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Covalent Repair of Molecular Recombinants in the Ligase-negative Amber Mutant of T4 Bacteriophage

A. W. KOZINSKI AND P. B. KOZINSKI

Department of Medical Genetics, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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Parent-to-progeny molecular recombination in T4 bacteriophage is followed by covalent repair which restores the strand integrity of the recombinant molecule (4). This observation was extended by proof of covalent repair in recombinant molecules arising as a result of interparental molecular exchanges (5, 10).

The addition of chloramphenicol (CM) at critical times after infection resolved the process of replication and recombination and revealed the following pattern. The addition of CM at approximately 5 min after infection allows semiconservative replication but prevents molecular recombination; the addition of CM at approximately 7 min after infection permits, in addition, molecular recombinations but prevents covalent repair of the recombined moiety. Only the addition of CM at about 10 min after infection allows the expression of an enzyme which restores the integrity of recombinant molecules, covalently joining light deoxyribonucleic acid and heavy deoxyribonucleic acid (DNA) subunits [light, DNA of normal density; heavy, DNA substituted with 5-bromodeoxyuridine (5-BU)]. Thus, a phage-coded repairing enzyme, "repairase," was postulated (6, 7). An enzyme, ligase, was isolated and purified from T4-infected bacteria (11). It was reasonable to assume that this was the enzyme responsible for the repair in the recombinant molecules.

The objective of this report was to examine parent-to-progeny recombination and covalent repair of phage-recombinant DNA molecules in *Escherichia coli* B (nonpermissive host) infected with ligase-negative T4 amber H39X (2). This strain was proven to not produce T4-coded ligase (1). The lack of repair of recombinant molecules could be interpreted as supporting evidence for the role of ligase in the repair of recombinant molecules. In contrast, abundant covalent repair leads to the assumption that the recombinant repairing enzyme "repairase" might still await purification.

DNA of T4 Am H39X, after injection into the nonpermissive *E. coli* B23, undergoes extensive breakages, first of the nonrandom nature, and secondly of the random type, which correlate with an extensive parent-to-progeny recombination. Resulting short fragments are able to replicate autonomously in the nonpermissive host (9). It was demonstrated in this study that, in the case of the ligase-negative T4 Am H39X mutant, extensive parent-to-progeny recombination produces a recombinant DNA molecule composed of parental-light and progeny-heavy subunits which are remarkably efficiently joined by a covalent bond. This observation leaves in doubt the role of ligase in the repair of the recombinant molecule and suggests that an enzyme which we called "repairase" is not identical with ligase.

TCG medium and the analytical procedures applied in this study were identical to those described in previous papers (7; A. W. Kozinski, Cold Spring Harbor Symp. Quant. Biol., *in press*).

E. coli B23, a nonpermissive host for T4 AmH 39X (plating efficiency 10^{-6}), was grown for two generations to 3×10^8 cells/ml in TCG medium supplemented with 5-BU, fluorodeoxyuridine, and uracil, and infected with a light, ^{32}P-labeled (specific activity, 5 mc/mgP) T4 AmH39X with a multiplicity of infection of 3.0. [A reciprocal experiment in which light bacteria were infected with heavy parental phage was also performed, and the results were similar to those presented in this report. However, Am H39X substituted with 5-BU is mostly dead, a feature common for most of the DNA-deficient amber mutants analyzed (3).] Part of the infected suspension at 5 and 10 min after infection was transferred to CM (100 μg/ml). At 30 min after infection, bacteria were quickly chilled in ethylenediaminetetraacetate buffer (pH 8) and sedimented, and DNA was extracted. The intracellular DNA was analyzed in a native and denatured form in a CsCl gradient, to which an adequate amount of light and heavy ^3H-labeled DNA was added as a reference (Fig. 1). From our data, we made the following conclusions. In the bacterial suspension to which no CM was added, a significant extent of parent-to-progeny recombination occurred. Recombination manifested itself by a displacement of parental-light DNA toward the heavy location.

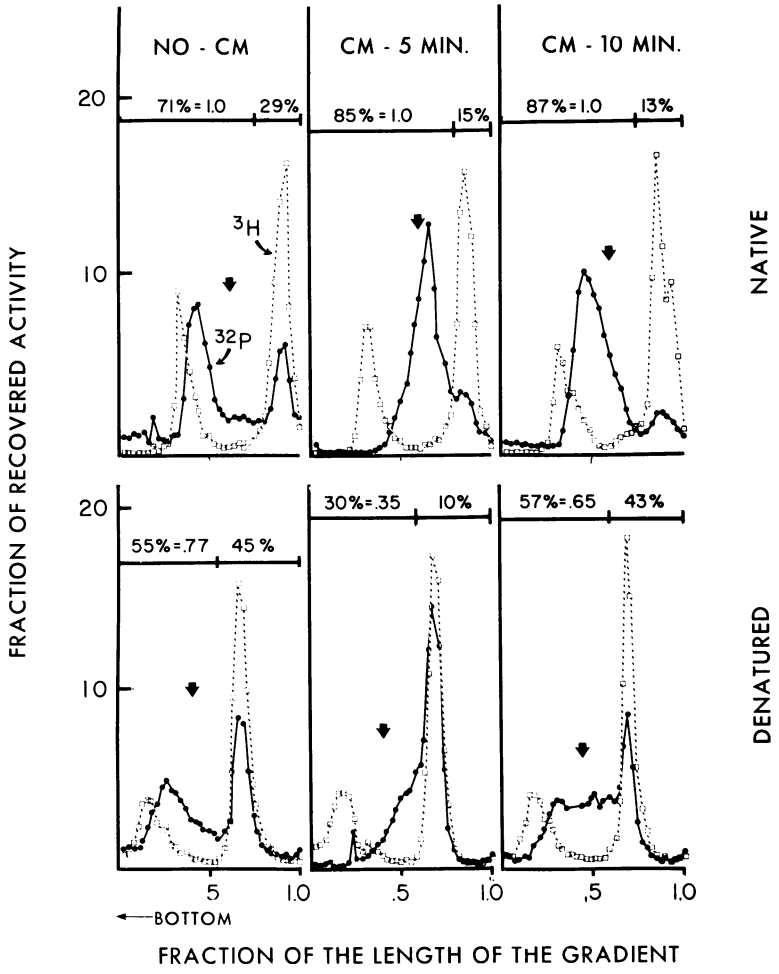

FIG. 1. *CsCl analysis of the intracellular fate of ^{32}P-labeled light amber T4 H39X DNA in heavy E. coli B host. The upper panel represents analysis of the native intracellular DNA; the lower panel shows the same DNA, denatured in the presence of 1% HCHO at 90 C for 3 min and analyzed in CsCl supplemented with 1% HCHO. Left column, no CM added; note extensive recombination and covalent repair, which causes most of the recombinants to band, after denaturation, on the heavier side of the arrow. Arrow represents midpoint between light and heavy ^{3}H reference and not the true hybrid location. Center column: note lack of parent-to-progeny recombination; replications proceed only to the hybrid density. (Light reference ^{3}H-DNA is an absolute marker which coincides with light ^{32}P-DNA; ^{3}H heavy reference DNA serves merely as a position marker; this is due mostly to a significant variation in the extent of uptake of 5-BU. In this experiment, reference heavy 5-BU DNA is nearly 100% substituted, whereas the substitution of T4 Am H39X was less; thus, the midpoint between reference light and heavy ^{3}H does not coincide with a hybrid represented in the center upper panel.) After denaturation, most of the radioactivity returns to the light location, indicating lack of covalent joining between light and heavy moieties. Right panel: fate of intracellular DNA in bacteria to which CM was added at 10 min after infection. In contrast to bacteria treated with CM at 5 min and similarly to bacteria receiving no CM, the main fraction of the DNA bands on the heavy side of the gradient, close to heavy reference DNA. After denaturation, pattern of the CsCl distribution is far less homogeneous than those of samples without CM, a large majority of the material banding now on the lighter side of the gradient. This indicates that, although there is a significant amount of covalent repair, a large fraction of the recombinants remain imperfectly repaired. Horizontal bar at top of each panel divides the moieties of the gradient into two classes, one coinciding with conservative, nonreplicating light reference, and one enclosing all of the rest of the DNA which is heavier than reference. Figures above the horizontal bar refer to summed amount of ^{32}P activity within the enclosed area. The summed activity of DNA heavier than reference prior to denaturation (upper panels) is assigned a value of 1; after denaturation (lower panels), this figure becomes smaller, permitting calculation of the extent of covalent repair resistant to heat; for the samples represented, this is 0.77, 0.35, and 0.65, respectively.*

Recombinant molecules, when denatured, did not separate into the light-radioactive and heavy-cold moiety, but retained average composition, still banding close to the heavy reference DNA. *This proves the establishment of a covalent bond* between light-radio-active-parental and heavy-progeny subunits in a recombinant molecule.

The addition of CM at 5 min after infection (center panels, Fig. 1) allowed semiconservative replication of injected DNA, which proceeds only to a hybrid density, in a CsCl gradient. Hybrid moiety, after denaturation, released parental strands mostly as a pure parental DNA, not associated with the heavy progeny moiety.

The addition of CM (right panels, Fig. 1) at 10 min after infection allowed extensive parent-to-progeny recombination, which, when analyzed in native form, displayed an extent of recombination similar to that of the suspensions not treated with CM. After denaturation, however, covalent repair appeared to be less pronounced. Thus, the pattern of events occurring after the addition of CM was, in Am H39X, quite similar to that described for wild phage, although its timing was somehow different.

The lack of separation of the light and heavy moieties after denaturation could be caused either by an end-to-end covalent joining or by an interstranded cross-linking between two complementary adjacent strands. However, this second theory was disproved by the following experiment. Recombinant molecules, supplemented with ^{3}H reference DNA after heat denaturation, were allowed to cool; after cooling, the mixture was digested with exonuclease I, and the kinetics of solubilization of both DNA types were determined. The kinetics of solubilization of both DNA types were found to be identical. Assuming interstranded cross-linking, one should expect efficient reannealing of recombinants and subsequent resistance toward the exonuclease I activity. This experiment proved covalent repair of an end-to-end type.

The calculation of the fractional contribution of parental material to a recombinant molecule was based on estimating locations of a majority of 5-BU-substituted progeny molecules, ^{32}P-containing moieties, and pure parental phage DNA (4). In a recent experiment, we were not able to directly measure the location of a majority of progeny molecules. Thus, as a reference, a heavy and light ^{3}H-labeled DNA was supplemented. The midpoint (arrow, Fig. 1) is not a location of hybrid, as the efficiency of 5-BU substitution varies considerably.

That the ^{32}P peak shown in the middle upper panel is a true hybrid was confirmed by sonic treatment, after which the density of the ^{32}P-containing material retained the same position in the CsCl gradient.

The location of heavy progeny DNA was then extrapolated in respect to the heavy ^{3}H reference; it was found to be 10 to 15%. In the nonpermissive host, the parental molecule, after breakage, was incorporated into short fragments of a molecular length between 0.05 and 0.15 phage equivalent length [for bacteria not treated with CM or for those treated at 10 min after infection; DNA in those where CM was added at 5 min remains to a large extent integral (A. W. Kozinski, Cold Spring Harbor Symp. Quant. Biol., *in press*)]. Thus, the total length of parental subunits, if uninterrupted, is of 0.005 to 0.015 phage equivalent length, and the frequency of recombination and total amount of covalently repaired joints per unit of length of the molecule are higher than in the wild phage.

In summary, parent-to-progeny recombination in Am H39X is followed by an efficient covalent bond repair between the subunits contributing to the recombinant molecule. This fact, not expected for a ligase-negative phage mutant, led to three possible explanations.

(i) The amber T4 X39 is not truly a ligase-negative mutant, but produces an enzyme which is nonextractable by the procedures applied by Weiss and Richardson.

(ii) The observed repair is performed gratuitously by the host ligase. For wild phage, there are significant contraindications for host-mediated repair (7), indicating that, when CM was added at the proper time, there was no repair of the recombinant molecule. Furthermore, the experiments of Tomizawa and Anraku (9) show lack of repair in some DNA-deficient Amber mutants. This is in contrast to nicks resulting from ultraviolet-damage excisions which are repaired very efficiently by a host enzyme in the presence of CM (8).

(iii) What we consider as the most likely hypothesis is that T4 phage ligase is not the enzyme involved in the repair of the recombinant molecule, but is required for the repair of single-stranded breaks occurring as a result of endonucleotic activity of phage-coded nickase. Facts of covalent repair of parent-to-progeny recombinant molecules presented in this and another report (A. W. Kozinski, Cold Spring Harbor Symp. Quant. Biol., *in press*) correlate well with proof for the covalent repair in the recombinant molecules resulting from parent-to-progeny exchanges presented by C. C. Richardson (Cold Spring Harbor Symp. Quant. Biol., *in press*).

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LITERATURE CITED

1. Fareed, G. C., and C. C. Richardson. 1967. Enzymatic breakage and joining of deoxyribonucleic acid. II. The structural gene for polynucleotide ligase in bacteriophage T4. Proc. Natl. Acad. Sci. U.S. **58**:665–672.
2. Hosoda, J. 1967. A mutant of bacteriophage T4 defective in α-glucosyl transferase. Biochem. Biophys. Res. Commun. **27**:294–298.
3. Kozinski, A. W., and Z. Z. Felgenhauer. 1967. Molecular recombination in T4 bacteriophage deoxyribonucleic acid. II. Single-strand breaks and exposure of uncomplemented areas as a prerequisite for recombination. J. Virol. **1**:1193–1202.
4. Kozinski, A. W., and P. B. Kozinski. 1963. Fragmentary transfer of ^{32}P-labeled parental DNA to progeny phage. III. The average size of the transferred parental fragment. Two cycle transfer. Repair of the polynucleotide chain after fragmentation. Virology **20**:213–229.
5. Kozinski, A. W., and P. B. Kozinski. 1964. Replicative fragmentation in T4 bacteriophage DNA. II. Biparental molecular recombination. Proc. Natl. Acad. Sci. U.S. **52**:211–218.
6. Kozinski, A. W., and P. B. Kozinski. 1965. Early intracellular events in the replication of T4 phage DNA. II. Partially replicated DNA. Proc. Natl. Acad. Sci. U.S. **54**:634–640.
7. Kozinski, A. W., P. B. Kozinski, and R. James. 1967. Molecular recombination in T4 bacteriophage deoxyribonucleic acid. I. Tertiary structure of early replicative and recombining deoxyribonucleic acid. J. Virol. **1**:758–770.
8. Kozinski, A. W., and Z. Lorkiewicz. 1967. Early intracellular events in the replication of T4 phage DNA. IV. Host-mediated single-stranded breaks and repair in ultraviolet-damaged T4 DNA. Proc. Natl. Acad. Sci. U.S. **58**:2109–2116.
9. Kozinski, A. W., and P. B. Kozinski. 1968. Autonomous replication of short DNA fragments in the ligase negative T4 AmH39X. Biochem. Biophys. Res. Commun. **33**:670–674.
10. Tomizawa, J., and N. Anraku. 1964. The molecular mechanism of genetic recombination in bacteriophage. J. Mol. Biol. **8**:508–515.
11. Weiss, B., and C. C. Richardson. 1967. Enzymatic breakage and joining of deoxyribonucleic acid. I. Repair of single-stranded breaks in DNA by an enzyme system from Escherichia coli infected with T4 bacteriophage. Proc. Natl. Acad. Sci. U.S. **57**:1021–1029.