Partial Replication of UV-Irradiated T4 Bacteriophage DNA Results in Amplification of Specific Genetic Areas

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Received 5 September 1980/Accepted 19 June 1981

Upon infection of Escherichia coli with bromodeoxyuridine-labeled T4 phage that had received 10 lethal hits of UV irradiation, a sizable amount of phage DNA was synthesized (approximately 36 phage equivalent units of DNA per infected bacterium), although very little multiplicity reactivation occurs. This progeny DNA was isolated and analyzed. This DNA was biased in its genetic representation, as shown by hybridization to cloned segments of the T4 genome immobilized on nitrocellulose filters. Preferentially amplified areas corresponded to regions containing origins of T4 DNA replication. The size of the progeny DNA increased with time after infection, possibly due to recombination between partial replicas and nonreplicated subunits or due to the gradual overcoming of the UV damage. As the size of the progeny DNA increased, all of the genes were more equally represented, resulting in a decrease in the genetic bias. Amplification of specific genetic areas was also observed upon infection with UV-irradiated, nonbromodeoxyuridine-substituted (light) phage. However, the genetic bias observed in this case was not as great as that observed with bromodeoxyuridine-substituted phage. This is most likely due to the higher efficiency of multiplicity reactivation of the light phage.

Bacteriophage T4 DNA replication has been shown to be initiated at multiple and specific sites on the T4 genome and to proceed bidirectionally (6). Two origins of replication (one in the area of genes 50-5 and the other, weaker one in the area of genes uvsW-29) have been identified by hybridization of early synthesized progeny DNA to cloned T4 segments immobilized on nitrocellulose filters (5). The pattern of the initiated areas remained the same for high or low multiplicity of infection and in the presence or absence of a density label (10; M. E. Halpern, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1981). More recently, gene amplification during T4 phage development was demonstrated after the infection of Escherichia coli with T4 phage (10). The term "gene amplification" in this context refers to the process by which specific genetic areas within a single genome are preferentially and repeatedly replicated, leading to the accumulation of partial replicas unattached to the parental molecule. This has been observed under two conditions. In the first, gene amplification was observed at very early times after infection, before the parental DNA molecule had completed one round of replication. In the second, chloramphenicol was added at early times after infection, which allowed greater amounts of the amplified DNA to be synthesized. It was then postulated that during certain stages of T4 phage development, there exist "signals" for the termination of DNA replication, which results in the amplification of specific genetic areas. At the present time, the exact nature of this signal, if it exists, is not known.

In this paper we will demonstrate that UV irradiation provides a mechanism by which DNA replication is terminated, thus resulting in the amplification of genetic areas which are endowed with origins of replication.

Most of the experiments were performed with bromodeoxyuridine (BUDR)-substituted (heavy) phage. The advantage of using these phage stems from the fact that heavy phage undergo very low levels of multiplicity reactivation (L. L. Restifo, T. Madera, S.-K. Ling, H. H. Vogelbacker, and A. W. Kozinski, manuscript in preparation), which would, of course, result in the formation of complete, undamaged genomes. The extensive damage to the heavy DNA is due to the chain of events that the parental molecule is subjected to after debromination of 5-bromo-

uracil to uracil by UV irradiation. In short, upon
infection of wild-type hosts, the irradiated parental molecule becomes extensively broken due to the excision of uracil by the host enzyme uracil glycosylase and due to subsequent digestion of the DNA by endonucleases (H. Vogelbacker and S.-K. Ling, Fed. Proc. 40:1764, 1981). In vitro, uracil glycosylase excises uracil from UV-irradiated heavy DNA, whereas, on the other hand, it has no effect on non-irradiated DNA. Upon infection of a uracil glycosylase mutant, UV-irradiated heavy T4 DNA undergoes much less breakdown and greater multiplicity reactivation than are observed in the wild-type host (Vogelbacker and Ling, Fed. Proc. 40:1764, 1981). It is out of the context of this paper to elaborate on these findings at this time, since the major objective of this paper is to demonstrate gene amplification upon infection with UV-irradiated heavy T4 phage.

(This research was done by H.H.V. in partial fulfillment of the requirements for a Ph.D. thesis, University of Pennsylvania.)

MATERIALS AND METHODS

Bacterial and phage strains. E. coli B23 was used in all of the experiments. The phage strain employed was the osmotic shock-resistant strain T4BO1. The PBR322 recombinant plasmids and the T4 genes they contain were: 663, genes 50-5; 669, genes 6-7; 657, genes 7-8; 660, genes 9-11; 659, genes 12-16; 654, gene 24; 656, genes uvsW-29 (designated in Fig. 4 and 5 as W-29); 625, gene 30; 658, genes 35-36; 653, genes 37-38; 621, gene 52; 627, genes 40-41; 624, genes 42-Bgt; and 622, gene 43. The PCRI recombinant plasmid, 46, contained the tRNA₅₅ gene (16).

Growth medium and preparation of phage stocks. The growth medium was TCG (11). The “heavy” medium and phage containing BuDR were prepared as previously described (7). In our hands, heavy phage were 85 to 95% viable. This was determined by two independent criteria: (i) by calculating the ratio of plating phage to the bacteria-killing particles; (ii) by comparing the total amount of phage DNA to the plating titer of the phage. All experiments were carried out at 37°C.

DNA extraction from infected cells. Samples from the infected-cell suspensions were diluted twofold in ice-cold LTL-EDTA (0.15 M NaCl, 0.01 M Tris-hydrochloride, 0.015 M EDTA, pH 7.4), and the cells were sedimented and resuspended in 1 ml of LTL-EDTA. For CsCl and Cs₂SO₄ gradient analysis, the DNA was extracted by the sodium dodecyl sulfate-panase-phenol method (9). The efficiency of recovery upon extraction is 100%. For alkaline sucrose gradient analysis, the DNA was released from the infected cells according to the LTL lysis procedure (13).

When used for labeling progeny DNA, [¹°H]thymidine was added as a “package,” which upon dilution into the experimental medium resulted in 5 µg of thymidine per ml (an adequate amount of [¹°H]thymidine to assure the desired specific activity), 5 µg of 5-fluorodeoxyuridine per ml, 5 µg of adenine per ml, and 10 µg of uracil per ml. CsCl and Cs₂SO₄ density gradients were prepared and run as previously described (3, 8). The alkaline sucrose gradients were prepared as 5 to 20% sucrose in 1 M NaCl-0.2 M NaOH and were centrifuged at 30,000 rpm for 3 h in a Beckman SW50.1 rotor.

The distance sedimented by the experimental DNA molecules relative to that sedimented by the reference (size 1) DNA (D₂/D₁) serves as a measure of strand integrity (1). The interpretation of D₂/D₁ as a measure of the number of random breaks incurred per strand was performed according to Litwin et al. (12).

UV irradiation of the bacteriophage was performed with a germicidal lamp (General Electric Co.) without a filter. During the UV irradiation, the phage were suspended in Tris-salt buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4). The number of UV irradiation hits received was calculated from the phage survival curves. One hit is the amount of UV irradiation that causes, on the average, one lethal event per phage. In conformity with the data of Carlson and Kozinski (3), the time it took to deliver 1 hit for heavy phage was equal to the time it took to deliver 0.5 hit for light phage.

The cloning and transfer of cloned T4 DNA segments to nitrocellulose filters have been described (16). There was approximately 2.4 µg of DNA charged onto each filter. The hybridization of the experimental DNA to the cloned DNA immobilized on nitrocellulose filters was performed according to the method of Denhardt (4). The experimental DNA labeled with [¹°H]thymidine was combined with [³²P]-labeled (specific activity, 1 mCi/mg) reference mature phage DNA. The hybridization mixtures consisted of 4 x 10⁶ cpm of H (approximately 0.04 µg of experimental DNA) and 2 x 10⁵ cpm of [³²P] (approximately 0.1 µg of reference DNA). The mixtures were then sonicated, denatured by heat (100°C for 15 min), and hybridized at 65°C for 48 h. For each moiety, the hybridization was performed in a single vial containing the nitrocellulose filters charged with the different cloned T4 genes. After hybridization, the filters were washed extensively with 3X SSC (1X SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and dried, and the hybridized radioactivity was counted in a toluene-based scintillation fluid in a liquid scintillation counter. The statistical error did not exceed 3% (5). (It should be noted that with this amount of radioactivity added, an uncharged filter or a filter charged with plasmid DNA alone registered 1.5 times the background for [³²P] and 2 times the background for [¹°H]. Both were considered when performing the calculations. We want to emphasize [5] that controls performed on numerous occasions revealed that the labeling of the proportion of the net weight of the DNA carrying either of the isotopes from 10:1 [experimental DNA:reference DNA] to 1:10 resulted in a proportional distribution of both of the isotopes hybridized to the cloned fragments. Finally, increasing the total amount of the DNA added to the set of filters by a factor of 10 did not alter the proportion of the two labels after hybridization. In the experiment documented in this paper, the lowest observed hybridized count was 150 cpm.) The results are expressed as
relative representations (RR) of genetic segment \( x \) in the progeny DNA hybridized. The \(^3\text{H}/\text{\( ^{32}\text{P} \)} \) ratio observed for a filter charged with a given genomic area, \( x \), was divided by the ratio of the sums of \(^3\text{H} \) and \(^{32}\text{P} \) hybridized to all of the nitrocellulose filters in a set:

\[
RR_x = \frac{\sum \text{H}_x/\sum \text{P}_x}{\sum \text{H}/\sum \text{P}} \tag{1}
\]

The expression of the data in this form allowed all of the experimental results to be drawn on a common scale depicting the RR of each genetic area in the progeny DNA (10).

**RESULTS**

Experiments were performed to determine the genetic representation of the progeny DNA produced after the infection of *E. coli* with UV-irradiated heavy phage. This was determined by hybridization of \([\text{\( ^3\text{H} \)}}\] thymidine-labeled newly synthesized DNA to cloned T4 DNA segments immobilized on nitrocellulose filters. In addition, the amount of DNA synthesized was measured and expressed as the number of phage equivalent units of DNA synthesized per infected cell. The proof of the phage nature of the DNA and the size of the progeny DNA synthesized is also documented.

Although the results were confirmed in numerous, independent experiments, we wish to emphasize that the results presented here were derived from a single experiment. This eliminates minor fluctuations in temporal events occurring between repeated experiments and allows fair comparison of different properties of exactly the same material.

Net amount of phage DNA synthesized in bacteria infected with UV-irradiated BUdR-labeled phage. It has previously been shown that there is a greater amount of DNA synthesized in cells infected with UV-irradiated T4 phage than one would expect if only the multiplicity-reactivated cells produced phage DNA (14). An even greater extent of synthesis is shown to occur after infection with UV-irradiated, BUdR-substituted phage.

Bacteria were grown to a concentration of \( 3 \times 10^8 \) cells per ml in a “light” medium and were infected at a multiplicity of infection of 8 with heavy phage which had received 10 lethal hits of UV irradiation. (Since almost 30% of the T4 genes code for proteins involved in DNA replication \([15]\), low multiplicity of infection experiments cannot be performed with phage which have received high doses of UV irradiation. For example, phage which receive 10 lethal hits of UV irradiation will have, on the average, three genes necessary for DNA replication damaged. This certainly will detrimentally affect phage DNA replication. Smaller doses of UV irradiation, on the other hand, will allow for a large number of survivors, thus rendering the interpretation of these types of experiments virtually impossible.) As a control, unirradiated heavy phage were also used to infect a separate portion of “light” bacteria. At 3.5 min after infection, \(^3\text{H}\)-labeled thymidine (specific activity, 10 mCi/mg) was added. At various times, samples were taken for the measurement of DNA synthesis and for the extraction of intracellular DNA.

Figure 1 shows the average amount of DNA synthesized per infected cell as a function of time after infection. Although no appreciable multiplicity reactivation (\(~3\%) \) is observed with heavy phage that have received 10 hits of UV irradiation, 36 phage equivalent units of DNA per infected bacterium were synthesized. This is approximately 10% of the level of DNA synthesis observed after infection with the same amount of unirradiated heavy phage. Therefore, in many infected cells which do not undergo multiplicity reactivation, a considerable level of DNA synthesis still occurs.

To confirm that all of the observed synthesis (i.e., \([\text{\( ^3\text{H} \)}}\] thymidine uptake) was that of phage DNA, part of the bacterial suspension was sampled at 25 min after infection, and the DNA was extracted and fractionated in a CsCl gradient. In this type of equilibrium gradient, bacterial DNA bands to the right, lighter side of the T4 reference DNA (2). All of the observed synthesis

![Fig. 1. Average amount of DNA synthesis in *E. coli* cells infected with BUdR-labeled T4 phage that received 0 and 10 hits of UV irradiation, expressed as the number of phage equivalent units (PEU) of DNA per infected cell as a function of time. DNA synthesis was determined by measuring the amount of \([\text{\( ^3\text{H} \)}}\] thymidine incorporated into the DNA. The actual figures may be higher due to the reutilization of host DNA breakdown products.](http://jvi.asm.org/ on July 3, 2017 by guest)
was phage DNA. Moreover, all of the newly synthesized DNA banded coincidently with light $^{32}$P-labeled T4 reference DNA (Fig. 2). This indicates that the synthesis was not primarily of a repair type ($^{3}$H label would band, in this case, close to the heavy location) but rather represented light copies of the heavy parental genome (or rather of its segments). Note that there is no detectable amount of $^{3}$H label at the location of the hybrid (one parental strand heavy, one progeny strand light). This might be indicative of two likely possibilities: (i) not all parental molecules participate in replication (or for that matter, not all infected bacteria produce partial progeny replicas); (ii) upon a limited number of rounds of replication, parental heavy strands still containing some nonexcised uracil become, belatedly, further degraded and thereby eliminated from the replicative DNA pool.

Size of progeny strands of UV-irradiated BUdR-labeled phage. For the determination of the size of the progeny strands of UV-irradiated heavy phage, samples were taken from the bacterial suspension at 10, 15, 25, and 40 min after infection and subjected to alkaline sucrose gradient analysis along with $^{32}$P-labeled reference DNA (referred to as size 1). At all four times sampled, there is significant displacement of the [$^{3}$H]DNA peak from the reference [$^{32}$P]-DNA peak (Fig. 3). This shows that the size of the progeny DNA synthesized after infection with UV-irradiated heavy phage is quite small.

![Graph](http://jvi.asm.org/)

**Fig. 2.** Cs$_{2}$SO$_{4}$ gradient analysis of DNA synthesized 25 min after infection of E. coli with BUdR-labeled T4 phage that had received 10 hits of UV irradiation. The DNA bands coincidently with the light $^{32}$P-labeled reference T4 DNA.
relative to the size of the intact strands. (Neutral sucrose gradient analysis was not performed because the extraction procedure could convert single-stranded nicks to double-cuts and, hence, give spurious results. In addition, recombinant molecules, because of their geometry [branched structures], will sediment differently in a neutral sucrose gradient than will double-stranded linear pieces of DNA of the same accumulated length [8].)

Interpretation of the sedimentation patterns is based on the graph of Litwin et al. (12) that relates that value of $D_2/D_1$ to a size distribution of fragments resulting from random interruptions in a unit length of DNA. For example, $D_2/D_1$ of the progeny DNA extracted 15 min after infection was 0.45. This is what would be expected if T4 DNA was subjected to a process which randomly introduced 15 cuts.

Because of the steepness of the standard curve in the region where the values of $D_2/D_1$ are less than 0.40, it is difficult to quantify precisely the size of the progeny DNA 10 min after infection, at which time $D_2/D_1$ was 0.38. This corresponds to at least 20 cuts.

As a function of time, the sizes of progeny molecules, derived from a high multiplicity of infection with UV-irradiated heavy phage, increase (Fig. 3, vertical comparison). The calculated number of randomly delivered interruptions (cuts) required to give the observed banding patterns changes progressively from greater than 20, for samples taken at 10 min, to 9, for samples taken at 40 min. We consider it reasonable to conclude that this progressive increase in size might result from recombination among members of the intracellular pool of DNA (see below and Fig. 6).

Gene representation in progeny DNA of UV-irradiated BUdR-labeled T4 phage as measured by hybridization to cloned T4 DNA segments. To analyze the gene representation in the progeny DNA accumulated upon infection with UV-irradiated heavy bacteriophage, we isolated the light moieties in the Cs$_2$SO$_4$ gradients from samples obtained 25 and 40 min after infection. The fractions were digested with pancreatic ribonuclease and then hybridized against $10^{-3} \text{ M} \text{ EDTA}$ and prepared for hybridization as described above. The moieties were then hybridized to each of 15 cloned genomic segments immobilized on nitrocellulose filters.

Scrutiny of Fig. 4 reveals a sizable bias in the gene representation. The most highly replicated areas (those of genes 50-5 and uvsW-29) are those which have been shown previously to contain origins of replication (5). In particular, the maximum-to-minimum ratio is 4.6 for samples taken 25 min after infection and 3.5 for samples taken 40 min after infection.

**Fig. 3.** Alkaline sucrose gradient analysis of DNA synthesized at 10, 15, 25, and 40 min after infection of E. coli with BUdR-labeled T4 phage that had received 10 hits of UV irradiation. The $^{32}P$ label represents size 1 T4 reference DNA. Note that the size of the DNA increases with time but remains smaller than the size 1 reference. $D_2/D_1$ for $^{3}H$-labeled progeny is at 10 min, 0.38; at 15 min, 0.45; at 25 min, 0.50; and at 40 min, 0.53. If one assumes assortment of sizes resulting from the random cuts, those $D_2/D_1$ values correspond to more than 20, 15, 12, and 9 cuts, respectively.

**Fig. 4.** Hybridization of progeny DNA to cloned genetic segments. $^{3}H$-labeled progeny DNA isolated 25 and 40 min after infection with BUdR-labeled T4 phage that had received 10 hits of UV irradiation was assayed. $[^3H]$thymidine-labeled DNA was isolated from preparative CsCl density gradients, dialyzed, mixed with $^{32}P$-labeled mature T4 reference DNA, sonicated, denatured, and hybridized to cloned T4 genetic areas immobilized on nitrocellulose filters. The vertical bars show the RR of each genetic area in the $^{3}H$-labeled progeny DNA. These bars are placed on the genetic map (given in kilobases and starting with the rII region) in the center of the region represented by each cloned fragment. The broken horizontal line indicates the theoretical RR that would be expected if all of the genes tested were equally represented in the progeny DNA (RR = 1). The maximum-to-minimum (max/min) value is a result of division of the highest RR observed by the lowest.
taken at 40 min after infection. (In other words, the areas most highly represented are produced in 4.6-fold- and 3.5-fold-greater quantities, respectively, than the areas least represented.) For the 40-min sample, calculation of the number of gene copies per cell showed that at this time, when 36 phage equivalent units of DNA had been synthesized (Fig. 1), the number of gene copies varied from 76 for genes of high RR to 21 for genes of low RR.

The following approach (10) was used:

\[ \text{copies } x = \frac{\text{H}^*_x/\text{P}^*_x \cdot (\text{PEU DNA/Ic})}{M} \]  

where copies \( x \) is the number of copies of genetic area \( x \) per cell; \( \text{H}^*_x/\text{P}^*_x \) is the ratio of \( ^3\text{H} \)-labeled progeny to \( ^{32}\text{P} \)-labeled reference DNA hybridized to a filter charged with cloned genetic segment \( x \); \( M \) is the \( ^3\text{H}/^{32}\text{P} \) ratio of DNA hybridized to a nitrocellulose filter charged with mature phage DNA; and PEU DNA/Ic is phage equivalent units of DNA per infected cell.

**Gene representation in progeny DNA of UV-irradiated light T4 phage as measured by hybridization to cloned T4 DNA segments.** All of the experiments described above were performed with heavy phage. Originally, we avoided using light phage, since they exhibit a sizable degree of multiplicity reactivation which would make the interpretation of our results more difficult; nevertheless, we were interested in examining the progeny DNA after a high multiplicity of infection with light phage irradiated with 15 lethal hits of UV irradiation. Progeny DNA from a sample obtained 20 min after infection was extracted for hybridization by the methods described in the previous experiments with heavy phage.

Although the maximum-to-minimum ratio of 2.2 is less pronounced than in the analogous experiment with heavy, irradiated phage, the pattern of differential hybridization resembles very well that documented in Fig. 4, i.e., peaks at genetic areas 50-5 and uvsW-29 (Fig. 5). Since reactivated phage must contain a normal, unbiased complement of genetic material and since there was a significant amount of multiplicity reactivation observed (24%), it is not surprising that the overall bias in the progeny is less pronounced. Nonetheless, we consider it justifiable to conclude that upon infection with light, UV-irradiated phage, there is also partial replication of “preferred” areas of the genome.

**DISCUSSION**

We conclude from the above results that in *E. coli* cells infected with UV-irradiated, BUDR-substituted T4 phage, a considerable amount of DNA synthesis occurs in cells not productively infected. This DNA is biased in its genetic representation, and the accumulated regions correspond to areas known to contain origins of T4 DNA replication as previously reported by Halpern et al. (5).

These data are consistent with the assumption that UV irradiation, acting upon heavy DNA, imposes termination (most likely by cuts occurring within the host cell) (Restifo et al., in preparation). This termination in turn results in the partial replication and amplification of those segments which are endowed with origins and, therefore, results in the more abundant accumulation of certain areas than of others. The possibility that the uptake patterns reflect a high versus low frequency of adenine-thymine base pairs in the different cloned fragments is out of the question, since, first, in the control cohybridization experiment with \(^7\text{H} \)-thymidine- and \(^{32}\text{P} \)-labeled DNA (random labeling), a straight line results with RR = 1 (5). In other words, all of the tested areas have, on the average, the same proportional amount of thymidine to phosphorus. Second, sonication of \(^7\text{H} \)-adenine- and \(^{32}\text{P} \)-labeled heavy DNA revealed no significant skew of the specific activities of the two labels after CsCl gradient analysis (6).

The other conceivable possibility which would affect our conclusion is the possibility of successful competition in the uptake of \(^7\text{H} \)-thymidine by host DNA degradation products (i.e., cold thymidine). This is highly unlikely, since it
would call for the hypothesis that the host DNA degradation products are preferentially channeled to certain areas of the T4 genome, and not to others. Moreover, this would not explain the hybridization patterns seen at early times after infection, e.g., the genetic bias in the progeny DNA at 6 min after infection when there is no measurable host reutilization.

Note that the overall pattern (the profile) of RR is qualitatively similar at 25 and 40 min after infection despite the decreased bias at the later time (Fig. 4). This decrease in the maximum-to-minimum ratio (bias) with time, considered in the light of the increasing progeny size documented above (Fig. 3), suggests that, due to recombinational events, fragments endowed with origins provide for the replication of fragments without them or with only weak origins (Fig. 6).

There is another explanation for this gradual increase in the size of the progeny subunits. One can assume that the damages are being eliminated and gradually repaired or that temporary blocks are being bypassed. Replication can then proceed, further encompassing more of the genome, resulting in longer progeny subunits more representative of genetic areas further away from the origins of replication.

In normal phage DNA there exist signals for the termination of replication, resulting in the amplification of specific genetic areas (10). At this time it is not clear what these termination signals are. We have now demonstrated that UV irradiation restricts replication to subunits containing origins which result in amplification of specific areas of the genome.

A series of experiments documented by Raysigui et al. (14) led to the conclusion that multiplicity reactivation among UV-inactivated phage occurs by partial replication followed by recombination of those partial replicas. This in turn, it was postulated, results in the reconstitution of a complete phage genome. It was hypothesized at that time, but not proven, that the areas endowed with origins of replication should replicate preferentially and repeatedly. Data presented in this paper demonstrate that, indeed, in the case of UV-irradiated heavy phage, specific genetic areas are repeatedly replicated.

**ACKNOWLEDGMENTS**

This research was supported by National Science Foundation grant PCM 78-19971 (awarded to A.W.K.). H.H.V. was supported by Public Health Service training grant T32GM 07229-06, and L.L.R. was supported by Medical Scientist training grant GM 07170 from the National Institutes of Health. This research resulted partially from the efforts of the students participating in the course (551) given by the Graduate Group in Genetics. The assistance of M. Leahy is greatly appreciated.

**LITERATURE CITED**


