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RNA Structure and Heterologous Recombination in the Double-Stranded RNA Bacteriophage φ6

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Bacteriophage φ6 has a genome of three segments of double-stranded RNA, designated L, M, and S. A 1.2-kbp kanamycin resistance gene was inserted into segment M but was shown to be genetically unstable because of a high recombination rate between segment M and the 3′ ends of segments S and L. The high rate of recombination is due to complementary homopolymer tracts bounding the *kan* gene. Removal of one arm of this potential hairpin stabilizes the insertion. The insertion of a 241- or 427-bp *lacZ′* gene into segment M leads to a stable Lac$^+$ phage. The insertion of the same genes bounded by complementary homopolymer arms leads to recombinational instability. A stable derivative of this phage was shown to have lost one of the homopolymer arms. Several other conditions foster recombination. The truncation of a genomic segment at the 3′ end prevents replication, but such a damaged molecule can be rescued by recombination. Similarly, insertion of the entire 3-kb *lacZ* gene prevents normal formation of virus, but the viral genes can be rescued by recombination. It appears that conditions leading to the retardation or absence of replication of a particular genomic segment facilitate recombinational rescue.

Bacteriophage φ6 infects the plant pathogen *Pseudomonas phaseolicola* (*P. syringae* pv. phaseolicola) HB10Y (HB10Y) (37). It has a genome of three different pieces of double-stranded RNA (dsRNA) packaged within a polyhedral nucleocapsid (NC) (34). The NC contains a procapsid that has RNA polymerase activity (30).

We have developed an in vitro genomic packaging system that utilizes single-stranded RNA (ssRNA) obtained from the in vitro transcription of viral NCs or RNA transcripts of plasmids carrying cDNA copies of the viral genomic segments. The RNA is packaged by procapsids isolated from *Escherichia coli* cultures that are expressing the cloned genes coding for the four proteins of the φ6 procapsids. These filled particles are capable of infecting spheroplasts. We have previously demonstrated that a transcript of segment M that contains an insertion of the gene for kanamycin resistance can be incorporated into the genome of viable phage (29). Virus that contains the kanamycin gene is genetically unstable, and virus preparations contain many particles that have lost part of the modified M segment (29). This loss was shown to be the result of heterologous recombination between the genomic segments, resulting in the replacement of the 3′ end of segment M with that of either segment S or segment L (25). In the present report, we describe an investigation of some of the factors that promote heterologous recombination in φ6. Heterologous recombination has been found in several ssRNA viruses, some with unit genomes and some with multipartite genomes (18, 31), mostly with positive-strand viruses but with negative-strand viruses as well (4). 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. Several viruses isolated in nature appear to have been formed by heterologous recombination.

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MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. *P. phaseolicola* HB10Y (HB10Y) (37) was used as the host for φ6 lysate preparations and for phage titration (Table 1). *E. coli* JM109 (39) was used for the propagation of recombinant plasmids.

Construction of plasmids. Plasmids derived from pT3T7 19U (Table 2) were used to prepare templates for the production of ssRNA packaging substrates for the formation of φ6 virus containing modified genomic segments (27). Plasmid pLM672 was the original construction that had the *kan* gene of pUC4K in a cDNA copy of genomic segment M (29). The *kan* gene is bounded by a *Pst*I site on each side as well as 12 G's on the 5′ side and 12 C's on the 3′ side. This cartridge was inserted into the *Pst*I site in the 3′ noncoding region of segment M (Fig. 1).

Plasmids pLM778 and pLM779 were prepared by partial cutting of plasmid pLM672 with *Pst*I and by digesting with exonuclease BAL 31. The resulting digests were ligated and tested for kanamycin resistance in *E. coli* JM109. Plasmids from resistant colonies were sequenced, and pLM778 and pLM779 were found to have deletions of 53 and 131 bases, respectively, at the 3′ ends of the *kan* gene. pLM778 is missing 10 bases of the *kan* insert, and pLM779 is missing only the homopolymer arm. In neither case was the 75-base terminal replication region (25) impaired.

Polymerase chain reaction (PCR) was used to prepare fragments of the *E. coli lacZ* gene bounded by *Eco*RV sites. *lacG* was synthesized on the template of pUC8 with primers OLM81 and OLM82 (Table 3). It contains the multiple cloning sites (MCS) of pUC8 but is trimmed to have only 241 nucleotides when it is cut with *Eco*RV. The *lacZ* fragment has 60 amino acids. *lacH* was synthesized on the template of pMC9 (21) with primers OLM81 and OLM102 (Table 3). The cartridge contains 427 nucleotides when it is cut with *Eco*RV. The *lacH* fragment has 133 amino acids. Although both cartridges are missing part of the *lac* operator, they are controlled by the *lac* repressor in plasmids that do not have an additional *lac* operator.

TABLE 1. Bacterial strains, phages, and plasmids

Bacterium or phage	Description	Reference or source
Bacteria		
P. phaseolicola HB10Y	Host of ϕ6	37
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) [F' *traD36 proAB lacI*^{q}ZΔ*m15*]	J. Messing
Phages		
ϕ6	Wild-type phage	37
ϕ6K1	ϕ6 containing *kan* in segment M	29
ϕ1767	Recombinant of M with 3′ end of S resulting from transfection with transcript of pLM655 truncated at *Pst*I site	This study
ϕ1789	Recombinant of M with 3′ end of S resulting from transfection with transcript of pLM780 which contains entire *lacZ* in the *Pst*I site	This study
ϕ1791	Recombinant of M with 3′ end of S resulting from transfection with transcript of pLM780 which contains entire *lacZ* in the *Pst*I site	This study
ϕ1797	Recombinant of M with 3′ end of L resulting from transfection with transcript of pLM656 truncated at the *Pst*I site	This study
ϕ1798	Recombinant of S with 3′ end of L resulting from transfection with transcript of pLM658 truncated at the *Cla*I site	This study
ϕ1800	Transfectant containing transcript of pLM789; *lacG* in M with homopolymer arms; 18 G's and 27 C's; unstable	This study
ϕ1807	Mutant of ϕ1800 that is stable and missing sequence from nucleotide 3498 to position −2 of the *lac* open reading frame	This study
ϕ1817	Transfectant containing transcript of pLM847; *lacH* in M with *Eco*RV cut ligated to blunted *Pst*I site; stable	This study
ϕ1819	Transfectant containing transcript of pLM844; *lacH* in M with homopolymer arms; 21 G's and 14 C's; unstable	This study

Plasmids pLM763 and pLM765 were prepared by inserting the *Eco*RV-cut PCR product of *lacG* into either the *Cla*I site of pLM759, which contains the cDNA copy of segment S (see Fig. 1) and is missing the vector *Cla*I site, or into the *Pst*I site of pLM656, which contains the cDNA copy of segment M. In both cases, the sites were blunted by treatment with T4 DNA polymerase (27).

Plasmid pLM780 was constructed by inserting the 3-kbp *Pst*I cartridge of *lacZ* from pMC1871 (7) into the *Pst*I site of pLM765 and selecting for a Lac$^+$ frameshift mutation.

Plasmid pLM789 was constructed by terminal deoxynucleotidyltransferase tailing of *lacG* with poly(dC) and annealing this material to plasmid pLM656 which was cut with *Pst*I and tailed with poly(dG) (32). After transformation of JM109, Lac$^+$ colonies were picked, and plasmids were screened by sequencing for homopolymer arms of various lengths. Plasmid pLM789 has *lacG* in its *Pst*I site bounded by 18 G's at the 5′ side and 27 C's at the 3′ side.

The derivatives of ϕ6 containing *lacG* formed fluorescent plaques when plated on a lawn of HB10Y carrying plasmid pLM746 (LM1034) in a medium containing 4-methylumbelliferyl-β-D-galactoside (MUG). Phages carrying *lacH* produced plaques with about 10 times the fluorescence of those carrying *lacG* and also resulted in blue plaques on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Fig. 2). Plasmid pLM746 is a derivative of

TABLE 2. Plasmids

Plasmid	Description	Reference or source
pGHS1	*Lactococcus* plasmid containing *E. coli lacZΔm15*	15
pT7T3 19U	*amp* P_{T7} P_{T3} *lacZ'*	Pharmacia
pUC-4K	*amp lacZ' kan* cartridge in *Pst*I site	38
pMC1871	Cartridge with most of *lacZ*	7
pLM254	*amp lacZ'* with pUC8 MCS; vector for pseudomonads	23
pLM655	*amp* P_{T7}; exact copy of segment M in pT7T3; 19 U's	29
pLM656	*amp* P_{T7}; exact copy of segment M in pT7T3; 19 U's	29
pLM658	*amp* P_{T7}; exact copy of segment S	12
pLM672	*amp* P_{T7}; *kan* gene in *Pst*I site of pLM656	29
pLM746	Insert of *lac*Ω in pLM254; for *lac* complementation	This study
pLM759	*amp* P_{T7}; exact copy of segment S; vector lacks *Cla*I site	This study
pLM763	*amp* P_{T7}; *lacG* in *Cla*I site of S; no homopolymer arms	This study
pLM765	*amp* P_{T7}; *lacG* in *Pst*I site of M; no homopolymer arms	This study
pLM778	pLM672 with 53-base deletion of 3′ homopolymer arm	This study
pLM779	pLM672 with 131-base deletion of 3′ homopolymer arm	This study
pLM780	*amp* P_{T7}; complete *lacZ* gene in *Pst*I site of M	This study
pLM789	*amp* P_{T7}; *lacG* in *Pst*I site of M with 18 G's on 5′ side and 27 C's on 3′ side	This study
pLM844	*amp* P_{T7}; *lacH* in *Pst*I site of M with 21 G's on 5′ side and 14 C's on 3′ side	This study
pLM847	*amp* P_{T7}; *lacH* in *Pst*I site of M; no homopolymer arms	This study

TABLE 3. Oligonucleotides for cloning and sequencing

Oligonucleotide name	Sequence	Description
OLM60	GATCTGCTGTCTCAACAGATC	Forward from 2847 in M (before *Eco*RV)
OLM62	CCCTCTAGAGAGAGAGCCCCCGAAG	Complementary to 3′ termini with *Xba*I site
OLM68	GCGAACTACGAAGGTAGAACG	Forward from 2400 in S
OLM70	GAAAGCCCGGCATCGATGTCGTTTGCA	S sequence from 2673 to 2694
OLM71	AACGACATCGATGCCGGGCTTTCTGCA	Complement of OLM70
OLM81	GAGCGGATATCAATTTCACACAGG	PCR primer for making *lacZ′* constructions; contains an *Eco*RV site and starts in *lac* operator
OLM82	CCCGATATCAGGCGCCATTCGCCATTCAGG	PCR primer for 3′ end of *lacG*; contains an *Eco*RV site and ends at same place as pUC8 *lacZ′*
OLM102	CCCGATATCAGCTTTCATCAACATTAAATGTGAGCGAG	PCR primer for 3′ end of *lacH*; contains an *Eco*RV site and ends at 133 amino acids of *lacZ*
M13	GTAAAACGACGGCCAGT	For sequencing pT7T3 19 U
RevM13	CAGGAAACAGCTATGAC	For sequencing pT7T3 19 U

shuttle vector pLM254 (23), into which we have inserted part of the *lacZΔm15* gene of plasmid pGSH1 (15) and part of the *lacZ* gene of plasmid pMC1871 (7). The plasmid has the wide host range of RSF1010 (1), the *lac* promoter *up* mutation of pLM254 (23), and the *lacZΔm15* gene, with the result that it is a general *lac* complementation plasmid for many gram-negative species (38).

In vitro synthesis of positive-sense transcripts by T7 polymerase. Plasmids were cut with endonuclease *Xba*I, and the resulting 5′ overhang was removed with mung bean nuclease before transcription with T7 RNA polymerase (27). Truncated segments were produced by cutting the plasmids with endonuclease *Cla*I or *Pst*I before the transcription reaction (Fig. 1). RNA was purified by extracting once with phenol: chloroform:isoamyl alcohol and then with chloroform: isoamyl alcohol.

Plasmid pLM931 contains a 27-base insert in the *Pst*I site of the cDNA copy of segment M, 5′ to a *lacH* insert. The 27-base insert is identical to the sequence around the *Cla*I site near the 3′ end of segment S. pLM844 contains the cDNA copy of segment M with *lacH* inserted in the *Pst*I site with homopolymer tails of 21 G's on the 5′ side and 14 C's on the 3′ side. The plasmid preparation was partially cut with *Pst*I and religated after blunting with T4 DNA polymerase to remove the 3′ *Pst*I site. The resulting plasmid, pLM927, was cut with *Pst*I and ligated in the presence of oligonucleotides OLM70 and OLM71. Plasmid pLM931 was found to contain the insert in the correct orientation. The orientation was confirmed by sequencing.

Preparation of ϕ6 ssRNA. ϕ6 ssRNA was synthesized by an in vitro transcription reaction essentially according to the method described by Emori et al. (9) with NCs prepared as described by Mindich et al. (23). The resulting mixture of ssRNA and dsRNA was fractionated on a cellulose column with elution buffers containing decreasing concentrations of ethanol according to the method described by Franklin (11). The ssRNA eluted at 20% ethanol was concentrated by ethanol precipitation and dissolved in water.

Preparation of procapsids. ϕ6 procapsids were prepared as described by Gottlieb et al. (13), with the expression plasmid pLM450 (14) propagated in *E. coli* JM109. The procapsid preparation that was used contained 400 μg of protein per ml as determined by the Bradford assay (5). Purified procapsids were divided in aliquots and frozen at −70°C. Aliquots were thawed just prior use.

Polymerase reaction conditions. The ϕ6 RNA polymerase reactions contained 50 mM Tris-Cl, pH 8.2, 3 mM $MgCl_2$, 100 mM NH_4O acetate, 20 mM NaCl, 5 mM KCl, 5 mM dithiothreitol, 0.1 mM Na_2 EDTA, 1 mM (each) ATP, GTP, CTP, and UTP, 5% polyethylene glycol 4000, and 40 mg of Macaloid per ml (10). To each 25-μl reaction mixture, 500 to 750 ng of ϕ6 ssRNA and 1.4 μg of procapsid protein were added. In the experiments in which cDNA-derived M segment RNA was used, the ϕ6 ssRNA was derived from mutant sus507 (3), which has an amber mutation in gene 6 of segment M. When cDNA-derived S segment RNA was used, RNA from sus297, which has an amber mutation in gene 12 of segment S, was used (22). The amount of the synthetic transcript was 5 to 10 μg. The reactions were routinely run at 28°C for 75 min.

Assembly of coat protein. Purified protein P8 was assembled onto the packaged procapsids in 31.5-μl mixtures containing 10 μl of the specific polymerase reaction mixtures and a standard amount of 2.2 μg of purified P8 (28). The final composition of the assembly mixture chosen (including the components carried over from the polymerase reaction and from the P8 preparation) was 15.9 mM Tris-Cl (pH 8.2), 0.95 mM $MgCl_2$, 0.21 mM Na_2EDTA, 6.3 mM NaCl, 97 mM KCl, 95 mM NH_4Cl, 1.6 mM dithiothreitol, 7.0 mM potassium phosphate (pH 7.4), 31.7 mM NH_4 acetate, 0.32 mM (each) of ATP, GTP, CTP, and UTP, 1.6% polyethylene glycol 4000, and 0.71 mM $CaCl_2$. The reaction mixture was incubated for 1 h at 24°C.

The NC infection. HB10Y cells were rendered competent for ϕ6 NC infection as described previously (26). To each 120-μl aliquot of cells, 20 μl of each coat protein assembly mixture was added, and the infection was allowed to proceed in 35-μl droplets on Millipore VSWP02500 filters on Luria-Bertani (LB) plates overlaid with LB top agar–3% lactose–20 mM potassium phosphate, pH 7.2, at room temperature for 60 min. Thereafter, the cells were washed once with 1 ml of the LKSB buffer (26) and resuspended in 100 μl of the same buffer, and infectious center titers were determined on HB10Y lawns. Control transfections with ssRNA from wild-type ϕ6 gave several thousand plaques, RNA from amber mutants gave few or no plaques, and mixtures of RNA from amber mutants and T7 polymerase transcripts of cDNA resulted in few to several hundred plaques, depending on the construction and the preparation.

Isolation of recombinational derivatives. Bacteriophage ϕ6K1, which contains the *kan* gene in segment M, was plated on a lawn of HB10Y. Most plaques were small and turbid. These contained the *kan* insert. Large clear plaques were found to contain recombinants. High-titer stocks were prepared by using HB10Y and the sloppy agar method (35). These preparations were extracted with phenol and analyzed

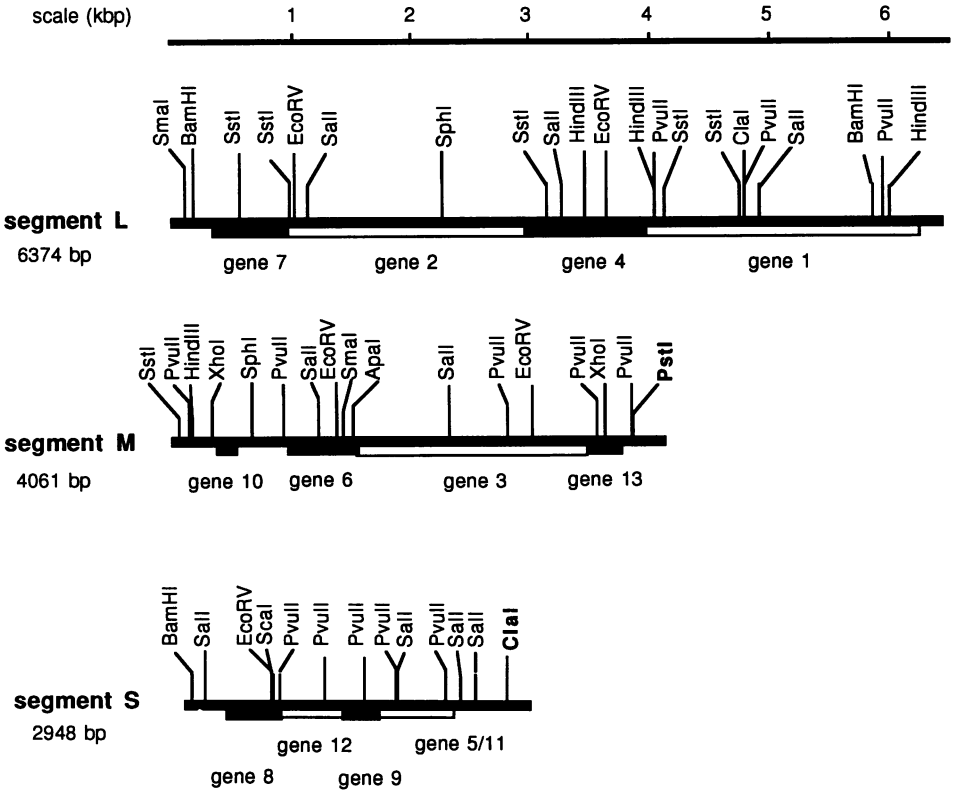

FIG. 1. Restriction maps of the cDNA copies of the three genomic segments of ϕ6. The *Pst*I site in M and the *Cla*I site in S that were used for gene insertions are shown in boldface type.

for the size of segment M by agarose gel electrophoresis (29). Virtually all such isolates showed unique migration patterns for segment M. Several preparations of interest were used to prepare large-volume lysates (8), which were used for the preparation of ssRNA.

Phages carrying the *kan* insert with only one homopolymer arm formed plaques of normal size. Plaques of all sizes were picked, and phage stocks were prepared as described above. All contained *kan* inserts.

Phages carrying *lac* inserts were screened by plating on LM1034 in the presence of MUG (50 μg/ml) or X-Gal (200 μg/ml). Nonfluorescent or white plaques were picked, and stocks were prepared.

PCR amplification of cDNA. Two methods were used for PCR amplification. In the first, in vitro transcripts of phages were incubated with oligonucleotide primer OLM62 (Table 3) and avian myeloblastosis virus reverse transcriptase (24). 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 PCR with oligonucleotide primer OLM60 (Table 3) along with OLM62. 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 ϕ1798 transcripts were amplified with OLM62 and OLM68, and the product was cloned into pT7T3 19U which was cut with *Sal*I and *Xba*I.

In the second method, a small preparation of phage containing about 10^{11} to 10^{12} PFU was extracted with phenol, the RNA was precipitated with ethanol, dissolved, and electrophoresed in 1% agarose, and the bands were electroeluted, precipitated, and dissolved in water. The RNA was then used as template for cDNA synthesis with either avian myeloblastosis virus reverse transcriptase or the Pharmacia kit with cloned murine leukemia virus reverse transcriptase. The resulting DNA was cut and ligated to pT7T3 19U.

DNA sequence analysis. Sequence analysis was performed by the dideoxy chain termination method (33) with bacteriophage T7 DNA polymerase (Sequenase; U.S. Biochemicals). The synthetic deoxyoligonucleotides used as primers were the standard M13 sequencing primer and the reverse M13 sequencing primer (Table 3). In all cases, the size of the recombination product as calculated for the sequence at the crossover site agreed with the size of the genomic segment as determined by gel electrophoresis.

RESULTS

Stability of *kan* in segment M. We have prepared a derivative of bacteriophage ϕ6, designated ϕ6K1, in which a gene for resistance to kanamycin has been inserted near the 3′ end of genomic segment M (Fig. 1) (29). This was accomplished by inserting a kanamycin resistance gene cartridge of pUC-4K (Table 2) into the *Pst*I site of a cDNA copy of the genomic segment that had been cloned into a T7 promoter vector called pT7T3 19U (Table 2). The cartridge is 1.2 kbp and is bounded by 12 G's and a *Pst*I site on the 5′ side and 12 C's and a *Pst*I site on the 3′ side. A transcript of the

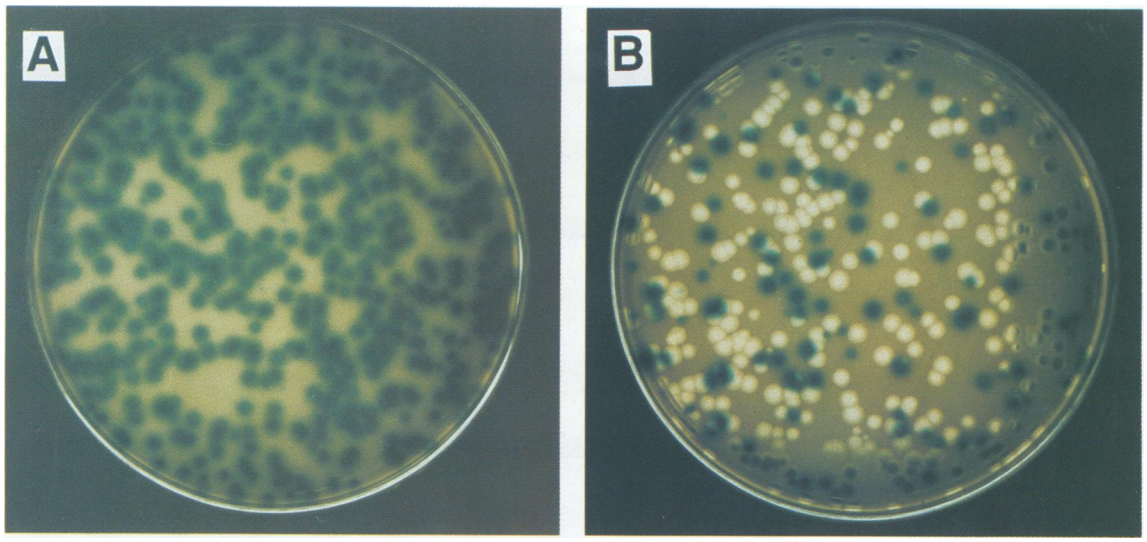

FIG. 2. Plaques of ϕ1817, which has *lacH* stably inserted into the *Pst*I site of segment M, on LM1034 in LB agar with X-Gal (A); plaques of ϕ1819, which is genetically unstable due to homopolymer arms at the position of the *lacH* insertion (B).

resulting plasmid, pLM672, was packaged along with purified ssRNA segments *s* and *l* into procapsids which were then coated with protein P8 and introduced into spheroplasts of the host bacterium. The resulting bacteriophage, ϕ6K1, formed small turbid plaques. Large clear plaques were observed at a frequency of 0.1 to 10%. Whereas ϕ6K1 contained normal-size segments S and L and a larger-than-normal segment M, the clear plaque forms had M segments with sizes varying from a few hundred base pairs larger than normal to several hundred base pairs smaller than normal. These M segments were shown to be the products of heterologous recombination in which the 3′ end of M was replaced by the 3′ end of segment S or L (25). Although the plaques formed by the phages containing the *kan* insert were smaller than normal, the phage yield was at normal levels, indicating that the high proportion of recombinants was not due to the low productivity of the insert-bearing phage.

To test the possibility that the instability of the *kan* gene was due to the homopolymer arms that bounded it, we prepared plasmids in which one of the homopolymer arms was removed by treatment with exonuclease BAL 31. Two plasmids (pLM778 and pLM779) were constructed. They had deletions at the 3′ ends of the *kan* gene that included a few bases in the *kan* insert and either 34 or 123 bases in segment M. Transcripts were prepared, packaged, and used to transfect HB10Y. Plaques were isolated, phage were prepared, and RNA was analyzed and shown to contain the *kan* gene. Whereas transcripts of the parent plasmid pLM672 resulted in genetically unstable phage, both of these plasmids resulted in phage that were completely stable. Whereas ϕ6K1 produced mainly small turbid plaques that contained the *kan* insert in segment M and large clear plaques that had lost the insert, the plaques formed by phages that had lost one homopolymer arm were larger and clearer than the ϕ6K1 plaques, and phage in all of the plaques, whether they were large or small, contained the *kan* insert.

Stability of *lac* in segment M. We constructed a Lac complementation system for pseudomonads by preparing a wide host range plasmid containing the ω fragment of the *lacZ* gene. This plasmid was designated pLM746 (Table 2). We then prepared plasmids with cDNA copies of segment M containing inserts of the α portion of the *lacZ* gene. One insert, which is derived from the pUC8 (38) MCS, is designated *lacG*. It is 241 bases long. Another insert, designated *lacH*, is 427 bases long. Transcripts from these plasmids were used in transfection experiments to produce ϕ6 containing parts of *lacZ*. Phage with *lacH* produced blue plaques on HB10Y containing pLM746 in the presence of X-Gal (Fig. 2A). Phage with *lacG* formed plaques that were fluorescent with MUG but were not blue with X-Gal. The level of β-galactosidase activity in HB10Y is dependent on the length of the α fragment. *lacG* has 60 amino acids, while *lacH* has 133 amino acids.

Phages with either of the two *lac* inserts were genetically stable. White or nonfluorescent plaques appeared with a frequency of about 0.1%. These plaques were found to contain phages with mutations to Lac^- rather than deletions of the *lac* insert. Plasmids were then prepared with inserts of *lacG* bounded by arms of poly(G) and poly(C). Phage produced by these transcripts were found to be genetically unstable. The instability increased according to the length of the homopolymer arms. Phage derived from the transcript of pLM789, which contained *lacG* bounded by 18 G's and 27 C's in addition to the *Pst*I sites, were so unstable that all fluorescent plaques contained mixed populations and could not be purified because progeny fluorescent plaques contained over 50% recombinants. A similar result was obtained with phages containing the *lacH* insert (Fig. 2B), in which homopolymer arms of 21 G's and 14 C's resulted in the same degree of instability. In general, the large Lac^- plaques had lost the *lac* insert, and analysis of the RNA by gel electrophoresis showed that the size of segment M was altered (Fig. 3). In all cases, the alteration was due to recombination. The phages that contained inserts bounded by homopolymer arms were able to reproduce well enough to yield stocks that had normal titers. The high proportion of recombinants was not due to the relative efficiencies of production between the insert-bearing strains and the recombinants, although the recombinants would eventually overwhelm the parental phages if stocks were propagated without plaque purification.

Occasionally, a large Lac^+ plaque formed by the unstable

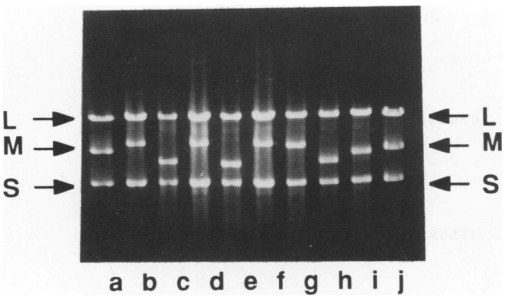

FIG. 3. Agarose gel electrophoresis of dsRNA extracted from phage samples derived from phage ϕ1800 that contains *lacG* bounded by homopolymer arms. RNAs in lanes a and j are from normal ϕ6; lane b, d, and f RNAs derive from Lac$^+$ plaques, whereas lane c, e, g, h, and i RNAs derive from Lac$^-$ plaques. Note that the Lac$^+$ phage have M segments with the same size (4,340 kbp), while the M segments of the recombinants vary in size.

phage was found to be stable. One of these, ϕ1807, was used as template for cDNA synthesis, PCR, cloning, and sequencing. It was found that a deletion had been formed at the 5′ end of the *lac* insert that had removed one of the homopolymer arms. In this case, there was no evidence of heterologous recombination but rather an internal deletion from position 3501 in segment M (which is after the Shine-Dalgarno sequence for gene 13) and the −2 position of the *lacG* open reading frame. This results in an intact open reading frame with an adequately placed Shine-Dalgarno sequence.

Therefore, it appeared that the homopolymer arms contributed to heterologous recombination and that inserts of 241, 427, and 1,200 bases without homopolymer arms were stable in the *Pst*I site of segment M. We then prepared transcripts containing the entire *lacZ* gene in the same site. Transfection experiments yielded very few plaques. The yield was reduced by about 100-fold relative to that for normal segment M. The plaques that were obtained were not Lac$^+$ on HB10Y, but some were Lac$^+$ on LM1034 (HB10Y with pLM746). RNA prepared from the phages showed that they were recombinants with sizes of segment M ranging from less than normal to several kilobases larger than normal (Fig. 4). Sequencing of several recombinants confirmed that they were the products of heterologous recombination (Fig. 5). It appears that the 3-kb size of the entire *lacZ* gene is too big to add to the 4 kb in segment M, but recombination does rescue a small percentage of the M segments.

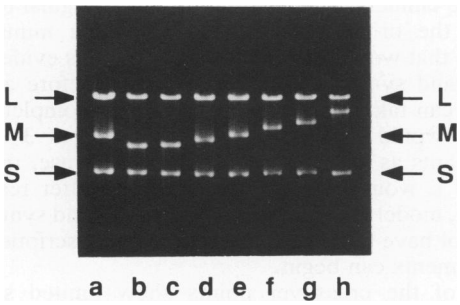

FIG. 4. Agarose gel electrophoresis of dsRNA extracted from phage samples derived from plaques produced by transfection with RNA derived from pLM780, which contains the entire *lacZ* gene in segment M. RNA in lane a is from normal ϕ6. RNAs in lanes b, c, d, e, f, g, and h are from recombinant plaques. None are Lac$^+$ alone; however, the phages used for lane g were Lac$^+$ on lawns of LM1034. The sequence of the crossover junction is shown in Fig. 5 (1791).

The effect of 3′ truncation. We have shown that the 5′ end of each genomic segment carries the information for packaging specificity and that segments that are missing their 3′ ends can be packaged but do not serve as templates for minus-strand synthesis (12). Transcripts of S copies that had been truncated at the *Cla*I site or M copies that had been truncated at the *Pst*I site were prepared (Fig. 1). The transcripts of the truncated segments were packaged with normal heterologous segments and used to transfect host cells. Plaques were obtained at a small percentage of the frequency found with normal transcripts. Phage stocks were prepared, and RNA was analyzed by gel electrophoresis. It was found that the plaques were all recombinants. Sequencing of cDNA copies of the RNA showed that they were all products of heterologous recombination. The crossover points were usually 5′ to the truncation site, although in one case (Fig. 5) the crossover point was at the presumptive end formed by the *Cla*I cut of the copy of segment S.

The effect of sequence identity on recombination. Although we find that in about half of the cases, there are only one or two overlapping bases at the crossover point, in the other half we find six or more identical bases. This suggests that the heterologous recombination system does in fact prefer regions with similar sequences. A test was devised to see whether sequence identity would stimulate recombination. We prepared a 27-nucleotide insert that is identical to the sequence around the *Cla*I site of segment S (Table 3). This insert was placed 5′ to a *lacH* insert bounded by homopolymer arms (21 G's and 14 C's) at the *Pst*I site of segment M in pLM844. The new plasmid was called pLM931. We knew that the *lac* construction is extremely unstable, and we expected that recombinants whose crossover sites utilized the identical sequences would all have a particular size. The phage containing *lac* and the duplication of the *Cla*I site was unstable, and white plaques appeared at a frequency of more than 50% in passaged small blue plaques. Small blue plaques were picked and replated. Eighteen white-plaque derivatives were picked, phage stocks were prepared, and RNA was analyzed by electrophoresis. It was found that most of the recombinant phages showed segment M sizes incompatible with the sizes expected for crossovers at the identical sequence. Several preparations looked as if they might be products of such an event on the basis of probing with oligonucleotides. However, sequence analysis showed that none of the crossovers occurred in the 27-base sequence. The absence of crossovers in the 27-nucleotide identity region suggests that the initiation of the crossover is not determined by sequence matching; instead, it appears that the matching influences the location of the crossover on the receptor strand.

DISCUSSION

Homologous recombination in several positive-strand RNA viruses, particularly in poliovirus (16, 17) and coronavirus (18, 19), has been demonstrated and studied. In addition, the products of heterologous recombination have been observed in virus isolates from the wild (see reference 18) or in experiments in which defective segments of segmented genome virus could be rescued (6). Although we have not explored the mechanism of the recombinational event, our results are consistent with the currently held copy choice model (17).

FIG. 5. Base sequences of recombination products around the crossover regions of representative recombinants. The sequence of the original segment that donates the 5′ end is underlined, and that of the original L or S segment that donates the 3′ end is shown below. The extent of sequence identity at the crossover point is indicated by the dark horizontal lines. Phages ϕ1767 and ϕ1797 were the products of transfections with truncated M segments. ϕ1798 was the product of a truncated S segment. ϕ1791 and ϕ1789 were the products of transfections with the entire *lacZ* gene inserted into the *Pst*I site of segment M. ϕ1791 has a crossover in the *lacZ* gene, and enough of the gene remains so that this phage is Lac^+ on ω-complementing strains. ϕ1789 has a crossover within the M sequence and has therefore lost the entire *lac* sequence.

The experiments that we have described in the present report show that the frequency of recombinants in a population of ϕ6 can be increased more than 1,000-fold by the incorporation of a sequence bounded by homopolymer G-C arms. Using the *lac* screening system, we find that the normal recombinant frequency is less than 1 in 1,000. We do not know whether the increase in recombination is due to the formation of a hairpin or to some other consequence of the homopolymer tracts; however, it seems likely that hairpins would be formed. The hairpins could promote recombination by providing a barrier to synthesis of minus-strand RNA, or they might prevent plus-strand genomic precursors from being completely packaged. The normal 3′ termini of the segments contain double-stranded structures (25), but they are probably much smaller than those formed by the homopolymer tracts.

Although it is difficult to precisely quantitate the relative frequencies of transfection, the level of rescue recombination that we find for truncated genomic precursors is about 1 to 10% of the expected number of plaques with intact RNA. This would imply that recombination is being facilitated in these cases, since we found that the frequency of recombinants was less than 1 per 1,000 in the absence structures that promote recombination. It is worth emphasizing that the recombinational rescue events that we report here take place during the first round of replication of the virus. The frequency is not amplified by selection during successive rounds of viral replication.

We have shown in other studies that the synthesis of minus strands does not take place until all three plus strands have been packaged by a procapsid (12). However, synthesis of minus strands on intact templates takes place even if the other packaged molecules are truncated and cannot replicate. This regulatory program might serve to coordinate the replication of the genomic segments so that their numbers remain equal; however, it may also serve to set up conditions that would favor recombinational rescue of genomic segments that are packaged with damaged 3′ ends.

The finding that the truncated genomic precursors can be rescued by recombination implies that the recombination is taking place during the synthesis of minus-strand RNA, since in the absence of minus-strand synthesis there is no transcription. Additionally, we have shown that the truncated segments do not support minus-strand synthesis (12). If the recombination is taking place during minus-strand synthesis, it is not clear how the recombinational complexes are resolved. Figure 6 shows the recombinant minus strand binding to both the donor and the recipient plus strands. Two possibilities are (Fig. 6, pathway A) that the chimeric minus strand leaves its original 3′ template spontaneously and (Fig. 6, pathway 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. There is evidence that minus-strand synthesis must occur on L before any transcription can take place (12). If it must be completed, then model B would not stand, since L donates 3′ ends to recombinants as well as S does. In such a case, the minus strand of L would not be complete until after resolution. However, model B would stand if minus-strand synthesis on L does not have to be completed before transcription of the other segments can begin.

Many of the crossover points show limited sequence identity between the two parental strands. This region is often 6 bases or more in length (Fig. 5). When we placed an insert of 27 bases of identity 5′ to the destabilizing region, we found no evidence of preferred crossing over in this region. Our tentative conclusion is that the launching region is determined by some condition in the donor strand and that this donor strand then seeks out a similar sequence on which

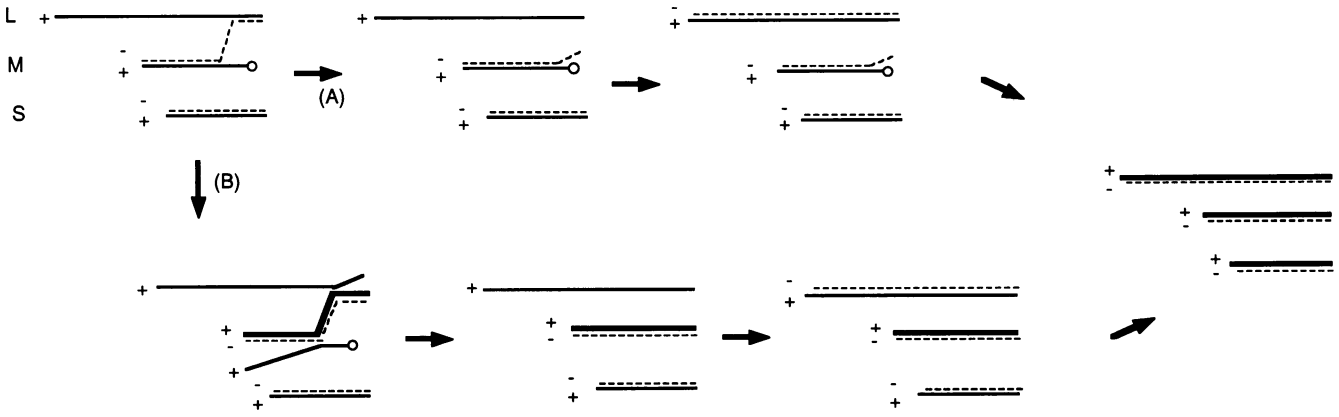

FIG. 6. Models for the resolution of recombinational intermediates. The diagram illustrates a situation in which the plus strand of M is truncated at the 3′ end and cannot serve as the template for minus-strand synthesis. Minus-strand synthesis occurs normally on S and begins on L. The nascent minus 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 the template for its normal complement. In pathway B, transcription (boldface line) of the recombinant structure leads to the displacement of the original template strands. The L template would then be free to serve as the template for normal L minus-strand synthesis.

to land. There would be a much greater probability that the exploring strand would land back on its proper template, but if another plus strand is available, it might be chosen. The sequence identity is not necessary but is preferred. Since the frequency of recombination is very low when the segments are intact and normal, there must be something special about the cases that promote recombination that is different from a situation in which one strand is simply replicating faster than another. It is possible that all three segments synthesize minus strands simultaneously in normal particles. Under these circumstances, the system might be very sensitive to a delay or absence of minus-strand synthesis on one of the segments.

There are many possibilities for matching sequences of six nucleotides in two heterologous strands. If a 6-base sequence at the end of the nascent chain had to match with a sequence within a region of a few hundred bases, the probability would be of the order 10% that it would find a matching site; however, if the matching sequence could be chosen from some part of the exploring chain (not necessarily the very end of it), the probability of a match would be much higher. One might ordinarily think that the nascent minus strand would be tightly bonded to the template. However, the situation may be similar to that proposed for nascent RNA that is formed by *E. coli* DNA-dependent RNA polymerase, wherein it appears that the newest part of the chain is not tightly associated with the template but rather is associated with the polymerase (36).

ϕ6 is the only dsRNA virus that has shown intermolecular recombination. Deletions and duplications have been found in rotavirus (20), and the mechanism of production of these intramolecular rearrangements may be similar to that for heterologous recombination; however, this remains undemonstrated. An internal duplication that was reported for segment 10 of human rotavirus (2) shows a 7-base direct repeat that seems reminiscent of the sequence relationships reported in the present paper and in our previous description of the ϕ6 recombination system (25). It seems likely that once it becomes possible to screen for intermolecular recombinants, they will be found in the other segmented dsRNA virus systems.

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