to be doubly infected. Of these, 18 barley and two maize plants were used for RT-PCR analysis. Chimeric RNAs were not detected in RNAs extracted from either of the two maize plants. However, chimeric RNAs were detected in extracts from 11 of 18 doubly infected barley plants. Of these 11 samples, 8 were tested for chimeric RNAs containing nucleotide sequences corresponding to each of the BSMV RNAs (α, β, and γ BSMV 5′ sequences). All three types of BSMV/MStV chimeric RNAs were recovered from five samples, only BSMVα/MStV and BSMVβ/MStV chimeric RNAs were detected from two plants, and from one plant only the BSMVγ/MStV chimeric RNA was detected.

Properties of the BSMV/MStV Chimeric RNAs. If as our data suggest, the RT-PCR products from doubly infected plants corresponded to chimeric RNAs originating from cap-snatching, then the MStV mRNAs should have short 5′ extensions ranging in size, but originating from the 5′ termini of the BSMV RNAs. The RT-PCR products corresponding to chimeric RNAs should be larger than those corresponding to only the 5′ terminus of MStV genomic RNAs. Furthermore, based on previous data showing that 5′-terminal extensions on *Tenuivirus* mRNAs range in size primarily from 10–15 nucleotides (12, 14, 28), at least some of the RT-PCR products generated from chimeric BSMV-MStV RNAs should contain more BSMV nucleotides than only the 10 5′-terminal BSMV nucleotides specified by the 5′ BSMV PCR primers. Therefore, the RT-PCR products were further characterized by PAGE and by nucleotide sequence analysis.

The sizes of the RT-PCR products corresponding to the chimeric BSMVβ/MStV NCP mRNA were compared with the RT-PCR products synthesized from RNAs extracted from plants infected singly by BSMV and MStV. The RT-PCR products representing the chimeric BSMVβ/MStV mRNA were larger when compared with RT-PCR products corresponding to only the MStV RNA 4 (Fig. 4, lanes 3 and 4). Additionally, the RT-PCR products representing the chimeric BSMVβ/MStV mRNAs appeared heterogeneous in size as compared with the discrete products corresponding to either of the nonchimeric BSMVβ or MStV RNA 4 (Fig. 4, compare lanes 2–4).

To better determine whether the RT-PCR products represented the chimeric BSMV/MStV RNAs, the RT-PCR products generated independently from two BSMV and MStV doubly infected plants, and from plants singly infected by either MStV or BSMV were cloned and sequenced (Table 2). All clones originating from the chimeric BSMV/MStV RNAs had the 10 BSMV nucleotides that also were present in the respective 5′ BSMV PCR primers. However, between these 10 5′-terminal BSMV nucleotides and the MStV RNA 4 nucleotide sequence most clones also had one to four additional nucleotides that also corresponded to the BSMV genomic RNA sequences (type a, c, d, e, i, j, m, n, p, q, s, and t; Table 2). Several clones, especially those corresponding to chimeric BSMVα/MStV RNA 4 (for example type a; Table 2), lacked nucleotides corresponding to the exact 5′ terminus of MStV RNA 4. In several cases, the origin of the nucleotides A and C

Table 2. Nucleotide sequences for BSMV/MStV chimeric RNA cDNA clones

Sequence type*		Freq†	Partial nucleotide sequence‡	
MStV RNA4		4		acacaaagtcCAGGGCATTTTACAATATTCTATTCAATCCACTATCATCGAAGGTACAAGATATG...
αBSMV		4	gtatgtaagt	TGCC...
BSMVα/MStV	a	15	gtatgtaagt	TGC----------**C**ACAAAGTCCA...
	b	3	gtatgtaagt	----------GGCATTTTAC...
	c	2	gtatgtaagt	TG----------**C**ACAAAGTCCA...
	d	2	gtatgtaagt	T------***ACCG***-----ACACAAAGTCCA...
	e	1	gtatgtaagt	TGCC----------ACACAAAGTCCA...
	f	1	gtatgtaagt	----------CCA...
	g	1	gtatgtaagt	----------**T**ACAAGATATG...
	h	1	gtatgtaagt	--------***C***-------ACACAAAGTCCA...
	i	1	gtatgtaagt	TGC----***AC***-------ACACAAAGTCCA...
βBSMV		2	gtaaaagaaa	AGGAACA...
BSMVβ/MStV	j	16	gtaaaagaaa	AGGA----------**ACA**CAAAGTCCA...
	k	2	gtaaaagaaa	----------**A**CACAAAGTCCA...
	l	2	gtaaaagaaa	----------***C***--ACACAAAGTCCA...
	m	2	gtaaaagaaa	A----------***CAC***--ACACAAAGTCCA...
	n	2	gtaaaagaaa	A----------***C***--ACACAAAGTCCA...
	o	1	gtaaaagaaa	---------***CACAC***--ACACAAAGTCCA...
	p	1	gtaaaagaaa	AGGA----------CAAAGTCCA...
γBSMV		3	gtatagcttg	AGCATTA...
BSMVγ/MStV	q	19	gtatagcttg	AG----------**CA**CAAAGTCCA...
	r	5	gtatagcttg	----------**A**CACAAAGTCCA...
	s	1	gtatagcttg	AG----------**CA**AAGTCCA...
	t	1	gtatagcttg	A----------ACACAAAGTCCA...

*Indicates the type of RNA that was used to generate RT-PCR clones and used for nucleotide sequence analysis.
†Indicates the number of clones of the corresponding type (i.e., MStV RNA 4) which were analyzed.
‡Shows the respective nucleotide sequence (as DNA). Nucleotides shown in lower case letters are attributed to the PCR primers. Dashes and spaces were added to aid in alignment of nucleotide sequences. Boldface and underlined letters indicate that the corresponding nucleotide could originate from either MStV or BSMV nucleotide sequences. Boldface and italicized letters indicate nucleotides that cannot be classified definitively as originating from MStV or BSMV.

between the 10 5′-terminal BSMV nucleotides and MStV RNA 4 sequences could not be conclusively determined because of sequence overlap between the BSMV and MStV RNAs (types a, c, j, k, q, and s). Two clones (type d; Table 2) had the nucleotides ACCG in the junction between the BSMV and MStV sequences. The origin of these nucleotides could not be directly attributed to either of the viral RNAs. Finally, some clones also appeared to have repeated segments consisting of the nucleotides A and/or C in the junction region (types h, i, l, m, n, and o; Table 2).

DISCUSSION

The data presented herein show that by using RT-PCR, *in vivo* generated MStV mRNAs with defined 5′ nucleotide extensions were detected. These extensions corresponded to the 5′-terminal sequences of the three BSMV RNAs, α, β, and γ. These chimeric RNAs were detected only from plants infected with both the *Tenuvirus*, MStV, and the *Hordeivirus*, BSMV, but not from RNA samples created *in vitro* by mixing RNAs from plants separately infected by MStV and BSMV. Thus, coinfection of barley plants with both BSMV and MStV was required for the *in vivo* generation of the chimeric RNAs.

Based on our current understanding of *Tenuivirus* replication and transcription, and that *Tenuivirus* mRNAs contain capped 5′ nonviral-encoded nucleotides at their 5′ termini (12–14, 28), the BSMV/MStV chimeric RNAs likely resulted from capped RNA-primed transcription, or cap-snatching. The α, β, and γ BSMV RNAs have capped 5′ termini. All three are efficient mRNAs and with the exception of the 5′-most three or four nucleotides, each differs in nucleotide composition (30–33). Our data suggest that in cells of the barley plants coinfected by BSMV and MStV, all three of the BSMV RNAs were able to serve as primer donors for the transcription of the MStV mRNAs. Previously, *in vitro* transcription assays have been used to demonstrate that defined capped RNAs can serve as primer donors (16, 23, 24). *In vivo* transcription/replication assays for the Bunyamwera virus S RNA, which utilized vaccinia virus as a helper virus, also provided evidence that suggested that defined mRNAs might serve as primer donors (15). The data presented herein clearly show that defined capped RNAs from a heterologous coinfecting virus can serve as primer donors for MStV mRNA transcription.

The nucleotide sequences of cloned cDNAs corresponding to the BSMV/MStV chimeric mRNAs displayed characteristics consistent with those identified previously for the nonviral 5′-terminal nucleotide sequences of mRNAs for viruses in the genus *Tenuivirus*, and for viruses in the *Bunyaviridae* (12–16). The mRNA 5′ nonviral extensions for viruses in the *Tenuivirus* genus ranged in size from 0–23 nucleotides, but most commonly 11–15 nonviral nucleotides were found (12, 14). Nucleotide sequence analysis and gel electrophoretic analysis of the RT-PCR products from chimeric BSMV/MStV RNAs showed that the fragments were composed of a narrow size range. In each case regardless of the donor BSMV RNA, the majority of the clones had extensions of 11–14 nucleotides: 78%, 81%, and 81% of the cloned RT-PCR products had 11–14 nucleotides directly attributable to the α, β, and γ BSMV RNAs, respectively. In addition, the range in the number of BSMV nucleotides found here clearly showed that the RT-PCR products contained more nucleotides than were present in the BSMV PCR primer. Each 5′ BSMV PCR primer had only 10 nucleotides identical to either of the BSMV α, β, or γ RNA termini. Yet, 21 of the 27 BSMVα/MStV clones had either one, two, three, or four additional nucleotides beyond the 5′ BSMV primer sequence, and these nucleotides were identical to those of the BSMV α RNA. The additional BSMV nucleotides were then followed by nucleotides corresponding to MStV RNA 4. Similarly, additional nucleotides attributable to the BSMV β and γ RNAs were found in 21 of 26 clones for each set of the 26 clones analyzed.

Another characteristic displayed by the cloned products and consistent with the previously characterized 5′ nonviral extensions (12, 14, 15) was variation in nucleotide sequence in the junction between the primer donor segment and the MStV RNA 4 nucleotide sequence. In all *Tenuivirus* genomic RNAs, the 3′ termini used as a template for transcription are 3′-UGUGUUU. . . -5′. For both MStV and the related *Tenuivirus* rice stripe virus, nucleotide sequence analysis of cloned cDNAs has shown variability of the nucleotides at the junction. Some clones lacked either one, two or three of the predicted 5′-most nucleotides complementary (i.e., ACA) to the template genomic RNA (12, 14). Furthermore, the mRNAs of the *Bunyaviridae* members typically lack one or three of the 5′ viral nucleotides (18, 27, 29, 39). Our analyses showed that 17, 1, and 20 of the clones obtained from the α, β, and γ BSMV/MStV chimeric RNAs, respectively, also lacked either one or three of the first 5′ nucleotides of the MStV RNA 4 sequence. In addition, several of our clones had repetitions of the nucleotides AC in the junction between the BSMV and the MStV nucleotides, another feature consistent with previously cloned 5′ nonviral extensions on the MStV and rice stripe virus mRNAs (12, 14).

For several members of the *Bunyaviridae*, the viral polymerase is proposed to undergo a "prime-and-realign" mechanism during RNA synthesis of both mRNAs and genomic RNAs (15, 29, 40). During prime-and-realign the 3′ nucleotide of a primer donor hydrogen bonds to a complementary nucleotide in the viral template RNA and a few of the nucleotides are elongated as governed by the viral template (29). The template is then realigned (29). The realignment can place the 3′ nucleotide of the primer donor in position −1 thus showing a precise junction between the donor and the viral sequence (29). But realignment of the 3′ nucleotide of the primer donor in other positions can result in the appearance of repeated viral-encoded nucleotides or the deletion of nucleotides corresponding to the viral template (29). Further support of the prime-and-realign mechanism has been obtained by *in vitro* transcription of the Germiston virus (*Bunyaviridae*) (41). When a globin mRNA was used as a donor for capped RNA-primed transcription, extra U or GU residues were found inserted between the primer sequence and the viral sequence (41). The U or GU residues were proposed to be inserted during elongation as directed by the viral template (3′-UCAUCA. . . -5′) but before realignment. Realignment then results in the apparent repetition of either U or GU residues between the donor and viral sequences (41). The prime-and-realign mechanism could explain the apparent repetition of some nucleotides in the junction between the BSMV and identifiable MStV sequences as well as the deletion of MStV encoded nucleotides found in the chimeric BSMV/MStV RNAs.

Overall our data also provide important information in regards to the location of MStV transcription within the cell. Capped RNA-primed transcription for the influenza virus occurs within the host cell nucleus (42). In contrast, replication and transcription for members of the *Bunyaviridae* and the genus *Tenuivirus* are thought to occur in the cytoplasm of infected host cells (22, 43–47). Because BSMV replicates in the cytoplasm, our data support cytoplasmically localized transcription and cap-snatching for the *Tenuivirus* MStV, similar to that for viruses in the *Bunyaviridae* (47). Our data also show that MStV and BSMV RNAs are not separately compartmentalized within a doubly infected cell so as to exclude interactions between these unrelated viruses.

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