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MOLECULAR STUDIES OF GENETIC RNA–RNA RECOMBINATION IN BROME MOSAIC VIRUS

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I. Introduction

It is well known that DNA-based organisms rearrange and repair their genomic DNA through recombination processes and that these rearrangements serve as a powerful source of variability and adaptation for these organisms. In RNA viruses genetic recombination is defined as any process leading to the exchange of information between viral RNAs (King, 1988). There are two types of recombination events: legitimate and illegitimate. While legitimate (homologous) recombination occurs between closely related sequences at corresponding positions, illegitimate (nonhomologous) recombination could happen at any position between unrelated RNA molecules (King, 1988). In order to differentiate between symmetrical and asymmetrical homologous

crosses, Lai (1992) defined the former as homologous recombination and the latter as aberrant homologous recombination.

In this chapter we use brome mosaic virus (BMV), a multicomponent plant RNA virus, as an example to discuss the progress in studying the mechanism of genetic recombination in positive-stranded RNA viruses. Studies described below summarize molecular approaches used to increase the frequency of recombination among BMV RNA segments and, more importantly, to target the sites of crossovers to specific BMV RNA regions. We demonstrate that the latter can be accomplished by introducing local complementarities to the recombining substrates.

II. Background Information

A. Recombination in Animal RNA Viruses

Sequence rearrangements which suggest the existence of recombination processes have been found in numerous animal RNA viral genomes. The phenomenon of recombination among mRNA viruses was first demonstrated experimentally in the picornaviruses (Hirst, 1962; Ledinko, 1963; Pringle, 1965) and more recently in coronaviruses (Lai *et al.*, 1985). The defined molecular mechanism of these processes is not known. By analysis of both intratypic and intertypic crosses involving the same genetic markers, Kirkegaard and Baltimore (1986) found that independent inhibition of the replication of parental strains had an opposite effect on the frequency of intratypic crosses, supporting a discontinuous copy choice-based mechanism. Romanova *et al.* (1986) and Tolskaya *et al.* (1988) determined the nucleotide sequences at crossover sites in the genome of a number of intertypic poliovirus recombinants. They found that recombination occurred within the genome segments which had the potential to form secondary structure elements (Romanova *et al.*, 1986; Tolskaya *et al.*, 1988). They proposed a model of recombination in which recombining RNA molecules form a local double-stranded structure. Banner and Lai (1991) found that within a hypovariant 1-kb region of mouse hepatitis coronavirus, the legitimate recombination was nearly random, but subsequent passages caused selection at certain locations. This indicated that, at least in coronaviruses, the recombination hot spots resulted from selection rather than from specific sequences. Weiss and Schlesinger (1991) used deleted and mutationally altered Sindbis virus RNAs to study recombination in a region spanning the junction between the nonstructural and structural protein genes. All recombi-

nants were found to be illegitimate and contained sequence insertions derived from the parental RNAs. One recombinant contained a stretch of non-Sindbis virus nucleotides (nt). The authors concluded that recombination between Sindbis virus RNAs may be analogous to that observed in bromoviruses (see Section II,B).

B. Recombination in Plant RNA Viruses

As in animal mRNA viruses, sequence rearrangements in plant virus RNA genomes and extensive relationships among various groups of plant RNA viruses both confirm the importance of RNA–RNA recombination in plant virus evolution. Based on nt sequence homologies, several superfamilies of positive-strand RNA viruses have been recently proposed (Goldbach, 1990). Natural RNA rearrangements have been identified in the genomes of alfalfa mosaic virus (Huisman *et al.*, 1989), beet necrotic yellow vein virus (Bouzoubaa *et al.*, 1991), bromoviruses (this review), hordeiviruses (Edwards *et al.*, 1992), luteoviruses (Mayo and Jolly, 1991), nepoviruses (Rott *et al.*, 1991), tobamoviruses (Shirako and Brakke, 1984), tobraviruses (Robinson *et al.*, 1987; Goulden *et al.*, 1991), and tombusviruses (Hillman *et al.*, 1987).

The recombination has been also confirmed experimentally. For example, a temperature-sensitive mutation in the RNA3 component of alfalfa mosaic virus acquired a 5′-terminal fragment from the RNA1 component during infection (Huisman *et al.*, 1989). An insertion mutant of tobacco mosaic virus easily lost one of its duplicate coat protein (CP) cistrons, probably by some form of looping-out (Dawson *et al.*, 1989). Cascone *et al.* (1990) have detected a recombination between the satellite and defective interfering (*DI*) RNAs of turnip crinkle virus. RNA rearrangements have also been postulated for virusoids (Keese and Symons, 1985; Symons *et al.*, 1985).

C. Molecular Biology of BMV

BMV has been used for years as a model to study single-stranded plant RNA viruses. Consequently, it is among the best molecularly characterized ones. The genome of BMV is divided into three RNA components, called RNA1, RNA2, and RNA3. There is a fourth RNA (RNA4), which is subgenomic and coencapsidates with RNA3 (Fig. 1). Nucleotide sequences of these RNAs are known (Ahlquist *et al.*, 1984a). One of the most-studied aspects of the BMV life cycle is the replication of its RNAs. Both *in vitro* (Ahlquist *et al.*, 1984b; Bujarski *et al.*, 1985, 1986; Dreher *et al.*, 1984) and *in vivo* (French and Ahlquist, 1987) studies discovered minus-strand synthesis promoters within the

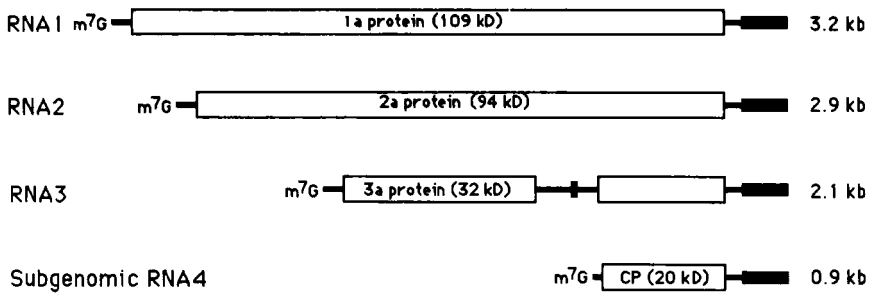


FIG. 1. Molecular organization of the brome mosaic virus genome. The open reading frames are boxed and labeled. The 3′-terminal sequences which share approximately 200 nucleotides among all BMV components are marked as solid boxes. A 20-nt oligo(A) tract is shown as a small vertical rectangle within the intercistronic region of RNA3.

3′-noncoding region, the last 134 nt of which form a tRNA-like structure (Ahlquist *et al.*, 1981; Rietveld *et al.*, 1983). This structure also interacts with several tRNA-specific enzymes (Haenni *et al.*, 1982). Other sequences responsible for the completion of the replication cycle have been identified on RNA3 within the intercistronic region and in the 5′-noncoding region (French and Ahlquist, 1987). The intercistronic region is comprised of the subgenomic RNA4 promoter (Marsh *et al.*, 1988; Miller *et al.*, 1985) as well as signals responsible for regulation of the plus-strand:minus-strand ratio (Marsh *et al.*, 1991c). The 5′-noncoding region contains internal regulatory sequence motifs (Marsh *et al.*, 1989) that are thought to interact with cellular transcriptase factors in order to initiate plus-strand RNA synthesis (Marsh *et al.*, 1991a). In addition, a replication signal has been identified on BMV RNA2 by Marsh *et al.* (1991b), who demonstrated that deletions of certain coding sequences inhibited the accumulation of this BMV RNA.

Another extensively studied aspect of BMV RNA replication is its replicase. Two viral replicase proteins, 1a and 2a, have been found to be encoded by RNA1 and RNA2, respectively, and the expression of both has been shown to be necessary for RNA replication in protoplasts (Kiberstis *et al.*, 1981). A template-dependent and-specific RNA polymerase has been isolated from BMV-infected barley leaves (Bujarski *et al.*, 1982; Miller and Hall, 1983). Recently, this crude extract has been purified significantly (Quadt and Jaspars, 1990), revealing that, in addition to 1a and 2a proteins, the active complex contained some host-encoded factors, one of them probably being an elongation factor (immunoprecipitated with anti-EIF-3 of wheat germ (Kao *et al.*, 1992; Ahlquist *et al.*, 1992). A comparative analysis of amino acid sequences

indicated that protein 1a has at least two domains: one for a helicase and one for a capping enzyme, whereas 2a represents the catalytic unit. Mutations and deletions in these proteins were used to identify the regions active in RNA replication as well as regions responsible for interaction between them (Kroner *et al.*, 1990; Traynor *et al.*, 1991). The latter has been recently confirmed by immunoprecipitation of the wild-type (wt) and truncated 1a–2a *in vitro*-translated protein complexes (Kao *et al.*, 1992).

The dicistronic BMV RNA3 component encodes the nonstructural 3a protein and the coat protein. Although the role of 3a protein is not completely documented, the studies of a genetic hybrid containing wt cowpea chlorotic mottle virus (CCMV) RNA3 (Bancroft, 1970), the exchanges of 3a ORFs between CCMV and either BMV (DeJong and Ahlquist, 1991) or sunn-hemp mosaic virus (DeJong and Ahlquist, 1992), and sequence comparison analyses (Dzianott and Bujarski, 1991; Romero *et al.*, 1992) all indicate that 3a functions as a movement protein. We have recently demonstrated that the introduction of certain amino acid substititions into the coat protein affected the systemic transport of the virus and the accumulation of the RNAs (S. Flasinski, A. M. Dzianott, S. Pratt, and J. J. Bujarski, 1993, unpublished observations). Deletion of the CP open reading frame (ORF), however, did not prevent the necrotic reactions on a local lesion host.

Relatively less information is available for two other members of the bromovirus family: broad bean mottle virus and CCMV. The nucleotide sequences of all the RNA segments as well as the infectious cDNA clones are available for these viruses (Dzianott and Bujarski, 1991; Romero *et al.*, 1992; Allison *et al.*, 1989). Computer analysis has revealed significant sequence homologies among all three corresponding bromovirus RNA components. Further studies have confirmed that pseudorecombinants between BMV and CCMV RNAs are infectious (Allison *et al.*, 1988). Also, it has been shown that some chimeric BMV/CCMV 2a proteins can function in viral RNA replication (Traynor and Ahlquist, 1990). As for BMV, a crude extract from CCMV-infected cowpea leaves had a bromovirus RNA-specific RNA-dependent RNA polymerase activity (Miller and Hall, 1984).

D. Previous Studies on Recombination in Bromoviruses

One of the most useful experimental systems in which to study recombination in plant viruses is bromoviruses. Since the RNA3 segment of bromoviruses does not provide trans-acting RNA replication factors, most of the recombination experiments have utilized the RNA3 mutants. Bujarski and Kaesberg (1986) have shown that a par-

tially debilitating BMV RNA3 mutant (designated M4) carrying a deletion in the 3′ RNA replication promoter was repaired *in vivo* by exchanges with the sequences of other BMV RNA components. A deletion in the 3′-noncoding region of the RNA2 segment has been repaired with a similar mechanism (Rao and Hall, 1990). The derivatives of the M4 mutant were used to generate and characterize a number of illegitimate BMV RNA3 recombinants (Bujarski and Dzianott, 1991; Nagy and Bujarski, 1992). These results revealed that the crossovers occurred near the regions potentially capable of forming double-stranded heteroduplexes. In order to investigate whether replication is required for recombination, Rao *et al.* (1990) and Ishikawa *et al.* (1991) have used nonamplifiable 3′ mutants of BMV RNA2 and RNA3, respectively. Both groups demonstrated that the replication activity of the accepting RNA molecule was not necessary for recombination.

Sequence data indicated that recombinational rearrangement had occurred near the 5′ end of a CCMV RNA3 segment (Allison *et al.*, 1989). Recombination among CCMV RNAs was later proved by Allison *et al.* (1990). The authors have shown that a pair of deletion CCMV RNA3 mutants coinfected with wt RNA1 and RNA2 was able to restore the wt RNA3. The crossovers occurred within the intercistronic overlapping region.

III. Recombination in the 3′-Noncoding Region of RNA3

A. Recombination Systems Used in These Studies

Extensive sequence homologies among the 3′-noncoding regions in three genomic BMV RNAs provide a convenient opportunity to study intersegment genetic recombination. The recombinational repair of the mutagenized 3′-terminal sequences was observed in both systemic (barley) and local lesion (*Chenopodium hybridum* or *C. quinoa*) hosts (Bujarski and Kaesberg, 1986; Rao *et al.*, 1990). Local lesion hosts provide a large number of independent infections and a reduced selection pressure. The majority of the work described here has been done with these local lesion hosts.

In vitro-transcribed BMV RNAs (Janda *et al.*, 1987) were inoculated mechanically. Progeny RNAs were isolated and characterized by Northern blots, amplified by a reverse transcription polymerase chain reaction (RT-PCR) protocol, and sequenced as described previously (Nagy and Bujarski, 1992).

M4 RNA3 (see Section II,D) generated some recombinants during prolonged infections. In order to increase the efficiency of recombinant

selection, we constructed a series of M4 derivatives that had their 3′-noncoding region duplicated (Nagy and Bujarski, 1992). We observed that one of the constructs (designed DM4) that contained the M4 deletion in both the internal (designated region B) and the external (designated region A) parts of the duplicated 3′-noncoding region recombined more readily in *C. hybridum* and in barley than M4 did (Fig. 2). DM4 was especially useful for further studies because (1) it generated both legitimate and illegitimate recombinants and (2) the region that participated in recombination (Fig. 2) was separated from the 3′-terminal region A that contained the cis-acting elements needed for replication.

In studying the effect of different sequences on recombination, a recombination vector (designated DM4CC6) was designed (Fig. 2). The idea was to develop an infectious RNA3 molecule stable in infection with a possibility of inserting sequences of interest and studying their recombinational activity. The duplicated 3′-noncoding region of DM4 was further extended with an insertion of a heterologous 3′-noncoding CCMV sequence. In contrast to DM4, DM4CC6 did not accumulate detectable levels of recombinants in *C. quinoa*. However, as shown in the following section, further insertion of recombinationally active sequences restored the recombinational ability of DM4CC6.

In addition to the above systems, nonreplicating RNA3 constructs were used to determine which strands were participating in recombination. Here, the minus-strand synthesis was debilitated by removing the functional promoter. Recombination can only use the plus strands to restore the active minus-strand promoter of the mutant RNA. If template switching were the mechanism, the above data would mean that recombination occurred during minus-strand synthesis of the donor RNA.

B. The Effect of Selection on Crossover Sites

The M4 and especially DM4 recombination systems generated a wide spectrum of legitimate and illegitimate recombinants (see Fig. 3). The distribution of crossover sites was not random. This suggested that the structure of the recombination substrates was important in directing the crossover events. Alternatively, natural selection processes could select for the best-adapted molecules from a random pool of recombinants. Replication competition between parental and recombinant RNA molecules was examined by inoculating barley protoplasts with *in vitro*-transcribed RNAs. The results demonstrated a direct correlation between the accumulation of recombinant molecules in single infections and their ability to compete with parental M4 and especially

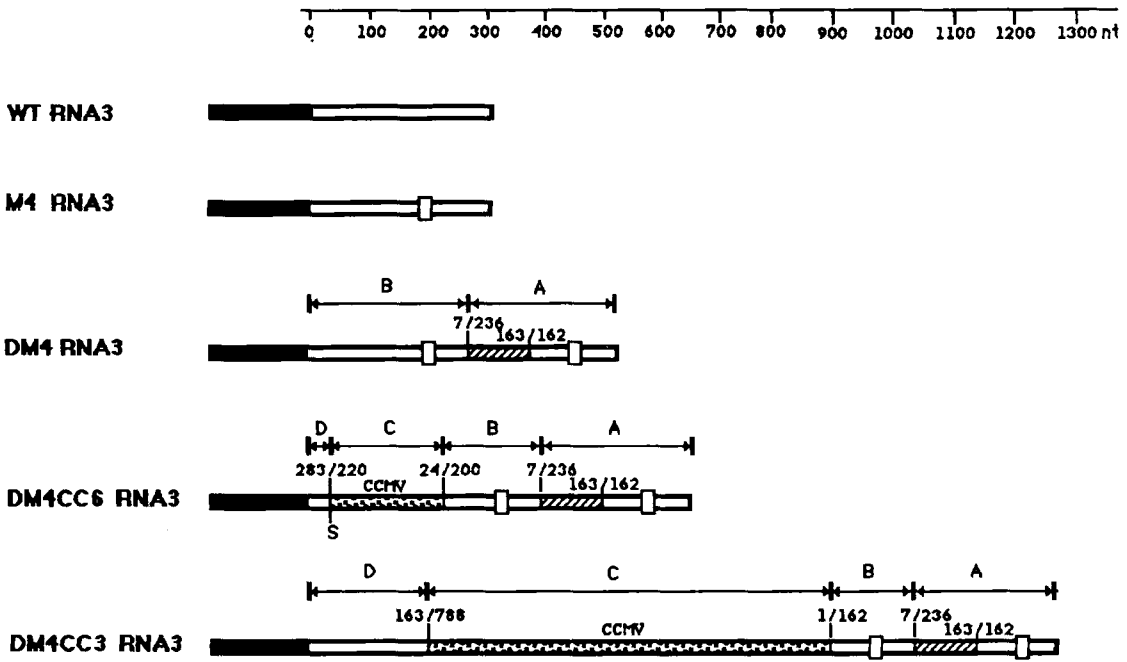


FIG. 2. BMV RNA3 constructs carrying mutations in the 3′-noncoding region. The C-terminal part of the coat protein cistron is shown by the solid box on the left. The original 3′-noncoding sequences of RNA3 are shown by open boxes. The position of the M4 deletion is depicted by a small rectangular open box. The RNA1 inserts are shown as cross-hatched boxes, whereas dotted boxes represent CCMV RNA3 inserts. The extended 3′-noncoding sequences are divided into regions designated by A–D. Essentially, regions A and B represent almost identical sequences. The exact positions of ligated inserts are indicated by numbers that represent the coordinates of published BMV and CCMV sequences (Ahlquist *et al.*, 1984a; Allison *et al.*, 1989).

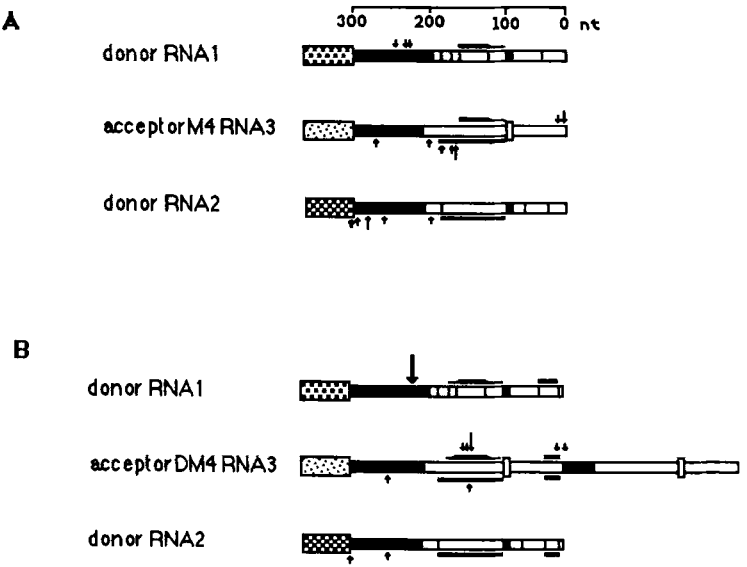


FIG. 3. Distribution of legitimate and illegitimate crossover sites on donor and acceptor BMV RNAs. (A) Crossovers generated by M4 infections. (B) Crossovers generated by DM4 infections. Downstream parts of the coding regions are shown by large dotted boxes on the left. The 3′-noncoding sequences which are not homologous among three BMV RNAs are shown as solid boxes, whereas the homologous regions are indicated as open boxes. Vertical lines inside the open boxes depict the location of marker nucleotide substitutions when compared to the acceptor RNA3 sequences. The small solid box depicts the location of the M4 marker deletion in the RNA3 segment. Regions involved in legitimate recombination are represented by horizontal bars, whereas the illegitimate crossovers are depicted by vertical arrows. The bars and the arrows above the boxes represent RNA1–RNA3 crossovers, while those below the boxes represent RNA2–RNA3 crossovers. The width of the bars and the length of the arrows are proportional to the frequency of the crossovers at that particular position.

DM4 RNAs in mixed infections. In fact, all the M4- and DM4-derived legitimate and illegitimate recombinant RNA3s examined, regardless of whether they had sequences derived from either RNA1 or RNA2, were very competitive with the parental mutants (Nagy and Bujarski, 1992). This indicated that progeny recombinants easily accumulated, because they easily outcompeted their parental RNAs.

C. Models of Illegitimate Recombination

Figure 3 shows that all legitimate crossover events occurred within the long (197- to 220-nt) 3′ region of M4 or DM4 (homologous among three BMV RNA components) and the corresponding part of either wt

RNA1 or wt RNA2. The illegitimate crosses were distributed differently. The majority of donor sites were located in the heterologous part of the 3′-noncoding region of wt RNA1 or wt RNA2. The acceptor sites were dispersed along the entire length of the 3′-noncoding region of M4 and region B of DM4. These characteristic differences between the legitimate and illegitimate events might reflect different recombination mechanisms. One advantage of studying illegitimate recombinants was the possibility of determining the exact position of crossovers. This helped us to investigate the mechanism of illegitimate recombination.

1. Heteroduplex Model

In order to investigate the structural requirements of recombination, the 3′-end nt sequences of 20 M4- or DM4-generated illegitimate recombinants were determined. This did not reveal any conserved sequence at the crossover sites. Sequence motifs found previously in several recombinants of turnip crinkle virus were not observed in BMV recombinants (Cascone *et al.*, 1990; Zhang *et al.*, 1991). However, sequence complementarities between the recombining RNAs were identified around the crossover sites (Fig. 4). As in poliovirus (Romanova *et al.*, 1986; Tolskaya *et al.*, 1988), this finding suggested that formation of a local heteroduplex between the recombination substrates might hold the RNA molecules together and thus promote recombination events. Examination of the free energy released during formation of such putative heteroduplexes showed that they are energetically permissible (Bujarski and Dzianott, 1991; Nagy and Bujarski, 1992).

We used an RNA3-based recombination vector construct, DM4CC6 (Fig. 1), to provide evidence on the heteroduplex-mediated recombination in BMV. A 66-not-long 3′-noncoding region of BMV RNA1 was inserted into the *Spe*I site of DM4CC6 in either sense or antisense orientations. This region of RNA1 was found to be recombinationally inactive with M4 and DM4 infections (Fig. 3). The heteroduplex model predicted that the RNA1-derived antisense region of the resulting PN2(−) construct (Fig. 5) would activate the corresponding RNA1 region as a donor sequence. Likewise, the model predicted that the sense orientation of the RNA1-derived region [construct PN2(+)] would not activate recombination. Indeed, illegitimate recombinants between PN2(−) and wt RNA1 components having crossovers within or in close vicinity of the antisense region were readily generated during infection on *C. quinoa*. No recombinants were obtained with PN2(+) (P. D. Nagy and J. J. Bujarski, 1993, unpublished observations).

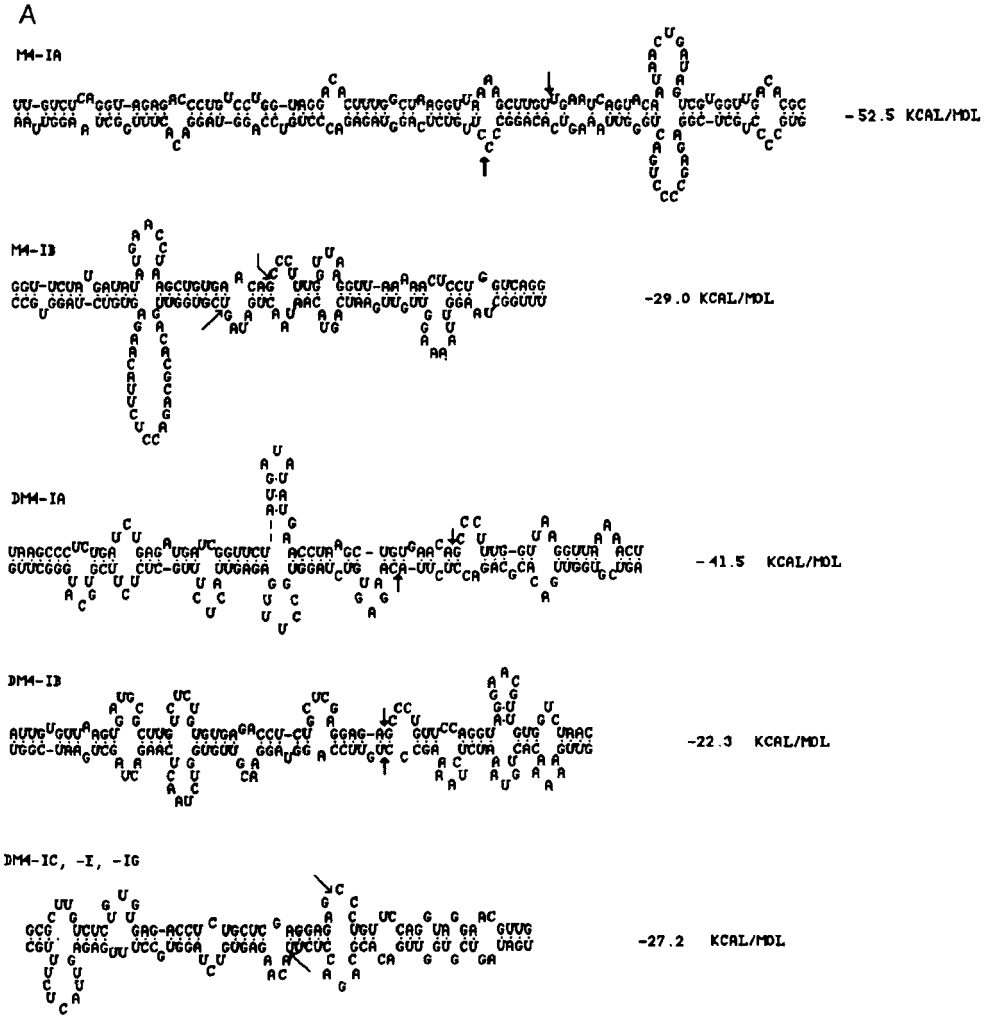


FIG. 4. Proposed complementary sequences between positive strands of BMV RNA recombination substrates, arranged as minimal-energy structures around illegitimate recombination sites (shown by arrows). Donor wt RNA1 or RNA2 sequences are shown in the upper lines, while acceptor mutant RNA3 sequences are shown in the lower lines. The RNAs are hybridized in opposite orientations. Each resulting recombinant contains top sequences up to the arrow and then continues with the bottom sequences 5′ of the bottom arrow. The base pairings are shown by dots. Free energies are indicated on the right-hand side.

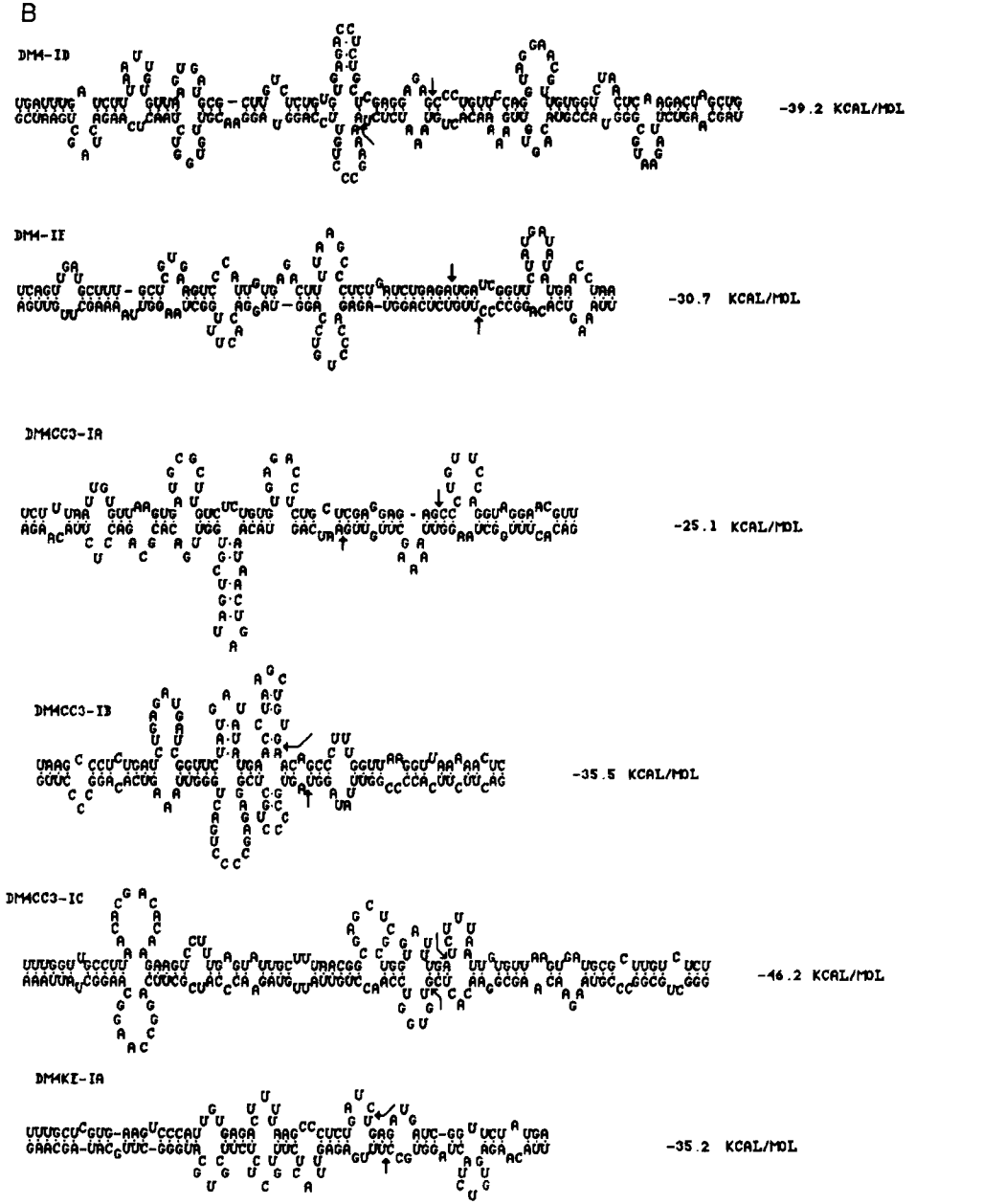
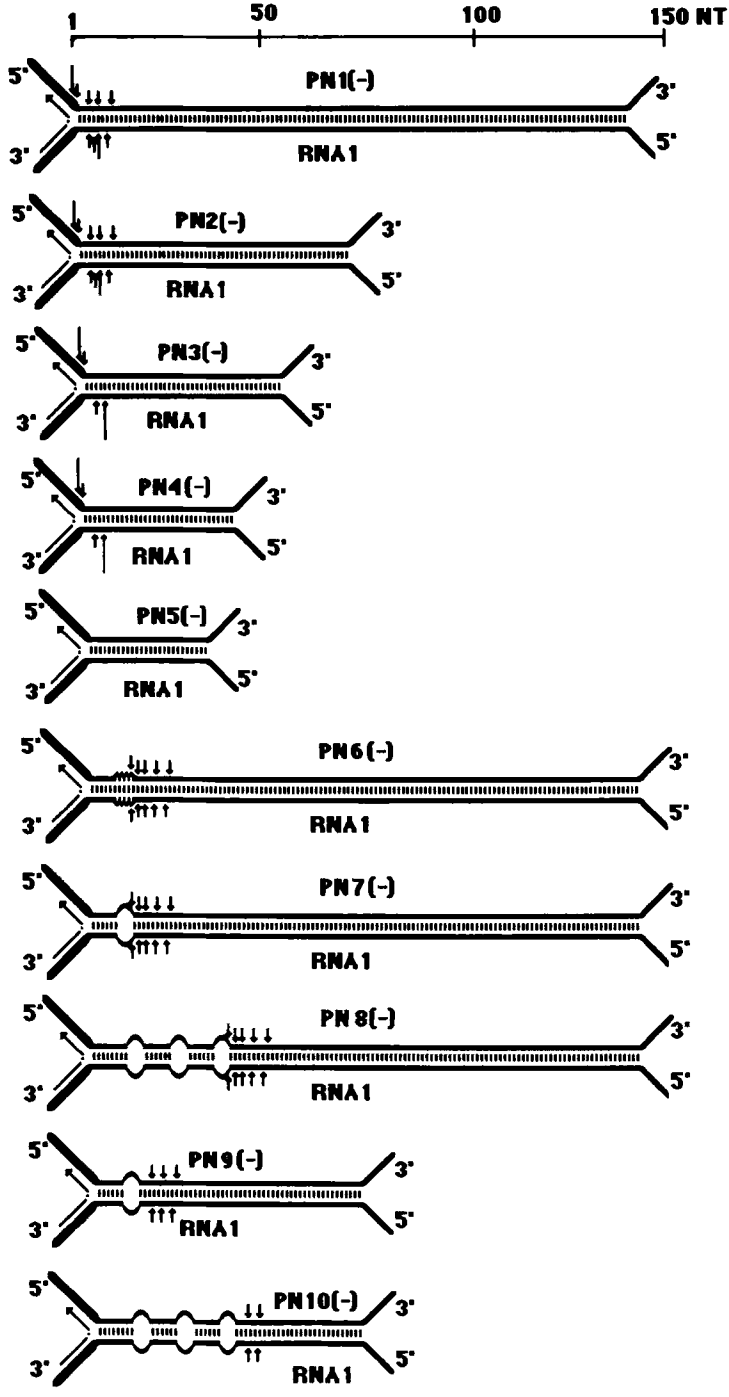


FIG. 4. (*Continued*)

A series of PN2(−) derivatives having 140-, 40-, 30-, and 20-nt-long RNA1-derived antisense regions was constructed to determine the length of the recombinationally active heteroduplex. We observed that the shortest antisense construct that generated recombinants contained a 30-nt antisense insert [NPN4(−), Fig. 5]. *In vitro* RNase protection experiments proved that the predicted heteroduplexes were easily formed between RNA1 and PN2(−) derivatives (P. D. Nagy and J. J. Bujarski, 1993, unpublished results).

2. Trimolecular Hybridization Model

The bimolecular heteroduplex model can explain the occurrence of illegitimate crossovers found for RNA3 mutants containing antisense RNA1 fragments. Since the recombinationally active region B of DM4 and the corresponding region of M4 were identical (except the deleted last 6 3′ nt), the heteroduplex model predicted that the two systems could generate similar recombinants. However, we observed the unusual distribution of crossover sites on donor RNA1 molecules obtained with DM4 infections. Specifically, we found that the majority of DM4-generated illegitimate recombinants contained the last 236 nt of RNA1. Since a short 74-nt RNA1-derived sequence (positions 162–236, counted from the 3′ end of wt RNA1) is present within region A in the DM4 construct (Fig. 2), a modified heteroduplex model can be proposed (Fig. 6). In this model three molecules interact. The first interaction is formed between a wt RNA1 plus strand and a DM4 region A minus strand. The latter could be a part of the DM4-replicative intermediate. This heteroduplex is called arm B and it is energetically strong, extending theoretically 216 nt. The formation of a strong arm B might hold the donor RNA1 in close vicinity of DM4 plus strands (which are also thought to be part of a DM4 replicative intermediate) and help to align and to form a short heteroduplex between plus strands of RNA1 and DM4. This heteroduplex is termed arm A. The arm A heteroduplex is similar to the heteroduplexes described previously for M4 and DM4 recombinants (Bujarski and Dzianott, 1991; Nagy and Bujarski, 1992) (Fig. 4), but it is energetically weaker than these. Arm C is formed between the plus and minus strands of DM4. The model predicts that crossovers occur between arms A and B by joining the donor RNA1 plus strand (at position 236) to the acceptor DM4 plus strand (the position is variable). The main difference between the bimolecular heteroduplex model and the "T-shape" model is that the latter includes a supporting minus-strand sequence. This may enforce the observed crossover preferences between the plus-strand recombination substrates. A three-component model was also proposed by Kuge *et al.*


1
50
100
150 NT
5'
3'
PN1(-)
RNA1
PN2(-)
PN3(-)
PN4(-)
PN5(-)
PN6(-)
PN7(-)
PN8(-)
PN9(-)
PN10(-)

(1986) to describe the generation of DI molecules in poliovirus. However, arm A is not included in that model.

In order to obtain experimental data supporting our tricomponent model, construct DM4 was modified to alter the putative structure of the recombination intermediates. Construct DM4CC3 (Fig. 2) contained the 3′-noncoding region of DM4 with a heterologous CCMV insertion at position 162 of DM4 region B. The CCMV sequence represented the last 788-nt part of the RNA3 segment (Allison *et al.*, 1989). Such a modification was expected to change arms A and C, but not arm B of the putative trimolecular heteroduplex formed between DM4 and RNA1. Since arm B was postulated to be the main force of recombination at position 236 of RNA1, the trimolecular model predicted that DM3CC3 should generate illegitimate recombinants that have the hotspot junction site of RNA1. When tested on *C. hybridum*, the CCMV insertion significantly decreased the generation of illegitimate crossovers observed before in DM4 infections. The isolation of the recombinant DM4CC3–IA (Fig. 6), having the invariable 236 nt from RNA1, supported the postulated role of arm B in recombination.

Further indirect supporting data were obtained from experiments using constructs DM4KE and DM4CC3KE, nonreplicative derivatives of DM4 and DM4CC3, respectively. DM4KE and DM4CC3KE were constructed by deleting 41 3′-end nt of region A of DM4 and DM4CC3, respectively. DM4KE and DM4CC3KE did not produce detectable amounts of minus strands (P. D. Nagy and J. J. Bujarski, 1992, unpublished observations). In the absence of minus strands of the acceptor molecules, arm B and thus the tricomponent intermediate could not be formed. Indeed, none of the 18 and 12 recombinants isolated from DM4KE and DM4CC3KE infections, respectively, had the donor site at

FIG. 5. Schematic representation of the heteroduplex regions formed between wt RNA1 and various RNA3 mutants. Vertical arrows indicate the location of illegitimate crossover sites. The height of the arrows is proportional to the number of recombinants generated at the particular location. Arrows between the RNA substrates on the left indicate the proposed direction of template switching (for details see text). All constructs are derivatives of DM4CC6 carrying an insert derived from RNA1 in antisense orientation. The basic construct PN1(−) contains a 140-nt sequence of RNA1 (positions 243–382, counted from the 3′ end, according to Ahlquist *et al.*, 1984a). Constructs PN2(−) through PN5(−) are right-side deletion derivatives of PN1(−) carrying 66-, 40-, 30-, and 20-nt antisense regions, respectively. PN6(−) was obtained by replacing four C residues with U residues at the left side of PN1(−). PN7(−) and PN8(−) were derived from PN1(−) by introducing one or three short heterologous sequences, respectively. PN9(−) and PN10(−) were generated as PN7(−) and PN8(−), but the basic construct was PN2(−). Watson–Crick base pairings are indicated by solid lines and small bars between the lines. G–U pairs are shown by jagged lines.

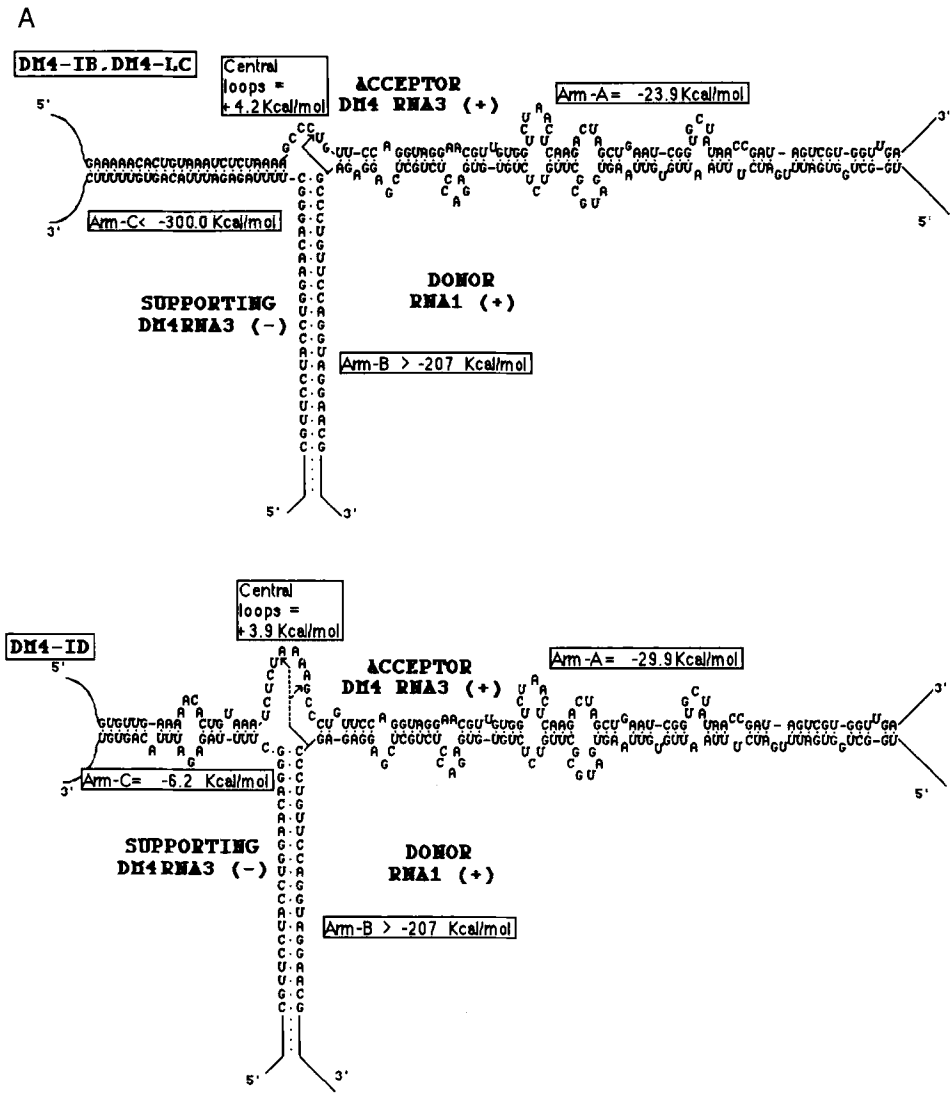


FIG. 6. Proposed trimolecular hybridization intermediates to explain preferences in the illegitimate crosses observed between DM4 RNA3 and wt BMV RNA1 molecules. Both plus and minus unwound portions of the replicative intermediate (RI) of parental DM4 and DM4CC3 RNA3 mutants, designated as acceptor (+) RNA3 and supporting (−) RNA3, respectively, hybridize to the donor BMV RNA1 plus strand. Since the two RNA3 mutants contain 74-nt portions of BMV RNA1 within their 3′-noncoding region (see Fig. 2), the hybridization is perfect within the 214-nt-long arm B (only the upper part of arm B is displayed). Arm A is generated by hybridization between upstream portions of the donor RNA1 molecule and the downstream parts of the acceptor plus strand of RNA3, and therefore is not perfect. Arm C arises by rehybridization of unwound parts of both

B

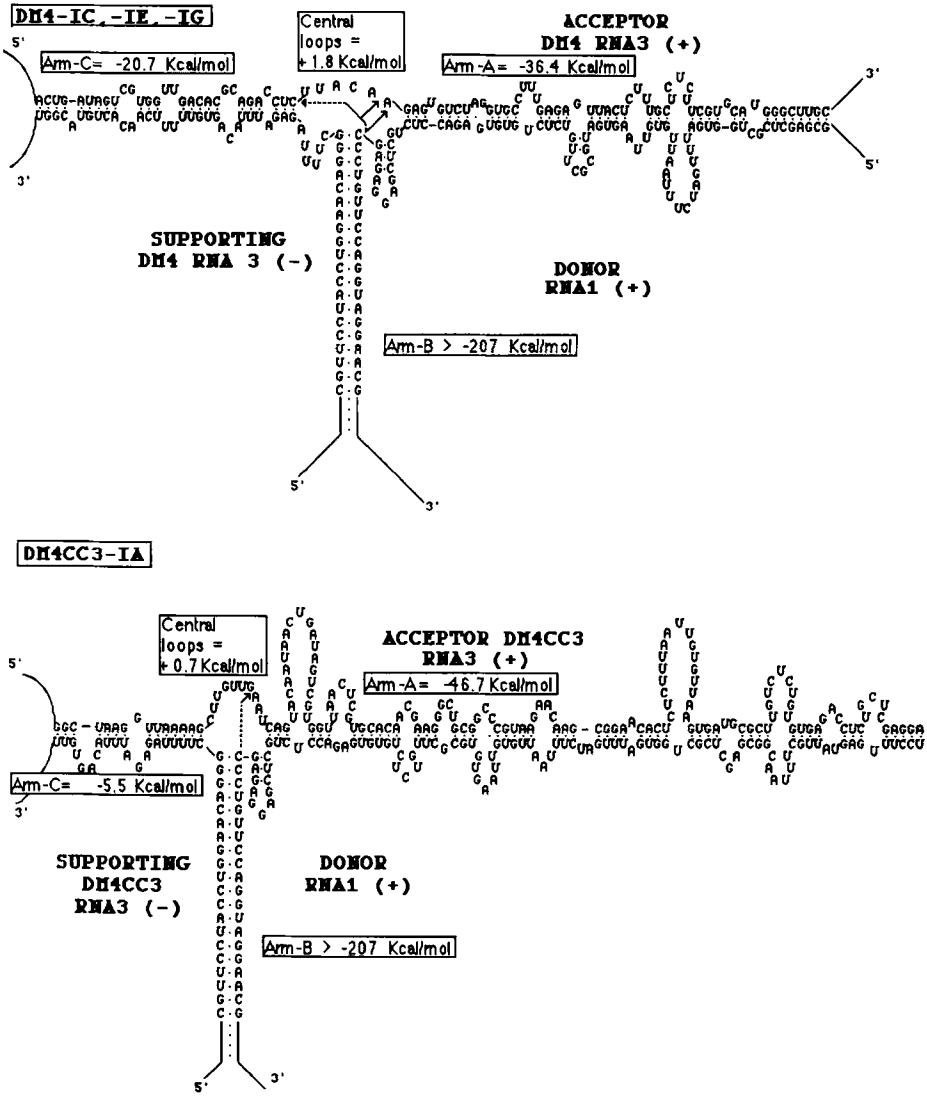


FIG. 6. *(Continued)*

strands from the acceptor mutant RNA3 RIs. The positioning of the arm C rehybridization is enforced by arm A but especially by arm B and, therefore, is not always perfect. The crossover sites are indicated by arrows.

position 236 of RNA1 (P. D. Nagy and J. J. Bujarski, 1992, unpublished observations).

In conclusion, the formation of the majority of illegitimate crossovers could be explained by local hybridization between two recombining RNA molecules. We postulate, however, that more complicated trimolecular interactions among certain RNA substrates might further affect the crossover events.

D. Mechanism of Illegitimate Recombination

Differentiation between the two favored recombination mechanisms, template switching (TS) and breakage and religation (B+R), is necessary to understand the molecular basis of recombination. To address this question, we took advantage of the fact that the location of illegitimate crossovers generated by PN2(−) and its derivatives could be determined. Moreover, we used the facts that in the above system the ability of heteroduplex formation between the templates was required for recombination and that only RNA3-derived recombinants were detected. The crossovers should occur at the left side of the heteroduplex if recombination occurs according to the TS model (Fig. 5). The right-side crossovers are less likely, because the unwinding activity of the replicase complex would destabilize the heteroduplex and finally would release the acceptor molecule. On the contrary, the B+R model predicts that the cleavage of both strands should occur within or at the right side of the heteroduplex in order to keep the RNA substrates together until ligation covalently joined them. Left-side digestions would release the long 5′ region of RNA3 and the 3′ RNA1 fragments because of the absence of a double-stranded anchor. The participation of positive or negative strands in recombination does not change the above predictions. The observation that the crossovers found in recombinants generated by PN1(−) to PN4(−) were located invariably at or close to the left side of the heteroduplex favors the TS mechanism. The shift in the location of crossovers in PN6(−) to PN10(−) infections is even more supportive (Fig. 5). This is because the replicase complex is expected to penetrate easier through the weakened or disrupted left side of heteroduplexes formed between PN6(−) to PN10(−) and RNA-1. Moreover, the presence of one to three nontemplate (mainly uridine residues) nucleotides at some crossover sites indicated the involvement of viral replicase through the enzyme stuttering during the resumption of RNA synthesis. A similar phenomenon has been described for turnip crinkle virus (Cascone *et al.*, 1990).

The above data favor the TS mechanism, in which the crossovers

occur during replication of viral RNA. Modifications of BMV 1a and/or 2a proteins may, therefore, influence recombination events. Our preliminary data indicate that a mutation in the helicase-like domain of BMV 1a protein significantly changed the location of crossover sites compared to the wt virus (P. D. Nagy, A. M. Dzianott, P. Ahlquist, and J. J. Bujarski, 1993, unpublished observations).

IV. Recombination in Other Regions of RNA3

A. Recombination Systems Used in These Studies

The results obtained for 3′-noncoding regions revealed the importance of local heteroduplexes for the promotion of crossover events. Since BMV replicase could operate differently at the 3′-noncoding initiating sites and at more upstream locations, we wanted to find out if a heteroduplex mechanism could induce recombination in other RNA3 regions. Sequence analysis revealed the possibility of local hybridizations between two molecules at numerous locations along the entire length of the RNA3 component (not shown). In order to map regions active in recombination, frame-shift mutations were introduced at several sites in the RNA3 molecule (Section IV,B). The involvement in recombination of the intercistronic region was further tested by using RNA3 mutants containing deletions in 3a or CP genes (Section IV,C). The recombination activity between pairs of these mutants was determined by coinoculation rearrangements was tested by inserting long palindromic sequences into RNA3 molecules (Section IV,D).

B. Complementation and Rearrangements among Frame-Shift Mutants

Frame-shift mutations were introduced at several locations of 3a and CP ORFs (Fig. 7A). Each of these mutants or their combinations (plus wt RNA1 and RNA2) were inoculated on *C. hybridum*, and the progeny RNA3 was tested for the presence of recombinants by sequencing of RT-PCR-amplified cDNA products (Nagy and Bujarski, 1992).

1. Complementation

Several coinoculation experiments demonstrated the possibility of complementation between CP and 3a ORF frame-shift mutants. The progeny RNA obtained from mutants A or B together with mutants C, D, E, F, or G revealed the accumulation of both parental RNA3 variants. In most cases it was possible to reinoculate progeny RNA from

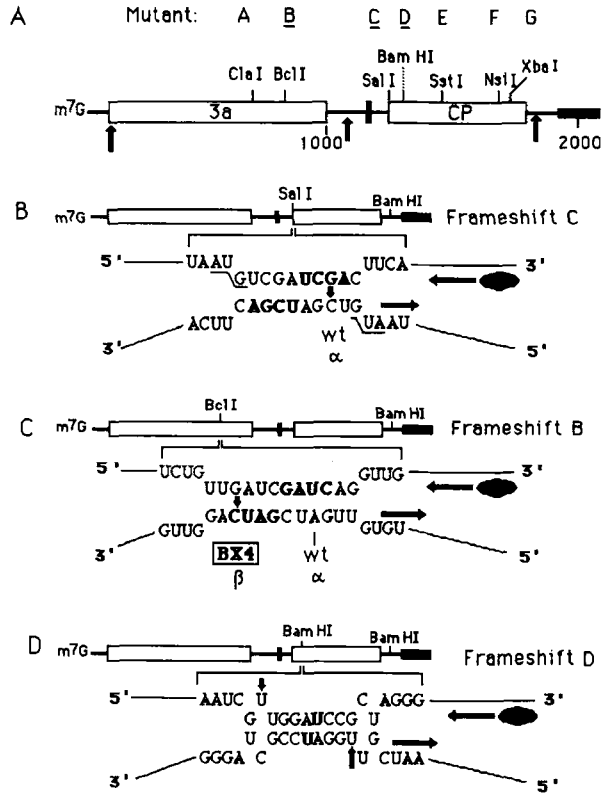


FIG. 7. (A) Frame-shift mutations in the coding regions of BMV RNA3. Mutants A, B, C, E, F, and G were obtained by the repair of cleaved restriction sites, whereas mutant D was obtained by site-directed mutagenesis. Each frame-shift mutant contained one of the three *Bam*HI restriction site marker mutations (indicated by arrows) which were introduced by site-directed mutagenesis. The mutants with which the recombinant RNA3 progeny was obtained are underlined. (B–D) The proposed heteroduplexes between two molecules of mutant C, mutant B, and mutant D, respectively. Frame-shift insertions are shown in boldface. Arrows indicate the direction of minus-strand RNA synthesis during template switching by the replicase complex (shown as an ellipse).

one plant to another several times without recombinant formation. Interestingly, the RNA3 mutants with a defective CP gene accumulated to higher levels than their counterparts with 3a ORF mutations. This probably reflected the differences in their replication and stability.

Complementation between movement protein and CP was also reported for other tripartite viruses. For example, alfalfa mosaic virus P3 (movement) and CP deletion mutants complemented viral infection

in replicase-expressing transgenic tobacco plants (van der Kuhl *et al.*, 1991). Also, in barley stripe mosaic hordeivirus, complementation was detected between two RNA γ-mutants (Petty *et al.*, 1990).

2. Conversion to wt Sequences

The majority of frame-shift mutants was not infectious to *C. hybridum* plants when inoculated alone. An exception was mutant C, which contained a 4-nt frame-shift insertion immediately after the first initiation codon of the CP ORF. The truncated but functional CP was synthesized starting from a second downstream AUG codon (Sacher and Ahlquist, 1989). Nearly 10% of the local lesions contained the repaired wt RNA3 molecules or contained a mixture of these and the parental C mutant. The presence of the marker mutation at the 3′ end of the recovered wt RNA3 excluded the possibility of contamination. The presence of the repaired wt RNAs could be explained by the fact that two identical molecules of mutant C form a 10-nt heteroduplex, shown in Fig. 7B. Local complementarity could have brought the two molecules together, which may have allowed the replicase switches from one template to another. If this switch occurred at the position marked "α" (between G and C), the 4-nt frame-shift insert would have been removed and the wt RNA3 restored. While switches at other positions would also have been possible, selection pressure may have led to accumulation of the wt recombinant molecules. However, one cannot exclude the possibility of intramolecular looping-out of the frame-shift sequences.

When mutant C was coinoculated with another RNA3 frame-shift variant (e.g., A, F, or G), more local lesions occurred. Again, most local lesions contained both parental RNA3 molecules, but a small fraction accumulated wt RNA3 components. Yet in another fraction of local lesions, mutant C derivatives were identified in which the frame-shift 4-nt insert was maintained but the first CP ORF AUG codon had been changed to ACG, UUG, or GUG. These mutants induced symptoms similar to wt on barley, a systemic BMV host, and readily reverted to wt RNA3 sequence. This reversion included the removal of the 4-nt C frame-shift mutation, which could have occurred through recombination events described in Fig. 7B. In addition, the mutated initiation codons reverted to the wt AUG, probably as a result of errors during BMV RNA replication. An alternative mechanism might involve the intramolecular looping-out of the 4-nt frame-shift insert.

3. Sequence Repetitions and Additions

Experiments that involved coinoculation of *C. hybridum* with mutant B, which contained a 4-nt frame-shift insert in the 3a ORF and

one of the CP ORF frame-shift mutants (C, E, F, or G) led to the selection of an RNA3 variant, designated B×4 (Fig. 7C). In contrast to mutant C, in which the 4-nt frame-shift mutation was deleted, in B×4 the initial GAUC frame-shift insertion was replaced by a triple repeat of this motif. The complementation with the CP mutants was required to initiate infection. Two to three passages through *C. hybridum* generated the B×4. The sequence of the inserted region and the presence of a marker mutation at the 3′ end indicated that B×4 might have been formed through rearrangements between two mutant B RNA3 molecules. In this case the switch could have occurred at β instead of at position α (Fig. 7C). The latter would restore the wt RNA3 molecule.

It is unclear why B×4 instead of wt RNA3 accumulated during infection. One explanation is that B×4 generated 3a protein, which had 4 additional amino acids near its C terminus. As compared to the wt virus, B×4 did not cause local lesions on the inoculated leaves, but spread systematically in *C. hybridum*. This is, perhaps, why B×4 outcompeted the wt RNA3.

Another illegitimate RNA3 variant, designated D1, was identified after coinoculation with CP frame-shift mutants D and G. Mutant D1 had two additional uridine residues 3 nt upstream of the frame-shift insert. This generated a CP molecule containing three amino acids (Phe, Gly, and Ser) which replaced the wt Trp23 and Thr24 entities. Generation of the D1 mutant can be also explained by an asymmetrical template switch within a short heteroduplex formed between two RNA3 molecules (Fig. 7D). However, a nontemplate addition of two uridine residues during replication of a single RNA3 molecule cannot be excluded.

C. Recombination among Deletion Mutants

Six deletion RNA3 mutants were constructed and tested for recombinational activity. Three had deletions in the 3a ORF and the other three had deletions in the CP ORF (Fig. 8). Only two of them, BB4 and SX1 were infectious when inoculated on *C. hybridum*. The amount of local lesions was about 0.1% of the number induced by similar amounts of wt RNA3. Apparently, the CP ORF and the CP itself were not necessary for the formation of necrotic lesions.

When pairs of the above deletion mutants were coinoculated, an increase in the number of local lesions was observed (Table I). The parental mutant with a deletion in the CP ORF constituted the major RNA3 fraction. Northern blots demonstrated that parental mutants BB4 or SX1 readily outcompeted the 3a ORF mutants and were stable after several passages through *C. hybridum* (not shown). This is proba-

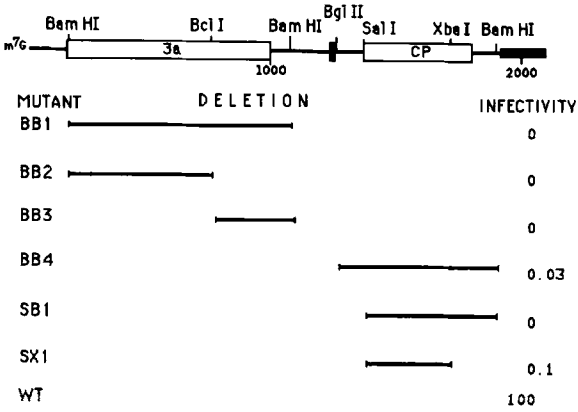


FIG. 8. BMV RNA3 deletion mutants used in this work. Individual regions were deleted by digestion at the indicated restriction sites. The infectivity of individual mutants is expressed as a percentage of local lesions generated by an equivalent amount of wt RNA3.

bly because the 3a ORF counterparts were no longer available for recombination. In addition to parental RNA3 mutants, wt RNA3 recombinants were found in the lesions induced with several combinations of deletion variants. The recombination frequency did not correlate with the length of the overlapping sequences. The recombinational repair of BMV RNA3 deletion mutants confirms the earlier

TABLE I
RECOMBINATION ACTIVITIES AMONG PAIRS OF RNA3 DELETION MUTANTS

Mutant pair Δ3a × ΔCP	Overlap (nt)	Infectivity (%)	Progeny RNA	Recombination rate (%)
BB1 × BB4	151	0.10	BB4	—
BB1 × SX1	182	0.10	SX1	—
BB2 × BB4	371	0.10	wt[a] + BB4	100
BB2 × SX1	402	0.15	wt[a] + SX1	50
			SX1[b]	—
BB3 × BB4	151	0.20	wt[a] + BB4	20
			BB4[b]	—
BB3 × SX1	182	0.20	SX1	—

[a]wt RNA3 represents more than 50% of the whole RNA3 pool.

[b]Nearly 20% of lesions contained only the indicated parental mutants. The infectivity is expressed as a fraction (%) of local lesions generated by equal amount of wt RNA3, whereas the recombination rate is defined as a fraction (%) of local lesions containing recombinants. The composition of progeny RNAs from local lesions was determined by the use of Northern blots and by RT-PCR analysis.

data of Allison *et al.* (1990), who observed recombination among CCMV RNA3 deletion mutants. They have proposed that the intercistronic oligo(A) segment promotes CCMV replicase to leave primary template and to resume on the second deletion mutant. Alternatively, the overlapping intercistronic sequences might form heteroduplex structures among recombining RNA molecules. Sequence analysis confirmed such a possibility for both BMV and CCMV RNA3 intercistronic regions (not shown).

D. Rearrangements at Palindromic Inserts

One possible mechanism by which the RNA molecules form local heteroduplex structures is through hybridization at the hairpin–loop palindromes. Theoretically, such structures are possible alternatives to intramolecular hairpin–loops (Romanova *et al.*, 1986). We have tested the stability of strong hairpin–loop insertions in BMV RNA3 during infection. Palindromic sequences of 44 or 92 nt were ligated at the *Sal*I site just upstream of the initiation codon of CP ORF (constructs L-1CP and L-2CP, respectively, in Fig. 9). In the L-3a construct the 44-nt palindromic sequence was inserted at the *Cla*I site of the 3a ORF. The third location involved the *Ban*II restriction site just downstream of the CP ORF. Here five repeats of the GACUAGUC palindromic sequence were ligated to generate the 40-nt secondary structure element (construct L-SP3′). As shown in Table II, L-1CP and L-2CP were infectious to *C. hybridum*, but construct L-3a was not infectious. The latter reflected the importance of the 3a protein for the BMV life cycle. Examination of progeny RNA3 molecules revealed that all local lesions induced with L-1CP and nearly 50% of lesions induced with L-2CP accumulated wt RNA3. In addition, RNA3 molecules containing partially removed inserts were detected. The infectivity of L-SP3′ was much higher compared to that of other deletion mutants. Interestingly, a population of progeny RNA3 in which one, two, or three GACUAGUC segments were deleted, accumulated. This could happen either by recombination between two hybridized antiparallel L-SP3′ RNA molecules or by looping-out events. There must be special, but as yet unrecognized, structural features responsible for the observed deletion patterns in such multipalindromic inserts.

V. Conclusion

BMV represents a well-established *in vivo* recombination system. We have demonstrated that sequence alterations introduced at the

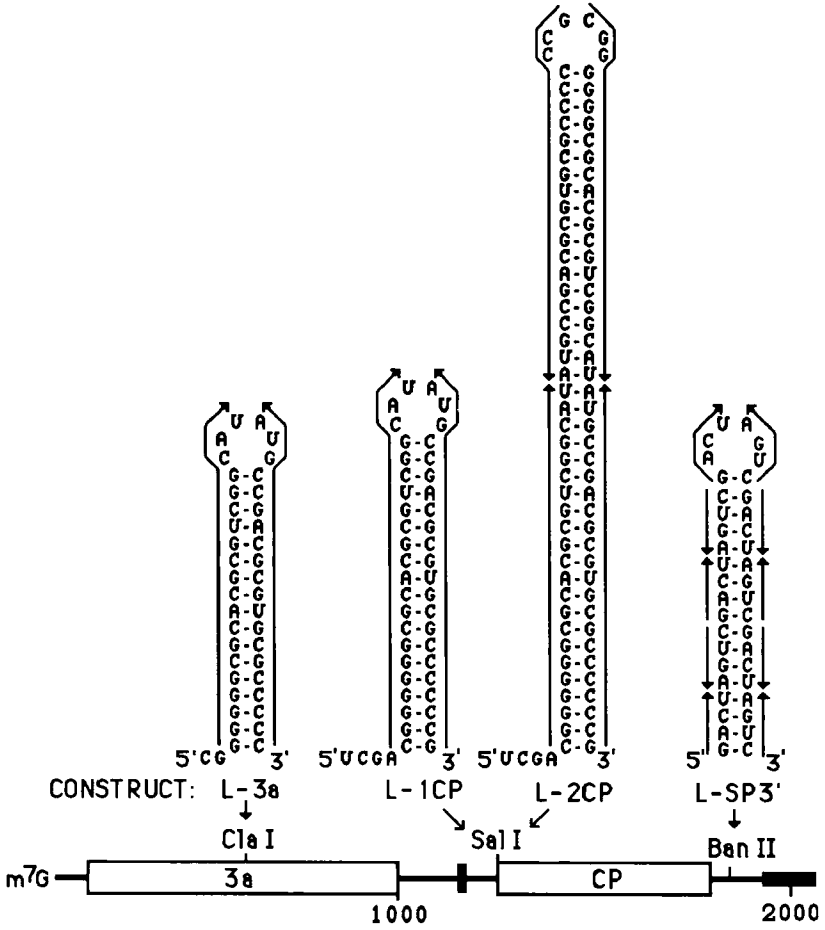


FIG. 9. Insertion of palindromic sequences at 3a protein, CP, and 3′-noncoding regions of BMV RNA3. Constructs L-3a and L-1CP contain essentially the same hairpin–loop element, whereas L-2CP has a duplication of that sequence. Construct L-SP3′ has a 5-fold repeat of the GACUAGUC *Spe*I linker sequence. Arrows depict reverse palindromic orientations.

3′-noncoding region of the RNA3 component were efficiently repaired by legitimate as well as illegitimate intersegment crosses. Sequence analysis of illegitimate sites suggested a role for local complementarity in recombination. Insertion of short antisense sequences of RNA1 into RNA3 allowed us to confirm, for the first time, that local heteroduplexes promote illegitimate crosses. We have also shown that the structure of the heteroduplex influenced the location of crosses, supporting the TS mechanism. Our heteroduplex-driven recombina-

TABLE II
STABILITY DURING INFECTION OF RNA3 MUTANTS CARRYING HETEROLOGOUS PALINDROMIC SEQUENCES[a]

Construct	Infectivity (%)	Nonparental progeny	% in RNA3 pool
C (4)	60	wt	10
L-1CP (48)	50	wt	100
L-2CP (92)	10	wt	50
		C1[b]	50
L-3a (42)	0	—	—
L-SP3′ (40)	100	32 nt[c]	40
		24 nt	40
		16 nt	20

[a]Please refer to Section IV,D and Fig. 9 for the structure of the mutants used.
[b]C1 variant has the first AUG CP ORF mutated to UUG and a 4-nt nonviral insert.
[c]The numbers indicate the size of inserts found among the RNA3 progeny derived from the parental L-SP3′ construct.

tion system will allow us to investigate RNA sequence requirements for recombination. It will also permit us to test various mutations and to map the regions on BMV replicase proteins which are involved in copy choice events.

With regard to recombination at other RNA3 locations, we have demonstrated efficient complementation among both frame-shift and deletion-containing BMV RNA3 mutants. The asymmetry in accumulation of the complementing CP over 3a protein mutants was observed. The complementation increased the sequence rearrangement processes, leading to the regeneration of wt RNAs or to the formation of new mutants. Mechanisms such as looping-out and recombinational crosses could be envisioned. The rearrangements in coding regions produced mutated proteins which provided new means for host–virus adaptation.

The removal of the palindromic inserts was very efficient and, in the case of multiple repeats, produced a population of deleted progeny. The intramolecular hairpin–loops and intermolecular local hybridizations can be considered as equivalent double-stranded structures exerting similar effects on template switching. Therefore, both systems could serve as convenient experimental models for molecular studies on the mechanism of genetic recombination in BMV and probably in other similar RNA viruses.

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