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Late Events in T4 Bacteriophage Production

I. Late DNA Replication Is Primarily Exponential

LINDA D. KOSTURKO[1] AND ANDRZEJ W. KOZINSKI*

Department of Human Genetics, University of Pennsylvania, Philadelphia, Pennsylvania 19174

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The possibility of a switch in the mechanism of T4 DNA replication, from an exponential-bidirectional mode at early stages to a nonexponential (rolling circle) mode at later stages of phage development, has been investigated. The conclusion that DNA replication does not involve such a change in mechanism for the majority of replicating molecules is based on the analysis of the clonal distribution of mutants specifically induced at late times after infection. The clonal distribution of mutants, induced by adding 5-bromodeoxyuridine to infected cells at a time when 100 phage equivalents of DNA had accumulated, fits the pattern predicted by exponential replication.

In recent years some light has been shed on the structure of T4 DNA during the initial rounds of replication: replication begins at several specific initiation sites along the DNA molecule (7); progeny DNA is synthesized from both template strands, in both the 3′ and the 5′ directions, from the initiation point toward both ends of the molecule (2). DNA synthesis at the 5′ end of the growing strand appears to lag behind synthesis at the 3′ end so that the distance from the initiation point to the 3′ end of the growing strand is usually longer than the distance from the initiation point to the 5′ end of the same strand (2). Eventually two daughter molecules are produced, each possessing one strand of the infecting parental DNA molecule (4).

At the present time, much data support this model for the initial rounds of replication, and presumably it could describe all subsequent rounds. However, the suggestion has been made that in the later rounds of replication, the structure of replicating DNA more closely resembles the rolling circle model proposed by Gilbert and Dressler (6). In support of this idea, Bernstein and Bernstein found some circular structures with one or more linear branches radiating from the circumference in autoradiographs of [^{3}H]thymidine-labeled, late-replicating T4 DNA (1). They suggested that at some time after the initiation of replication the mode of replication switches from an exponential to a nonexponential rolling circle model.

One difference between these two mechanisms is their predictions of the clonal distribution of mutants. With exponential replication, which seems to govern the initial round for T4 DNA, one molecule replicates to produce two copies, each of which in turn produces two copies, and so on. A mutation occurring during one round of replication would be repeatedly duplicated in each subsequent round of replication, since any strand of DNA can serve as a template for further replication. The number of mutant molecules, and consequently mutant phage, per clone depends on the number of rounds of replication after the mutational event in such a way that the number frequency of clones of size 2^k is inversely proportional to 2^k, where k is the number of rounds of replication after the mutation (12).

The rolling circle model (6) is a nonexponential mode of replication; one DNA strand serves as the template for successive rounds of replication so that each copy is produced independently of the last. A mutation occurring in a progeny molecule during nonexponential replication, as in the rolling circle model, yields only one mutant copy of DNA. If the mutation rate is so low as to allow only one mutation per infected cell, then a mutation in a progeny (nontemplate strand) will yield only one mutant phage in that cell, if replication proceeds by this model. If a template strand is mutated then all subsequent copies will carry the mutation. The number of mutant phage produced in a cell in which a template molecule is mutated depends on the number of rounds of replication after the mutation of the template, but the distribution of mutant clones would be very different from the

[1] Present address: Yale University, Sterling Chemistry Laboratory, New Haven, Conn. 06520.

distribution expected if replication were exponential. Examination of the distribution of mutant clones should distinguish between the two possible models of T4 DNA replication.

Luria observed the clonal distribution of spontaneous mutants and concluded that, overall, phage replication was exponential (12). Moreover, his data on clones of small size best fit the expected results of exponential replication, indicating that the last few rounds of replication in which a molecule participates before encapsidation are exponential rather than linear. To emphasize the results of Luria on the clonal distribution of spontaneous mutants, the experiment described below was designed to test whether mutations specifically induced in the later stages of replication would show a clonal distribution characteristic of exponential DNA replication.

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MATERIALS AND METHODS

Phage strains. T4B*r*AP72-O_1^r is the result of a cross of T4BO_1^r and T4B*r*AP72, an *rII* mutant of T4B obtained through the courtesy of J. Drake.

For the genetic cross, *Escherichia coli* BB was grown to 3×10^8/ml and infected with T4BO_1^r and T4B*r*AP72 at multiplicities of infection of 5 and 1, respectively. At 90 min after infection the cells were lysed with chloroform, and the lysate was plated on a lawn of *E. coli* B23. Several hundred of the plaques that formed possessing an *rII* phenotype were transferred with sterile toothpicks to two plates: (i) a plate with a lawn of *E. coli* K(λ); (ii) a plate with a lawn of *E. coli*. BB. Several of those plaques, confirmed to have an *rII* phenotype, were transferred from the BB plate to H broth with chloroform. A portion (0.1 ml) of this resuspension was mixed with 1 ml of saturated CsCl in 0.3 M KCl-nutrient broth (8 g of KCl/liter) and allowed to equilibrate at 4 C for 30 min. The mixture was then diluted 1:5 with KCl-nutrient broth and allowed to equilibrate for 15 min at 4 C. Both this dilution and the original resuspension were further diluted with H broth and plated on a lawn of BB. A resuspension which was as resistant to osmotic shock as a control stock of T4BO_1^r was chosen for further experimentation, and the stock of phage produced from it was called T4B*r*AP72-O_1^r.

Bacterial strains. *E. coli* B23, K(λ), and BB were used in this study.

Media and growth conditions. Basic media, ^{32}P labeling, and growth conditions were identical to those described previously (8, 9). All experiments were performed at 37 C at a cell concentration of 3×10^8/ml in Tris-Casamino Acids-glucose medium. Revertants were induced by adding to infected cells a mixture of 200 μg of 5-bromodeoxyuridine (5-BUdR), 5 μg of 5-fluorodeoxyuridine, and 20 μg of uracil per ml of culture.

RESULTS

A suitable mutagen for this test is 5-BUdR; since 5-BUdR acts after incorporation into DNA during synthesis, the probability of a mutation in the template strand itself is very small. The use of an *rII* mutant of T4 whose reversion rate is 10^4 larger in the presence of 5-BUdR (5) allows the neglect of noninduced mutations. Since in only 1 case out of 10^4 will a mutant clone result from spontaneous reversion of the *rII* gene, all clones observed can be considered as resulting from reversions induced by the 5-BUdR. Therefore it is not necessary to consider those spontaneous mutations which might occur early in replication, before the pulse of 5-BUdR and while template molecules may still be synthesized, nor late in replication in template molecules, after they may have begun to function as rolling circles. Thus, if reversions are induced at late times, a model of nonexponential replication predicts the occurrence of clones of size one only, since presumably all templates have been formed before the mutagen 5-BUdR is added. However, if replication is exponential, an exponential distribution of clone sizes should result (Fig. 1).

To insure that the mutagen is not added while template molecules are still being synthesized, the moment at which DNA replication may be supposed to switch from one to the other must be arbitrarily chosen. However, a fair estimate can be made from the following considerations. Unless lysis is prevented, a T4-infected cell starts producing mature phage at a time when approximately 100 phage equivalents (PE) of DNA have been synthesized (11). There could be no more than 50 rolling circles at this time, since the products of replication are observed to be concatemers. One might then expect that the mode of DNA replication could change no later than the time when 50 PE of DNA have been synthesized per cell.

E. coli BB was infected at a multiplicity of 5 (the fraction of uninfected cells was measured as less than 1%) with T4B*r*AP72-O_1^r. At 4 min after infection $^{32}PO_4$ was added to the medium to a final specific activity of 0.1 mCi/mg of P. At this time, and at various times throughout the experiment, samples of this culture were taken to measure the extent of DNA synthesis. Relevant data are shown in Fig. 2, which should be referred to for the number of PE synthesized.

At 12 min after infection, chloramphenicol (CM) was added to the culture to a final concentration of 150 μg/ml. CM is an antibiotic that inhibits protein synthesis, and the addition of CM at this late time prevents maturation of

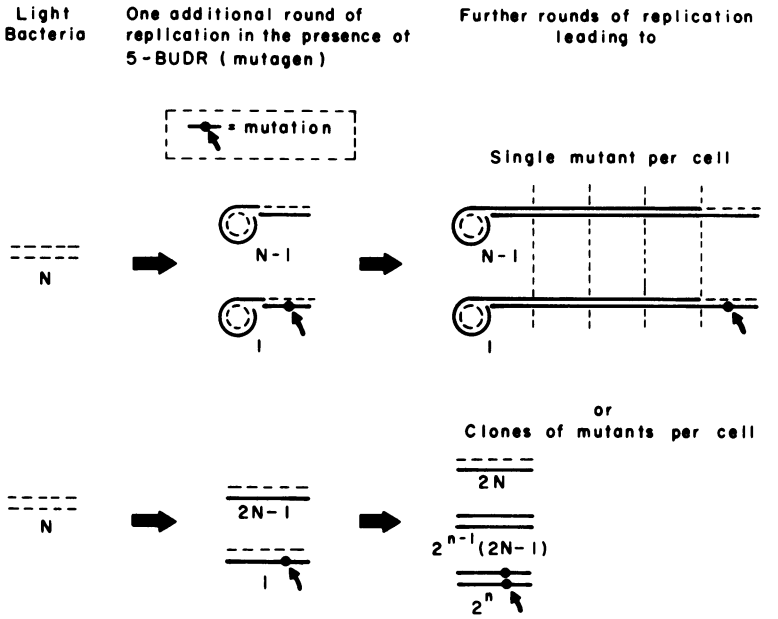

FIG. 1. *Comparison of the distribution of mutants in the infected cell expected for the rolling circle model or for an exponential model of replication. N is the initial number of PE of DNA replicating, and n is the number of rounds of replication after the mutational event. Rolling circle replication will result in only one mutant per cell, whereas exponential replication will produce 2^n mutants per cell. The dotted lines divide the rolling circle concatemer in PE lengths.*

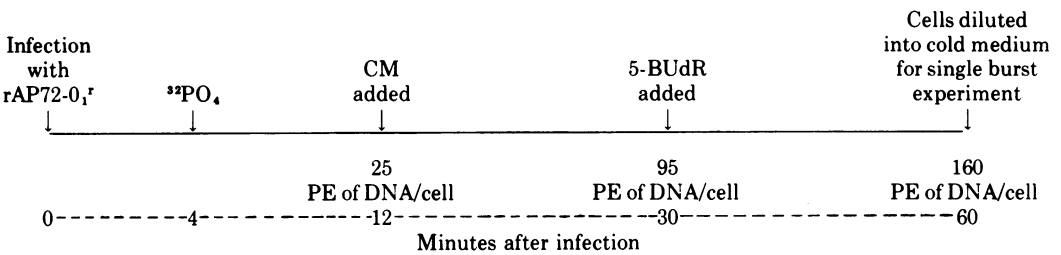

FIG. 2. *Flow chart of experiment A. At the times indicated, the amount of $^{32}PO_4$ taken up to DNA was measured as the counts present in acid-precipitable, alkali-resistant material. The data is presented as PE of DNA per infected cell.*

phage but allows DNA replication and recombination to continue (11, 13). By adding CM to the culture at this time, the duration of the 5-BUdR pulse could be prolonged, allowing the synthesis of more DNA and consequently increasing the opportunity for reversions to occur.

The culture was fed 5-BUdR at 30 min postinfection. At 60 min the cells were diluted with chilled medium to a final cell concentration such that the probability that 1 ml contained more than one cell-containing revertant phage was only 0.01. This concentration was estimated from the frequency of cells yielding revertants (approximately 5×10^{-3}), a number calculated from previous experiments. [The plating on lawns of *E. coli* K(λ) and on lawns of *E. coli* B23 resulting from this experiment demonstrated that in the lysate the frequency of revertants (the ratio of plaque formers on K to plaque formers on B) was 1.4×10^{-5}, as compared with the frequency of 1.4×10^{-7} of a control lysate which received no 5-BUdR.]

After dilution, while still stored on ice, the samples were distributed in 1.0-ml portions to sterile tubes. The tubes were incubated at 37 C for 5 to 8 h and then stored in the dark at 4 C until their contents could be plated. For plating, 2 ml of 2× top layer agar (1.33 times the normal concentration of top layer constituents) (45 C) and 3×10^8 (0.2 ml) detector bacteria,

K(λ) at 0 C, were added to each of the above tubes, which had been prewarmed to 37 C; the entire contents of the tube were poured over bottom layer agar. The plates were incubated overnight at 37 C. Clone size was scored as the number of plaques per plate. The clonal distribution is shown in Fig. 3 as the log (clones containing n or more revertants) versus log(n). The line is the theoretical result expected if replication is exponential: $\sum_{\geq n} Y_n = 2mN/n$ (12).

At the time of addition of 5-BUdR to this sample, 95 PE of DNA per cell had been synthesized; this is well above the upper limit of the estimated size of the DNA pool allowed for a switch over in this mode of replication (50 PE). The addition of CM at 12 min after infection should not inhibit a transition to a rolling circle mode; replicating rolling circles produce concatenated structures, and it is known that such a late addition of CM does not inhibit the formation of DNA concatemers, nor does it interfere with the process of recombination (8, 10, 12). In addition, the results of this experiment agree quite well with those of Luria (12), which were obtained without the addition of CM.

We see that the clonal distribution compares very well with the result expected for exponential replication. If rolling circle replication was the predominant model in late T4 DNA replication, all clones observed would contain only a single mutant, and the graph of that result would be a single point at $n = 1$. Since 5-BUdR would only be expected to cause mutations in progeny DNA, template strands should not be mutated, so that no larger size clones would be expected. The data support the conclusion that the rolling circle model does not describe the later stages of T4 DNA replication and that for the majority of replicating molecules late T4 DNA is exponential.

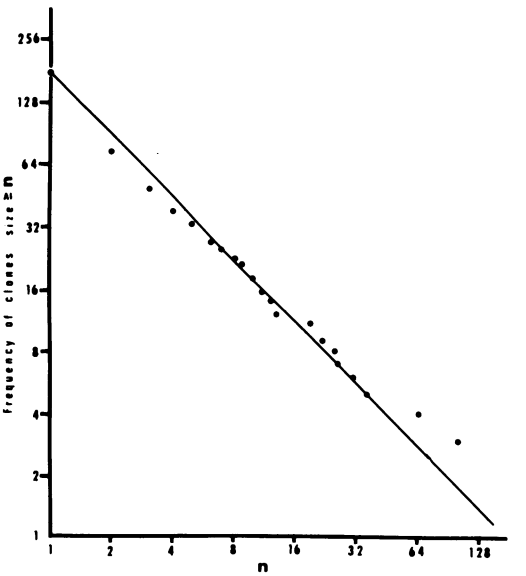

FIG. 3. *Clonal distribution of revertants induced by the addition of mutagen (5-BUdR) to cells at the time when nearly 100 PE of DNA, on the average, had been synthesized per infected cell. The solid line indicates a line of slope = −1, which is the theoretical result expected if replication is exponential. The points represent the experimental data of two experiments in which the size of 189 clones was measured and tabulated.*

DISCUSSION

Previous experiments have shown that in the early stages of T4 DNA replication, DNA synthesis begins at multiple, specific initiation sites (2, 7) and proceeds bidirectionally (2), resulting in an exponential duplication of parental molecules (12). In this last respect (exponential, rather than nonexponential, duplication) at least, late T4 DNA replication seems to resemble the initial stage.

The experiment described here examined the clonal distribution of 5-BUdR-induced revertants of an *rII* mutant of T4, where reversion is specifically induced in the late stages of replication, when concatemers exist. The observed clonal distribution is compatible with an exponential mode of replication, indicating that long after concatemers of DNA are formed, and when some 100 PE of progeny DNA have been synthesized, replication largely continues in an exponential mode. This does not mean that no rolling circle replication exists in the later stages of T4 DNA replication, but only that the majority of replicating molecules replicates in an exponential manner.

Bernstein and Bernstein examined autoradiographs of late replicating T4 DNA and observed that a small percentage of the molecules they examined involved circular structures (1). They suggested that late replication of T4 DNA might occur according to the rolling circle model. However, Doermann has pointed out (3) that the structures observed by Bernstein and Bernstein could have been produced by recombination rather than by rolling circle replication.

In presenting the models for this experiment, no distinction is made between reversions produced during ordinary replication and those produced during repair-replication, for exam-

ple, those that might be induced during the repair of gaps in recombinant molecules. Such a distinction could be misleading, since the conclusions depend on the size distribution of mutant clones, which is independent of the type of synthesis (ordinary replication or repair-replication) which produced the reversions. The distribution of mutant clone size depends only on the manner in which DNA is replicated after the mutational event. If a reversion occurred in a progeny DNA molecule during repair replication, and if no further replication of that molecule occurred, as expected if replication were nonexponential, then that mutation would result in a clone of size one. On the other hand, if replication were exponential, a reversion occurring during repair-replication could be duplicated repeatedly to produce a clone of larger size.

In either of these two cases, those mutant clones resulting from mutations during repair-replication would have no special effect on the overall distribution of clone sizes. However, if replication were nonexponential and repair-replication produced a reversion in a template molecule, rather than a progeny molecule, some effect on the results might be anticipated. To estimate the size of this effect, we must estimate how frequently mutations occur during repair-replication of recombinant molecules.

With a few assumptions and experimentally derived parameters (see Appendix), the fraction of observed mutant clones that could have resulted from reversions during repair-replication is estimated to be 8.5%. If replication were linear and template molecules participated in recombination, then 91.5% of all reversions would produce clones of size one, whereas 8.5% of all reversions would produce clones of larger sizes. However, the distribution of sizes of these larger clones would be random rather than exponential because the size of a clone would be equal to the number of rounds of replication after the reversion during repair of the template. This number depends on the time at which the recombinational event occurred, and since these events are presumably random with time, the distribution of clones of size greater than one should be random.

In this experiment 111 of 189 observed clones, or only 58% of the total, were size one, and the distribution of clones greater than one in size fits well to that predicted for exponential replication. Even if replication was assumed to be exponential an excess of clones of size one would be expected for this experiment, since half of the reversions which remain heterozygous (with the reversion on the sense strand) would make a clone of size one in addition to the homozygous revertant particles. Yet there are fewer clones of size one (only 111) than would be expected if replication were linear, that is, 173. This estimate suggests that the results of this experiment cannot be accommodated by a model in which repair-replication causes reversions in molecules which replicate in a nonexponential manner.

The evaluation of the data presented here depends on a comparison with the theoretical result that, in the case of exponential replication, $\log Y_n = -1 (\log n)$, where Y_n is the frequency of clones containing n or more mutants. This relation derives from an exponential model of replication in which one molecule produces two molecules, each of which produces two more, and so on, together with the additional assumption that replication is not synchronized.

A better mathematical description of phage DNA replication was presented by Steinberg and Stahl (14), who assumed the existence of a steady-state pool of vegetative DNA. Their model proposed that whereas the pool of DNA available for replication increased in size with each round of replication, the simultaneous process of maturation removed DNA from the pool at the same rate. The model was used to generate a theoretical clonal distribution of spontaneous mutants with the stipulation that any mutants occurring during the eclipse period, before maturation begins, would be ignored. If the later stages of T4 DNA replication resembled a nonexponential mechanism, such as the rolling circle model, the switch to this mode from the earlier exponential model might be expected to temporally precede the onset of phage maturation. The implication is that the size distribution of clones, arising from spontaneous mutations occurring after the eclipse period, generated by the Steinberg-Stahl model, which assumes that late replication proceeds exponentially, should reflect the change in the mode of replication by exhibiting a difference between itself and the size distribution of clones obtained experimentally by Luria. A plot, as $\log Y_n$ versus $\log n$, of the data generated by this model fits very well to the experimental data of Luria (12), except where n is large. (This deviation is expected, since the largest size clones arise from the earliest induced mutations.) The Steinberg-Stahl model suggests that the data of Luria also support the conclusion of this experiment, that the distribution of clone sizes of mutants specifically induced in the later stages of T4 DNA replication

fails to support a nonexponential description of the main body of T4 DNA replication. A small amount of nonexponential replication may occur, but the simplest conclusion is that the primary mode of T4 DNA replication is exponential.

APPENDIX

To estimate the fraction of observed mutant clones that can be attributed to reversions arising during repair-replication, three assumptions should be considered: (i) cuts in DNA leading to recombination are random in time; (ii) all cuts lead to recombinational events and the time between the cuts that produce the recombinational fragment and the repair of the gap between those fragments is very small; and (iii) recombinational cuts are random along the DNA molecule.

The number of recombinational events and repairs per PE length of DNA can be estimated from the measured contribution of parental DNA to a progeny molecule. The parent-to-progeny contribution is 7%, which is delivered as one strand of a double-stranded hybrid parent-progeny DNA fragment (8). If the average double-stranded fragment is 14% of the mass of a PE of DNA, then seven such fragments can be cut per PE length of DNA, and six cuts are required to produce those seven fragments. Thus on the average there are six recombinational events per unit length of DNA.

Recombining fragments are not blunt ended but have free single-stranded ends (10). When two fragments recombine their single-stranded ends overlap, pairing homologous regions (9). The region of nonhomology remains as a single-stranded "gap" to be repaired. To estimate the number of recombinational events that will leave the point *rII* mutation open for repair, the size of the gap to be repaired must be estimated.

The recombining fragments are on the average 14% PE in length; therefore, the average size of the single-stranded end will be half that, or 7% PE in length. When two such fragments recombine, the average size of the gap left is half the length of the single-stranded end, or 3.5% PE in length. Therefore, every cut leads to a gap 3.5% PE in length to be repaired.

Now, a cut for recombination can be made anywhere along the DNA molecule (assumption iii). T4 DNA contains about 200,000 base pairs, and a gap 3.5% PE in length will cover 7,000 base pairs. Therefore 7,000 gaps with unique end points will include one particular base pair in the T4 DNA molecule. There are 193,000 unique gaps that can be formed in the T4 DNA molecule; therefore 7,000/193,000 or 3.65% of all gaps left by recombinational events will expose the *rII* point mutation to repair.

The maximum number of template molecules possible with linear replication has been estimated as 50, based on the size of T4 DNA concatemers (14). The time period during which such molecules could have participated in recombination covers the period from their inception to 60 minutes postinfection, when CM was removed and maturation began.

Since recombinational events occur randomly with time (assumption i), the fraction occurring during the 30-min 5-BUdR pulse is: (50 molecules) (6 recombinational events/molecule) (1/60 min) (30 min) = 150 recombinational events. The fraction of 150 recombinational events that would involve repair of the region surrounding the *rII* point mutation is simply 3.65% or 5.5. The number of reversions due to repair-replication is equal to the number of copies of the region around the *rII* point mutation made by repair-replication during the 5-BUdR pulse, 5.5, multiplied by the reversion rate. Since the reversion rate, the fraction of replication events resulting in a reversion, is the same for ordinary replication as for repair-replication, the fraction of reversions attributable to repair-replication is equal to 5.5 divided by the total number of copies of the region surrounding the rII point mutation made during the 5-BUdR pulse (160 - 95 = 65). Therefore, the percentage of observed reversions due to repair-replication = $5.5/65 \times 100 = 8.5\%$.

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