

## Recombination and Polymerase Error Facilitate Restoration of Infectivity in Brome Mosaic Virus

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Received 12 August 1992/Accepted 2 November 1992

The tRNA-like structure present in the 3' noncoding region of each of the four virion RNAs of brome mosaic virus possesses a conserved A-67-U-A-65 (<sup>67</sup>AUA<sup>65</sup>) sequence. Four mutations in this region (<sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>GAA<sup>65</sup>, and <sup>67</sup>CAA<sup>65</sup>, each with a double base change, and <sup>67</sup>GUA<sup>65</sup>, containing a single point mutation), previously shown in vitro to be defective in minus-strand promoter function, were introduced into full-length genomic RNAs 2 and 3, and their replicative competence was analyzed in barley protoplasts. All four RNA 3 mutants were capable of replication, although progeny plus-sense RNA 3 accumulation was only 12 to 42% of that of the wild type. Replication of RNA 2 transcripts bearing these mutations was even more severely debilitated; the accumulation of each mutant progeny plus-strand RNA 2 was <10% of that of the wild type. Analysis of mutant RNA 3 progeny recovered from local lesions induced in *Chenopodium hybridum* and systemic infections in barley (*Hordeum vulgare*) plants revealed that the mutant base at position 67 from the 3' end had in each case been modified to an A. These changes generated RNAs with functional pseudorevertant (<sup>67</sup>AAA<sup>65</sup> for mutants <sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>GAA<sup>65</sup>, and <sup>67</sup>CAA<sup>65</sup>) or revertant (<sup>67</sup>GUA<sup>65</sup> → <sup>67</sup>AUA<sup>65</sup>) sequences. In most instances, the presence of internal markers permitted discrimination between polymerase error and RNA recombination as the process by which sequence restoration occurred. The pseudorevertant sequence was found to be capable of persistence during subsequent propagation in plants when present on RNA 3 but not when present on RNA 2. These data document the fluidity of the RNA genome and reveal situations in which polymerase error or recombination can function preferentially to restore an optimal sequence. They also support the concept that RNA viruses frequently exist as quasispecies and have implications concerning evolutionary strategies for positive-strand RNA viruses.

Viruses containing a positive-sense RNA genome constitute an abundant group of pathogens infecting humans, animals, and economically important crop plants. In natural infections, accurate copying of viral genomes by polymerases is important for effective replication and subsequent establishment of infection in host cells. Whereas DNA viruses have a low ( $10^{-8}$  to  $10^{-11}$ ) error rate (49) as a result of polymerase proofreading activity, no such correction mechanism is present in RNA-dependent RNA replication and misincorporation frequencies of  $10^{-3}$  to  $10^{-4}$  are typical (25, 49). A consequence of the inherent error frequency in RNA synthesis is the generation of highly heterogeneous populations held together in a dynamic equilibrium; such heterologous mixtures of related genomic sequences have been referred to as quasispecies (11), and natural selection is likely to maintain the most favorable consensus sequence for overall genome replication (9, 36). Heterogeneity as a result of polymerase error has been observed in RNA viruses of animals (49) and bacteria (10) and in plant viruses (5, 20, 51) and their satellites (12, 28). In addition to polymerase error, genetic variation in RNA viruses results from legitimate and illegitimate recombination; both mechanisms contribute to the rapid evolution of RNA viruses (29).

The genome of brome mosaic virus (BMV), the type member of the bromovirus group of plant viruses, is divided among three single-stranded, positive-sense RNAs (30). RNAs 1 and 2 encode nonstructural proteins required for viral replication (1, 19, 39) that share sequence similarities

with proteins encoded by several other plant and animal viruses (2, 21). BMV RNA 3 is dicistronic; it encodes a nonstructural 32-kDa protein with putative transport function and a 20-kDa capsid protein, neither of which is essential for RNA replication in protoplasts (19, 39) but both of which are required for systemic spread in barley plants (45). The coat protein gene is expressed from RNA 4, a subgenomic mRNA (18, 34).

The aminoacylatable 3' noncoding sequences of BMV RNAs can be folded to mimic a tRNA-like structure (44) and harbor recognition signals not only for the initiation and synthesis of minus strands but also for interaction in vitro and in vivo with several tRNA-specific enzymes (22, 32). Using a template-dependent and template-specific RNA-dependent RNA polymerases isolated from infected barley leaves, Dreher and Hall (14, 15) mapped the functional regions within the tRNA-like structure that are required for minus-strand synthesis. Subsequent experiments utilizing a barley protoplast system (31) and infectious RNA transcripts synthesized in vitro from T7-based full-length cDNA clones demonstrated the telomeric nature of the 3' CCA<sub>OH</sub> terminus (38) and the dispensability of tyrosylation for viral replication (16). However, BMV RNA 3 mutants that were replication defective but capable of supporting progeny RNA accumulation in protoplasts at levels approaching 40% of the wild-type level were unable to establish systemic infections and failed to undergo recombination (16). Similar mutants with mutations that severely debilitated replication of RNA 2 without having a detectable effect on accumulation of the other viral RNAs in protoplasts (39) were swiftly corrected to functional recombinants by homologous recombination with 3' termini from either RNA 1 or RNA 3 (43).

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In early experiments to discriminate among the functions of the tRNA-like 3' end of BMV RNAs, mutations were introduced into the putative tyrosine anticodon -AUA- at positions 65 through 67 from the 3' end ( $^{67}\text{AUA}^{65}$ ) (13). It now appears that the stem-loop containing this sequence may not be the anticodon arm analog of the tRNA-like structure (37), and the mutations were found to drastically debilitate minus-strand promoter activity without an appreciable effect on aminoacylation functions (13). Additionally, because previous studies have revealed marked differences in the effects of individual mutations present on RNAs 2 and 3 (39, 40), we placed the various mutant sequences on both RNAs and analyzed the replication competence of the mutants in protoplasts and the infectivity of the mutants in barley and *Chenopodium hybridum* plants. These studies revealed (i) that  $^{67}\text{AUA}^{65}$  is a major sequence determinant for the recognition of viral replicase, (ii) that *cis* and *trans* interactions between the 3' region and its upstream genomic RNA context are important, and finally (iii) that RNA viruses frequently exist as quasispecies and two major processes (polymerase error and recombination) are responsible for the conversion of lethal mutations to biologically functional sequences.

## MATERIALS AND METHODS

**General recombinant DNA materials and methodology.** Restriction enzymes, human placental RNase inhibitor (RNAGuard), T7 RNA polymerase, cap analog m<sup>7</sup>GpppG, and plasmid pT7/T3 were obtained from New England Biolabs, Boehringer-Mannheim, Pharmacia, or Bethesda Research Laboratories. Radioactive materials were purchased from NEN. Enzymes were used essentially as recommended by the manufacturer. Plasmids were grown, purified, and analyzed by standard methods (47). All other chemicals used were of reagent grade.

**Transfer of 3'-terminal mutations into BMV genomic RNAs and production of infectious RNA transcripts.** Four mutations previously introduced into the loop region of arm C of the 3' tRNA-like structure (Fig. 1) (13) were introduced into full-length cDNA clones of BMV RNAs 2 (pT7B2) and 3 [pT7B3(Tth)] as *Hind*III-*Bam*HI fragments (16, 38, 39) and confirmed by dideoxy sequencing. Three of the four mutations were initially designated A32, A48, and A60 (13) but are named more descriptively here as  $^{67}\text{UAA}^{65}$ ,  $^{67}\text{GAA}^{65}$ , and  $^{67}\text{CAA}^{65}$ , respectively. Full-length genomic cDNA clones pT7B1 and pT7B2, or mutant derivatives of pT7B2, were linearized with *Bam*HI for the production of runoff transcripts, whereas pT7B3(Tth) and its mutant derivatives were linearized with *Tth*111I before transcription (16, 38, 39). Capped full-length RNA transcripts were synthesized in vitro by using T7 RNA polymerase as described by Dreher et al. (16). Before the barley protoplasts were inoculated, transcripts were freed of DNA template by precipitation with 2.6 M LiCl (16, 38).

**Isolation and transfection of barley protoplasts.** Protoplasts were isolated from barley (*Hordeum vulgare* cv. Dickson) leaves (31) and approximately  $2.5 \times 10^5$  cells were transfected with the desired combination of transcripts by using polyethylene glycol (16, 38). Inocula consisted of 1  $\mu\text{g}$  of each of the capped genomic transcripts (B1, B2, B3, and mutant derivatives). After inoculation, the protoplasts were incubated for 20 h at room temperature under lights.

**Analysis of progeny RNA from protoplasts.** Samples of the total RNA isolated from protoplasts by sodium dodecyl sulfate (SDS)-phenol extraction (31) were denatured with

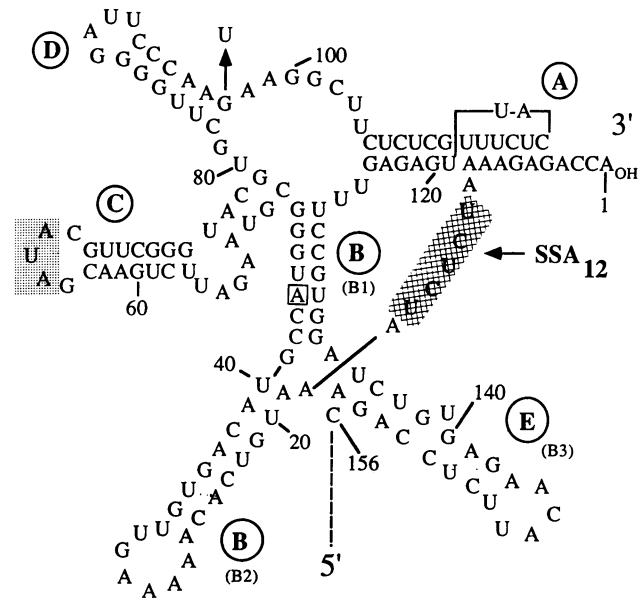


FIG. 1. Schematic representation of the 3' tRNA-like structure of BMV RNA 3 based on that of Perret et al. (37). The stippled box in arm C indicates the region in which mutations were introduced. In vitro transcripts of the three mutants bearing  $^{67}\text{UAA}^{65}$ ,  $^{67}\text{GAA}^{65}$ , and  $^{67}\text{CAA}^{65}$  also varied from wild-type  $^{67}\text{AUA}^{65}$  by a single-base substitution,  $^{97}\text{G} \rightarrow ^{97}\text{U}$  (indicated by an arrow). The cross-hatched region indicates the alteration of  $^{16}\text{UCUCU}^{12}$  to  $^{16}\text{AAAAA}^{12}$ , yielding the marker sequence  $\text{SSA}_{12}$  ( $^{19}\text{AAAAAAAAAAAAA}^8$ ). Within the 3' 134 nucleotides, RNA 2 is distinguished from RNA 3 by the presence of a G instead of an A at position 44 (boxed).

glyoxal, electrophoresed in 1% agarose, and transferred electrophoretically to nylon filters (16, 38). Prehybridization and hybridization conditions were as described by Dreher et al. (16).  $^{32}\text{P}$ -labeled RNA probes specific for BMV plus- and minus-sense RNAs were transcribed from plasmid pT73TR by using either T7 or T3 RNA polymerase (38–40). Since this plasmid contains a 3' cDNA fragment of about 200 nucleotides that is conserved among the four RNAs of BMV, probes corresponding to this sequence could be used to detect all of the BMV RNAs. The relative yields of wild-type and mutant progeny RNAs 2 and 3 were quantitated by densitometry of the Northern RNA blot autoradiographs. Correction for differences in inoculation efficiency and protoplast viability was made by normalization of the yield of progeny RNA 2 against the densitometric value obtained for RNA 1 progeny and normalization of the yield of progeny RNA 3 against the value obtained for RNAs 1 and 2 (38–40). The accumulation of total progeny (the sum of the band intensities of all viral RNAs) was estimated as described by Duggal et al. (17).

**Inoculation of barley and *C. hybridum* plants.** Capped transcripts of genome polarity were synthesized in vitro from wild-type and mutant templates (16) and adjusted to 100  $\mu\text{g}/\text{ml}$  in a buffer containing 50 mM Tris-HCl (pH 7.0), 250 mM NaCl, 5 mM EDTA, and 3 mg of bentonite per ml (43). Approximately 6-day-old barley and *C. hybridum* plants at the two-leaf stage (previously kept in the dark for 12 h) were mechanically inoculated with 10  $\mu\text{l}$  of this mixture. Control inoculations were performed with mixtures containing wild-type RNA transcripts.

**Virus purification from barley plants.** Approximately 12 to

TABLE 1. In vitro properties and accumulation in barley protoplasts of mutant progeny plus- and minus-sense RNAs 2 and 3

Mutant	In vitro properties <sup>a</sup> (% of wild-type value)			In vivo replication <sup>b</sup> (% of wild-type value)			
	Tyrosylation	Adenylation	Minus-strand promoter	RNA 2		RNA 3	
				Plus sense	Minus sense	Plus sense	Minus sense
<sup>67</sup> AUA <sup>65</sup>	100	100	100	100	100	100	100
<sup>67</sup> UAA <sup>65</sup>	100	45	15	<10	<10	12	10
<sup>67</sup> GAA <sup>65</sup>	108	47	7	<10	<10	12	8
<sup>67</sup> CAA <sup>65</sup>	94	53	18	<10	<10	16	12
<sup>67</sup> GUA <sup>65</sup>	104	100	6	<10	<10	42	8
<sup>67</sup> AAA <sup>65</sup>	ND <sup>c</sup>	ND	ND	<10	ND	55	ND

<sup>a</sup> Data from Dreher et al. (13–15).

<sup>b</sup> In vivo replication data are averages of at least four independent inoculations of barley protoplasts. Progeny plus- and minus-sense RNAs 2 and 3 were assayed by densitometry of Northern blots as described in Materials and Methods.

<sup>c</sup> ND, not determined.

15 days after inoculation, systemically infected barley leaves were harvested and virus was isolated by grinding the leaves in a buffer containing 0.5 M sodium acetate, 0.08 M magnesium acetate (pH 4.5), and 0.144 M  $\beta$ -mercaptoethanol. The homogenate was centrifuged in a Beckman JA20 rotor for 20 min at 10,000 rpm, and the resulting supernatant was emulsified with an equal volume of chloroform. The particulate material was separated by centrifugation in a Beckman JA20 rotor at 15,000 rpm for 15 min, and the upper phase was overlaid on 5 ml of 10% sucrose prepared in virus suspension buffer (50 mM sodium acetate, 8 mM magnesium acetate [pH 4.5]) and centrifuged in a Beckman VTI<sub>60</sub> rotor at 55,000 rpm for 60 min. The pellet was dissolved in virus suspension buffer, and RNA was extracted with SDS-phenol (31).

**Isolation of progeny RNA from *C. hybridum* plants.** Single discrete lesions were excised with a sterile cork borer, and RNA was extracted with SDS-phenol (43). Northern hybridization (16, 38) was used to analyze the levels of progeny RNA in each excised lesion.

**Sequence analysis of progeny RNA.** Progeny RNAs isolated from barley plants or *C. hybridum* were suspended in sterile distilled water. Reverse transcriptase and a primer [d(5' GACATGGTCTCTTTAG 3')] complementary to the 3' end of each of the four BMV RNAs were used to synthesize first-strand cDNA corresponding to progeny RNAs 2 and 3. The resulting cDNAs were converted to double-stranded forms by 50 polymerase chain reaction (PCR) cycles (46) with 5' primers corresponding to approximately 450 bases from the 3' end of the minus-strand of BMV RNA 2 [d(5' AGGACCACACAACGCTTG 3')] or BMV RNA 3 [d(5' CGGACGCCTTTCGAGGGG 3')] and *Taq* polymerase as specified by the manufacturer (Perkin Elmer Cetus, Norwalk, Conn.). A single cycle consisted of 1 min at 92°C for denaturation, 2 min at 50°C for annealing, and 2 min at 72°C for elongation. The PCR product was treated with Klenow fragment to extend the incomplete DNA fragments, modified with T4 polynucleotide kinase, and cloned into the *Sma*I site of the pT7/T3lacZ (Pharmacia) as described previously (38, 43). As a control for possible artifacts (such as base substitutions or recombination) introduced by PCR amplification, RNA isolated from wild-type infections (in barley or *C. hybridum*) was similarly amplified and cloned in the same plasmid and shown not to bear any sequence modifications. Unless otherwise mentioned, six to eight clones were picked for each cDNA construct for sequence analysis. Since sequence changes were seen in the progeny only at the positions under investigation, random errors during reverse transcription or PCR amplification did not obscure the

findings. The DNA sequences of the inserts were determined by the chain-termination procedure with Sequenase (U.S. Biochemical) and synthetic primers corresponding to either T7 or T3 promoter regions (43).

## RESULTS

**Expression of BMV RNA 3 variants in barley protoplasts.** In previous studies, Dreher and Hall (14, 15) characterized tyrosylation, adenylation, and minus-strand promoter functions in vitro for four mutations (<sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>GAA<sup>65</sup>, <sup>67</sup>CAA<sup>65</sup>, and <sup>67</sup>GUA<sup>65</sup>) introduced into the <sup>67</sup>AUA<sup>65</sup> triplet sequence present in arm C of the 3' tRNA-like structure of BMV RNA 3 (Fig. 1). Each mutation resulted in a large decrease of minus-strand synthesis but had little or no effect on aminoacylation (Table 1). The replication of full-length RNA 3 bearing these mutations was tested in protoplasts by cotransfection with wild-type RNAs 1 and 2. The levels of progeny minus-strand accumulation for all four RNA 3 mutants were depressed compared with that for wild-type RNA 3 and similar to their relative minus-strand promoter activities determined in vitro; the accumulation of mutant progeny plus-strand RNAs ranged from 12 to 42% of the wild-type value (Fig. 2; Table 1).

**Expression of BMV RNA 2 variants in barley protoplasts.** Since BMV RNA 2 encodes one of the *trans*-acting proteins essential for viral replication (19, 39), it appeared likely that the depression of minus-strand promoter activity seen in vitro for the <sup>67</sup>AUA<sup>65</sup>-region mutations on RNA 2 would affect viral replication in vivo. This was found to be the case; accumulation of RNA 2 progeny in barley protoplasts transfected with inocula containing each of the RNA 2 mutants was less than 10% of the wild-type value (Fig. 3; Table 1). Despite the dramatic decrease in RNA 2 progeny accumulation, there was no detectable effect on accumulation of the other BMV RNAs (Fig. 3). This is consistent with previous findings (39), suggesting that the p2a protein translated from RNA 2 is very stable or is required in only small amounts.

**Effect of BMV RNA 3 variants on infections in plants.** Although the proteins encoded by BMV RNA 3 are not essential for replication in barley protoplasts, the presence of RNA 3 stimulates overall replication and results in a 100-fold excess of plus strands over minus strands, compared with the near parity for plus- and minus-strand accumulation in its absence (35, 42). These effects undoubtedly contribute to the obligatory presence of RNA 3 for systemic spread in whole plants (45). Consequently, BMV RNA 3 mutants 5'PsK and  $\psi$ GG, which are severely debilitated in

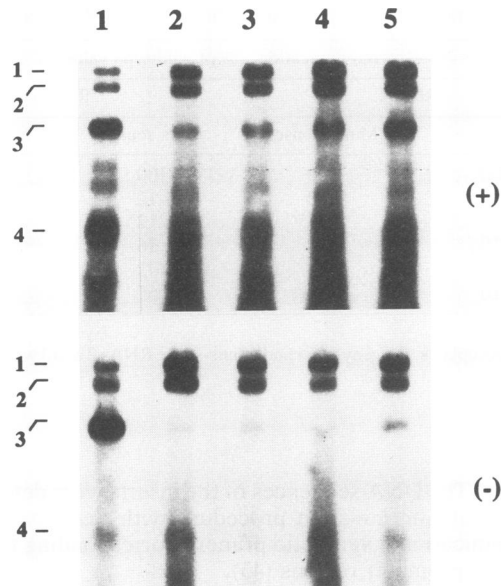


FIG. 2. Expression of BMV RNA 3 variants in barley protoplasts. Shown are Northern blots of progeny plus and minus-sense RNA samples isolated from barley protoplasts transfected with wild-type RNAs 1 and 2 and the following RNA 3 transcripts (lanes): 1, wild-type  $67\text{AUA}^{65}$ ; 2,  $67\text{UAA}^{65}$ ; 3,  $67\text{GAA}^{65}$ ; 4,  $67\text{CAA}^{65}$ ; 5,  $67\text{GUA}^{65}$ . RNAs extracted from approximately  $2.5 \times 10^5$  barley protoplasts were electrophoresed in 1% agarose gels after denaturation with glyoxal and transferred to nylon membranes. The blots were then hybridized with  $^{32}\text{P}$ -labeled minus- and plus-sense RNA probes representing the homologous 3' regions present on each of the four BMV RNAs. The positions of the four wild-type BMV RNAs are shown on the left. Blots were exposed for 1 h to detect plus-sense RNAs and 48 h for minus-sense RNAs.

replication (20 and 40% of the wild-type value, respectively) failed to establish efficient systemic infections in barley plants and yielded no evidence for recombination (16). BMV RNA 3 sequences bearing mutations in the  $67\text{AUA}^{65}$  loop (Fig. 1) are unlikely to have significant effects on the surrounding tRNA-like structure but give decreases in progeny accumulation (Table 1) similar to those observed for the 5' PsK and  $\psi\text{GG}$  mutations (16). Consequently, it was of

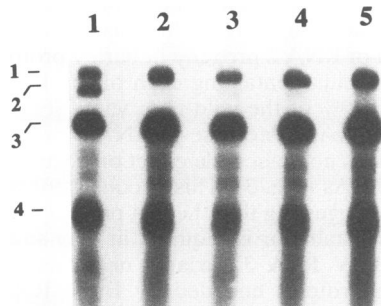


FIG. 3. Expression of BMV RNA 2 variants in barley protoplasts. Shown are Northern blots of progeny plus-sense RNA samples isolated from barley protoplasts transfected with wild-type RNAs 1 and 3 and the following (lanes): 1, RNA 2 bearing the 200 3'-terminal nucleotides from wild-type RNA 3; 2,  $67\text{UAA}^{65}$  RNA 2; 3,  $67\text{GAA}^{65}$  RNA 2; 4,  $67\text{CAA}^{65}$  RNA 2; 5,  $67\text{GUA}^{65}$  RNA 2. The positions of the four wild-type BMV RNAs are shown on the left. Blots were exposed for 1 h to detect plus-sense RNAs.

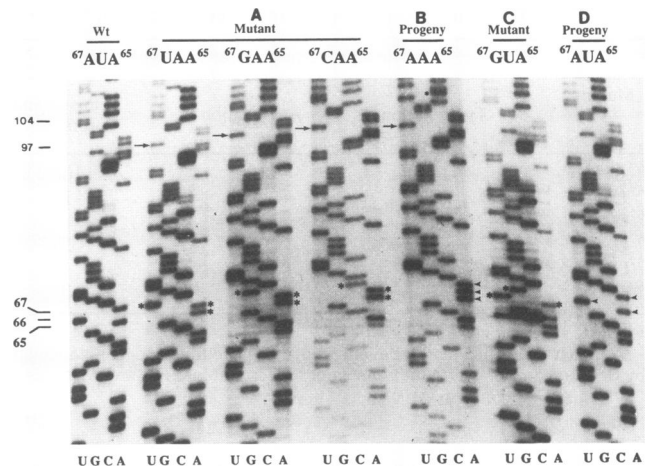


FIG. 4. Sequence analysis of progeny RNA from *C. hybridum* plants. (A) The sequence of mutant BMV RNA 3 cDNA clones bearing three double-base changes ( $67\text{UAA}^{65}$ ,  $67\text{GAA}^{65}$ , and  $67\text{CAA}^{65}$ ) introduced into the wild-type  $67\text{AUA}^{65}$  region is shown. These sequences also differ from the wild type (Wt) by a single-base substitution,  $97\text{G} \rightarrow 97\text{U}$  (arrow). (B) Representative progeny containing the pseudoreversion sequence ( $67\text{AAA}^{65}$ ; arrowheads) isolated from *C. hybridum* plants infected with  $67\text{GAA}^{65}$ . The arrow indicates conservation of base  $97\text{U}$ , and the asterisk indicates the second mutation site ( $104\text{U} \rightarrow 104\text{C}$ ) found in the progeny RNA of mutant  $67\text{GAA}^{65}$  (see the text). (C) The sequence of mutant BMV RNA 3 cDNA clone bearing a single-base substitution at position 67 ( $67\text{GUA}^{65}$ ) is shown. (D) Representative revertant sequence (arrowheads) recovered from *C. hybridum* plants inoculated with the  $67\text{GUA}^{65}$  mutant. Although the sequences shown were derived from cDNA clones, they are labeled according to the corresponding RNA sequences of the supplied RNA transcripts or recovered progeny. The numbers on the left represent the bases at which mutations (marked by asterisks in panels A and C) were introduced. Numbering is from the 3' terminus.

interest to see whether BMV RNA 3 transcripts bearing mutations in the  $67\text{AUA}^{65}$  sequence could induce local lesions in *C. hybridum* or support systemic infections in barley.

Three ( $67\text{UAA}^{65}$ ,  $67\text{GAA}^{65}$ , and  $67\text{CAA}^{65}$ ) of the four RNA 3 mutants contain U at position 97 (Fig. 1 and 4). In vitro, this is a functionally neutral mutation from the consensus G found at this position (14, 15) and serves as a useful marker. In separate experiments (41), we have found that inoculations containing RNA 3 bearing both  $\text{U}^{97}$  and  $\text{A}^{132}$  substitutions (for  $\text{G}^{132}$ ) yielded progeny levels in protoplasts and plants that were similar to those yielded by the wild-type sequence.

**Infection of *C. hybridum* with RNA 3 mutants.** Local lesions were produced on *C. hybridum* after inoculation with RNA mixtures containing each of the four RNA 3 mutants (Table 2). Compared with the number in wild-type infections, very few lesions developed from transfections containing the RNA 3 mutants (Table 2), and the time course for their appearance was delayed by 3 days. However, after 6 days, the lesion size and morphology were similar to those of lesions induced by inocula containing all three wild-type transcripts. Quantitative Northern blot analysis revealed that the level of RNA 3 in each lesion was similar to that of the wild-type control (data not shown). This suggested that sequence modifications were involved in the production of infectious progeny, so sequence analysis of RNA 3 progeny

TABLE 2. Characterization of progeny from barley and *C. hybridum* plants

Inoculum	No. of samples		Sequence recovered	Likely process	Evidence and comments
	Observed	Analyzed			
<b>RNA 2 mutants</b>					
<i>C. hybridum</i>					
Wild type	>100 lesions	2 lesions	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	None	Wild-type sequence retained
<sup>67</sup> CAA <sup>65</sup> + U <sup>97</sup>	6 lesions	5 lesions	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Recombination	Absence of U <sup>97</sup>
		1 lesion	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup>	Polymerase error	Retention of U <sup>97</sup> (Fig. 4B)
<sup>67</sup> GAA <sup>65</sup> + U <sup>97</sup>	6 lesions	6 lesions	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Recombination	Absence of U <sup>97</sup>
<sup>67</sup> GUA <sup>65</sup> + G <sup>97</sup>	9 lesions	3 lesions	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Recombination or reversion	No markers, difficult to discriminate the likely process (Fig. 4D)
		1 lesion	<sup>67</sup> AUA <sup>75</sup> + G <sup>97</sup>	Recombination	Presence of U <sup>43</sup> and G <sup>44</sup>
<sup>67</sup> UAA <sup>65</sup> + U <sup>97</sup>	6 lesions	6 lesions	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Recombination	Absence of U <sup>97</sup>
<i>H. vulgare</i>					
<sup>67</sup> CAA <sup>65</sup> + U <sup>97</sup>	10 plants	10 plants	None	No symptoms seen	Remained uninfected
<sup>67</sup> GAA <sup>65</sup> + U <sup>97</sup>	10 plants	10 plants	None	No symptoms seen	Remained uninfected
<sup>67</sup> GUA <sup>65</sup> + G <sup>97</sup>	10 plants	10 plants	None	No symptoms seen	Remained uninfected
<sup>67</sup> UAA <sup>65</sup> + U <sup>97</sup>	10 plants	10 plants	None	No symptoms seen	Remained uninfected
<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup>	5 plants	5 plants	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Recombination	Absence of U <sup>97</sup> and C <sup>104</sup> , progeny recovered from inoculated leaves only
<b>RNA 3 mutants</b>					
<i>C. hybridum</i>					
<sup>67</sup> CAA <sup>65</sup> + U <sup>97</sup>	7 lesions	1 lesion	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Recombination	Presence of G <sup>44</sup>
		3 lesions	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup>	Polymerase error	Retention of U <sup>97</sup> (Fig. 4B)
<sup>67</sup> GAA <sup>65</sup> + U <sup>97</sup>	13 lesions	3 lesions	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup>	Polymerase error	Retention of U <sup>97</sup> (Fig. 4B)
		1 lesion	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup>	Polymerase error	Also contained C <sup>104</sup> (Fig. 4B)
<sup>67</sup> GUA <sup>65</sup> + G <sup>97</sup>	16 lesions	5 lesions	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Reversion	Absence of G <sup>44</sup>
<sup>67</sup> UAA <sup>65</sup> + U <sup>97</sup>	5 lesions	1 lesion	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Recombination	Presence of G <sup>44</sup>
		3 lesions	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup>	Polymerase error	Retention of U <sup>97</sup> (Fig. 4B)
<i>H. vulgare</i>					
<sup>67</sup> CAA <sup>65</sup> + U <sup>97</sup>	10 plants	10 plants	None	No symptoms	All 10 plants remained uninfected
<sup>67</sup> GAA <sup>65</sup> + U <sup>97</sup>	10 plants	10 plants	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup>	Polymerase error	Only 1 plant infected, 9 plants remained uninfected (Fig. 4B)
<sup>67</sup> GUA <sup>65</sup> + G <sup>97</sup>	10 plants	10 plants	<sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup>	Reversion	4 plants infected; 6 plants remained uninfected (Fig. 4D)
<sup>67</sup> UAA <sup>65</sup> + U <sup>97</sup>	10 plants	10 plants	None	No symptoms	All 10 plants remained uninfected
<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup>	5 plants	4 plants	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup>	Systemic infection	All 4 plants retained <sup>67</sup> AAA <sup>65</sup> , U <sup>97</sup> , and C <sup>104</sup>
Wild-type RNA 1 + <sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup> RNA 2 + <sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup> mutants					
No infectivity in <i>C. hybridum</i> or <i>H. vulgare</i>					

was undertaken for a total of 17 discrete single lesions (four each for <sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>CAA<sup>65</sup>, and <sup>67</sup>GAA<sup>65</sup> and five for <sup>67</sup>GUA<sup>65</sup>; Table 2). Sequence analysis of progeny from nine single-lesion isolates (three each for mutants <sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>CAA<sup>65</sup>, and <sup>67</sup>GAA<sup>65</sup>) revealed that, in each case, base 67 had been modified to an A, yielding <sup>67</sup>AAA<sup>65</sup> (Table 2; Fig. 4B). RNA 3 progeny bearing the <sup>67</sup>AAA<sup>65</sup> sequence are classified as pseudorevertants because, although they are biologically functional, this sequence differs from those of the input mutations (<sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>CAA<sup>65</sup>, and <sup>67</sup>GAA<sup>65</sup>) and the wild type (<sup>67</sup>AUA<sup>65</sup>). The progeny from one lesion resulting from the mutant <sup>67</sup>GAA<sup>65</sup> contained a second-site mutation of U<sup>104</sup> → C<sup>104</sup> in addition to <sup>67</sup>AAA<sup>65</sup> and U<sup>97</sup> (Table 2; Fig. 4B). Characterization of progeny from five lesions resulting from inocula containing <sup>67</sup>GUA<sup>65</sup> RNA 3 revealed that reversion to the wild-type sequence (<sup>67</sup>AUA<sup>65</sup>) had occurred (Table 2; Fig. 4D). The conservation of U<sup>97</sup> (for mutants <sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>CAA<sup>65</sup>, and <sup>67</sup>GAA<sup>65</sup>) and the absence of bases diagnostic for the 3' termini of either RNA-1 or RNA-2 showed that recombination was not responsible for restoring infectivity. However, in two instances (one each for mutants <sup>67</sup>UAA<sup>65</sup> and <sup>67</sup>CAA<sup>65</sup>), the absence of U<sup>43</sup>, U<sup>97</sup>, and C<sup>131</sup> and the presence of G<sup>44</sup> and <sup>67</sup>AUA<sup>65</sup> in progeny sequences indicated that homologous recombination with the 3'-terminal sequences of wild-type

RNA 2 had occurred (Table 2). Progeny RNA 3 obtained from two independent lesions resulting from control inoculations maintained the wild-type 3' noncoding sequence.

**Infection of barley plants with RNA 3 mutants.** Only 1 of 10 barley plants (from two independent experiments) inoculated with transcripts of wild-type RNAs 1 and 2 and the <sup>67</sup>GAA<sup>65</sup> RNA 3 mutant displayed mosaic symptoms characteristic of BMV infection (Table 2). The onset of mosaic symptoms was delayed by 1 week compared with the onset of symptoms in control inoculations containing all three wild-type transcripts. Northern analysis of inoculated or noninoculated upper leaves from the remaining nine symptomless plants failed to detect any viral RNA. However, of 10 plants inoculated with <sup>67</sup>GUA<sup>65</sup> RNA 3, 4 produced symptoms with kinetics similar to those observed for wild-type infections (Table 2). The other six plants remained symptomless for at least 3 weeks, and no viral RNA was detected in either inoculated or noninoculated leaves. Neither systemic disease symptoms nor viral RNA was evident in 20 barley plants inoculated with mixtures containing transcripts of either <sup>67</sup>UAA<sup>65</sup> RNA 3 or <sup>67</sup>CAA<sup>65</sup> RNA 3.

The yields of virus purified from barley plants infected with the RNA 3 mutant derivatives <sup>67</sup>GAA<sup>65</sup> and <sup>67</sup>GUA<sup>65</sup> were similar to those obtained from wild-type infections. To determine whether the progeny RNAs retained the intro-

TABLE 3. Discrimination between recombination and polymerase error in *C. hybridum*<sup>a</sup>

Lesion no.	Sequence recovered	Likely process	Evidence and comments
1	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup> <sup>67</sup> AUA <sup>65</sup> + SSA <sub>12</sub>	None Recombination	Represents inoculum RNA; 2 of 12 clones contained this sequence Presence of SSA <sub>12</sub> marker; 10 of 12 clones contained this sequence (Fig. 6)
2	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup> <sup>67</sup> AUA <sup>65</sup> + SSA <sub>12</sub>	None Recombination	Represents inoculum RNA; 2 of 12 clones contained this sequence Presence of SSA <sub>12</sub> marker; 10 of 12 clones contained this sequence (Fig. 6)
3	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup> <sup>67</sup> AAA <sup>65</sup> + G <sup>97</sup> + U <sup>104</sup> <sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup> + U <sup>104</sup>	None Polymerase error Polymerase error	Represents inoculum RNA; 2 of 12 clones contained this sequence (Fig. 7A) Bases 97 and 104 reverted to wild type; 2 of 12 clones contained this sequence (Fig. 7B) Base 66 was modified to wild type; 8 of 12 clones contained this sequence (Fig. 7C)
4	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup> <sup>67</sup> AAA <sup>65</sup> + G <sup>97</sup> + U <sup>104</sup>	None Polymerase error	Represents inoculum RNA; 2 of 12 clones contained this sequence Bases 97 and 104 reverted to wild type; 2 of 12 clones contained this sequence
5	<sup>67</sup> AAA <sup>65</sup> + U <sup>97</sup> + C <sup>104</sup> <sup>67</sup> AUA <sup>65</sup> + G <sup>97</sup> + U <sup>104</sup> <sup>67</sup> AUA <sup>65</sup> + SSA <sub>12</sub>	None Polymerase error Recombination	Represents inoculum RNA; 2 of 12 clones contained this sequence Bases 97 and 104 reverted to wild type; 4 of 12 clones contained this sequence Presence of SSA <sub>12</sub> marker; 6 of 12 clones contained this sequence

<sup>a</sup> One-week-old *C. hybridum* plants were inoculated with a mixture of in vitro transcripts (150 µg/ml) containing wild-type RNA 1 + <sup>67</sup>AAA<sup>65</sup> RNA 2 + SSA<sub>12</sub> RNA 3. A total of five lesions appeared on two inoculated leaves. Total RNA was isolated, and sequences corresponding to BMV RNA 2 were amplified by PCR and cloned for sequence verification as described in Materials and Methods.

duced mutations, cDNA libraries corresponding to the appropriate regions of each progeny RNA 3 were constructed by PCR. Dideoxynucleotide sequencing of several cDNA clones corresponding to progeny of <sup>67</sup>GAA<sup>65</sup> RNA 3 revealed the presence of pseudoreversion sequences similar to those recovered from the local lesion host (Fig. 4B). Progeny RNA 3 isolated from all four barley plants inoculated with <sup>67</sup>GUA<sup>65</sup> RNA 3 appeared to be true revertants; they contained the <sup>67</sup>AUA<sup>65</sup> sequence and lacked bases diagnostic for RNA 1 or 2 (Fig. 4D; Table 2). Progeny RNA 3 obtained from control inoculations maintained the wild-type 3' non-coding sequence.

**Effect of BMV RNA-2 variants on local lesion and systemic infections.** *C. hybridum* plants inoculated with wild-type RNAs 1 and 3 and each of the RNA 2 <sup>67</sup>AUA<sup>65</sup> mutants developed local lesions (Table 2). As found with the same mutations on RNA 3, the appearance and development of lesions were delayed. A total of 22 single lesions were isolated for sequence verification. In contrast to the low replication found for the four RNA 2 variants in protoplasts (Fig. 3), Northern hybridization analysis of total RNA isolated from these single lesions yielded levels of RNA 2 similar to those of the wild-type control (data not shown). Sequence analysis of cDNA clones obtained by PCR for 17 single lesions resulting from the mutants <sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>GAA<sup>65</sup>, and <sup>67</sup>CAA<sup>65</sup> revealed that homologous recombination had taken place between the supplied RNA 2 mutants and 3' sequences from RNA 3 (Table 2). In each case, the presence of the wild-type <sup>67</sup>AUA<sup>65</sup> sequence and the absence of the marker base U<sup>97</sup> (for mutants <sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>GAA<sup>65</sup>, and <sup>67</sup>CAA<sup>65</sup>) indicated that recombination with the 3' end of RNA 3 had occurred by strand switching of polymerase upstream of U<sup>97</sup>. However, a single lesion resulting from an inoculum containing <sup>67</sup>CAA<sup>65</sup> RNA 2 yielded progeny RNA that contained the pseudorevertant sequence <sup>67</sup>AAA<sup>65</sup> and U<sup>97</sup>. The fact that no RNA 2 sequences containing G<sup>44</sup> were recovered eliminates the possibility that the transcripts used for inoculation were contaminated with wild-type RNA 2.

Progeny from three of the four single-lesion isolates re-

sulting from an inoculum containing <sup>67</sup>GUA<sup>65</sup> RNA 2 yielded progeny RNA with the wild-type <sup>67</sup>AUA<sup>65</sup> sequence (Table 2). Other than the <sup>67</sup>GUA<sup>65</sup> mutation, the 3' end of this RNA 2 variant is identical to that of RNA 3. Consequently, it was not possible to determine whether these progeny represented revertants or recombinants. However, in one case it was clear that recombination with wild-type RNA 1 had occurred downstream of base 131, since U<sup>43</sup> and G<sup>44</sup> were present and C<sup>131</sup> was absent.

In contrast to the situation in the local lesion host, no symptoms developed over a period of 3 weeks in barley plants after inoculation with any of the RNA 2 <sup>67</sup>AUA<sup>65</sup> variants (Table 3). Northern hybridization and PCR analysis of total RNA isolated from either primary leaves or uninoculated secondary leaves failed to detect BMV RNAs. Control plants inoculated with all three wild-type transcripts developed normal infections.

**Synergistic depression of progeny accumulation in protoplasts when both RNAs 2 and 3 bear the <sup>67</sup>AAA<sup>65</sup> pseudorevertant sequence.** The pseudorevertant RNA 3 3' sequence <sup>67</sup>AAA<sup>65</sup> was cloned from progeny RNAs obtained from *C. hybridum* plants transfected with inocula containing <sup>67</sup>GAA<sup>65</sup> RNA 3 (Fig. 4B) and transferred to RNAs 2 and 3 so that the effect of this pseudorevertant sequence on their replication could be examined. In barley protoplasts, <sup>67</sup>AAA<sup>65</sup> RNA 3 plus-strand progeny accumulated to 55% of the wild-type level (Fig. 5, lane 5); however, replication of <sup>67</sup>AAA<sup>65</sup> RNA-2 was greatly debilitated, its progeny accumulating to less than 10% of the wild-type level (Fig. 5, lane 4). Total RNA progeny accumulation resulting from transfections containing either the <sup>67</sup>AAA<sup>65</sup> RNA 2 or the <sup>67</sup>AAA<sup>65</sup> RNA 3 mutant was also depressed, being less than 75% of the wild-type control level (Fig. 5, lanes 4 and 5). Interestingly, transfections containing wild-type RNA 1 and both RNAs 2 and 3 bearing the <sup>67</sup>AAA<sup>65</sup> mutation yielded only 15% of the total progeny detected for wild-type infections (Fig. 5, lane 6), and individual progeny levels for RNAs 2 and 3 were too low to permit accurate quantification. These



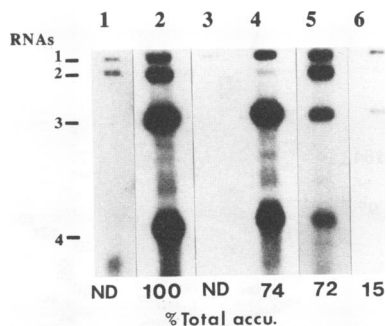


FIG. 5. Expression in barley protoplasts of  $^{67}\text{AAA}^{65}$  RNA 2 and  $^{67}\text{AAA}^{65}$  RNA 3. Shown is an autoradiograph from the Northern blot analysis of progeny plus-sense RNA samples isolated from barley protoplasts transfected with the following (lanes): 1, wild-type RNAs 1 and 2; 2, wild-type RNAs 1, 2, and 3; 3, wild-type RNA 1 and  $^{67}\text{AAA}^{65}$  RNA 2; 4, wild-type RNA 1,  $^{67}\text{AAA}^{65}$  RNA 2, and wild-type RNA 3; 5, wild-type RNAs 1 and 2 and  $^{67}\text{AAA}^{65}$  RNA 3; 6, wild-type RNA 1,  $^{67}\text{AAA}^{65}$  RNA 2, and  $^{67}\text{AAA}^{65}$  RNA 3. Lanes 1, 3, and 6 were exposed for 4 h, and lanes 2, 4, and 5 were exposed for 45 min. The positions of the four wild-type BMV RNAs are shown on the left. The numbers shown at the bottom of each lane represent the percentage of total accumulation values with respect to that of wild type. ND, not determined.

results suggest a synergistic interaction between RNAs 2 and 3 in progeny production.

**Persistence in vivo of the  $^{67}\text{AAA}^{65}$  pseudorevertant sequence in the absence of recombination.** The persistence of the pseudorevertant sequence during replication in planta was investigated by using *C. hybridum* and barley plants. Barley plants inoculated with mixtures containing wild-type RNAs 1 and 3 and  $^{67}\text{AAA}^{65}$  RNA 2 remained symptomless over a 2-week postinoculation period. Although no signal was detected in RNA from uninoculated upper leaves, Northern analysis of total RNA isolated from the inoculated leaves revealed the presence of all four BMV RNAs, confirming that replication of inoculum RNA had occurred. Sequencing of the tRNA-like structure of progeny RNA 2 isolated from the inoculated barley leaves revealed 3'-terminal sequences that were indistinguishable from that of wild-type RNA 3 (data not shown). Since reversion by polymerase error would require events at three locations ( $\text{A}^{66}$ ,  $\text{U}^{97}$ , and  $\text{C}^{104}$ ), it is likely that sequence correction was acquired by homologous recombination.

Each of five barley plants inoculated with wild-type RNAs 1 and 2 and  $^{67}\text{AAA}^{65}$  RNA 3 showed systemic symptoms. Sequence analysis of progeny RNA 3 revealed the presence of  $^{67}\text{AAA}^{65}$ ,  $\text{U}^{97}$ , and  $\text{C}^{104}$  (Table 2), confirming that the pseudorevertant sequence was preserved during replication in planta and is apparently stable when present on the RNA 3 component.

*C. hybridum* infected with inoculum containing  $^{67}\text{AAA}^{65}$  RNA 2 yielded only a small number of local lesions. Although the onset of these lesions was delayed by 3 days compared with that in wild-type controls, Northern hybridization revealed levels of progeny RNA 2 that were similar to that from wild-type lesions and sequence analysis (data not shown) confirmed that the wild-type sequence had been restored. These results suggest that, as in the case of barley, recombination resulted in reversion to the wild type. Inoculation of *C. hybridum* with mixtures containing  $^{67}\text{AAA}^{65}$  RNA 3 resulted in the formation of numerous lesions whose onset paralleled that of wild-type lesions, but sequence analysis of the progeny showed that the pseudorevertant

sequence was maintained, together with the markers  $\text{U}^{97}$  and  $\text{C}^{104}$ .

When barley or *C. hybridum* plants were inoculated with a mixture of transcripts containing wild-type RNA 1 together with  $^{67}\text{AAA}^{65}$  RNA 2 and  $^{67}\text{AAA}^{65}$  RNA 3, no evidence of systemic infection was seen on barley, nor did any lesions develop on *C. hybridum* (Table 2). These observations, plus the fact that no viral RNA was detected by Northern blot analysis of these plants, revealed that the synergistic depression of replication caused by the presence of this mutation on RNAs 2 and 3 renders the virus incapable of infecting the hosts systemically.

**Evidence for both homologous recombination and reversion of mutant sequences in single lesions.** The experiments described above did not permit distinction between polymerase error and recombination as the mechanism for restoration of the wild-type sequence in progeny RNA 2 isolated from lesions on *C. hybridum* inoculated with wild-type RNAs 1 and 3 and  $^{67}\text{AAA}^{65}$  RNA 2. To discriminate between these processes,  $\text{SSA}_{12}$  RNA 3 was substituted for wild-type RNA 3 in the inoculum to provide a marker sequence. This mutant (14) bears the sequence modification  $^{16}\text{UCUCU}^{12} \rightarrow ^{16}\text{AAAAA}^{12}$  (giving a stretch of 12 A residues between positions 7 and 20 from the 3' end; Fig. 1) and has no significant effect on replication and accumulation of RNA 3 in protoplasts (41).

*C. hybridum* plants were inoculated with wild-type RNA 1,  $^{67}\text{AAA}^{65}$  RNA 2, and  $\text{SSA}_{12}$  RNA 3. Five lesions resulted, although their onset was delayed by 6 days compared with that of lesions induced by wild-type transcripts. Total RNA was isolated from each lesion, and the sequence of the 3' 450 nucleotides was determined by dideoxynucleotide sequencing of progeny RNA 2 cDNA copies amplified by PCR. An average of 12 separate clones were sequenced for each cDNA construct from each lesion. In each case, the sequence upstream from the tRNA-like structure was indistinguishable from that of wild-type RNA 2 (Fig. 6) but sequence heterogeneity was clearly evident in the 3' tRNA-like region (Table 3).

Sequencing of progeny RNA 2 isolated from two independent lesions (Table 3, lesions 1 and 2) revealed that 90% of the clones contained 3' termini containing the  $\text{SSA}_{12}$  RNA 3 marker sequence, confirming that recombination had occurred between the 3' termini of inoculum  $^{67}\text{AAA}^{65}$  RNA 2 and  $\text{SSA}_{12}$  RNA 3 (Fig. 6). Interestingly, in each lesion, the length of the A-residue stretch had been extended, to 14 in one lesion (Fig. 6A) and 20 in the other (Fig. 6B). Elongation of the A tracts probably resulted from RNA polymerase stuttering (24, 50).

The absence of the internal marker bases ( $\text{U}^{97}$  and  $\text{C}^{104}$ ) present in the  $^{67}\text{AAA}^{65}$  RNA 2 used as an inoculum showed that strand switching of the RNA polymerase had occurred upstream of base  $\text{C}^{104}$ . The remaining 10% of the clones contained the mutant ( $^{67}\text{AAA}^{65}$ ) RNA 2 sequence present in the inoculum. No other sequence rearrangements were observed. Similar characterization of progeny RNA 2 from two other lesions (Table 3, lesions 3 and 4) revealed that each contained three different RNA 2 populations: (i) 2 of 12 clones sequenced contained RNA 2 sequences with 3' termini indistinguishable from that of the inoculum RNA (Fig. 7A); (ii) the 3' termini of two additional clones revealed the presence of the input  $^{67}\text{AAA}^{65}$  sequence, but the wild-type bases  $\text{G}^{97}$  and  $\text{U}^{104}$  were substituted for the internal marker bases  $\text{U}^{97}$  and  $\text{C}^{104}$  present in the  $^{67}\text{AAA}^{65}$  RNA 2 inoculum (Fig. 7B); (iii) the remaining 8 clones contained progeny RNA 2 with 3' termini indistinguishable from that of wild-

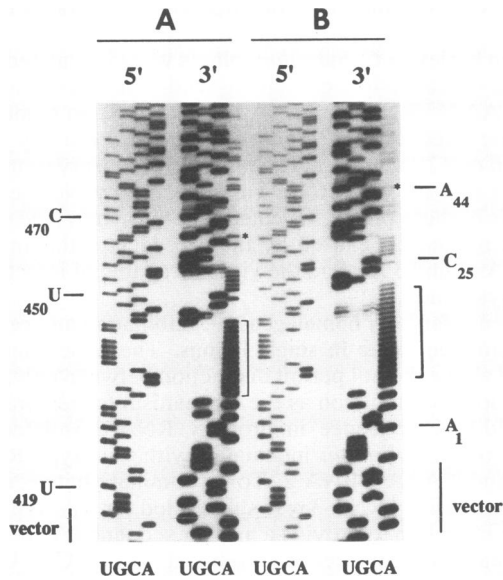


FIG. 6. Sequence analysis of recombinant progeny RNA 2 recovered from two independent local lesions (A and B) induced in *C. hybridum* plants inoculated with wild-type RNA 1,  $^{67}\text{AAA}^{65}$  RNA 2, and  $\text{SSA}_{12}$  RNA 3. The relevant portions of 5'- and 3'-terminal sequences present in a 450-nucleotide PCR product were shown. The marker sequence  $\text{SSA}_{12}$  and the additional A residues present in RNA progeny are bracketed. The diagnostic base present at position 44 in the 3' terminus of wild-type RNA 3 is marked by an asterisk. Numbering is from the 3' terminus. Although the sequences shown were derived from cDNA clones, they are labeled according to the corresponding RNA sequences of the recovered progeny.

type RNA 3 (Fig. 7C). It is noteworthy that the progeny characterized (Fig. 7) from these two lesions contained neither wild-type RNA 2 nor RNA 2 bearing the  $\text{SSA}_{12}$  3' marker sequence. This indicates that the pseudoreversion sequence present in the inoculum RNA 2 had reverted to the wild type. Additionally,  $\text{A}^{44}$  (present in the supplied  $^{67}\text{AAA}^{65}$  RNA 2 transcripts) was maintained in the progeny, further confirming that no recombination had taken place. There is no possibility that this arose from contaminating wild-type RNA 3, because all RNA 3 progeny retained the  $\text{SSA}_{12}$  3' marker sequence. Sequence analysis of progeny RNA 2 from the fifth lesion obtained (Table 3) revealed that it contained both recombinant and revertant RNA 2 progeny sequences similar to those shown in Fig. 6 and 7, respectively.

## DISCUSSION

It is well established that the lack of proofreading ability of RNA-dependent RNA polymerase leads to fluidity in RNA genomes, evidenced by the presence of quasispecies in viral progeny (11). Despite the presence of these quasispecies, viral genomes of a given isolate retain a consensus sequence. To our knowledge, little molecular evidence has been advanced previously to identify the mechanisms by which the consensus sequence is maintained. A simplistic explanation is that the consensus sequence always outcompetes mutant sequences. However, for certain functions important to the virus life cycle this appears to be untenable. For example, we have shown that specific mutations in the BMV RNA 3' sequences give rise to templates that are better than the

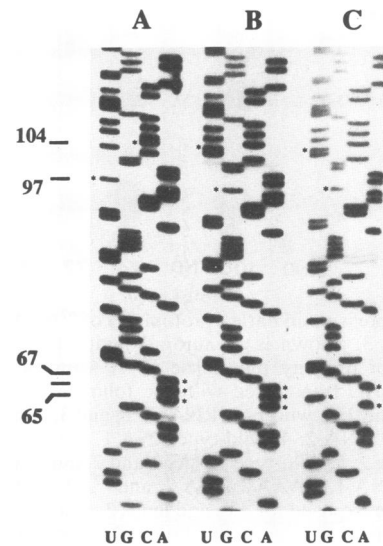


FIG. 7. Autoradiograph showing the sequences recovered from a single lesion of *C. hybridum* to demonstrate the stepwise reversion of input pseudorevertant sequence. (A) Sequence of the input inoculum RNA 2 containing  $^{67}\text{AAA}^{65}$ ,  $^{97}\text{U}$ , and  $^{104}\text{C}$  (positions marked); (B) modification of  $^{97}\text{U}$  to  $^{97}\text{G}$  and  $^{104}\text{C}$  to  $^{104}\text{U}$ ; (C) modification of  $^{66}\text{A}$  to  $^{66}\text{U}$ . The modifications in panels B and C yielded complete reversion to the wild type. Numbering is from the 3' terminus. Although the sequences shown were derived from cDNA clones, they are labeled according to the corresponding RNA sequences of the recovered progeny.

wild-type for minus-strand initiation (6) or for host nucleotidyl transferase (15).

Data from inoculations containing several discrete mutations (Tables 2 and 3) provide insight to the mechanisms by which the consensus sequence is restored. Reversion, pseudoreversion, and recombination events were all identified. The sites chosen for this study (Fig. 1) are important for recognition by the virus replicase (Fig. 2 and 3), and strong selection undoubtedly exists against debilitating changes at such sites. Nevertheless, we observed the persistence of some mutations, suggesting that they caused insufficient impairment to be effectively selected against over the generations investigated. It will be of interest to see whether these mutations persist or revert to the wild type after multiple passages. Persistent mutations that give rise to phenotypic changes (or modified host range) are undoubtedly represented in nature as strains and isolates: several epidemics of influenza virus A have been attributed to modifications of a single or a few bases resulting from polymerase error (52). In plant viruses, single-nucleotide changes can significantly alter pathogenicity and host specificity (48), thereby providing an environment favorable for the evolution of new pathogens.

**Sequence specificity of the  $^{67}\text{AUA}^{65}$  loop region in BMV replication.** The *in vivo* analyses reported here (Table 1; Fig. 2 and 3) confirm previous *in vitro* studies showing that the  $^{67}\text{AUA}^{65}$  sequence is an important recognition site for minus-strand promotion (14). Previously studied mutations in the tRNA-like region affecting minus-strand promoter activity (5'PsK and  $\Delta\text{knob}$  [14]) probably induce structural alterations. In contrast, the mutations studied here are present in a loop region and probably have little or no effect on the tRNA-like conformation. Additionally, it is unlikely that



they lead to RNA instability, because we have previously shown that the entire tRNA-like structure can be deleted with no effect on RNA degradation (39); further, transcripts bearing these mutations were found to have an mRNA template function identical to that of wild-type transcripts (41). Consequently, the substantial difference in RNA 3 plus-strand progeny levels in protoplasts transfected with <sup>67</sup>GUA<sup>65</sup> RNA 3 or <sup>67</sup>UAA<sup>65</sup> RNA 3 (42 or 12% of wild-type levels; Table 1) indicates a requirement for sequence specificity within this loop, which is thought to be involved in the binding of core polymerase or host proteins involved in viral replication (23). These observations show how strongly the mutation of a single nucleotide can influence viral replication. The results also indicate that although the presence of a purine at position 67 in the case of RNA 3 permits substantial levels of replication (Table 1), any substitution at this position greatly debilitates replication of RNA 2 (<10% of the wild-type replication level). When present on RNA 2 or 3, these differential effects of similar mutations extend previous observations on *cis* interactions between the 3' tRNA-like structure and genomic upstream regions (17).

**Sequence restoration in planta by polymerase error.** Propagation of the four replication-deficient BMV RNA 3 variants in planta resulted in a change at position 67 of each mutant that yielded a biologically functional pseudorevertant (<sup>67</sup>AAA<sup>65</sup> for mutants <sup>67</sup>UAA<sup>65</sup>, <sup>67</sup>GAA<sup>65</sup>, and <sup>67</sup>CAA<sup>65</sup>) or revertant sequence (<sup>67</sup>AUA<sup>65</sup> for mutant <sup>67</sup>GUA<sup>65</sup>). Because the observed base substitution does not result from (i) misincorporation during *in vitro* transcription (since the mutant transcripts are defective in replication when tested in protoplasts; Fig. 2), (ii) a PCR artifact (since several single lesions derived from independent transcript preparations yielded identical progeny sequences), (iii) a repair mechanism (since no proofreading activity is associated with RNA polymerases [49]), or (iv) the action of an "RNA duplex unwindase" activity (since this specifically replaces A with G [33]), it is virtually certain that it does result from the action of the error-prone RNA-dependent RNA polymerase.

The majority of mutations arising in the <sup>67</sup>AUA<sup>65</sup> region of either BMV RNA 2 or 3 are nonviable (Table 2), and their correction early after infection is necessary to generate replication-competent sequences. Since simultaneous correction of two adjacent nucleotides by polymerase error probably occurs much less frequently than does that for a single base, initial selection in planta is likely to include RNA sequences containing single-base changes that ameliorate replication. In the case of <sup>67</sup>CAA<sup>65</sup> RNA 3, any change other than A at position 67 is nonviable, since RNAs 3 bearing either <sup>67</sup>UAA<sup>65</sup> or <sup>67</sup>GAA<sup>65</sup> were found to be incompetent in replication in protoplasts (Fig. 2). Subsequent propagation in planta of the partially repaired <sup>67</sup>AAA<sup>65</sup> sequence permitted further base replacements through polymerase error, and wild-type progeny containing reversions at two and even three sites were recovered (Fig. 7).

**Evidence for *trans* interactions among BMV RNAs.** The synergistic effect of the pseudorevertant (<sup>67</sup>AAA<sup>65</sup>) sequence when present on both RNAs 2 and 3 (Fig. 5, lane 6) is especially interesting. Translation *in vitro* of each of the RNA 2 and RNA 3 variants yielded amounts of product similar to that obtained from wild-type sequences (41), indicating that the reduction in overall accumulation of viral RNAs is not due to decreased translatability of the mutant RNAs. Additionally, when protoplasts were transfected with <sup>67</sup>AAA<sup>65</sup> RNA 2, there was only a 26% decrease in total progeny accumulation (Fig. 5, lane 4), indicating that sufficient *trans*-acting factor was translated from the low levels

of <sup>67</sup>AAA<sup>65</sup> RNA 2 present. These observations are in accord with previous results showing that as little as 1.9 ng of RNA 2 produces sufficient protein 2a to support the replication of RNAs 1, 3, and 4 (39). Hence, the substantial decrease (85%; Fig. 5, lane 6) in total progeny RNA resulting from inoculations containing <sup>67</sup>AAA<sup>65</sup> RNA 2 and <sup>67</sup>AAA<sup>65</sup> RNA 3 probably results from *trans* interactions among the 3'-terminal sequences of BMV genomic RNAs.

The existence of intermolecular and intramolecular interactions among BMV RNAs has now been adduced from several observations. For example, whereas RNA 3 bearing the Δ5' mutation (a deletion of nucleotides 135 through 156, which lie immediately upstream of the tRNA-like structure [14]) had little effect on overall replication of BMV RNAs; RNA 2 bearing this mutation interfered in *trans*, drastically reducing progeny accumulation of the entire BMV genome (40). Additional support for the existence of inter- and intramolecular RNA interactions affecting viral replication derives from recent experiments in which the 3' tRNA-like structures were permuted among the three BMV RNAs (17). Notably, transfection of protoplasts with BMV RNAs 1, 2, and 3, all bearing the RNA 1 3' sequence, yielded only 15% of the progeny from transfection with the wild type. Taken together, these observations permit speculation that localized sequences throughout the viral genome contribute significantly to the tertiary and quaternary structures of individual viral RNAs that can differentially recruit host- and virus-encoded factors involved in the life cycle of the virus. Furthermore, such intra- and intermolecular RNA interactions are likely to permit strand switching during minus-strand replication, thereby facilitating the frequent recombination found for BMV RNAs (8, 43), which can assist in maintaining the consensus genome.

**Sequence restoration in planta by recombination.** RNA recombination has been recognized as an important process in the evolutionary variation of animal viruses (26), and recent molecular evidence suggests that RNA recombination also occurs frequently in plant viruses (3, 4, 7-9, 43). Characterization of several progeny RNA 2 sequences in this study (Tables 2 and 3) clearly indicates that recombination is the major mechanism responsible for the generation of biologically functional sequences (Fig. 6). The data in Tables 2 and 3 also support our previous observations (43) that homologous recombination occurs more frequently than does nonhomologous recombination (7). Since all recombinants characterized in this study involve precise recombination (Table 2; Fig. 6), we believe that the copy-choice mechanism, in which the viral RNA polymerase switches templates during negative-strand synthesis (27), best fits the observations. Furthermore, a majority of recombination events between supplied mutant RNA 2 and wild-type RNA 3 (Table 2) probably result from the abundant accumulation of nascent RNA 3 negative strands (RNA 3 replicates to higher levels than does RNA 1 [35]), since these represent the most available template donors for recombination by the copy-choice mechanism.

Despite the existence of multiple mechanisms for genetic variation among viruses, an important question that still remains to be addressed is whether evolution of novel pathogens is a rare or frequent event. It has previously been surmised that RNA viruses are subjected to greater sequence drift than are DNA viruses and hence are not suitable as vectors (51). However, recent experimental evidence indicate that fidelity of tobacco mosaic virus replication bearing foreign sequences was much higher and that the sequence drift is less of an evolutionary force in tobacco

mosaic virus than had been anticipated (25). In the present investigation, although we did not calculate the actual mutation accumulation rate in BMV, the data show that polymerase error and recombination act rapidly to modify mutant sequences debilitated for replication in a given host. They also show that modification to the consensus sequence can occur via a several-step process (in the case of the pseudorevertant; Fig. 7) and that the functional pseudorevertant sequence can persist over several replication cycles. The pseudorevertant we identified represents a specific example of a quasispecies, and it seems apparent that occasions will arise when the genome function contained in the modified sequence will be enhanced through better interaction with host functions. It is also conceivable that the modified interaction can lead to infectivity in a new host variety or species when chance events result in the novel sequence being inoculated into what proves to be a receptive host. Thus, although our data do not provide direct evidence relating to the frequency with which new pathogens arise, they do provide definitive evidence for the mechanisms by which maintenance of a consensus genome is achieved and by which modified genome sequences can be generated.

#### ACKNOWLEDGMENTS

We thank Lisa Pleasants and Naomi Trevino for excellent technical assistance and Clayton Huntley, Fred Lahser, Rohit Duggal, and Valerian Dolja for critical reading of the manuscript.

This work was supported in part by NSF grant DBM-8921023 and by the Texas A&M Office of University Research Services.

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