Virology **286,** 113–118 (2001)
doi:10.1006/viro.2001.0995, available online at http://www.idealibrary.com on IDEAL®

Reverse Genetics and Recombination in Φ8, a dsRNA Bacteriophage

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Received February 19, 2001; returned to author for revision March 29, 2001; accepted May 4, 2001

Bacteriophage Φ8 has a genome of three dsRNA segments. It is able to acquire plasmid transcripts of cDNA copies of the genomic segments as replacements of its resident chromosomes. It is also able to effect recombination between the plasmid transcripts and the resident chromosomes. Depending upon the extent of sequence identity between the plasmid transcript and the resident chromosome, the recombination can be homologous or heterologous. Homologous recombination has not previously been reported for viruses with double-stranded RNA genomes. © 2001 Academic Press

Key Words: bacteriophage; dsRNA; recombination; *Pseudomonas.*

Bacteriophage Φ8 is similar in structure and genome organization to bacteriophage Φ6, which contains three segments of double-stranded RNA (dsRNA) (Hoogstraten *et al.,* 2000; Semancik *et al.,* 1973) packaged inside a procapsid which is covered by a lipid-containing membrane composed of additional viral proteins (Vidaver *et al.,* 1973). The genome of Φ6 has been cloned and sequenced and the life cycle and structure of the phage has been the subject of considerable investigation (Butcher *et al.,* 1997; de Haas *et al.,* 1999; Mindich, 1999a). Φ6 has been shown to carry out a heterologous recombination process that results in the 3′ end of the plus strand of one genomic segment replacing the 3′ end of another genomic segment (Qiao *et al.,* 1997). This recombination takes place by template switching during minus-strand synthesis and results in an average of 3.6-base identity at the crossover point (Qiao *et al.,* 1997). In this paper we show that Φ8 is capable of both this type of heterologous recombination and homologous recombination. In the latter case, the recombination event results in an exact fit of the new sequence into the continuity of the resident sequence. Because of the availability of reverse genetic techniques in Φ6 and Φ8 it is possible to manipulate the systems so as to clarify some of the parameters involved. We have established a system wherein plasmid transcripts are acquired by a deletion mutant of phage Φ8. We test the consequences of sequence identity between the transcript and the phage on the frequency of homologous versus heterologous recombination. We also test the consequences of the transcripts containing a packaging sequence (Gottlieb *et al.,* 1994), *pac,* on the frequency of recombination. *Pac* sequences are near the 5′ ends of plus strands and are unique for each segment (Gottlieb *et al.,* 1994).

There is evidence of template switching involving heterologous recombination in the *Reoviridae,* a class of viruses of eukaryotic organisms that contain genomes of 10, 11, or 12 dsRNA genomic segments. These events lead to either duplications or deletions within genomic segments with the tell-tale limited sequence identity at the crossover points (Desselberger, 1996). The recombination process cannot be studied in depth in the *Reoviridae* because manipulation of the genomes is not yet possible.

RESULTS

Preparation of the recombination tester phage 21 (*ϕ*2756)

Our goal was to prepare a phage that would recombine with plasmid transcripts in a manner that would facilitate the distinction between homologous and heterologous recombination. Plasmid pLM2764 was prepared to contain a cDNA copy of segment M of Φ8 in plasmid pLM350, which is a shuttle vector between *Escherichia coli* and pseudomonads. The sequence of segment M was modified so that the two 3′-terminal genes, F and G, were deleted and the *lacα* was inserted after gene 3b (Fig. 1). This plasmid was transformed into *P. syringae* strain LM2489, which is a host for Φ8.

We had previously prepared another deletion plasmid, designated pLM2599, which had a deletion in gene 3a caused by removing the sequence between the *Nco*I sites at 1578 and 2268. It also had a *kan* gene inserted at its 3′ end. The phage derived from this plasmid, *ϕ*2745, was able to plate on host strains that carried a plasmid that expressed genes 3a and 3b. It was, therefore, able

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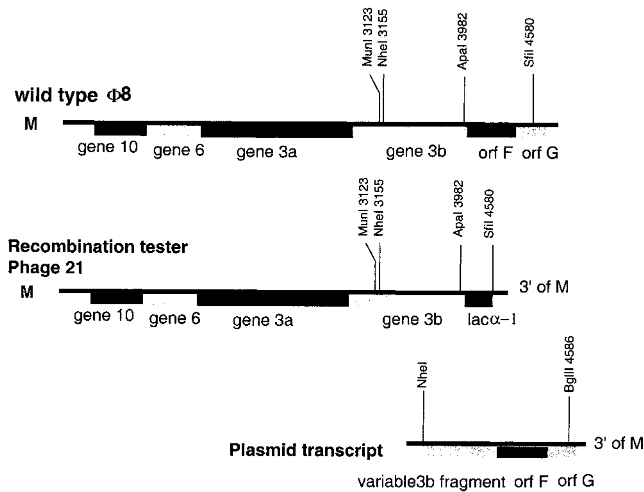

FIG. 1. Map of the cDNA copy of Φ8 M and the deletion construct, pLM2764, that was incorporated into tester phage 21 with *lacα* replacing genes F and G. The transcript of plasmids containing genes F and G and parts of gene 3b (pLM2797, 2799, 2801) is also shown. The relevant restriction sites are shown.

to plate on a strain carrying plasmid pLM2764, described above. During propagation on this strain, the plasmid transcript could be acquired to produce tester phage 21, which has genes F and G replaced by *lacα*. This phage was able to propagate on strains carrying a plasmid that expressed genes F and G and was able to acquire transcripts of these plasmids by recombination.

Preparation of the donor plasmids and acquisition of transcripts

We prepared cDNA fragments that included the 3′ end of segment M with genes F and G and parts of gene 3b (Fig. 1, plasmid transcript). The 3b sequences ranged from the largest, which included the sequence from the normal *Nhe*I site at 3155 to the end of 3b at 4048 (an overlap of 894 nucleotides), to fragments that had the *Nhe*I sites at 3395, 3570, or 3810, which made for overlaps of 648, 479, or 239 nucleotides with the gene 3b in the phage. These were ligated into plasmid pLM350 along with an *Mun*I/*Nhe*I fragment at the *Eco*RI site. The plasmid transcripts were produced from the vector *lac* promoter and did not contain the normal *pac* sequences for Φ8 segment M packaging. Host cells carrying these plasmids were capable of complementing the FG deletion in *ϕ*2756. Upon propagating the phage on these strains, we found that among the phage produced were a number that were able to plate on normal host cells without a complementing plasmid. The frequency of these phages ranged from 10^{-8} to 10^{-7}. Plaques were picked and RNA was prepared from the viral preparations. The phages produced on the strain with the overlap of 239 bases all had abnormal sizes for genomic segment M (Fig. 2, lanes g and h). The other preparations had about 50% normal-sized segments and 50% abnor-

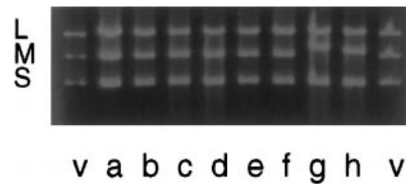

FIG. 2. dsRNA prepared from *ϕ*2756 and selected recombinants. Lane V, wild-type Φ8. Phages that have acquired transcript with sequence overlaps of 894 bases (a, b), 648 bases (c, d), 479 bases (e, f), and 239 bases (g, h) are shown. The M segments of lanes g and h are abnormal while the others are the size of wild-type virus.

mal (Fig. 2, lanes a, b, c, d, e, and f). The normal-sized M segments were subjected to RT-PCR and the resulting DNA was cloned and sequenced and in all cases was found to be of completely normal sequence, indicating that the recombination was homologous.

Other examples of recombination in Φ8

We prepared a cDNA copy of segment M with the gene for kanamycin resistance (*kan*) in the *Sfi*I site with in gene G. Although we have identified a protein product of gene G, it appears that either this gene is not necessary or a truncated form is functional. The acquisition of this transcript was selected by propagating wild-type phage on this strain, filtering, and then plating onto host cells in the presence of kanamycin in order to select for carrier state kanamycin-resistant colonies (Onodera *et al.,* 1992). The colonies could be purified, and phage (Φ2645) could be isolated from the supernatant fluid of cultures. These phage preparations were somewhat unstable genetically (about 10% loss) and *kan* deletants could be isolated when the phages were plated on strains that complemented genes F and G. When the cDNA copies of the *kan* deletants were sequenced it was found that they had lost the *kan* gene by template switching from either segment S or segment L. The sequences of two examples, Δ2 and Δ3, are shown in Fig. 3. These are typical of heterologous recombination events described for Φ6 (Qiao *et al.,* 1997).

Deletion phage Δ2 could be complemented by a number of different plasmids. It is defective in genes F and G

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M 4201   GCTGGGCTATACCCTATGTTGGCGCGCTTTCCAGGTGCCC
Δ3       GCTGGGCTATACCCTATGTTGGCGCGCTCCTCAGCGTAGA
S 2747   GTCTCGTGATACTGGGGGCGGAATCGCTCCTCAGCGTAGA
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M 4201   GCTGGGCTATACCCTATGTTGGCGCGCTTTCCAGGTGCCC
Δ2       GCTGGGCTATACCCTATGTTGGCTTAGTCGATACCATCGA
L 6476   TAACAGCAGTTGCAATCAACCGCTTAGTCGATACCATCGA
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FIG. 3. Sequences at the crossover points in heterologous recombinants that have lost *kan*. The boxed sequences show limited sequence identity at the crossover points.

as the crossover from segment L is in gene F (Fig. 3). A number of isolates that could be propagated on strains without plasmids were obtained and all were found to be the result of homologous recombination. The donating plasmids ranged from those whose transcripts started at the normal *Nhe*I site, as used in the case of *ϕ*2756, to larger overlaps such as pLM2504, which starts at the *Bam*HI site at nucleotide 215 (Fig. 1). The donating transcript could have no *pac* site as in the case of pLM2504 or could have the *pac* site of Φ6 (pLM2446).

The role of *pac* sites in recombination frequencies

Packaging is rather tightly regulated in Φ6, of which only one of each plus strand is packaged in the order s:m:l if the molecules are of normal size (Mindich, 1999a). Recombination is usually found between different genomic segments. In the case of recombination between plasmid transcripts and the viral genome, there might be specific consequences in response to the presence or absence or specificity of the *pac* site. Having no *pac* site should mean that the probability of the transcript being acquired by the particles is very low. But having a *pac* site of the same specificity as the recipient segment might involve interference. We prepared plasmids with the 648-base overlap in gene 3b, but with *pac* sites for either segment S or M at the 5′ end. These plasmids were able to complement the defect in the recombination tester phage 21 (*ϕ*2756). The frequencies of acquisition of transcript were found to be higher in the case of transcripts with *pac* sequences, but the differences were only about 10-fold, which was less than expected. The frequency of acquisition without a *pac* sequence was about 1×10^{-8} while the frequency for a transcript with an S sequence was 3×10^{-7} and for an M sequence it was 1×10^{-7}. The recombinants were analyzed to determine the relative frequencies of heterologous and homologous recombination. It was found that the presence of a *pac* sequence did not make much of a difference and that there was no evidence of interference in the case in which the plasmid transcript had the same *pac* sequence as the recipient segment.

The role of a normal 3′ end in the plasmid transcript on acquisition

In order for the plasmid transcript to serve as an initial template, it requires a sequence similar to that of the normal 3′ end (Qiao *et al.,* 1997). A transcript that has a normal 5′ end with a *pac* sequence can be rescued by template switching, but in the present set of experiments we are requiring that the transcript serve as the first template. In fact, we found that plasmid transcripts that lack both a normal 3′ and a normal 5′ sequence (pLM2572) do not get acquired although they can complement deletions.

DISCUSSION

RNA recombination has been described in many viral systems (Lai, 1992). The current consensus is that the mechanism involves template switching during RNA synthesis (Kirkegaard and Baltimore, 1986). It is likely that the precise mechanisms vary with different viruses because the details of RNA synthesis differ rather greatly. For example, most viruses replicate their RNA in pools where many RNA molecules can interact, but where the concentration of RNA is not very high. In other systems such as Φ6 and Φ8, the replication takes place within a particle, the procapsid, and only those molecules which have been packaged can participate, but they are in very close proximity (Mindich, 1999a). Recombination in Φ6 was shown to be promoted by having the recipient RNA strand unable to initiate its template function (Onodera *et al.,* 1993). One can imagine a situation in which there is an availability of nascent RNA ends that have become freed of their original templates and polymerase that can now land on new templates that have been unable to initiate minus-strand synthesis (Figs. 4C, 4D, and 4E). If minus-strand synthesis has already taken place on a prospective recipient template, there would be no place for the nascent chain to land. In the present study, we are collecting recombinants that occur in particles in which all the strands can serve as templates. Therefore the frequency of recombination is very low, to an extent because of this condition and also because the recombination depends upon the packaging of plasmid transcripts. Whereas the frequency of recombination can be about 1% when phage are assembled *in vitro* with one of the strands unable to initiate its minus-strand synthesis (Qiao *et al.,* 1997), in the present study the frequency of recombination is on the order of 10^{-7}.

The polymerase of Φ6 has recently been crystallized and its atomic structure determined (Butcher *et al.,* 2000, 2001). A striking feature of this structure is that the path of the template through the protein is completely enclosed, implying that the enzyme is completely precessive and that the 3′ end of the template enters the polymerase. This would suggest that the recipient template that cannot initiate can, however, pass through the polymerase so that when template switching does occur, it can take place at any point on the template 3′ of the position where the polymerase is located (Figs. 4D and 4E). The annealed nascent chain and new template would back up so that the 3′ end of the nascent chain would be in the catalytic site (Fig. 4F). Template switching would involve the movement of the nascent chain to a new template without bringing along the polymerase, which would be locked onto the previous template. Φ6 and Φ8 have 12 polymerase molecules per procapsid, so we assume that each RNA is complexed with a different polymerase molecule. Our assumption is that, in the present case, recombination takes place onto templates

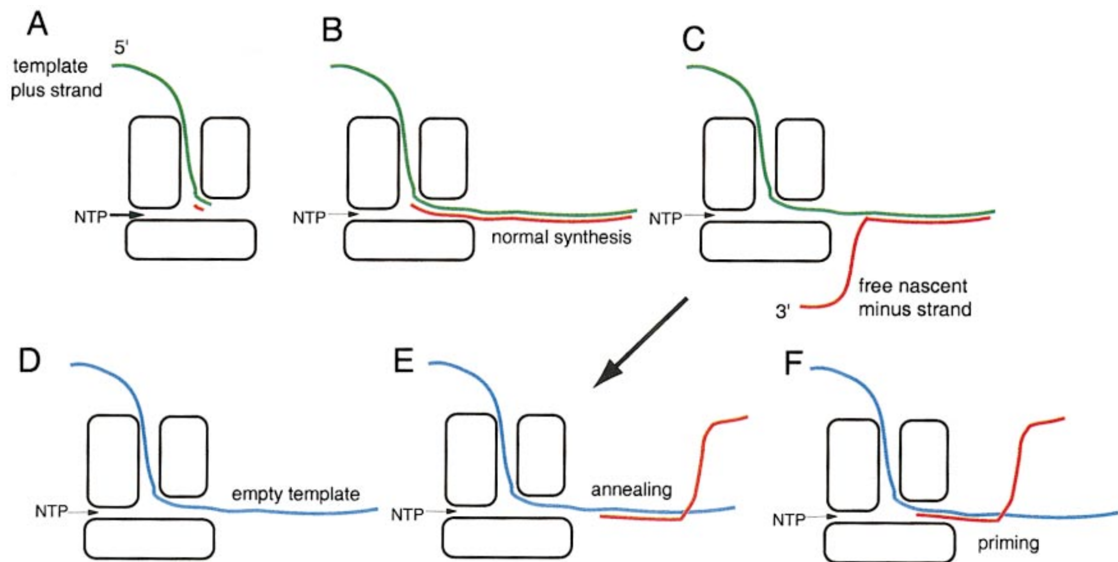

FIG. 4. Proposed model for the process of template switching recombination in Φ8. Normally, the 3′ end of the template plus strand (green) enters the polymerase and is arrested near the site of nascent minus-strand chain formation (A); as the minus strand (red) is synthesized the dsRNA leaves the polymerase through the exit pore (B). The 3′ end of the nascent chain can displace from the template (C). It can reanneal to the template or it can anneal to an empty template (blue) (E). A plus-strand RNA that does not have a proper 3′ end can enter the polymerase, but the template is not arrested at the catalytic site (D). Instead, it passes through the polymerase and can scan backward and forward. When a nascent minus strand anneals to the empty template and the template moves back into the polymerase, the nascent chain can act as a primer to start minus-strand synthesis on the empty template (F). The result is a recombinant minus strand.

that have slipped through the polymerase without initiating minus-strand synthesis (Fig. 4D). These should be rare events. It is also possible that the recipient templates are molecules that inadvertently lost their 3′ ends due to the action of cellular nucleases (Mindich *et al.,* 1994; Onodera *et al.,* 1993).

The experimental approaches for studying recombination in Φ6 were primarily oriented toward studying heterologous recombination (Mindich, 1996). In a few cases, attempts were made to test for homologous recombination. In one case, we inserted a limited sequence from segment S into the *lacα* gene located in segment M and looked for an increase in launching from segment S to rescue this M segment if the 3′ end was missing. The sequence identity was only 27 nucleotides and no effect was seen. According to the results that we report in this paper, no effect would be expected. Recombination in Φ6 takes place during minus-strand synthesis and does not show particular preferences in terms of launching points from the original template or landing points in the second one as long as there is no selection against deletions (Qiao *et al.,* 1997). When the nascent chain and the template share about 400 nucleotides of identity the chances for a homologous crossover may simply improve. If the annealing involves such a large amount of sequence, it does not necessarily imply that there is this much free nascent chain; one could imagine that after initial pairing the nascent chain unwinds from the original template and winds onto the new template in a manner similar to that seen in the movement of crossover junctions in strand invasion models of DNA recombination (Kowalczykowski *et al.,* 1994).

It was somewhat surprising to find that plasmid transcripts missing *pac* sequences could be acquired by Φ8. Packaging in Φ6 is very stringent; however, there is accumulating evidence that transcripts without *pac* sequences can be acquired by Φ6 at very low frequencies. The *pac* sequences in Φ6 are about 200 nucleotides in length (Gottlieb *et al.,* 1994) and extend to about nucleotide 300. Preliminary studies in our laboratory indicate that the Φ8 *pac* sequences are shorter and in the case of S and L extend only to about nucleotide 140.

Recombination in retroviruses is both homologous and heterologous, with homologous recombination being more frequent (Zhang and Temin, 1994). The polymerase in this case is reverse transcriptase and template switching is a normal part of retrovirus replication. The choice between homologous and heterologous recombination is influenced by the length of sequence identity at the crossover junction; however, sequence identities of as little as 40 nucleotides made significant differences in the choice between the two forms of recombination (Zhang and Temin, 1994).

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids

LM2489 is a rough derivative of *P. syringae pv. phaseolicola* HB10Y (Vidaver *et al.,* 1973) and was used as the primary host for plating Φ8 and Φ6. LM2509 is a deriv-

TABLE 1

Plasmids Used in This Study

Plasmid	Characteristics
pLM2446	Φ8 M from *Xho*I at 91 to 3′ end with *pac* sequence of Φ6 M from 5′ to *Xho*I at 307
pLM2504	Φ8 M from *Bam*HI at 215 to 3′ end
pLM2572	Φ8 M from *Bam*HI at 215 to *Sfi*I site at 4580
pLM2599	Φ8 M with *Nco*I deletion and insertion of *kan* in *Sfi* site at 4580
pLM2764	Φ8 M with genes F and G replaced by *lac*α
pLM2793	*Mun*I at 3123 to 3′ end; no *pac* seq
pLM2797	*Nhe*I at 3395 to 3′ end; no *pac* seq
pLM2799	*Nhe*I at 3570 to 3′ end; no *pac* seq
pLM2801	*Nhe*I at 3810 to 3′ end; no *pac* seq
pLM2823	*Nhe*I at 3570 to 3′ end; *pac* seq of M
pLM2830	*Nhe*I at 3570 to 3′ end; *pac* seq of S

ative of LM2489 that lacks pili and is resistant to Φ6, but sensitive to Φ8.

Plasmid pLM1454 is a derivative of the cloning vector pT7T3 19U (Pharmacia). It was used for the cloning of cDNA copies of phage RNA produced by RT-PCR. Plasmids used in this study are listed in Table 1. They are all derivatives of plasmid pLM350, which is a derivative of pLM254, which is a shuttle vector that replicates in *E. coli* and pseudomonads (Mindich *et al.,* 1985). Details of the construction of the plasmids are available from the authors.

Media

The media used were LC and M8 (Sinclair *et al.,* 1976). Ampicillin plates contained 200 μg of ampicillin per milliliter in LC agar.

Enzymes and chemicals

All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, T4 polynucleotide kinase, Klenow enzyme, and exonuclease BAL-31 were purchased from Promega, New England Biolabs, and Boehringer Mannheim.

Acquisition of transcripts

We have taken advantage of a special property of Φ8 for the preparation of the various phage constructions. The virus is able to acquire plasmid transcripts from infected cells and to incorporate these into its genome as substitutions for defective genomic segments. The acquisition results in genomic segments with their proper 5′ and 3′ sequences as long as these are in the transcript, even though there might be extra sequence in the transcript as it is synthesized (Mindich, 1999b). Plasmid transcripts can be acquired as new genomic segments or they can recombine with existing segments to form novel structures (Onodera *et al.,* 1995).

The M segment of the Φ8 recombination tester phage, 21, has the structure described in Fig. 1. It has a deletion of genes F and G, which are replaced by the sequence of *lac*α. Phage 21 was plated on a lawn of LM2489 carrying plasmids listed in Table 1. These plasmids were able to complement the deletion, and confluent lysis of the lawns resulted. The soft agar from these plates was collected and centrifuged. The titers of the resulting virus preparations were determined on complementing and noncomplementing host cells. Plaques that appear on plates with noncomplementing hosts must be recombinants since the plasmid transcripts do not contain the complete sequence of segment M. Plaques were picked from the noncomplementing plates and high-titer stocks were prepared. RNA was extracted from these stocks and subjected to gel analysis to determine whether the sizes of the M genomic segments were normal. In the cases in which homologous recombination was suspected, the M segments were isolated and subjected to RT-PCR, cloned, and sequenced at the NYU Skirball Institute sequencing facility.

RT-PCR

dsRNA was isolated from crude phage preparations by first treatment with DNase, then phenol extraction, and electroelution of the RNA from agarose gels. The RNA was then denatured and annealed to primer and incubated with AMV reverse transcriptase. The products were then subjected to PCR with *Pwo* DNA polymerase (Boehringer Mannheim) (Mindich *et al.,* 1999). The resulting DNA products were cloned into pT7T319U and sequenced.

ACKNOWLEDGMENTS

We greatly appreciate discussion with the members of the Bamford and Stuart laboratories about the structure of the Φ6 polymerase prior to publication. This work was supported by Grant GM31709 from the National Institutes of Health.

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