Mechanics of Frameshift Mutagenesis in Bacteriophage T4: Role of Chromosome Tips*

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Communicated by S. Spiegelman, December 29, 1969

Abstract. In contrast to observations in bacteria and fungi, frameshift mutations in bacteriophage T4 do not arise during genetic recombination. Nascent mutants, captured in the heterozygous condition, exhibit properties which indicate that the new lesions are located at the extreme tips of the chromosomes, and are segregated by a combination of recombination and replication.

Frameshift mutations consist of additions and deletions of small numbers of base pairs, and may involve from 1 to at least 20 residues. They are efficiently induced and reverted by various acridines, and appear first in a heterozygous condition. The mutagenic acridines intercalate between adjacent base pairs. Inhibition of DNA replication by 5-fluoro-deoxyuridine, however, did not abolish proflavin mutagenesis in bacteriophage T4, although it effectively eliminates base analog mutagenesis.

Acridines are highly mutagenic for bacteriophage T4, but are very weak mutagens for bacteriophage λ and for bacteria. T4 is a rapidly recombining organism, whereas is not. It was therefore suggested that acridines produce frameshift mutations by inducing unequal crossing over. This hypothesis was supported by the discovery of correlations between frameshift mutagenesis and diploidy in the bacterium Escherichia coli, and between frameshift mutagenesis and recombination of outside markers in the yeast Saccharomyces cerevisiae. The correlation was less evident in T4, however: newly arisen frameshift mutations appeared to be associated with recombination of outside markers, but these markers were recovered in the heterozygous condition, and the heterozygous regions were much longer than typical recombinational overlaps. Furthermore, results to be presented here show a striking lack of correlation in T4 between frameshift mutagenesis and recombination of very close outside markers. It should also be emphasized that contemporary theory of the mechanics of recombination does not easily encompass a process of unequal crossing over directed by agents which intercalate between adjacent base pairs.

Since frameshift mutants frequently revert by intracistronic suppressor mutations, the revertant polypeptides may contain multiple amino acid substitutions, including deletions and additions. Comparisons of wild-type and re-
vertant amino acid sequences permit partial reconstructions of the corresponding mRNA sequences.\textsuperscript{23, 24} A close examination of such mRNA sequences suggested that frameshift mutations tend to arise near localized base pair sequence redundancies.\textsuperscript{23} A specific model of frameshift mutagenesis, involving errors of repair, was offered to explain this result. In this model, the seed event consists of a strand interruption in a double-stranded DNA molecule, and the final event consists of the closure, by repair enzymes, of a locally mispaired configuration. The mutagenic acidines were further postulated to stabilize the (spontaneously) mispaired region, thus promoting its fixation before a return to the normal configuration could occur.

This scheme explains the ability of ultraviolet irradiation to induce frameshift mutations,\textsuperscript{25}--\textsuperscript{27} since photochemical lesions lead both to excision repair\textsuperscript{28} and to gap production during DNA replication.\textsuperscript{29} The greatly increased mutagenicity of acidines bearing an alkylating group\textsuperscript{6} may also be explained by supposing that coupling is thereby achieved between alkylation repair and acridine action.

The model also explains correlations between frameshift mutagenesis and recombination, in which strand interruptions necessarily occur.\textsuperscript{22} The lack of such a correlation in T4, however, requires a special explanation. T4 chromosomes are circularly permuted, the chromosomal termini occupying all positions on the genetic map.\textsuperscript{30, 31} These ends may represent exceptionally long-lived strand interruptions, where frameshift mutations may arise with unusually high frequencies. The resulting mutational heterozygotes would be complex, however, because T4 chromosomes also exhibit terminal redundancy.\textsuperscript{31} The length of the redundancies, corresponding to several thousand base pairs,\textsuperscript{15, 32, 33} easily explains the extensive heterozygosity observed in the vicinity of newly arisen frameshift mutations when markers are present.\textsuperscript{9} The main purpose of the present study is to probe the structure of these mutational heterozygotes.

Materials and Methods. Escherichia coli and bacteriophage T4B were used throughout; most strains have been described previously.\textsuperscript{25} In addition to KB cells, the following λ lysogens were used: the highly rII-restrictive strain OP33 from Dr. R. Friedman; and the amber-suppressing strain QA1 and ochre-suppressing strain CA165 from Dr. S. Brenner.

Unless otherwise indicated, experiments were performed at 37°C using L broth (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.1% glucose, and 1% NaCl), or M9CA (0.3% KH\textsubscript{2}PO\textsubscript{4}, 0.6% Na\textsubscript{2}HPO\textsubscript{4}, 0.1% NH\textsubscript{4}Cl, 0.4% glucose, 0.0133% MgSO\textsubscript{4}, 0.4% Difco casamino acids, and 10\textsuperscript{−4} M FeCl\textsubscript{3}). L broth was adjusted to pH 7.8 for experiments involving proflavin mutagenesis. The bottom agar for plaque assays contained 1% tryptone, 0.1% yeast extract, 0.02% glucose, 0.5% NaCl, and 1% agar; the top agar contained 1% tryptone, 0.233% yeast extract, 0.0466% glucose, 0.765% NaCl, and 0.65% agar. T4 stocks were grown and assayed on BB cells. Revertant and recombinant r\textsuperscript{+} particles among an excess of r particles were scored using KB, QA1, or CA165 cells as selective indicators.

Proflavin dihydrochloride was obtained from Mann Research Laboratories. 5-Fluorodeoxyuridine (F\textsubscript{d}U\textsubscript{R}) was a gift from Hoffmann-LaRoche, Inc.

Results and Discussion. Tests for a correlation between frameshift mutagenesis and recombination: The first test was designed to reveal coincidences between the reversion of a frameshift (fs) mutation and the recombination of a pair of very closely linked outside amber markers (Table 1).\textsuperscript{34} Because of the
presence of "barriers" between \( fs \) and each of the outside markers,\(^2 \) reversion can only occur by frameshift mutations between \( am1 \) and \( am2 \), and the amber markers themselves cannot be rendered invisible by a reading frameshift.\(^3\) Both crosses were performed under identical conditions and in the presence of proflavin, which stimulates recombination in \( T4 \).\(^8 \) In the absence of correlation between recombination and frameshift mutagenesis, \( \frac{(am1{^+}fs^{+}am2^{+})/(am1^{+}am2^{+})}{fs^{+}} = 1 \). We observed a value of 0.94; values from about 5 to 700 were observed in \( S.\) \( cerevisiae \).\(^1\)

**Table 1. Test for correlation between frameshift mutagenesis and recombination.**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Product</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>( am1 fs am2^{+} \times am1^{+} fs am2 )</td>
<td>( am1^{+}fs^{+}am2^{+} )</td>
<td>( 1.02 \times 10^{-7} )</td>
</tr>
<tr>
<td>( am1 am2^{+} \times am1^{+} am2 )</td>
<td>( am1^{+}fs^{+}am2^{+} )</td>
<td>( 6.56 \times 10^{-4} )</td>
</tr>
<tr>
<td>( am1^{+}am2^{+} )</td>
<td>( am1^{+}am2^{+} )</td>
<td>( 1.66 \times 10^{-2} )</td>
</tr>
</tbody>
</table>

The double mutants \( rEM84\rUV353 \) (\( am1 fs \)) and \( rUV353\rHB233 \) (\( fs am2 \)) were constructed by recombination; \( fs \) is the central mutation. Log phase BB cells were infected with an average of five of each \( T4 \) parental type. At \( t = 3 \) min, anti-\( T4 \) serum was added to inactivate unadsorbed particles. At \( t = 8 \) proflavin was added to 10 \( \mu \)g/ml, and chloramphenicol to 250 \( \mu \)g/ml. At \( t = 18 \) the complexes were washed with cold broth on millipore HA filters, and resuspended in warm broth. They were incubated for an additional 30 min and then lysed with chloroform. The lysates were plated on QA1 cells to assay \( f s^{+} \) revertants regardless of their outside marker configuration (\( am1^{+}fs^{+}am2^{+} \)), on EB or OP33 cells to measure \( am1^{+}fs^{+}am2^{+} \) and \( am1^{+}am2^{+} \), and on BB cells to measure total particles. Between 14 and 52% of \( f s^{+} \) revertants are distinguishable as false revertants by their plaque morphology, depending upon which cells are used for selective plating. Plaque morphology tests were therefore performed upon adequate samples of all revertant populations, and the calculations employed only the frequencies of apparently wild-type revertants. We have also corrected for small differences in efficiencies of plating on the various indicators.

A qualitatively different test was performed by a method which reduces recombination frequencies in a local region of the genome.\(^30 \) If a cross is made between a minority parent (one particle per cell) and a majority parent (many particles per cell), most mating events involving the minority parent will also involve the majority parent. If the majority parent carries a deletion shorter than the average length of the homologous pairing region, then recombination within the region of the minority parent corresponding to the deletion is sharply reduced. The crosses are described in Table 2. Because of the presence of "barriers,"\(^2 \) \( rUV353^{+} \) revertants cannot arise from frameshift mutations outside of the region covered by the very short deletion \( r196 \). The very long deletion

**Table 2. Frameshift mutagenesis with suppression of recombination.**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Multiplicities</th>
<th>Burst sizes</th>
<th>Revertant Frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
<td>Corrected</td>
</tr>
<tr>
<td>( rUV353 )</td>
<td>11.6</td>
<td>83</td>
<td>( 2.2 \times 10^{-4} )</td>
</tr>
<tr>
<td>( rUV353 )</td>
<td>0.39</td>
<td>97</td>
<td>( 2.5 \times 10^{-4} )</td>
</tr>
<tr>
<td>( rUV353 \times r196 )</td>
<td>0.39 + 9.9</td>
<td>38</td>
<td>( 5.1 \times 10^{-7} )</td>
</tr>
<tr>
<td>( rUV353 \times r1272 )</td>
<td>0.39 + 11.2</td>
<td>77</td>
<td>( 7.0 \times 10^{-7} )</td>
</tr>
</tbody>
</table>

At \( t = 6 \) min after infection of log phase BB cells, proflavin was added to 8 \( \mu \)g/ml. At \( t = 20 \) the complexes were diluted 80-fold into broth. After 30 min of incubation lysis was completed with chloroform. Revertants were assayed on OP33 cells, and total particles on BB cells. The frequency of revertants was about \( 1.6 \times 10^{-4} \) in the \( rUV353 \) stock, and was less than \( 3 \times 10^{-5} \) in the deletion mutant stocks. Observed revertant frequencies were corrected for dilution of \( rUV353 \) by the majority parent by assuming that the relative yield of \( rUV353 \) was equal to its relative input; thus \( (5.1 \times 10^{-7})(0.39 + 9.9)/0.39 = 1.4 \times 10^{-4} \).
r1272 is considerably longer than the average length of the pairing region, and does not inhibit recombination in the vicinity of the frameshift mutation carried by the minority parent. When the crosses were performed in the presence of proflavin, reversion of the frameshift mutation was not significantly inhibited by the majority parent carrying the short deletion. (A small reduction was observed with r196, but this was also associated with a reduced burst size, and may reflect a fortuitously deleterious effect of the stock. Full coupling between frameshift mutagenesis and recombination would have produced an approximately 20-fold reduction in the corrected revertant frequency.) We therefore conclude that frameshift mutation in T4 is rarely a consequence of recombination.

**Properties of frameshift mutational heterozygotes:** T4 can exhibit heterozygosity because of terminal redundancy, strand noncomplementarity, or "clumping" (particles exhibiting anomalously large sedimentation coefficients, but not otherwise characterized). We wished to determine whether frameshift mutational heterozygotes resemble terminal redundancy or internal heteroduplex heterozygotes (Fig. 1).

![Diagram](https://via.placeholder.com/150)

**Fig. 1.**—Heterozygotes of bacteriophage T4. Parallel horizontal lines represent complementary DNA strands. Cistrons are delineated by parallel vertical lines. A mutant configuration is indicated by a dot, the wild type by +. The mutational heterozygote depicted would result from forward mutation, not reversion.

The T4 rII region must function early in the latent period if the rII+ phenotype is to be expressed. It is therefore likely that r/r+ internal heteroduplex heterozygotes, if formed randomly with respect to the strand containing the r allele, will function successfully half the time (when the r+ allele is located on the transcribed strand). Terminal redundancy r/r+ heterozygotes, on the other hand, should always function successfully, except when the end of the chromosome falls within the r+ cistron. Internal heteroduplex heterozygotes were constructed in several r × r+ crosses using point r mutants together with FUdR to inhibit replication-dependent segregation of the recombinational heterozygotes. Terminal redundancy heterozygotes were constructed in the cross r × r+ using a deletion r mutant. Some of these lysates were centrifuged through D2O/broth gradients in order to remove "clumping" heterozygotes. Frameshift mutational heterozygotes were constructed by infecting cells with wild-type T4, treating briefly with proflavin, and harvesting the first particles to be matured after the end of the proflavin treatment. The ability of each type of heterozygote to function in a restrictive host was tested by preadsorbing the lysates either to permissive B cells or to nonpermissive KB cells at very low particle-to-cell ratios, and scoring heterozygotes by their characteristic mottled plaque morphologies.
The results appear in Table 3. As expected, about half the internal heteroduplex heterozygotes\(^3\) and nearly all the terminal redundancy heterozygotes multiplied in the nonpermissive cells. All the proflavin-induced mutational heterozygotes multiplied in the nonpermissive cells. We therefore conclude that most frameshift mutations in T4 arise at or near the ends of chromosomes (Fig. 1). The \(r\) mutants induced by proflavin are predominantly \(r\)II, and tests of the mutational heterozygotes appearing in this experiment revealed that about 75 per cent contained \(r\)II mutants not susceptible to reversion by base analogs.

If “heterozygotes” (mottled plaque formers) were to arise because of mutations induced during the first cycle of growth in B or KB cells by proflavin carried

<table>
<thead>
<tr>
<th>Type of heterozygote</th>
<th>Mutant</th>
<th>Heterozygote Frequency after Preadsorption on</th>
<th>Transmission coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recombinational heteroduplex</td>
<td>UV187 (A:T)</td>
<td>0.059 (1099)</td>
<td>0.032 (1021)</td>
</tr>
<tr>
<td></td>
<td>UV1 (A:T)</td>
<td>0.106 (139)</td>
<td>0.054 (182)</td>
</tr>
<tr>
<td></td>
<td>UV2 (fs)</td>
<td>0.086 (177)</td>
<td>0.054 (195)</td>
</tr>
<tr>
<td></td>
<td>UV13 (G:C)</td>
<td>0.132 (102)</td>
<td>0.057 (216)</td>
</tr>
<tr>
<td></td>
<td>UV44 (fs)</td>
<td>0.088 (53)</td>
<td>0.040 (127)</td>
</tr>
<tr>
<td></td>
<td>UV55 (G:C)</td>
<td>0.135 (85)</td>
<td>0.077 (148)</td>
</tr>
<tr>
<td></td>
<td>UV76 (A:T)</td>
<td>0.112 (74)</td>
<td>0.062 (101)</td>
</tr>
<tr>
<td></td>
<td>UV109 (A:T)</td>
<td>0.101 (225)</td>
<td>0.067 (203)</td>
</tr>
<tr>
<td></td>
<td>UV183 (A:T)</td>
<td>0.084 (99)</td>
<td>0.046 (157)</td>
</tr>
<tr>
<td></td>
<td>UV279 (A:T)</td>
<td>0.100 (261)</td>
<td>0.057 (293)</td>
</tr>
<tr>
<td>Terminal redundancy</td>
<td>168 (deletion)</td>
<td>0.0038 (90)</td>
<td>0.0033 (191)</td>
</tr>
<tr>
<td>Profavin mutational</td>
<td>—</td>
<td>0.00061 (65)</td>
<td>0.00067 (55)</td>
</tr>
</tbody>
</table>

**Recombinational heteroduplex heterozygotes:** Log phase B cells were suspended in M9CA supplemented with 25 \(\mu\)g/ml l-tryptophan, 20 \(\mu\)g/ml FUDR, and 40 \(\mu\)g/ml uracil. After 2 min at 37\(^o\)C they were infected with an average of about five particles each of T4\(^{r}\) and of rII mutant (fs = frameshift; A:T = adenine-thymine base pair at the mutational site; G:C = guanine-5-hydroxymethylcytosine base pair). After 9 more min, chloramphenicol was added to 200 \(\mu\)g/ml. After 3 hr the complexes were chilled, washed twice by centrifugation, and resuspended at 10\(^6\)/ml in M9CA containing 12 \(\mu\)g/ml FUDR and 24 \(\mu\)g/ml uracil. After 60 min they were lysed with chloroform.

**Terminal redundancy heterozygotes:** Log phase B cells in broth were infected with an average of five particles each of \(r\) and an rII deletion mutant. After 30 min lysis was completed with chloroform.

**Profavin mutational heterozygotes:** Log phase BB cells in broth were infected with an average of five particles of T4\(^{r}\). At 3 min anti-T4 serum was added to inactivate unadsorbed particles. At 8 min proflavin was added to 10 \(\mu\)g/ml. At 18 min the complexes were washed with cold broth on Millipore HA filters and resuspended at 10\(^6\)/ml in warm broth. After 8 more min they were lysed with chloroform.

**Purification of lysates:** Lysates were centrifuged to remove cell debris. The UV187 and r168 lysates were then concentrated by centrifugation at 30,000 rpm for 60 min at room temperature in a Spincio SW30 rotor. The pellets were resuspended overnight in broth, layered on linear 0 → 100% D2O/broth gradients, and centrifuged for 15 min at 20,000 rpm in the SW30 rotor. Peak 0.2-ml fractions were used.

**Preadsorption:** Log phase cells in M9CA were supplemented with 36 \(\mu\)g/ml l-tryptophan and 0.007 M KCN. After 2 min at 26\(^o\)C, an equal volume of lysate was added (an average of 1–6 \(\times\) 10\(^4\) particles per cell). After 8 min, anti-T4 serum was added to inactivate unadsorbed particles. After 8 more min, 24 \(\mu\)g/ml FUDR and 40 \(\mu\)g/ml uracil were added. Complexes were immediately centrifuged and resuspended in M9CA containing 12 \(\mu\)g/ml FUDR and 24 \(\mu\)g/ml uracil. After 12 min, 200 \(\mu\)g/ml chloramphenicol was added. After an additional 13 min the complexes were centrifuged and resuspended in chilled broth containing 3 \(\times\) 10\(^6\) B cells per ml, and 0.1 ml samples were rapidly plated. Mottled plaques were scored after 15 hr at 37\(^o\).

**Heterozygote frequencies** are expressed per total \(r\) and \(r\)\(^+\) progeny. Total numbers of mottled plaques counted are given in parentheses.
over by treated wild-type phages, then all mutational heterozygotes would appear to multiply in nonpermissive cells. To test this possibility, wild-type particles were soaked for two hours at 44° in broth containing 50 µg/ml proflavin, about five times the usual concentration used in vivo. These particles become partially inactivated (average survival 69%) and extremely sensitive to white light (two 15-w white fluorescent lights 6 in from the samples producing about one lethal hit per minute to survivals on the order of 10⁻⁴), indicating that the proflavin extensively penetrated all of the particles. When 57,800 untreated particles were plated on B cells, 20 r and 10 mottled plaques appeared, of which 13 and 7, respectively, were rII. When 69,100 proflavin-treated but unirradiated particles were plated, 17 r and 12 mottled plaques appeared, of which 10 and 4, respectively, were rII. Mottled plaques are therefore unlikely to be produced by carried-over mutagen.

We have also attempted to examine the properties of mutational heterozygotes associated with the reversion of frameshift mutations. Revertants were induced and assayed using the procedures described in Table 3. All revertants, however, appeared as homozygotes, despite the fact that revertants of base-pair substitution rII mutants are readily captured in the heterozygous condition.

Conclusions. Our results are conveniently interpreted by assuming that frameshift mutations in bacteriophage T4 arise nearly exclusively at the tips of chromosomes in the form of heteroduplex terminal redundancy heterozygotes. For reversion to occur, a chromosome must terminate very near to the mutational site. The resulting r⁺ strand therefore represents only a fragment of a cistron. Before it can function, it must at the very least be recombined into an intact cistron. Since the rII function is required early in the latent period, the hypothetical frameshift reversion mutational heterozygotes are not likely to survive in nonpermissive cells. We imagine that a process like that shown in Figure 2 is required for the generation of homozygous frameshift mutations in bacteriophage T4. The critical role of the tips of T4 chromosomes was predicted by Streisinger et al. 23

**Fig. 2.** Segregation of a T4 frameshift mutational heterozygote. Symbols are as in Fig. 1. The heterozygote depicted would result from reversion, not forward mutation.

The independence of frameshift mutagenesis and recombination in T4 is consistent with the hypothesis that frameshift mutations arise as anomalies of repair. Any agent which leads to strand nicking in DNA should therefore produce frameshift mutations. This is true not only of ultraviolet irradiation and the ICR compounds, but probably also of alkylation and of photodynamic action. These agents, however, are rather weak frameshift mutagens in T4, where internal nicks appear to be much less mutagenic than chromosome tips.
These results by themselves do not offer insights into the enzymatic basis of frameshift mutagenesis. The suggestion that acridines act as mutagens because they stabilize improbable mispaired configurations is not likely to be critically tested until a suitable in vitro system becomes available. However, it already conflicts with the observation that the mutagenicity of various acridines does not correlate with their effects upon the melting temperature of DNA, and with the nonmutagenic character of several agents which stabilize DNA in vitro. Furthermore, this suggestion requires that acridines stabilize mispaired regions more effectively than correctly paired regions, for which no evidence is available. An alternative hypothesis is that acridines, whether intercalated or not, interact directly with repair enzymes, either to stimulate the fixation of mispaired configurations or to cause the enzymes themselves to make mistakes.

It is a pleasure to acknowledge the assistance in these experiments of Isidora Albrecht, Effie Bailey, Lynne Bartenstein, and Susan Forsberg, as well as the refreshing criticisms of Frank Stahl. The experiment described in Table 2 was suggested in 1964 by Gunther Stent; it turned out to be difficult.

* Supported by grant E59 from the American Cancer Society, grant AI04886 from the National Institute of Allergies and Infectious Diseases, NIH, and grant GB6998 from the National Science Foundation.

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Some of this variation must be due to sampling errors, since we usually scored fewer than 200 mottled plaques for both numerator and denominator. In addition, part of the variation could reflect the "repair" of heterozygotes, providing that repair erased one type of heterozygote more often than the reciprocal type. M. Meselson (personal communication) has detected polarized repair in bacteriophage λ.

40 We commonly observed that preadsorption of r particles to KB cells resulted in transmission coefficients (due to leakiness) of about 10⁻¹. This is much greater than the frequency of induced revertants. When the infected KB cells were plated on a mixture of 6 x 10⁷ KB cells plus 2 x 10⁷ B cells per plate, revertants formed clear plaques which were easily distinguished among a large excess of turbid r plaques. When analyzed, the clear plaques sometimes contained a considerable fraction of r particles. This effect, however, was independent of prior proflavin mutagenesis, and was particularly obvious when the r mutant exhibited a high spontaneous reversion rate. We therefore believe that some clear plaques arose from r particles because of selection on the plate, and not from heterozygotic revertants.
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