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 phenomenon was studied in only a few viral species. Among them, bromovirus (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 weak point: it could not really explain the role of RNA structure in nonhomologous recombination.


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Abbreviations: BMV, bromovirus; 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.
RNA viruses and retroviruses are the only known living species which use RNA to store their genetic information. Another feature common to both types of viruses is their enormous genetic variability [1, 2]. There is a lot of data suggesting that an individual RNA virus 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 species used to classify higher organisms does not apply to RNA-based viruses [3–5].

Several processes contribute to the continuous generation of new viral variants. Point mutations are most frequently introduced into the viral genome during the replication process (because of the low fidelity of RNA dependent polymerases that lack proofreading activity) [6–10] while RNA recombination is responsible for some more profound 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 between different RNA viruses has been demonstrated. Additionally, it was shown that viral RNA can recombine 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 major factor responsible for the emergence of new, often dangerous viral strains or species.

Basing on the structure and function of RNA molecules, two general types of genetic RNA recombination were distinguished: homologous and nonhomologous [12]. Homologous RNA recombination involves two identical or similar molecules and is called precise if recombinant junction sites are located accurately at corresponding nucleotides or imprecise when the junction sites occupy different positions within recombining molecules. As a result of precise crossovers parental molecules are regenerated, whereas imprecise combination produces molecules in which some sequences are duplicated or deleted. Nonhomologous recombination crossovers occur between two different RNA molecules, and so the resultant recombinants differ significantly from the parental molecules.

Despite extensive studies, the molecular mechanism of RNA recombination is still not well understood. Most of the data collected with different experimental systems suggest that recombination crossovers occur according to the copy-choice hypothesis [11, 18–20]. This hypothesis postulates that recombinants are formed during replication, when the viral replicase-nascent strand complex switches from one RNA template (called RNA donor) to another (called RNA acceptor). The molecular basis of template switching events is better understandable if they involve two homologous RNA molecules, when the nascent strand synthesized on the RNA donor is complementary to the RNA acceptor [21, 22]. The problem becomes more complicated if nonhomologous recombination is taken into consideration. According to the definition, recombination of this type occurs between two RNA molecules of different sequences. The generated recombinants differ distinctly from the parental molecules, and being dysfunctional, they rarely accumulate in vivo. Some data suggest that viral polymerases use promoter-like structures to switch from the donor to the acceptor template [23]. Other results emphasize the role of local hybridization (formation 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 recombine according to an alternative mechanism, that is by template breakage and rejoining [28]. First, cleavage of the viral genome generates 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 bromo 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 cross overs are roughly randomly distributed...
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 mutienzymatic 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 for motion by discontinuous transcription presumes that viral polymerase is naturally selected to mediate template-switching events. Indeed, studies in 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 promoter-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) postulated 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-
A. 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. Mechanism of nonhomologous recombination

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.
participate 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 (GUCUC and GUCCC, respectively) at the junction sites.

In order to identify the structural motifs involved in nonhomologous recombination between BMV RNAs, the nucleotide sequences...
of the parental and recombinant molecules within and at the vicinity of the junction sites were assessed carefully. Results of the undertaken analysis suggested that nonhomologous recombination occurs in a site-specific manner if the local RNA–RNA heteroduplex is accompanied by specifically positioned short homologous sequences (regions h in Fig. 3B)

To determine whether the same RNA structural motifs were involved in other recombination 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 regions, and generated almost or completely symmetrical recombinants (re-

Figure 2. Distribution of the nonhomologous crossovers occurring in vivo between BMV RNA1 and PN3(−) or PN8(−) RNA3.

In both heteroduplexes the upper sequence represents the (+) strand of PN3(−) or PN8(−) RNA3 (the region where RAS is located) while the lower sequence represents the corresponding (complementary) fragment of the (+)RNA1 strand. The junction sites of each recombinant are marked with arrows (pointing to the last nucleotide coming from wt RNA1 and the first nucleotide from the RNA3 derivative) and with the same letter. Numbers accompanying the letters indicate how often the given recombinant was identified.

[53]. Regions h are placed in such a way that the heteroduplex formed by recombining molecules can adopt two alternative structures, either a full-length duplex or a shorter duplex 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, however, be the only factor allowing viral replicase to switch from one RNA template to another. The hairpin 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 between the nascent strand (synthesized on the RNA1 donor) and the acceptor template.

In addition, the preference of BMV replicase to switch after certain nucleotides were investigated [53]. It was found that in 42% of recombinants a U was the last nucleotide coming from the donor template, 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 hairpin 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 suitably in vitro system in which recombinationally active regions of BMV RNAs were applied as donor and acceptor template plates (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

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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 hairpin on the RNA3 template (the structure below). Such structural flexibility results from the presence of short homologous sequences specifically positioned in recombining molecules (the shaded 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 in the hairpin 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 in vivo in 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.
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 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 recombinant 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).
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 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 reinitiated.
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.

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