Heterologous Recombination in the Double-Stranded RNA Bacteriophage $\phi 6$

LEONARD MINDICH,* XUEYING QIAO, SHIROH ONODERA, PAUL GOTTLIEB, AND JEFFREY STRASSMAN

Department of Microbiology, The Public Health Research Institute, New York, New York 10016

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Bacteriophage $\phi 6$ contains three double-stranded RNA genomic segments. We have constructed a virus with an insertion of a kanamycin resistance gene in genomic RNA segment M. The virus forms small, turbid plaques, and its genome is unstable. Virus from a single plaque contained from about 0.1 to 10% large clear-plaque forms of the virus; these were usually missing the kanamycin resistance gene, and in many cases, the resulting segment M was larger or smaller than its normal size. Sequence analysis of the genomic RNA of the apparent deletions showed that they were formed by recombination events between segment M and either segment S or L. These heterologous recombination events resulted in the loss of the kanamycin resistance gene from segment M and the replacement of the 3' end of segment M with the 3' end of segment S or L. Although the 3' ends of the single-stranded RNA transcripts of the genomic segments appear to have extensive secondary structure, the sequences at the 3' ends are not involved in the specificity of genomic packaging.

Bacteriophage $\phi 6$ infects the plant pathogen *Pseudomonas phaseolicola* (*Pseudomonas syringae* pv. *phaseolicola*) HB10Y (21). Three separate pieces of double-stranded RNA compose its genome, which is located inside a polyhedral nucleocapsid (19). The nucleocapsid has RNA polymerase activity (17) and is covered by a lipid-containing membrane (21).

We have developed an in vitro genomic packaging system that utilizes single-stranded RNA (ssRNA) obtained from the in vitro transcription of viral nucleocapsids or RNA transcripts of plasmids carrying cDNA copies of the viral genomic segments. The RNA is packaged by procapsids formed in *Escherichia coli* cultures that are expressing the cloned genes for $\phi 6$ procapsids. We have previously demonstrated that a recombinant transcript of segment M that contains an insertion of the gene for kanamycin resistance (*kan*) can be incorporated into the genome of viable phage (16).

Virus that contains the *kan* gene is genetically unstable, and virus preparations contain particles that have lost part of the recombinant M segment (16). For this study, we have examined the sequence of the apparent deletants and show that they arise by recombination between heterologous segments.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. HB10Y (21) was used as the host for $\phi 6$ lysate preparations and for phage titration (Table 1).

Isolation of recombination derivatives. Bacteriophage $\phi 6K1$, which contains the *kan* gene in segment M, was plated on a lawn of HB10Y, and large clear plaques were picked. High-titer stocks were prepared by using HB10Y and the sloppy agar method (20). These preparations were extracted with phenol and analyzed for the size of segment M by agarose electrophoresis (16). Virtually all such isolates showed unique migration patterns for segment M. Several preparations of interest were used to prepare large-volume

lysates (4), which were used for the preparation of ssRNA. Phages $\phi 1690$, $\phi 1691$, and $\phi 1703$ were prepared this way.

In one case, we found that a carrier state culture of HB10Y (16) that was being maintained on kanamycin-containing medium contained a genomic segment S that migrated faster than normal. Dilutions of a phage stock, *sus325* (14), that contained an amber mutation in gene 5 of segment S (Fig. 1) were plated on this strain, and the resulting plaques were tested for the appearance of wild-type gene 5 function. One of these plaques was designated $\phi 1712$. Analysis of the RNA of $\phi 1712$ showed that segment S had the rapid migration characteristic of the RNA in the carrier state cells. Analysis of the sequence of this segment S showed that it contained a deletion. Segment M was shown to be derived from the superinfecting phage.

Supernatant fluid of the carrier state culture yielded very few plaques. One such plaque was used to prepare a phage stock, and it was found that this virus, $\phi 1713$, contained the faster-migrating segment S and a segment M that was slower migrating than normal but faster than that of $\phi 6K1$. The segment M of $\phi 1713$ was found to be a recombinant between segment M and the deletion derivative of segment S.

Preparation of $\phi 6$ ssRNA. $\phi 6$ ssRNA was synthesized by an in vitro transcription reaction essentially according to Emori et al. (5), using nucleocapsids prepared as described by Mindich et al. (14).

PCR amplification of cDNA. In vitro transcripts of phages $\phi 1690$, $\phi 1691$, $\phi 1703$, and $\phi 1713$ were incubated with oligonucleotide primer OLM62 (Table 2) and avian myeloblastosis virus reverse transcriptase (15). OLM62 is complementary to the 3' end of all of the genomic segments and contains a tail with an *Xba*I site. The product was extracted with phenol, precipitated with ethanol, and dissolved in DNA buffer. This DNA was then used as template for the polymerase chain reaction (PCR), using oligonucleotide primer OLM60 (Table 2) along with OLM62. The protocol for PCR was 37°C for 2 min, 72°C for 3 min, and 94°C for 1 min, repeated for 35 cycles. The amplified DNA was then cut at the *Xba*I site and at *Eco*RV, which is centered at nucleotides 2978 to 2979 in gene 3 of segment M (Fig. 1). This DNA was then cloned into plasmid pT7T3 19U (Pharmacia) that had been cut with *Xba*I and *Sma*I. Phage $\phi 1712$ transcripts were

* Corresponding author.

TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Remarks	Reference or source
Bacterial strains		
<i>P. phaseolicola</i> HB10Y	Host of $\phi 6$	21
<i>E. coli</i> JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1</i> $\lambda^- \Delta(lac-proAB)$ (F' <i>traD36 proAB lacI</i> ^Z ΔM15)	J. Messing
Phages		
$\phi 6$	Wild-type phage	21
K1	$\phi 6$ containing <i>kan</i> in segment M	16
$\phi 1690$	Recombinant of M with 3' L	This study
$\phi 1703$	Recombinant of M with 3' L	This study
$\phi 1691$	Recombinant of M with 3' S	This study
$\phi 1712$	Deletion near 3' end of S	This study
$\phi 1713$	Recombinant of M with 3' end of $\phi 1712$ S	This study
Plasmids		
pT7T3 19U	<i>amp</i> , P _{T7} , P _{T3} , <i>lacZ'</i>	Pharmacia
pUC-4K	<i>amp</i> , <i>lacZ'</i> , <i>kan</i> cartridge in <i>PstI</i> site	22
pLM656	<i>amp</i> , P _{T7} ; exact copy of segment M in pT7T3 19U	16
pLM672	<i>amp</i> , P _{T7} , <i>kan</i> gene in <i>PstI</i> site of pLM656	16

amplified with OLM62 and OLM68, and the product was cloned into pT7T3 19U that was cut with *SalI* and *XbaI*.

DNA sequence analysis. Sequence analysis was performed by the dideoxy-chain termination method (18), using bacteriophage T7 DNA polymerase (Sequenase; U.S. Biochemical). The synthetic deoxyoligonucleotides used as primers were the standard M13 sequencing primer and the reverse M13 sequencing primer (Table 2).

RESULTS

We have prepared a derivative of bacteriophage $\phi 6$ designated $\phi 6K1$, in which a *kan* gene has been inserted near the 3' end of genomic segment M (Fig. 1) (16). This was accomplished by inserting a *kan* gene cartridge of pUC-4K (Table 1) into the *PstI* site of a cDNA copy of the genomic segment that had been cloned into a T7 promoter vector called pT7T3 19U (Table 1). A transcript of the resulting plasmid, pLM672, was packaged along with purified segments s and l into procapsids, which were then coated with protein P8 and introduced into spheroplasts of the host bacterium. The resulting bacteriophage, $\phi 6K1$, formed small turbid plaques. Large clear plaques were observed at a frequency of 0.1 to 10%. Whereas $\phi 6K1$ contained normal-size segments S and L and a larger than normal segment M, the clear-plaque forms had M segments of sizes varying from a few hundred base pairs larger than normal to several hundred base pairs smaller than normal (16).

cDNA copies of the 3' ends of segment M of the various apparent deletants were prepared by first synthesizing in vitro transcripts of purified nucleocapsids and then incubating the cDNA with reverse transcriptase, using an oligonucleotide primer that is complementary to the three segments.

The cDNA was then amplified by PCR, using the same terminal primer along with one that primes the reaction in the opposite direction starting inside of gene 3. This material was cloned into pT7T3 19U, and the sequence of the DNA was determined (see Materials and Methods).

It was found that the deletants did not have the 3' ends of the M segment. Of three deletants that were examined, two had the 3' end of segment L and one had the 3' end of segment S. In each case, the point of crossover was 5' to the point of insertion of the *kan* gene. In two of the cases, the point of exchange was downstream of gene 13, which ends at nucleotide 3722; in one of the exchanges with segment L ($\phi 1703$), the crossover was inside but near the end of gene 3 at nucleotide 3403, resulting in a read-through that elongated protein P3 by 14 amino acids and the elimination of gene 13. The normal end of gene 3 is at nucleotide 3416. The points of crossover showed little sequence identity. In two cases we found only two identical bases in the crossover region, and in two others we found six identical bases (Fig. 2 and 3). Two of the recombination events took place at the same point in segment M, but in one case with segment L and in the other with segment S (Fig. 3).

Cells infected by $\phi 6K1$ can form carrier states in which the culture becomes kanamycin resistant because it carries the replicating phage as an episome (16). Many of these cell lines lose the ability to form phage while maintaining the ability to replicate the viral genome. In one such case, the size of segment S was found to be reduced. This smaller genomic segment was still functional in that it could be rescued into a superinfecting phage that had an amber mutation in gene 5 of segment S. The resulting phage was designated $\phi 1712$. It was determined that the deletion was near the 3' end of the

TABLE 2. Oligonucleotides for cloning and sequencing

Name	Sequence	Remarks
OLM60	GATCTGCTGTCTCAACAGATC	Complementary to minus strand of M around nucleotide 2847 (before <i>EcoRV</i>)
OLM62	CCCTCTAGAGAGAGAGCCCCCGAAG	Complementary to plus-strand 3' termini with <i>XbaI</i> site
OLM68	GCGAACTACGAAGGTAGAACG	Complementary to minus strand of S around nucleotide 2400
M13	GTAACACGACGGCCAGT	For sequencing pT7T3 19U
RevM13	CAGGAAACAGCTATGAC	For sequencing pT7T3 19U

FIG. 1. Restriction maps of the cDNA copies of the three genomic segments of $\phi 6$ and segment M of $\phi 6K1$ which was derived from a transcript of pLM672.

segment, and sequencing showed that it was an internal deletion of 189 nucleotides in the 3' noncoding region (Fig. 2).

This carrier state culture was found to produce phage at a level of several orders of magnitude less than normal carrier state cells. One plaque produced by these cells, $\phi 1713$, was analyzed and found to be a recombinant between segment M and the 3' end of the segment S containing a deletion described above. The recombinant virus has an M segment with the 3' end of S but containing the 189-bp deletion. The crossover point in this recombinant had a six-base identity between the M and S sequences at the point of crossover (Fig. 2 and 3).

Several controls were performed to verify that the recombinants were not artifactual products of PCR. The molecular weights of the recombinant M segments as well as the deletion in segment S ($\phi 1712$) were estimated on the basis of their electrophoretic mobilities in agarose gels. These estimated molecular weights agreed very closely with the molecular weights calculated on the basis of the crossover sequence coordinates (Fig. 2). The molecular weights were within the expected error of estimation of molecular weights by electrophoretic mobility. The electrophoretic behavior of recombinant M segments can be seen in a previous publication (16), in which phage $\phi 1703$ is listed as sample d3 in Fig. 2.

In addition, we have confirmed some of the rearrangements by probing the viral RNA with radioactive oligonucleotides complementary to limited regions of the $\phi 6$ genome. As an example, a probe complementary to the region at nucleotide 2600 in segment S did not react with segment S of $\phi 1712$, where we found a deletion of this region. A probe

complementary to nucleotide 2400 did react with segment S of $\phi 1712$ and also reacted with both segment S and segment M of $\phi 1713$ as expected on the basis of the data in Fig. 2 (data not shown).

DISCUSSION

Homologous recombination has been demonstrated and studied in several positive-strand RNA viruses, particularly in poliovirus (9, 11) and coronaviruses (12). In addition, the products of heterologous recombination have been observed in virus isolates from the wild (10) or in experiments in which defective segments of segmented genome virus could be rescued (3). The formation of deletions or duplications in genomic RNA segments may be mediated by mechanisms similar to those responsible for recombination. Duplications in the genome of rotavirus have been reported (6, 13). Recombination is clearly useful for nonsegmented RNA viruses, both for facilitating genetic diversity and for repair. Heterologous recombination can be useful for repair and also for making more dramatic changes in viral genomes than caused by homologous recombination.

The results that we report appear very reminiscent of those reported for brome mosaic virus (3). In both cases, there is exchange of heterologous ends. The $\phi 6$ recombination is not completely dependent upon sequence identity but appears to use it. The brome mosaic virus system might involve a more complex interaction between the recombining segments (2).

We report in this paper that the genomic segments of $\phi 6$ can recombine through regions of very limited identity. The recombination appears to occur at a high frequency. However, the frequency of recombination must be much higher than what we observe, since we select only plaque-forming virus. This requires a crossover within the 400 nucleotides between the end of gene 3 and insertion site of *kan*, whereas segment M contains 4.1 kb. It is not yet known whether this reflects a normal behavior or is induced by the nature of the gene that we have inserted into genomic segment M. The inserted *kan* gene adds 1.2 kbp to the 4 kbp already contained in genomic segment M (16). In addition, there is an 18-bp high-G+C, potential hairpin structure at the point of insertion. Preliminary results suggest that the homopolymer arms are increasing the recombination rate by at least 10-fold (15a).

The characterization of the recombination products has given useful insights into the requirements for $\phi 6$ development and packaging. The most important finding is that the sequences at the 3' ends of the genomic segments do not define the specificity of packaging of the individual segments. Since virus with segment M containing a 3' end that is identical to that of either the L segment or the S segment packages all segments perfectly, it is clear that the segments are not differentiated by sequence in this region.

We have examined the 3' ends for secondary structure and found a striking indication of structural conservation among the three segments (Fig. 4). Within the terminal 75 nucleotides of the three segments, the only differences are those that are in the presumed loops of hairpins or, if in the stems of hairpins, balanced by additional changes that maintain the base pairing. Although the structure shows a number of hairpin stems, it does not have a tRNA-like terminus as found for some other viruses such as brome mosaic virus (1). The 3' ends of the segments are necessary for the synthesis of minus strands (7a). The elaborate structure might play a

FIG. 2. Diagrams of the recombination products. The restriction map of the cDNA copy of segment M is shown along with schematic diagrams of the recombinant segments. The 5' ends of the recombinants are not shown, and the recombinant 3' ends are not drawn to scale. The identical nucleotides at the crossover junctions are shown, as well as the length of the heterologous replacement of the 3' end. ϕ 1712-S shows the deleted form of segment S in ϕ 1712. This should be compared with segment S in Fig. 1. The 3' end of this segment is found in the recombinant ϕ 1713. The sizes of the rearranged genomic segments are listed in the box as calculated on the basis of the sequences determined on the PCR material and as estimated from the electrophoretic mobility of the viral RNA segments.

role in polymerase recognition or function, but might also be involved in protection from cellular nucleases.

The reported frequencies of both homologous and heterologous recombination are usually very low. The high frequency found in this study might well be explicable in terms of the replication mechanism of ϕ 6. The replication of ϕ 6 RNA takes place within a procapsid (8). The three genomic segments are present in close proximity.

The appearance of an internal deletion as in ϕ 1712 could be due to an event involving a single RNA molecule or could be due to a recombination event between two different RNA molecules. On the basis of unpublished studies that we have conducted on the replication of ϕ 6, we believe that the entry of two different RNA molecules of the same genomic segment into a procapsid would be an impossible or very rare event. If this is so, we should find that biparental homologous recombination events are nonexistent or rare.

If the recombination is a normal occurrence, we might ask why the viral genome is not identical at the 3' ends. Although we do not see any deficiency in the virus with identical ends in two segments, it is possible that there is some small advantage offered by the natural sequence diversity. It is also possible that the sequence is undergoing constant variation and what we see is the result of recombination, which

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UCUCUAAUGGAGACCAACCCGCGUCUUCAGCUUGAGUCGCGUCGCGC M
UCUCUAAUGGAGACCAACCCGCGUCUUGGCUUGGCGCCACAACCGACAU 1690
UGGUGAUAAGCAACGCUUGGGCAUGGUUGGCGCCACAACCGACAU L

AGACCAACCCGCGUCUUCAGCUUGAGUCGCGUCGUUACGCUUGC M
AGACCAACCCGCGUCUUCAGCUUGGCUUGGCUUGGCUUGGCUUGGCUUGG 1691
GGAUUGAUCACCGUUCGCGAGCUUGGCUUGGCUUGGCUUGGCUUGGCUUGG S

UAAGUACGCGGGCCUGUAAGGAGUCGUAUGGCGUCGUAACAAUCC M
UAAGUACGCGGGCCUGUAAGGAGUAGGCGUCUUGGCGUCGCGUCGCGUC 1703
GCCGCGUCGCGGCUUCGAGUAGGCGUCUUGGCGUCGCGUCGCGUC L

UCUCUAAUGGAGACCAACCCGCGUCUUCAGCUUGAGUCGCGUCGCGC M
UCUCUAAUGGAGACCAACCCGCGUCUUGGCGUUGGCGUUGGCGUUGGCGU 1713
ACCCGAAGCAAAGCGAGGCGCGUCGCGGGCGUUGGCGUCGCGC S

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FIG. 3. Base sequences of recombination products around the crossover region. The sequence of the original M segment is underlined, and that of the original L or S segment is shown below. The extent of sequence identity at the crossover point is indicated by the dark horizontal lines. The numbers refer to the individual recombinant phages depicted in Fig. 2. Note that both 1690 and 1713 have recombined at the same point in segment M.

FIG. 4. Presumed secondary structure of ssRNA transcripts of the $\phi 6$ genomic segments. 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. The crossovers in all of the recombinants except $\phi 1691$ are outside of this diagram. The crossover point in $\phi 1691$ is in the loop of the nonidentical hairpin of segment S, with AGCUUG identical in S and M.

would tend to make the termini of the segments identical, and mutation, which would make them dissimilar. We are currently preparing substrates that will facilitate the investigation of the mechanism of the $\phi 6$ recombination.

The recombinational results have cleared a minor problem in $\phi 6$ structure in that one of the recombinants is missing gene 13 (7) entirely. This mutant has normal structure and development. On this basis, we can say that P13 is not necessary for $\phi 6$ membrane structure or for phage development. We do not know whether P13 expression benefits the virus under special circumstances.

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