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Heterologous recombination in the segmented dsRNA genome of bacteriophage Φ6

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The genome of bacteriophage Φ6 is composed of three unique segments of double-stranded RNA packaged within a procapsid. One segment can recombine with another in regions that share little sequence similarity. Although the recombination is therefore heterologous, the crossover points usually consist of two to six identical nucleotides. The frequency of recombinants is enhanced by conditions that prevent or hinder the minus strand synthesis of a single plus strand segment. Recombination serves as a repair system as well as a means of changing the genetic structure of the virus. The reaction can be studied in an in-vitro packaging and replication system involving purified procapsids and ssRNA. Although there are striking differences in the mechanisms of recombination in RNA viruses, there are also strong similarities. All seem to use a copy-choice template switching action for recombination. The Φ6 system is a useful model for the recombination of other segmented double-stranded RNA viruses such as the Reoviridae.

Key words: heterologous recombination / segmented genome / bacteriophage Φ6 / dsRNA

General background of viral RNA recombination

Recombination in RNA viruses was first described in 1963 by Hirst and Ledinko.[1,2] They demonstrated the exchange of poliovirus genes for guanidine resistance and resistance to a serum inhibitory activity. These findings were extended by the demonstration of recombination between temperature-sensitive mutants of poliovirus.[3] The original experiments were somewhat of a *tour de force* in that the recombinant frequencies were very close to those of revertants of the mutations. RNA genetic systems show greater mutation rates than DNA systems. The results of Kirkegaard and Baltimore[4] support the idea that recombination in poliovirus is due to template switching during polymerization. In that case, it was indicated that switching takes place during minus strand synthesis. The current consensus view is that RNA recombination in viruses involves copy choice, but this view lacks extensive experimental support.[5,6] Recombination has been demonstrated and studied in several other positive strand RNA viruses, particularly in coronavirus.[7,8] The recombination in most of these cases appears to take place between regions with extensive sequence identity and is therefore homologous. Rates of recombination between markers are low and proportional to physical distance. Recombination frequencies for intratypic crosses with poliovirus were of the order of 10^{-3} for markers separated by a few hundred bases to about 1% for markers that are several kb apart.[3,4] The rate of recombination in coronavirus is very high,[9] but its genome is very large (31kb) and its rate of recombination per nucleotide seems to be similar to that of poliovirus.[8] It is also possible that the high recombination rate in coronavirus is related to the leader-primed transcription, which in itself, is a form of aberrant homologous recombination.[10] Coronavirus appears to produce both homologous and heterologous recombinants.

Heterologous recombination has been found in several single stranded RNA viruses, some with genomes that are unit and some that are multipartite.[8,11] Most of these are positive strand viruses but some are negative strand viruses.[12] This type of recombination appears to constitute a mechanism for the rescue of segments with damaged 3' ends. It also is a means of enlarging the genome of the virus. As the sequencing of RNA viral genomes has progressed, it has become clear that some viruses isolated from nature are the products of a recombination between rather different viruses. Several indications of natural heterologous recombination have become apparent. There is evidence that sindbis virus and eastern equine encephalitis virus or their ancestors recombined at some point to form western equine encephalitis virus.[13] Although there is no demonstration of RNA recombination in influenza virus crosses, evidence for heterologous recombination has been found in the sequence of a

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small defective interfering (DI) segment.[14] Heterologous recombinants of flu were found as a consequence of transfection experiments.[12] Some contaminating products of in-vitro RNA synthesis by viral polymerases have also shown signs that they are the products of heterologous recombination. This is true of the RNA phage Qβ[15] and for sindbis virus[16] where chimeras between viral genome and tRNA have been found. Although bacteriophage Qβ has not been shown to produce recombinants in crosses, the minivariant RNA species that appear in Qβ polymerase preparations or even in the air of Qβ laboratories seem to have been formed by heterologous recombination events.

Heterologous recombination has been found to occur between plasmid transcripts and viral genomes containing deletions. In many cases, these events are very rare, but because the deletions do not revert, the low frequency events can be selected. Qβ has also been shown to recombine with plasmid transcript.[17] Recombination has been reported between plant RNA viruses and transcripts in transgenic plants.[18] The formation of defective interfering (DI) genomic segments has been found in many RNA viruses, plus, minus and ds. These can be the results of polymerase skipping or heterologous recombination between identical segments. It seems likely that the mechanisms for polymerase skipping and heterologous recombination might be the same.

A clear case of heterologous recombination within a cross was described in the tripartite brome mosaic virus.[19] A virus with a defect in segment 3 was capable of local but not systemic infection. Variants that had regained the capability for generalized infection were isolated, and these variants were found to have reconstituted the 3' end of the defective segment 3 from that of segment 1 or 2 by heterologous recombination. Many of the recombinants were products of homologous crossing over in the 3' regions, but heterologous recombinants were also found.

Bacteriophage Φ6

Bacteriphage Φ6 exhibits heterologous RNA recombination and is quite easy to study due to the development of an in-vitro packaging and replication system. Φ6 has a genome of three segments of double-stranded RNA (dsRNA) packaged within a single procapsid[20,21] (Figures 1 and 2) It is the only bacteriophage known to have a dsRNA genome, although there are a number of viruses with dsRNA that infect eukaryotes. A polyhedral procapsid is formed from four proteins; P1, P2, P4 and P7, early in infection.[22] Protein P2 has the sequence motifs characteristic of viral RNA polymerases.[23] Therefore, the procapsid is not only a structural element but also the polymerase complex for the virus. The procapsid is able to package plus strand transcripts of the three genomic segments. After packaging, the three plus strands serve as templates for the synthesis of minus strands and result in three molecules of dsRNA in each procapsid. The three genomic segments have 18 identical bases at the 5' end of the plus strands, with the exception that base 2 is G in segments S and M, and U in segment L. The 18 base sequence is necessary for packaging and for control of transcription. Following the 18 base conserved sequences are non-conserved, segment-specific regions of about 200 bases that are involved in genomic packaging and the control of minus strand synthesis.[24] At the 3' ends there is a sequence of 75 bases that is structured with four hairpins. The sequence and the structure are highly conserved (Figure 3) Each of the segments contains four or five genes and the coding sequences are interior to those involved in packaging or RNA synthesis[26] (Figure 2).

An in-vitro genomic packaging system has made it feasible to prepare virus with modifications in the

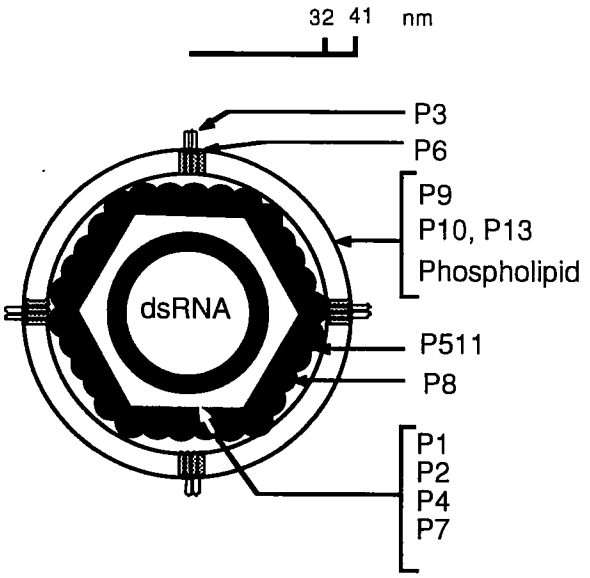

Figure 1. Diagram of the structure of Φ6. The three pieces of genomic dsRNA are found inside a dodecahedral procapsid structure composed of P1, P2, P4 and P7. This is enclosed by a shell of P8. Outside of this is the membrane composed of phospholipids and proteins P9, P10, P13, P6 and P3. P5 is a lysozyme.[26]

genomic segments. It has been possible to produce specific base changes, deletions and insertions into the genome.[25,27,28] The gene for kanamycin resistance from Tn903 has been inserted into the 3' noncoding regions of the three segments. (ref 25 and Mindich *et al*, unpublished). The gene for the α fragment of *lacZ* of *E. coli* has also been inserted into the same regions (ref 29 and Mindich *et al*, unpublished).

Procapsids composed of proteins P1, P2, P4 and P7 can be produced in cultures of *E. coli* carrying plasmids that contain cDNA copies of the Φ6 genomic segment L. These procapsids resemble structures formed during normal infection of its host *Pseudomonas phaseolicola* (Figure 4). They can be isolated and perform the expected functions of normal procapsids *in vitro*. They are capable of packaging single-stranded plus strands of the Φ6 genome in the absence of minus strand synthesis. If all three segments are packaged, minus strand synthesis takes place.[30] This results in dsRNA in the particles. The dsRNA is able to support the synthesis of plus strand molecules both *in vitro* and *in vivo*. The procapsids that have packaged and replicated viral RNA *in vitro* can infect spheroplasts of the host organism if they are first coated with protein P8. In this way, live virus can be isolated with engineered genetic changes.[27]

The first indication of recombination in Φ6 was the observation that a *kan* insertion into the *PstI* site of the 3' non coding region of segment M (Figure 2) was

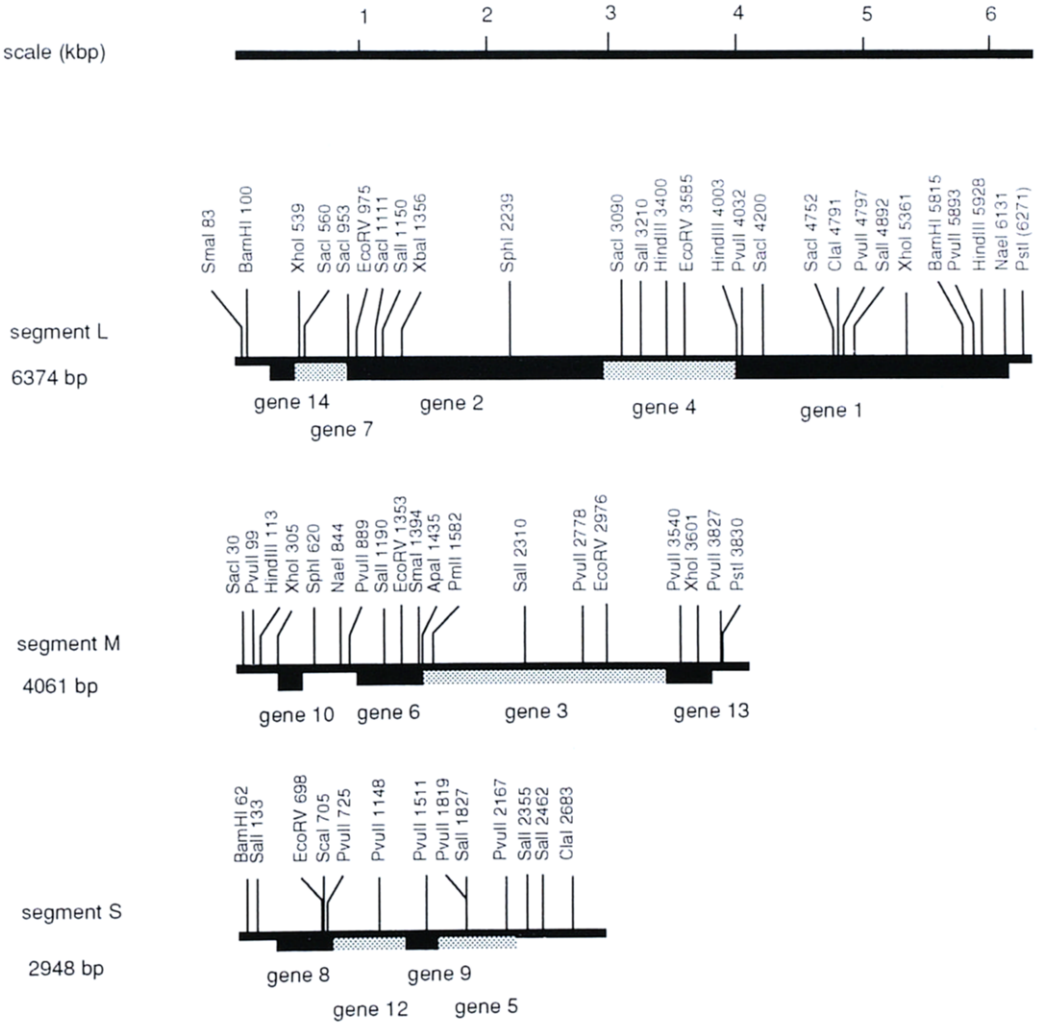

Figure 2. Restriction map of the cDNA copies of the genomic segments of Φ6. Genes are identified below the segments.[26,41] The sequences necessary for packaging are located in the 5' non-coding regions (left). The sequences necessary for minus strand synthesis are at the 3' ends.

very unstable. In some cases the gene was lost in half the phages within a few rounds of replication. Sequence analysis showed that the gene was not simply deleted but replaced with the 3' end of one of the other segments.[25] The replacement was not reciprocal. It was hypothesized that the reason for the genetic instability of the *kan* gene was the complementary homopolymer arms bounding the *kan* gene.[29] These arms contained 12 G's or C's and a *PstI* site. Removing one of the arms led to a stabilization of the *kan* gene. This idea was confirmed by experiments with the *lac* insert. It was found that inserting the gene for the α fragment of the *lacz* gene in the non-coding region at the 3' end of segment M resulted in a genetically stable virus (Figure 5A). When plated on a host strain carrying a complementing *lacZ* ω fragment the phage formed blue plaques in the presence of X-gal. Less than one in one thousand plaques were white and these were due to mutations, not to recombination. However, when the same gene was bounded by complementary homopolymer tracts, the construction became unstable (Figure 5B). The degree of instability increased with the size of the complementary arms.

The segments with the inserts bounded by long homopolymer tracts were not as good templates for

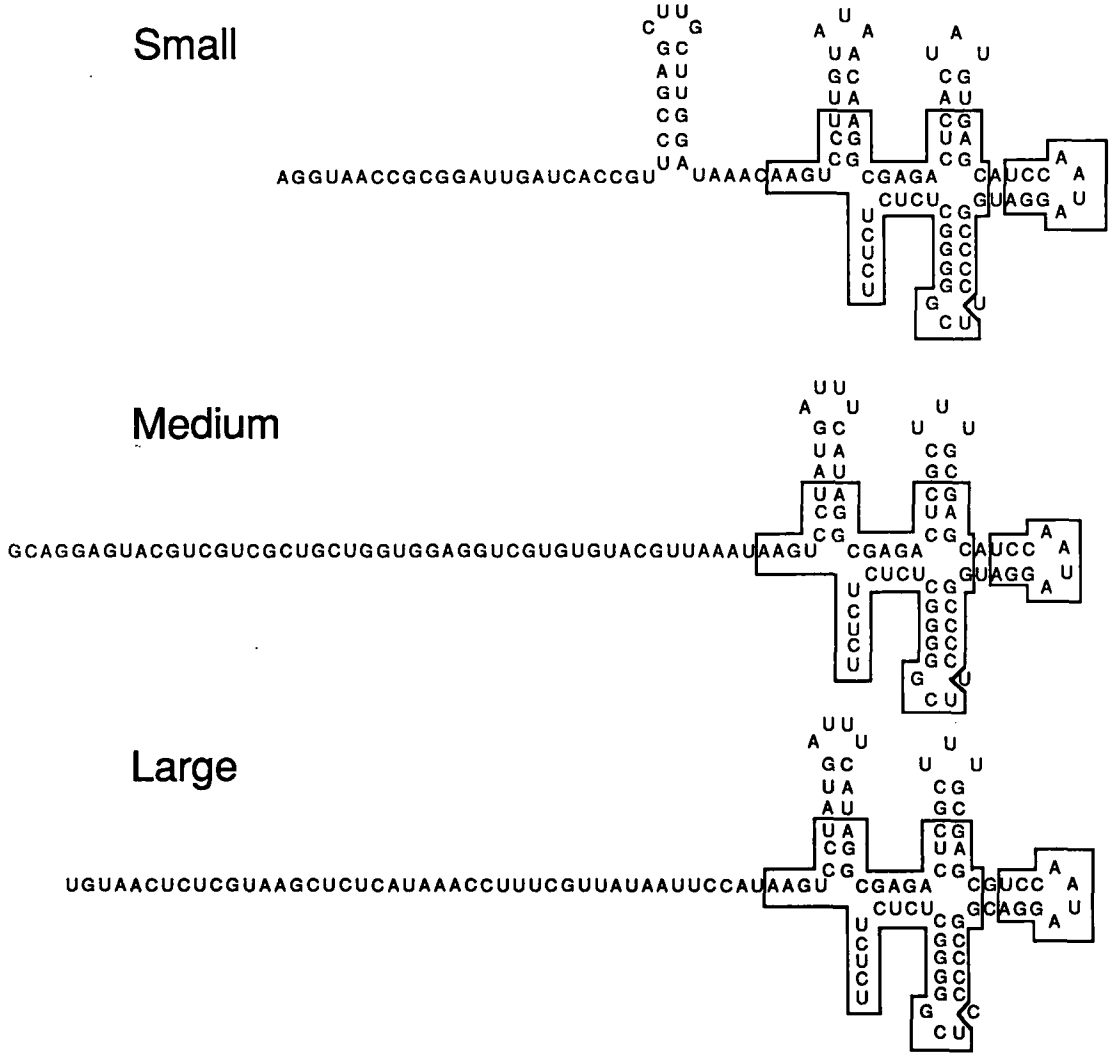

Figure 3. Presumed secondary structure at the 3' ends of the ssRNA transcripts of the Φ6 genomic segments.[25] The region of similarity covers the terminal 75 nucleotides, and bases that are identical in all three transcripts are boxed. Note that the bases that are not identical maintain the predicted secondary structure.

minus strand synthesis as were segments with normal structure. This presumably results from alteration in the secondary rather than the primary structure of the RNA. We have established, in unpublished experiments, that the altered secondary structure prevents complete entry of the RNA into the procapsids. The 3' end remains vulnerable to ribonuclease. The presence of the homopolymer arms increases the frequency of recombination at least 1000-fold. The insertion of the entire 3kb *lacZ* gene into segment M also induced high levels of recombination. In this case it was not clear whether the extra RNA prevented normal packaging or whether it interfered with minus strand synthesis.[29] The products of heterologous recombination of Φ6 vary in the size of the recombinant segments. Since there are extensive dispensible regions in each segment, it is possible to have segments that are shorter than normal or longer (Figure 6). Recombinants that alter the coding regions are less likely to be seen unless the phage is propagated on strains that can complement defects in the genome.

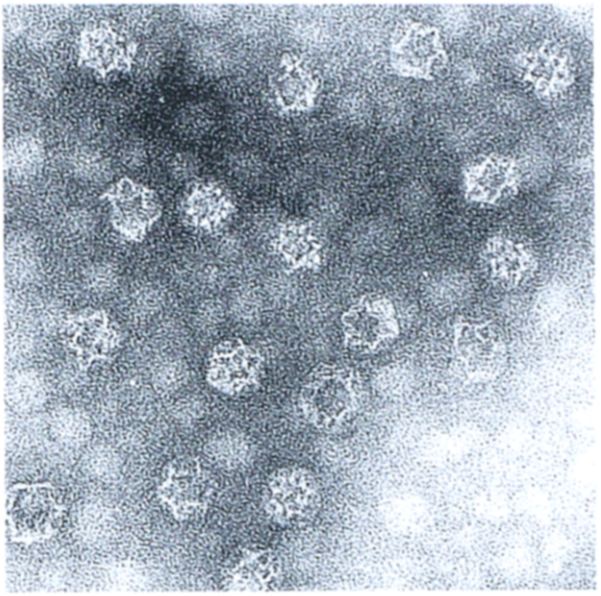

Figure 4. Electron micrograph of procapsids isolated from a strain of *E. coli* carrying a plasmid with a cDNA copy of segment L. The sample is stained with ammonium molybdate.[42]

Plus strands that are missing the normal 3' conserved sequence do not usually serve as templates for minus strand synthesis. Minus strand synthesis requires the presence of each of the three genomic segment plus strands in the procapsid particle, but the plus strands do not have to contain proper 3' ends in order to stimulate minus strand synthesis in trans. If

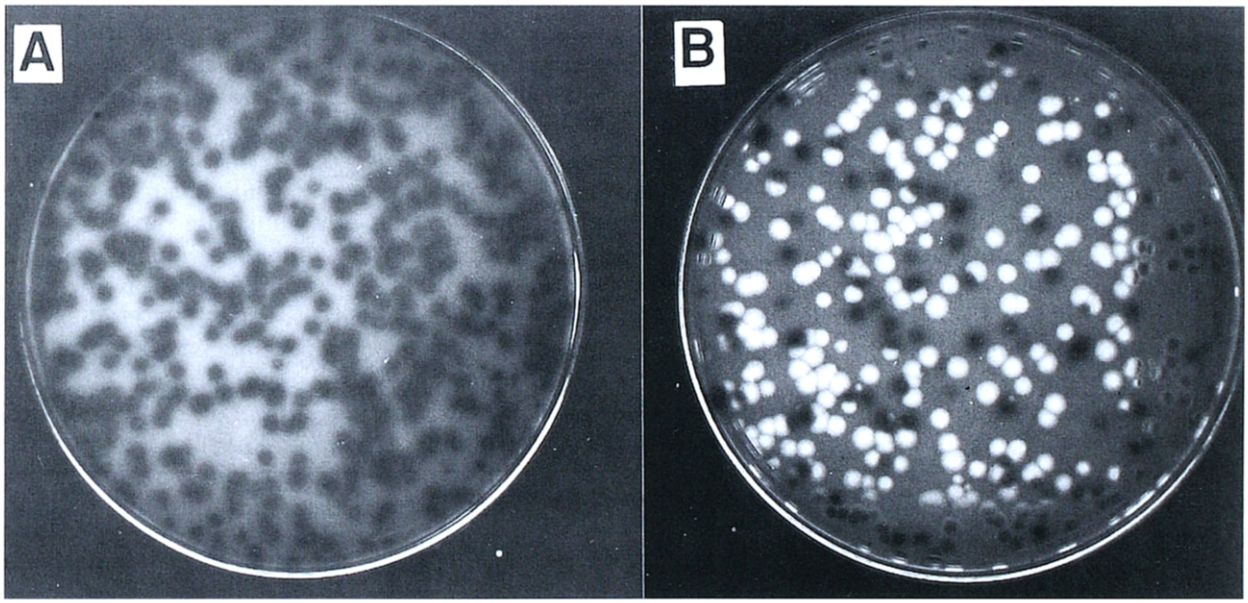

Figure 5. Blue plaques of Φ1817 which has *lacH* stably inserted into the *PstI* site of segment M, on an indicator strain in LB agar with X-gal (A); plaques of Φ1819, which is genetically unstable due to homopolymer arms at the position of the *lacH* insertion (B).[29] Most of the plaques in (B) have lost the *lacH* insert by recombination and are therefore white. The indicator strain is LM1034, a host strain that carries a plasmid that contains a *lacΩ* gene that complements the *lacα* gene in the phage.

two normal plus strands are packaged along with a third that is truncated at the 3' end, the two normal strands will serve as templates for minus strand synthesis, but the truncated one will not.[31] If such a packaging mix is used in a transfection assay, one finds plaques at a frequency of a few percent of that found with three normal plus strands. The phage in the plaques are all recombinants that have repaired the truncated 3' end by replacing it with that of one of the two intact segments. The finding of recombinants as products of transfection indicates that the recombination event is taking place within the procapsid particle during minus strand synthesis. Since minus strand synthesis occurs in the procapsid, and plus strands that lack the proper 3' ends cannot serve as templates for minus strand synthesis unless they are repaired, we infer that recombinational repair must have taken place in the procapsid. It is not known if recombination can take place during plus strand synthesis (transcription).

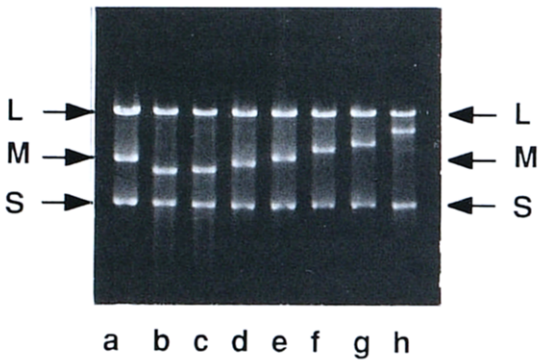

Figure 6. Agarose gel electrophoresis of dsRNA extracted from phage samples derived from plaques produced by transfection with RNA derived from pLM780 which contains the entire *lacZ* gene in segment M. RNA in lane a is from normal Φ6. Lanes b, c, d, e, f, g and h are from recombinant plaques. None are Lac^+ alone; however the phages used for lane g were Lac^+ on lawns of the indicator strain LM1034.

The requirement for all three segments to be packaged before minus strand synthesis begins is likely to be important for the precise packaging that is manifested by the Φ6 system. However, this requirement also serves to set up a situation where a segment with a damaged 3' end has the opportunity to be repaired by copy choice switching of the polymerase. If the segments would start minus strand synthesis before complete packaging, the damaged segment might enter the particle too late to be repaired.

If the recombination is taking place during minus strand synthesis, it is not clear how the recombinational complexes are resolved. That is to say, how do the chimeric minus strands detach from their two templates? Figure 7 shows the recombinant minus strand binding to both the donor and recipient plus strands. Two possibilities are that (A) the recombinant minus strand leaves its original 3' template spontaneously or (B) that transcription of new plus strands displaces the chimeric minus strand from its original template allowing the original template to reinitiate minus strand synthesis that would now be normal. It is important to keep in mind that all Φ6

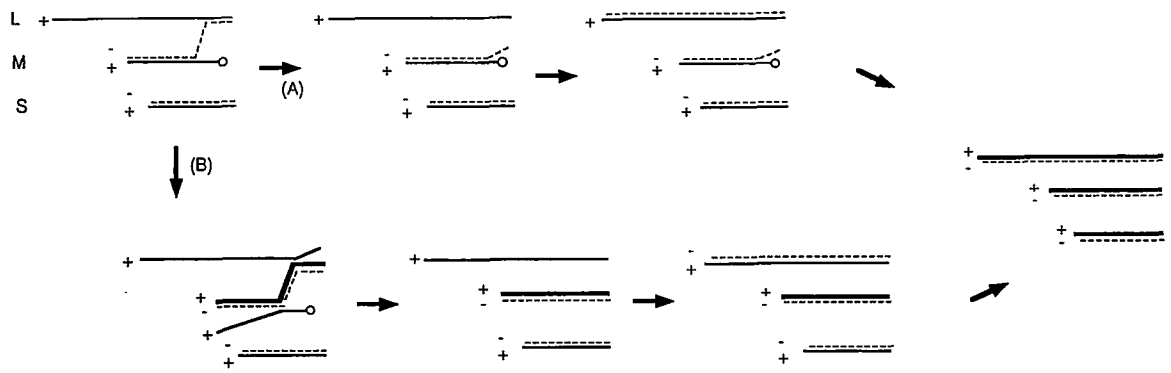

Figure 7. Models for the resolution of recombinational intermediates. The diagram illustrates a situation where the plus strand of M is truncated at the 3' end and cannot serve as a template for minus strand synthesis. Minus strand synthesis occurs normally on S and begins on L. The nascent minus strand on L leaves its original template to copy segment M. In pathway (A) the new recombinant strand spontaneously leaves its L template and L can serve as template for its normal complement. In pathway (B) transcription (bold line) of the recombinant structure leads to the displacement of the original template strands. The L template would then be free to serve as template for normal L minus strand synthesis. The segments are displayed so that the 5' ends of the plus strands are at the left.

RNA synthesis takes place within procapsids. Distinguishing between these models awaits the development of a direct analysis of the products of recombination in the in-vitro packaging and replication system.

It appears that heterologous recombination is a rare event in normal Φ6 replication, but that conditions that lead to a decrease in the rate of minus strand synthesis or prevent minus strand synthesis promote recombination. Some of these conditions promote the switch of the polymerase and the nascent chain to a new template. Sequencing of the crossover regions of recombinants showed that the recombination events took place between regions of limited sequence identity. At the crossover points there were usually from two to six identical bases in both the donor and recipient segments.[29] Recombination was not enhanced by creating longer stretches of identity. For example, an insert of 27 nucleotides of segment S was inserted upstream of a *lac* insert in segment M. Recombinants were isolated and sequenced. None of about 30 recombinants had crossed over in the 27 base identity. This suggests that the polymerase detaches from the original template without regard to identity in the other strands and only after detachment does the complex search for a place to land. We have not found any features of sequence or structure that promote 'launching'. We believe that the detachment of the nascent chain from the template may be a normal part of synthesis, but that recombination involves the offering of a new template onto which the nascent chain can land, it is noteworthy that heterologous recombination takes place in retroviruses through the mediation of reverse transcriptase and the sites of recombination also seem to favor those with short sequence identity.[32]

There is a significant apparent difference between Φ6 and other RNA viruses in the physical relationship between the RNA template and the polymerase. In the case of Φ6, the polymerase is a component of the procapsid. While P2 has the sequence motifs characteristic of viral RNA polymerases, it is not free in the particle, but is a component of the structure. For most RNA viruses, with the possible exception of the retroviruses, the polymerase is likely to be free in solution or attached to cellular structures. The fixed nature of the polymerase in Φ6 could cause problems as the enzyme tracks along the template. One possibility is that the template both translocates and rotates relative to the active site of the polymerase as new bases are added to the minus strand. Alternatively, the active site moves relative to other parts of the enzyme, and such movement allows it to track around the template. Presumably any such movement is limited, and continuous synthesis under this model would involve cycles of active site extension and retraction coincident with saltotory movements of the template. This is reminiscent of the 'inchworm' model of transcription of *E. coli* RNA polymerase.[33] One might imagine that the catalytic unit of the polymerase could extend enough to account for one full turn of the newly forming double stranded structure. Following each complete rotation the polymerase and nascent chain detach from the template and re-establish the proper place by matching sequence. If there were another template strand in close proximity, it might be possible for the polymerase to switch to it. There are hundreds of possibilities for matching sequences of six nucleotides in two heterologous strands. One might imagine that the template switching is a very rare occurrence unless the other template is unable to start on its own. It might be that under normal circumstances, all three segments are replicating simultaneously so that there would not be empty template available for polymerase jumping. If polymerase were extending and retracting, one might imagine that it would have difficulty in dealing with closely apposed repeats. We made a construction with four bases repeated six times to see if it would generate frame shifts that could be detected by a loss of LacZ activity. No frame shifting was observed in several thousand plaques. This result implies that such a 'resetting' of the nascent chain is rather precise.

There is another condition that favors heterologous recombination in Φ6. If any 5' part of the 75 nucleotide 3' conserved sequence of a genomic segment is deleted, the segment is then subject to high rates of recombination. This occurs despite the fact that in many of these cases the rate of minus strand synthesis *in vitro* appears to be normal.[34] The reason for the increase in recombination is not known; however our working model is that these deletions lead to a susceptibility to degradation *in vivo* at the 3' end. If this were to happen, the damaged segments would be equivalent to the truncated segments described above and would be packaged but unable to serve as templates for RNA synthesis unless rescued by recombination. The role of the secondary structures at the 3' ends of the segments is not clear, although it must be important. Many RNA viruses have similar structures and the possibility of these structures having a role in the stability of the molecules has been raised before.

The frequency of recombination can be very high in Φ6. It is considerably higher than that found for other RNA viruses. This is probably due to the fact that the process is taking place within the procapsid where the concentrations of RNA are extremely high. It is very difficult to measure the recombination frequencies for Φ6 because it is taking place within the genome of a single particle. The cross continues to operate as the plaques develop. Nevertheless, we find recombination frequencies of the order of 50 percent within the distance from the *PstI* site in segment M to the end of gene 3. This is a distance of about 300 bases. Whereas poliovirus and coronavirus show frequencies of about 0.1 % per 100 nucleotides, the frequency for Φ6 seems to be about one-hundred-fold greater. But this high level of recombination is only seen when 'provoked'. Under ordinary conditions, the recombination frequency for Φ6 is probably lower than that described for the other RNA viruses. Recombination in Φ6 does not seem to be promoted by sequence identity or complementarity. It has been suggested that recombination in some of the tripartite plant viruses is favored near complementary sequences. It has been proposed that complexes may be formed between the donor and recipient segments.[35,36] A similar relationship has been reported for poliovirus.[37] This type of interaction may reflect the difficulty in getting segments in close proximity during plus or minus strand synthesis. In the case of turnip crinkle virus, recombination appears to be site-specific and is stimulated by the presence of a stem-loop structure[38] that resembles the start site for transcription. This seems to be a unique mechanism.

The system that we have used selects recombinants in the 3' non-coding regions of the Φ6 genomic segments. Recombination in the coding regions would not usually be seen in our experiments. We have also found evidence of recombination events in the 5' non-coding regions. In order to demonstrate recombination in the 5' regions we have had to use a system in which the virus incorporates plasmid transcripts into its genome as extra segments. Phage containing deletions in vital genes can grow on host strains carrying plasmids with cDNA copies of the missing genes. In some cases the virus has been able to pick up the transcripts of these plasmids and incorporate them into their genomes, either as a fourth segment or as a replacement for the deletion segment. However, the new segment must always have the same 5' packaging sequence as the segment that contains the deletion. If the transcript lacks the normal 3' end, it can acquire it by heterologous recombination. If it lacks one of the three normal 5' ends it will not be picked up. If the transcript has the same 5' end as the deletion bearing segment it will be picked up at a high frequency. If it has the 5' end of one of the other segments, the pick up will be at a low frequency. Upon examination it was found that the 5' end of a transcript that originally had a heterologous packaging sequence can be exchanged by recombination so that it now has the same sequence as the 5' end of the deletion bearing segment. This is interpreted as follows: the transcript can be picked up by a procapsid *in vivo* as long as it has the 5' end of one of the segments. In an infected cell it will not matter if the correct complement of genes is in a particular particle. If the segment lacks a normal 3' end, it will be acquired easily as has been shown. However to produce a virus particle that can form a plaque, the newly acquired segment must have a proper 5' end for its segment class and only recombinants that have done this will be viable.

Recombination has not been shown definitively for the other dsRNA viruses. In the case of the reoviridae, segments have been found to have deletions or duplications of parts of genes. In many cases, the duplications look very much like products of heterologous recombination in that they show limited sequence identity at the junction points. Deletions and duplications have been found in rotavirus[39] and the mechanism of production of these intramolecular rearrangements may be similar to that for heterologous recombination, but this remains to be demonstrated. An internal, tandem duplication of part of a gene in segment 10 of human rotavirus[40] shows a seven base direct repeat between the two crossover sites that is reminiscent of the sequence relationships described for Φ6. It seems likely that once it becomes feasible to screen for intermolecular recombinants, they will be found in the other segmented dsRNA virus systems.

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