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An *in Vitro* System for the Investigation of Heterologous RNA Recombination

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Bacteriophage Φ6 has a genome of three segments of double-stranded RNA enclosed in a polyhedral procapsid. Purified procapsids are capable of the specific packaging of viral plus strands and the synthesis of their complementary minus strands. The genomic segments of Φ6 are capable of heterologous recombination. We have prepared an *in vitro* system containing purified procapsids that is capable of packaging plus strands of the genomic segments and synthesizing minus strands on these templates. The system generates heterologous recombination products when stimulated by having one of the plus strands incapable of serving as a template for minus strand synthesis. Recombinants were produced upon transfection of spheroplasts with the *in vitro* packaged and replicated RNA. Sites of recombination were not found to be localized in particular regions of either the donor or the recipient strands.

INTRODUCTION

Bacteriophage Φ6 has a genome composed of three unique segments of double-stranded RNA packaged in a polyhedral procapsid composed of four proteins, P1, P2, P4, and P7 (Mindich, 1988). Empty procapsids are capable of packaging plus strand transcripts of the genome. These serve as templates for the synthesis of minus strands, resulting in dsRNA, which in turn serves as template for the synthesis of plus strands (Gottlieb *et al.,* 1990). Each segment has a specific packaging sequence of about 200 nucleotides near the 5′ end of the plus strands (Gottlieb *et al.,* 1994). These sequences are sufficient for packaging into procapsids. Packaging shows a dependence relationship in that segment S can be packaged alone, but M depends upon S and L depends upon M and S (Frilander and Bamford, 1995; Qiao *et al.,* 1995). Minus strand synthesis of the three segments requires the prior packaging of segment L (Frilander *et al.,* 1995). The normal 3′ ends of the plus strands are necessary for their serving as templates for minus strand synthesis. If a segment is packaged without its normal 3′ end, it can potentiate the minus strand synthesis of the other two segments, but it will not serve as a template for its own minus strand synthesis (Frilander *et al.,* 1992). Under such conditions, a process of heterologous recombination can take place: minus strand synthesis initiating at the 3′ end of one of the other plus strand templates switches in midcourse to the initiation-defective plus strand template to generate a recombinant minus strand that is replication-competent (Onodera *et al.,* 1993).

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Our studies of this process of heterologous recombination have so far involved constructions that produce viable phage on normal host cells. In several cases we have inserted reporter genes such as *lac* or *kan* into the 3′ noncoding regions of segments and observed genetic instability caused by structures such as hairpins (Onodera *et al.,* 1993). These recombination events were limited to the 3′ ends of the segments. In other experiments we isolated recombinants at the 5′ ends of the segments (Onodera *et al.,* 1995). In the present study, we have developed an *in vitro* system for the investigation of the Φ6 heterologous recombination. Although the final products are plaque forming phages, the recombination event takes place *in vitro* during minus strand synthesis. Since the host strain carries a plasmid that complements mutations in gene 3, the products of recombination can include those that have lost substantial portions of segment M.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids

Escherichia coli strain JM109 (Yanisch-Perron *et al.,* 1985) was used for the propagation of all plasmids. Plasmid pLM450 contains a cDNA copy of genomic segment L of Φ6, encoding the four procapsid proteins (Gottlieb *et al.,* 1988) (Fig. 1). Constructions were done with the derivatives of plasmids pLM687, pLM656, and pLM659, which were prepared from pT7T3 19U (Table 1). These plasmids replicate in *E. coli* and have T7 promoters placed so as to yield faithful transcript copies of the Φ6 genomic segments. *Pseudomonas phaseolicola* HB10Y (HB) is the normal host of Φ6. Strain LM527 is strain HB carrying plasmid pLM380, which complements mutants in gene 3 of segment M (Fig. 1). Many of the transfection

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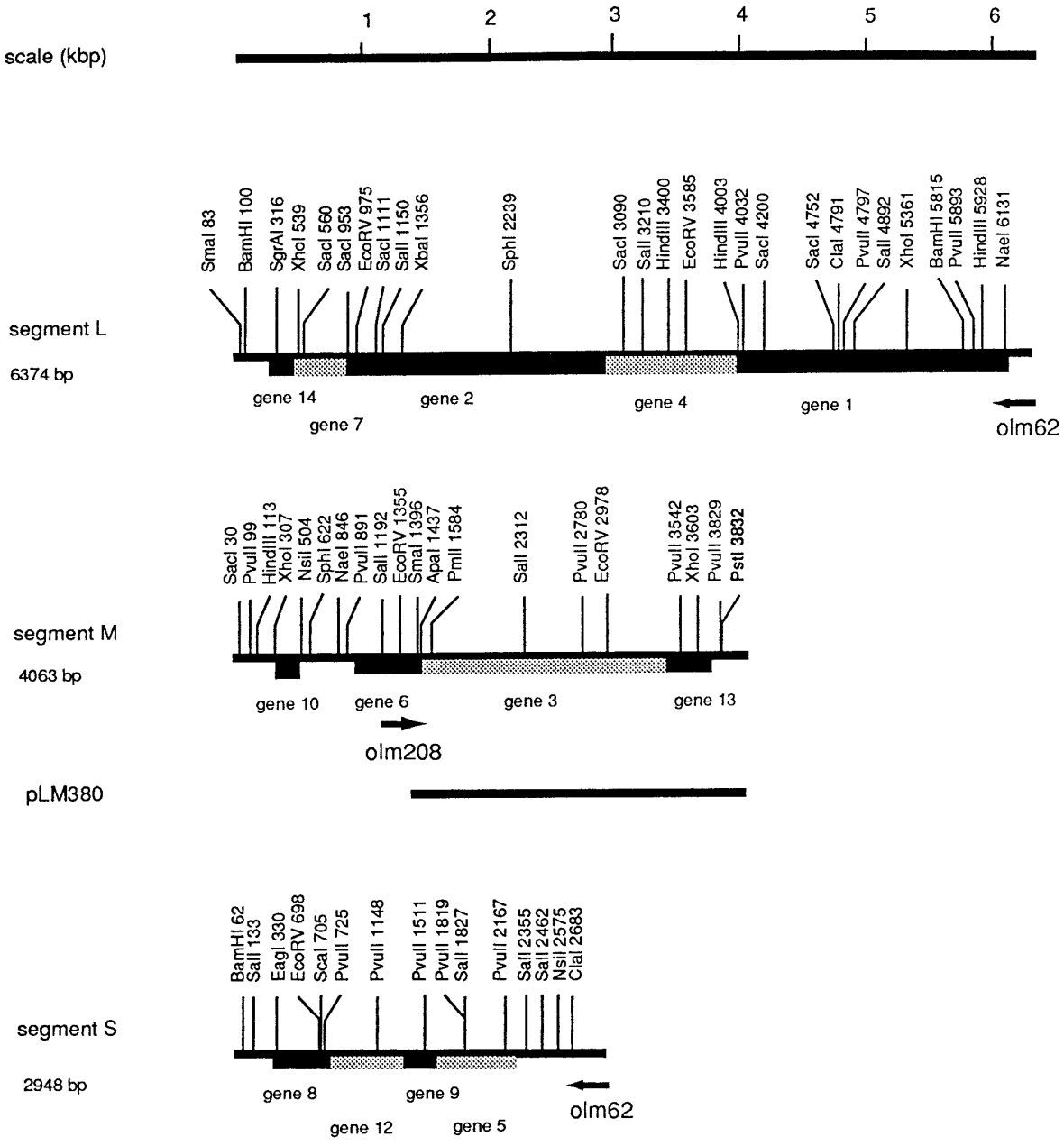

FIG. 1. Map of the cDNA copies of the three genomic segments of Φ6. The *Pst*I site that is truncated in M for the recombination experiment is shown in boldface type. Oligonucleotide olm208 was used as the primer for reverse transcription. The PCR used both olm62 and olm208; this combination will only amplify recombinant molecules. Plasmid pLM380 was used for complementing recombinant phage that had lost the activity of gene 3. Recombination was therefore expected from nucleotide 1472 to nucleotide 3833 in segment M. One recombinant was found to have crossed over near the end of gene 6 at position 1443.

experiments utilized ssRNA derived from an amber mutant of Φ6 in gene 6 of segment M that is designated as *sus507* (Lehman and Mindich, 1979).

Preparation of procapsids

Procapsids were isolated from *E. coli* JM109 containing plasmid pLM450. They were purified from French press lysates produced at 7000 psi according to Gottlieb et al. (1988) and Qiao *et al.* (1995). Purified procapsids were divided into aliquots and frozen at −70°. Aliquots were thawed immediately prior to use.

In vitro synthesis of plus sense transcripts by T7 polymerase

Plasmids were cut with the restriction endonuclease *Xba*I. The *Xba*I site is located at the 3′ end of the cDNA inserts. The 5′ overhang resulting from the *Xba*I digestion was blunted with mung-bean nuclease so that the runoff

TABLE 1

cDNA Plasmids

Plasmid	Segment	Remarks	Reference or source
pT7T3 19U	Vector	*amp*, P_{T7}, P_{T3}, *lacZ*; cloning vector for *E. coli*	Pharmacia
pLM254	Vector	*amp*, *lacZ*; shuttle vector for *E. coli* and pseudomonads	(Mindich *et al.,* 1985)
pLM380	M	gene 3 in pLM254	(Mindich *et al.,* 1985)
pLM656	M (4063 bp)	*amp*, P_{T7}; exact copy of segment M in pT7T3 19U	(Olkkonen *et al.,* 1990)
pLM659	S (2948 bp)	*amp*, P_{T7}; exact copy of segment S in pT7T3 19U	(Frilander *et al.,* 1992)
pLM687	L (6374 bp)	*amp*, P_{T7}; exact copy of segment L in pT7T3 19U	(Mindich *et al.,* 1994)

transcript would have a 3′ end identical to that of the viral transcripts. The plasmids were transcribed with T7 RNA polymerase as described previously (Olkkonen *et al.,* 1990). The polymerase reaction contained 2 m*M* each of UTP, ATP, GTP, and CTP. The RNA was purified by filtering through G-50 Sephadex spin columns (Boehringer Mannheim).

The recombination assay

Procapsids were incubated with a mixture of plus strand RNA from a transcription reaction using nucleocapsids of sus507, an amber mutant in gene 6, and a copy of segment M that was truncated at the *Pst*I site. The reaction volume was 12.5 μl. After 90 min of incubation, the reaction was diluted to 31 μl in a mixture that contained 0.7 m*M* calcium ions and about 2 μg protein P8 (Olkkonen *et al.,* 1990). After 60 min of incubation at 24° the mixture was added to protoplasts of *Ps. phaseolicola* strain LM527 and incubation continued as described previously. The protoplasts were then plated on a lawn of LM527 and plaques were picked the next day. Procapsids containing only RNA from *sus507* did not result in any plaques, as the reversion frequency was just below the threshold of our plating. The amber mutant was not complemented by pLM380 as this plasmid does not contain an intact gene 6. The expectation was that all plaques would be due to recombination that would supply a 3′ end to segment M and that recombinants whose crossover regions resulted in a defective gene 3 would be complemented by plasmid pLM380.

Plaques were picked and used to prepare single plate stocks by plating on lawns of LM527 in 0.6% agar. The top agar was collected and spun at 14,000 rpm for 15 min. The supernatant was adjusted to 10% PEG and 0.5 *M* NaCl and centrifuged. The pellet was suspended in 100 μl Buffer A (1 m*M* magnesium, 10 m*M* potassium phosphate, pH 7.5). Proteinase K was added to a final concentration of 200 μg/ml. The sample was extracted with phenol after 16 h of incubation at 23°. RNA was precipitated with ethanol and dissolved in 50 μl of DNA buffer (10 m*M* Tris, pH 8, 0.1 m*M* EDTA). Gel analysis of the RNA samples revealed a wide range of sizes for segment M (Fig. 2).

Reverse transcriptase reaction

About 3 μg of RNA in 20 μl water was mixed with 200 ng of oligonucleotide primer OLM208, which is identical to the sequence of segment M from nucleotides 1335 to 1353. The mixture was heated at 100° for 4 min and quickly cooled to 42° and 12 μl was mixed with 4 μl RT buffer (Promega, 5×), 2 μl dNTP (2 m*M* each), and 2 μl AMV reverse transcriptase (5 units). The mixture was incubated at 42° for 1 h.

PCR

Ten microliters of the RT-mix was supplemented with 0.2 m*M* dNTP, 1 μg OLM208, 1 μg OLM62 (complementary to the 3′ ends of all three segments and containing an *Xba*I site), 2.5 units of *Taq* DNA polymerase, and PCR buffer components (Perkin–Elmer) in 50 μl. The mixture was taken through 30 cycles of 94° for 1 min, 60° for 30 sec, and 72° for 3 min. The final product was extracted with phenol, ethanol precipitated, and dissolved in 20 μl DNA buffer.

Cloning and sequencing

The PCR products were cut with *Sma*I and *Xba*I and ligated to vector pT7T3 19U that had been cut with *Pst*I and blunted with T4 DNA polymerase and then cut with *Xba*I. The ligates were then introduced into *E. coli* JM109 by transformation and white colonies were picked from LB plates containing ampicillin and XGal. Small DNA preparations were made and the plasmids were analyzed to determine their restriction site patterns. On the basis of the preliminary restriction mapping, oligonucleotide primers were chosen for the sequencing of the crossover regions of the recombinants. Sequencing was done with Sequenase (U.S. Biochemical) with the buffers suggested by the manufacturer. In all cases, the calculated size of the recombinant segment M was compared with that found by direct gel analysis of the RNA and found to be of less than a 100- or 200-nucleotide difference. In a few cases, where the recombinant molecules were larger than 4 kb, it was necessary to use other nucleotide primers to successfully clone the cDNA copies.

The effects of incubation conditions on virus yield and recombination frequency

The conditions for minus strand synthesis and plus strand synthesis have been investigated and optimized (Gottlieb *et al.,* 1990; Van Dijk *et al.,* 1995). The optimal conditions for packaging and synthesis are not necessarily the optimal conditions for yield of virus in the transfection reaction or for the yield of recombination products. We have found that optimal transfection is achieved if the concentration of each NTP in the minus strand synthesis reaction is 0.3 m*M* rather than the 1 m*M* that is optimal for both minus and plus strand synthesis. Using this condition, it is now possible to produce several thousand plaques from each transfection reaction with RNA derived from transcription of cDNA plasmids.

RESULTS

Isolation of phage recombinants

Heterologous recombination is normally rare in Φ6. The frequency of recombinations that would eliminate a *lac* gene inserted into the noncoding region of segment M is less than one in several thousand. However, when the insert is bounded by a hairpin structure, it is lost at a frequency of several percent or higher. When exact copies of segments S and L were packaged along with a segment M that was truncated at the *Pst*I site, transfection into spheroplasts resulted in plaque-forming virions at a frequency about 1% of that obtained with three intact segments (Onodera *et al.,* 1993). All of the plaques were the products of heterologous recombination. An important point is that the composition of each plaque was homogeneous. This suggests that the recombination event that led to the rescue of the phage happened only once and that this event must have occurred during minus strand synthesis. These results led us to expect that the frequency of heterologous recombination in our *in vitro* reaction should be on the order of 1% or greater; since, in the case of the viruses that are plating on normal host cells, it is necessary that the recombination take place in the noncoding 3′ region of segment M. In the present experiments, where we are plating phage on host cells that contain plasmids that complement gene 3, we have collected recombinants that result in interrupted genes as well.

We have already shown that viable phage can be constructed with large deletions in their genomic segments if the missing genes are complemented by expression from cDNA plasmids (Onodera *et al.,* 1995). This suggested that it would be possible to investigate the sites of recombination by plating the products of the *in vitro* minus strand synthesis on strains carrying plasmids that would complement phages that have lost parts of their coding sequences. Plasmid pLM380 contains a full cDNA copy of gene 3 and is able to complement phages that are either mutant in gene 3 or have gene 3 missing (Fig. 1). Gene 13 had previously been shown to be dispensable (Mindich *et al.,* 1992). Plus strand RNA was prepared from a nonsense mutant (*sus507*) of gene 6. Procapsids that contained normal proteins P1, P2, P4, and P7 were isolated from *E. coli* and incubated with this RNA along with either a normal transcript of segment M or a transcript that was truncated at the *Pst*I site. The *Pst*I site is in the noncoding 3′ region of the plus strand of segment M (Fig. 1). The procapsids were incubated with protein P8 after minus strand synthesis and then used to infect spheroplasts of a strain carrying plasmid pLM380. In representative reactions the mixture with normal segment M yielded about 3000 plaques, the mixtures with the truncated segment M yielded about 50 plaques, and the mixtures with only the *sus507* transcripts yielded no plaques. The plaques derived from the truncated segment were further tested for the ability to propagate on a host strain without the complementing plasmid and we found that between 5 and 30% were able to form plaques. This indicated that the site of recombination was predominantly within gene 3. The actual size of the noncoding region up to the *Pst*I site and the size of gene 3 are 420 and 1945 nucleotides, respectively. Therefore, the noncoding region is about 18% of the total sequence of gene 3 and the noncoding region. Although the numbers are not sufficient to allow statements about the differences in the ratios, it is clear that recombination is not limited to the noncoding region or to the 3′ end of the genomic segment.

When the plaques derived from normal segment M were tested in the same way, it was found that about 1% could not grow on the host without a plasmid. RNA from these phages was prepared and analyzed by gel electrophoresis to determine whether the M segments were of normal size or of variable sizes that would indicate recombination. It was found that the sizes of the M segments were normal, indicating that the low frequency of defective phages was due to mutational defects. We have found that phages derived from transcription of plasmids with T7 RNA polymerase have a significant level of defects (from one to several percent). Phages formed from *in vitro* transcription of viral nucleocapsids show fewer than 0.4% mutations in gene 3.

Points of launching and landing

Forty-seven recombinant plaques were analyzed so as to determine the points of donor launching in segments S or L and the points of reception in segment M. The migration patterns of the RNA are shown for a representative group in Fig. 2. The results of the determinations are shown in Fig. 3. In the upper part of Fig. 3 it can be seen that the launching positions in L are broadly distributed, with no particular concentration. The points of reception in M are concentrated toward the middle of

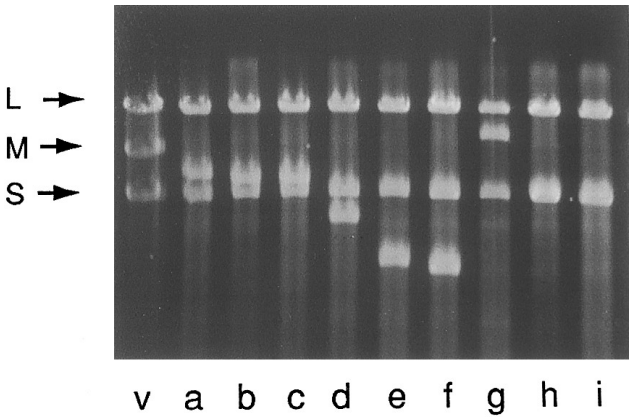

FIG. 2. Agarose gel electrophoresis of dsRNA isolated from viral products of transfection when segment M was truncated at the *Pst*I site. Normal viral RNA is shown in lane v. The other lanes contain RNA derived from recombinants. The samples shown in lanes a, b, c, d, e, f, g, h, and i were TN11, TN12, TN46, TN13, TN14, TN15, TNA, TN16 and TN42, respectively.

segment M, with very few occurring near the 3′ end or at the point of truncation. In the lower half of the figure one can see that the launching positions from segment S are not particularly concentrated either. The points of reception in M are again concentrated near the middle of the segment. The bottom pattern shows the lengths of the recombinant molecules. It is clear that there is no preferred size and most molecules are smaller than normal, although some are larger than the normal size of M. An analysis of the relationship between the region of launching and the landing region in segment M showed that there was no preferred landing region for launches from a particular region of either S or L (not shown). The broad distribution of launch points suggests that there is not a strong signal at the onset of minus strand synthesis to promote recombination. The frequency of recombination is enhanced at least 10- or 20-fold by the truncation of the recipient segment; yet the donated strands originate throughout their period of synthesis. A similar experiment in which the RNA of segments S and L were from plasmid transcripts rather than the viral nucleocapsid of *sus507* gave similar results in that most of the recombinants had crossed over within gene 3. A transfection experiment in which segment M was not truncated but contained a *lac* insert bounded by a hairpin structure (Onodera *et al.,* 1993) also yielded

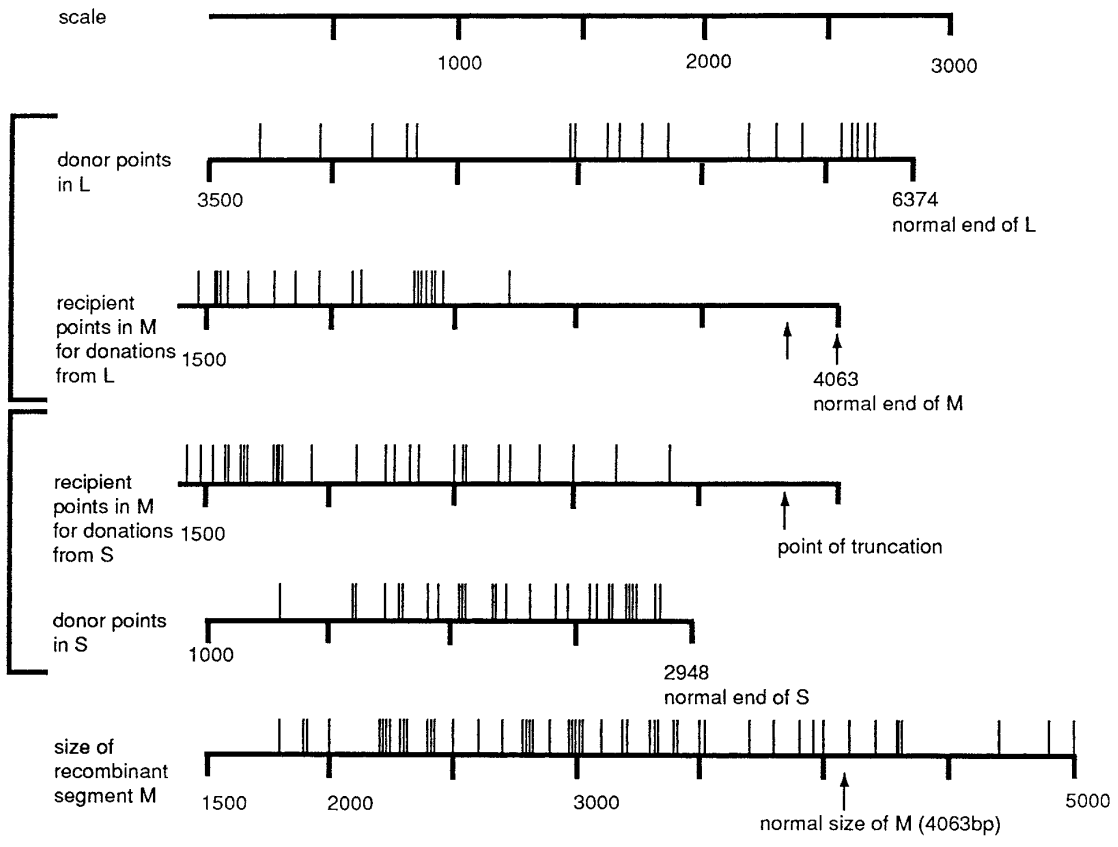

FIG. 3. The locations of crossover regions in the recombinant phages. RNA samples including those from Fig. 2 were used as templates to prepare cDNA, which was then amplified and cloned into vectors and sequenced. The positions of the crossover points in terms of their donor origins in segments S or L are shown, as well as the positions in segment M where they were received. At the bottom of the figure, the sizes of the recombinant M segments are shown. Note that the sizes do not concentrate at the normal size of M. All illustrations show plus strands with the 5′ end at the left.

recombinants that were primarily crossing over within gene 3 when the host strain was able to complement gene 3. However, the segments containing hairpin structures are extremely unstable and seem to evolve into forms that eventually produce recombinants that are sometimes limited to either the end of gene 3 or the region downstream from it. These recombinations are very difficult to study as they are occurring during several rounds of replication.

Analysis of the crossover region

We had previously shown that the crossover regions were characterized by limited sequence identity between the donor and recipient strands (Mindich *et al.,* 1992; Onodera *et al.,* 1993). The identity ranged from zero to about 10 bases. In the present study of 47 recombinant molecules we have found that the arithmetic mean of the number of identical bases was 3.6 and the mode was 3. The range was from zero to 12. Some examples of sequences at the crossover regions are shown in Fig. 4. As a result of collecting and analyzing a larger number of recombination products we have noticed a new feature of the sequences at the crossover regions. We find that in about half of the cases, there are gaps between the point of crossover and an upstream sequence identity; where the sequence of the gap is usually that of the recipient strand. RNA TN37 (in Fig. 4) shows a 7-base identity at the crossover point with a rather clean transition between the sequences of segments M and S. In the cases of TN31, TN34, TN43, and TN15 we can see that the sequence between the two regions of identity is the sequence of segment M. We found that in 17 cases, there was an upstream identity of 2 or more bases within 5 bases of the crossover point. In only one of these cases was the intervening sequence that of the donor strand. In 12 cases where the two regions of identity were separated by 1 or 2 bases, the intervening sequence was always that of the middle segment. These findings suggest that the donor strand is trimmed back after landing on the new template.

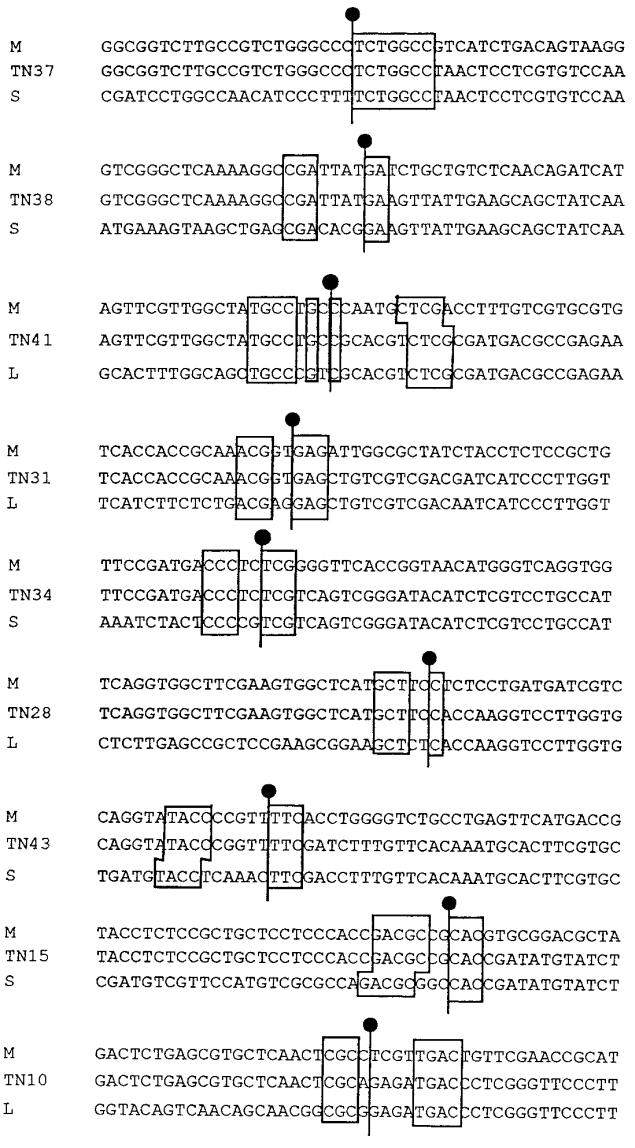

FIG. 4. Sequence analysis of crossover regions. In each case the recombination product is shown in the middle row. The donor of the 5′ sequence (M) is shown above and the donor of the 3′ sequence (S or L) is shown below the recombinant sequence. The boxes enclose the sequence identity at or near the crossover points. In some cases the sequence identity is not exactly at the crossover point. The crossover points, or the leftmost bases of the identity regions, are indicated with a vertical line.

DISCUSSION

Recombination in RNA systems has so far been studied in infected cells. In the case of Φ6 the recombination and replication of RNA takes place inside the procapsid. Because we can perform packaging and replication *in vitro* it is possible to carry out recombination *in vitro* as well. Our experiments involve the plating of the procapsids on host cells to obtain plaques. This implies a limitation in that only viable recombination products are assayed. Molecules that are too large and molecules that lack gene 6 will not be propagated. However, the range of sizes of segment M that we isolate is large enough so that we can make conclusions as to the distribution of both launching and receiving points in the Φ6 recombination process. It is clear that there is no propensity to form recombinant molecules of normal size and it is clear that there are no particular hot spots for either launching or landing. *In vivo* recombination systems in which the recombination products are collected by RT-PCR amplification of RNA isolated from cells have an advantage in that they are independent of packaging; however, they are subject to variation due to differences in replication rate and differences in ease of amplification. The system that we have used is independent of rate of replication in that each recombinant yields only one plaque and the

yield is independent of amplification in that the recombinant RNA is isolated and measured before RT-PCR amplification.

The finding of a region of limited sequence identity at the crossover point in Φ6 recombinants is significant. The average length of this identity is 3.6 bases, while the probability of a single base identity occurring by chance is only 0.25. Previous studies have shown that regions of sequence identity or complementarity of 27 nucleotides do not promote recombination in Φ6 (Mindich, 1995). Some positive strand RNA viruses have been reported to show an association of complementary sequences near the crossover points in recombinants. This was originally reported for poliovirus and subsequently found for brome mosaic virus (Romanova *et al.,* 1986; Bujarski and Dzianott, 1991; Nagy and Bujarski, 1993). These viruses do not exhibit the limited sequence identity that we see with Φ6 at the crossover points of heterologous recombination. We have not seen a relationship involving complementary sequences in the case of Φ6 (Mindich, 1995). In the cases of poliovirus and brome mosaic virus, it might be that the association between two recombining molecules is promoted by having complementary sequence. The RNA molecules might not be at high concentrations in the viroplasm. In the case of Φ6, the RNA molecules are already packaged in procapsids and their proximity is great. They might be as close to each other as necessary to facilitate recombination.

The heterologous recombination that is seen in our experiments must be taking place during minus strand synthesis. A plus strand without a proper 3′ end is not a substrate for minus strand synthesis (Frilander *et al.*, 1992), and there can be no additional plus strand synthesis without first forming the minus strand template. We find that the composition of plaques formed in our experiments with truncated segments is always homogeneous, indicating that the recombination is taking place once in the infecting particle. The simplest model for the recombination would be one in which a nascent minus strand is displaced from its template along with polymerase and lands on a new template. The polymerase would then continue extending the nascent chain on the new template. The nascent chain might be completely single stranded, or it might be that only the leading edge is single stranded while the 5′ end is still bonded with its original template. The leading edge of the nascent chain would anneal to the new template at a region of limited sequence identity. Our results suggest that there is often additional sequence identity upstream of the crossover point, but that the intervening sequence prefers that of the recipient strand. An intriguing possibility is that the nascent chain anneals to both regions and that it is then digested back before synthesis begins. RNA polymerases of both eukaryotes and prokaryotes show an activity that truncates the leading edges of nascent transcripts under special conditions (Surratt *et al.,* 1991). In the case of *E. coli* RNA polymerase, two accessory proteins, GreA and GreB, have been identified as facilitating this process (Borukhov *et al.,* 1993). However, it has recently been shown that the RNA polymerase itself has the ability to truncate the nascent chain at high pH (Orlova *et al.,* 1995).

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