HOMOLOGOUS RECOMBINATION IN Q-BETA RNA BACTERIOPHAGE

THESIS

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Q-Beta phage RNAs with inactivating insertion (8 base) or deletion (17 base) mutations within their replicase genes were transfected into *Escherichia coli* spheroplasts containing Qß replicase provided in *trans* by a resident plasmid. Replicase-defective (Rep^-) Qß phage produced by these spheroplasts were unable to form plaques on cells lacking this plasmid. When individual Rep^- phage were isolated and grown to high titer in cells containing plasmid-derived Qß replicase, revertant Qß phage (Rep^+), with the original mutation (insertion or deletion) repaired, were obtained at a frequency of ca. 1×10^{-8}. RNA recombination via a "template-switching" mechanism involving Qß replicase, the mutant phage genome, and the plasmid-derived replicase mRNA was shown to be the primary means by which these mutant phages reverted to wild-type.

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LIST OF TERMS, DEFINITIONS AND ABBREVIATIONS

Apa I	-A restriction site and an enzyme.
Bacteriophage	-A virus that infects bacteria.
Bgl I	-A restriction site and an enzyme.
cDNA	-Complementary Deoxyribonucleic acid.
Homologous-Recombination	-Genetic exchange between DNA or RNA molecules that have identical or nearly identical base sequences.
in-trans	-Biological activity provided by a second component and limited by diffusion.
in-vitro	-Outside a living organism; pertaining to a cell free environment.
in-vivo	-Within a living organism; pertaining to conditions within living cells.
Minus-strand	-Complementary to the genomic strand of the RNA virus plus-strand.
mRNA	-Messenger Ribonucleic acid.
Plasmid	-An extrachromosomal genetic element in bacteria composed of a circular double-stranded DNA.

PFU	-Plaque Forming Units; the number of viral infections initiated from a given sample.
PEG	-Polyethylene glycol.
Plus-strand	-Positive-strand; the infectious genomic RNA of the virus, which can serve as a mRNA.
Rep^-	-Phenotypicly replicase defective phage.
Rep^+	-Phenotypicly replicase independent phage.
Replicase	-An RNA-dependent RNA polymerase that is highly specific for the viral RNA.
Reverse-transcriptase	-An RNA-dependent DNA polymerase that catalyze the synthesis of DNA from an RNA template.
SDS	-Sodium dodecyl sulfate.
Speroplast	-A bacterial cell that is largely, but not entirely, freed of its cell wall.
Silent-mutation	-A mutation that does not result in a detectable phenotypic effect.
Sal I	-A restriction site and an enzyme.
Sma I	-A restriction site and an enzyme.
SnaB I	-A restriction site and an enzyme.
Stu I	-A restriction site and an enzyme.

Translation-frameshift	-A mutation that leads to an alteration in the normal relation between nucleic acid and amino-acid sequence in the corresponding protein.
Tranfomation	-The process of introducing purified DNA (plasmid) into a cell
Transfection	-The process of introducing purified viral genetic material (DNA or RNA) into a cell

INTRODUCTION

The RNA bacteriophage(s) of *Escherichia Coli (E. coli)* are some of the simplest organisms in nature. Their genetic material consists of a single-stranded polycistronic messenger-sense RNA (36). They have a highly efficient replicative system and any mutational changes within their genome could result in disruptions in their replication and infectivity. From an evolutionary point of view, it would be interesting to know what mechanism(s) viruses use to rectify deleterious mutations that occur within their genomes. RNA viruses must possess some mechanisms which stabilize and extend (evolve) their genetic information. Genetic recombination in RNA viruses is one such process in which the exchange of information between genomic RNA molecules helps alleviate mutations within the genome (17). This process has been observed in some plant and animal RNA viruses and appears to occur via a mechanism involving "template switching" between homologous regions of genetic RNAs (1, 2, 4, 13, 14, 17, 37)

The first evidence of recombination in RNA viruses was observed in poliovirus more than twenty years ago (7, 14, 17). Since that time, a number of other RNA viruses, including plant RNA

viruses have been demonstrated to undergo homologous RNA recombination (1, 2, 4, 10, 14, 17, 18, 27, 37). Many workers have reported that RNA phages do not undergo RNA recombination (10, 14, 17). However, Munishkin and co-workers (25, 26) have recently reported several non-homologous recombination events in Qß RNA replication templates. The report will present evidence that homologous recombination can occur in Qß bacteriophage as well. The mechanism for these recombination events appears similar to that of other viral systems.

Qß and its replicase (RNA-dependent RNA polymerase) have been long considered one of the best characterized RNA replication systems for RNA viruses (3). Recent experiments from several workers have demonstrated that phage Qß RNAs can be amplified *in vivo* by Qß replicase expressed in *trans* from a plasmid (13, 24, 30). By supplying Qß replicase in *trans* from a plasmid, various mutations can be introduced in the replicase gene of a cDNA copy of the Qß RNA. Mutant RNAs can then be produced by transcription (23) and transfected into spheroplasts containing Qß replicase provided by a plasmid. Products of these transfections can be assayed for the production of infectious virus in order to test the effect of mutations on replication (32).

In this fashion, Shaklee and co-workers developed a simple system of plaque assays to assess the complementation of Qß replicase mutants by plasmid derived Qß replicase (32, 33). In a

preliminary study (32) these workers were able to complement a lethal mutation (17 nucleotide deletion) within the the replicase gene of Qß genomic RNA. The replicase dependent phages (Rep^-) were amplified to high titer and, in most cases, were still replicase dependent and maintained their lethal mutation. However, Shaklee observed (32) that a small number of the amplified phage had lost their dependence on plasmid derived Qß replicase and somehow replaced the missing (deleted) nucleotides. In other words, these phage had become replicase independent or Rep^+. In contrast, Mills et al (24) described similar experiments with deletion and insertion mutants within the genomic Qß replicase gene, but reported no evidence of reversion in any of their mutant phage isolates.

The present report describes an investigation using two distinct replicase mutants of Qß phage in which reversion (Rep^- to Rep^+ phenotypic change) was observed at an appreciable frequency. To ensure that contamination was not the cause of the apparent reversion, silent mutations were introduced into the replicase gene of the plasmid supplied in *trans*. By sequence analysis of revertant phage, it was demonstrate that the revertant phage contained the silent marker mutation. This finding plus the report by Munishkin *et al* (25, 26), suggests that RNA recombination is one mechanism utilized by RNA phage to repair and evolve their genomes. This mechanism was not previously considered as a possible means by

which RNA phages could repair or evolve their genomes (10).

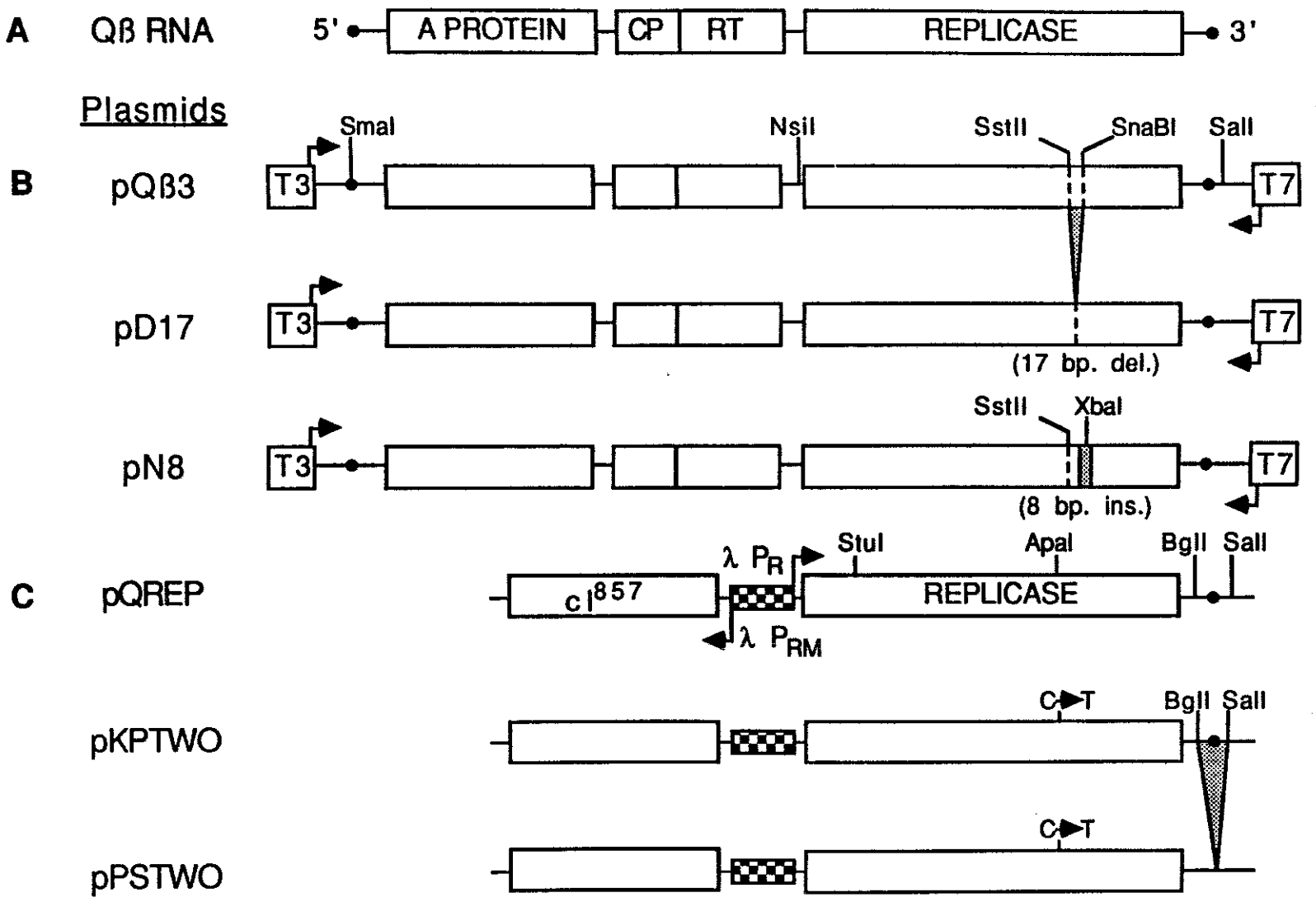
MATERIALS AND METHODS

Materials. Restriction enzymes and other enzymes required for these studies were purchased from New England Biolabs (Tozer, MA), GIBCO BRL (Grand Island, NY), or Promega (Madison, WI). Reverse transcriptase was obtained from Life Sciences, Inc. (St. Petersburg, FL). Synthetic oligonucleotides were obtained from several sources: Midland Certified Reagent Company (Midland, TX), Ed Meenhan and John Atkins of the University of Utah, and by in-house preparation on a Milligen/Biosearch Cyclone^{TM} Plus Synthesizer. The oligonucleotides were purified on 20% polyacrylamide/8M urea gels prior to use (16). *E coli* HB101 [*supE44 hsdS*20($r_B^- m_B^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20*xyl*-5 *mtl*-1, F^-] was the bacterial strain used for standard transformations, production of plasmids, and as a transfection recipient in many of the procedures described below. *E. coli* Q13 (*rna*-19 *his*-95 *tyrA*6 *relA*1 *pnp*-13 *spoT*1 *metB*1, Hfr) was used as an indicator host for wild-type Qß bacteriophage infection. These strains were kindly provided by Dr. Barbara Bachmann of the *E. coli* Genetic Stock Center (New Haven, CT). This strain was also used, following transformation with a

replicase expression plasmid, in order to complement replicase-defective mutants of Qß. *E. coli* JM109 [*endA*1 *recA*1 *gyrA*96 *thi hsdR*17($r_k^- m_k^-$) *relA*1 *supE*44 I^- D(*lac-pro*AB), (F', *traD*36 *pro*AB *lacI*^{q}ZDM15)] a recombination minus strain, *E. coli* BMH 71-18 mut S [*thi supE* D(*lac-pro*AB) (*mut*S::Tn10) (F', *proA*$^+$*B*$^+$ *lacI*^{q}ZDM15)] a repair minus strain, and M13R408 helper phage were used in the site-directed mutagenesis of the Qß replicase expression plasmid and were obtained as part of the Altered Sites^{TM} mutagenesis system from Promega (Madison, WI). Plasmids pQß3 and pQREP (Fig. 1) were used as starting materials for the construction of other plasmids described in this report. pQß3 is a full-length cDNA copy of Qß RNA and pQREP is a plasmid that contains only the replicase gene from the Qß genome. The latter plasmid has a thermal induction site that can result in replicase overproduction when *E.coli* transformed with the plasmid are incubated at the induction temperature (37° C). The construction of these plasmids have been described elsewhere (30, 33).

General Procedures. The methods employed for plasmid construction, bacterial transformation, and plasmid preparations were standard techniques and have been described in detail elsewhere (19). Sequencing of plasmids to confirm site directed mutagenesis was carried out on alkaline-denatured plasmid DNAs

Figure 1. Transcription and expression plasmids containing phage Qß cDNAs. (A) Depiction of Qß genomic RNA and encoded proteins. CP and RT correspond to the coat protein and read-through protein, respectively. (B) Linear depiction of relevant regions of full-length phage transcription plasmids containing parental (pQß3) and mutant (pD17 and pN8) Qß cDNAs. Position and orientation of T3 and T7 RNA polymerase promoters are indicated. The Sma I and Sal I sites indicated in pQß3 are present, but not shown in pD17 and pN8. (C) Linear depiction of relevant regions of Qß replicase expression plasmids. The checkered area indicates the promoter/operator region of the phage λ expression vector used in these studies. The expression vector and temperature-sensitive cI857 allele were originally obtained courtesy of Queen (28) and modified by Miglietta (22). The plasmid indicated as pQREP was named pREP in a previous communication (33). In each representation [(A), (B), and (C)], a closed circle (•) indicates the presence of a complete 5' or 3' terminal sequence. Restriction sites used in plasmid constructions and *in vitro* transcription are indicated. Shaded triangles indicate the areas deleted in going from one plasmid to another. The shaded rectangle in pN8 indicates the insertion position of an 8 base-pair Xba I linker. Arrows generally indicate the direction of transcription from a given promoter. In plasmids pKPTWO and pPSTWO, the arrows indicate the C to T marker mutation placed into the Qß replicase gene.


A
Qß RNA
5'
A PROTEIN
CP
RT
REPLICASE
3'
Plasmids
B
pQß3
SmaI
NsiI
SstII
SnaBI
SalI
T3
T7
pD17
(17 bp. del.)
pN8
XbaI
(8 bp. ins.)
C
pQREP
λ P_R
StuI
ApaI
BglI
cI857
λ P_RM
pKPTWO
C▶T
pPSTWO

(6). Transcript RNAs (plus-strands) were prepared by digesting the cDNA plasmids with *Sal* I and RNAs synthesized by addition of T3 RNA polymerase and nucleoside triphosphates to the reaction. Minus-strands RNAs were prepared in a similar fashion, except *Sma*I and T7 RNA polymerase were utilized (see Fig. 1). For sequencing plus-strand RNAs , RNAs were annealed to a primer oligonucleotides. Sequencing was then carried out by primer extension using reverse transcriptase (34). All liquid cultures and agar (1%) plates involved in procedures for amplification of phage particles and plaque assay (area of cell lysis on a bacterial lawn) were prepared with LB broth (19) containing 10 mM $CaCl_2$. Ampicillin (100 μg/ml) was added to cultures and plates as required for maintenance of plasmids.

Constructions of lethal Qß mutants. pD17 and pN8 were plasmids prepared by Shaklee (29) from pQß3 (see Fig. 1). They contain lethal mutations within the replicase gene (*rep*$^-$) that render resultant phage unable to replicate without replicase supplied in *trans*. pD17 contains a 17 nucleotide deletion between the *Sst* II and *Sna* B I sites at bases 3805 and 3822 of the Qß RNA genome, respectively (20). Similarly, pN8 contains a 8 base insertion at the *Sna* B I site (20). Construction of these plasmids is described in detail elsewhere (27).

Construction of a replicase plasmid with a silent point mutation. pKPTWO was prepared from pQREP but contains a silent point mutation in the replicase gene. To produce this point mutant, the Altered Sites^{TM} *in vitro* mutagenesis system was utilized (Promega). pQREP (see Fig. 1) was double digested with *Stu* I/*Sal* I and the 1550 base-pair DNA fragment (insert) was isolated and ligated to *Sma* I/*Sal* I pSELECT-1 (vector) (Promega). The reaction products were used to transform competent *E. coli* JM109. Tetracycline-resistant clones were screened by restriction enzyme analysis and appropriate isolates infected with M13R408 helper phage. Single-stranded M13 phagemids were isolated from the polyethylene glycol (PEG) pellet of the resultant culture supernatant after 6 hrs of infection, protein removed by multiple extractions with phenol/chloroform (19), and DNA precipitated with ethanol. A mutagenic oligonucleotide complementary to bases 3777 to 3813 of the Qß genome (20), but containing a C to T substitution at base 3796, (5'GGTCCTAATCAATCCTTTTGCGAAAAACCGCGGGTG3'), was annealed to this single-strand DNA template along with an oligonucleotide provided by the manufacturer which repairs the defective ampicillin-resistance gene in pSELECT-1. Following second-strand DNA synthesis with T4 DNA polymerase, reaction products were transformed into competent *E. coli* BMH 71-18 mut S strain. After outgrowth of this mixed culture in the presence of ampicillin,

plasmids were isolated by the alkaline lysis procedure (19), and used to re-transform competent HB101 cells. Individual ampicillin-resistant clones were screened by sequencing to confirm the presence of the desired mutation in the Qß replicase gene. Plasmid pKPTWO (Fig. 1) was then prepared by ligating the 550 base-pair *Apa* I/*Sal* I fragment isolated from the mutant plasmid above to the 4900 base-pair *Apa* I/*Sal* I vector obtained from double-digestion of pQREP. Qß replicase activity in cells containing pKPTWO was confirmed as described in RESULTS.

To prepare plasmid pPSTWO (Fig. 1), pKPTWO was first digested with *Bgl* I and the protruding 3' ends removed with T4 DNA polymerase. Resultant DNAs were digested with *Apa* I and the 460 base-pair intervening fragment isolated on low-melting point agarose. A separate aliquot of pKPTWO was digested with *Sal* I and the 3' recessed fragment ends filled with T4 DNA polymerase. The sample was then digested with *Apa* I and the resultant 4900 base-pair fragment ligated to the fragment isolated above. The ligation reaction products were transformed into competent HB101 cells and ampicillin-resistant clones screened by restriction enzyme analysis, confirming the elimination of the *Bgl* I and *Sal* I sites in pPSTWO (Fig. 1). Qß replicase activity in cells harboring pPSTWO was demonstrated as described in RESULTS.

Complementation assays. Minus-strand RNA transcripts were prepared from plasmids pQß3, pD17, and pN8 by methods similar to those used for plus-strand RNAs (see general procedures). The DNA templates were removed by DNAse (31) and the concentration of RNA determined by spectrophotometric methods (19). About 1-5 µg of RNA from each sample was than inserted (transfected) into HB101 spheroplasts containing pQREP (30, 33). After a 3 hour incubation, serial dilutions were made of the supernatant of each sample. Wild-type or replicase independent Qß phages (Rep^+) resulting from these transfections were titered on lawns of *E. coli* Q13 strain, while replicase dependent Qß phages (Rep^-) were titered on lawns of Q13 transformed with plasmid pQREP or pKPTWO. Aliquots of each dilution (100 µl) were plated on the appropriate lawn and incubated overnight to assay for bacteriophage plaque formation.

Amplification and reversion analysis of Qß mutants. For amplification of mutant phages, a culture media of bacteria containing Qß replicase had to be prepared. An overnight culture of Q13(pQREP) or Q13(pKPTWO), grown at 28° C (non-induced temperature) in LB broth was diluted 1:50 with the same media and pre-warmed to 37°C (partial induction condition). The culture was then grown to mid-log phase (OD_{600} = 0.25). To amplify and test for reversion of Rep^- Qß mutants, individual Rep^- phage plaques from

Q13(pQREP) or Q13(pKPTW0) titer plates were lanced with sterile toothpicks and shaken into 1 ml of LB broth ("Round 1" isolates). Each sample was tested for spontaneous reversion by spot testing 10 µl on lawns of Q13. Samples that did not show Rep^+ characteristics (no plaque formation) were than subsequently amplified. "Round 1" isolates (0.5 ml of each) was used to inoculate (infect) 1 ml aliquots of the mid-log phase culture and these incubated for 3 hours 37°C. The samples were spun-down at low-speed to pellet debris and chilled overnight. "Round 2" samples (1 ml aliquots) were then used to infect 10 mls of Q13(pQREP) or Q13(pKPTWO) culture as above. The supernatants from the latter samples were then termed "Round 3" isolates. Individual mutant isolates from round 1 to 3 were titered for both Rep^+ and Rep^- Qß phages on *E. coli* Q13 and Q13(pKPTWO) lawns, respectively. In experiments with pPSTWO, the mutant phages were amplified in rounds 2 and 3 by infection of cultures of *E. coli* Q13(pPSTWO) (see Table 3).

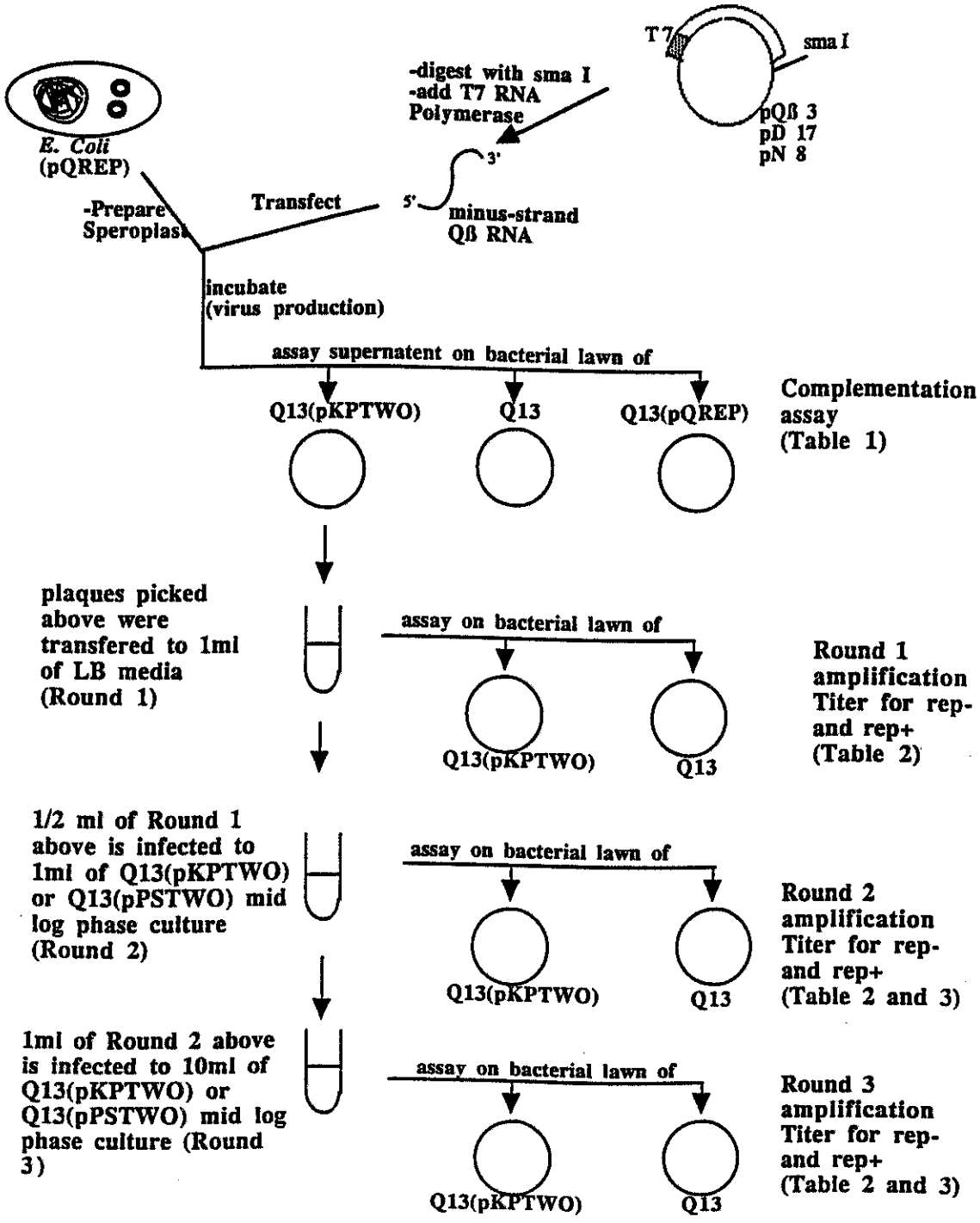
Sequence analysis of Qß mutants and wild-type revertants. An oligonucleotide complementary to bases 3823-3848 of the Qß genome (20) was used as primer for reverse transcriptase sequencing of RNAs extracted from Rep^- and revertant (wild-type or Rep^+) Qß phage. To sequence these RNAs, individual revertant or

Rep$^-$ Qß phage were picked as above and amplified through two additional rounds in their respective culture (with or without Qß replicase provided in *trans*). The phage present in 5 mls of supernatant were precipitated with one third volume of 40% PEG in 2 M NaCl and the pellet dissolved in 500 µl of 10 mM TrisHCl, pH 7.6. Following addition of 100 µl of 10% SDS, the sample was extracted twice with equal volumes of phenol, twice with an equal volume of phenol/chloroform, and the RNA precipitated with 3 volumes of ethanol (19). RNA pellets were redissolved in 50 µl of 10 mM Tris-HCl, pH 7.6. Typically, 1 to 2 µl of this solution (ca. 2 µg of RNA) was used in sequencing. Sequencing was carried out by the procedure of Seeburg and Chen (6).

RESULTS

Experimental Design. Figure 2 illustrates the overall outline of the experiments described in this study. Briefly, minus-strand RNAs of pQß3, pD17 and pN8 (Fig. 1) were transfected into HB101 containing plasmid derived Qß replicase [HB101(pQREP)]. After 3 hours of incubation (37°), the supernatant solutions were assayed for infections viral particles on Q13, Q13(pQREP), and Q13(pKPTWO) lawns (Table 1). Plaques formed (areas of cell lysis) on the bacterial

Figure 2. Experimental Design



lawns were picked and amplified two rounds in either Q13(pKPTWO) or Q13(pPSTWO). The supernatent from each round of infection were assayed on lawns of Q13 and Q13(pKPTWO) (Tables 2 and 3) to titer for Rep^+ and Rep^- phages, respectively. Revertants (Rep^+ phage obtained from the second round of amplification) were picked and amplified in *E. coli* Q13 two rounds and the genomic RNA sequence determined in the area of the original mutation (Fig 3). The experiments are describe in greater detail below.

Complementation of Qß mutants by plasmid-derived Qß replicase. The first objective of this experiment was to see if the lethal mutations constructed in pD17 and pN8 (Fig. 1) could be complemented by plasmid derived Qß replicase. Previous work by Mills *et al* (24) established that insertion and deletion mutations within the Qß replicase gene could be complemented if replicase was supplied in *trans*. pD17 and pN8 contain a 17 base-pair deletion and an 8 base-pair insertion, respectively. Both of these mutations were designed to cause a translational frameshift during replicase translation, thus creating a replicase-defective phage genome. Minus-strand RNAs of pD17 and pN8 were used to transfect an *E. coli* strain containing a replicase producing plasmid, based on previous work showing that minus-strands were actively replicated to plus-strand RNAs by these cells (30, 33).

TABLE 1

Complementation of Qß Mutants by Plasmid-derived Qß Replicase

RNA source	PFU/µg RNA		
	Q13	Q13/pQREP	Q13/pKPTWO
pQß3	4.9×10^5	7.9×10^5	4.8×10^5
pD17	0	3.7×10^4	3.1×10^4
pN8	0	2.4×10^5	5.3×10^5

Table 1 presents typical results and demonstrates that both mutations could be complemented in *trans* by plasmid-derived Qß replicase to yield Rep$^-$ Qß phage. Wild-type minus-strands from pQß3 resulted in the synthesis of wild-type Rep$^+$ Qß phages. Thus this sample showed plaques on all three Q13 cell lawns. Defective minus-strands from pD17 and pN8 yielded only Rep$^-$ Qß phages [plaques only on Q13(pQREP) and Q13(pKPTWO)] and thus showed plaques only on cells with Qß replicase provided by an expression plasmid. These experiments were often done in duplicate or triplicate and differences of 2-fold in titer were often observed. These differences were not considered significant in analyzing the results. It was observed however, that the deletion mutation of pD17 minus-strands resulted in a reproducible 5- to 10-fold decrease in Rep$^-$ phage yield, relative to minus-strands of either the insertion mutant or the wild-type (Table 1). There was no significant differences in plaque sizes observed in any of the phage lawns. It thus appears that the

truncated version of Qß replicase from pD17 and pN8, formed due to their translational frameshift, did not interfere with *trans* replicase binding and replication of these defective RNAs.

Qß mutant amplification yields wild-type recombinants. In preliminary experiments with pD17 transfections (not shown), plaques from the Q13(pQREP) lawn titer plate (Table 1) were lanced with sterile tooth-pick and shaken into 1 ml aliquots of LB-broth. Aliquots (10 ml) of each of these isolates were then spotted onto a Q13 bacterial lawn. Surprisingly, about 60% of the isolates tested in this fashion gave plaques, indicating reversion of some Rep^- phage to a Rep^+ phenotype. In a similar manner each of these Rep^+ plaques was picked and subsequently amplified in *E. coli* strain Q13. The total RNA was isolated (see Methods) and the nucleotide sequence determined in the region of the original 17 base deletion. This analysis showed that all Rep^+ samples had the 17 nucleotides replaced and had a wild-type rep^+ sequence (not shown). These experiments were repeated, however this time plaques that were negative in the spot test, were amplified through two rounds on Q13(pQREP) (see Methods) and subsequently the supernatant assayed for Rep^+ and Rep^- phage on Q13 and Q13(pQREP) cell lawns, respectively. It was now observed that revertants appeared in all isolates following round 2 and 3 of amplification. Six isolated plaques

were picked from among these revertants and subsequently amplified and sequenced in a similar manner. Again, all six isolates showed a similar wild-type sequence with the 17 bases restored (not shown). To account for these findings, it was postulated, RNA recombination involving mRNA of the replicase and plus-strand RNA of the Qß mutant template was responsible for reversion of the phage (Rep^- to Rep^+ phenotypic change) and the restoration of the 17 missing nucleotides. Another possibility considered however, was that contamination with wild-type phage may have affected the results and led to the Rep^+ phage present in each of the amplified samples.

To test the hypothesis that recombination was responsible for the reversion observed, and to eliminate contamination as a possible explanation, pQREP with a silent point mutation as marker (pKPTWO) was constructed (Fig 1). This sequence alteration, located 11 bases to the 5' side of the deletion made in pD17, was designed to change one phenylalanine codon (UUC) for another (UUU). This provides a silent marker for analysis of recombination events involving pKPTWO and/or its replicase mRNA. Results from Table 1 showed that pKPTWO had the ability to complement replicase function in Rep^- Qß mutants of pD17 and pN8.

To investigate recombination, 5 individual Rep^- plaques of both the deletion (pD17) and insertion (pN8) mutant were isolated and

amplified to high titer by two subsequent rounds of infection on *E. coli* Q13(pKPTW0). Samples 1-5 were mutant phage isolates obtained from the pD17 transfection, and samples 6-10 represented isolates resulting from pN8 (Table 2). Titers of Rep^+ and Rep^- Qß phage present in all rounds of amplification were then determined on Q13 and Q13(pKPTW0) lawns, respectively. Table 2 shows the phage titers that were determined for each sample following each round of amplification.

The results demonstrated that each of the 10 samples contained significant numbers of Rep^+ revertants following amplification of the mutant phages (Table 2) just as in the preliminary experiments. Plaques from Round 1 samples, were spot-tested and shown to be free of revertants prior to the experiment presented in Table 2. Samples 1-10, which showed no revertants in round 1 showed ca. 10^2 to 10^5 Rep^+ revertants after only one additional cycle of infection in Q13(pKPTWO). The overall number of Rep^- phage in each sample also increased substantially during round 2 amplification (ca. 4 orders of magnitude). Rep^+revertants phage were observed in the round 2 samples at a frequency which ranged from 2×10^{-6} (sample 3) to 3×10^{-9} (sample 4). Round 3 of amplification appeared to increase the numbers of both Rep^- and Rep^+ Qß in each sample about 10-fold relative to round 2 isolates, but did not significantly alter the frequency of Rep^+ occurrence in any of the samples. It was thus concluded that reversion to the Rep^+

TABLE 2

Phage Titers Resulting from Amplification of Qß Rep⁻ Mutants [a]

Sample	Round 1		Round 2		Round 3	
	Rep+	Rep⁻	Rep+	Rep⁻	Rep+	Rep⁻
1	0	8.0×10^6	4.4×10^3	2.2×10^{10}	4.0×10^4	1.2×10^{12}
2	0	9.1×10^7	3.1×10^3	6.0×10^{11}	6.4×10^4	4.5×10^{12}
3	0	3.0×10^7	2.0×10^5	3.6×10^{11}	2.4×10^6	8.0×10^{12}
4	0	4.5×10^7	1.5×10^3	6.7×10^{11}	1.0×10^4	2.6×10^{12}
5	0	4.2×10^7	2.4×10^3	5.9×10^{11}	3.1×10^4	3.5×10^{12}
6	0	1.9×10^7	1.0×10^3	2.6×10^{11}	1.0×10^4	9.4×10^{12}
7	0	5.8×10^7	7.5×10^2	2.8×10^{11}	3.1×10^3	3.4×10^{12}
8	0	1.0×10^8	1.0×10^3	3.8×10^{11}	2.0×10^4	8.9×10^{12}
9	0	5.0×10^7	4.6×10^3	1.3×10^{11}	7.7×10^4	9.9×10^{11}
10	0	3.6×10^7	2.4×10^3	3.4×10^{11}	2.9×10^4	6.2×10^{12}

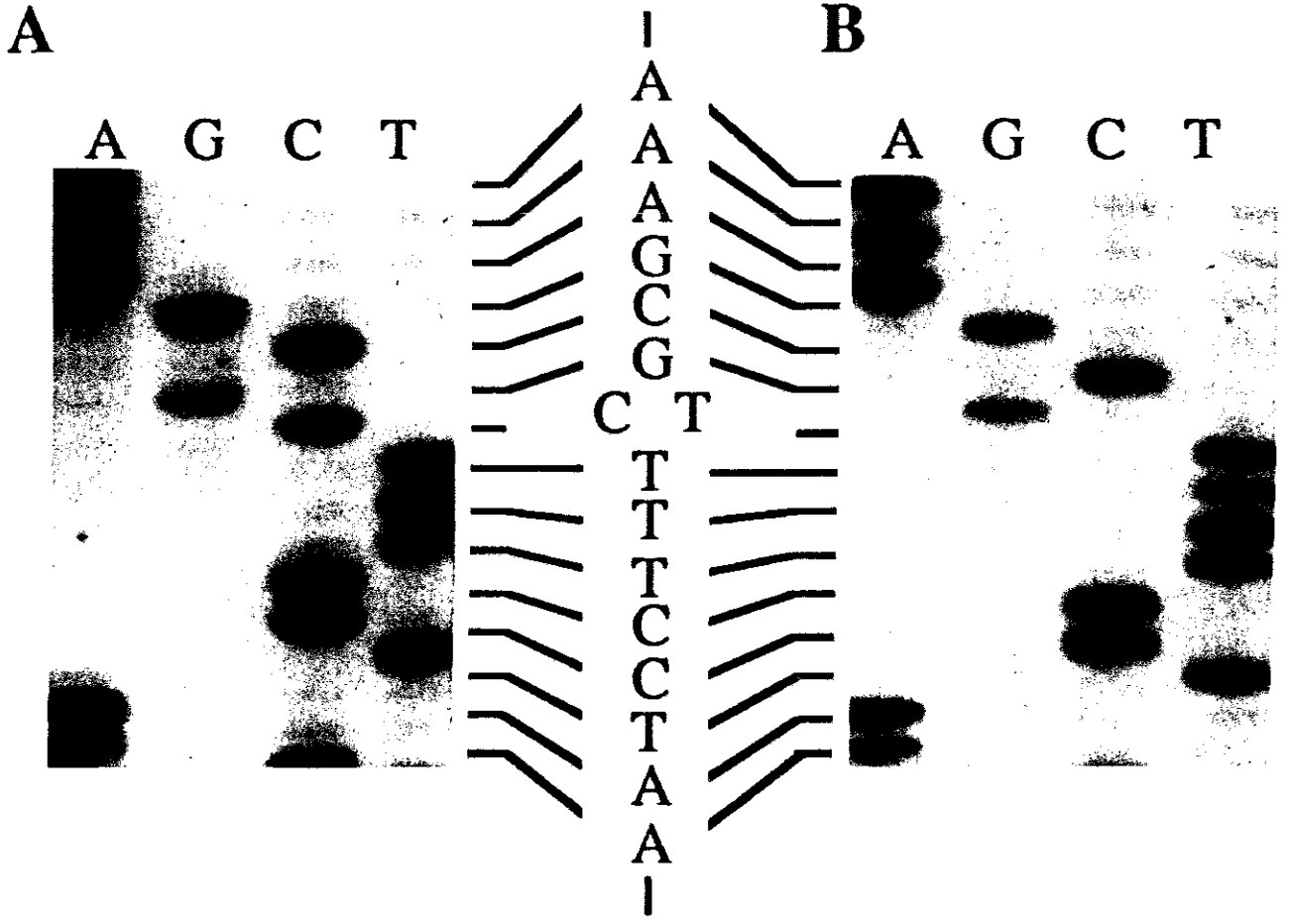
[a] Data represent the total PFU of each type of phage present in each sample following the round of amplification indicated. Although *rep* $^+$ phage were not found in any of the samples where "0" is indicated, only 100 µl of the undiluted sample was assayed in each case. The possibility exists that some *rep* $^+$ phage were present in other aliquots of these samples.

phenotype was a common feature of Rep⁻ Qß mutants and occurred at a similar, but variable frequency in both sets of mutants analyzed (pD17 and pN8).

Two revertant plaques from each isolate following round 2 amplification (single plaques on Q13 cell lawns) were then amplified to high titer on *E. coli* Q13. The phage and genomic RNA were isolated and sequenced in the area of the mutations. The sequence of one such sample, as well as the sequence determined from a separately derived wild-type Qß RNA, is presented in Figure 3. The results showed that the point mutation originally introduced into plasmid pKPTWO was now present (rescued) in the RNA sequence of all of the 20 samples sequenced. In addition, the 17 base deletion of pD17 and the 8 base insertion of pN8 were corrected (repaired) in the sequences determined for these revertants (not shown). Since the point mutation was originally placed only in plasmid pKPTWO, the data showed that recombination with the plasmid or with its replicase mRNA during round 2 amplification was responsible for the reversion of these Rep⁻ Qß mutants. Thus the data supported a recombination hypothesis, but did not support a hypothesis involving contamination with wild-type Qß phage.

Requirement for recombination and reversion of Qß mutants. The results from Table 2 and the sequence analysis showed that recombination occurred at a regular frequency during

Figure 3. Genome sequence analysis in mutated region of phage Qß RNAs. (A) Sequence of wild-type Qß genomic RNA. (B) Sequence of a revertant Qß genome isolated as described in the text. Note that although anti-sense DNA was synthesized in the reverse transcriptase reactions of (A) and (B), the autoradiograph of the gel was rotated 180° for this representation in order to show a "sense" DNA sequence. (C) The DNA nucleotide sequence obtained in (A) and (B) is indicated along with the amino acids encoded by the corresponding bases in Qß genomic RNA. Nucleotide base numbers were assigned according to Mekler (20).



C

3787 3801

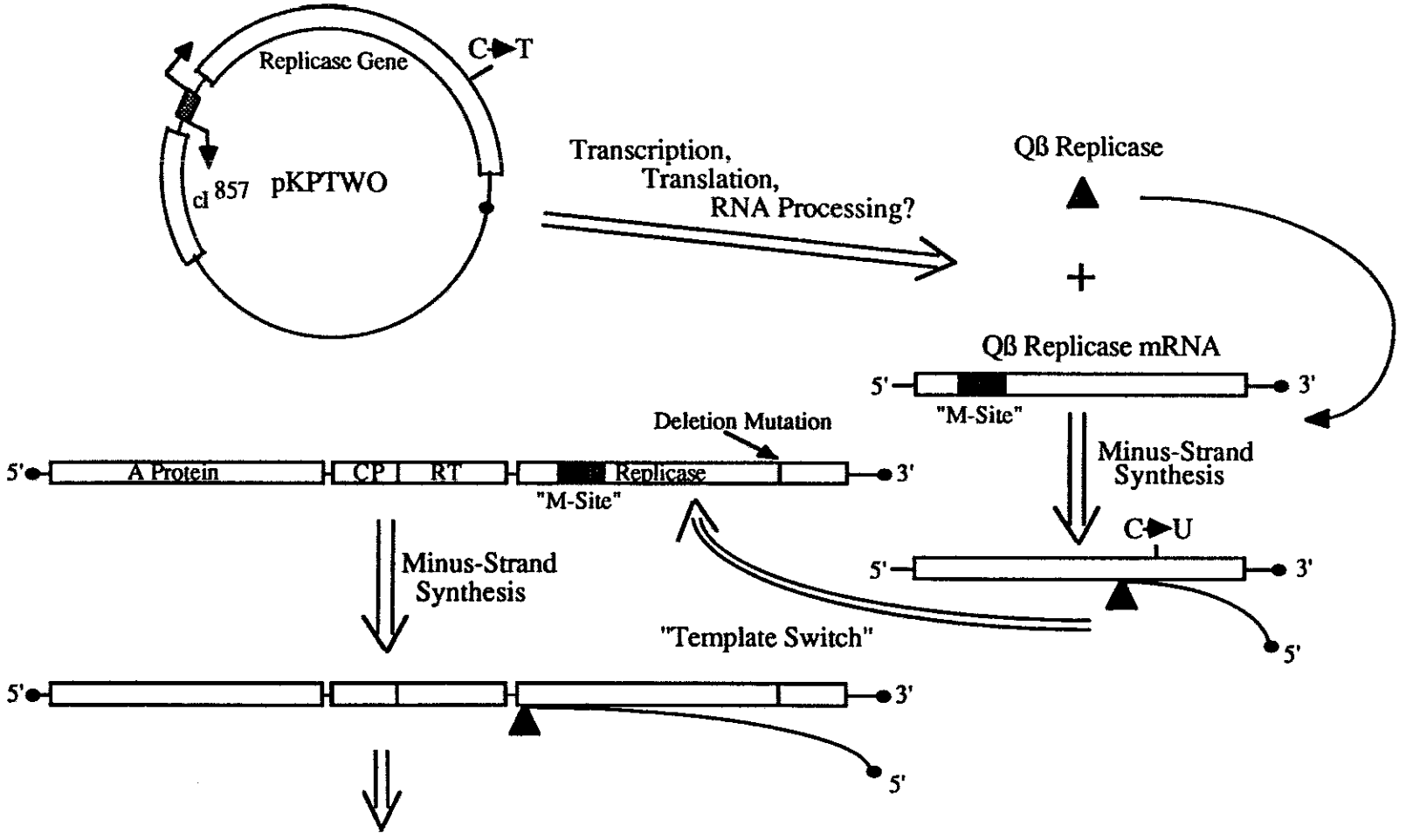
— AAT CCT TT C/T GCG AAA —

Asn Pro Phe Ala Lys

Rep$^-$ phage amplification. The mechanism by which this recombination occurred however, was not determined. Infectious mutant phage were produced because replicase was provided in *trans*. The replicase, translated from the plasmid, transcribes plus-strands from the minus-strand Qß RNA. Subsequently, minus-strands are transcribed from the 3' terminus using the plus-strand RNA as template (3). In this fashion mutant phage were subsequently produced in large quantities. Based on this replication mechanism, a recombination mechanism was proposed (Fig. 4). According to this model, if recombination is to occur, transcription of the minus-strand will initiate on the plasmid derived mRNA of the replicase gene and subsequently switch templates to a homologous (complementary) region of the Rep$^-$ phage genome beyond the lethal deletion or insertion mutation. The resultant recombinant RNA subsequently produces Rep$^+$ phages. Template switching mechanisms similar to this have been proposed in polio virus and other RNA viruses (14, 17, 18). Figure 4 depicts a model showing how template switching by Qß replicase could result in the recombinant, revertant phage obtained in our experiment.

In order to test the proposed recombination mechanism, a deletion was made in the 3' non-coding region of the replicase expression plasmid pKPTWO. Figure 1 C depicts the resultant plasmid, pPSTWO, and indicates the 80-base region deleted from the 3' end of the replicase cDNA. The 3' non-coding region has been described

Figure 4. Template switching model for recombination/reversion of Qß rep$^-$ mutants. Following translation and assembly, Qß replicase (RNA-directed RNA-Polymerase) from pKPTWO binds to an internal binding site ("M-Site") and the 3' non-coding region of a template (36) and initiates transcription of minus-strands. In genetic RNA recombination however, the Qß replicase binds to the Qß replicase mRNA of pKPTWO and initiates minus strand synthesis. The replicase, with nascent minus-strand attached, then switches templates to the mutant Qß genome beyond the deletion mutation.



Synthesis *in vivo* of Recombinant Minus- and Positive-Strand Qß RNAs

as a necessary region for infection (24) and minus-strand transcription cannot be initiated if the 3' non-coding region in not present (36). pPSTWO allows normal translation of replicase from its mRNA but, no viable minus-strands can be initiated by the replicase from its 3' terminus.

As in earlier experiments, 5 individual Rep^- plaques of both the deletion (pD17) and insertion (pN8) mutant were isolated and amplified to high titer by two subsequent rounds of infection on *E. coli* Q13/pPSTWO. Samples 11-15 were mutant phage isolates from pD17, while samples 16-20 were from pN8 (Table 3). Titers of Rep^+ and Rep^- present in each sample following each of the 3 rounds of amplification were then determined as before. Table 3 shows the phage titers that were determined following amplification of these samples on Q13/pPSTWO cells.

The results of Table 3 showed some very interesting results. The amplification of Rep^- phages in samples 11-16 between round 1, round 2 and round 3 was similar to that of table 2 (pKPTWO) but, there were no Rep^+ revertants observed in any of these isolates. Revertants were observed however, in all of the insertion mutant samples (16-20). While the overall numbers of Rep^- Qß phage in the pN8 isolates also increased substantially during rounds 2 and 3 of amplification (similar to the pD17 samples), Rep^+ phage were observed in each of the latter samples.

TABLE 3

Phage Titers Resulting from Amplification of Qß Rep⁻ Mutants [a]

Sample	Round 1		Round 2		Round 3	
	Rep+	Rep⁻	Rep+	Rep⁻	Rep+	Rep⁻
11	0	3.3×10^6	0	4.4×10^{10}	0	1.3×10^{12}
12	0	2.0×10^7	0	1.4×10^{11}	0	2.8×10^{11}
13	0	4.1×10^7	0	1.1×10^{11}	0	2.3×10^{12}
14	0	2.4×10^7	0	1.6×10^{11}	0	5.0×10^{11}
15	0	6.5×10^6	0	4.8×10^{10}	0	8.8×10^{10}
16	0	6.2×10^6	5.1×10^2	1.4×10^{10}	2.2×10^3	3.0×10^{11}
17	0	1.2×10^6	2.1×10^2	2.0×10^{11}	2.1×10^3	3.4×10^{11}
18	0	4.2×10^7	2.1×10^4	7.7×10^{10}	1.9×10^5	2.6×10^{11}
19	0	1.8×10^6	4.5×10^2	1.1×10^{11}	1.1×10^3	5.8×10^{11}
20	0	7.8×10^6	7.0×10^4	2.1×10^{11}	1.0×10^5	3.8×10^{11}

[a] Data represent the total PFU of each type of phage present in each sample following the round of amplification indicated. Although *rep* $^+$ phage were not found in any of the samples where "0" is indicated, only 100 µl of the undiluted sample was assayed in each case. The possibility exists that some *rep* $^+$ phage were present in other aliquots of these samples.

The frequency of occurrence of Rep^+ revertants in these round 2 samples, relative to Rep^- phage in the same isolate were comparable to that seen previously (Table 2), ranging from 3×10^{-7} (sample 20) to 1×10^{-9} (sample 17). Round 3 again, appeared to increase the numbers of both Rep^- and Rep^+ Qß in each sample, by about 10-fold relative to round 2 isolates, but did not significantly alter the frequency of Rep^+ occurrence in any of the samples (Table 3).

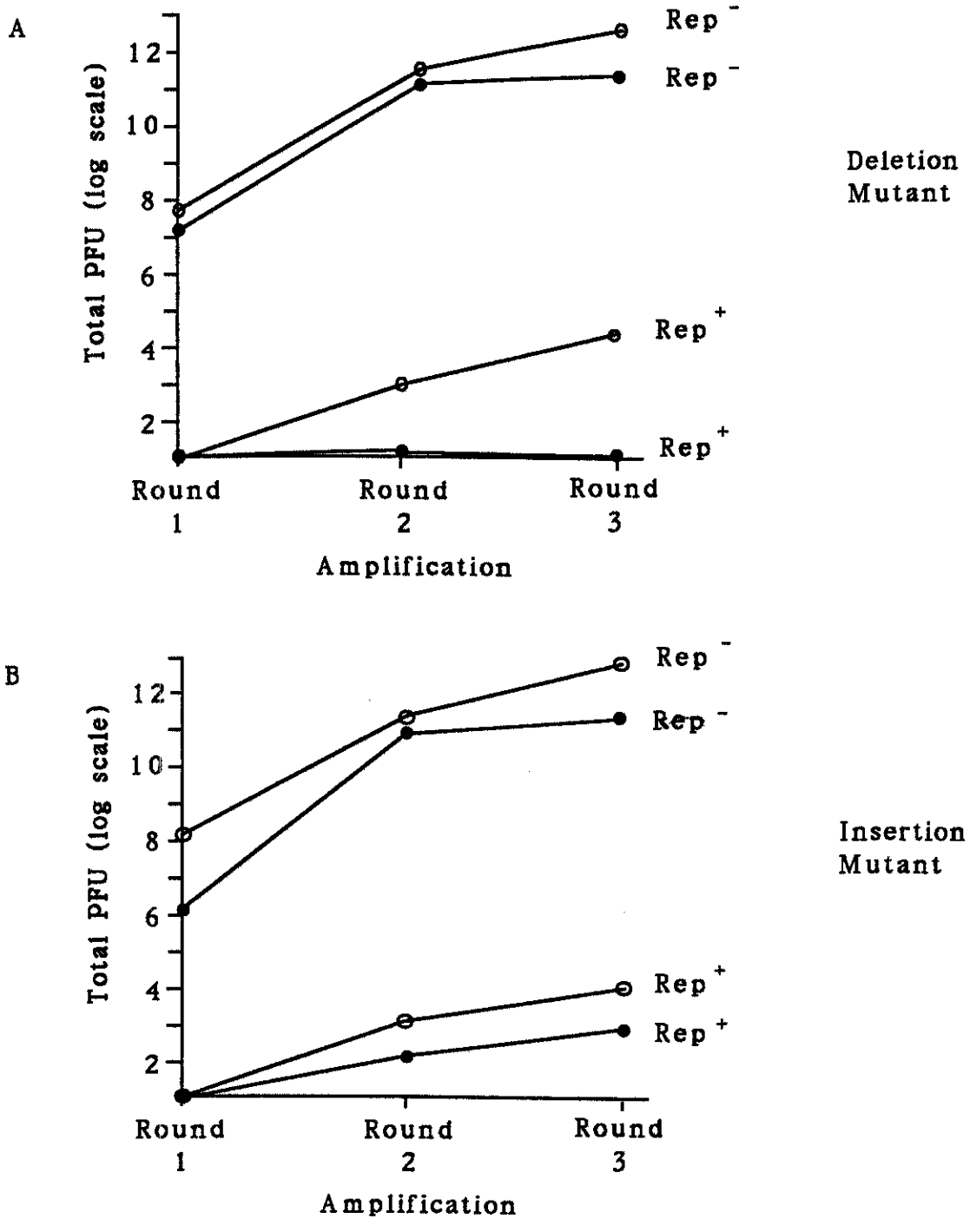
The reversion of of the pN8 phage mutants was unanticipated (samples 16-20). Two revertant phage from each of samples 16-20 (isolated from single plaques on Q13 cell lawns) were amplified to high titer on *E. coli* Q13, total RNA isolated, and the nucleotide sequence determined in the area of mutation. Of the 10 revertants sequenced in this fashion, 7 contained the wild-type sequence (20). This result suggested that another mechanism, such as deletion of the 8 base insert, was responsible for reversion of these samples. The other 3 revertants (1 from sample 16, 2 from sample 20) were found to contain the silent point mutation, C to U, present as a marker in the pPSTWO plasmid. It thus appears that recombination was involved in their generation. The specific mechanism responsible for the generation of these latter phage is not completely understood, but it appears to be a distinct recombination mechanism from that presented in Figure 4.

DISCUSSION

The marker mutation rescued in all the revertants from Table 2 is a clear indication of recombination in the Qß system. In spite of literature suggesting that RNA phage do not undergo genetic recombination (12), non-homologous recombination between Qß RNA and $tRNA^{Asp}$ has been observed (25, 26). In addition, a wide variety of plant and animal RNA viruses have now been demonstrated to undergo recombination (1, 2, 4, 10, 14 17, 18, 37). The occurance of homologous and non-homologous recombination is also well documented in retroviruses and template-switching events have been described in their replication and recombination (14, 35). There appears to be an universal mechanism under-lying both homologous and non-homologous recombination among RNA viral systems. Copy choice or template-switching has been reported to be an inherent activity of viral RNA-dependent RNA polymerases (5, 7, 10, 14, 16, 17, 27). Results from the present experiments suggest that homologous recombination in bacteriophage Qß occurs by a similar template-switching mechanism (Fig. 4).

Figure 5 is a replot of data combined from Tables 2 and 3. Samples 2 and 12 were chosen as typical representatives for the deletion mutants, while samples 8 and 17 were the representatives

Figure 5. Replot of data from Tables 2 and 3



Open circles are data from Table 2 pKPTWO amplification (samples 2 and 8).
Closed circles are data from Table 3 pPSTWO amplification (sample12 and 17).

chosen for the insertion mutants. The reversion events noticed in Table 2 and 3 for each mutant isolate were observed in the second round of amplification. This fact is a direct consequence of there being nearly equal numbers of phage and bacteria present at the beginning of this amplification [ca. 1 x 10^8 Rep$^-$ phage measured as round 1 titer, and ca. 2 x 10^8 bacteria present in 1 ml of early-log culture (19)]. Thus, a near maximum number of infections are occurring during round 2 of infection and this is reflected in the nearly 4-log increase in Rep$^-$ phage present at the end of the amplification [estimates of the Qß burst size range from 10,000 to 40,000 (38)]. Round 3 amplification, in contrast, only provided a 10-fold increase in infectable bacteria and thus only a 10 fold rise in phage titer was observed following this amplification. Reversion of the deletion mutants (Fig. 5A) was exactly as predicted from the model presented in Figure 4. Results presented here are the first demonstration of "homologous" RNA phage recombination, since only homologous recombination could provide the deletion mutant of Qß with an intact replicase gene and explain other aspects of our results . The 17 base deleted from pD17 was exactly repaired in each of the revertants sequenced (samples 1-5). It is only by homologous recombination that deleted regions can be exactly repaired. Additionally, based on the template switching model, recombination could be eliminated or significantly reduced if the 3' transcription initiation region was eliminated from the Qß replicase mRNA of

pPSTWO. Results from Table 3 (Fig. 5A) showed no revertants when these samples were amplified on Q13(pPSTWO) and this indicates that deletion mutants recombine-revert by the template-switching mechanism shown in Figure 4.

Reversion of the insertion mutants (Fig. 5B), on the other hand, appeared to occur by other mechanisms including the template-switching recombination mechanism of Figure 4. The marker rescue and the perfect deletion of the deleterious insertion (Table 2), seems to suggest a recombinant type reversion. However, reversion at a frequency not significantly lower was seen upon removal of the plasmid mRNA 3' non-coding region of the replicase gene (compare Tables 2 and 3, and Fig. 5B). Thus, other mechanisms such as deletion must have been involved in the the reversion of samples 16-20. Sequence analysis of several revertants from these samples showed a perfect deletion of the inserted 8 base insertion (seven of ten samples), without the presence of the marker mutation, supporting this contention. However, three revertants obtained from samples 16 and 20 contained the marker point mutation (Table 3) indicating recombination was involved in their generation, rather than (or in addition to) deletion. Although deletion of the inserted sequence was expected to contribute to the reversion of these mutants, it was not predicted that the frequency of occurrence of the two different reversion pathways (deletion vs. recombination) would be so similar

(compare Tables 2 and 3, and Fig. 5 b). Also no explanation can be provided for the observation of recombination in the insertion mutants amplified on Q13(pPSTWO). It is interesting to speculate however, that generation of single revertant in this case could stimulate further recombination events, including double-crossovers not observed with the deletion mutants (samples 11-15 and Table 3). This would provide an explanation for the observation of recombinant phage in samples 16 and 20 (Table 3) and for the fact that only recombinant phage (no wild-type sequences) were noted in the 5 revertants sequenced from samples 6-10 (Table 2).

RNA viruses are well known for their extremely high mutation rates (14, 17). Thus, in the absence of recombination, the potential exists for deleterious mutations to accumulate, leading to decreased fitness of the population. The ability to recombine may thus be advantageous in repairing lesions created by mutations (14, 17). It could be argued that RNA phage recombination, as reported here, is a low-frequency event and would thus not significantly contribute to RNA phage evolution and/or phage genome stabilization. RNA recombination frequencies reported in Table 2 and 3 are probably an underestimate of the actual recombination frequencies in nature however, since it must be noted that in the preliminary experiments, about sixty percent of the samples showed spontaneous reversion when spot tested for Rep^+. By choosing isolates for the latter experiments that did not revert spontaneously on the spot test, in

effect, selective pressure was used to to select strains that showed slow reversion. In spite of this prejudice in our experimental design, RNA recombination in this system took only 3 hours (one cycle of infection). This, plus the fact, that there are tremendous numbers of *E. coli* and RNA phage in nature (9), and that both homologous and non-homologous recombination have been demonstrated in Qß, supports the contention that RNA recombination could make a significant contribution to both the genetic alteration and repair of RNA phage genomes.

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