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This paper is dedicated to Professor Maciej Wiewiórowski
Review

RNA recombination in brome mosaic virus, a model plus strand RNA virus

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Studies on the molecular mechanism of genetic recombination in RNA viruses have progressed at the time when experimental systems of efficient recombination crossovers were established. The system of brome mosaic virus (BMV) represents one of the most useful and most advanced tools for investigation of the molecular aspects of the mechanism of RNA–RNA recombination events. By using engineered BMV RNA components, the occurrence of both homologous and nonhomologous crosses were demonstrated among the segments of the BMV RNA genome. Studies show that the two types of crossovers require different RNA signal sequences and that both types depend upon the participation of BMV replicase proteins. Mutations in the two BMV-encoded replicase polypeptides (proteins 1a and 2a) reveal that their different regions participate in homologous and in nonhomologous crossovers. Based on all these data, it is most likely that homologous and nonhomologous recombinant crosses do occur *via* two different types of template switching events (copy-choice mechanism) where viral replicase complex changes RNA templates during viral RNA

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Abbreviations: BMV, brome mosaic virus; CCMV, cowpea chlorotic mottle virus; CMV, cucumber mosaic virus; CP, coat protein; FMDV, food and moth disease virus; kb, thousand of nucleotides (nts); MHV, mouse hepatitis virus; ORF, open reading frame; PPV, plum pox virus; PVY, potato virus Y; RdRp, RNA-dependent RNA polymerase; TCV, turnip crinkle virus; TMV, tobacco mosaic virus; ts, temperature-sensitive; TS, template switching; UTR, untranslated region; wt, wild-type.

replication at distinct signal sequences. In this review we discuss various aspects of the mechanism of RNA recombination in BMV and we emphasize future projections of this research.

RNA viruses are the only living species utilizing RNA as their genetic material. In this respect, RNA viruses differ from DNA-based viruses, as for the later the basic mechanisms of genetic variability resemble those which are observed in the host DNA-based organisms. Many animal and the majority of plant virus groups and families consist of RNA viruses. The variability of genome organization among the RNA viruses is enormous (Gibbs *et al.*, 1997). In fact, the potential for variation of the RNA genome is so large, that terms of classic population biology hardly describe RNA viruses. Instead, a term of quasispecies was proposed to reflect the nature of RNA virus populations (Domingo *et al.*, 1995; Eigen, 1996; Eigen & Biebricher, 1988; Holland *et al.*, 1992; Moya & Garcia-Arenal, 1995).

The evidence for the role of recombination in the evolution of RNA viruses is overwhelmingly visible in the phylogenetic relationships among the variety of virus groups (Dolja & Carrington, 1992; Morozov *et al.*, 1989). The complete nucleotide sequences of the genomic RNAs of a large number of RNA viruses belonging to different virus groups have been obtained. This made possible comparisons of viral families at the molecular level which revealed the relatedness of various RNA viruses. In turn, comparison of the sequence data have led to a greater understanding of various aspects of the RNA virus evolution. Those aspects include genome organization of RNA viruses (Strauss & Strauss, 1988; Zaccomer *et al.*, 1995), amino-acid sequence similarities in viral proteins (Goldbach, 1986; Hodgman & Zimmern, 1987; Zaccomer *et al.*, 1995), and the differentiation of RNA genomes (Holland *et al.*, 1982; Smith & Inglis, 1987; Steinhauer & Holland, 1987; Zaccomer *et al.*, 1995). All these studies document RNA recombination as one of the major driving forces of RNA virus evolution.

In general, it is believed that RNA–RNA recombination occurs between RNA molecules without DNA intermediates. This is because the life cycle in RNA viruses does not involve any DNA steps. The RNA recombination events can occur among different viral RNAs (derived from either different viruses or from the same virus) and involve either homologous or nonhomologous crossover events (Bujarski *et al.*, 1994; Lai, 1992; Nagy & Simon, 1997). The former occurs between two nearly identical RNAs (or within nearly identical RNA regions) while the latter occurs between nonrelated RNAs (or dissimilar regions).

GENETIC RECOMBINATION IN RNA VIRUSES

Natural sequence rearrangements were found in RNA viruses of animals, plants and in RNA phages. The examples being: alfalfa mosaic virus (Huisman *et al.*, 1989), beet necrotic yellow vein virus (Bouzoubaa *et al.*, 1991), bromoviruses (see below), hordeiviruses (Edwards *et al.*, 1992), luteoviruses (Mayo & Jolly, 1991), nepoviruses (Rott *et al.*, 1991), tobamoviruses (Shirako & Brakke, 1984), tobraviruses (Robinson *et al.*, 1987), tombusviruses (Hillman *et al.*, 1987), and turnip crinkle virus (TCV) (Cascone *et al.*, 1990). This by itself confirms that RNA recombination events are common among various groups of RNA viruses.

There are also examples in which host-derived sequences have been identified in viral RNAs, i.e. an ubiquitin-coding sequence in bovine diarrhea virus (Meyers *et al.*, 1991), a sequence from 28S rRNA inserted in the hemagglutinin gene of an influenza virus (Khatchikian *et al.*, 1989), and a tRNA sequence in Sindbis virus RNA (Monroe & Schlesinger, 1983). For plant viruses, several

potato leafroll virus isolates contain sequences homologous to an exon of tobacco chloroplast (Mayo & Jolly, 1991). Recently, acquisition of chloroplast sequences during RNA recombination were observed for BMV (Nagy & Bujarski, 1994). Allison *et al.* (1989), and Green & Allison (1994) demonstrated recombination between viral RNA and transgenic plant transcripts using an overlapping pair of deletion mutants in coding regions of CCMV RNA3. Also, a deletion mutant of red clover necrotic mosaic virus was restored by recombination with transgenically expressed viral RNA (Lommel & Xiong, 1991). Inefficient crossovers were observed between the transgenic coat protein mRNA and the mutated genomic RNA of potato virus Y potexvirus (PVY) that lacked the 3′ noncoding region (3′UTR) (Jakab *et al.*, 1997). This supports the earlier observations that the 3′ UTR plays a significant role in RNA recombination. Homologous recombination events between the transcripts of coat protein-defective plum pox potyvirus (PPV) and the functional PPV coat protein mRNA expressed in transgenic *Nicotiana benthamiana* plants were observed (Maiss *et al.*, 1997). The crossover events were not efficient, most likely due to complementation by functional CP. This demonstrates that complementation might reduce selection pressure on the accumulation of recombinants.

Several recent reviews have described the molecular studies on the mechanisms of recombination (Lai, 1992; Pilipenko *et al.*, 1995; Simon & Bujarski, 1994). The experimental systems (described in two issues of *Seminars of Virology*, vol. 7/6, 1996 and 8/2, 1997), with which RNA recombination could be studied in RNA viruses include those for plant viruses: BMV, TCV, tombusviruses, closteroviridae as well as those for animal RNA viruses: coronaviruses, picornaviruses, alphaviruses, nodaviruses, bacteriophage $\Theta\beta$, vesicular stomatitis virus, and retroviruses. In particular for plant RNA viruses, nonhomologous recombination has been induced in alfalfa mosaic virus where a temperature-sensitive (ts) mutation in the RNA3 component acquired a 5′ terminal fragment from the RNA1 component during infection (Huissman *et al.*, 1989). Also, an insertion mutant of tobacco mosaic virus easily lost one of its duplicate coat protein cistrons through recombination (Dawson *et al.*, 1989). In TCV, nonhomologous recombination was observed between satellite RNAs (Cascone *et al.*, 1993), between satellite and genomic RNAs (Zhang *et al.*, 1991), and between defective interfering (DI) and satellite RNAs (Cascone *et al.*, 1993). Here, downstream crossovers cluster at a highly structured promoter region, suggesting the occurrence of recombination during plus strand synthesis. In tomato bushy stunt tombusvirus, White & Morris (1994) demonstrated recombination between two types of defective RNAs.

Genetic recombination among animal plus strand RNA viruses was first observed in picornaviruses: poliovirus (Emini *et al.*, 1984; Hirst, 1962; Ledinko, 1963; Tolskaya *et al.*, 1988; Tolskaya *et al.*, 1983) and foot-and-mouth-disease virus (FMDV) (King *et al.*, 1982; McCahon *et al.*, 1985; Saunders *et al.*, 1985). Kirkegaard & Baltimore (1986) demonstrated that homologous recombination in poliovirus occurs *via* a copy-choice mechanism. Romanova *et al.* (1985; 1986) and Tolskaya *et al.* (1983; 1988) found that recombination occurred within regions having the potential to form double-stranded structures.

In coronaviruses, homologous recombination was detected using mouse hepatitis coronavirus (MHV) (Keck *et al.*, 1987; 1988; Lai *et al.*, 1985). Banner & Lai (1991) have found crossover hot spots after serial passages of MHV, demonstrating the importance of selection in coronavirus recombination. Zhang & Lai (1994) have shown that the process of transcription priming in MHV (that might be analogous to RNA recombination) leads to the heterogeneity of leader-mRNA fusions.

The involvement of specific protein-protein or protein-RNA interactions rather than RNA-RNA interactions was suggested.

Weiss & Schlesinger (1991) observed the regeneration of the Sindbis virus genome by crossovers among two RNA fragments. They concluded that recombination between Sindbis virus RNAs may be analogous to that observed in bromoviruses.

Nonhomologous recombination was noticed between two components of flock house nodavirus (Li & Ball, 1993). The authors found short (5–7 nt) homologous sequences at the junction sites. In BMV, such short homologies supported very inefficient crossovers between RNAs 2 and 3 (Nagy & Bujarski, 1993).

Homologous recombination was observed between the RNAs of bacteriophage $\Theta\beta$ expressed as mRNAs from plasmids cotransformed into *E. coli* (Palasingam & Shaklee, 1992). The exact crossover sites could not be detected due to long regions of sequence identity between the marker mutations.

Genetic recombination has been recognized in other types of RNA viruses, as well. For instance, in influenza virus, a negative-strand multipartite RNA virus, the 5′ crossovers occur in the ribonucleo-protein transfection system (Bergman *et al.*, 1992); in retroviruses, recombination occurs by changing templates during reverse transcription (Pathak & Hu, 1997; Hu & Temin, 1990; Stuhlmann & Berg, 1992). In Φ6 phage, the 3′ nonhomologous crosses among the double-stranded RNA segments have been observed within virions (Onodera *et al.*, 1993).

One of the most useful experimental systems with which to study recombination in RNA viruses are bromoviruses. In this review we describe the discovery, development, current status and future plans obtained with the BMV recombination system.

MOLECULAR BIOLOGY OF BROME MOSAIC VIRUS

The genus Bromovirus includes brome mosaic bromovirus (BMV), broad bean mottle bromovirus (BBMV), and cowpea chlorotic mottle bromovirus (CCMV). BMV has been used for years as a model for positive strand RNA viruses and, consequently, it is among the best molecularly characterized viruses (DeJong & Ahlquist, 1991). The bromovirus genome is divided into three RNA components (RNA-1, RNA-2 and RNA-3). There is a fourth RNA (RNA-4), which is subgenomic and co-encapsidates with RNA-3 (Fig. 1). Nucleotide sequences of the genomic RNAs for bromoviruses are known (Allison *et al.*, 1988; Dzianott & Bujarski, 1991). Full-length cDNA clones of BMV (Ahlquist *et al.*, 1984a; Ahlquist *et al.*, 1984b; Janda *et al.*, 1987), CCMV (Allison *et al.*, 1988), and BBMV (Pogany *et al.*, 1994), from which infectious RNA

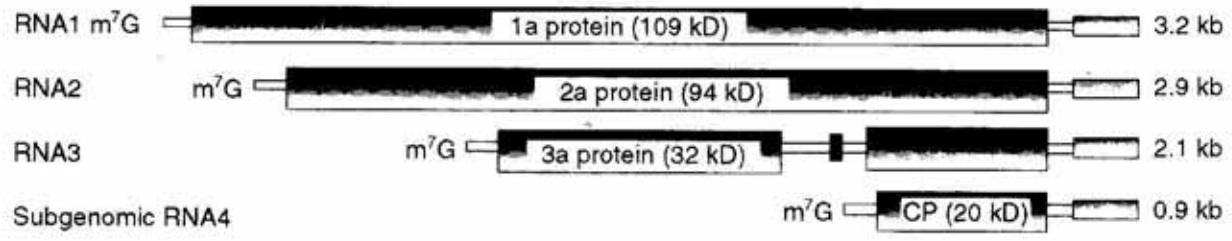

Figure 1. Molecular organization of the bromovirus genome.

The open reading frames are boxed and labeled. RNAs 1 and 2 encode two replicase proteins (1a and 2a) while RNA3 encodes the 3a movement protein and the coat protein. The 3′ terminal sequences, which include approximately 200 nucleotides common to all BMV RNA components, are marked as boxes on the right. A 20 nt oligo(A) tract is shown as a small vertical rectangle within the intercistronic region of RNA3.

can be synthesized *in vitro*, are available. The *in vitro* self-cleavage process can generate infectious BMV transcripts (Dzianott & Bujarski, 1989).

Both *in vitro* and *in vivo* studies of BMV RNA3 replication identified minus strand synthesis promoters within the 3' noncoding tRNA-like structure (Miller *et al.*, 1986). Other replication signals have been identified within the intercistronic and 5' noncoding regions of RNA3. The intercistronic region comprises the subgenomic RNA4 promoter (Marsh *et al.*, 1988), as well as signals regulating the plus to minus-strand ratio (Marsh *et al.*, 1991). The 5' noncoding region contains plus strand RNA synthesis regulatory sequences (Marsh *et al.*, 1988; 1989; Pogue & Hall, 1992). Replication signals have also been identified on BMV RNA-2 (Marsh *et al.*, 1991).

A purified template-dependent and -specific BMV RNA polymerase (RdRp) preparation has been isolated (Hall *et al.*, 1982; Quadt & Jaspars, 1990). It contained the 1a and 2a viral proteins and some yet to be recognized host factors (Quadt *et al.*, 1993). Based upon amino-acid sequence comparisons, protein 1a helicase and capping enzyme domains were recognized while the 2a protein was classified as RNA polymerase (Ahlquist *et al.*, 1985). Mutations and deletions in these proteins were used to identify the regions active in RNA replication (Kroner *et al.*, 1989; Lahser *et al.*, 1993; Traynor & Ahlquist, 1990; Traynor *et al.*, 1991) as well as regions responsible for interaction between both polypeptides (Kao & Ahlquist, 1992; Kao *et al.*, 1992). A chimeric 1a/2a protein supported RNA synthesis only when the unmodified 1a was also present (unpublished results from P. Ahlquist laboratory). This suggested the presence of two forms of 1a (a bound and an unbound form) in the replicase complex. An active BMV RdRp has been isolated from 1a/2a expressing yeast cells, provided that there was at least one replicatable BMV RNA component present (Quadt *et al.*, 1995). This suggests that RNA–protein binding is required to assemble an active RdRp complex.

GENETIC RECOMBINATION IN BMV

Discovery of RNA recombination in BMV

It has been suggested that bromoviruses and other tricornaviruses were derived from a monopartite ancestor virus by recombination. Also, sequence similarity of the 3' noncoding region among bromovirus genomic RNAs can be explained by recombination. That recombination could indeed occur between the segments of a tricornavirus was first demonstrated by Bujarski & Kaesberg (1986). A partially debilitated BMV RNA3 mutant (designated M4) that had a short deletion in the 3' replication promoter was repaired *in vivo* by acquiring sequences from the remaining wild type BMV RNA1 or RNA2 components. Rao & Hall (1993) demonstrated that RNA recombination was as important as nucleotide substitutions caused by polymerase errors for the repair of 3' noncoding region of BMV RNAs. In addition to these intraviral recombination processes, interviral recombination has been observed to occur in the 3' noncoding region, when a mixture of all wild type genomic RNAs of BMV and CCMV, except BMV RNA3 that has been substituted by a M4 RNA3 duplication derivative (designated DM4, Nagy & Bujarski, 1992), was co-inoculated on *C. quinoa* plants (Nagy & Bujarski, unpublished). As a result of inefficient crosses with CCMV RNA3, the DM4 has been repaired by acquiring the CCMV 3' noncoding region.

Homologous and nonhomologous recombinants

In order to understand the mechanism(s) of genetic recombination in BMV, a number of recombinants, obtained by using M4 or DM4 RNA3 mutants, were characterized (Nagy & Bujarski, 1992). Basically, two types of recom-

binants, homologous and nonhomologous, were found. They differed from one another in several features, as follows:

- (i) Contrary to nonhomologous recombination homologous recombinants had the cross sites at corresponding positions on the recombination substrates.
- (ii) An analysis of homologous recombinants revealed sequence homology at and near the sites of crossovers. Neither sequence homology nor specific RNA motifs were found at the sites of crossovers in nonhomologous recombinants. However, sequence complementarity of the recombination substrates with (+) strand polarity near the crossover sites were observed (Bujarski & Dzianott, 1991; Nagy & Bujarski, 1992). Examination of the free energy released during formation of such putative heteroduplexes showed that they were energetically permissible (Bujarski & Dzianott, 1991; Nagy & Bujarski, 1992). This observation suggested that hybridization between RNA templates helps to bring the recombining RNAs together, thus facilitating nonhomologous crosses.
- (iii) Homologous recombinants were isolated 5–10 times more often than nonhomologous ones. This suggested that either homologous recombinants were generated more frequently or that better adapted homologous recombinants outcompeted the nonhomologous ones. It has been demonstrated in barley protoplasts that homologous and nonhomologous recombinants expressed similar competitiveness (Nagy & Bujarski, 1992). Therefore, the former possibility is more likely to be responsible for the observed differences.

All the above differences supported the idea that the formation of homologous and nonhomologous recombinants utilizes different mechanisms.

Construction of recombination vectors

Generation of recombinants by using the above described M4, DM4 or nonreplicating RNA3 mutants was inefficient. To overcome this problem, RNA3-based recombination vector were designed. The idea was to develop an infectious RNA3 molecule (RNA3 component is dispensible for viral replication) stable in infection with a possibility to insert sequences of interest in order to study their recombinational activity. The vector, designated PN0, is a DM4-RNA3 derivative that contains additional CCMV 3′ noncoding se-

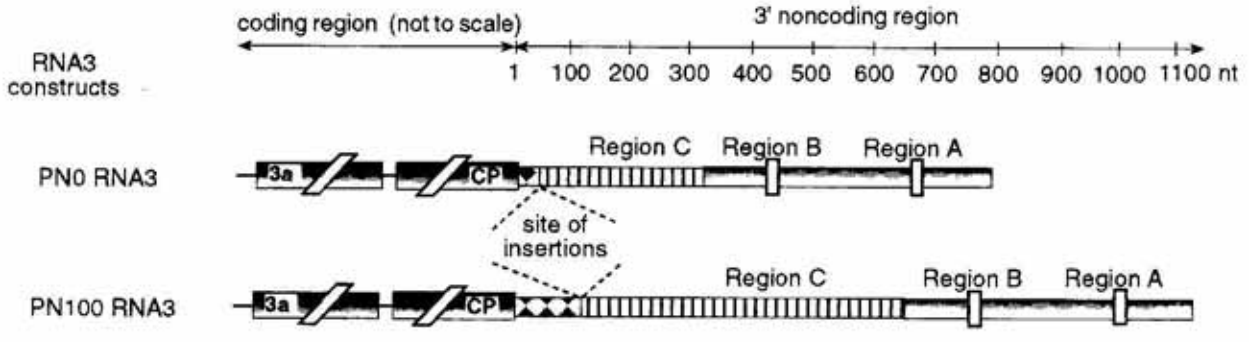

Figure 2. Recombination vectors used in the BMV recombination system.

The picture shows schematic representation of the 3′ noncoding regions in the vector BMV RNA-3 derived constructs. The 3′ sequences (nearly 600 and 1200 nt long, respectively) consist of three segments: the very 3′ 216 nt long sequence (region A) is a functional chimera derived from the 3′ sequences of RNA1 and RNA3 while region B has RNA3 specific and common 3′ sequences with a deletion of last 6 nt. Both elements have the M4 deletion (represented by small rectangular open boxes). Region C contains a 197 or 765 nt (in PN0 and PN100, respectively) 3′ terminal sequence of CCMV RNA3 except the last 23 nt. Because of the 3′ deletion in region B, RNA replication can initiate only from the terminal region A. Arrows depict the sites where the recombinationally-active sequences of interest can be inserted into these vectors.

quences (Fig. 2). Although it accumulated poorly in *C. quinoa*, PN0 did not generate either homologous or nonhomologous recombinants (Nagy & Bujarski, 1993). However, as described below, the recombination ability of PN0 could be "activated" by inserting sequences that fulfill the requirements of the recombination mechanism. This vector was used to characterize sequences active in nonhomologous recombination (see below). In a similar way, a PN100 RNA3 derivative was constructed as a suitable vector for the study of homologous recombination (Fig. 2).

Mechanism of recombination in BMV: *cis*-acting factors

Heteroduplex-driven crossovers

Insertion of recombinationally active sequences into the PN0 recombination vector induced intersegment recombination with high frequency. As importantly, this allowed for targeting the junction sites in both RNAs, thus leading to the generation of viable and competitive RNA3 recombinants. For instance, inserting 66 nt sequence complementary to RNA1 into the cloning site of PN0 RNA3 [PN2(-)] induced the generation of RNA1/RNA3 recombinants (Fig. 3). On the contrary, insertion of the corresponding region in direct orientation did not give recombinants at detectable frequency (Nagy & Bujarski, 1993).

In addition to demonstrating the usefulness of a recombination vector system, the above studies also supported crucial evidence for the heteroduplex-based nonhomologous recombination. This is because the junction sites clustered (were targeted) at one side of the short heteroduplexed regions (Fig. 3). Also the formation of local (approximately 66 nt) double-stranded region between the RNA1-derived antisense sequence in PN2(-) RNA3 and wt RNA1 was demonstrated by nuclease protection assay (Nagy & Bujarski, 1993; Bujarski *et al.*, 1994; Bujarski & Nagy, 1994). No protected fragment was detected for PN2(+) RNA3 that had the RNA1-derived insert in direct orientation.

The above system unravelled the molecular means for establishing a targeted RNA recombination system. This allowed for further characterization of structural requirements of recombination at both RNA and protein levels (see below). To define the length requirement of the heteroduplexed region in nonhomologous recombination, a series of PN0 derivatives with RNA1-derived complementary inserts of 20–140 nt was tested *in vivo* (Nagy & Bujarski, 1993). This revealed that longer heteroduplexed regions support recombination at a higher frequency than shorter ones. Also, shorter than 30 nt heteroduplexed regions [see PN5(-) in Fig. 3] did not induce RNA1/RNA3 recombination at detectable level.

Analysis of junction sites revealed that they were clustered at the 'left side' of the heteroduplex region. The most plausible model to explain such distribution, and the observed recombination frequency in those experiments is a replicase-driven template switching (TS) model (Fig. 4). This mechanism proposes that the replicase enzyme (RdRp) has difficulties to pass through the heteroduplex region. Due to replicase pausing, occasional TS events occur on to the complementary region of the acceptor strand. This is supported by a "right-ward" shift of junction sites into the energetically more stable parts of the heteroduplex region after destabilizing the 'left-side' of the heteroduplex region. This was achieved by replacing four G–C base pairs with G–Us or when mismatch-mutations were introduced to create one or three, respectively, "bubble" structures [as an example, see PN8(-) in Fig. 3] (Nagy & Bujarski, 1993).

Overall, this demonstrated that the structure and/or stability of the heteroduplexed region can influence both the frequency of recombination and the sites of junctions. The majority of recombinants had the junctions

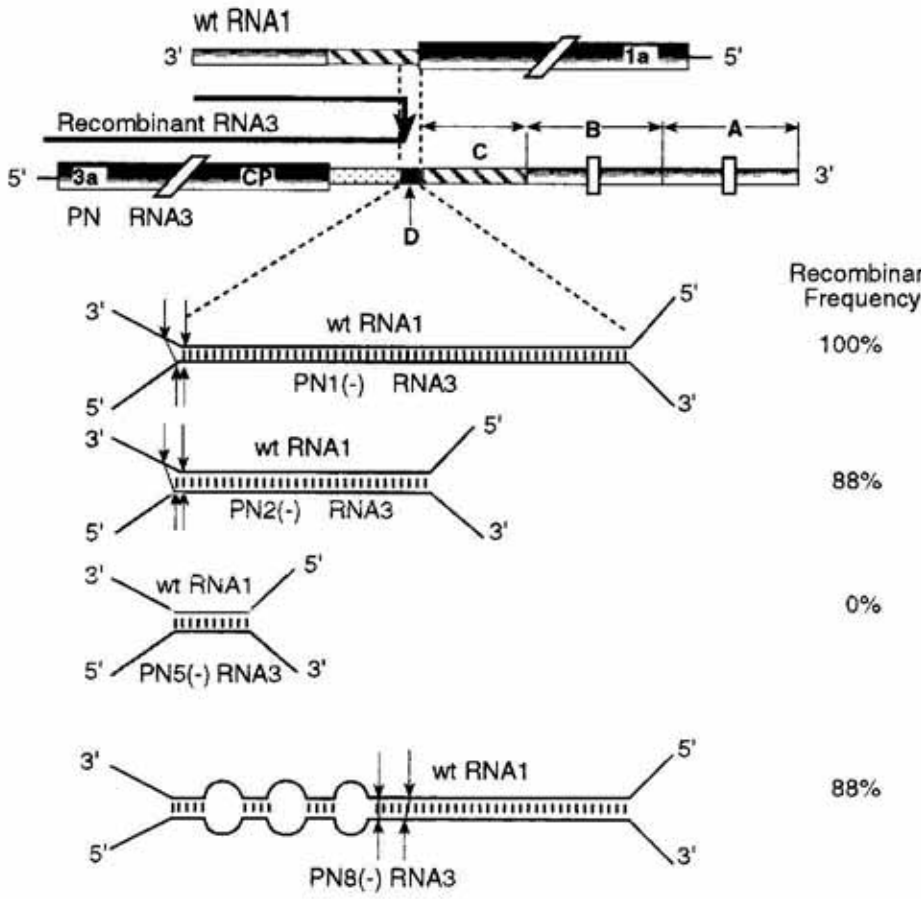

Figure 3. Schematic representation of the parental RNAs and the heteroduplex structures formed between them.

The 3′ noncoding region of the RNA3 constructs contains four regions: the 3′ half is a duplicated BMV 3′ end sequence with short deletions (regions A and B), while region C is derived from CCMV. The upstream portion of the 3′ noncoding sequence contains the RNA1-derived region in complementary orientation (region D). The most frequent junction sites are shown by arrows. The incidence of recombination (recombination frequency in percent) is measured in local lesions of *C. quinoa* and is shown on the right.

juxtaposed at or near counterpart nucleotides within the heteroduplex, while others had the donor and the acceptor sites more apart from each other. This can be explained by temporary formation of nonstandard or alternative double-stranded structures right before or during the TS events. The involvement of 3–10 nt sequence homologies in the junction site selection, or conformational changes within the replicase complex at certain positions (for example 'inchworming' movement along the template similar to those proposed for other RNA polymerases; see Chamberlin, 1995) are the other possible factors involved.

In total, according to the model, the formation of heteroduplexes between the RNA templates can facilitate TS events (Fig. 4). This is due to (i) bringing the RNA substrates into a close proximity within the cells and/or (ii) the structure or the stability of the heteroduplex regions which cause a "road block" slowing down or stalling the approaching replicase at or within the double-stranded region. It has been proposed that the replicase switches between templates in a processive manner by sliding under the heteroduplex region towards the opposite side of the acceptor RNA. (Bujarski & Nagy, 1994). In essence, the outcome is analogous to looping out events at strong secondary structures during RNA replication.

Homology-driven crossovers

The first comparative analysis of homologous and nonhomologous recombinants in BMV revealed several important differences, such as the selection of crossover sites (regions), the degree of precision of crossover events and the frequency of their generation with homologous recombinants isolated 5–10 times more frequently than nonhomologous ones (Bujarski *et al.*, 1994). Also, the imprecise nature of crossover events mediated in the heteroduplex system (see above) made it difficult to explain how this structure could

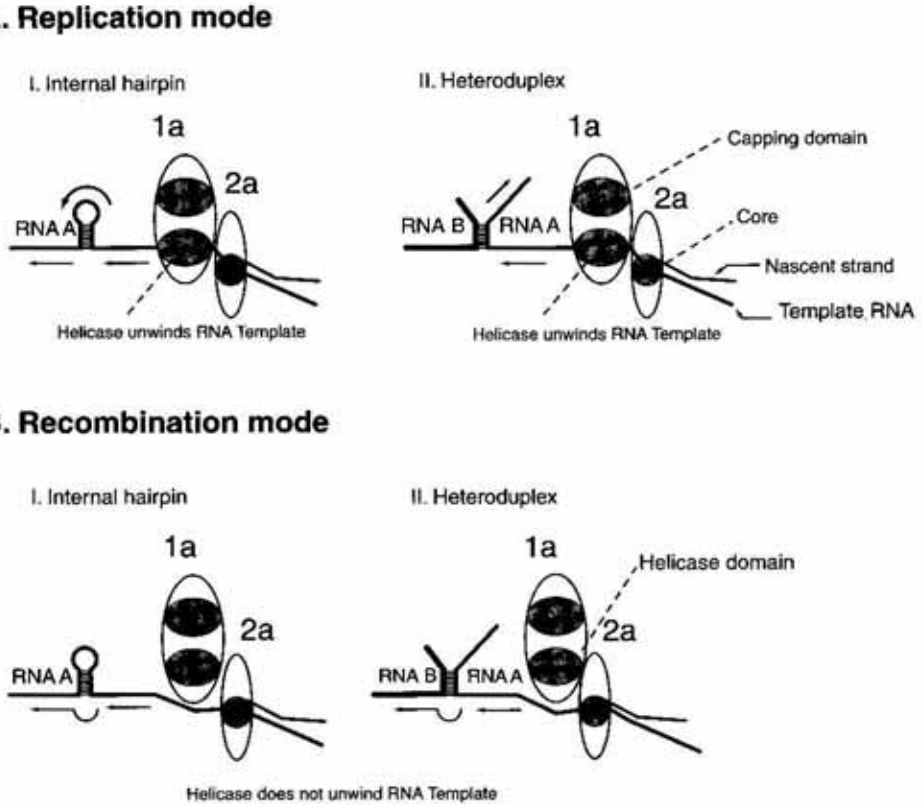

Figure 4. A model of the mechanism of heteroduplex-mediated recombination in BMV.

Proteins 1a and 2a (represented by ellipses), which contain functional domains (represented by smaller dotted ellipses) of nucleotidyl transferase, helicase and core RNA polymerase, interact with each other and with host factors (represented by a smaller solid circle). The figure shows the two modes the replicase enzyme may operate near double-stranded (either intramolecular hairpins or intermolecular heteroduplex) structures: replication mode and recombination mode. During replication mode, the RNA (represented by a solid line) interacts with both helicase and core domains. Helicase unwinds double-stranded regions and the original RNA template is copied through. During recombination mode, the conformation of the complex is different so that the RNA interacts only with the 2a core domain. In this case, undissociated double-stranded regions "slide" through the RNA polymerase active site. The arrows indicate the direction of replicase migration.

facilitate the formation of homologous recombinants with precise crossovers. All the above observations suggested that the sequence and/or structure requirements of homologous RNA recombination is different from that of nonhomologous RNA recombination in BMV. Detailed testing of homologous recombination is a rather difficult task, because the homologous junction sites cannot be pointed out precisely due to (i) the presence of sequence identity in the region of crossovers (that included the conserved 3′ sequences) and (ii) the precision of the events.

Some of the above difficulties were overcome by the use of an RNA3 recombination vector (designated PN100) similar to that used in the heteroduplex-mediated recombination studies (Fig. 2). Insertion of a 60 nt RNA2-derived region (designated region R) into PN100 in direct orientation (sense orientation) resulted in an RNA3 construct (PN-H26, Fig. 5A) that efficiently recombined with wt RNA2. Most of the crossovers were of homologous type that occurred precisely within the common region (Nagy & Bujarski, 1995). The use of 5′ nested deletions within region R in RNA3 defined that 15 or longer sequence identity between RNA2 and RNA3 can support efficient generation of RNA2/RNA3 homologous recombinants, while 5 and 9 nt long common regions supported only reduced levels of homologous recombination. No homolo-

gous RNA2/RNA3 recombinants were detected when the length of sequence identity was 4 or none. Mismatch mutations were introduced into region R in RNA3 to provide markers for localization of junction sites (Fig. 5B). The presence of four (in total) or more mismatch-mutations, however, reduced drastically the frequency of crossovers and also caused a shift in the location of crossovers towards nonmutagenized portion of region R (Nagy & Bujarski, 1995). Surprisingly, the predicted secondary structures in region R in the above mismatch-mutants, the observed homologous recombination frequencies and the distribution of junction sites (recombination profile) did not correlate (Fig. 5B), arguing that the secondary structures at the hot-spot regions play limited, if any, role in homologous recombination. This represented another piece of evidence that homologous and nonhomologus recombination utilize different RNA intermediates during the recombination events.

The use of the above homologous recombination system allowed for the isolation of imprecise recombinants having mismatched, deleted, inserted or nontemplated nucleotides within the crossover region. These sequence changes were not present in the parental RNAs and were not introduced during the RT-PCR reaction, cloning and sequencing (Nagy & Bujarski, 1995). The changes were almost invariably located in the crossover region since they were harbored by RNA3 marker mutation at the 5′ side and the RNA2 marker mutation from the 3′ side. This strongly suggests that they were introduced during the crossover events and they point at the actual crossover sites.

The above sequence changes were not distributed randomly along the entire length of region R, but rather they concentrated within two short stretches that contained mainly A and U nucleotides (Nagy & Bujarski, 1995). These observations led to a working model which predicted that the AU-rich stretches in the homologous regions can cause imprecise crossover events. The imprecision may be due to either misalignment between the RNA templates, replicase stuttering (nontemplated addition of several nucleotides), nucleotide misincorporation and their different combinations within the AU-stretches (Nagy & Bujarski, 1996).

This model was tested by increasing the length of one of the AU-rich stretches in both RNA2 and RNA3. The reasoning was that the presence of long AU-rich sequences should favor the imprecise crossover events. Accordingly, duplication and triplication of the length of the AU-rich regions resulted in a remarkable increase in isolation of imprecise recombinants (as high as 86% of all homologous recombinants can be imprecise).

In addition to recognition of the sequence requirements of imprecise homologous recombination, the above experiments suggested that AU-rich sequences are likely playing important roles during the homologous crossover events. To address this question, about 30 nt artificial AU-rich sequences were introduced 3′ to a defined, virally-derived sequence (region R′, that correspond to the 5′ 23 nt of region R, see Fig. 5A) into both RNA2 and RNA3 vectors (Fig. 6A). These experiments revealed that insertion of each of these AU-rich sequences increased the frequency of homologous recombination significantly. Also, a shift of crossover sites to the AU-rich regions was observed. Inserting one of the AU-rich sequences 5′ to region R′ neither increased the frequency of crossovers significantly nor changed the distribution of crossovers (Fig. 6A) Nagy & Bujarski (1997). When the AU-rich sequence was present in one of the RNAs (either RNA2 or RNA3) at downstream location, moderate levels of recombination, similar to those without AU-rich sequences in both RNAs, was observed (Fig. 6A–B) (Nagy & Bujarski, 1997).

Further experiments surprisingly, however, demonstrated that AU-rich sequences alone in both RNA2 and RNA3 supported only low level of homologous recombination (Fig. 6B).

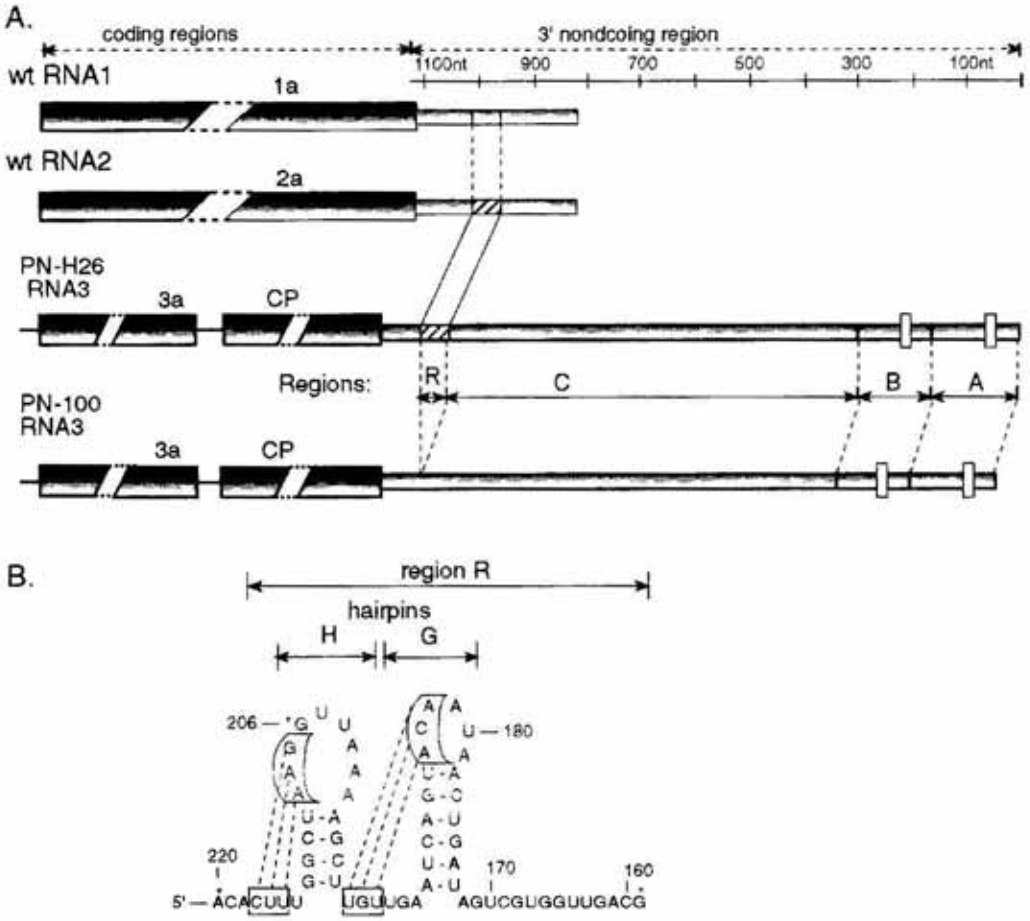

Figure 5. Homologous recombination system in BMV.

A. Schematic representation of the 3′-noncoding region of wild-type BMV RNAs 1–2 and PN-H26 and PN-H100 RNA3 constructs.

The PN100 RNA-3 mutant contain defective 3′ noncoding region that consists of three 3′ noncoding sequence-elements shown by horizontal double-headed arrows (regions A–C, described previously). The location of two viable M4 deletions is shown by rectangular small boxes. PN-H26 RNA3, a derivative of PN100, contains a 60 nt long RNA3-derived sequence (marked as region R, positions 160–219 of wt RNA3, counted from the 3′ end) inserted in sense orientation upstream to region C of PN100. Sequence of region R in PN-H26 is identical with the corresponding RNA2 sequence (enclosed by solid lines), except at position 206. This sequence is less similar to that present in RNA1.

B. A computer-predicted secondary structure of the 60 nt region R in PN-H26.

Nucleotide numbering corresponds to wt RNA3 sequences (positions 160–219, counted from the 3′ end). The boxed nucleotides within the two hairpins, connected by dotted lines, indicate two putative pseudoknot structures that correspond to hairpins H and G in the wt RNA3, as proposed by Lahser *et al.* (1993). Asterisks depict marker mutations that are different in PN-H26 and wt RNA2.

Also, combination of constructs with AU-rich sequences present at downstream location and R′ present in either RNA2 or RNA3, gave rise to recombinants at reduced level. Thus, the 5′ region R′ and the 3′ AU-rich sequence must be present on both RNAs for efficient homologous recombination to occur (Fig. 6B–C, E).

R′ sequences were replaced in both RNAs with several different, both artificial and virally-derived sequences (Fig. 6D) (Nagy & Bujarski, 1997). Interestingly, several unre-

lated GC-rich sequences or sequences with close to average AU content increased the frequency of homologous recombination when compared to AU-rich sequences alone. In contrast, purely AU-rich sequences at upstream positions increased only slightly the frequency of events, likely due to the duplication of their length. Since several sequences that had different nucleotide orders but with similar nucleotide compositions showed comparable effect on homologous recombination (Fig. 6C, E), we postulate that these sequences act as recombination activators: The downstream AU-rich region is the "core" region, and the

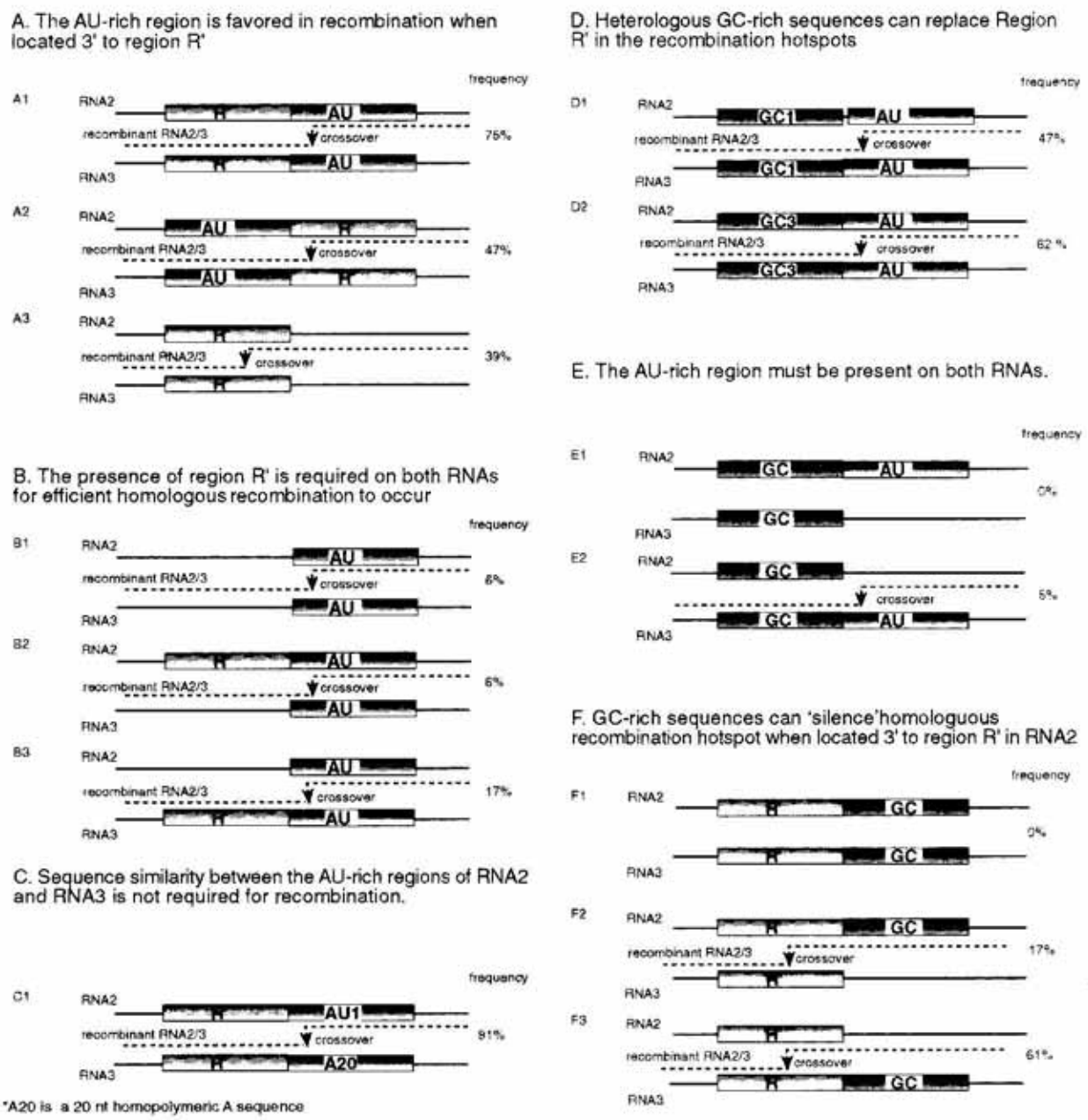

Figure 6. Schematic representation of the common sequences in the parental RNA2 and RNA3 constructs.

Each box represents approximately 30 bases which are either A+U-rich, or G+C-rich or close to average (R'). The incidence of homologous recombination was measured in local lesions of *C. quinoa*. The favored location of junction sites is depicted by an arrow.

upstream GC-rich region is the 'enhancer' region. The role of these regions can be explained if one assumes that homologous recombination mainly occurs during the positive strand synthesis (see below).

According to a proposed model, the 5′ GC-rich (or average) and the downstream AU-rich sequences play different roles in the donor and acceptor RNAs (Fig. 7). The AU-rich sequences in the donor RNA may cause replicase pausing and/or facilitate the release (departure) of the 3′ end of the nascent strand due to the weak A–U base-pairing. The GC-rich upstream portion of the nascent strand may facilitate the "landing" on the complementary region ("landing site") of the acceptor RNA due to strong G–C base pairing. The AU-rich sequence in the acceptor RNA may play a rather unique role: it can facilitate the opening of the partially double-stranded replication intermediate (RI) *via* local "bubble structure" formation. The experiments shown in Fig. 6A–F are in accordance with this model which predicts that omitting any of the components of the homologous recombination activators in RNA2 and/or RNA3 should reduce recombination.

By testing sequences flanking the above recombination activators it was shown that long (between 20–30 nt) GC-rich sequences located only at downstream positions (but not in upstream positions) in both RNA2 and RNA3 or only in RNA2, reduced significantly the incidence of recombination at upstream locations (Fig. 6F) (Nagy & Bujarski, 1997). Thus, basically the downstream GC-rich sequences acted as homologous recombination silencers on the upstream hot-spot regions. Based on the homologous recombination model shown in Fig. 7, the downstream GC-rich sequence in the acceptor RNA (RNA2 if recombination occurs during positive strand synthesis) can interfere with the formation of appropriate recombination intermediates *via*, for instance, inhibiting the formation of the "bubble" structure. Alternatively, or in addition, the landing replicase (presumably in association with the nascent strand) might have difficulties during re-initiating the strand synthesis on the acceptor RNA due to the close downstream location of GC-rich region. The latter might form a rather stable intermolecular base-paired structure that can be difficult to unwind during replicase-landing and/or re-initiation (Fig. 7) (Nagy & Bujarski, 1997).

The above studies contributed to our understanding of RNA sequence/structure requirements of recombination in BMV and defined for the first time the differences between homologous and nonhomologous recombination. They allowed for prediction of recombination hot-spots (Nagy *et al.*, 1998) and also gave us highly efficient recombination systems in which the role of replicase proteins could be tested (see below).

Mechanism of recombination: *trans*-acting factors

Protein 1α-helicase

Comparison of RNA sequences and structures at the crossover sites, the distribution (location) of crossovers, the presence of non-templated nucleotides at the crossover sites in some recombinants, all suggested a replicase-driven TS mechanism for both homologous and nonhomologous recombination. Further evidence for the role of replicase proteins in recombination was obtained by using BMV genomic RNAs with mutations within their open reading frames. First, three viable 1a protein mutants with two amino-acid insertions within the putative helicase region were tested using the heteroduplex system of Fig. 3A. The recombinants isolated from infections with one of the mutants (designated PK19 RNA1 that is temperature sensitive in replication) showed a characteristic 5′ shift in crossover sites towards energetically less stable portions of the RNA1-RNA3

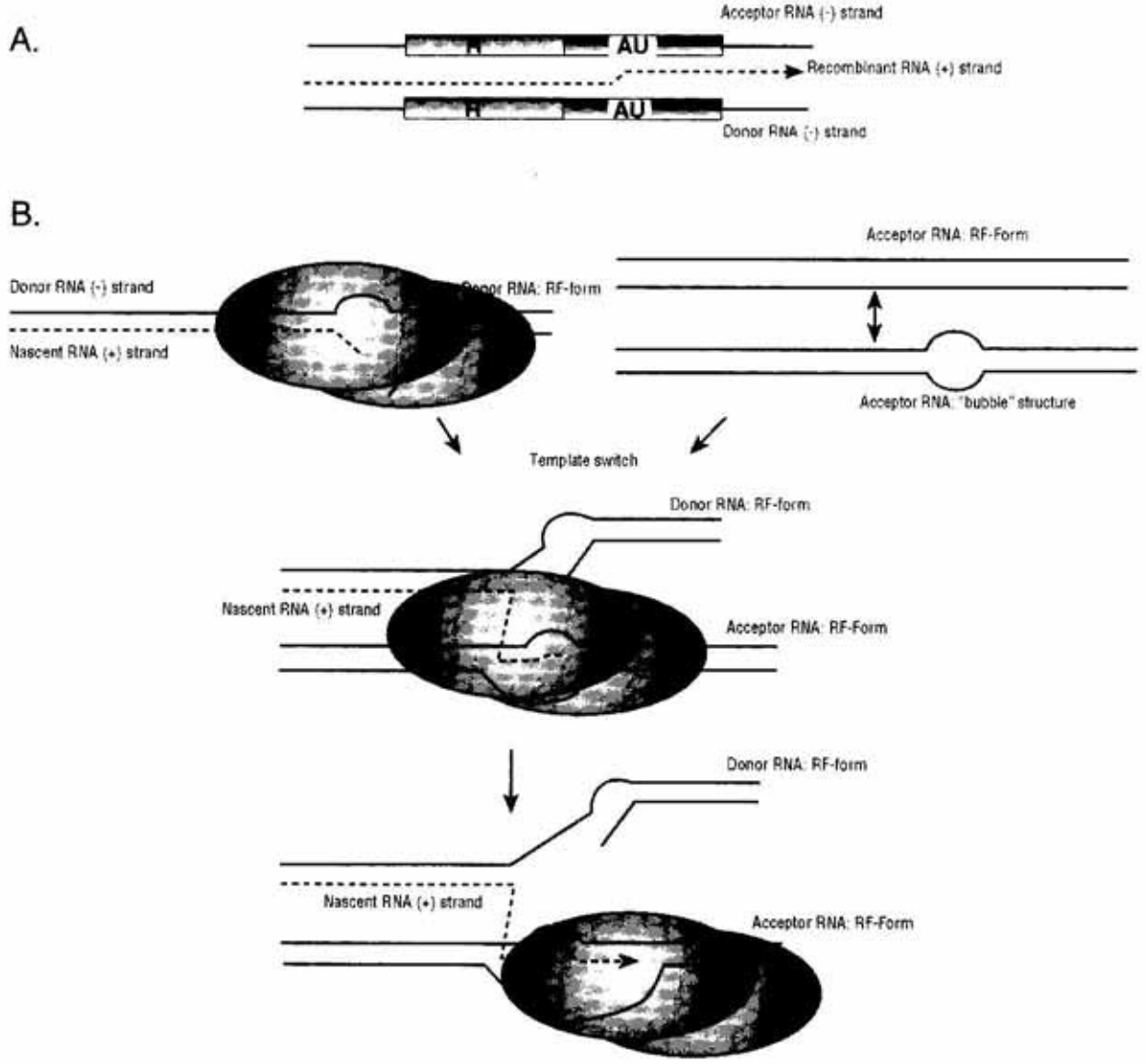

Figure 7. A BMV replicase template switching model of homologous recombination. (A) The RNA strands and regions that are postulated to participate in the recombination events. (B) The proposed structure of the recombination intermediates during the homologous recombination event.

The BMV replicase is thought to switch templates in the AU-rich regions that can form temporary "bubble structures". The GC-rich or average-content regions are postulated to stabilize the recombination intermediate formation *via* base pairing between the aborted nascent strand and the acceptor strand. See further details in the text.

heteroduplex at the elevated (but still permissible) temperature. This suggested that the helicase domain of 1a fails to carry out unwinding of (or pauses at) heteroduplex regions. When the progress of the RdRp on the original template is temporary impeded, the enzyme might have an increased opportunity to switch and copy the adjacent strand present in the heteroduplexed RNAs (Nagy *et al.*, 1995). The three helicase mutants tested at the permissive temperature showed different alterations in the distribution of recombination sites and/or variable effects on the incidence of nontemplated nucleotides (mainly U residues) at recombination sites.

Another interesting observation with these helicase mutants was that they all increased the frequency of recombination as compared to wt RNA1. RNA1/RNA3 recombinants were generated, though only at low 5% level, with

an RNA3 mutant [PN5(−)] carrying only 20 nt sequence complementary to RNA1 when using PK19 RNA1 at the elevated temperature, but not with the wt RNA1, further strengthened the above conclusion (Nagy & Bujarski, 1997).

Protein 2a–RNA polymerase

A single viable amino-acid mutation within the core domain of the polymerase, protein 2a (designated DR7), had the opposite effect on the heteroduplex-mediated nonhomologous recombination because it inhibited the frequency of recombination below the level of detection (Figlerowicz *et al.*, 1997). In contrast, the frequency of homologous events was comparable in infections with DR7 or wt RNA2. However, the frequency of imprecise homologous recombinants was doubled and also the crossover sites were shifted towards 5′ regions in DR7 infections, as compared to wild type.

Two viable mutations (designated MF-II and MF-V) in the N-terminal portion of the 2a protein, that is known to interact with the C-terminal domain of 1a were generated and tested in recombination. The heteroduplex-mediated nonhomologous recombinants were generated at reduced frequency in infections with either MF-II or MF-V as compared to wt RNA2. This and the above data with DR7 demonstrated that mutations in the 2a protein can reduce the frequency of heteroduplex-mediated recombination. Using the above RNA2 mutants in the homologous recombination system revealed that in the presence of MF-II, homologous recombinants were generated at a high frequency. In contrast, in infections with MF-V RNA2, the homologous recombination frequency was reduced markedly, as compared to wild type. (Figlerowicz *et al.*, 1998). Also, a 3′ shift in distribution of junctions was observed in homologous recombinants obtained with MF-V. Thus, the nature and characteristics of both homologous and nonhomologous recombinants were influenced by mutations in the polymerase gene of BMV. This gives a crucial support for a replicase-driven TS mechanism operating in both systems.

A noteworthy observation of the above studies is that mutations in the 2a replicase gene of BMV can influence the frequency of homologous and nonhomologous recombination differently. This further supports the above conclusions that the two recombination types utilize different recombination intermediates. Also, in general, it was more difficult to find RNA2 mutants that supported reduced frequency of homologous recombination than those inhibiting the frequency of nonhomologous recombination, when compared to wt RNA2. We speculate that the extensive base pairing between the nascent strand and the acceptor strand at the replicase "docking site" stabilizes the homologous recombination intermediates ("recombinosome", see below), while similar base pairing does not exist for the heteroduplex system. We propose the term "recombinosome" to describe a complex between the recombining RNAs, the replicase and other (putative) factors involved in recombination. The stability of the recombinosome (the time required for TS events before the disassembly of the recombinosome takes place) is likely influenced by both the RNA components and the replicase.

In conclusion, these data demonstrate that different domains of the polymerase 2a protein are responsible for and/or affecting differently BMV replication, nonhomologous recombination and homologous recombination. This opens up the possibility to engineer recombinant RNA viruses which will recombine at reduced (or non-detectable) level, thus increasing the stability as well as safety of viral expression vectors.

Further studies

Testing various 1a and 2a BMV replicase mutants is being continued in order to identify/map the sites on these proteins that are

involved in RNA recombination. We predict that many of these mutants will be suitable for biochemical characterization and will allow us to understand the mechanism of recombination. For instance, with these studies it should be possible to find out whether genetic recombination is needed for the maintenance of BMV RNA genome during virus life cycle. One can assume that the virus utilizes homologous recombination for repairing its genomic RNA and to remove RNA replication errors. Thus, it should be useful to check BMV replicase mutants in passaging through host plants which would allow us to test the accumulation of errogenous variants during virus replication.

It is important to study whether host (cellular) factors participate in recombination. It is likely that host factors are the crucial components of BMV replicase, and as such they may influence the template switching properties of viral replicase complex. Since these factors differ among the hosts, they may cause differences in the ability of RNA viruses to undergo RNA recombination.

We plan to study host factors using well characterized genetic systems. One such system is represented by *Arabidopsis thaliana*: apparently some of the *Arabidopsis* ecotypes can function as hosts for BMV. We will study the frequency of BMV RNA recombination in various *Arabidopsis* ecotypes. Subsequent mapping of participating gene(s) should allow us for further understanding of the relationship of recombination pathways and the host. Another potential system to be employed for these purposes is *Saccharomyces cerevisiae*, the yeast. Yeast has been demonstrated to support BMV replication. The use of well characterized yeast mutants should mapping of the participating host factors.

Possible practical applications of the host studies would include looking for such a host variant that does not support virus recombination. One may expect that the lack of recombination abilities not only prevents the formation of new virus strains but also may erradicate infectious viral agents due to accumulation of errogeneous variants.

CONCLUSIONS AND PERSPECTIVES

Positive-stranded RNA viruses cause many diseases in humans, livestock and crop plants. Genetic RNA recombination is a topic of intrinsic interest for scientists studying biodiversity, evolution, mutation and replication of these viruses. Some unanswered questions are: why do certain RNA viruses show rapid recombination or produce defective interfering (DI) RNAs (King, 1988; Roosinck, 1997) while many others recombine infrequently. The expression of viral sequences in transgenic organisms raises many questions about recombination between distantly related viruses. This is considered a risk to the environment as well as to human and animal health. There is an urgent need for general criteria describing recombination so the chance of the occurrence of new viral pathogens can be predicted. Quite often, dangerous viral infections are caused by newly emerging strains (species) due to an unbalanced host/virus adaptation.

Our study will not only allow for a better understanding of the above problems but will help to solve them. For instance, characterization of the recombinationally active sequences might provide greater utility for directing crossovers into specific RNA targets during viral infection (Nagy & Bujarski, 1993), or for stabilization (destabilization) of desired (undesired) RNA sequences. Also, understanding the mechanism of RNA recombination may help researchers to predict the occurrence of new viral pathogens, to deliver genes in such a way that makes the generation of (unwanted) recombinants unfavorable, or, quite the reverse, to destabilize unwanted viral or host messages.

The BMV recombination system offers unique opportunities to study the mechanism of RNA recombination. Unlike any other

known RNA recombination system, BMV supports three types of crossovers: homologous, imprecise (aberrant) homologous, and nonhomologous. This permits not only separate studies on these distinct events but also their direct comparison. The tripartite character of the BMV RNA genome provides a useful means for distinguishing between the effects coming from the donor and from the acceptor RNA molecules. There is a great deal of tolerance for modifications within the 3′ noncoding regions of BMV RNAs, which allows for the construction of the RNA recombination vectors. By using these vectors we have demonstrated for the first time that homologous crossovers require different sequence features than nonhomologous events, and that the sites of crossovers can be targeted with appropriate signal inserts. The extensive knowledge of BMV RNA replication makes the BMV system more likely to advance quickly in studying the phenomenon of RNA recombination.

BMV serves as a model virus for recombination in other alphavirus-like viruses (that include important pathogens of humans, animals and plants). Indeed, nonhomologous recombinants obtained with Sindbis virus were similar to those observed in BMV (Weiss & Schlesinger, 1991). Also, the proposed role of local heteroduplexes in poliovirus RNA recombination (Romanova *et al.*, 1985; 1986) has been confirmed by using BMV.

The experimental RNA recombination systems in many other RNA viruses have not reached the level of development that has been reached for BMV. This is partly because some systems (e.g., picornaviruses, $\Theta\beta$) rely on crossovers within large homologous sequences so the exact recombination sites cannot be determined, and a strong selection for functional ORFs does not allow for visualization of less precise crosses. For other systems, e.g. coronaviruses, full-length transcribable cDNA clones are not yet available and the virus is not well characterized molecularly; poliovirus genomic RNA does not tolerate modifications within the key elements involved in recombination; influenza virus does not support recombination very efficiently; and in TCV, recombination requires promoter-type sequences, thus representing special site-directed events.

In general, the specific characteristics of RNA recombination may depend upon many features, including those related to the RNA structure, to the properties of viral RdRp (e.g., RNA/replicase binding, the effectiveness of template unwinding (helicase activity), the efficiency of the RNA catalytic site, the RdRp host factors), or to other viral or cellular RNAs or proteins. For instance, there are data revealing a host dependence of RNA recombination (King, 1988; Lai 1992). This might reflect the differences in the properties of viral replicase due to the variability of host factors. In BMV, our data reveal that not all homologous sequences can support homologous recombination apparently reflecting the existence of features/factors in this process. Also, not every complementary sequence can support high frequency of nonhomologous recombination. It is then imperative to continue with characterization of these factors to progress with our understanding of the mechanism(s) of recombination.

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