Genetic Recombination in Brome Mosaic Virus: Effect of Sequence and Replication of RNA on Accumulation of Recombinants

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Received 22 June 1992/Accepted 10 August 1992

In order to facilitate the isolation of recombinants in brome mosaic virus, a series of duplication mutants with alterations in the RNA3 3' noncoding region has been engineered. The distribution of crossovers, which was observed to be dependent on the parental RNA3 sequence, supported the role of RNA structure in recombination. However, a negative correlation between replication of the parental RNA3 constructs and the accumulation of recombinant progeny confirmed the role of selection.

Sequence rearrangements in viral RNA genomes (3, 6, 16, 26–28, 32, 34, 35) and the relationship between various groups of RNA viruses (15, 36) both confirm the importance of RNA-RNA recombination in the evolution of viruses. RNA recombination in animal viruses has been extensively studied (4, 21, 22, 24, 25, 33, 37, 39) and was demonstrated with several plant virus groups, including alfalfa mosaic virus (17, 38), bromoviruses (2, 9, 10, 18, 30, 31), carmoviruses (11, 40), and tobamoviruses (12, 29). Many of these studies emphasize the importance of RNA structure for recombinant formation. For instance, in brome mosaic virus (BMV), a tripartite positive-stranded plant RNA virus (1), the introduction of a partially debilitating 3' deletion (designated M4) into the RNA3 segment has demonstrated in vivo exchanges among the three BMV RNA components (9, 10). Both legitimate and illegitimate RNA3 recombinants were identified (9). Since the crossovers concentrated near the regions capable of local hybridizations among BMV RNAs, a role for RNA structure in recombination was proposed. However, recent work with mouse hepatitis coronavirus has demonstrated that in the absence of selection pressure the distribution of recombination sites was almost random. Serial passages in tissue culture led to the occurrence of recombination hot spots. This drew attention to the role of natural selection in recombination (5).

To further study the relationship between natural selection, RNA sequences, and recombination, we used a series of M4 RNA3 mutants replicating at different rates (Fig. 1). Mutants were isolated by duplication of the 3' noncoding regions in such a way that the 3'-terminal region A served as a minus-strand promoter (7, 13, 14). In the basic construct (designated DW), both regions contained wild-type (wt) sequences, while M4W, WM4, and DM4 derivatives had the M4 deletion introduced in region A, region B, and in both regions, respectively, and SW had a significantly shorter region B. Previously characterized illegitimate recombinants 1 and 3 (described in reference 9) were used to obtain full-length M4W and SW RNA3 constructs, respectively. Construct DW was created by replacing the *KpnI-EcoRI* fragment of pB3TP7 (described in reference 19) with a corresponding fragment from recombinant 1 (9). To obtain

DM4, a unique *HincII* site of pGEM3-zf(–) was removed by digestion with *HincII* and *HindIII*; digestion was followed by filling in with the Klenow polymerase and religation. The resulting pGEM3(H) plasmid was used to subclone an *XbaI-EcoRI* fragment from recombinant 1 (9). Afterwards, a *HincII-EcoRI* fragment from the M4 construct (described in reference 8) was inserted. This generated a DM4(XE) plasmid which was utilized as a source of the *XbaI-EcoRI* fragment that substituted an analogous *XbaI-EcoRI* wt fragment in pB3TP7. Construct WM4 was generated by inserting the largest *KpnI-EcoRI* fragment of DM4 between these sites in pB3TP7.

To test for infectivity, a mixture of transcribed wt RNA1, wt RNA2, and one of the individual RNA3 mutants was inoculated on host plants, as described previously (19). These three BMV RNA segments were synthesized from plasmids pB1TP3, pB2TP5, and the engineered pB3TP7, respectively (a generous gift of Paul Ahlquist, University of Wisconsin, Madison) (19). As shown in Table 1, all the RNA3 constructs were infectious to both *Chenopodium hybridum* (local lesion) and barley (systemic) hosts; all replicated in barley protoplasts. To characterize the emerging recombinants, progeny RNAs were amplified by a reverse transcriptase-polymerase chain reaction (RT-PCR) procedure similar to that of Kawasaki (20). The first cDNA strand was synthesized in a 20- μ l reaction mixture containing 4 μ l of the total RNA extract (obtained from the local-lesion leaf tissue by phenol-chloroform extraction with a buffer composed of 1% sodium dodecyl sulfate, 50 mM glycine, 50 mM NaCl, and 10 mM EDTA, pH 9.0), 1 mM each deoxynucleoside triphosphate (dNTP), 1 U of RNasin per μ l, 25 pM first-strand synthesis primers (5' CAGTGAAT TCTGGTCTCTTTTAGAGATTTACAG 3' [primer 1] or 5' CAGTGAATTCGAACCCCTAAGGGTCTTCC 3' [primer 3]), 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 3 mM MgCl₂, and 200 U of mouse mammary leukemia virus RT. After incubation at 55°C for 40 min, the RT was inactivated at 95°C for 5 min, the volume was adjusted to 50 μ l, and the mixture was subjected to PCR. The reaction mixture contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl₂, 1 mM each dNTP, 25 pM (each) first-strand and second-strand (5' CTGAAGCAGTGCCTGCTAAGGCGGTC 3' [primer 2]) primers, and 1 U of *Taq* DNA polymerase (Cetus). After 40 thermocycles (94°C for 1 min, 60°C for 1 min, and 72°C for 2

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FIG. 1. Structures of the 3' noncoding regions in parental RNA3 mutants. The terminal and internal sequences are shown as region A and region B, respectively, and the boundary between the two regions is marked by a small black box. The end of the coat protein cistron is shown as a solid box on the left. The wt RNA3 sequences are shown as open boxes, while those originating from either RNA1 or RNA2 are shown as crosshatched boxes. The position of the M4 deletion is indicated by a small vertical rectangular open box. The locations of junction sites between individual BMV RNA sequences are marked by arrows and identified by numbers corresponding to the last (top number) and the first (bottom number) nucleotides, counted from the 3' end (1). The locations of restriction sites are marked as follows: E, *EcoRI*; H, *HincII*; K, *KpnI*; X, *XbaI*.

min), the resulting cDNA was analyzed in 1% agarose gels. The PCR-amplified DNA was digested with *XbaI* and *EcoRI* restriction enzymes, ligated between these sites into the pGEM3-zf(-) vector (Promega Corp.), and sequenced in both directions.

To test for possible PCR-generated recombination artifacts, a mixture of transcribed wt RNA1 and RNA2 plus one of the RNA3 constructs shown in Fig. 1 was amplified with primer 1 (which hybridized to the very 3' end of all BMV RNAs) and the RNA3-specific primer 2. We observed that only the expected region of RNA3 was amplified (data not shown). In contrast, when primer 2 was used together with primer 3 (representing the deleted M4 region), no DNA product was detected from a mixture of transcribed wt RNA1 and RNA2 plus either DM4 or M4 RNA3. Also, comparative analyses revealed that the recombinant RNA3 molecules detected by Northern (RNA) blot corresponded in size to the RT-PCR cDNA products (not shown). All these

data indicated that there was only a minimal possibility of generating recombinant molecules in RT-PCR reactions.

Figure 2 shows the types of recombinants characterized during this work. We observed that, in general, legitimate recombinants emerged more frequently than the illegitimate ones. Of the 57 identified legitimate recombinants, 15 had the crossovers located in an upstream part of region B, 4 had them within central region B, and 38 had them near the region B 3' end (Fig. 2A). One recombinant (M4W-LC) had two legitimate junctions, and another recombinant (M4-IA) had one legitimate and one illegitimate junction. All crossovers occurred within the highly homologous 3'-terminal 193 nucleotides of RNA1 or 219 nucleotides of RNA2 donor segments. Among illegitimate recombinants, all but M4-IA donor sequences were derived from upstream nonhomologous regions of RNA1 or RNA2 (Fig. 2B). In the previously characterized M4-derived illegitimate recombinants, the most-frequent acceptor sites were at positions 7 to 12 and 171 to 175 (Fig. 2B) (9, 10). In the case of DM4, many of the acceptor sites were concentrated at internal positions between nucleotides 143 and 147, and there was a clear donor RNA1 crossover hot spot at position 236 or 237. One recombinant was isolated several times from local lesions in separate plants, further demonstrating the crossover preferences.

Among the three BMV host systems used in this study, systematically infected barley plants supported the highest number of viral replication cycles, while barley protoplasts supported the lowest. Consequently, the probability of the better-replicating recombinants competing more efficiently than the parental RNAs was the lowest for barley protoplasts and the highest for barley plants. Indeed, as illustrated in Table 1, it was more difficult to detect recombinants in barley protoplasts than in barley plants. Only two constructs, DM4 and WM4, generated recombinants in primary infections in barley or *C. hybridum*. Since DM4 and WM4 replicated less readily in barley protoplasts than the other constructs (Table 1), the observed recombination rate suggested the importance of natural selection in recombination. To examine how natural selection influenced the accumulation of recombinants, we compared the replication rates and the competition efficiencies of parental and recombinant RNAs in protoplast infections. Four representative parental RNA3 molecules (DM4, M4, M4W, and SW) and six representative recombinants, four illegitimate (DM4-IC, DM4-IF, M4W, and SW) and two legitimate (DM4-LA and DM4-LB), were selected for these studies. Barley protoplast cells (1.5×10^5) were inoculated with mixtures containing 1.0 μg each of the wt BMV RNA1, wt RNA2, and one or two RNA3 variants. The following RNA3 quantities were used in double inoculations: 1.0 μg each for 1:1 ratios, 1.0 and 0.5 μg for 2:1 ratios, and 1.0 and 0.1 μg for 10:1 ratios. After incubation for 24 h at 24°C, total RNA was extracted, separated in 1% agarose gels, transferred to a nylon membrane (Hybond; Amersham), and hybridized to a ^{32}P -radiolabeled BMV RNA strand-specific probe under conditions described by Kroner et al. (23). When the replication of the individual RNA3 variants was examined (Fig. 3A), it became apparent that all tested illegitimate and legitimate recombinants, regardless of having the 3' end derived from RNA1 or RNA2, accumulated to almost wt levels (lanes 3 to 8). In contrast, those with terminal M4 deletions (M4 and DM4; Fig. 3A, lanes 1 and 2) accumulated to significantly reduced levels (Table 1). Inoculations with mixtures of two RNA3 variants revealed that the recombinants readily competed more efficiently than both DM4 and M4 RNAs, even at a 10:1

TABLE 1. Characterization of parental BMV RNA3 mutants by infectivity, replication, and recombination

Mutant	% Infectivity ^a in:		% Replication ^b in:				% Recombination ^c in:		
	<i>C. hybridum</i>	Barley	Barley protoplasts		Barley plants		<i>C. hybridum</i>	Barley plants	Barley protoplasts
			RNA3	RNA4	RNA3	RNA4			
None (wt)	100	100	100	100	100	100	0 (1)	0 (5)	ND ^d
M4	75	71	50	70	45	65	0 (1)	20 (2)	ND
M4W	48	42	85	65	55	5	0 (1)	70 (3)	0 (3)
DM4	30	29	30	15	0	0	85 (1)	100 (1)	50 (3)
WM4	39	29	30	15	0	0	100 (1)	100 (1)	50 (3)
DW	44	42	90	60	50	5	0 (1)	80 (3)	0 (3)
SW	55	57	95	80	80	30	0 (1)	0 (5)	0 (3)

^a Infectivity is expressed as the percentage of local lesions formed in *C. hybridum* or the percentage of systematically infected barley plants compared with the infectivity of the wt BMV.

^b Replication is expressed as the mutant-to-wt ratio of RNA accumulated in either barley protoplasts or whole barley plants. Protoplast values are average values derived from densitometry analysis of Northern blots from two independent inoculations. Barley values are derived from densitometry of photographs of ethidium bromide-stained agarose gels of virion RNA preparations (not shown). Samples were harvested 14 days postinoculation.

^c Recombination is expressed as a fraction (percentage) of recombinant-containing lesions on *C. hybridum* or as a percentage of systemically infected barley plants which contained recombinant progeny RNA3 molecules. Progeny RNAs were amplified by RT-PCR and were cloned and sequenced as described in the text. For the protoplasts, recombination was estimated by Northern blot analysis, as described in the text. The observed differences in size between the parental and recombinant molecules were utilized to detect recombinant molecules on Northern blots. Numbers in parentheses indicate the number of passages required to detect the observed recombination level.

^d ND, not determined.

DM4-recombinant ratio (Fig. 3B, lanes 15 to 21 and 24 to 29). M4 competed more efficiently than DM4 at a 1:1 ratio (Fig. 3B, lane 23) but did not at a 2:1 ratio (lane 22). This indicated that M4 accumulated slightly better than DM4. On the other

hand, both M4W and SW competed efficiently with the tested recombinants (Fig. 3B, lanes 4 to 7 and 12 to 14, respectively). SW competed more efficiently than M4 (lane 9). In summary, the results of the competition assays de-

FIG. 2. Structures of 3' noncoding regions of legitimate (A) and illegitimate (B) recombinants. The names of the recombinants reflect their origination from parental RNA3 mutants (e.g., M4 and M4W, etc.) and indicate their legitimate (L) or illegitimate (I) character. The end of the coat protein cistron is marked as a solid box on the left. The regions where homologous recombination events occurred are enclosed in dotted boxes. Individual sequences and other elements are depicted as in Fig. 1. RNAX indicates that, because of sequence identity, the 3'-terminal region can be derived by crossings between any of the BMV RNAs. The locations of illegitimate and legitimate crossover sites are marked by arrows and numbered from the 3' end. The numbers of independent lesions or plants found to contain given recombinants are shown; ♦♦ indicates that the marked recombinants can originate either from intramolecular rearrangements or from intermolecular recombination events.

FIG. 3. Results from tests of efficiency of competition between parental RNA3 constructs and progeny recombinants. Barley protoplasts were inoculated with mixtures containing 1 μ g of wt RNA1, 1 μ g of wt RNA2, and either a single (A) or two different (B) RNA3 mutant transcripts, as indicated above each lane, and were incubated at 24°C for 24 h. The amounts of RNA3 mutant inocula (in micrograms) are shown. Total RNA from approximately 3×10^4 protoplast cells was loaded onto the gel, except for one-third of that amount loaded for wt virus (lane 8 in panel A). Accumulation of BMV RNAs was determined by Northern hybridization analysis, as described in the text. The structures of individual RNA3 variants are shown schematically in Fig. 1 and 2. The migration of BMV RNA segments is indicated on the right.

scribed above confirmed that newly emerging recombinants could be efficiently selected over M4 and DM4 parental RNAs. In contrast, one can expect much more time to be necessary to accumulate detectable recombinant levels in M4W- and SW-infected plants.

All our results cannot, however, be explained by the selection for better-replicating (accumulating) recombinants. For instance, M4 and DM4 mutants had almost identical acceptor regions (region B) and demonstrated similar low competition efficiencies in protoplasts (Fig. 3). Since the two constructs had the potential for generating similar recombinants, the observed differences in the location of crossover sites (Fig. 2) (9) must have originated from structural effects. It is possible, for example, that the terminal noncoding sequences affected the internal sequences, exposing some previously unexposed parts of the acceptor region B for recombination. Also, in the past we have observed that crossovers accumulated at regions potentially forming local heteroduplexes (9). Analyses of a large number of recombinants available from this work confirmed that energetically stable heteroduplexes could be arranged around all crossover sites (data not shown). Since similar heteroduplexes could be drawn with M4 and DM4 at the same position, the observed preferences for donor crossovers at nucleotide 236 of RNA1 in DM4-derived recombinants could not be explained. A possible explanation is that DM4 has an RNA1 sequence (nucleotides 162 to 236) in region A. This sequence could facilitate hybridization between part of the unwound RNA1 negative strand of the DM4 replicative intermediate (DM4 RI) and the corresponding region of the donor RNA1 positive strand. We postulate that this, in conjunction with another hybridization between upstream portions of RNA1 and DM4 RI positive strands, can form a trimolecular intermediate, thus increasing the probability of crossovers between DM4 and RNA1 at position 236 (27a).

In conclusion, our studies have demonstrated that accumulation of BMV recombinants is determined by several interrelated elements, including RNA structure and natural selection. It remains to be seen whether similar factors operate in other RNA virus systems.

We thank Theo Dreher, Roy French, R. Meganathan, and Sandra Guy for valuable comments and suggestions during the writing of the manuscript; Paul Ahlquist for providing infectious cDNA clones of

wt BMV RNA components; and Mohammed Ashraf for synthesis of deoxyoligonucleotide primers.

This work was supported by Public Health Service grant AI26769-01A2 from the National Institute of Infectious Diseases and by the Plant Molecular Biology Center at Northern Illinois University.

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