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Targeting of the site of nonhomologous genetic recombination in brome mosaic virus

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Summary. The genome of brome mosaic virus (BMV) consists of three positive strand RNA segments that share a high degree of sequence homology in the 3′ noncoding region. The phenomenon of both homologous and nonhomologous intersegment RNA-RNA recombination has been demonstrated within the 3′ noncoding region of BMV RNAs. It has been postulated that nonhomologous crossovers occur at local heteroduplexes formed between the recombining BMV RNA substrates of the same polarity and that the formation of double-stranded regions facilitates strand switching by the replicase. To test the hypothesis of hybridization-mediated recombination in BMV, RNA-3 constructs carrying short antisense RNA1-derived sequences have been used to induce nonhomologous recombination events between RNA-1 and RNA-3 at or near the site of hybridization. We find that both the incidence of recombination and the location of recombinant junctions depends on the structure and the stability of heteroduplexes. Furthermore, our preliminary results demonstrate that mutations in the helicase-like domain of BMV protein 1a affect the location of recombinant junctions. This provides experimental evidence that BMV replicase protein 1a participates in recombination.

Introduction

The genome of brome mosaic virus (BMV) includes three RNA segments that share a high degree of sequence homology within portions of their 3′ noncoding regions [1]. The phenomenon of RNA-RNA recombination in BMV was first demonstrated within the 3′ noncoding region when a systemic BMV host was inoculated with a mixture of wild type (wt) RNA1, wt RNA2 and mutated RNA3 (designated M4) [2]. Both homologous (legitimate) and nonhomologous (illegitimate) recombinants

were identified. Characterization of a large number of nonhomologous crossovers suggested that recombining BMV RNA substrates of the same polarity can form local heteroduplexes at the crossover sites [3, 4]. We postulated that the creation of double-stranded regions facilitated strand switching by the replicase [3, 4].

As in BMV, the formation of local heteroduplex structures at crossover sites was proposed for poliovirus [5, 6]. In coronaviruses, some form of discontinuous mechanism has been suggested to explain high frequency recombination in mouse hepatitis virus (MHV) [7]. Based on the observation that sequences at crossover sites were similar to the recognition sequences of turnip crinkle virus (TCV) replicase, reassociation of polymerase complex has been proposed to be responsible for recombination in TCV [8]. Among alphaviruses, a mutationally altered Sindbis virus RNAs induced illegitimate recombinants that contained both virally-derived and nonviral insertions [9].

A major goal of this research was to test the hypothesis of hybridization-mediated recombination in BMV. RNA-3 constructs carrying short antisense RNA-1 sequences have been used to induce recombination events between RNA-1 and RNA-3 at or near the site of hybridization. We find that both the incidence of recombination and the location of recombinant junctions depends on the structure and stability of heteroduplexes formed between the recombining RNAs. In addition, we provide preliminary results demonstrating that certain mutations in helicase domain of BMV protein 1a affected the location of recombinant junctions. This supported the involvement of BMV replicase in recombination.

Effect of sequence modifications in the RNA3 3′ noncoding region on recombination

The M4 RNA-3 deletion mutant generated recombinant molecules with a low frequency, when tested on *Chenopodium hybridum* local lesion host [4]. However, another RNA-3 mutant (designated DM4), which contained duplication of the M4 3′ noncoding region (regions A and B in Fig. 1 and [3, 4]), recombined at high levels. The DM4-generated recombinant molecules had the crossovers (indicated by arrows in Fig. 1) distributed along the length of region B. In order to make possible studies on sequences required for recombination, the DM4 construct was modified by insertion of a 197 bp CCMV RNA-3 3′ noncoding fragment (region C in PN0 construct). Our previous data revealed the role of selection in BMV recombination [4]. We hypothesized that, due to the extended length and the possibility of competition for BMV replicase proteins [10], progeny recombinants, which contain the CCMV insert, will not be viable. Consequently, the crossover events should be directed

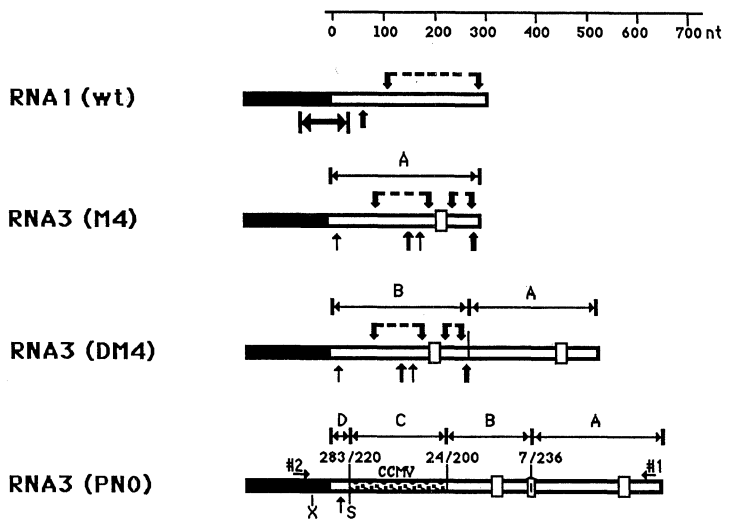

Fig. 1. Schematic representation of the 3′ terminal noncoding regions in the wt BMV RNA1 and in three mutated BMV RNA3 (*M4*, *DM4*, and *PN0*). The DM4 RNA-3 mutant has a duplicated 3′ noncoding region (open boxes designated as regions A and B), each containing a 20 nt deletion (designated as *M4*, see [29]) between nucleotides 81–100 (shown by a small vertical rectangular open box). The PN0 has an additional 197 nt 3′ noncoding region of CCMV RNA-3 (region C; cross-hatched box). Regions B and C lack the last 6 and 23 essential nucleotides (marked by the smallest rectangular box), respectively. The numbers above PN0 RNA-3 show the positions of the first and the last nucleotides of each region, counted from the 3′ end. The sites of crossovers are marked by either above (legitimate recombination) or below (illegitimate recombination) arrows. *S* and *X* mark the position of Spe I and Xba I restriction sites. Coding regions (not to scale) are represented by solid boxes. The location of the RNA-1 sequence that was used as antisense insert in PN constructs (see Fig. 2) is marked by a double horizontal arrow below the RNA-1 molecule

upstream to the CCMV insert. That the upstream region of BMV RNA3 had been inefficient in recombination in M4 and DM4 infections suggested that PN0 might be inefficient in recombination, as well. As predicted, none of the PN0-induced local lesions on *C. quinoa* contained recombinants (Fig. 1). In *C. hybridum*, 5% local lesions accumulated recombinants, all having crossovers at predicted upstream locations (marked by arrows in Fig. 1). These results demonstrated that the crossovers could occur only inefficiently at upstream locations and that the elimination of the 3′ half of the CCMV insert was important for recombinant accumulation.

Antisense sequences direct the site of crossovers

We speculated that the recombination activity of PN0 would be restored by insertion of sequences that fulfill the structural requirements of recombination. To test our hypothesis that local hybridization facilitates

crossover events, a 66 nucleotide sequence derived from RNA-1 was inserted at the SpeI restriction site of region D, in both the sense or antisense orientation. Figure 2 demonstrates that the corresponding antisense construct PN2(−) accumulated recombinants and that the crossovers were localized within or near the targeted sequence (shown by arrows). Northern blot analysis revealed that the recombinant RNA-3 components accumulated to high levels within the lesions (data not shown).

The antisense inserts have been further modified in order to test the effect of their length and sequence composition on recombination. Constructs PN1(−) and PN3(−) through PN5(−) contained 140, 40, 30 and 20 bp antisense RNA1 cDNA fragments nested at a common 243 RNA-1 position. PN1(−), PN3(−), and PN4(−), but not PN5(−), generated recombinant RNA3 progeny in *C. quinoa* (Fig. 2). This demonstrated that an antisense sequence longer than 20 nt is necessary to promote efficient recombination. The incidence of recombination depended on the length of the antisense sequence, whereas the recombination crossovers clustered at several hot spots within or near the left side of the putative heteroduplex (using the convention shown in Fig. 2). The latter suggested that the left side of the heteroduplex is associated with recombination.

In order to test the involvement of left side of the heteroduplex as well as the role of selection in recombination, sequence alterations were introduced outside the hot spot sequences observed for PN1(−) through PN4(−) recombinants. The PN6(−) construct was derived from PN1(−) by substitution of four C residues with four U residues. A shift of the crossover sites towards the central part of the duplex was observed. The C to U modifications in PN6(−) were located downstream of the PN1(−) through PN4(−) recombination hot spots. Therefore, the region where the recombination hot spots for PN1(−) through PN4(−) occurred, was not mutated in PN6(−). Consequently, the fact that none of the PN6(−)-induced crossovers occurred at the same positions as those generated by PN1(−) through PN4(−) could not be explained by selection. This conclusion was supported by results obtained with PN7(−) through PN10(−) constructs. Plasmid PN7(−) had a 5-base deletion in the upstream part of the antisense region and a 6-base heterologous sequence that disrupted the left side of heteroduplex (Fig. 2). The crossover sites in PN7(−)-generated recombinants were shifted further towards the central part of the heteroduplex. The PN8(−) construct, which contained three downstream mismatches (Fig. 2), generated recombinants with crossovers shifted even more into the central part of heteroduplex. That crossovers generated by some PN constructs were not observed for the other PN constructs, although all PN constructs

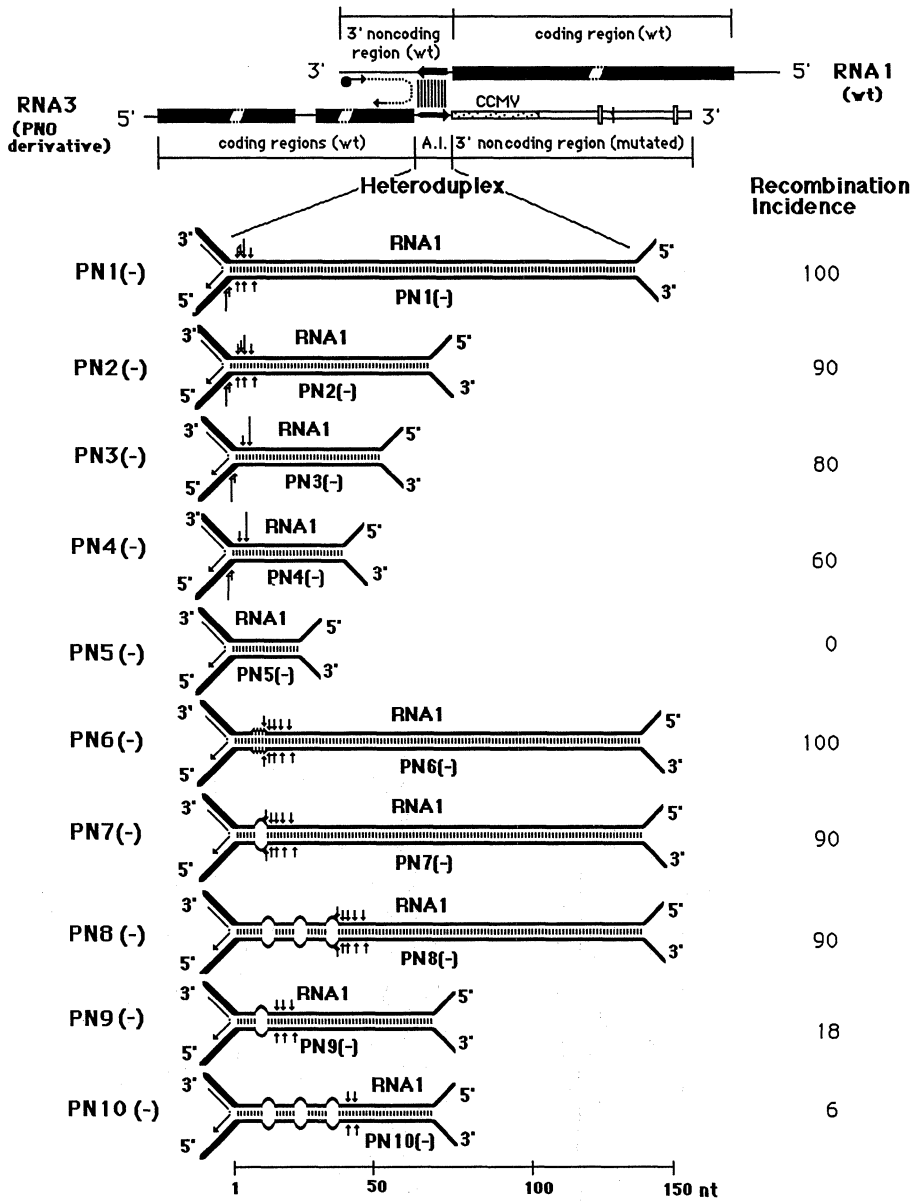

Fig. 2. Top: Schematic representation of the wt RNA-1 molecules as hybridized to antisense inserts in PN1(−) through PN10(−) RNA-3 constructs (marked by a central cluster of short vertical lines). The viral replicase (represented by a small solid circle) initiates at the 3′ end of the wt RNA-1 and switches to the RNA-3 sequences at the left side of the heteroduplex (following the dotted line). Bottom: Magnification of the heteroduplex region (represented by double lines) and location of crossover sites (represented by vertical arrows). The curbed region on the left side of the PN6(−) double line depicts the area of C to U mutations. One or three loops in PN7(−) through PN10(−) double lines represent mismatch mutations. The recombination incidence is defined as the percentage of local lesions on *C. quinoa* that accumulated the recombinants

had recombinationally active sequences, suggested that the crossovers were primarily determined by the structure of the antisense region at the recombination sites.

In order to imitate previously proposed partial hybridizations naturally occurring between recombining BMV RNAs at the sites of crossovers [3, 4], constructs PN9(−) and PN10(−) were designed (Fig. 2). They could form energetically much weaker heteroduplexes with RNA1 than did the PN1(−) through PN8(−). Indeed, PN9(−) and PN10(−) demonstrated a significantly reduced recombination incidence. As our model predicted, recombinant crossovers were located within sequences capable of heteroduplex formation.

Model of heteroduplex-mediated template switches by BMV replicase

Based on the results described above we speculate that the replicase can operate in two modes: regular RNA synthesis mode (A) and aberrant (recombinant) RNA synthesis mode (B) (Fig. 3). During regular RNA synthesis, the helicase domain of the replicase complex (in protein 1a) precedes that of viral RNA polymerase (in 2a protein) while moving across the RNA template. This enables the helicase portion to unwind double-stranded regions. Such factors as temperature, substrate or RNA template concentration, among other, might contribute to the mainte-

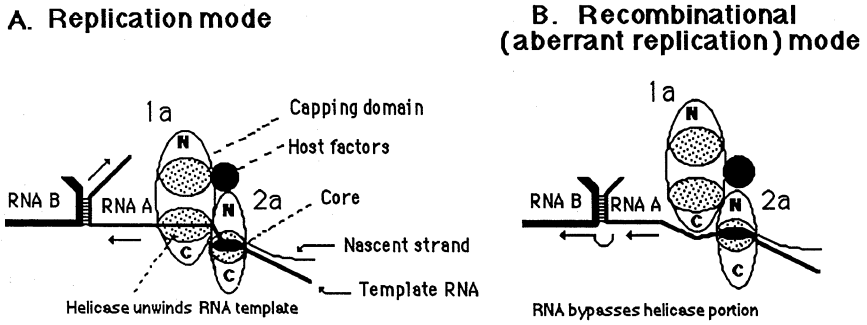

Fig. 3. The model of strand switching by BMV replicase. Proteins 1a and 2a (represented by ellipses) contain functional domains (represented by smaller internal ellipses) of nucleotidyl transferase, helicase and core RNA polymerase. The proteins interact with each other and with host factors (represented by a smaller solid circle). During replication mode (**A**), the RNA template (represented by a solid line) interacts with both helicase and core RNA polymerase domains. Helicase unwinds double-stranded (heteroduplex) regions and the original RNA template is copied through. During recombinational (aberrant replication) mode (**B**), the conformation of the replicase complex is different so that the RNA can interact only with the 2a core domain. In this case, the RNA polymerase passes through undissociated double-stranded regions synthesizing a recombinant nascent strand. The arrows indicate the direction of replicase migration

nance of the proper conformation of the replicase complex. The noncovalent nature of 1a–2a binding over long regions has been demonstrated recently [11, 12]. Conformational fluctuations may occasionally release the RNA template from the helicase portion, thus promoting aberrant RNA replication. In such a case, double-stranded RNA structures bypass the helicase and "slide" directly through the active site of the RNA polymerase component. The removal of double-stranded structures would result in generation of recombinants with the crossovers clustered at one side of the heteroduplex.

Discussion

The results reported in this communication provide the first direct evidence that local complementarity between viral RNA molecules can induce recombination events. The crossovers occurred between nonconserved, upstream parts of the 3′ noncoding regions in wt RNA1 and in mutated RNA3. This generated different-than-wt-size RNA3 recombinants, with most of the 3′ noncoding region taken from the RNA1 segment. Hence, these recombinants can be defined as nonhomologous (illegitimate) ones. Our system does not explain homologous recombination events, as defined by Lai [13]. One can envision the involvement of heteroduplexes in homologous recombination if double-stranded regions were formed by palindromic RNA sequences. It remains to be demonstrated whether the heteroduplex-driven mechanism could operate in other regions on the BMV genome or within the genomes of other RNA viruses.

Clustering of crossovers at the left side of the putative heteroduplex supports the hypothesis of replicase strand switching mechanism. Such a mechanism is also supported by preliminary data indicating that mutations within the helicase-like domain of BMV replicase 1a protein affected the recombination incidence, the incorporation of nontemplated nucleotides, and the number of asymmetric crosses (P. D. Nagy et al., unpubl. res.). BMV RNA replication involves multiple functions, including template recognition and binding for positive- and negative-strand synthesis, subgenomic promoter recognition and binding, initiation and elongation of RNA synthesis, strand separation, and capping. If the model shown in Fig. 3 is correct, template binding, strand separation and elongation of RNA synthesis are the steps most likely involved in recombination. The molecular details of the operation of RNA polymerase around double-stranded regions are not known. One possibility is that the enzyme detaches just before and reattaches right after double-stranded structure. However, our data did not reveal conserved sequences at crossover sites, neither sequences resembling BMV RNA genomic or

subgenomic promoters. We speculate that BMV replicase may not be able to reattach de novo at random internal RNA positions. Therefore, the enzyme relocations may occur inside the replicase complex, so that the flanking RNA template sequences are still being held by the replicase portions. Although the model predicts that the 2a protein component switches the RNA templates that have been released from the 1a protein portion, both proteins might participate in these events. Kinetically, the model presumes that strand switching represents a unimolecular RNA copying reaction.

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