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Diverse Mechanisms of RNA Recombination

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Abstract—Recombination is widespread among RNA viruses, but many molecular mechanisms of this phenomenon are still poorly understood. It was believed until recently that the only possible mechanism of RNA recombination is replicative template switching, with synthesis of a complementary strand starting on one viral RNA molecule and being completed on another. The newly synthesized RNA is a primary recombinant molecule in this case. Recent studies have revealed other mechanisms of replicative RNA recombination. In addition, recombination between the genomes of RNA viruses can be nonreplicative, resulting from a joining of preexisting parental molecules. Recombination is a potent tool providing for both the variation and conservation of the genome in RNA viruses. Replicative and nonreplicative mechanisms may contribute differently to each of these evolutionary processes. In the form of *trans* splicing, nonreplicative recombination of cell RNAs plays an important role in at least some organisms. It is conceivable that RNA recombination continues to contribute to the evolution of DNA genomes.

Key words: viruses, RNA genome, recombination, nonreplicative recombination, evolution

INTRODUCTION

The genomes of RNA viruses change and evolve like the genomes of all other biological organisms. The major cause of their variation is the infidelity of template replication, which is partly determined by the fact that viral RNA-dependent RNA polymerases (RdRPs) lack proofreading activity [1, 2]. Rough estimates showed that, on average, one mutation arises in every newly synthesized molecule of viral RNA. The mutation rate is so high in some viruses (e.g., in picornaviruses) that its slight increase already suffices to dramatically reduce or completely abolish the viability of the virus because of a high probability of substantial genetic lesions (mutation catastrophe) [3, 4].

In addition, replication of viral RNA genomes is accompanied by covalent rearrangements: deletions, duplications, and recombination. An illustrative example of deletions is provided by defective interfering (DI) genomes, which accumulate in a virus population upon high-multiplicity infections and lack a fragment of the sequence coding for viral proteins [5–7]. Short duplications and deletions arise, in some cases, as pseudoreversions in response to artificial damaging mutations, as observed for the poliovirus and Theiler's murine encephalomyelitis virus [8–11]. Duplications also reserve a place in the evolutionary history of viruses: for instance, the genome of the foot-and-mouth disease virus codes for three highly similar variants of the replicative protein VPg [12]. There are grounds for believing that an extended sequence (more than 100 nt in size) was duplicated in the 5'-untranslated region (5UTR) of the genome of an enterovirus precursor. An additional extended duplication took place more recently in another region of 5UTR in the bovine enterovirus [13].

A special role in the variation of RNA viruses is played by recombination, the generation of new genomes from two or more parental RNAs. Recombination between viral RNA molecules was observed for the first time as early as in the 1960s in the poliovirus [14, 15]. Two related but phenotypically different strains were used as parents. A minor portion of the virus progeny resulting from co-infection with the two strains expressed characters of both parents. A similar method was used soon afterwards to detect recombination in the foot-and-mouth disease virus [16]. The recombination rate proved to vary among different pairs of poliovirus mutants [17]. On the assumption that the recombination rate is proportional to the distance between the corresponding mutations, a genetic (linkage) map was constructed for the polioviral genome. The map proved to be additive, testifying again to the existence of RNA recombination. It is indeed hardly conceivable that a different mechanism associates the genetic distance between mutations with the frequency of the corresponding double mutants.

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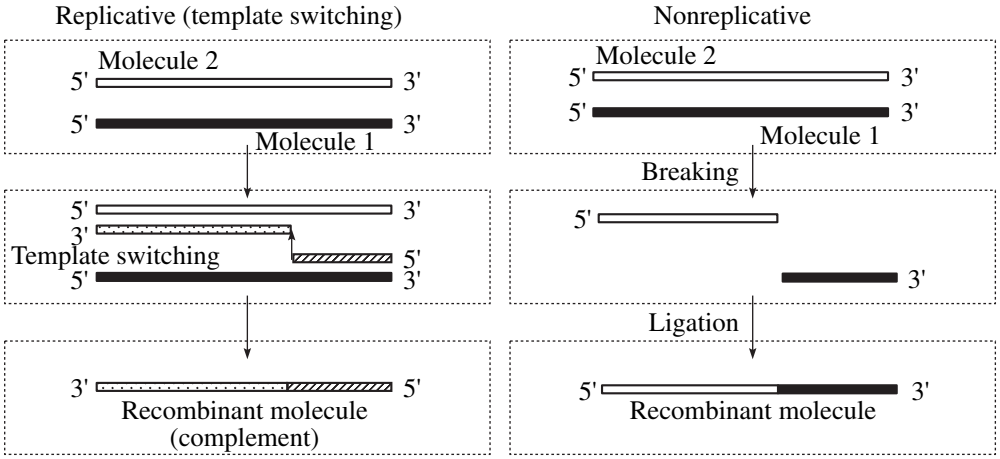

Fig. 1. Two types of RNA recombination. Parental RNAs are shown with open and black bars; nascent daughter strands, with dotted or crosshatched bars.

Direct biochemical evidence for the inheritance of information from two parental viral RNAs was obtained in the early 1980s. Proteins of recombinants resulting from crossing polioviruses of different serotypes were assayed by partial proteolysis and isoelectric focusing and proved to originate from different strains [18, 19]. Convincing evidence for RNA recombination was provided by RNA sequencing [20–25]. Early studies of RNA recombination were described in detail elsewhere [26–28].

Recombination is characteristic of most, if not all, RNA viruses of animals [28–31], plants [32, 33], and microorganisms [34, 35], but its rate considerably varies among different viruses. Some viruses with a negative RNA genome (e.g., hantaviruses [31]) have a low recombination rate. In some flaviviruses, RNA recombination has not been detected so far [36]. In many RNA viruses, however, recombination occurs at a high rate under experimental conditions and is widespread in nature. For instance, there are grounds for believing that recombination events have taken place in the evolutionary history of all currently circulating enteroviruses [37–43].

RNA recombination can be observed not only *in vivo* but also in cell-free systems [35, 44–48].

Mutations and covalent rearrangements of RNA genomes contribute to their diversity, a resource providing for the evolution of RNA viruses. Here, we summarize the current views of the mechanisms and biological significance of RNA recombination as a major generator of this resource, with special emphasis on the recent progress in the field.

REPLICATIVE RNA RECOMBINATION WITH TEMPLATE SWITCHING

Intermolecular RNA recombination, as well as deletions and duplications, can theoretically proceed via two fundamentally different mechanisms, replicative and nonreplicative. Recombination is associated with RNA synthesis in the former case, while recombinant molecules are generated at the postsynthetic (postreplicative) level in the latter.

According to the model of replicative template switching, synthesis of a complementary strand starts on one RNA molecule and is completed on another; i.e., a newly synthesized molecule is a primary recombinant (Fig. 1). Several variants are conceivable for the transfer of the nascent strand from one template to another. The strand can be removed from the primary template as a result of premature termination and can be transferred to another template with or without RdRP. A donor RNA fragment can result from degradation of a previously synthesized RNA; in this case, the start and end of generation of a recombinant molecule are temporally separate.

Cooper *et al.* [26, 49] were the first to propose template switching as a mechanism of homologous (i.e., exact) recombination in polioviruses. One of the ideas underlying this hypothesis was simple and quite convincing: the enzymes that covalently join RNA molecules were unknown at that time. Kirkegaard and Baltimore [25] showed that replication is indeed essential for the generation of recombinants, providing experimental support for the hypothesis. It was concluded on circumstantial evidence that, in the system used, template switching took place predominantly during synthesis of the (–) RNA strand on the template of the viral (+) RNA strand. Although the arguments were open to criticism [50, 51], the model of template switching was extrapolated to other viral systems [28]

and was considered to be a synonym of the replicative mechanism, which, in turn, was thought to be the only possible way of RNA recombination. The replicative model is supported, though indirectly, by the fact that mutations altering the proteins of the replication complex affect the efficiency of recombination [52–54].

The possibility of template switching was confirmed in experiments with purified RdRPs of the poliovirus [55], the bovine viral diarrhea virus (BVDV) [48], and several plant viruses [47, 48]. It is essential for template switching that RdRP is capable of using the 3' end of the incomplete nascent RNA strand as a primer to be elongated on a new template. Such activity was demonstrated for RdRPs of at least some RNA viruses (e.g., see [56–60]).

Although the model of template switching is widely accepted, the molecular mechanisms underlying this phenomenon are still incompletely understood. In particular, three key questions are open. First, why is elongation on the first template interrupted? Second, does the incomplete nascent strand dissociate alone or together with RdRP? Third, how is the site of the acceptor template chosen to resume synthesis? These problems still lack ultimate answers and are a matter of more or less justified speculation.

It is quite conceivable that elongation pauses, which are possibly determined by some elements of RNA secondary structure, are among the factors favoring premature termination of the nascent RNA strand and its dissociation from the template [24, 53, 61–63]. Possibly, the nascent strand dissociates more easily at RNA regions enriched in U and A [64]. Moreover, premature termination and dissociation may be caused by degradation of the template, for instance, in AU-rich, poorly structured regions [65] or by erroneous addition of a mismatching nucleotide by RNA polymerase [66]. In the latter case, termination and dissociation of the incomplete nascent strand can be regarded as a special type of proofreading.

A commonly accepted model of template switching suggests dissociation of the elongation complex. It was assumed, however, that replicative rearrangements arise without dissociation as well. According to one hypothesis, regions of two parental RNA molecules are held together via complementary interactions with a third, supporting, molecule, while a recombinant molecule is generated when RdRP passes from one parental template onto the adjacent region of the second template (Fig. 2) [7]. Another hypothesis suggests that RdRP sometimes slides back on the template, releasing a short unpaired 3'-terminal region of the nascent strand. When this region is anchored on the same or another template, resumed elongation results in deletion/duplication or recombination, respectively. This mechanism possibly underlies the origin of some short deletions [66]. Note that the capability to slide back on the template is well known for DNA-dependent RNA polymerases [68, 69].

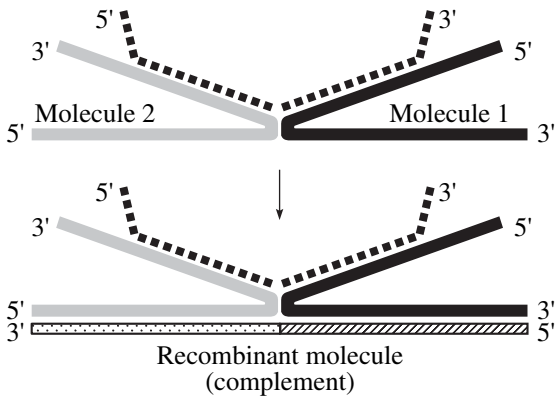

Fig. 2. Model of RNA recombination with a supporting molecule (dashed line).

The major role in choosing the site to resume RNA synthesis on the second template is played by RdRP, the 3' end of the nascent RNA strand, or both. One of the existing hypothesis suggests that RdRP, along with the nascent RNA strand, binds to its recognition site, a replicative *cis* element, on the second template. This hypothesis is based on the fact that crossover sites cluster in the vicinity of promoters or replicative enhancers in the genomes of some plant viruses [47, 70–73]. Mutations of such elements impair the efficiency of recombination. It is possible that recognition of replicative *cis* elements by viral RdRP contributes to the choice of crossover site during recombination in alphaviruses [74] and coronaviruses [75], although, in the latter, a considerable role in choosing the landing site is played by complementary interactions between the 3' end of the nascent strand and intergenic repeats of the template [76]. The clustering of crossover sites in the vicinity of replicative *cis* elements provides evidence, though circumstantial, in favor of template switching.

It is clear, however, that such an association with replicative *cis* elements is absent when crossover sites are relatively uniformly distributed throughout the genome, as is the case with picornaviruses. It is thought that the choice of anchorage site is facilitated when the 3' end of the nascent strand is complementary to a region of the second template [24, 25]. Yet complementary sites may each consist of a few nucleotides, and an erroneous landing is highly probable because of the abundance of short direct repeats in RNAs. A factor theoretically capable of bringing correct (homologous) regions of two templates together is the formation of a heteroduplex between two direct repeats corresponding to hairpins [24, 61].

The effect of temperature on the distribution of crossover sites [77] is probably associated with its effect on the secondary structure of RNA and, consequently, on termination/dissociation of the primary

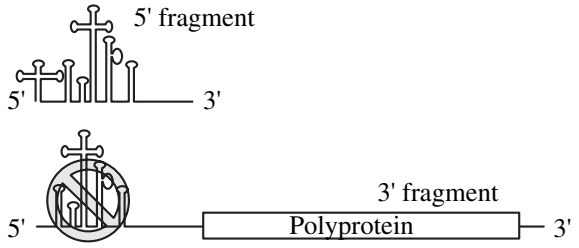

Fig. 3. Polioviral RNA fragments that complemented each other and were used in experiments on recombination at 5UTR. Replicative and translational regulatory elements of the 3' fragment were deleted or inactivated by mutations.

elongation complex or the association of the incomplete nascent strand with another template.

RNA RECOMBINATION INVOLVING REPLICASE OF PHAGE Qβ

Template switching is not the only possible mechanism of RNA recombination. An alternative model was advanced on the basis of experimental data on replication of phage Qβ RNA in a cell-free system [78]. The system contained only highly purified Qβ replicase and ribonucleoside triphosphates and allowed exponential replication both for phage Qβ RNA and for small RQ RNAs, natural satellites of the phage [79]. To study RNA recombination with this system, experiments were performed with 5' and 3' fragments of a satellite RQ RNA. The fragments complemented each other and, in themselves, could not be exponentially replicated by Qβ replicase. It is essential for replication that both ends of a molecule contain necessary *cis* elements, which could be achieved only via a fusion of two molecules into one as a result of recombination. The reaction products were analyzed by a molecular colony technique [80]: an RNA sample was applied to agarose gel containing Qβ replicase; gels were covered with a nylon membrane impregnated with a solution of four NTPs; and generation of molecules capable of replication was inferred from the formation of RNA colonies (clones), which are detectable, for instance, with ethidium bromide.

Detection of colonies directly demonstrated for the first time that RNA recombination does not necessarily involve DNA intermediates (DNA synthesis was impossible because of the absence of dNTPs), nor does it require any protein other than Qβ replicase [78]. The mechanism of recombination in the above system differs from template switching. First, recombinants were nonhomologous, notwithstanding the homology of overlapping ends, which were added to the fragments on purpose. Only homologous recombinants were produced in a control sample containing not only the same reagents, but also reverse transcriptase, which is capable of jumping from one template to another. Second, a hydroxyl group at the 3' terminus of the 5' fragment was critical for efficient recombination. Most recombinants contained the full-length 5' fragment and a part of the 3' fragment. Recombination was observed even when RNA fragments of opposite polarities were used as partners [60].

To explain the above findings, a mechanism was proposed that is similar to the mechanism of splicing and suggests that the 3'-hydroxyl group of one fragment attacks a phosphodiester bond of the other [60, 78]. This new type of RNA recombination is considered to be replicative, because it depends on Qβ replicase [81] and RNA synthesis [60]. It is thought that, during synthesis, replicase assumes a certain active conformation, which allows it to catalyze the above transetherification reaction [60].

NONREPLICATIVE RNA RECOMBINATION PRESUMABLY REQUIRING CELL PROTEINS

Data are continuously accumulating that viruses possess a fundamentally different, nonreplicative, mechanism(s) of RNA recombination. In particular, this is evident from experiments with the poliovirus. The polioviral genome is a single-stranded RNA of about 7.5 kb in size and of a positive polarity, being thereby capable of functioning as a template in translation. This RNA contains a single extended open reading frame and codes for a precursor polyprotein, whose partial proteolysis by viral proteases yields viral proteins [82]. Translation of viral RNA is initiated via a cap-independent mechanism, unusual for eukaryotic mRNA: the polioviral RNA lacks a cap, and the ribosome binds to what is known as the internal ribosome entry site (IRES) in the 5UTR [83, 84]. Replication of this RNA requires at least three *cis*-acting replicative elements, which are at both ends and in the internal region of the molecule, as well as RdRP and some other proteins encoded by the viral genome [85]. Replication of the polioviral RNA, along with other steps of virus reproduction, occurs in the cytoplasm.

The possibility of nonreplicative recombination was first studied with pairs of polioviral RNA fragments. One fragment in a pair contained the near full-length 5UTR and lacked the coding region; the other had an intact open reading frame coding for the polyprotein, while the translational and replicative elements of the 5UTR were removed or inactivated (Fig. 3) [86]. Introduced together into cells, these fragments allowed generation of a viable recombinant virus progeny. Most recombinants were a result of imprecise (nonhomologous) recombination. The changes observed in the 5UTR did not affect functionally significant regulatory elements but were restricted to a region where the primary structure can be dramatically rearranged without impairing the infectivity of

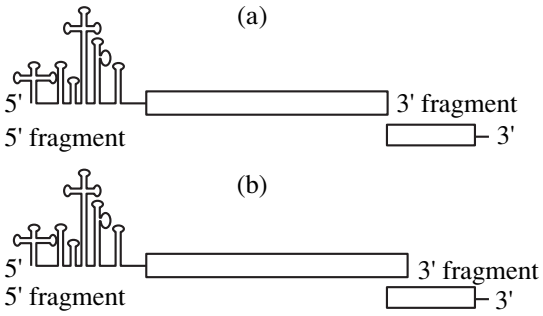

Fig. 4. Polioviral RNA fragments with a break in the sequence coding for RNA polymerase. The fragments (a) precisely complement each other or (b) have an overlap.

the virus [8, 9, 87–89]. Since the fragments were not in themselves capable of replication or translation, recombinant genomes could arise only by a nonreplicative mechanism.

The possibility of nonreplicative RNA recombination was demonstrated even more rigorously with pairs of RNA fragments corresponding to the polioviral RNA with a break in the RdRP-coding region (Fig. 4) [90]. Since each fragment contained only a part of the gene coding for RdRP, this enzyme, which is essential for virus replication, could be synthesized only after recombination.

In one variant, the RdRP gene lacked a single phosphodiester bond (Fig. 4a). Simple ligation would restore the integrity of the genome in this case. Cotransfection of virus-sensitive cells with two partners yielded viable viruses. The efficiency of ligation was virtually independent of whether the fragments could form heteroduplexes in which the nucleotides to be ligated were close together. It was only necessary that the 5' partner have a 3'-phosphate and the 3' partner have a 5'-OH group. RNA ligases that join a 3'-phosphorylated nucleotide with a 5'-hydroxylated nucleotide are still unknown, suggesting preliminary activation of the partner ends by cell enzymes. Known RNA ligases utilize as partners either terminal 2',3'-cyclophosphates with a 5'-phosphate or a 5'-hydroxyl group [91–94] or 3'-OH with 5'-phosphate (or 5'-triphosphate) [95, 96]. It is possible to assume that, in the cell, the ends of the fragments are converted into the form suited to RNA ligases. One of the possible variants of such activation is cyclization of the 3'-terminal phosphate of the 5' fragment by RNA-3'-phosphate cyclase [97, 98]. However, conversion of the 3'-terminal phosphate of the 5' fragment into 2',3'-cyclophosphate did not increase the efficiency of ligation [90]. Thus, cyclization of the 3'-terminal phosphate was either not essential or nor limiting for ligation under the conditions used.

Fragments of other pairs had an overlap (Fig. 4b). The sequence coding for the active enzyme could be restored only as a result of precise (homologous)

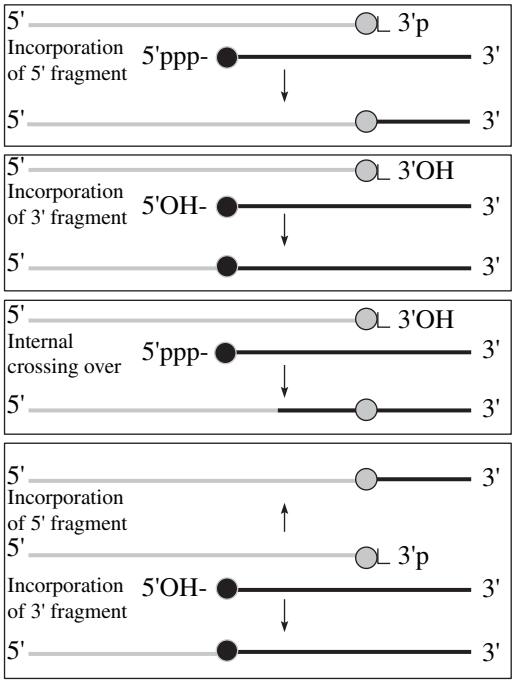

Fig. 5. Dependence of the preferential structure of the recombinant genome on the chemical structure of the terminal nucleotides of 5' (gray) and 3' (black) fragments. Circles show the corresponding marker terminal mutations.

recombination. Therefore, it was necessary that the extra segment(s) be deleted from one or both fragments to produce a viable genome. Such deletions were indeed found in viable viruses resulting from cotransfection.

An interesting association was observed between the location of crossover sites and the structure of the terminal nucleotides in partners (Fig. 5). When both fragments used for transfection had phosphorylated terminal nucleotides (the 5' fragment had a monophosphorylated 3' end and the 3' fragment had a tri-, di-, or monosphosphorylated 5' end), the full-length 5' fragment was incorporated into the genome in most recombinants. When both fragments had dephosphorylated ends, it was the full-length 3' fragment that was incorporated into the genome in most recombinants. In other words, the 3'-phosphorylated nucleotide of a 5' fragment and the 5'-hydroxylated nucleotide of a 3' fragment were capable of finding the correct site on the RNA partner and integrating into it to yield a perfect recombinant genome. When the 5' fragment was dephosphorylated and the 3' fragment phosphorylated, the crossover site was within the overlap; i.e., precise internal crossing over was observed in this case.

The available data are insufficient for proposing a mechanism of recombination observed in the above experiments. Moreover, it is still unclear whether terminal incorporation and internal crossing over proceed via the same or different mechanisms. It seems

probable only that the reaction(s) requires activation of one or both partners and involves cell enzymes.

Another open question is how the accuracy of recombination is achieved. It is most plausible that crossover sites are to some extent promiscuous (or at least have many possible locations) and that the precision of recombination is determined by selection of viable variants. First, in the experiments on recombination at the 5UTR, precise recombination was not essential for generation of viable genomes and crossover sites were distributed throughout the permissible region in RNA [86]. Second, inserts of one or two triplets were observed even for recombination at the RdRP-coding region, which was expected to require strong homology at the crossover site [90]. Third, recombination sometimes yielded malformed, though still viable, genomes with a mosaic RdRP gene and additional inserts in the 3UTR [90].

Although occurring in many regions of viral RNA, nonreplicative recombination does not necessarily proceeds in different regions with similar efficiencies. Indeed, crossover sites cluster in hot spots when recombination takes place at the 5UTR, regardless of whether the 5' or the 3' full-length partner is incorporated or recombination is internal ([86]; E.V. Belousov *et al.*, unpublished data). The factors responsible for such selectivity are still unknown. It is possible to assume that hot spots correspond to sites of preferential cleavage of the recombining partners by nucleases or cryptic ribozymes or, alternatively, to sites of preferential ligation (or incorporation into a polynucleotide chain).

Similar data were recently obtained with the BVDV, which belongs to the genus *Pestivirus* of the family Flaviviridae. Like the polioviral RNA, the genome of this cytoplasmic virus is a single-stranded RNA of about 12.5 kb, which functions as a translational template and contains a single open reading frame coding for a polyprotein precursor of all viral proteins. The 5UTR and the 3UTR of the BVDV RNA harbor *cis* elements essential for replication and translation, including IRES [99]. Infection with the BVDV is usually asymptomatic. In some cases, however, it has severe complications and a lethal outcome. Then two virus variants, or a virus pair, can be isolated from affected animals: one is noncytopathogenic and causes asymptomatic infection and the other is cytopathogenic and causes death [100]. Cytopathogenic variants commonly originate from noncytopathogenic viruses as a result of genome rearrangements (recombination). It is experiments with the BVDV that provided additional evidence for nonreplicative RNA recombination [101]. Transfection was performed with overlapping fragments of the BVDV RNA, which lacked different parts of the RdRP-coding segment. It is clear that such fragments could not be replicated by themselves. However, cotransfection yielded viable recombinant viruses. Both homologous and nonhomologous recombination was observed, with viral RdRP being altered by inserts and deletions in the latter case.

"SPONTANEOUS" RNA RECOMBINATION

Nonreplicative RNA recombination probably involves cell enzymes in the above cases. However, there are variants of recombination that seem to require no macromolecules other than RNA partners. To prevent recombination depending on Qβ replicase (see above), RQ RNA fragments were incubated in the absence of this enzyme and NTPs (the medium contained Mg^{2+}) or the 5' fragment was oxidized and lacked the 3'-OH group, which is essential for the replicase-dependent mechanism. Recombinants were generated again, although the generation rate was three orders of magnitude lower than in the presence of replicase [81]. Such "spontaneous" recombination occurred only between internal regions of the partners, and the crossover sites were distributed fairly uniformly.

These findings made it possible to assume that the capability of exchanging fragments is a general property of RNA molecules [81]. However, such exchanges are rare: their rate was estimated at 10^{-9} recombination events per nucleotide per hour in the above experiments.

COVALENT RNA REARRANGEMENTS CATALYZED BY SPLICEOSOMES AND RIBOZYMES

It has been known for a long time that nonreplicative covalent bonding between cell RNAs or their fragments takes place during splicing of various types [102–104] and some variants of RNA editing [96]. The main mechanisms of these processes are briefly considered below in order to compare them with nonreplicative recombination of viral RNA genomes and to note some differences and similarities.

Intramolecular (*cis*) splicing is most common and consists in excision of internal segments (introns) from a primary RNA transcript and ligation of the other segments (exons) of the same molecule. One mechanism of splicing is intricate and specialized and involves spliceosomes, which consist of tens of proteins and half a dozen low-molecular-weight RNAs. Another mechanism is due to the catalytic (ribozyme) activity of RNAs themselves. In this case, a ribozyme is in an intron and its activity is determined, in particular, by the specific secondary and tertiary structures of the RNA molecule. In addition to the complex specialized machinery (spliceosomes and ribozyme-containing introns), splicing requires recognition of several oligonucleotide signals located both in introns and in exons. Natural splicing is strongly site-specific,

involving certain sites of an RNA molecule. One molecule can harbor several specific sites, which open up the possibility of alternative splicing [105].

The molecular mechanisms of splicing are beyond the scope of this review. However, the problem of interest is clearly connected with *trans* splicing, a specific splicing variant that involves the joining of segments belonging to different RNA transcripts. It is possible to consider *trans* splicing as a variant of nonreplicative RNA recombination. *Trans* splicing was detected in various organisms but is especially common in protozoans. *Trans* splicing can proceed both via the spliceosome-dependent [106] and, at least under laboratory conditions, via the ribozyme [107] mechanisms. Ribozymes are indeed capable of catalyzing partial reactions underlying *trans* splicing. For instance, some ribozymes introduce breaks in RNA [103, 108] and thereby generate potential partners for subsequent recombination. The capability of RNA ligation is also characteristic of natural ribozymes [109, 110] and ribozymes obtained by artificial selection *in vitro* [111–113].

Recent interest in *trans* splicing is due to two circumstances. On the one hand, its ribozyme variant may provide a model for important processes associated with the prebiotic stage of the evolution of the RNA world. On the other hand, artificial *trans* splicing is promising for correcting pathologically changed RNA molecules and, consequently, can be used for treatment and prevention of various disorders.

Several variants are known for artificial *trans* splicing catalyzed by ribozymes. One of the RNA partners can be covalently linked with a ribozyme, while the other, free, partner must contain a short oligonucleotide recognizable by the given ribozyme. As a result of the ribozyme activity, the partners are joined in a single molecule and the ribozyme is released. This mechanism was successfully used to restore the integrity of a truncated *lac*Z mRNA [107] and to correct the coding potential of several other modified mRNAs [114–116]. In another variant, a ribozyme (a self-splicing group II intron) was cleaved into two components. Its 5'-terminal fragment was ligated with the 3' end of exon 1 and the 3'terminal fragment was ligated with the 5' end of exon 2. As a result of its function, the two exons were joined together and the intron was released [117].

One more variant of *trans* splicing involves two RNA partners and a ribozyme as separate molecules. For instance, the yeast group II intron catalyzes reciprocal *trans* splicing of two RNAs containing hexanucleotide sites for intron binding. The reaction yields two chimeric RNAs, one containing the 5' end of one RNA partner and the 3' end of the other and the second one, vice versa [118]. Recombinant RNAs were similarly obtained with *Tetrahymena* and *Azoarcus* group I introns acting as ribozymes [119]. The *Azoarcus* ribozyme performed the reaction with a high yield and imposed minimal requirements on the structure of recombination partners: the partners must only contain trinucleotides complementary to the corresponding sites of the intron.

A specific variant of the covalent joining of RNA molecules is reverse splicing, that is, insertion of an intron between two exons [120, 121]. The reaction is not strongly specific: in addition to precise homing, an intron can be inserted in other sites that meet the minimal requirement of the presence of a specific tetranucleotide [122]. Reverse splicing probably contributes to the spreading of introns [123, 124].

Both the spliceosome-dependent and the ribozyme variants were studied in attempts to employ *trans* splicing in gene therapy. In the former case, cells are transformed with a construct designed to insert a therapeutic exon into a pathologically changed endogenous RNA or to substitute it for a pathological exon [125]. Another approach is introducing a donor of a correcting RNA sequence along with a covalently bound ribozyme to catalyze *trans* splicing. Though less efficient, this method of correcting RNA molecules is independent of the exon–intron structure of the target RNA [126]. A study was made of the possibility of using ribozyme-dependent *trans* splicing for treating persistent infection with the hepatitis C virus. A ribozyme was ligated with a fragment of the viral IRES and the mRNA coding for the diphtheria toxin. As a result of *trans* splicing with the viral RNA, the exogenous mRNA acquires the functional IRES and synthesis of the diphtheria toxin causes death of the infected cell [127].

Thus, the covalent joining of the fragments of different RNAs by means of *trans* splicing requires a sophisticated machinery (spliceosomes or ribozymes). Under natural conditions, *trans* splicing is highly specific and joins only exons possessing the proper *cis* signals. Yet the signals themselves are quite simple, providing for the possibility of nonspecific reactions [122]. In any case, the necessity for an intricate machinery and a relatively high site specificity differentiate *trans* splicing from the known types of nonreplicative RNA recombination.

POSSIBLE PLACE AND ROLE OF REPLICATIVE AND NONREPLICATIVE RNA RECOMBINATION

Recombination plays a dual role in the evolution of RNA viruses. On the one hand, recombination facilitates elimination of harmful mutations arising during replication of viral RNA and thereby provides a potent mechanism for stabilizing the genome [28, 128–130]. Homologous recombination is probably responsible for maintaining the conservation of the 3' ends of plant RNA viruses [131–134]. On the other hand, RNA

recombination is an important factor in the variation of viruses, providing for the acquisition of qualitatively new genetic information as a result of transferring functionally significant modules from one virus to another or from the host to a virus [28, 30, 135, 136].

While these general ideas are unquestionable, the biological significance of individual recombination events occurring in nature is still incompletely understood. For instance, intertypic recombinants are rapidly generated and become dominant in the intestine of recipients of the Sabin poliomyelitis vaccine, which contains polioviruses of three serotypes [137, 138]. This finding suggests a selective advantage of intertypic recombinants over the parental viruses. Vaccination is performed with attenuated viruses, and recombination may eliminate the attenuating mutations. This circumstance is probably responsible for the predominance of intertypic recombinants among viruses isolated from rare patients with paralytic poliomyelitis developing as a result of vaccination [139–141]. Under certain conditions (e.g., when population immunity is low), derivatives of vaccine viruses become capable of wide circulation. So far, all known long-circulating (which cause outbreaks of infection) derivatives of poliovirus vaccine strains are recombinants that contain RNA regions acquired either from wild-type polioviruses or related enteroviruses [142]. On the other hand, it cannot be excluded that, owing to its high rate, recombination is likely to occur when two compatible partners meet and that further fixation of recombinants is due to random bottlenecking rather than selection.

In addition, it is difficult to determine whether particular natural recombination events are replicative or nonreplicative. It is possible to assume from general observations (e.g., the low processivity of viral RdRP) that, for example, replicative recombination is more common than nonreplicative recombination in coronaviruses. The clustering of crossover sites in the vicinity of replication promoters or enhancers suggests the same situation for some plant viruses [47, 73]. However, it is still impossible to compare the frequency of the two mechanisms of recombination for other viruses, even for the best-studied system of polioviral infection.

The type of recombination is difficult to identify even when viral RNA fragments, rather than viruses, are recombination partners. It is most interesting in the context of this review that viable recombinants are generated from RNA partners incapable of self-replication. Such a situation was described, in particular, for overlapping RNA fragments of the Sindbis [143] and rubella [144] viruses. One fragment comprised about two-thirds of the genome from its 5' end and coded for nonstructural proteins, and the other coded for structural proteins. Cotransfection with these fragments yielded recombinants, and a considerable portion of these was a result of imprecise (nonhomologous) recombination. These findings were interpreted in terms of the replicative model, because the 5' fragment was capable of directing RdRP synthesis. Although possible, this interpretation is still questionable because, first, RdRP of the rubella virus is insufficiently effective *in trans* [145, 146]. Second, it seems unnatural that the portion of nonhomologous crossovers is rather high, while there are extended identical sequences corresponding to the overlap of the two fragments. Thus, the nonreplicative origin of recombinants cannot be excluded in these cases.

Data on the incorporation of cell nucleic acids into viral RNAs are of particular interest. The evolutionary relatedness of virus and cell genes was demonstrated quite convincingly [147]. Here, we will only consider relatively recent cases of the acquisition of host genes or gene fragments by RNA viruses.

The BVDV provides the most illustrative example. As already noted, this virus usually causes asymptomatic infection both in cultured cells and in animals. However, a pair of related isolates can be obtained from animals with lethal infection: one isolate is a cytopathogenic recombinant and the major disease-producing factor [100]. Many cytopathogenic variants result from insertion of various cell nucleotide sequences into the viral genome. Viral RNA most commonly acquires the ubiquitin gene [100, 148, 149], which plays an important role in protein degradation within the cell. In addition, integration into the viral genome was observed for other host sequences such as genes coding for ubiquitin-like proteins involved in nucleocytoplasmic transport [150] and regulation of the cell cycle [151], other intracellular transport components [152, 153], a chaperone [100], and the ribosomal protein S27a [154]. Such inserts affect the processing of the viral polyprotein and thereby change the virus phenotype.

Other examples of cell nucleotide sequences integrated into viral RNAs are a fragment of the 28S rRNA in the genome of the influenza virus [155] and a sequence similar to an exon of tobacco chloroplast RNA in the RNA genome of the potato leafroll virus [156].

Recombination between host and viral RNAs was also observed under laboratory conditions. Structures originating from cell tRNAs were found in the 5'-terminal region of several DI genomes of the Sindbis virus [157] and in small RQ RNAs from *Escherichia coli* cells infected with phage Qβ [158]. Fragments of other host genes were also detected in RQ RNAs. A pseudorevertant was isolated from cells transfected with a mutant RNA transcript of the poliovirus and proved to contain an insert partly (14 out of 15 nt) identical to a region of the host 28S rRNA [159]. Since the identical region is also present in the *E. coli* 23S rRNA, it cannot be excluded that recombination between the viral RNA and the contaminant bacterial

rRNA occurred during the experiment. Working with nonpurified polioviral transcripts, we observed a fragment of the *E. coli* 23S rRNA inserted into the 5UTR of the polioviral RNA (unpublished data).

Although incorporation of host nucleotide sequences into viral RNAs can be explained in terms of template switching, the nonreplicative mechanism is equally possible in such cases. The nonhomologous character of the corresponding recombination events provides additional circumstantial evidence in favor of the nonreplicative mechanism.

CONCLUSIONS

Covalent rearrangements of RNA are widespread and play an important biological role. Intramolecular or intermolecular splicing is a classical example of natural rearrangements in cell RNAs. Although splicing can be determined by different mechanisms (depending on spliceosomes or ribozymes), its important features are fairly strong site specificity and the involvement of complex macromolecular structures. Recombination between viral RNAs represents a special type of RNA rearrangements. RNA recombination can proceed through various—replicative and postreplicative (nonreplicative)—mechanisms. In turn, the mechanisms of replicative and nonreplicative RNA recombination can vary. Some of them are probably similar to the processes of splicing, while others are based on different principles. In most cases, RNA recombination shows no appreciable site specificity and does not require conserved or intricate RNA structures.

Recombination continues to play an important role in the variation and evolution of RNA viruses, facilitating the exchange of genes (or their fragments) between different viruses or between viruses and host cells. On the other hand, recombination performs an opposite function, maintaining the stability of viral RNA genomes and eliminating unfavorable mutations.

In addition, nonreplicative recombination between viral RNAs has another aspect, going beyond the scope of virology. It is appealing to assume that the uninfected cell also provides room for some of the processes underlying nonreplicative recombination and differing from canonical exon–intron rearrangements associated with intramolecular or intermolecular splicing. If nonspecific covalent joining of cell RNAs or their fragments does occur, then a natural step on the road is to consider the possibility of fixation of chimeric RNA sequences in chromosomal DNA via reverse transcription. In other words, it is possible to assume that some of the mechanisms of nonreplicative RNA recombination play an important role in the evolution of not only viral, but also cell genomes [51, 90].

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