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Review

How RNA viruses exchange their genetic material★✪

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One of the most unusual features of RNA viruses is their enormous genetic variability. Among the different processes contributing to the continuous generation of new viral variants RNA recombination is of special importance. This process has been observed for human, animal, plant and bacterial viruses. The collected data reveal a great susceptibility of RNA viruses to recombination. They also indicate that genetic RNA recombination (especially the nonhomologous one) is a major factor responsible for the emergence of new viral strains or species.

Although the formation and accumulation of viral recombinants was observed in numerous RNA viruses, the molecular basis of this phenome non was studied in only a few viral species. Among them, brome mosaic virus (BMV), a model (+)RNA virus offers the best opportunities to investigate various aspects of genetic RNA recombination *in vivo*. Unlike any other, the BMV-based system enables homologous and nonhomologous recombination studies at both the protein and RNA levels. As a consequence, BMV is the virus for which the structural requirements for genetic RNA recombination have been most precisely established. Nevertheless, the previously proposed model of genetic recombination in BMV still had one weakness: it could not really explain the role of RNA structure in nonhomologous recombination. Recent dis-

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Abbreviations: BMV, brome mosaic virus; FMDV, foot-and-mouth disease virus; MHV, mouse hepatitis virus; TCV, turnip crinkle virus; RAS, recombinationally active sequence; HIV RT, human immunodeficiency virus reverse transcriptase; wt, wild type; nt, nucleotide.

cov er ies con cern ing the lat ter prob lem give us a chance to fill this gap. That is why in this re view we pres ent and thor oughly dis cuss all re sults con cern ing nonhomologous re com bi na tion in BMV that have been ob tained un til now.

RNA viruses and retroviruses are the only known liv ing spe cies which use RNA to store their genetic information. Another feature com mon to both types of vi ruses is their enormous ge netic vari abil ity [1, 2]. There is a lot of data sug gest ing that an in di vid ual RNA vi rus or retrovirus does not form a homogenous population but rather a set of different viral variants. That is why, according to some authors, the term spe cies used to clas sify higher organisms does not apply to RNA-based viruses [3–5].

Sev eral pro cesses con trib ute to the con tin uous generation of new viral variants. Point mutations are most frequently introduced into the viral genome during the replication pro cess (be cause of the low fi del ity of RNA dependent polymerases that lack proofreading activity) [6–10] while RNA recombination is re spon si ble for some more pro found changes within the viral genome (sequence deletion/insertion or strand exchange) [11–14]. The latter process was observed for human, animal, plant and bacterial viruses [12]. Exchange of the genetic material between the same viruses, between different viral strains or be tween dif fer ent vi ruses has been dem onstrated. Additionally, it was shown that viral RNA can re com bine with host RNA [15, 16] as well as with transgenic mRNA that is formed in cells expressing viral genes [17]. The data above revealed that RNA recombination is a ma jor fac tor re spon si ble for the emer gence of new, of ten dan ger ous vi ral strains or spe cies.

Basing on the struc ture and func tion of RNA molecules, two general types of genetic RNA recombination were distinguished: homologous and nonhomologous [12]. Homologous RNA re com bi na tion in volves two iden ti cal or similar molecules and is called precise if recombinant junction sites are located ac curately at the cor re spond ing nu cleo tides or imprecise when the junc tion sites oc cupy dif ferent positions within recombining molecules. As a re sult of pre cise cross overs pa ren tal mol e cules are re gen er ated, whereas im pre cise recombination produces molecules in which some sequences are duplicated or deleted. Nonhomologous re com bi na tion cross overs oc cur between two different RNA molecules, and so the resultant recombinants differ significantly from the parental molecules.

Despite extensive studies, the molecular mech a nism of RNA re com bi na tion is still not well understood. Most of the data collected with different experimental systems suggest that recombination crossovers occur according to the copy-choice hy poth e sis [11, 18–20]. This hy poth e sis pos tu lates that recombinants are formed dur ing rep li ca tion, when the vi ral replicase-nascent strand complex switches from one RNA tem plate (called RNA do nor) to an other (called RNA ac cep tor). The mo lec u lar basis of template switching events is better understandable if they involve two homologous RNA mol e cules, when the na scent strand syn the sized on the RNA do nor is com ple mentary to the RNA acceptor [21, 22]. The problem becomes more complicated if nonhomologous recombination is taken into consid er ation. Ac cord ing to the def i ni tion, re com bi na tion of this type oc curs be tween two RNA molecules of different sequences. The gen erated recombinants differ distinctly from the parental molecules, and being dysfunctional, they rarely ac cu mu late *in vivo*. Some data suggest that viral polymerases use promoter-like struc tures to switch from the do nor to the acceptor tem plate [23]. Other re sults em pha size the role of lo cal hy brid iza tion (for ma tion of local heteroduplexes) between recombining molecules [24, 25], leader sequences [26] or template breakage [11, 27]. Recently, it was shown that in some cases RNA can re com bine according to an alternative mechanism, that is by template breakage and rejoining [28]. First, cleav age of the vi ral ge nome gen er ates two RNA fragments. Next the 3′ end of the

newly formed molecule can be joined with another RNA in the ligation or transesterification process.

During the last decade several experimental systems for RNA recombination studies have been developed. However, only that created with brome mosaic virus (BMV) reached the level enabling comprehensive investigations of nonhomologous and homologous recombination at both the RNA and protein levels. As a result, the BMV-based system was applied to elaborate a model of genetic recombination in (+)RNA viruses (described in details in our earlier review [22]). This model presumes that template switching events are mediated by viral replicase and it well explains how homologous recombination is affected by the RNA structure. However, the data collected at that time did not entitle us to make some general conclusions on the role of RNA structure in nonhomologous recombination. Recent discoveries concerning the latter problem let us significantly improve our model by supplying its missing part. That is why in this review all the available results concerning the role of RNA structure in template switching by BMV replicase between nonhomologous RNA molecules are extensively discussed, whereas other aspects of genetic RNA recombination that were thoroughly described in several earlier reviews [12, 14, 22, 29] are only shortly presented.

EXPERIMENTAL SYSTEMS FOR GENETIC RNA RECOMBINATION STUDIES

Although genetic RNA recombination has been well documented in numerous RNA viruses, its molecular mechanism has been examined in only a few viral species. Such a situation well illustrates how difficult it is to create an efficient system for genetic RNA recombination studies. To investigate the molecular basis of this process we use an especially well-established experimental system developed with BMV. But before we present our recent observations concerning genetic RNA recombination in BMV we would like to describe shortly the most important results obtained with other viruses.

RECOMBINATION IN POLIOVIRUS AND FOOT-AND-MOUTH DISEASE VIRUS (FMDV)

Both poliovirus and FMDV belong to picornaviruses — small, icosahedral, single-stranded (+)RNA viruses infecting humans and animals [30]. Investigations conducted with picornaviruses are of special historical importance. They created proper grounds for further RNA recombination studies, causing recombination to become a recognized fact in the RNA world. Already in the early 1960s it was shown that mixed infections with two strains of poliovirus, each carrying a specific genetic marker, resulted in progeny exhibiting simultaneously both features characteristic to parental viruses [31, 32]. A similar phenomenon was observed for FMDV [33]. The obtained data suggested that about 10–20% of viral genomes undergo recombination during a single replication cycle [29]. Moreover, Kirkegaard and Baltimore discovered that suppression of poliovirus genome replication inhibits RNA recombination [18]. This observation provided the first experimental evidence supporting the copy-choice mechanism of RNA recombination. The proposed model for genetic recombination in picornaviruses assumes that template switching events occur preferentially but not exclusively during the (–) strand synthesis. If RNA polymerization is interrupted (because of RNA secondary structure or nucleotide misincorporation), incomplete RNA may leave the donor template and its synthesis can be resumed on another template. Recombination crossovers are roughly randomly distrib-

uted along genomic RNA, while recombination frequency strongly depends on the extent of similarity between parental RNAs [34, 35].

RECOMBINATION IN MOUSE HEPATITIS VIRUS (MHV)

MHV is a member of coronaviruses – single-stranded (+)RNA viruses with an extremely large non-segmented genome (from 27 to 31 kb) comprising 7 to 10 genes [30]. Studies involving MHV disclosed a very interesting but, at the same time, complicated picture of genetic RNA recombination in coronaviruses. In spite of some differences in genome organization, each coronavirus encodes a huge RNA-dependent RNA polymerase of about 750–800 kDa [30]. In fact, this protein should be classified as a multienzymatic complex as it displays several activities needed for viral genome expression and replication. In coronaviruses, each gene is expressed from a separate mRNA. Interestingly, mRNA molecules are synthesized in discontinuous transcription resembling the RNA recombination process. First, RNA polymerase synthesizes a 70–90 nt leader sequence (derived from the 5′-end of genomic RNA). Then the polymerase-leader complex leaves the template and restarts RNA synthesis on one of the intergenic transcription promoters. The resultant mRNAs have a leader sequence at the 5′-end and are 3′-coterminal. However, only the first gene located at the 5′-end of mRNA is used as a template for protein synthesis [29]. The above described mechanism of coronavirus mRNA formation by discontinuous transcription presumes that viral polymerase is naturally selected to mediate template switching events. Indeed, studies involving MHV demonstrated that about 25% of genomic molecules are recombinants [36, 37]. They are most likely formed according to the copy-choice mechanism [12]. Especially frequent recombination crossovers are observed in the MHV hypervariable region (within the envelope protein encoding sequence) [38]. It was postulated that recombination is induced by RNA polymerase pausing on the donor template. The coronavirus polymerase may pause because of RNA secondary structure, RNA breakage or a protein binding to the donor template. However, the question of the factor that transfers the polymerase-nascent strand complex to the acceptor template is still open [12]. At present, several possibilities are taken into consideration. The polymerase-nascent strand complex may recognize specific RNA motifs imitating transcription promoters or it may bind to the RNA–protein complex on the acceptor template. In the latter case, recombination can be mediated by RNA–protein or protein–protein interactions [12].

RECOMBINATION IN TURNIP CRINKLE VIRUS (TCV)

TCV, a small spherical virus of plants is a member of carmoviruses. Its genome is composed of a single-stranded (+)RNA molecule, 4 kb in length [30]. In addition, the TCV genome is frequently accompanied by subviral RNA, i.e. satellite RNA (sat-RNA) and defective interfering RNA (DI-RNA), which require the genomic molecule for replication and packaging [39–41]. Simon and coworkers demonstrated that all three kinds of RNA (genomic, sat- and DI-RNA) can participate in the recombination process [42–44]. They also found that recombination crossovers are not distributed randomly along the TCV genome (as in picornaviruses) but they are clustered only in some regions [43, 45]. The undertaken analysis of recombinant junction sites revealed the role of specific RNA sequences and structural motifs in recombination [23, 43, 45]. As a result, it was proposed that TCV recombinants are formed according to the copy-choice hypothesis during (+)RNA strand synthesis. Initially, the replicase-nascent strand complex is released from the donor template. Then it

binds to one of the promoter-like structures present on the RNA acceptor and resumes nascent strand elongation on the new template. In addition, local hybridization between the 3′-end of the nascent strand and RNA acceptor may enhance recombination events, although it is not definitely required [23, 45].

RECOMBINATION IN OTHER RNA VIRUSES

There are several other RNA viruses in which genetic recombination was investigated and some interesting, preliminary observations were made. Mindich and coworkers found that in bacteriophage Φ6 (double-stranded RNA virus with a three-segmented genome) recombination may occur inside procapsids, where viral polymerase synthesizes dsRNA using (+)RNA strand as a template [46, 47]. Recombination events involve different RNA segments since they were classified as nonhomologous, although recombinant junction sites are usually located within short regions of homology between recombining molecules [46, 47]. Similar observations were made during nodavirus studies [48]. However, in the latter viruses two other factors, in addition to local homology, seem to influence template switching by viral polymerase: template secondary structure, which may bring recombinant junction sites close together, and promotor-like sequences, which can directly bind viral polymerase. Different results were obtained by Raju and coworkers who found that in Sindbis virus homologous sequences are not required for recombination to occur [49]. Recombinant junction sites are randomly distributed along donor and acceptor templates in a similar way as in picornaviruses.

In general, most of the data collected suggest that RNA recombination occurs according to the copy-choice hypothesis. The location of recombinant junction sites and recombination frequency depend on the specific properties of viral replicases and on the primary and secondary structure of the recombining molecules.

BMV-BASED RECOMBINATION SYSTEM

Brome mosaic virus is a positive-sense RNA virus of plants [50]. The BMV genome is composed of three RNA molecules called RNA1, RNA2 and RNA3. All three BMV RNAs possess an almost identical 3′-noncoding region with a tRNA-like structure at the very end. RNA1 and RNA2 encode BMV replicase proteins 1a and 2a, respectively, while RNA3 encodes movement and coat proteins. BMV was the first plant RNA virus for which genetic RNA recombination was observed [51]. It was demonstrated that BMV can support the formation of both types of recombinants: homologous [52] and nonhomologous [25]. These observations allowed the development of an efficient BMV-based recombination system described in detail in Fig. 1.

ROLE OF BMV-POLYMERASE IN RNA RECOMBINATION

The models of homologous and nonhomologous recombination (presented in Fig. 1) posited that recombinants are formed according to the copy-choice hypothesis [25, 52]. Both mechanisms postulated the involvement of viral polymerase in strand transfer, although at that time there was no evidence confirming this idea.

To find some conclusive data supporting the mechanisms above, we tested whether mutations within BMV encoded polymerase (2a protein) might influence the recombination process. First, basing on the available biochemical and crystallographic data, five motifs conserved in RNA dependent RNA or DNA polymerases were recognized in the 2a protein. To determine which of them partici-

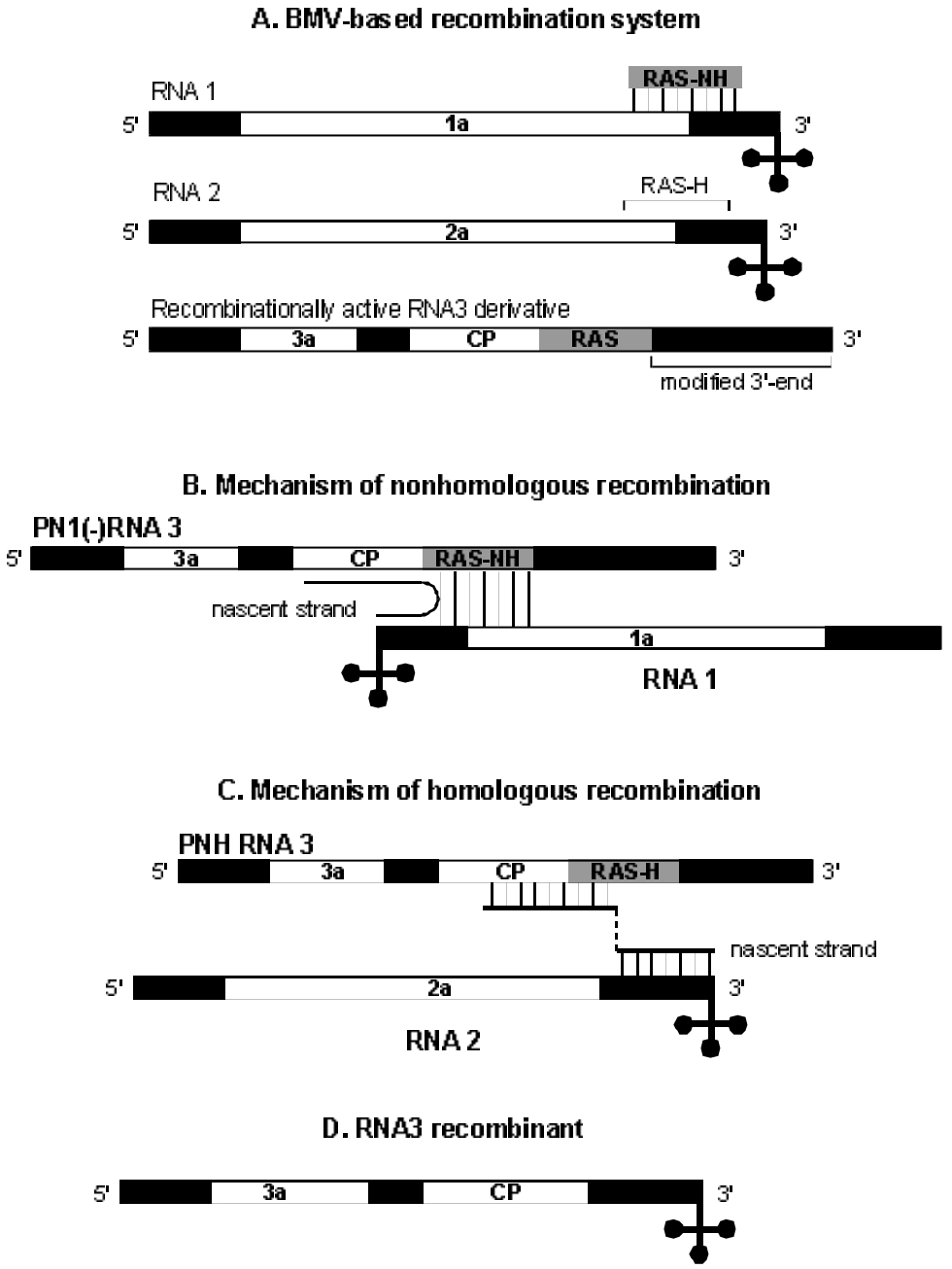

Figure 1. BMV-based recombination system.

Particular fragments of the BMV genome are marked as follows: 3′, 5′ and intercistronic noncoding regions are black; coding regions are white; recombinationally active sequences introduced into the RNA3 recombination vector are gray. **A**. Our studies of genetic RNA recombination involve specially prepared BMV mutant, in which the 3′-noncoding portion of RNA3 was modified (modified RNA3 is called a recombination vector). Mutated virus possesses the entire genetic information necessary for BMV development (its genome is composed of wtRNA1, wtRNA2 and RNA3 in which the coding and 5′-uncoding regions are unchanged), but replicates and accumulates to a visibly lower level than the wt form. Such a BMV mutant is stable in infected cells and starts to recombine if a recombinationally active sequence (abbreviated RAS) is inserted into the vector molecule just between the coding region and modified 3′-end. **B. Nonhomologous recombination**. Frequent nonhomologous recombination crossovers were observed when a 140 nt sequence complementary to the 3′-portion of RNA1 (sequence called RAS-NH) was introduced into the recombination vector. The presence of RAS-NH in the RNA3 derivative (named PN1(–)RNA3) allowed local RNA1-RNA3 hybridization (duplex formation) that efficiently mediated recombination events. The proposed mechanism of nonhomologous recombination between genomic BMV RNAs postulates that recombinants are formed according to the copy choice hypothesis. BMV polymerase cannot unwind the local RNA1-RNA3 duplex and switches from the donor to the acceptor template. **C. Homologous recombination.** Ho-

pate in RNA recombination, selected regions of 2a were mutagenized. Modifications were introduced within the polymerase catalytic center and the putative fingers domain (involved in RNA-protein interactions). Moreover, the N-terminal domain responsible for 1a–2a binding within BMV replicase was also mutagenized [19, 20].

Because recombinants are most likely formed during RNA replication, only those 2a protein mutants that did not affect the latter process were used for further studies. First, we found a single amino-acid mutation within the core polymerase domain of the 2a protein that inhibited nonhomologous RNA recombination without affecting the frequency of homologous crossovers [19]. This demonstrated that viral polymerase participates in the process studied and suggested that different mechanisms operate in homologous and nonhomologous recombination.

During further studies, two domains involved in genetic RNA recombination were identified: the N-terminal and putative "fingers" domains. Generally, our data indicate that by introducing specific modifications into viral polymerase one can influence the frequency of homologous and nonhomologous recombination as well as the location and precision of homologous crossovers [19, 20]. We also observed that the location of nonhomologous crossovers depends mostly on the structure of recombining molecules.

ROLE OF RNA STRUCTURE IN NONHOMOLOGOUS RECOMBINATION BETWEEN BMV RNAs

The model of nonhomologous recombination in BMV presumes that local hybridization between recombining molecules (RNA1/RNA3 heteroduplex formation) efficiently mediates template switching by the viral replication complex [25]. To increase our knowledge on the mechanism of nonhomologous RNA recombination large numbers of BMV recombinants were thoroughly examined [53]. All analyzed recombinants raised as a result of crossovers between wt RNA1 and modified RNA3 [19, 20, 25]. To obtain them ten various RNA3 derivatives were used: the previously described PN1(–) RNA3 (constructed by RAS-NH insertion into the recombination vector) and its nine derivatives serially named PN2(–) to PN10(–) RNA3 [25]. We observed that in most RNA1/RNA3 duplexes recombinant junction sites were distributed randomly within local double stranded regions. However, some duplexes supported recombination in a site-specific manner (Fig. 2) [25, 53]. For example, each infection with PN3(–) RNA3 resulted in recombinant A or B formation. They have the same length and differ from each other by a single nucleotide (GU**CU**CC and GU**CC**CC, respectively) at the junction sites.

In order to identify the structural motifs involved in nonhomologous recombination between BMV RNAs, the nucleotide sequences

Legend to Fig. 1. Continued

mologous BMV recombinants were generated when a 60 nt sequence from the 3′-portion of RNA2 (sequence called RAS-H) was inserted into the recombination vector. The presence of homologous sequences in RNA2 and RNA3 does not lead to their interaction, however, the nascent strand synthesized on the donor template can be complementary to the acceptor. The postulated mechanism of homologous recombination in BMV assumes that homologous recombinants are formed when viral replicase is paused within the region of local homology between RNA2 and RNA3 and the 3′-end of the newly synthesized RNA is released. During the next step, the 3′-end of the nascent strand hybridizes to the acceptor template and BMV replicase uses it as a primer to resume RNA synthesis. **D. BMV recombinants.** Both nonhomologous and homologous recombination repairs mutated virus. In the RNA3 recombinants generated the highly modified 3′-end is replaced by the 3′-noncoding fragment, conserved in all BMV RNAs, coming from RNA1 (nonhomologous recombination) or RNA2 (homologous recombination). Recombinants replicate and accumulate better than parental RNA3 molecules so that the latter are outcompeted from the infected cells.

of the parental and recombinant molecules within and at the vi cin ity of the junc tion sites were as sessed care fully. Re sults of the un dertaken anal y sis sug gested that nonhomologous recombination occurs in a site-specific manner if the lo cal RNA–RNA heteroduplex is accom pa nied by spe cif i cally po si tioned short ho mologous sequences (regions h in Fig. 3B) [53]. Re gions h are placed in such a way that the heteroduplex formed by re com bin ing molecules can adopt two alternative structures, ei ther a full-length du plex or a shorter du plex followed by a hairpin on RNA3. As shown in Fig. 3B, the formation of the hairpin brings both junction sites close to each other. This may not, how ever, be the only fac tor al low ing viral replicase to switch from one RNA template to an other. The hair pin formed when viral replicase begins to penetrate the RNA1–RNA3 heteroduplex may pause the BMV replicase, while short homologous sequences (10–11 nt region h) generate the complementarity be tween the na scent strand (syn the sized on the RNA1 do nor) and the acceptor template.

To de ter mine whether the same RNA structural motifs were involved in other re com bi nant formation the remaining recombination hot-spots found for wt RNA1 and PN1(–) to PN10(–) RNA3 were analyzed (Fig. 3C) [25, 53]. They were located within local double-stranded re gions, and gen er ated al most or completely symmetrical recombinants (re-

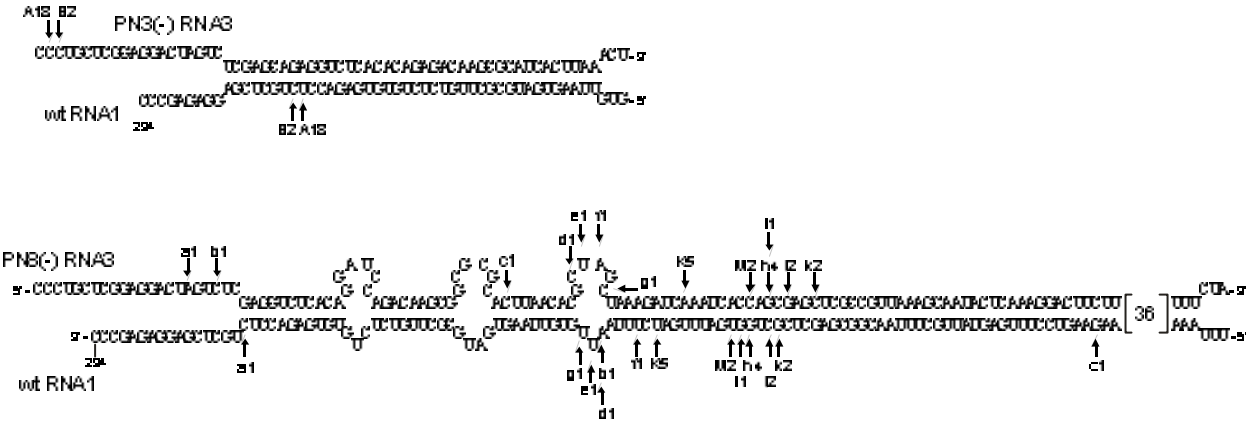

Figure 2. Distribution of the nonhomologous crossovers occur ring *in vivo* between BMV RNA1 and PN3(–) or PN8(–) RNA3.

In both heteroduplexes the up per se quence rep re sents the (+) strand of PN3(–) or PN8(–) RNA3 (the re gion where RAS is lo cated) while the lower se quence rep re sents the cor re spond ing (com ple men tary) frag ment of the (+)RNA1 strand. The junc tion sites of each re com bi nant are marked with ar rows (point ing to the last nu cleo tide com ing from wt RNA1 and the first nu cle o tide from the RNA3 de riv a tive) and with the same let ter. Num bers ac com pany ing the let ters in di cate how of ten the given re com bi nant was iden ti fied.

com binants for which both re com bi nant junction sites were lo cated ex actly at the base-paring nucleotides or were close to each other). How ever, only one re com bi nant (E) con tained a short com ple men tary se quence be tween the (+) na scent strand of RNA3 and (–)RNA1. The remaining most frequently observed recombinants had junction sites located close to each other within the A-U rich regions of the heteroduplex [19, 20, 25, 53].

In ad di tion, the pref er ence of BMV replicase to switch af ter cer tain nu cleo tides were in ves tigated [53]. It was found that in 42% of recombinants a U was the last nu cle o tide coming from the do nor tem plate, in 30% it was an A while only in 18% and 10% it was a C or a G, respectively. Apparently, template switching

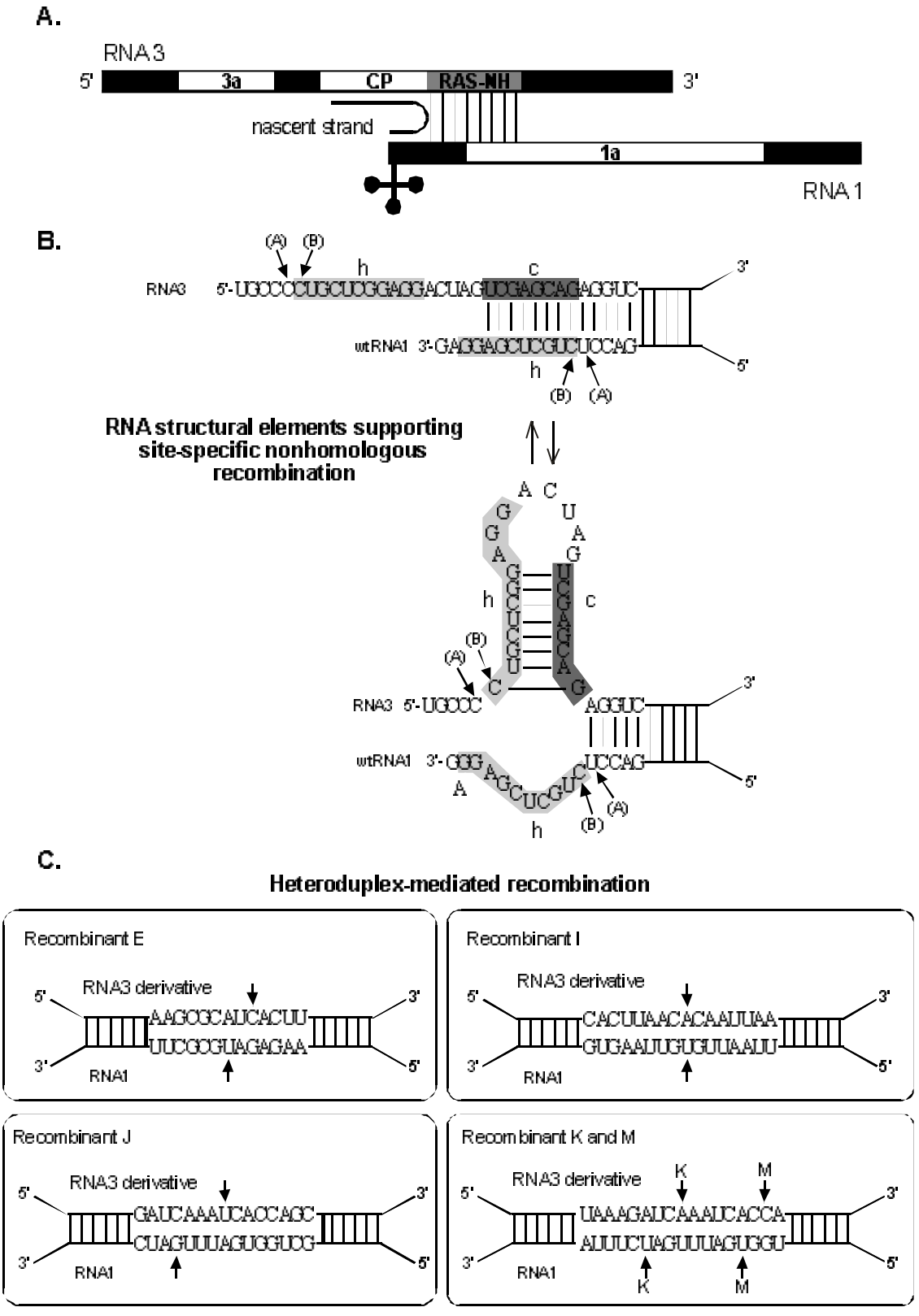

Figure 3. RNA structural elements supporting site-specific and heteroduplex-mediated nonhomologous recombination.

A. A schematic description of the RNA1–RNA3 heteroduplex that mediates recombination crossovers. **B.** Site-specific nonhomologous recombination. Sequences involved in site-specific recombination are highlighted, while the remaining fragment of the heteroduplex is represented by lines (the total length of the RNA1/RNA3 duplex may vary from 30 to 140 nt). Hybridization between RNA molecules is depicted by short lines. It was observed

by BMV replicase most frequently occurs after nucleotides forming the weaker A-U base-pairs (72% of crossovers). An analogous dependence was not observed for the first nucleotide coming from the acceptor template: in 48% of recombinants it was an A or a U while in 52% of recombinants a G or a C [53].

In general, the undertaken analysis suggested that there are two different types of nonhomologous recombination: site-specific, which generates asymmetrical recombinants A and B, and heteroduplex-mediated, producing almost or completely symmetrical recombinants. The former occurs if the local RNA–RNA heteroduplex is accompanied by specifically positioned short homologous sequences, while the latter depends on local RNA–RNA hybridization only [53].

In order to obtain experimental evidence supporting the observations above, a new RNA3 derivative named Mag1-RNA3 was made [53]. It was prepared by inserting a 137 nt portion of RAS-NH into the RNA3 recombination vector. As a result, Mag1-RNA3 and RNA1 were able to form a local double-stranded structure possessing all the putative elements supporting both heteroduplex-mediated and site-specific nonhomologous recombination (Fig. 4). Introducing specific modifications into Mag1-RNA3 we demonstrated that all three elements, i.e. the heteroduplex structure, short homologous sequences and a hair pin on RNA3 (which forms when BMV replicase unwinds a few first base-pairs of the heteroduplex) are required to target nonhomologous crossovers in a site-specific manner [53]. The data obtained with the Mag1-RNA3 derivatives also showed that the primary and/or secondary structure of the sequences involved in heteroduplex formation rather than the length of the double-stranded region plays the most important role in heteroduplex-mediated recombination [53]. In addition, our results suggested that sequences at the vicinity of the heteroduplex also influence the process studied.

NONHOMOLOGOUS RNA RECOMBINATION MEDIATED BY HUMAN IMMUNODEFICIENCY VIRUS REVERSE TRANSCRIPTASE

To determine how universal our observations are we decided to test if the identified elements of RNA structure are able to induce template switching by other viral polymerases. To this end, we created a suitable *in vitro* system in which recombinationally active regions of BMV RNAs were applied as donor and acceptor templates (Fig. 5) [54].

Among different viral polymerases that could potentially be used in our system, human immunodeficiency virus reverse transcriptase (HIV RT) was chosen. This enzyme is naturally selected to mediate recombination events, because HIV RT has to switch from one genomic RNA to another to produce double-stranded DNA that is integrated into the host genome. Additionally, the molecular

Legend to Fig. 3. Continued

that all RNA1–RNA3 heteroduplexes mediating site-specific recombination in BMV have a common left-hand portion that can adopt two different conformations. Recombining molecules can form a full-length duplex (the upper structure) or a shorter duplex with a hair pin on the RNA3 template (the structure below). Such structural flexibility results from the presence of short homologous sequences specifically positioned in recombining molecules (the shadowed sequences marked with an "h"). The region h is placed at the left-hand end of the heteroduplex in RNA1 and just before the heteroduplex in RNA3. The portion of RNA3 involved either in the heteroduplex or the hair pin stem formation is shadowed and marked with a "c". The arrows with letters indicate the junction sites of recombinants (A and B) generated as a result of site-specific crossovers. **C.** Heteroduplex-mediated recombination. Location of the junction sites (marked with arrows and with letters) found in recombinants that, beside A and B, were the most frequently identified during BMV infections involving the PN1(–) to PN10(–) RNA3 derivatives. Local RNA1/RNA3 heteroduplexes are represented by lines and the sequences near the recombination hot-spots are highlighted.

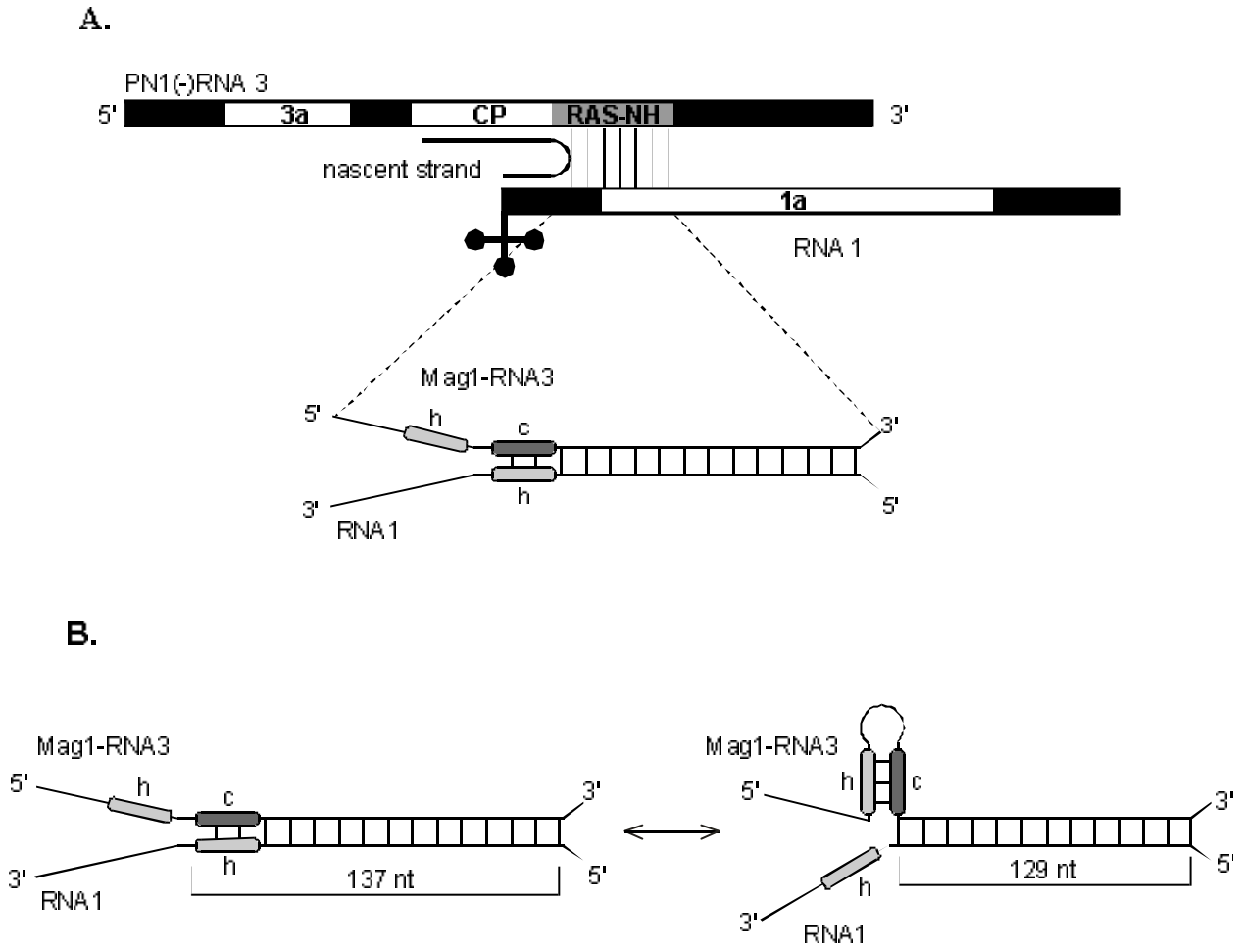

Figure 4. Construction of the universal recombinationally active Mag1-RNA3 derivative.

A. Mag1-RNA3 was prepared by inserting a 137 nt RAS complementary to RNA1 (between positions 2856 and 2992) into the recombination vector. As a result, Mag1-RNA3 and RNA1 were able to form a local double-stranded structure possessing all the putative elements supporting both heteroduplex-mediated and site-specific nonhomologous recombination. **B.** The left-hand portion of the RNA1/Mag1-RNA3 heteroduplex is identical to that of shown in Fig. 3B. That is why the heteroduplex can exist in two different conformations (as a full-length duplex or as a shorter duplex followed by a hairpin on the acceptor template).

structure of HIV RT has been solved by X-ray diffraction and that can be especially useful for further studies on the mechanism of RNA recombination.

The template switching abilities of HIV–RT were examined in primer extension reactions involving RNA donor (RNA1-NH) and acceptor (Mar1-RNA3 or its modified derivative) templates, and the donor template-specific primer A (see Fig. 5) [54]. An analysis of the reverse transcription products demonstrated that local hybridization between the donor and acceptor molecules (the heteroduplex structure supporting nonhomologous recombination in BMV [25]) pauses the primer extension reaction very effectively. However, the heteroduplex itself was not able to efficiently mediate *in vitro* recombination crossovers with HIV–RT. We observed that only those donor and acceptor templates which support site-specific crossovers in BMV ensured template switching by HIV–RT [54].

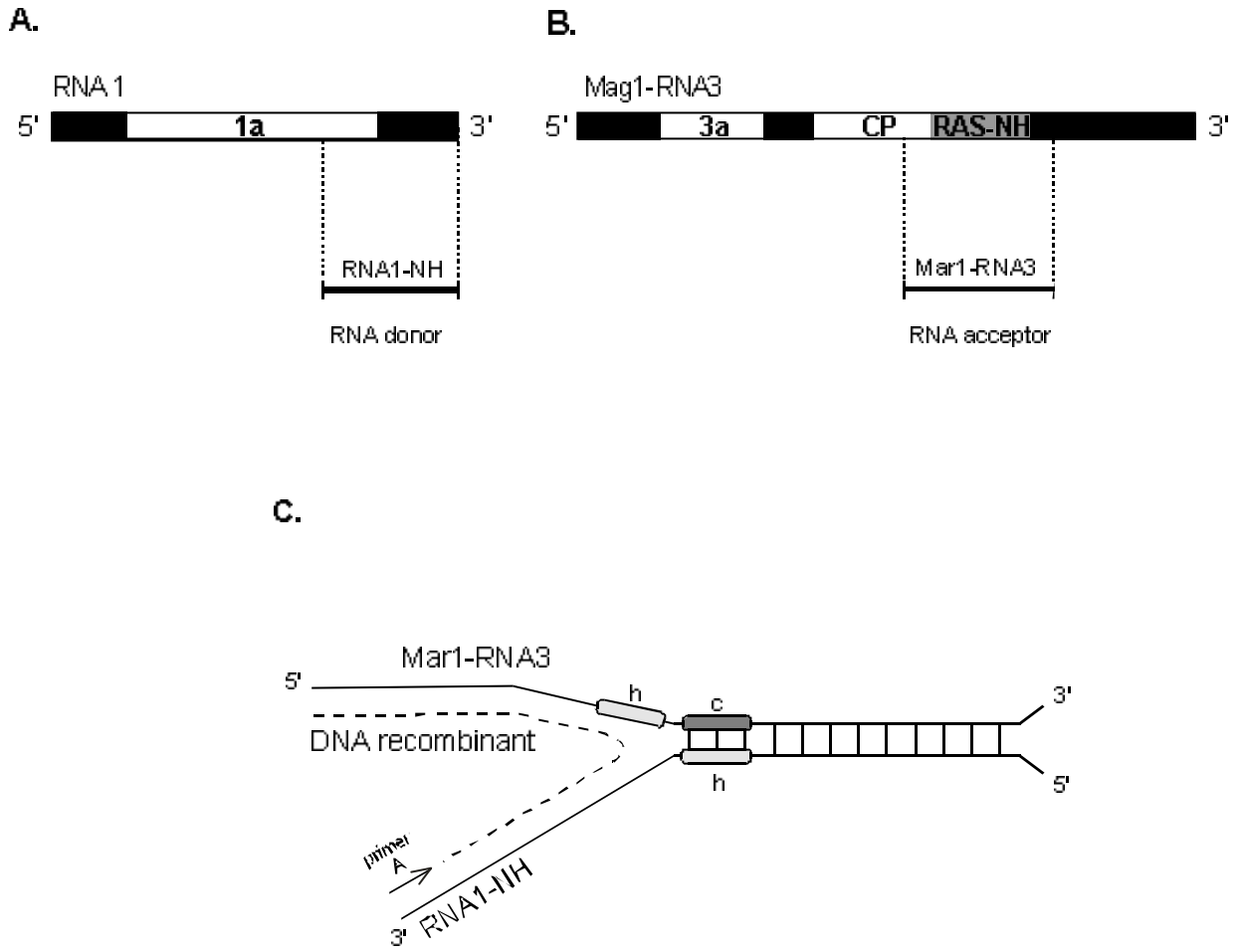

Figure 5. Nonhomologous recombination system *in vitro*.

The previously identified recombinationally active fragments of BMV RNAs were used in our system as donor (RNA1-NH) and acceptor (Mar1-RNA3) molecules. **A.** The RNA1-NH donor template corresponds to the 403 nt fragment of BMV RNA1 (between 3′ positions 1 and 403). **B**. The basic Mar1-RNA3 acceptor template is derived from Mag1-RNA3 (a 446 nt fragment of Mag1 RNA3). **C**. RNA1-NH donor and Mar1-RNA3 acceptor templates are capable of forming a local double-stranded region where, according to earlier data, site-specific and heteroduplex-mediated crossovers occur. The template switching ability of HIV–RT was tested in primer extension reactions involving the donor and acceptor molecules and the donor specific primer A (marked with an arrow). The acceptor and donor templates were constructed in such a way that the recombinants generated (dotted line) should be longer than each of the parental molecules (if formed according to the mechanism observed for BMV they should be at least 500 nt long). To examine the mechanism of template switching by HIV RT specific modifications were introduced into the Mar1-RNA3 acceptor molecule [54].

MECHANISM OF NONHOMOLOGOUS RNA RECOMBINATION

The data presented above suggest that BMV replicase and HIV–RT use an identical or similar mechanism to produce nonhomologous recombinants in a site-specific manner. The following scenario of BMV replicase/HIV–RT template switching is proposed to explain why both the presence and proper positioning of the identified RNA structural elements are required to generate recombinants (Fig. 6). RNA/DNA synthesis begins at the 3′-end of the donor template. BMV replicase/HIV–RT encounters the heteroduplex and unwinds a few first base-pairs, inducing hairpin formation on the acceptor template. Homologous sequences h are positioned in such a way that both heteroduplex unwinding and hairpin formation may occur simultaneously, allowing BMV replicase/HIV–RT to continue RNA/DNA strand elongation. However, the enzyme

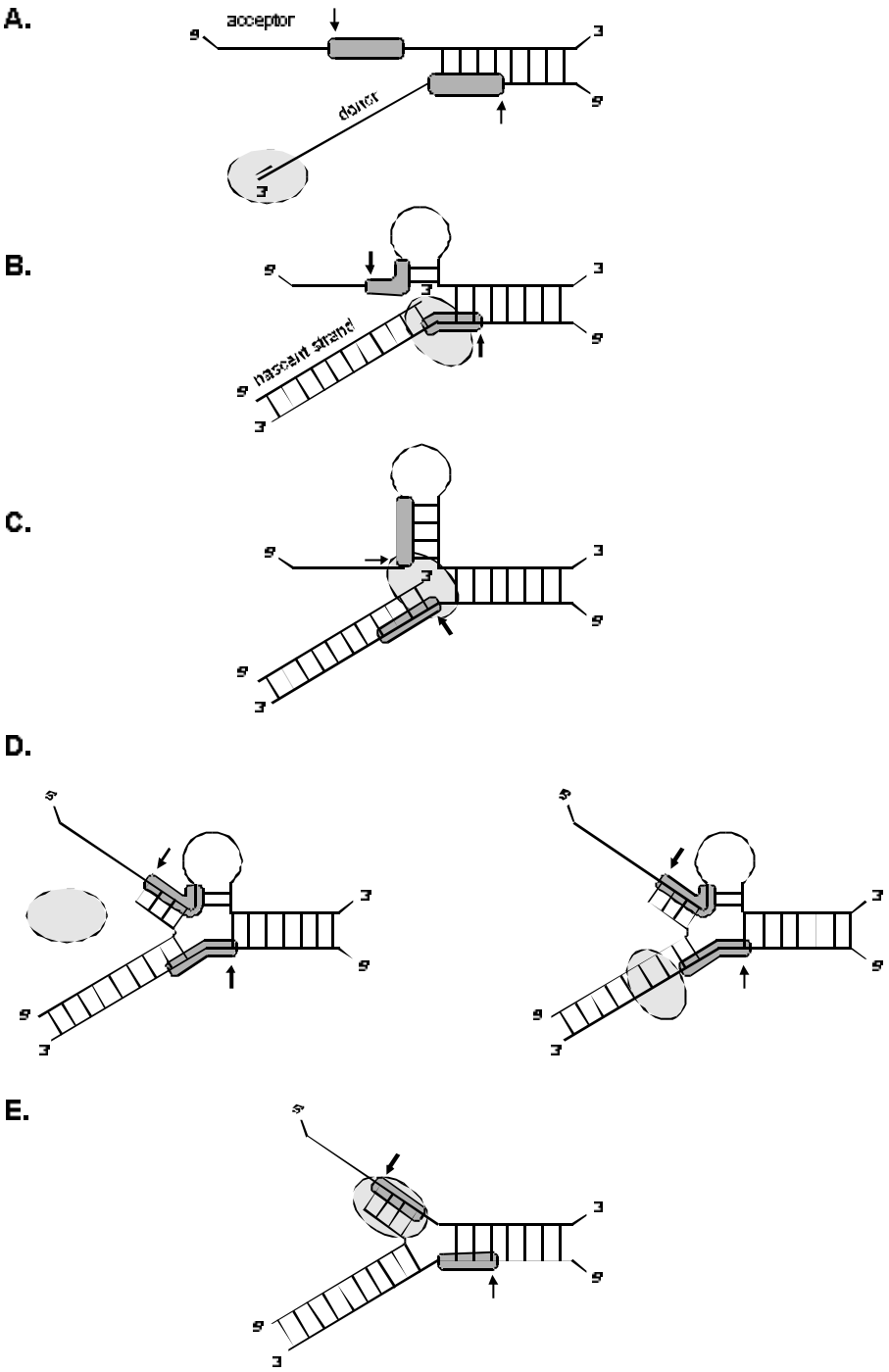

Figure 6. Putative mechanism of site-specific template switching by BMV replicase and HIV–RT.

Solid lines represent donor and acceptor RNA templates and the nascent strand (RNA or ssDNA). Short vertical lines symbolize strand hybridization. Homologous sequences present in the donor and acceptor templates are boxed. Arrows indicate the location of recombinant junction sites. **A**. Viral polymerase (symbolized by the gray oval) starts RNA or DNA synthesis at the 3′-end of the donor template. **B**. The enzyme encounters the heteroduplex structure and unwinds a few first base-pairs, inducing hairpin formation on the acceptor template. This allows the polymerase to continue nascent strand extension. **C**. The enzyme pauses within the double-stranded region (at the 5′-end of sequence h in the donor molecule). The generated hairpin brings recombinant junction sites close together. **D**. Polymerase pausing causes the enzyme to dissociate from the donor template (left-hand figure) or moves backward (right-hand figure). At the same time the heteroduplex begins to reconstruct (simultaneously the hairpin stem unwinds) while the released 3′-end of the nascent strand hybridizes to the 5′-end of sequence h located in the acceptor template. **E**. Viral polymerase hybridizes to the nascent strand-acceptor complex or moves forward and switches to the acceptor. Finally, nascent strand synthesis is re-initiated.

is paused after a while within the stable portion of the duplex (at the 3′-end of the sequence h). This may cause BMV replicase/HIV–RT and the 3′-end of the extended primer to be released from the donor (separately or as a complex) or BMV replicase/HIV–RT may move backward along the template releasing the 3′-end of the nascent strand (as it was proposed for the transcription complex [55]). While BMV replicase/HIV–RT and the 3′-end of the nascent strand leave the pausing site, a full-length duplex can be restored. At the same time, the 3′-end of the newly synthesized RNA/DNA may hybridize with the acceptor, since hairpin formation brings a suitable portion of acceptor sequence h very close to the pausing site. During the next step, BMV replicase/HIV–RT may hybridize to the nascent strand-acceptor complex (if the enzyme was released from the donor) or move forward and switch to the acceptor during a continuous process (if BMV replicase/HIV–RT backward sliding occurred earlier) and resume primer extension on Mag1- or Mar1-RNA3.

However, the results presented here cannot precisely explain the formation of recombinants with both junction sites located within the heteroduplex (heteroduplex-mediated crossovers generated in the BMV-based system). One may assume that they are also formed according to the copy-choice mechanism. During the first stage, the replication complex could pause because of strong donor-acceptor hybridization. But it still remains unclear how the viral replicase and the nascent strand are transferred from one template to another, and which elements in RNA and/or protein structure can mediate such a process (especially for recombinants whose junction sites are located far from each other within the heteroduplex structure). The presented data strongly suggest that donor-acceptor hybridization itself does not always ensure template switching by viral replicase. Nonhomologous RNA recombination also depends on the primary and secondary structure of the hybridized sequences as well as sequences proximal to the heteroduplex.

Moreover, the clustering of recombination crossovers within AU-rich regions, which are especially susceptible to breakage [56, 57], suggests that template-switching events may be additionally enhanced by RNA cleavage. Local unwinding of the RNA1-RNA3 duplex (so called breathing of the double-stranded structure) preferentially occurs within AU-rich regions and generates short, single-stranded RNA fragments. At the same time, it was shown that an A-U phosphodiester bond is about 50 times less stable than a C-G and 100 times less stable than a G-G when within a single-stranded region [58, 59]. RNA cleavage may serve as a strong pausing signal for viral replicase. In addition, the breakage of RNA continuity may help the replication complex to leave the donor template.

On the other hand, it cannot be excluded that heteroduplex-mediated recombination occurs by RNA breakage and rejoining. This opinion is supported by the fact that similar to splicing, heteroduplex-mediated recombination depends on RNA secondary structure. Therefore, further studies are required to demonstrate whether the same or different mechanisms operate in heteroduplex-mediated and site-specific nonhomologous RNA recombination.

REFERENCES

1. Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & Van de Pol, S. (1982) Rapid evolution of RNA genomes. *Science* **215**, 1577–1585.

2. Pathak, V.K. & Hu, W.-S. (1997) „Might as well Jump!“ Template switching by retroviral reverse transcriptase, defective genome formation and recombination. *Semin. Virol.* **8**, 141–150.

3. Holland, J., DelaTorre, J.C. & Steinhauer, D.A. (1992) RNA virus populations as quasispecies; in *Genetic Diversity of RNA Viruses*. (Holland, J., ed.) pp. 1–20, Springer Verlag.

4. Domingo, E., Holland, J., Biebricher, C. & Eigen, M. (1995) Quasi-species: The concept and the word; in *Molecular Basis of Virus Evolution* (Gibbs, A.G., Calisher, C.H. & Garcia-Arenal, F., eds.) pp.181–191, Cambridge University Press, Cambridge.

5. Eigen, M. (1996) On the nature of virus quasispecies. *Trends Microbiol.* **4**, 216–217.

6. Steinhauer, D.A. & Holland, J.J. (1983) Rapid evolution of RNA viruses. *Annu. Rev. Microbiol*. **41**, 409–433.

7. Smith, D.B. & Inglis, S.C. (1987) The mutation rate and variability of eucaryotic viruses: An analytical review. *J. Gen. Virol*. **68**, 2729–2740.

8. Pathak, V.K. & Temin, H.M. (1990) Broad spectrum of *in vivo* forward mutations, hypermutations, and hotspots in a retroviral shuttle vector after a single replication cycle: Substitutions, frameshifts, and hypermutations. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6019–6023.

9. Pathak, V.K. & Temin, H.M. (1990) Broad spectrum of *in vivo* forward mutations, hypermutations, and hotspots in a retroviral shuttle vector after a single replication cycle: Deletions and deletions with insertions. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6024–6028.

10. Kim, T., Mudry, R.A., Jr., Rexrode, C.A. & Pathak, V.K. (1996) Retroviral mutation rates and A-to-G hypermutation during different stages of retroviral replication. *J. Virol*. **70**, 7594–7602.

11. Coffin, J.M. (1979) Structure, replication and recombination of retrovirus genomes. Some unifying hypotheses. *J. Gen. Virol*. **199**, 47–59.

12. Lai, M.M.C. (1992) RNA recombination in animal and plant viruses. *Microbiol. Rev*. **56**, 61–79.

13. Zhang, J. & Temin, H.M. (1993) Rate and mechanism of nonhomologous recombination during a single cycle of retroviral replication. *Science* **259**, 234–238.

14. Simon, A.E. & Bujarski, J.J. (1994) RNA-RNA recombination and evolution in virus-infected plants. *Annu. Rev. Phytopathol*. **32**, 337–362.

15. Khatchikian, D., Orlich, M. & Rott, R. (1989) Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the hemagglutinin gene of an influenza virus. *Nature* **340**, 156–157.

16. Meyers, G., Tautz, N., Dubovi, E.J. & Thiel, H.-J. (1991) Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. *Virology* **180**, 602–616.

17. Green, A.M. & Allison, R.F. (1994) Recombination between viral RNA and transgenic plant transcripts. *Science* **263**, 1423–1425.

18. Kirkegaard, K. & Baltimore, D. (1986) The mechanism of RNA recombination in poliovirus. *Cell* **47**, 433–443.

19. Figlerowicz, M., Nagy, P.D. & Bujarski, J.J. (1997) A mutation in the putative RNA polymerase gene inhibits nonhomologous, but not homologous, genetic recombination in RNA virus. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2073–2078.

20. Figlerowicz, M., Nagy, P.D., Tang, N., Kao, C.C. & Bujarski, J.J. (1998) Mutations in the N-terminus of the brome mosaic virus polymerase affect genetic RNA-RNA recombination. *J. Virol*. **72**, 9192–9200.

21. Stuhlmann, H. & Berg, P. (1992) Homologous recombination of copackage retrovirus RNAs during reverse transcription. *J. Virol*. **66**, 2378–2381.

22. Figlerowicz, M. & Bujarski, J.J. (1998) RNA recombination in brome mosaic virus, a model

plus stranded RNA virus. *Acta Bioch. Polon.* **45**, 1–23.

23. Nagy, P.D., Zhang, C. & Simon, A.E. (1998) Dissecting RNA recombination *in vitro*: Role of RNA sequences and the viral replicase. *EMBO J.* **17**, 2392–2403.

24. Romanova, L.I., Blinov, V.M., Tolskaya, E.A., Viktorova, E.G., Kolesnikova, M.S., Guseva, E.A. & Agol, V.I. (1986) The primary structure of crossovers regions of intertypic poliovirus recombinants: A model of recombination between RNA genomes. *Virology* **155**, 202–213.

25. Nagy, P.D. & Bujarski, J.J. (1993) Targeting the site of RNA-RNA recombination in brome mosaic virus with antisense sequences. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6390–6394.

26. Zhang, X. & Lai, M.M.C. (1994) Unusual heterogeneity of leader-mRNA fusion in a murine coronavirus: Implications for the mechanism of RNA transcription and recombination. *J. Virol.* **68**, 6626–6633.

27. Vogt, P.K. (1971) The genome of avian RNA tumor viruses: A discussion of four models; in *Possible Episomes in Eukaryotes* (Sylvestri, L., ed.) pp. 35–41, North-Holland, Amsterdam.

28. Chetverin, A.B., Chetverina, H.V., Demidenko, A.A. & Ugarov, V.I. (1997) Nonhomologous RNA recombination in a cell-free system: Evidence for a transesterification mechanism guided by secondary structure. *Cell* **88**, 503–513.

29. King, A.M.Q. (1988) Genetic recombination in positive strand RNA viruses; in *RNA Genetics* (Domingo, P., Holland, J.J. & Ahlquist, P., eds.) vol. 2, pp. 149–165, CRC Press, Boca Raton.

30. *Fields Virology* (1996) (Fields, B.M., ed.) 3rd edn., Lippincott-Raven Publishers, Philadelphia, New Jork.

31. Hirst, G.K. (1962) Genetic recombination with Newcastle disease virus, poliovirus and in influenza. *Cold Spring Harbor Symp. Qant. Biol.* **27**, 303–309.

32. Ledinko, N. (1963) Genetic recombination with poliovirus type 1 studies of crosses between a normal horse serum-resistant mutant and several guanidine-resistant mutants of the same strain. *Virology* **20**, 107–119.

33. Pringle, C.R. (1965) Evidence of genetic recombination in foot-and-mouth disease virus. *Virology* **25**, 48–54.

34. Jarvis, T.C. & Kirkegaard, K. (1992) Poliovirus RNA recombination: Mechanistic studies in the absence of selection. *EMBO J.* **11**, 3135–3145.

35. Pilipenco, E.V., Gmyl, A.P. & Agol, V.I. (1995) A model for rearrangements in RNA genomes. *Nucleic Acids Res.* **23**, 1870–1875.

36. Fu, K. & Baric, R.S. (1992) Evidence for variable rates of recombination in MHV genome. *Virology* **189**, 88–102.

37. Fu, K. & Baric, R.S. (1994) Map locations of mouse hepatitis virus temperature-sensitive mutants: Confirmation of variable rates of recombination. *J. Virol.* **68**, 7458–7466.

38. Banner, L.R., Keck, J.G. & Lai, M.M.-C. (1990) A clustering of RNA recombination sites adjacent to a hyper-variable region of the peplomer gene of murine coronavirus. *Virology* **175**, 548–555.

39. Simon, A.E. & Howell, S.H. (1986) The virulent satellite RNA of turnip crinkle virus has a major domain homologous to the 3′-end of the helper virus genome. *EMBO J.* **7**, 3423–3428.

40. Oh, J.-W., Kong, Q., Song, C., Carpenter, C.D. & Simon, A.E. (1995) Open reading frames of turnip crinkle virus involved in satellite symptom expression and incompatibility with *Arabidopsis thaliana* ecotype Dijon. *Mol. Plant Microbe Interact.* **8**, 979–987.

41. Li, X.H., Heaton, L.A., Morris, T.J. & Simon, A.E. (1989) Turnip crinkle virus defective interfering RNAs intensify viral symptoms and

are generated *de novo*. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9173–9177.

42. Cascone, P.J., Carpenter, C.D., Li, X.H. & Simon, A.E. (1990) RNA recombination between satellite RNAs of turnip crinkle virus. *EMBO J.* **9**, 1709–1715.

43. Cascone, P.J., Haydar, T.F. & Simon, A.E. (1993) Sequences and structures required for recombination between virus-assisted RNAs. *Science* **260**, 801–805.

44. Zhang, C., Cascone, P.J. & Simon, A.E. (1991) Recombination between satellite and genomic RNAs of turnip crinkle virus. *Virology* **184**, 791–794.

45. Carpenter, C.D., Oh, J.-W., Zhang, C. & Simon, A.E. (1995) Involvement of a stem-loop structure in the location of junction sites in viral RNA recombination. *J. Mol. Biol.* **245**, 608–622.

46. Mindich, L., Qiao, X., Onodera, S., Gottlieb, P. & Strassman, J. (1992) Heterologous recombination in the double-stranded RNA bacteriophage Φ6. *J. Virol*. **66**, 2605–2610.

47. Onodera, S., Qiao, X., Gottlieb, P., Strassman, J., Frilander, M. & Mindich, L. (1993) RNA structure and heterologous recombination in the double-stranded RNA bacteriophage Φ6. *J. Virol*. **67**, 4914–4922.

48. Li, Y. & Ball, L.A. (1993) Non-homologous RNA recombination during negative strand synthesis of flock house virus RNA. *J Virol*. **67**, 3854–3860.

49. Raju, R., Subramaniam, S.V. & Hajjou, M. (1995) Genesis of Sindbis virus by *in vivo* recombination of nonreplicative RNA precursors. *J. Virol*. **69**, 7391–7401.

50. Ahlquist, P. (1992) Bromovirus RNA replication and transcription. *Curr. Opin. Genet. Dev.* **2**, 71–76.

51. Bujarski, J.J. & Kaesberg, P. (1986) Genetic recombination in a multipartite plant virus *Nature* **321**, 528–531.

52. Nagy, P.D. & Bujarski, J.J. (1995) Efficient system of homologous RNA recombination in brome mosaic virus: Sequence and structure requirements and accuracy of crossovers. *J. Virol*. **69**, 131–140.

53. Figlerowicz, M. (2000) Role of RNA structure in heteroduplex-mediated and site-specific nonhomologous recombination in brome mosaic virus. *Nucleic Acids Res.* **28**, 1714–1723.

54 Figlerowicz, M. & Bibi³³o, A. (2000) RNA motifs mediating *in vivo* site-specific nonhomologous recombination in (+) RNA virus enforce *in vitro* nonhomologous crossovers with HIV-1 reverse transcriptase. *RNA* **6**, 339–351.

55. von Hippel, P.H. (1998) An integrated model of the transcription complex in elongation, termination and editing. *Science* **281**, 660–665.

56. Bibi³³o, A., Figlerowicz, M. & Kierzek, R. (1999) The non-enzymatic hydrolysis of oligoribonucleotides. VI. The role of biogenic polyamines. *Nucleic Acids Res.* **27**, 3931–3937.

57. Bibi³³o, A., Figlerowicz, M. & Kierzek, R. (1999) Nonenzymatic hydrolysis of oligoribonucleotides. VII Structural elements affecting hydrolysis. *Nucleotides Nucleosides* **19**, 977–994.

58. Kierzek, R. (1992) Hydrolysis of oligoribonucleotides: Influence of sequence and length. *Nucleic Acids Res.* **20**, 5073–5077.

59. Kierzek, R. (1992) Nonenzymatic hydrolysis of oligoribonucleotides. *Nucleic Acids Res*. **20**, 5079–5084.