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Réponses du Dr C. Vélot aux allégations du Pr Fischer suite à l'expertise du Dr Vélot concernant les risques sur les vaccins de nouvelle génération contre la Covid-19

1 *« Il y a confusion entre les évènements de recombinaison telle qu'observés dans la nature et ce qui pourrait se passer chez un sujet vacciné. L'ARN vaccinal a une demi vie très courte, il est principalement capté par les cellules du système immunitaire et induit ainsi la réponse immune. »*

Tout d'abord les cellules qui reçoivent le matériel vaccinant sont essentiellement des cellules musculaires (injection intramusculaire), voire des cellules dermiques ou des cellules du tissu conjonctif (tissu qui enveloppe nos organes et notamment nos faisceaux musculaires), mais certainement pas nos cellules immunitaires. Ce sont donc les cellules précitées qui vont prendre en charge le matériel génétique viral injecté et fabriquer la protéine Spike (protéine de surface du virus Sars-Cov2), et c'est cette production de protéine virale (antigène) par les cellules musculaires notamment qui va engendrer la réponse immunitaire. Et heureusement d'ailleurs que ce ne sont pas les cellules immunitaires qui captent le matériel génétique viral pour produire elles-mêmes la protéine Spike, car cela conduirait sans aucun doute à une maladie auto-immune. Les cellules immunitaires fabriquent les anticorps dirigés contre les antigènes, mais bien sûr pas les antigènes eux-mêmes.

Concernant la demi-vie de l'ARN vaccinal, si elle était à ce point courte que cet ARN ne puisse recombiner avec un éventuel autre ARN viral infectant, cela signifierait qu'il n'aurait même pas le temps d'être traduit en protéine Spike.

La demi-vie d'un ARN messager humain (temps nécessaire pour que 50% de cet ARN disparaisse) varie entre 30 min et 24H. Pour un ARN de virus humain, on se situe plutôt vers le haut de la fourchette. En effet, la stabilité d'un ARN va dépendre de son taux de traduction car les machineries de traduction (les ribosomes), en lisant l'ARN (pour le traduire), le protègent. Or un ARN viral a vocation à être beaucoup traduit.

2 *« Cet ARN ne peut être retrotranscrit en ADN pour donner lieu à événement de recombinaison/insertion ».*

La recombinaison virale existe aussi bien entre ADN viraux d'une part qu'entre ARN viraux d'autre part. Et dans ce dernier cas, cela ne nécessite nullement une rétrotranscription — c'est à dire une conversion de l'ARN viral en ADN. Les ARN viraux recombinent directement. Comment le Pr. Fischer peut-il ignorer cela alors que c'est connu depuis les années 1990 ? Pour en attester, voir ci-dessous un article scientifique de 1992, publié dans une grande revue scientifique internationale à comité de lecture — *Microbiological Reviews* —, et intitulé « *RNA recombination in animal and plant viruses* ». (Version en ligne)

3 ***« La probabilité d'infection virale d'une cellule porteuse de l'ARNm vaccinal est très faible compte tenu de la 1/2 vie de cet ARNm (et des autres virus cités). De plus la capacité de recombinaison devrait parvenir du virus infectant selon un mécanisme hypothétique ».***

Concernant la demi-vie de l'ARNm viral, j'ai déjà répondu au point 1. J'ajoute que la rencontre entre le matériel génétique viral vaccinant d'une part, et celui d'un virus infectant d'autre part, n'est pas restreinte au seul cas où l'infection par un autre virus surviendrait strictement dans le créneau où l'ARN viral vaccinant est présent dans nos cellules. C'est également vrai dans le cas d'une infection qui précède la vaccination (et qui bien sûr est encore présente au moment de la vaccination). Certes, cela restreint la probabilité de rencontre entre les deux matériels génétiques viraux. Mais, une fois de plus, ne perdons pas de vue qu'il s'agit de vaccination massive, c'est-à-dire concernant des effectifs colossaux. La probabilité que de tels évènements se produisent n'est donc pas nulle. Et on ne peut en faire l'économie dès lors que les conséquences ne seraient pas que pour la personne vaccinée chez laquelle émergerait un tel virus recombinant plus virulent ou plus contagieux : ce risque doit être apprécié à l'échelle de la population.

Quant à « *la capacité de recombinaison qui devrait parvenir du virus infectant selon un mécanisme hypothétique* », de quoi parle le Pr. Fischer ? Quel mécanisme hypothétique ? On n'est pas dans la science fiction : encore une fois, ces mécanismes sont connus depuis les années 1990 !

4 ***« À noter que l'ARN a déjà été utilisé chez l'homme dans des essais de vaccination anti-cancer et sous formes d'oligonucléotides anti sens et siRNA et ce à des doses beaucoup plus élevées et répétées, sans que de tels évènements aient été observés. Par ailleurs de tels événements n'ont été reportés, ni lors de l'utilisation de virus inactivés (qui contiennent ARN et ADN !) ni atténués et ce depuis des dizaines d'années alors qu'ils pourraient avoir été injectés dans des cellules porteuses d'un virus... »***

Tout d'abord, de tels évènements ne peuvent évidemment pas se produire avec des vaccins utilisant des virus inactivés ou atténués puisque dans ces cas, le matériel génétique viral n'est pas délivré dans nos cellules. C'est justement l'originalité des vaccins nouvelle génération dits « vaccins génétiques », que de délivrer le matériel génétique viral dans nos cellules pour leur faire fabriquer elles-mêmes l'antigène.

Quant aux stratégies cliniques antérieures consistant à délivrer du matériel génétique dans nos cellules, et dont parle le Pr. Fischer — à savoir les essais cliniques d'immunothérapie, — il s'agit de traitements s'adressant à des patients atteints de cancers. Le but est d'essayer de leur faire développer des anticorps dirigés spécifiquement contre des protéines humaines présentes à la surface de leurs cellules cancéreuses (dans le but de détruire celles-ci). Le Pr Fischer parle abusivement de « *vaccination anti-cancers* ». Ce terme est totalement inapproprié et crée la confusion. Il ne s'agit en rien d'une vaccination, d'une part car ce n'est pas une démarche préventive mais curative, et d'autre part car il ne s'agit pas de lutter contre un agent infectieux mais contre certaines de nos propres cellules (devenues cancéreuses).

Non seulement ces patients sont bien sûr prêts à accepter des effets secondaires – même importants – dès lors que le rapport bénéfices/risques reste favorable, mais il s'agit d'effectifs extrêmement réduits. Ces essais n'ont donc rien à voir avec une véritable campagne de vaccination qui s'adresse à des personnes en bonne santé et en très grand nombre, en particulier dans le cas de la Covid qui concerne la population mondiale.

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RNA Recombination in Animal and Plant Viruses

MICHAEL M. C. LAI

Howard Hughes Medical Institute and Department of Microbiology, University of Southern California School of Medicine, Los Angeles, California 90033

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INTRODUCTION

RNA viruses are known to undergo rapid genetic change. The most common mechanism of change is through nucleotide substitutions resulting from the purportedly high error frequency of RNA synthesis (37, 94). This leads to the concept of RNA viruses being "quasispecies," which consist of collections of virion RNAs with slightly divergent nucleotide sequences (27). Furthermore, it accounts for the sequence drift of various RNA viruses under certain selection pressures (92, 93). RNA viruses that contain segmented genomes also can undergo genetic evolution by reassortment of the RNA segments. This mechanism accounts for antigenic shift and the selection of certain phenotypes in influenza virus, rotavirus, bluetongue virus, and others (28, 80). An additional mechanism, which occurs less frequently and has not been as well appreciated, is RNA-RNA recombination, which involves the exchange of genetic information between nonsegmented RNAs. The ability of RNA viruses to undergo genetic recombination so far has been demonstrated only for a few viruses. Among these, only picornaviruses and coronaviruses can recombine at a frequency that is easily detectable. The apparent rarity of genetic recombination by RNA viruses contrasts with the frequent generation of defective interfering (DI) RNA, which can be viewed as one form of nonhomologous recombination. DI RNA has been demonstrated in almost every RNA virus. Therefore, RNA-RNA recombination theoretically will occur in most RNA viruses. Indeed, by using more sophisticated methodology and different conditions, the phenomenon of RNA recombination has been demonstrated in an increasing number of viruses. Sequencing and structural characterization of the genomic RNAs of animal and plant viruses also have suggested that many natural strains of RNA viruses were generated by genetic recombination between related or even unrelated viruses. Furthermore, comparisons of the genetic structure of different RNA viruses suggested that many viruses have undergone RNA rearrangement during virus evolution; these rearrangements are best explained by RNA

recombination. Thus, RNA recombination is being recognized increasingly as an important and general phenomenon in the biology of RNA viruses.

This review will focus on different types of homologous RNA recombination, which result in intact and fully functional chimeric RNA, as well as structurally defective but replication-competent hybrid RNA. Nonhomologous RNA recombination, which involves unrelated RNA molecules, will also be discussed. However, the generation of DI RNA from wild-type viruses will not be dealt with, although its mechanism appears to be similar to that of RNA recombination. Retrovirus recombination is a different phenomenon from RNA recombination, but its mechanism is remarkably similar to that of RNA recombination. It will be briefly discussed for comparative purposes. I will review the pertinent facts on genetic recombination in RNA viruses, but will not present an exhaustive review of the literature. In addition, I will offer perspectives on the mechanism and biological significance of RNA recombination.

HISTORY OF STUDIES OF RNA RECOMBINATION

Genetic recombination involving the exchange of sequences between two nonsegmented RNA genomes was first described for poliovirus by Hirst (36) and Ledinko (64) in the 1960s. By using polioviruses possessing different genetic markers, e.g., resistance to horse or cattle serum and resistance to guanidine, possible recombinant viruses which had acquired the resistant phenotypes of both parental viruses were isolated. Since the frequency of isolation of such viruses during mixed infections was higher than that of spontaneous mutations during single infections, it was assumed that they were the products of recombination. This was the first indication that RNA viruses with nonsegmented genomes could undergo recombination, although it was not known at the time that these viruses contained a single-stranded, nonsegmented RNA genome and that RNA recombination would turn out to be an exception rather than the rule. Using a similar approach, Pringle subsequently showed that foot-and-mouth disease virus (FMDV), which, like poliovirus, is a member of picornavirus family, was able to undergo genetic recombination (83). Cooper subsequently used a collection of temperature-sensitive (*ts*) mutants of poliovirus and determined the recombination frequency between different pairs of *ts* mutants (20, 21). He was able to derive a recombination map for poliovirus, which was linear and additive between different *ts* markers. In these early studies, the occurrence of recombination could be inferred only from the detection of double mutants during mixed infections, which arose at a frequency in excess of spontaneous mutations during single infections. The possibility of enhancement of the mutation frequency of the parental viruses by mixed infection was not rigorously ruled out. The definitive proof of RNA recombination finally came from biochemical analyses of the potential FMDV recombinants. By using FMDV strains with distinguishable protein structure and RNA sequences, it was established unequivocally by biochemical analysis that the putative recombinant viruses indeed contained genetic sequences derived from both parental viruses (53). Thus, RNA recombination was established as a bona fide genetic phenomenon.

The second RNA virus family that was shown to undergo genetic recombination is coronavirus. Recombinant viruses were initially isolated by using a combination of the classical methods of mixed infections with two *ts* mutants and subsequent identification of the ts^+ viruses by biochemical characterization (59). Coronaviruses have since turned out to have a capacity to undergo recombination at an extremely high frequency (8, 69). Subsequently, a plant virus, brome mosaic virus (BMV), was shown to recombine in plants under certain circumstances (15). In recent years, there have been an increasing number of reports indicating the occurrence of recombination in various families of RNA viruses. Thus, recombination among RNA viruses appears to be more general than was previously recognized.

On the other hand, there have been many reports indicating the failure of RNA viruses to recombine; for instance, Newcastle disease virus, vesicular stomatitis virus, and RNA phages (37, 39, 81) failed to yield recombinants when classical genetic approaches were used. These results indicated that the frequency of RNA recombination, if it was present at all, was not higher than the frequency of spontaneous mutations in these viruses. Thus, there are probably genetic constraints on the occurrence of RNA recombination in many viruses.

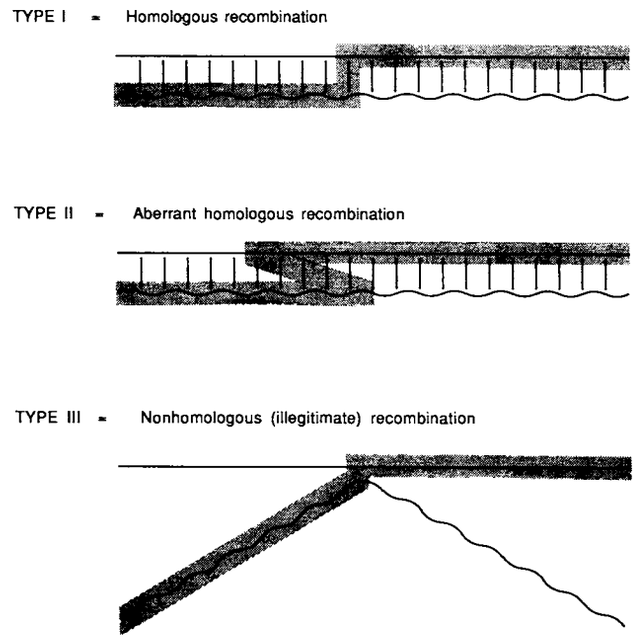

FIG. 1. Types of RNA recombination. The shaded lines represent recombinant RNA molecules. The vertical lines represent homologous nucleotides.

TYPES OF RNA RECOMBINATION

Classical genetic studies on picornavirus and coronavirus recombination indicated that all of the recombinants resulted from genetic crossing over at precisely homologous or comparable sites on the two parental RNAs involved. More recent studies on recombinants of other animal and plant viruses, however, showed the prevalence of other types of recombination, some of which are unique to RNA recombination and are not seen in DNA recombination. On the basis of the nature of the RNAs involved and sites of crossing over, RNA recombination can be classified into three types (Fig. 1).

Type I (Homologous) Recombination

This type of recombination involves two similar or closely related RNA molecules with extensive sequence homology; crossovers occur at sites perfectly matched between the two RNAs so that the recombinant RNAs retain the exact sequence and structural organization of the parental RNA molecules. Thus "homologous" refers to not only the presence of sequence homology between the two parental RNAs, but also the occurrence of crossovers at homologous or comparable sites on the two molecules. Sequence homology between the two RNAs must be present around, although not necessarily at, the crossover sites. Most of the RNA recombinations involving full-length viral genomes, such as picornavirus recombination, are of this type.

Type II (Aberrant Homologous) Recombination

Similar to type I recombination, type II recombination involves two RNA molecules with similar sequences. However, in contrast to type I recombination, crossovers occur not at the homologous or comparable sites, but at unrelated although usually nearby sites, on each parental RNA molecule. As a result, recombinant RNA contains sequence duplication or deletion and, in some cases, even insertion of nucleotides of unknown origin. The basis for the selection of crossover sites in this type of recombination is not clear. In most aberrant homologous recombination, there appears to be no sequence homology immediately around the crossover sites on the two RNA molecules. This type of recombination is unique to RNA recombination. In some genetic crosses, aberrant homologous recombination was the only type of recombination, even though sequence homology existed between the two RNA molecules involved. This type of recombination is particularly common when defective RNAs area involved in recombination.

Type III (Nonhomologous or Illegitimate) Recombination

Type III recombination occurs on RNA molecules which do not show any sequence homology. Thus, the basis of selection of recombination sites is unclear. One possibility is that the crossover sites on the two RNAs share similar secondary structure. This type of recombination is relatively infrequent in RNA recombination, but in DNA recombination it occurs at a much higher frequency than homologous recombination. This type of recombination may account for gene rearrangements, insertions, and deletions observed in RNA viruses.

HOMOLOGOUS RECOMBINATION IN PICORNAVIRUSES

Picornaviruses contain a single-stranded, positive-sense RNA of approximately 7 kb (for a review, see reference 88). The 5′ end of the genome contains a covalently attached protein, VPg, and the 3′ end contains poly(A). The genome consists of a single open reading frame, which starts at approximately 700 nucleotides from the 5′ end and terminates near the 3′ end of the genome. This single open reading frame is translated into one polyprotein, which subsequently is processed into multiple viral structural and nonstructural proteins. The structural proteins are encoded from the 5′ end of the open reading frame, whereas the remaining sequences encode virus-specific proteases and other proteins involved in RNA synthesis. The replication of RNA is carried out by a virus-specific RNA polymerase and appears to involve VPg protein. RNA synthesis takes place in the cellular membrane fraction. There is asymmetry of positive-strand and negative-strand RNA synthesis, with positive-strand RNA being the predominant product. It is not clear whether the polymerase complex contains host cell proteins. A detailed discussion of picornavirus recombination can also be found in a recent review (51).

Properties of Recombinants Obtained

Recombination has been demonstrated to occur between closely related picornavirus strains as well as between more distantly related strains (52, 70, 71, 99). The types of recombinants obtained depended on the selection markers used. Typically, recombination was demonstrated in mixed infections with two parental viruses which possessed different genetic markers, e.g., temperature sensitivity in replication, resistance to guanidine (82) or horse serum treatments (36), and resistance to antibody neutralization (82). Recombination was presumed to have occurred if the yield of phenotypically wild-type virus during mixed infections was higher than that during single infections with either of the parental viruses. On the basis of the assumption that the farther apart the two selection markers, the higher the probability that recombination can occur, genetic recombination maps, which denote recombination frequencies and genetic distances between various genetic markers, have been obtained for poliovirus and FMDV (20, 60, 72). These recombination maps show that genetic recombination frequency is additive in proportion to the distance between the genetic markers. Also, the genetic markers could be arranged in a linear fashion based on their recombination frequency. Of course, these recombination maps were based on the assumption that recombination was completely random and that there were no recombination hot spots or negative interference between different temperature-sensitive markers. Furthermore, it had to be assumed that complementation between temperature-sensitive mutants did not lead to enhancement of reversion of temperature-sensitive phenotype of the parental viruses. Since most pairs of *ts* mutants used led to the generation of recombinants, it was assumed that recombination occurred randomly. Unfortunately, there was no independent physical mapping to determine the precise sites of the genetic markers used in these studies. Therefore the genetic distances and recombination frequencies determined in these recombination maps may not be entirely correct and should be viewed with some caution.

Recombination appeared to occur throughout the entire genome (1, 51). This observation suggested that recombination is general and not site specific. By using two strains of viruses with proteins of different electrophoretic mobilities, it was possible to map roughly the crossover sites of various recombinant viruses on the RNA genome (53, 70, 71). It was shown that although crossovers could occur anywhere within the entire genome, no recombination was detected in the genetic regions encoding capsid proteins VP1 and VP3 (51). The failure to detect recombination within this region could be due to the physical properties of the genetic markers used in these studies. Alternatively, a more interesting interpretation is that recombination within this region may lead to nonfunctional or unstable gene products and, as a result, the recombinant virus was selected against. By performing biochemical analysis of the protein structure and RNA sequences of the ts^+ viruses isolated from coinfection

with two *ts* mutants, it was shown that most of the ts^+ viruses isolated were real recombinants and not the revertants of the parental viruses (71, 72). These findings suggested that the recombination frequency was much higher than the reversion frequency of the *ts* mutants used and thus provided assurance that the recombination maps obtained by earlier investigators and based on the frequencies of "wild-type" viruses during mixed infections (20, 60, 72) were valid. Most of the recombinants had a single crossover between the two selection markers, but recombinants with multiple crossover sites also have been obtained. The latter could be the result of a single recombination event or of multiple rounds of recombination (51, 52).

Recombination occurred more readily between the more closely related viruses and was shown to decrease as the genetic relationship between the two viruses diverged (55, 70, 71, 99). In a series of studies on recombination between different FMDV strains (intertypic recombination), most of the recombination sites were localized within the 3′ half of the RNA genome, i.e., the regions encoding nonstructural proteins (71). It was not clear whether intertypic recombination occurred preferentially at these sites or whether these were the results of the selection markers used.

So far, picornaviruses have been demonstrated to undergo only type I (homologous) recombination in both intratypic and intertypic recombination. Although recombination has been shown to occur throughout the entire genome of picornaviruses, with some exceptions as noted above (51), some studies did suggest the clustering of recombination sites in some hot spots. In a study on intertypic poliovirus recombination, recombination was shown to occur in the RNA regions where RNA could potentially form a secondary structure, allowing the two parental RNAs of different origins to complex and thereby forcing recombination to occur (87, 99). A very similar mechanism involving complementary sequences between the two RNAs also has been proposed to explain the generation of DI RNA and deletion, rearrangement, and recombination of poliovirus RNA (56). These studies predicted that recombination could occur only at restricted RNA sites. However, in another study examining poliovirus recombination sites between two selection markers (guanidine dependence and temperature sensitivity), it was shown that recombination could occur almost randomly between these two markers without sequence preference (55). Therefore, it is not certain whether picornavirus recombination requires any specific sequences. The reconciliation of these two alternatives will be discussed below in the section on the mechanism of RNA recombination.

Recombination In Vivo

The above discussion focused on recombinants which were isolated during experimental infections in tissue culture and required the use of certain selection markers. Thus, these recombinants were obtained under artificial selection pressures. Does recombination occur in natural infections? The answer came from unexpected findings during the examination of viruses isolated from children receiving poliovirus vaccines. Kew and Nottay (49) reported the isolation of a recombinant virus which contained sequences derived from all three serotypes of poliovirus vaccine strains as a result of two crossovers. This finding indicates that recombinants can appear spontaneously during natural infections. Subsequently, additional recombinants have been isolated from poliovirus vaccinees. In an interesting longitudinal study (76) of the polioviruses isolated from a child after administration of the oral poliovirus vaccines, recombinant viruses could be isolated as early as 8 days after vaccination. Additional recombinant viruses continued to emerge as late as 7 weeks postvaccination. Most isolates were intertypic recombinants between type 2 and 3 polioviruses, and all of the crossover sites were localized in the 3′ nonstructural protein genes (76). These observations suggest that RNA recombination occurs frequently among polioviruses and is a natural mechanism in the evolution of the virus. Whether or not the emergence of these recombinant viruses is the result of selective growth advantages of these viruses in the gastrointestinal tract has not been studied. The potential of polioviruses to undergo recombination in the gastrointestinal tract raised the issue of the stability and safety of the vaccine. Similar studies have not been reported for FMDV.

Recombination Frequency

By using resistance to inactivation by horse and cattle serum as genetic markers, the recombination frequency between two polioviruses was first determined to be approximately 0.2 to 0.4% (36, 64). With a series of *ts*-mutants, Cooper has shown that between the two most distant *ts* lesions of poliovirus, the recombination frequency was approximately 2% (20, 21). Using other selection markers, i.e., resistance to guanidine and a *ts* mutant, which are separated by approximately 1.5 to 3 kb in physical distance on the genome, McCahon et al. obtained a recombination frequency of 0.92% for FMDV (70, 72). Assuming that reciprocal recombination occurred at equal efficiency, King argued from these data that the recombination frequency should be between 10 and 20% for the entire FMDV genome (51). Kirkegaard and Baltimore also found a recombination frequency of 0.13% between two genetic markers (guanidine dependence and a *ts* growth phenotype) which were separated by 190 nucleotides in the poliovirus genome (55). Assuming that this region was not a recombination hot spot, the recombination frequency for the entire poliovirus genome could be extrapolated to be close to 20% (51). However, extrapolation based on such a small region is most probably unreliable. If the recombination frequency were really this high, one would expect to see multiple crossovers in many recombinant viruses. This has not been the case, although not enough recombinants have been studied to fully assess this possibility. These recombination frequencies were determined for intratypic recombination (72). When polioviruses of different serotypes were studied for recombination (intertypic recombination), recombination frequencies were considerably lower (55, 71, 99). For example, the recombination frequency between Mahoney type 1 and Lansing type 2 polioviruses between the two markers discussed above (guanidine dependence and a *ts* growth phenotype) was 170-fold lower than for the comparable intratypic recombination. These results suggest that recombination requires some sequence homology between the participating RNA strands.

HOMOLOGOUS RECOMBINATION IN CORONAVIRUSES

Coronaviruses contain a positive-sense RNA genome of 31 kb (for a review, see reference 58). This genome size is unusually large, almost twice the size of the next largest viral RNA (paramyxovirus). The RNA contains seven to eight

genes; three or four encode structural proteins, and the remainder encode nonstructural proteins. Unlike in picornaviruses, the structural protein genes are interspersed between the nonstructural protein genes. The first gene at the 5′ end of the genome is nearly 22 kb long (65) and makes up almost two-thirds of the entire genome. Each of the gene products is translated from individual mRNA species, which have a 3′-coterminal, nested-set structure; i.e., the sequences of the small mRNAs are included completely within the larger mRNAs. Each mRNA also contains a stretch of leader sequence of approximately 70 nucleotides, which is derived from the 5′ end of the genome. RNA synthesis in coronavirus appears to be carried out by a virus-specific RNA polymerase, which is derived from the gene products of the 5′-most gene. The polymerase first transcribes genomic RNA into negative-sense RNA, which then serves as a template for the synthesis of genomic and subgenomic mRNAs. Both genomic and subgenomic negative-sense RNAs are present. At least late in the infection, subgenomic mRNAs are transcribed from the negative-sense subgenomic RNAs. Because of the presence of the leader RNA, coronavirus RNA synthesis must involve some kind of splicing or a discontinuous transcription process.

Properties of Recombinants Obtained

The first coronavirus recombinant was isolated by selecting ts^+ progeny virus from a mixed infection of *ts* mutants belonging to two different mouse hepatitis virus (MHV) strains (59). Subsequently, additional recombinants were obtained by using different pairs of *ts* mutants and other selection markers, including monoclonal antibody neutralization resistance phenotypes and the ability of some viruses to induce cell-cell fusion (47, 48, 68, 69). One striking feature of these recombinants is that some of them appeared to have undergone more than one crossover event, indicating that coronavirus may undergo recombination at a very high frequency. The combination of these selection markers allowed the isolation of a wide range of recombinants. Although the crossover sites of most of the recombinants obtained initially clustered at the 5′ end of the genome (48, 59, 69), multiple recombination sites were subsequently detected over almost the entire genome (47, 68). Most strikingly, many of the recombinants not only have crossovers between the two selection markers, but also have crossover sites located outside the selection markers used. Since the latter crossover events were not specifically enriched by selection, their frequent detection could be explained only by the high frequency of their occurrence, such that no selection pressure was needed. For instance, in a recombination study between a *ts* mutant and a wild-type virus, with neutralizing monoclonal antibodies against the wild-type virus, all of the recombinant viruses isolated at the nonpermissive temperature had a crossover site between the two markers (68); also, almost half of them had one or more additional crossover sites outside the genes where the selection markers were localized (Fig. 2). In another series of recombination studies between MHV-2, which does not cause cell fusion, and a *ts* mutant of A59, which causes cell fusion at permissive temperatures, not only did the recombinants obtained have crossovers in the spike protein gene where the selection markers lie, but also half of them had additional crossovers in the 3′-end genes, which encode both the nucleocapsid protein and membrane protein (47). The latter crossover events were not under the selection pressures applied in that study.

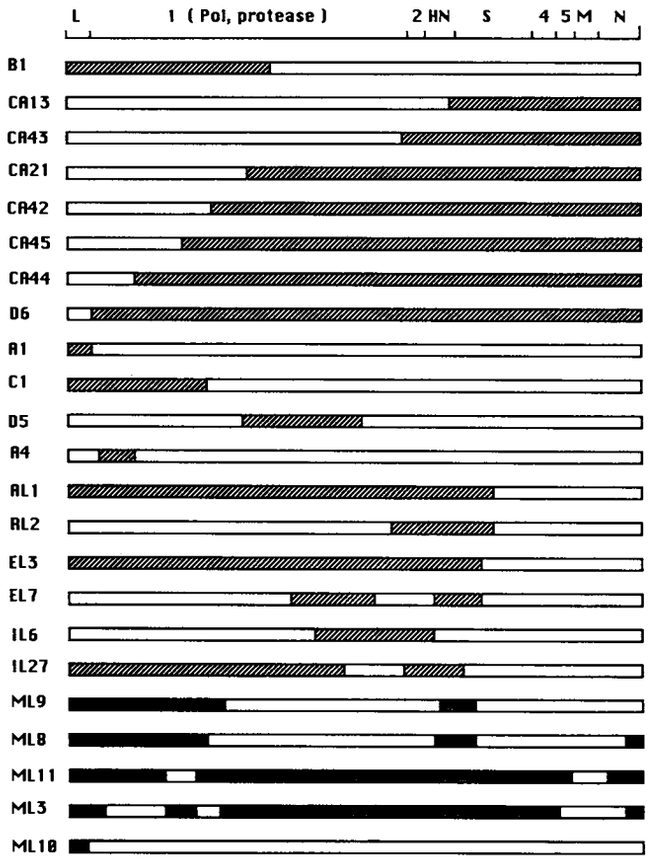

FIG. 2. Genetic structure of the representative coronavirus recombinants. The data are compiled from reference 46 to 48, 59, 68, and 69. Symbols: ▭, A59; ▨, JHM; ▩, MHV-2.

The results of these recombination studies indicate that coronavirus RNA recombination has several unique features. First, multiple crossover events appear to occur much more frequently in coronavirus recombination than in picornavirus recombination. It is not clear whether this is a result of the larger size of the RNA genome or a reflection of an inherently higher recombination frequency in coronavirus. One telltale sign of a higher recombination frequency in coronavirus is the frequent isolation of recombinants with crossover sites outside the two selection markers used, indicating that recombinants can be detected readily without applying selection pressure. Second, the occurrence of recombination may be constrained by the genetic incompatibility of the parental viruses; for instance, recombination occurred frequently in the 3′-end genes between A59 and MHV-2 strains (47), but not between other combinations of virus strains. Whether this genetic constraint on recombination is the result of nucleotide sequences in the parental viruses or the functional instability of the hybrid molecules generated in the recombinants is not clear. Third, in certain situations, recombinant viruses can have evolutionary advantages and become the predominant population. In a study involving *ts* mutants of A59 and a wild-type JHM, the progeny viruses isolated at the nonpermissive temperature for the parental *ts* A59 outgrew both of the parental viruses and became the predominant virus after only two tissue culture passages (69). This provides a striking example of the functional role of RNA recombination in virus evolution.

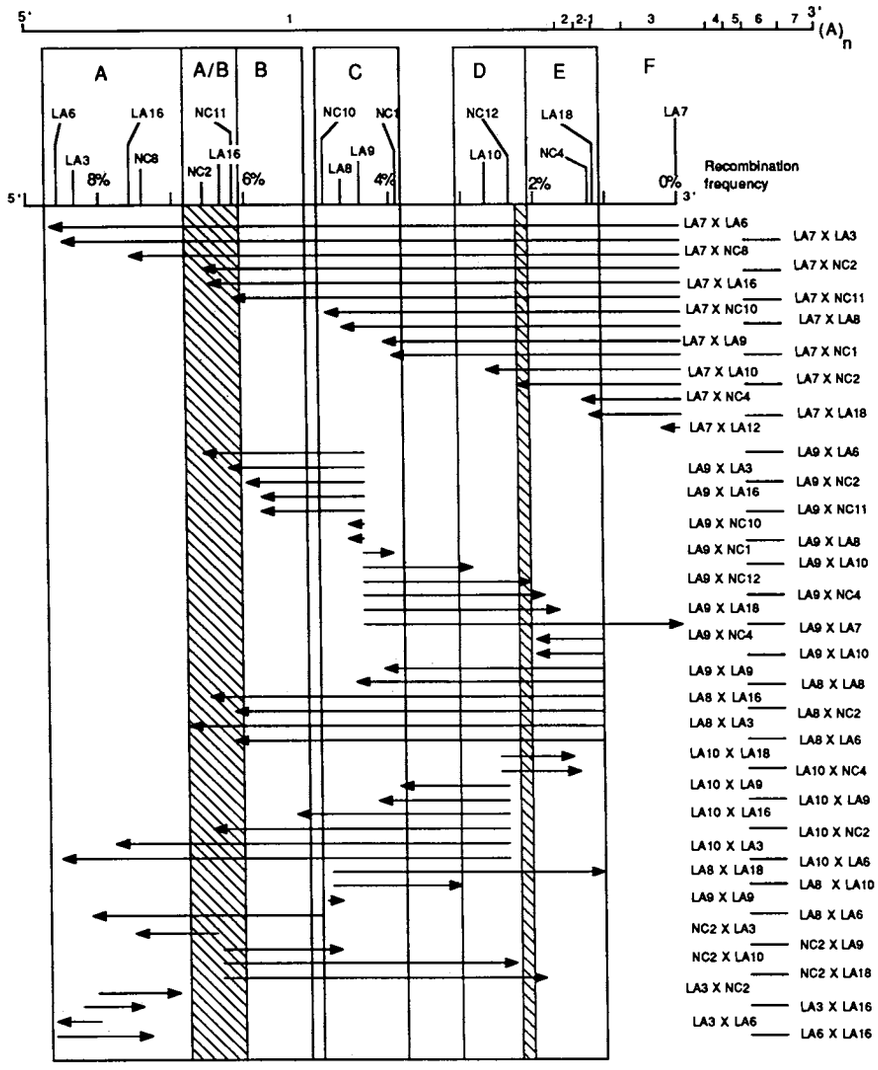

FIG. 3. Genetic recombination map of the coronavirus MHV. The percentages represent the frequency of recombination between each pair of *ts* mutants. Modified from reference 8 with permission. The genetic map of MHV RNA in relation to the *ts* markers and recombination frequencies is shown above (58).

On the basis of the recombination frequencies between different pairs of *ts* mutants, a linear genetic recombination map has been obtained (8) (Fig. 3). Recombination frequencies are additive, and the genetic recombination map is linear, consistent with the genetic structure of the coronavirus RNA genome. However, because of the lack of independent biochemical evidence for the genetic location of these *ts* lesions, the genetic distances determined in this recombination map must be viewed with caution. It is conceivable that some sites are hot spots for recombination, thus distorting the distances between the markers in these regions. So far, only homologous recombination has been demonstrated for coronaviruses.

Recombination In Vivo

Although there is no clear evidence that coronavirus recombination occurs during natural infections in humans and other animals, the potential occurrence of such recombination has been demonstrated in a study conducted in my laboratory (46). Two MHV strains were inoculated into mouse brain; recombinants were detected among the progeny virus isolated form the infected mice at a frequency of approximately 4.5%. The types of recombinants obtained were similar to those observed in tissue culture infection, i.e., recombinants with a crossover site between the two selection markers, but also with unexpected crossovers outside the markers used. This study suggests that recombination can occur in animals and that its frequency is similar to that observed in tissue culture infections. Thus, recombinant viruses could play a role during natural coronavirus infections.

Sequencing of different natural isolates of an avian coronavirus, infectious bronchitis virus, revealed that different regions of the spike and membrane protein genes have evolved at different rates, with different domains having closer homology to other infectious bronchitis virus isolates in a noncoordinated manner (17a, 57). One interpretation of

this finding is that recombination has occurred within the gene between different viruses, exchanging different domains and thus resulting in different degrees of genetic relatedness within the gene between the viruses. This observation suggests that coronavirus recombination could occur in natural viral infections.

Recombination Frequency

As indicated above, several studies of coronavirus recombination have suggested that the recombination frequency of coronavirus was of a sufficient level that recombinants could be isolated without application of selection pressure. Using a series of *ts* mutants, Baric et al. have determined the intratypic recombination frequencies for coronavirus (8). The two most distant *ts* markers had a recombination frequency of 8.7% (Fig. 3). Since one of the *ts* mutants studied appeared to have a lesion in a structural protein gene and the other was probably at the 5′ end of the genome, these two *ts* markers were likely to be separated by approximately 20 kb. On the assumption that recombination was completely random and occurred reciprocally, the recombination frequency for the entire coronavirus genome was extrapolated to be nearly 25% (8). This value is probably more accurate than the estimate for picornavirus recombination frequency, since the reported recombination frequency of coronavirus was determined for a much larger genetic region; thus, the extrapolation has more validity. Of course, this percentage may be compromised by the possibilities that certain regions are hot spots for recombination and that certain recombinants might have growth advantages over the parental viruses, as suggested by Makino et al. (69). These occurrences would inflate the observed recombination frequency. Such a recombination frequency is translated into roughly 1% recombination per 1,300 nucleotides for coronavirus RNA and compares favorably with the estimate of 1% recombination frequency per 1,700 nucleotides for poliovirus RNA (8, 21). These recombination frequencies are actually in the same range as in DNA recombination. Earlier studies suggested that recombination frequency is roughly 1% per 200 bp for T4 phage and per 1,750 nucleotides for *Escherichia coli* DNA (35). Thus, RNA recombination appears to occur at a much higher frequency than is commonly realized. The intertypic recombination frequency has not been determined for coronavirus.

RECOMBINATION IN ALPHAVIRUSES

Alphaviruses contain a positive-sense, single-stranded RNA of approximately 12 kb (for reviews, see references 89 and 95). The 5′ two-thirds of the RNA genome encodes nonstructural proteins, whereas the 3′ one-third encodes structural proteins. The four nonstructural proteins are translated from the genomic 49S RNA, whereas the structural proteins are encoded from a subgenomic 26S RNA, which is initiated from an intergenic site located in the 3′ half of the RNA genome. The nonstructural proteins, which include RNA polymerases, first synthesize a negative-sense copy of the genomic RNA, which is in turn used as a template for the synthesis of the positive-sense genomic and subgenomic RNAs.

By using classical genetic approaches with *ts* mutants, attempts have been made to detect genetic recombination in Sindbis virus. The frequency of occurrence of ts^+ virus during coinfections with two *ts* mutants was not higher than that during single infections with either parent virus (81). Thus, it was thought that Sindbis virus could not undergo RNA recombination. However, recombination has been demonstrated recently between RNAs of Sindbis virus (103). This study was performed by using Sindbis virus RNAs that contain intact nonstructural protein genes but defective structural protein genes and RNAs that contain the *cis*-acting sequences required for replication and encapsidation and intact structural protein genes but defective nonstructural protein genes. When two RNAs were cotransfected into susceptible cells, the RNA segments complemented each other and both replicated (30). In addition, some of the RNAs recombined in the region containing the overlapping sequences between them and yielded autonomously replicating infectious genomic RNA (103). Many of the recombinants had sequence insertions, deletions, or other rearrangements with respect to the parental viral sequences at the putative sites of crossover, and many of them had multiple crossovers. These sequence rearrangements suggest an aberrant homologous (type II) recombination mechanism or represent the results of additional rearrangements after recombination. This is the first evidence of recombination between Sindbis virus RNAs. Therefore, the previous failures (81) to detect recombinants were most probably due to a low frequency of homologous recombination, such that recombinants could not be detected over the background of parental viruses. The sequences at the crossover sites did not reveal any homology between the two parental RNAs, despite the presence of homologous regions elsewhere between them. Why type II recombination was favored over homologous recombination in this case is not clear.

Although homologous recombination in alphaviruses has not been demonstrated in a tissue culture system, evidence has suggested its occurrence during natural infections. Sequence analysis of western equine encephalitis virus (WEEV) RNA revealed that most of its RNA sequences are homologous to those of eastern equine encephalitis virus (EEEV), a New World alphavirus. However, much of the 3′-end sequences, which encode structural proteins, have a closer homology to Sindbis virus, an Old World alphavirus, whereas the extreme 3′-end noncoding region of WEEV appears more homologous to EEEV (32). Thus, WEEV could have been derived from EEEV and Sindbis virus as a result of double crossovers. Therefore, homologous recombination can potentially occur during natural virus infections. It is interesting that one of the putative crossover sites in WEEV RNA was located in the middle of the structural protein-coding region, such that the structural proteins of WEEV have two different origins, with the capsid protein gene being derived from EEEV and the rest of the structural proteins being derived from Sindbis virus. However, probably during the process of selection subsequent to recombination, the sequence of the capsid protein gene has mutated to the extent that it resembles that of Sindbis virus; as a result, all of the structural proteins in WEEV have sequences similar to Sindbis virus (32). This observation raised the interesting possibility that the structural proteins of Sindbis virus and EEEV were functionally incompatible and thus that the original recombinant was genetically unstable. This possibility may explain the rarity of homologous recombination.

RECOMBINATION IN PLANT VIRUSES

RNA recombination in plant viruses was first demonstrated for brome mosaic virus (BMV) (15). In recent years,

an increasing number of other plant viruses also have been shown to undergo recombination.

Bromoviruses

BMV, a member of the bromovirus family, contains three RNA segments, of 3.2, 2.9, and 2.1 kb, which share nearly 200 identical nucleotides at the 3′-end noncoding region (for a review, see reference 25). Each RNA encodes a single protein, except for RNA-3, which has two open reading frames. The second open reading frame of RNA-3 encodes the coat protein and is expressed through a separate subgenomic mRNA (RNA-4). The 5′- and 3′-end regions contain regulatory signals for the replication of these RNAs. RNA-1 and RNA-2 can replicate independently of RNA-3, whereas RNA-3 can replicate only with the help of both RNA-1 and RNA-2. Neither of the gene products of RNA-3 is required for RNA replication, but they are required for viral assembly and spread.

RNA recombination was first demonstrated in an experiment in which barley plants were inoculated with a mixture of in vitro-transcribed wild-type BMV RNA-1 and RNA-2 and a mutant RNA-3, which contained a deletion in the 3′-end common sequence but was replication competent (15). After a prolonged period following RNA inoculation, a novel RNA-3 with a size equivalent to that of the wild-type RNA-3 was detected. Sequencing of this new RNA showed that most of the novel RNA-3 contained the complete RNA-3 sequence similar to that of the wild-type RNA, apparently resulting from recombination between the mutant RNA-3 and either RNA-1 or RNA-2 in the 3′-noncoding region common to all the three RNAs. Most of the RNAs have a perfectly restored RNA-3 sequence, representing homologous recombination, but some of the RNA molecules contain duplications of part of the 3′-noncoding region, apparently as a result of type II recombination. This provided the first example of RNA recombination in plant viruses. The generation of the recombinant RNAs in this system was relatively inefficient and was not observed with similar mutant RNAs under other conditions (26).

The ability of BMV RNAs to undergo recombination also has been demonstrated by using a deletion mutant of RNA-2 together with wild-type RNA-1 and RNA-3 in infection of protoplast or *Chenopodium hybridum* (84, 85). These systems allowed the generation of recombinants between the defective RNA-2 and either RNA-1 or RNA-3 in the 3′-end homologous regions at relatively high efficiency (85). Most of the recombinants obtained were the results of homologous recombination. Interestingly, in this case, the mutant RNA-2 was replication defective and yet was able to recombine with other RNAs to generate replication-competent RNAs.

In all of these studies on BMV RNA recombination, homologous recombination appeared to be the rule. However, type II (aberrant homologous) recombination also has been observed. The mechanism of generation of BMV type II recombinants has recently been more thoroughly studied (14). This study involved the same deletion mutant of RNA-3 as previously used (15); the mutant was inoculated together with the wild-type RNA-1 or RNA-2 into barley seedlings (14). Recombination between mutant RNA-3 and RNA-1 or RNA-2 was examined. Although homologous recombinants predominated in most infected plants, aberrant homologous recombinants were detected in some of the plants. Some of these recombinants underwent additional recombination when they were passaged further in additional plants (14). All of these recombinants have crossover sites within the 3′-homologous region, but crossover sites were variable in different recombinants. The crossover sites on the two parental RNAs were usually staggered so that different degrees of sequence duplication in the 3′-noncoding region were noted in different recombinants. The analysis of the primary sequences surrounding the different crossover sites did not reveal any common motif; however, the sequences upstream and downstream of most of the recombination sites on the two parental RNAs could be aligned to form double-stranded heteroduplexes, which were postulated to cause RNA replicase to switch templates during RNA synthesis (14). Very similar RNA structures also have been postulated to be involved in poliovirus RNA recombination (87). The factor which promoted type II recombinants in some plants but not other were not known.

The second virus in the bromovirus family shown to undergo RNA recombination was cowpea chlorotic mottle virus (3). Similar to BMV, most of the deletion mutants of cowpea chlorotic mottle virus RNA-3 could undergo RNA replication when coinfected with wild-type RNA-1 and RNA-2. However, systemic spread of viral infection was blocked if the coding regions of RNA-3 were defective. When two RNA-3 mutants with different deletions in the coding region were coinfected with wild-type RNA-1 and RNA-2, systemic infection could occur as a result of recombination between the two RNA-3 mutants. Most of the recombinant RNAs represented homologous recombination, but some aberrant homologous recombinations resulting in sequence duplication also were detected (3). The recombination in this case occurred between two RNA-3 mutants, in contrast to the BMV recombination studies (14, 15, 84, 85), in which recombination occurred between different RNA segments in the homologous regions.

Recombination probably also has occurred during the natural evolution of BMV and cowpea chlorotic mottle virus. The RNA sequences of these two viruses show some features in their genomic organization which suggested the involvement of recombination events during their evolution (2).

Carmoviruses

The second plant virus family to be shown to undergo recombination was carmovirus, exemplified by turnip crinkle virus (TCV) (17). In contrast to the bromovirus family, TCV contains a single-stranded, positive-sense RNA of 4 kb. The virus frequently contains satellite RNAs of various sizes and structures, which require helper virus RNA for their replication. One of the most frequently observed satellite RNAs is RNA C (355 nucleotides), which is highly virulent, and its presence can exacerbate the symptoms of TCV infection (78). Sequence analysis of RNA C showed that it consisted of two distinct parts with different origins. The 5′ part (189 nucleotides) was identical to nearly the complete sequence of an avirulent satellite RNA D, whereas the 3′ part (166 nucleotides) contained sequences identical to two discontinuous regions of the genomic RNA of TCV helper virus (Fig. 4) (90). Thus, RNA C was apparently derived from three recombination events between the satellite RNA D and the helper virus TCV. These recombination events were probably of type III since there was no apparent sequence homology at the postulated recombination sites. This provided the evidence for the natural occurrence of nonhomologous recombination in TCV.

This type of recombination has been reproduced in an experimental infection of plants with several replication-

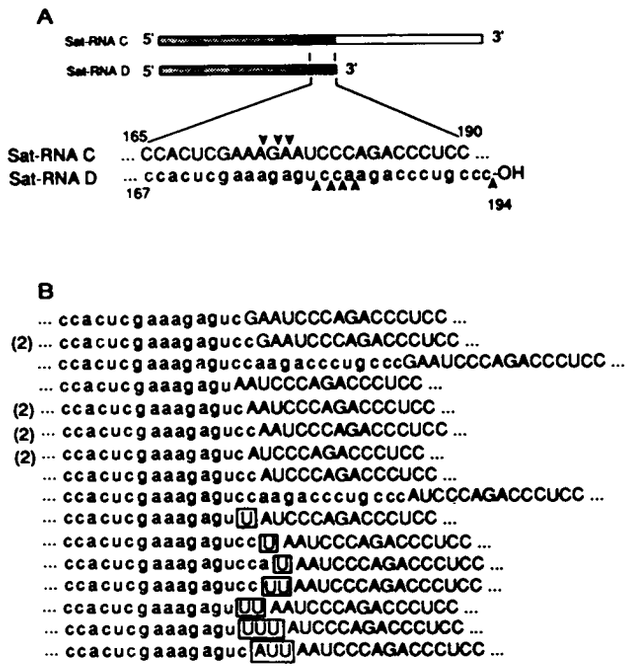

FIG. 4. RNA sequences of the recombinants between satellite RNA C and RNA D of TCV. (A) Parental RNA. Arrows indicate crossover points in different recombinants. (B) Sequences around the crossover sites of the various recombinant RNAs. Boxed nucleotides represent nontemplated nucleotides not present in either of the parental RNAs. The data are from reference 17, with permission.

defective mutants of RNA C and a helper TCV which also contains a satellite RNA D (17). All of the RNA C mutants used in this study had different types of mutations in the region identical to that of RNA D. At 2 to 3 weeks postinoculation, a new RNA species larger than the original mutant RNA C appeared. Sequence analysis of this RNA showed that it represented a mixture of diverse recombinants between RNA C and RNA D. The recombination sites of all of the recombinants were localized within a short region near the end of RNA D, but different RNAs had crossovers at different nucleotides. Surprisingly, although the sequences of RNA C and D were homologous around the sites of the crossovers, all of the recombinants obtained were the results of aberrant homologous recombination. Thus, each of them had a sequence duplication of a few nucleotides at the crossover site (Fig. 4). In addition, several recombinants had an insertion of one to three nucleotides (U, UU, UUU, or AUU), which were not present in the RNA of either parent, at the crossover sites. The presence of these nontemplated nucleotides suggested that recombination probably occurred during RNA synthesis. Since no artificial selection markers were used in this study, the finding that all of the recombination sites were within a small region of RNA suggested that the selection of recombination sites was not random; on the other hand, recombination did not require precise sequence homology at the crossover sites. The different crossover sites in the TCV recombinants contained several different kinds of sequence motifs: motif I was identical to the sequence located near the 5' end of the TCV genomic RNA, and motif II was also found at the 5' end of the satellite RNAs and DI RNAs (17). Recently, a third motif was found in a class of recombinant RNA between RNA D and genomic RNA of TCV (106). The detection of motifs I and II prompted the suggestion that TCV recombination occurred by a replicase-driven copy choice mechanism; i.e., the recombination sites contain the signal for RNA replication, which provided the recognition signal for RNA replicase during template switching (17). However, why replicase will stop at these sites was not explained. The reason why type II recombination was favored over homologous recombination in this case is not clear.

Alfalfa Mosaic Virus

Alfalfa mosaic virus contains a tripartite, positive-sense RNA genome, similar to the structure of bromovirus RNA. RNA-1 and RNA-2 are required for RNA replication, but RNA-3, which encodes two proteins, P3 and coat protein, is required for movement of the virus in plants. RNA sequencing of a temperature-sensitive mutant of alfalfa mosaic virus has shown that this mutant contains an RNA-3 which had acquired a 5'-terminal fragment from its own RNA-1 (41), suggesting the involvement of RNA recombination in the evolution of this virus. More recently, RNA recombination also has been demonstrated in an experimental infection of tobacco plants with alfalfa mosaic virus RNAs (101). This study was carried out with a transgenic tobacco plant which expressed the gene products of RNA-1 and RNA-2. A mutant RNA-3, with a deletion in the P3 gene, could replicate in this transgenic plant, whereas mutants with deletions in the CP gene could not replicate. When these two types of deletion mutants were coinoculated into this plant, systemic viral infection developed and a full-length RNA-3, similar to that of the wild-type RNA-3, became detectable after two viral passages in the plant (101). Although the structure of this new RNA has not been examined, this study provided a strong indication that recombination between the deletion mutants of RNA-3 had occurred. This could be homologous or aberrant homologous recombination. Interestingly, with some deletion mutants recombination did not occur, but the RNAs could nevertheless cause systemic diseases because of complementation between the mutant RNAs. The reason for the lack of recombination in these cases is not understood; these RNAs share common sequences, which should have allowed recombination to occur.

Other Plant Viruses

RNA recombination has not been directly demonstrated in other plant viruses. However, sequence analysis of natural isolates of some viruses suggested that at least some additional plant viruses have undergone RNA recombination during natural infections. One example is tobacco rattle virus, which is a tobravirus. RNA sequences of several natural isolates suggested that some of the tobacco rattle viruses were derived from recombination between viruses. For example, RNA-2 of strain PLB consisted of a 5'-terminal sequence identical to that of RNA-2 of strain PSG and a 3'-terminal sequence identical to that of the RNA-1 of strain PLB (4). In addition, two European isolates of tobravirus may contain sequences derived from two taxonomically distinct tobraviruses, i.e., tobacco rattle virus and pea early-browning virus (86).

These examples demonstrate that the ability of plant viruses to undergo RNA recombination is more common than generally realized.

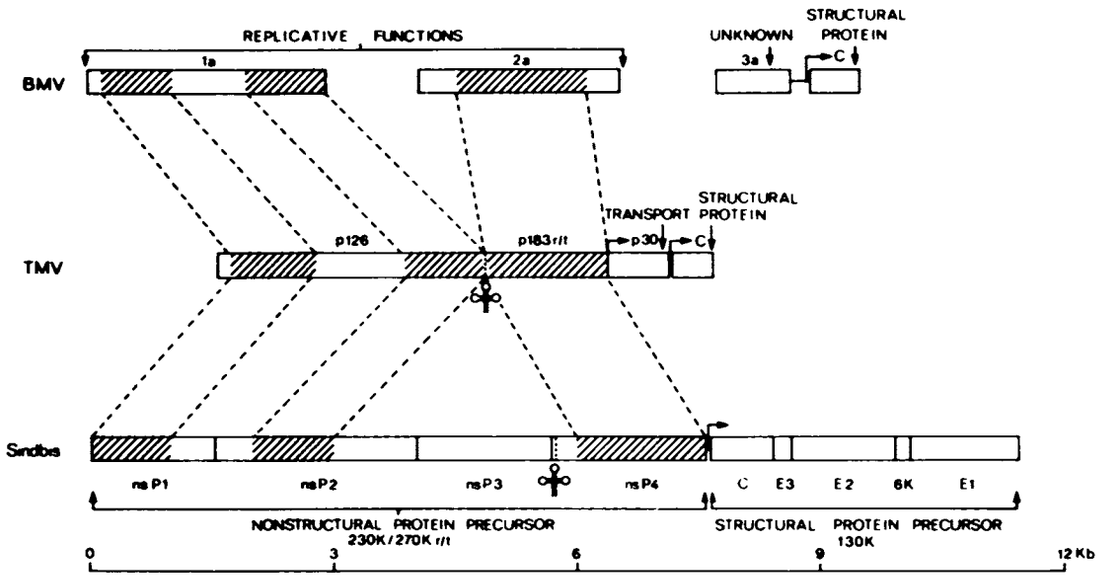

FIG. 5. Structural organization of the RNA genomes of BMV, TMV, and Sindbis virus. The data are from reference 107, with permission.

NONHOMOLOGOUS (ILLEGITIMATE) RECOMBINATION

From the discussion above, it is clear that, aside from picornaviruses and coronaviruses, most RNA recombination events in animal and plant viruses are not homologous recombinations, particularly when defective RNAs are involved. This is striking since these RNAs generally have homologous sequences between them. Thus, there appears to be no stringent requirement for sequence homology at the sites of crossover. This property of RNA recombination raised the possibility that even viruses or RNAs without apparent sequence homology might undergo recombination under some conditions. Such a property would give RNA viruses an added flexibility in recombination. This is type III (nonhomologous or illegitimate) recombination.

Nonhomologous Recombination between Distantly Related Viruses

Although it has never been demonstrated that viruses belonging to different families can recombine in tissue culture or animal infections, sequence analyses of viral RNAs suggest that many viruses have acquired genes by recombination with distantly related viruses. One example is coronaviruses, some of which contain a hemagglutinin-esterase (HE) gene (67, 105). This gene is present only in bovine, murine, and one of the human coronaviruses, but not in avian or porcine viruses (54, 105). It shares 30% amino acid sequence homology with the HA1 hemagglutinin protein of influenza C virus (67), which has both hemagglutinin and esterase activities. The esterase activity is preserved in coronavirus, whereas the hemagglutinin activity is less well preserved and is present only in some coronaviruses (54, 102, 105). Furthermore, this protein appears to be nonessential for coronavirus replication. In fact, this gene is easily mutated or deleted during virus passage in tissue culture or animal infections (104a). These findings suggest that the gene was most probably obtained fortuitously by coronavirus and was not an original part of the viral genome. The most likely explanation is that this gene resulted from recombination between coronavirus and influenza C virus (67). Since there is no apparent sequence homology, aside from the HE sequence, between coronavirus and influenza C virus, the acquisition of this gene by coronavirus from influenza C virus, or vice versa, could represent nonhomologous recombination. The homology of the HE gene between coronavirus and influenza C virus was obvious only at the amino acid level, but not at the nucleotide level (67); therefore, recombination probably occurred between their ancestral viruses. So why do some coronaviruses lack this gene? Deletion by virtue of intramolecular recombination is a plausible explanation, since coronavirus genes are flanked by homologous intergenic sequences (58). An alternative possibility is that the putative recombination between coronaviruses and influenza C virus occurred after speciation of coronaviruses; the divergence of the HE gene then may have occurred rapidly since this is a nonessential gene. Indeed, we have recently shown that this gene diverged unusually rapidly during viral infections in animals (104a).

Comparison of RNA sequences and genomic organizations of different viruses indicate that many possess conserved functional domains in their genomes and yet have very dissimilar genomic organizations (31, 33, 96, 97, 107). For instance, BMV has three separate RNA segments, whereas tobacco mosaic virus (TMV) contains a single molecule of RNA; however, amino acid sequence analysis suggests that several of the proteins in BMV and TMV probably have a common ancestral origin (33, 107) (Fig. 5). Some of these conserved sequences are even present in Sindbis virus, which is an animal virus (97). Thus, an intriguing possibility is that the different RNA segments of BMV might have been linked to form the single RNA genome of TMV. This possibility has been suggested recently by an interesting experiment, which showed that the nonstructural and structural protein genes of Sindbis virus could be expressed separately in two different RNA segments and could complement each other in *trans* to form a virus with segmented RNAs (30). Therefore, Sindbis virus,

which contains a single molecule of RNA, can actually function as a virus with two segmented RNAs. This suggests that a nonsegmented RNA could be derived from segmented RNAs by a recombination mechanism, which most probably involves nonhomologous sequences. Of course, these data also are compatible with the concept that segmented RNA viruses are derived from nonsegmented RNA viruses by RNA breakage. Even if this were the case, separating the single piece of RNA into replication-competent RNA segments must involve recombination of these RNA pieces with the replication regulatory elements. Thus, it is likely that RNA recombination has played a significant role in the evolution of these viruses. A similar relationship also exists between cowpea mosaic virus, which is a plant comovirus with two RNA segments, and poliovirus, which has a single segment of RNA (97, 107). The structural organizations of these two viruses are extraordinarily similar, except that their genes are divided between two RNA segments in cowpea mosaic virus.

Frequently, gene order within the genome is different between related RNA viruses, suggesting that gene rearrangement has occurred during the evolution of these viruses. For instance, between the coronaviruses MHV and infectious bronchitis virus, the gene order for the membrane protein and a nonstructural protein (*ns5*) is reversed (58). This gene rearrangement could be the result of RNA recombination involving RNA molecules of the same virus. There are many other examples of this kind of gene rearrangement. For example, some of the functional domains of the polymerase proteins in rubella virus and other alphaviruses are reversed (24). The same is true between the plant viruses cowpea chlorotic mottle virus and tobacco rattle virus (4) and among various viroids (48a). More interestingly, a nonstructural protein gene (*ns2*) is present in the genome of some species of coronaviruses as an independent gene (59), but is present in Berne virus, which is a torovirus belonging to the coronavirus superfamily, as part of the polymerase gene (91). Thus, the location and organization of this gene in these two viral genomes are entirely different. Significantly, this gene itself has probably been derived by coronaviruses by nonhomologous recombination from an unknown source, since it is also present in only a few, but not all, coronaviruses (58, 67). Thus, this gene appears to be subject to frequent recombination. In addition to this *ns* gene, Berne virus and coronavirus might, during their evolution, have undergone another nonhomologous recombination even, which involved the HE gene. The genetic locations of the HE gene on the genomes of Berne virus and coronavirus are drastically different (91), suggesting that this gene has been rearranged, possibly by a nonhomologous recombination event. Since this gene is evolutionarily related to the same gene in influenza C virus (67), an interesting question is whether this gene, like the *ns2* gene, has a special property to allow for its frequent involvement in RNA recombination and whether Berne virus derived the gene independently of coronaviruses.

The sequence rearrangements observed in these viruses could be the result of nonhomologous recombination; however, some of these also could be the result of homologous recombination involving a short stretch of homologous sequence. For instance, in coronaviruses, each gene is flanked by similar intergenic sequences (58). The flanking sequences would make each viral gene a gene cassette, which can recombine and rearrange with ease within the viral genome.

Recombination between Viruses and Cellular Genes

RNA viruses also can incorporate cellular genes by recombination. The most interesting example is bovine viral diarrhea virus, a pestivirus containing a positive-stranded RNA genome of 12.5 kb, whose viral genes are expressed as a polyprotein (19). This virus causes mucosal disease in cows, which is usually associated with a noncytopathogenic bovine viral diarrhea virus; however, cytopathogenic bovine viral diarrhea virus could always be isolated from the diseased animals (12). Many of the cytopathogenic virus strains were found to have inserted some cellular genes in a region encoding a nonstructural protein (19, 74). One of the most frequently observed insertions was the ubiquitin gene (74). The insertion sites and the inserted sequences in different cytopathogenic bovine viral diarrhea virus isolates differed (74), and there was no obvious consensus sequence at the sites of insertion. Therefore, this was most probably derived by a nonhomologous recombination. The incorporated sequences were expressed as part of the viral proteins (74) and may be associated with the cytopathogenicity of the virus. Since the coding strand of the ubiquitin gene was incorporated into the viral genome, the recombination probably occurred during the synthesis of negative-strand RNA, if pestivirus recombination occurs by a copy choice mechanism (see below). The reproducibility of this phenomenon suggests that some unknown features of RNAs facilitated this recombination and that recombinants had selective advantages.

A similar recombination between a viral RNA and a cellular gene also has been found in influenza virus (50). A segment of 28S rRNA was incorporated into the hemagglutinin gene of an influenza virus, leading to increased viral pathogenicity. These examples demonstrated the occurrence of nonhomologous recombination between RNA viruses and cellular genes. Such recombination may have fortuitously led to increased pathogenicity, and the recombinant virus was selected during virus passages in animals. This phenomenon of nonhomologous RNA recombination may be an important mechanism of virus evolution.

In an additional example, some DI RNAs of Sindbis virus have incorporated tRNA sequences at their 5′ ends (77, 100). Although the mechanism of DI RNA synthesis may not be entirely identical to that of RNA recombination, this may represent another form of nonhomologous RNA recombination between viral and cellular RNAs. Some satellite RNAs of TCV (discussed above) also contain nonviral sequences of unknown origin (17), which also were probably derived by nonhomologous recombination.

Nonhomologous Recombination in RNA Bacteriophages

Although a discussion of bacteriophage recombination is outside the scope of this review, the recent discovery of RNA recombination in bacteriophages (79) gave additional insights into RNA recombination in animal and plant viruses. When genetic approaches were used, recombination was never found between genomes of RNA bacteriophages (39). This failure to detect recombinants could have been due to the high mutation rate of RNA genomes. Recently, however, a small RNA product of spontaneous synthesis by a so-called template-free Qβ replicase preparation was shown to be composed of two regions of different origins (79). It contains, at the 5′ end, 80 nucleotides derived from the coat protein cistron of the Qβ phage RNA and, at the 3′ end, the 3′-terminal 33 nucleotides of *E. coli* $tRNA_1^{Asp}$ (79).

This represents the first and only recombinant RNA detected in prokaryotic system. It most probably was the result of nonhomologous recombination. The structure of this RNA is reminiscent of the Sindbis virus DI RNA containing tRNA sequences at the 5′ end (77, 100). Whether the generation of the recombinant RNA is the general property of Qβ replicase is not known. Besides, homologous recombination remains to be demonstrated in RNA phages.

MECHANISM OF RNA RECOMBINATION

Copy Choice versus Breakage and Rejoining

In considering the mechanism of RNA recombination, I would like to discuss several issues which are pertinent to the formulation of our current model. Unlike DNA recombination, which usually involves double-stranded DNA and can occur by either a breakage-and-rejoining mechanism (73) or, more rarely, a copy choice mechanism (13), only single-stranded RNA has been shown to undergo RNA recombination so far. Thus, the mechanism could be fundamentally different for DNA recombination and RNA recombination. The breakage-and-rejoining mechanism operates mainly with double-stranded DNA; nevertheless, this mechanism could conceivably operate in single-stranded RNA viruses in the double-stranded replicative intermediate RNA, thus mimicking DNA recombination and involving a Holliday junction structure during crossover (38). Alternatively, the breakage-and-rejoining mechanism in RNA viruses could operate in single-stranded RNA by a *trans*-splicing type of mechanism (98), cutting and joining two single-stranded RNAs. However, the latter mechanism usually is site specific, in contrast to most RNA recombinations. Nevertheless, this breakage-and-rejoining mechanism has not been rigorously ruled out for RNA viruses and may occur under some circumstances. Indeed it has been invoked to explain certain types of nonhomologous recombination in BMV (14). Conceivably, when one of the RNAs involved in recombination does not replicate (e.g., mutant RNAs without a replication signal) or replicates by a different mechanism (e.g., cellular RNA), there is a possibility that recombination involves a breakage-and-rejoining mechanism. However, there has been no direct experimental proof in support of this mechanism.

In contrast, there is strong evidence supporting the copy choice mechanism for the majority of RNA recombination, which involves a polymerase jumping from one template to another during RNA synthesis, as initially proposed by Cooper et al. (22). The hallmarks of this model are that recombination occurs only during RNA synthesis and that the parental RNA strand is not physically transferred to the progeny recombinant molecules. The first requirement of the copy choice mechanism has been demonstrated by several pieces of experimental evidence, whereas the second criterion has not been demonstrated to date. The copy choice mechanism is reminiscent of recombination in retroviruses (18, 40, 43).

Evidence in Support of Copy Choice Mechanism

The most direct evidence in support of the copy choice mechanism of RNA recombination came from the study of Kirkegaard and Baltimore (55), which demonstrated that RNA synthesis was required for poliovirus recombination. They studied RNA recombination between a wild-type poliovirus and a double-mutant virus, which was guanidine resistant and temperature sensitive. The RNA synthesis (of both positive- and negative-stranded RNAs) of wild-type virus could be inhibited by guanidine, whereas the negative-strand RNA synthesis of the mutant was inhibited at the nonpermissive temperature. Furthermore, the *ts* marker was located at the 3′ side of the guanidine resistance site. Thus, recombinant viruses which were resistant to guanidine and grew well at both 39 and 32°C must contain the 5′sequence of the mutant virus and the 3′ sequence of the wild-type virus, with the crossover site being located between the two selection markers. It was shown that when RNA synthesis by the wild-type virus was inhibited by guanidine, no RNA recombination leading to virus with the desired phenotype occurred. However, when negative-strand RNA synthesis of the double mutant was inhibited at the nonpermissive temperature, the desired RNA recombinants were still obtained at a high frequency. This result suggested that RNA replication of at least the wild-type virus was required for the generation of the desired recombinant viruses. This result is most consistent with the copy choice mechanism, since the breakage-and-rejoining mechanism would not predict such a differential effect of RNA replication on recombination (55). Furthermore, the finding that the negative-strand RNA synthesis of the wild-type virus, but not the mutant virus, was required suggested that RNA recombination occurred during negative-strand RNA synthesis, based on the consideration of the direction of RNA synthesis needed for the generation of this type of recombinant (55). This study provided the most direct evidence that RNA replication is required for RNA recombination.

Another piece of evidence came from the study of the aberrant nonhomologous recombination of TCV, which occasionally incorporated nontemplated nucleotides at the sites of crossing over (17). This finding is most consistent with the interpretation that RNA synthesis occurs simultaneously with recombination events.

The copy choice mechanism of RNA recombination is also indirectly supported by the structural analysis of a DI RNA of influenza virus (29). This RNA has been shown to consist of several discontiguous regions, some of which were derived from RNA-1 and others from RNA-3. Thus, it represents a true recombinant RNA between two different RNA molecules, probably resulting from nonhomologous recombination, although both RNAs belong to the same virus. Since the generation of DI RNA is generally thought to result from polymerase jumping caused by RNA secondary structures during RNA transcription (63), the presence of this recombinant DI RNA suggests that at least type III nonhomologous recombination involves a copy choice mechanism.

Physical Requirements of Template Switching

The copy choice mechanism involves template switching during RNA synthesis. How does this occur during RNA synthesis for recombination to take place? Several requirements must be met: (i) RNA polymerase must pause during RNA synthesis and then dissociate from the original template: (ii) another RNA template must be in close proximity for the polymerase to switch to; and (iii) there must be some physical features of the new template that allow the polymerase to bind and continue RNA synthesis. Several characteristics of RNA synthesis by RNA viruses fulfill these requirements.

(i) Transcriptional pausing has been demonstrated in RNA-dependent RNA synthesis in RNA phages and in DNA-dependent RNA synthesis in bacteria and DNA

phages (44, 61, 75). RNA polymerases usually pause at regions of strong secondary structure, which are the rate-limiting sites in RNA synthesis. Thus, the reactions of DNA-dependent RNA polymerases or RNA-dependent RNA polymerases are inherently discontinuous. Similarly, in the retroviruses, which also undergo a high frequency of recombination, reverse transcriptase also proceeds discontinuously with frequent transcriptional stops at sites of strong secondary structure (34). Conceivably, some pausing during transcription will result in the release of the RNA polymerase and nascent RNA transcript from the templates. Such incomplete RNA transcripts have been detected in coronavirus-infected cells (10). The sizes of these RNA transcripts suggest that they result from the pausing of RNA polymerase at sites of strong secondary structure. In addition, some of these RNA transcripts were dissociated from the template RNA (10). The detection of such incomplete transcripts dissociated from their template suggests that RNA polymerases are not strongly processive. Nonprocessivity is also a characteristic of reverse transcriptase and may account for the high frequency of recombination in retroviruses (18, 40). Conceivably, the ability of viruses to undergo recombination may correlate with the degree of nonprocessivity of their RNA polymerases. It should be noted that transcriptional pausing can be caused by the secondary structure of the RNA transcript as well as that of the RNA template.

(ii) The close proximity of different RNA templates is necessary for recombination to occur. In retroviruses, this is made possible by a physical linkage of two RNA templates in heterozygotes, thus facilitating template switching (18). In most RNA viruses, RNA synthesis occurs in membrane-associated compartments, thus allowing a local concentration of RNA molecules (11, 16, 23, 62). It also has been proposed that the complementary sequence in the secondary structure of RNA could allow two different RNA molecules to form a heteroduplex (14, 56, 87). This double-stranded region could not only serve as a transcriptional pausing site, but also bring the two RNA molecules physically close together. These sites have been shown to be the favored sites of recombination in poliovirus (87, 99) and BMV (14). It has been suggested that recombination in poliovirus occurs mainly during negative-strand RNA synthesis (55). This is consistent with the fact that positive-strand RNA is much more abundant than negative-strand RNA, thus providing more abundant templates for potential copy choice recombination.

(iii) The mechanism by which RNA polymerase would associate with another template is of considerable interest. There are two possible scenarios. The first is that a nascent RNA transcript dissociates from the transcription complex and then, as a free RNA, binds to a different template by means of sequence complementarity. The second is that RNA polymerase and transcription machinery, including the nascent RNA transcript, switch, as a whole, to a new template. The recognition of the binding site would not be determined by specific sequences, but rather by common RNA secondary structure. These two alternatives, or a combination of them, have not been distinguished. There is no doubt that homologous sequences facilitate RNA recombination. Indeed, closely related virus strains recombine at a much higher frequency than do more distantly related viruses (55). Furthermore, in homologous recombination, the hybrid RNA molecules often represent faithful switching of templates without any deletions or duplications at the crossover sites. Thus, the dissociated RNA intermediates appear to have faithfully realigned with the new template RNA. However, aberrant homologous (type II) recombination occurs very frequently in many viruses. Most remarkably, when defective RNAs were involved, aberrant homologous recombination occurred more frequently than homologous recombination (14, 17, 103). In the Kirkegaard and Baltimore study on poliovirus recombination (55) and our study on coronavirus recombination (7), crossovers could occur between neighboring nucleotides which were different between the two parental RNAs. Thus, it appears that nucleotides need not be perfectly aligned at the crossover site, although the presence of homologous sequences in the neighborhood helps the transcription complex to land. These observations are more compatible with the notion that template switching takes place by the polymerase complex as a whole and that the sequence alignment between the template RNA and the switching RNA transcript occurs at some distance from the crossover site. Even in the absence of homologous sequence, the polymerase complex may bind to a site where there is a similar secondary structure.

In the aberrant homologous recombination of TCV, some of the crossover sites appeared to correspond to the recognition sequence for RNA replicase (17). These sites may serve as the reassociation sites of the transcription complex. Thus, in any type of recombination, sequence homology or specific motif may provide the binding signal for the dissociated transcription complex, which includes nascent RNA transcripts.

Selection of Recombination Sites

Additional issues must be considered before a model of recombination can be proposed. (i) Are there hot spots of RNA recombination? Does RNA recombination occur more frequently at sites of strong secondary structure of RNA? Intuitively, this seems to be a predicted outcome if RNA recombination is precipitated by transcriptional pausing. In several recombination studies, recombinational hot spots have been demonstrated. One was the study of poliovirus recombination in tissue culture (87, 99). The crossovers occurred more frequently at sites of strong RNA secondary structure. A similar conclusion also has been made with FMDV recombinants (104). It was shown that coronavirus recombination occurred more frequently within a hypervariable region, in which deletions commonly occur after virus passage in tissue culture or animals (6). Thus, the same RNA secondary structure, i.e., strong stem-loop structures, may be responsible for both deletions and recombination by causing a pause in RNA transcription. Then why did polioviruses recombine almost randomly within a small region examined, without apparent sequence preference (55)? These apparent contradictions can be reconciled by proposing that nascent RNA transcripts may undergo nucleolytic cleavage before switching to a different RNA template. As a result, the crossover sites may not necessarily correspond to the sites of strong secondary structure of RNA. We have recently transfected an in vitro-transcribed RNA representing the 5′ end of the MHV genome into MHV-infected cells and found that this RNA could recombine with MHV genomic RNA. Interestingly, the recombination sites were not at the end of the transfected RNA, but were at various internal sites, suggesting that the transfected RNAs have been processed prior to recombination (65a). This RNA cleavage may be required for recombination. Indeed, in DNA recombination systems, the presence of free DNA termini has been shown to promote DNA recombination

(66). Thus, when the entire RNA is considered, there are recombinational hot spots which correspond to the transcriptional pausing sites or polymerase-binding motifs. However, within a defined region around the transcriptional pausing site, crossovers can occur at many different sites without sequence specificity.

(ii) A serious point for consideration is that most of the recombination studies were based on the analysis of the viruses harvested from mixed infections. Frequently, the progeny virus isolated had to be passaged further before sufficient virus titers were reached. Thus, the recombinants isolated usually represent viruses which grow well under these conditions and may represent several rounds of recombination and subsequent selection. Indeed, in a study of coronavirus recombination between MHV-2 and A59 viruses, the recombinants obtained initially were fusion positive, but after two passages they became fusion-defective, suggesting that additional recombination had occurred (47). On the basis of these considerations, it is likely that the recombinants obtained in most of the studies reflected the results of selection and not necessarily the actual mechanism of recombination. We have recently conducted a study to examine RNA recombination in the absence of artificial selection pressure. Two MHVs were coinfected into a susceptible cell line, and both the intracellular RNA and the virus released were screened for recombinants by using the polymerase chain reaction with primers specific for recombinants. It was found that recombination sites were distributed almost evenly among all of the potential crossover sites (7). These findings were in sharp contrast to the clustered crossover sites in recombinant viruses isolated by using conventional selection markers (6). When the viruses were passaged further in tissue culture, the crossover sites of most of the surviving recombinants became clustered in a very restricted region of the genome. This study suggests that recombination may occur more randomly than was previously realized, and the types of recombinants isolated in any study may represent only those which have selective advantages under the conditions used. In some cases, recombinants may acquire a hybrid protein which is unstable or functionally deficient. Such recombinants would not survive under culture conditions. This may explain why coronavirus recombinants have clustered crossover sites in the hypervariable region of the spike protein gene (6), since that region may be more tolerant of structural changes. It may also explain why recombination has not been observed in certain capsid protein genes of picornaviruses (51). Also, in WEEV, the capsid protein gene sequence appears to have undergone further evolution after recombination between EEEV and Sindbis virus (32). These data suggest that the types of recombinants isolated most probably reflect the results of selection. Therefore, in cases such as the recombinants derived from Sindbis virus DI RNAs (103), some of the duplications and rearrangements near the crossover sites may have occurred after recombination.

Model of the Mechanism of Copy Choice RNA Recombination

On the basis of the above considerations, a copy choice model of RNA recombination can be visualized as shown in Fig. 6. This model proposes that RNA replication pauses at sites of secondary structure (step 1). Some transcription complexes, which consist of the nascent RNA, RNA polymerase, and transcription factors, dissociate from their templates (step 2). The nascent RNA chain then binds to a different template at the comparable site because of sequence complementarity. This binding site is proposed to be upstream of the transcription complex (step 3). As a result, the transcription complex is directed to the template RNA at either the homologous site (step 4A), if the new template has the same secondary structure as the original RNA template, or a different site (step 4B), if the secondary structure of the template differs. Thus, the transcription complex itself does not determine the site of recombination. This binding may involve the transcription complex and the nucleocapsid protein or transcription-related proteins (perhaps cellular in origin). The 3′ end of the nascent RNA transcript may undergo some processing before RNA transcription starts again. Thus, even though pausing may occur only at RNA sites with strong secondary structure, the crossover sites may be random within this region because of the different extents of 3′-end processing. Viruses which undergo RNA recombination may possess a polymerase with nonprocessive properties, thus allowing the nascent RNA transcription products that arise by pausing of the polymerase to dissociate from the RNA template during RNA synthesis.

Several features of this copy choice model require additional comments. (i) Since RNA synthesis does not normally start from the middle of an RNA template, RNA polymerase is unlikely to be separated from its nascent RNA transcript and subsequently rejoin the RNA template. It is more likely that the transcription complex dissociates as a whole complex, consisting of multiple viral and cellular factors, or at least as part of the complex.

(ii) Since RNA recombination could occur at sites where there is no sequence homology, the nascent transcripts do not have to align with the template precisely at the crossover site in order for RNA synthesis to elongate on the nascent transcript. Then how does sequence homology between the nascent transcripts and the template RNA promote recombination? The pairing of these two RNAs most probably occurs outside (upstream) of the transcription complex (step 3). This binding may help orient the transcription complex to a different RNA template. Depending on the secondary structure of the transcript or template around the binding site, the transcription complex may bind to the template RNA at the homologous (step 4A) or nonhomologous (step 4B) sites. This will explain why aberrant homologous recombination appeared to be more common when two defective RNAs (e.g., Sindbis virus DI [103]) were involved in recombination since they have different overall genetic structure and thus the two RNAs may fold differently despite the presence of common sequences. This may also explain the presence of nontemplated nucleotides at sites of crossovers seen in TCV recombination (17), since the binding site of the transcription complex may not be specific and may allow some wobbling or stuttering during resumption of RNA synthesis. In the case of nonhomologous recombination, in which RNAs involved do not have sequence homology, the binding of the nascent transcript could be directed by RNA secondary structure.

(iii) To account for the presence of multiple recombination sites within a short stretch of sequences observed in poliovirus and coronavirus (7, 55), the nascent transcript, which is generated as a result of pausing at a presumably specific site, may be processed at the 3′ end before it is involved in RNA synthesis again. This could occur via an endo- or exonuclease. In coronavirus transcription, it has been pos-

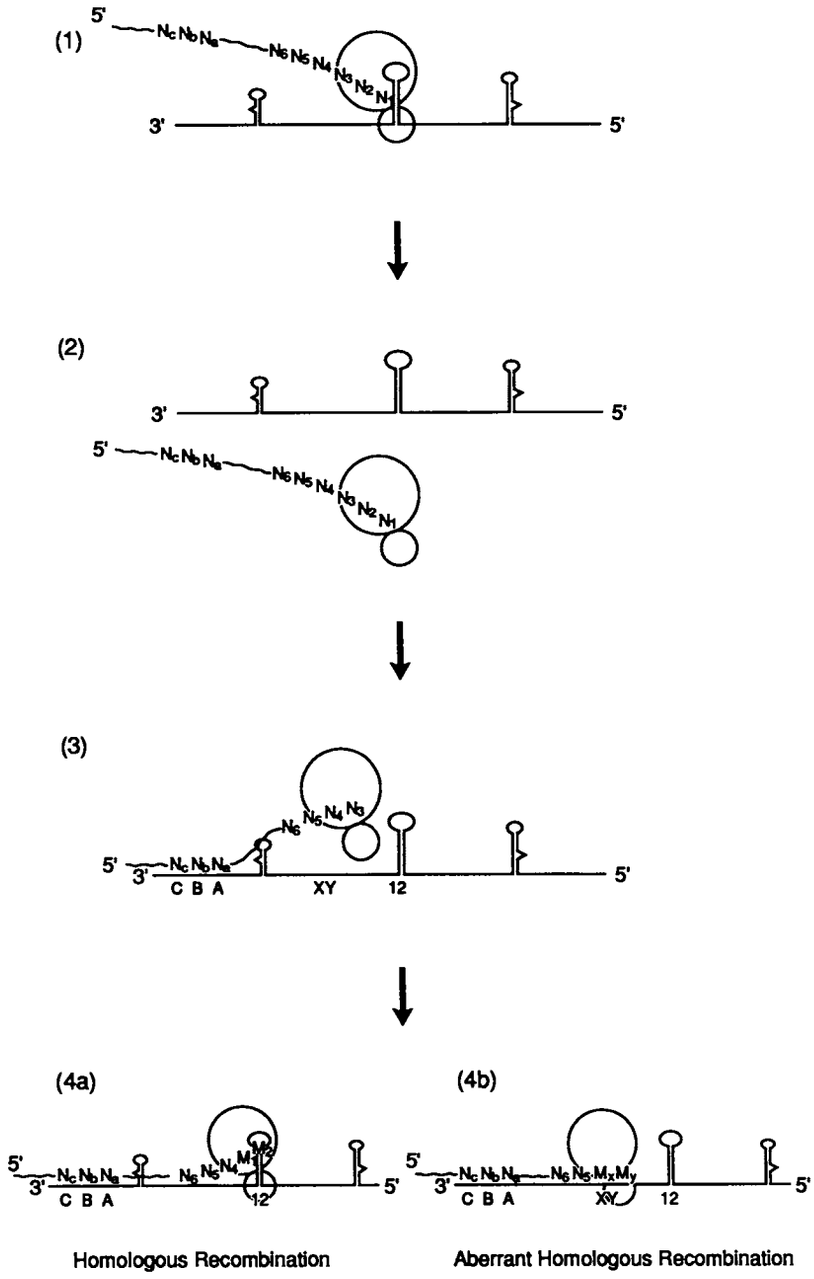

FIG. 6. Proposed copy choice model of recombination. (Step 1) Transcription pauses at the site of a stem-loop structure. (Step 2) The transcription complex is dissociated from the RNA template. (Step 3) The nascent transcript binds to a different template RNA at an upstream site via complementary sequences between the nascent RNA and template RNA. Some of the nucleotides at the 3′ end of the nascent transcript may be cleaved. (Step 4) The transcription complex binds to the template RNA. If the template RNA has a structure similar to the original RNA, the transcription complex would bind to the same site and homologous recombination would result (Step 4A). If not, the transcription complex may bind to a different site and aberrant homologous recombination would occur (Step 4B). The wavy lines represent nascent RNA transcripts. Circles denote the transcription complex, including polymerase. N's represent the nascent RNA sequence from the original template, while M's represent newly incorporated nucleotides copied from a different template. The difference between the template RNAs in Steps 4A and 4B is the presence or absence of the first stem loop structure.

tulated that the leader RNA is specifically processed by a nuclease before it is used for transcription (5).

These features may be experimentally testable. Indeed, it has been reported that at least some of the nascent transcripts of coronavirus RNA were associated with the nucleocapsid protein (9), supporting point (i) discussed above. Very recently, it has been shown that cleavage of nascent RNA can indeed occur within the prokaryotic transcription complex (97a), supporting point (iii) discussed above.

BIOLOGICAL SIGNIFICANCE OF RNA RECOMBINATION

The demonstration of RNA recombination in an increasing number of viruses suggests that it is a more general phenomenon than was previously realized. However, only a few viruses undergo homologous RNA recombination at a detectable frequency, and aberrant homologous recombination predominates under special circumstances. Thus, RNA recombination must be providing viruses with certain selective advantages.

(i) RNA recombination may be a mechanism to eliminate errors in RNA synthesis. RNA viruses often have a very high mutation frequency, because of a high error rate during RNA synthesis (92, 94). Neither RNA polymerases nor reverse transcriptase have proofreading activities; RNA viruses must therefore use different strategies to counter the deleterious effects of genetic mutation. For example, viruses with a segmented RNA genome probably can overcome these problems by reassortment of RNA segments, whereas some RNA viruses with nonsegmented genomes can use genetic complementation. However, because picornaviruses synthesize a polyprotein and some of its gene products function in *cis*, genetic complementation occurs only inefficiently. RNA recombination may provide an alternative mechanism in lieu of genetic complementation. This may also be true of retroviruses. On the other hand, coronaviruses may have a need for recombination because of the extremely large size of its RNA genome, which ranges from 27 to 31 kb. The large size of coronavirus RNA makes it particularly prone to errors during RNA synthesis. RNA recombination may generate diverse RNA molecules from which a functional RNA is selected.

(ii) RNA recombination is a mechanism for virus evolution. It allows the virus to adapt to different environments. For instance, in children receiving oral poliovirus vaccines, recombinants rapidly emerge as the predominant virus population which is different from the parental vaccine strains. The reproducibility of such a phenomenon suggests that it must be providing a powerful selection tool for the virus and may be essential for poliovirus survival in the human gastrointestinal tract. Other naturally arising recombinant viruses, e.g., WEEV, may have been generated more fortuitously; nevertheless, this mechanism allows for the emergence of new virus strains.

(iii) On the other hand, RNA recombination may lead to the convergence of virus strains. For instance, virus strains with different biological properties can potentially become homogeneous by virtue of repeated recombination and subsequent selection of a dominant recombinant virus. This concept, however, poses a potential problem. If viruses such as poliovirus undergo recombination so readily, how can they maintain multiple serotypes? There must be additional evolutionary pressure to select against this genetic convergence.

(iv) Nonhomologous recombination provides the virus with additional genetic tools to expand its biological repertoire by generating rearrangements of viral genes or inserting cellular or other viral genes.

IMPLICATIONS OF RNA RECOMBINATION ON THE STUDY OF RNA VIRUSES

RNA recombination also has practical consequences for the study of RNA viruses. (i) RNA recombination is an important parameter to consider in vaccination with live, attenuated viruses. As indicated by the poliovirus vaccines, recombination can generate new viruses among vaccine strains and possibly also between vaccine viruses and other viruses. These recombinant viruses, fortunately, have not been found to have serious consequences on the vaccines so far. However, they potentially could have deleterious effects. This possibility has been suggested by isolation of recombinant DNA viruses. For example, a highly virulent herpes simplex virus has been generated by recombination between two avirulent viruses during infection in animals (42). Similar observations also have been made with pseudorabies virus (45).

(ii) RNA recombination could potentially be used as a genetic tool in the construction of desired virus mutant, particularly for those in which no infectious cDNA clones are available. For instance, because of the extremely large genome size of coronaviruses, it is unlikely that an infectious cDNA clone will be readily available. The future development of a recombination system between replicating virus and transfected RNA fragments may allow the circumvention of this problem and provide a way to construct a desired virus strain.

(iii) Although RNA recombination has been demonstrated in only a few RNA viruses so far, it is likely to be more widespread. With proper experimental systems and selection procedures, it is likely that recombinants will be detected in many other virus systems. Sindbis virus, for example, was not thought to undergo recombination until recently, when recombination was demonstrated. Similar discoveries are expected to be made in an increasing number of viruses.

There are still many other issues to be resolved in our understanding of RNA recombination. For instance, which enzymatic properties of RNA polymerase determine the frequency of RNA recombination, and which other proteins are involved? The development of an in vitro recombination system may facilitate such an understanding. Also, the availability of the infectious RNA transcripts of the genetically engineered cDNA copies of various viruses and their DI RNAs will probably facilitate our understanding of the mechanism of RNA recombination. With an increasing realization of the importance of RNA recombination, these and other issues will be more rigorously considered in the study of RNA viruses and the development of vaccines.

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