Effects of UV Irradiation on the Fate of 5-Bromodeoxyuridine-Substituted Bacteriophage T4 DNA

LINDA L. RESTIFO, HELEN H. VOGELBACKER, THOMAS MADARA, SHUI-KUEN LING, AND
ANDRZEJ W. KOZINSKI*

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

Received 28 December 1982/Accepted 12 April 1983

We have carried out a series of experiments designed to characterize the impact of UV irradiation (260 nm) on 5-bromodeoxyuridine-labeled (heavy) T4 bacteriophage, both before and after infection of *Escherichia coli*. In many respects, these effects differ greatly from those previously described for non-density-labeled (light) phage. Moreover, our results have led us to propose a model for a novel mechanism of host-mediated repair synthesis, in which excision of UV-damaged areas is followed by initiation of replication, strand displacement, and a considerable amount of DNA replication. UV irradiation of 5-bromodeoxyuridine-labeled phage results in single-stranded breaks in a linear, dose-dependent manner (1.3 to 1.5 breaks per genomic strand per lethal hit). This damage does not interfere with injection of the phage genome, but some of the UV-irradiated heavy phage DNA undergoes additional intracellular breakdown (also dose dependent). However, a minority (25%) of the injected parental DNA is protected, maintaining its preinjection size. This protected moiety is associated with a replicative complex of DNA and proteins, and is more efficiently replicated than is the parental DNA not so associated. Most of the progeny DNA is also found with the replicative complex. The 5-bromodeoxyuridine of heavy phage DNA is debrominated by UV irradiation, resulting in uracil which is removed by host uracil glycosylase. Unlike the simple gap-filling repair synthesis after infection with UV-irradiated light phage, the repair replication of UV-irradiated heavy phage is extensive as determined by density shift of the parental label in CsCl gradients. The newly synthesized segments are covalently attached to the parental fragments. The repair replication takes place even in the presence of chloramphenicol, a protein synthesis inhibitor, suggesting it is host mediated. Furthermore, the extent of the repair replication is greater at higher doses of UV irradiation applied to the heavy phage. This abundant synthesis results ultimately in dispersion of the parental sequences as short stretches in the midst of long segments of newly synthesized progeny DNA. Together, the extensive replication and the resulting distribution pattern of parental sequences, without significant solubilization of parental label, are most consistent with a model of repair synthesis in which the leading strand displaces, rather than ligates to, the encountered 5' end.

Since this paper describes the events ensuing from UV irradiation (260 nm) of 5-bromodeoxyuridine (BUdR)-substituted (heavy) T4 bacteriophage, it will be useful to review the structural and functional consequences of irradiating non-density-labeled (light) phage. Studies from this laboratory have shown (13) that UV irradiation of light phage causes, in addition to thymine dimer formation, cross-linking of the DNA, approximately 0.1 cross-link per genome per lethal hit. Note, however, that before infection no single- or double-stranded breaks can be found in the irradiated phage DNA (13). Upon infection with UV-irradiated light phage, the parental

DNA acquires approximately one single-stranded break per lethal hit (16). These breaks are believed to be introduced adjacent to the thymine dimers by the phage-coded ν gene product since single-stranded cutting is not observed in ν mutants (13).

The nicking of the light DNA is followed by excision of the damage and repair synthesis to fill in the gap, resulting in a majority of unit length molecules as evidenced by alkaline sucrose gradient analysis (13). Of importance to the data we will present below is the fact that these gaps must be short since the repair synthesis, when carried out in density-labeled bacteria,

causes no density shift of parental label in CsCl gradients (21; E. Shahn, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1965).

The gap-filling repair replication of UV-irradiated light phage occurs even in the presence of chloramphenicol, suggesting a role of host-coded functions. In particular, it requires DNA polymerase I since host strains defective in this enzyme show poor repair of UV-induced damage to light phage DNA (2). Upon removal of chloramphenicol from the medium and further incubation, the repaired DNA is highly degraded, probably by phage-coded enzymes recognizing the unmodified cytosine residues inserted during host-mediated repair (2).

In host cells multiply infected with UV-irradiated light phage, complete phage genomes can be reconstituted, resulting in a productive infection. We have shown that this phenomenon, multiplicity reactivation, occurs by repeated partial replication of undamaged areas followed by recombination of those partial replicas (21) and not by interparental recombination as had previously been supposed. The level of DNA synthesis under these conditions is greater than can be accounted for by multiplicity reactivation alone. The parental contribution to the progeny DNA molecule was shown to be in the form of short, semi-conservatively replicated subunits, covalently linked to the progeny DNA (21). The length of these parental subunits became smaller with increasing doses of UV irradiation (21; E. Shahn, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1965). Therefore, the presence of the parental DNA in the progeny phage is not due to the scavenging of the damaged parental nucleotides or to the ligation of conservative (unreplicated) double-stranded parental subunits to the progeny DNA.

This paper is the second in a series examining the consequences of UV irradiation on heavy T4 bacteriophage. In an earlier paper (15), we documented a number of properties of the progeny DNA synthesized after infection with such phage. In particular, it was shown (15) that the gene representation of the progeny DNA was biased in favor of the genetic areas containing the origins of DNA replication (3), reflecting amplification of these areas. Over the course of the infection, the size of such progeny DNA increased while the genetic bias decreased, probably reflecting recombination among smaller progeny segments. CsCl gradient analysis indicated most of the newly synthesized progeny DNA was not covalently joined to the heavy parental DNA (15).

The experiments described below focus attention on the fate of the heavy parental DNA after UV irradiation and subsequent infection. We will first document the damages inflicted directly on the irradiated phage DNA. Our data will

further show that the events after infection are the result of a competition between two opposing forces which either protect against or initiate further breakdown of the already damaged irradiated DNA. This protection appears to allow an extensive repair replication which is host mediated. It results in a scattering of very short segments of parental sequences among long stretches of progeny DNA to which the former are covalently joined. The balance (or imbalance) between the degradative and protective processes may provide an explanation for differences we have observed in the level of multiplicity reactivation between heavy and light UV-irradiated phage.

More important, these results have led us to propose a new model of repair replication which differs significantly from classical gap-filling excision repair. In the latter case, the maximal progeny contribution to the repaired molecule can be only 50%. The model described below is reminiscent of replication late in T4 infection at which time recombinational intersections can act as sites of initiation of DNA synthesis (4).

(This research is partially in fulfillment of the requirements for a Ph.D. thesis of Helen H. Vogelbacker.)

MATERIALS AND METHODS

Escherichia coli strain B-23 was used in all of the experiments. The bacteriophage used was the osmotic shock-resistant strain T4BO₁^r.

The growth medium was TCG (14). The isotope labeling, the heavy medium, and the phage containing BUdR were prepared as previously described (8). The percent substitution of BUdR for thymidine in heavy phage was approximately 95%. The heavy phage (we will designate the DNA of such phage as HH DNA) used in our experiments were between 80 and 95% viable as determined by two independent criteria: (i) calculating the ratio of plating titer to the concentration of bacteria-killing particles and (ii) comparing the total amount of phage DNA to the plating titer of the phage. When infections were carried out in the presence of chloramphenicol, it was added to a final concentration of 200 µg/ml. Test of inhibition of protein synthesis was performed previously by measuring the uptake of [³H]leucine. The observed uptake was less than 1% of that in the untreated control.

When used for labeling the progeny DNA, [³H]thymidine was included in a package, which upon dilution into the experimental medium resulted in 5 µg of thymidine per ml, an adequate amount of [³H]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. All experiments were carried out at 37°C.

In preparation for DNA extraction, samples of suspensions of infected cells were diluted twofold in ice-cold LTL-EDTA (0.15 M NaCl, 0.01 M Tris-hydrochloride, 0.015 M EDTA, pH 7.4). The cells were then sedimented and suspended in 1 ml of LTL-EDTA. For CsCl gradient analysis, the DNA was extracted by the

sodium dodecyl sulfate-pronase-phenol method (11). The efficiency of phage DNA recovery upon extraction is 100%. For alkaline sucrose gradient analysis, the DNA was released from the cells according to the LTL lysis procedure (19), followed by digestion and concomitant denaturation of DNA with 0.1 M NaOH at 37°C for 10 min. For the isolation of intracellular phage DNA associated with proteins, a gentle lysis procedure using lysozyme and Triton detergent was performed as previously described (19).

CsCl density gradients were prepared and run as previously described (9). The denaturation of the DNA for CsCl gradient analysis was accomplished by adding KOH to a final concentration of 0.1 M, followed by neutralization with KH_2PO_4 . This step of neutralization is important, since centrifugation of T4 DNA in CsCl gradients containing KOH (or NaOH) at a concentration of 0.1 M results in the introduction of numerous single-stranded breaks (E. Shahn, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1965). The observed distribution of the parental label in a CsCl gradient was used to calculate the percentage of parental DNA which had replicated (R). The efficiency of replication (E) was calculated as previously described (21), accepting as 100% efficiency the extent of DNA replication observed in infections with unirradiated phage. The E value for UV-irradiated phage is expressed as a percentage of the replication of the unirradiated phage:

$$E = (R^{\text{UV}}/R^{\text{non-UV}}) \times 100\%$$

Alkaline sucrose gradients were prepared as 5 to 20% sucrose in 1 M NaCl and 0.2 M NaOH and were centrifuged at 30,000 rpm for 3 h in an SW50.1 rotor. Unit-length T4 or, where appropriate, T7 phage DNA was added as a size reference. The distance sedimented in alkaline sucrose gradients by the experimental DNA relative to that sedimented by the unit-length reference DNA (D_2/D_1) serves as a measure of strand integrity. The interpretation of D_2/D_1 as a measure of the number of single-stranded breaks incurred by genomic-length T4 DNA, as a result of processes acting at random locations along the molecule, was performed according to Litwin et al. (16). It should be noted, however, that this method of analysis cannot distinguish between true single-stranded breaks and alkali-labile areas.

The fast-sedimenting DNA-protein complexes were isolated in neutral sucrose gradients overlaid with 1 ml (a pad) of saturated sucrose solution. (In this paper, the term "padded neutral sucrose gradient" will be used when referring to such gradients.) The gradients were run at 15,000 rpm for 90 min in an SW50.1 rotor.

UV irradiation (260 nm) of the bacteriophage was performed with a germicidal lamp (General Electric) without a filter. During the UV irradiation, the phage were suspended in Tris-salt buffer (0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4). The number of UV irradiation hits received was calculated from the survival curves of the irradiated phage. One hit is the amount of UV irradiation causing, on average, one lethal event per phage, where the probability distribution is Poissonian. In conformity with the data of Carlson and Kozinski (1), the time required to deliver 1 hit to heavy phage is equal to the time required to deliver 0.5 hits to light phage.

The sonication of DNA was performed in a Raytheon sonicator. The molecular weights of the resulting DNA fragments ranged between 5×10^5 and 1×10^6 .

RESULTS

Effect of UV irradiation on the integrity of BUdR-labeled T4 DNA. ^{32}P -labeled heavy phage were exposed to 0, 5, or 10 lethal hits of UV (260 nm) irradiation. The DNA was released from the phage by alkaline lysis, and ^3H -labeled, unit-length heavy T4 DNA was added as a reference. The DNA was sedimented through alkaline sucrose gradients. As expected, the DNA from unirradiated phage sedimented coincidentally with the reference DNA (Fig. 1A). However, the DNA from UV-irradiated phage was significantly displaced from the reference DNA. This displacement was dose dependent. In particular, D_2/D_1 equaled 0.56 for the DNA of heavy phage exposed to 5 lethal hits (Fig. 1B). This ratio corresponds to seven randomly distributed single-stranded breaks (16). For the DNA of heavy phage exposed to 10 lethal hits, D_2/D_1 equaled 0.45 (Fig. 1C), corresponding to 15 single-stranded breaks.

We wished also to assay for the production of double-stranded breaks in the DNA of irradiated heavy phage. Of some concern was the possibility of artifactually introducing or promoting double-stranded breaks during the phenol extraction of native DNA from UV-damaged phage. To avoid this potential problem, the ^{32}P -labeled heavy DNA was first extracted from phage and then exposed to 5 or 10 lethal hits of UV irradiation. The irradiated DNA was examined in native form in neutral sucrose gradients along with ^3H -labeled, unit-length heavy T4 DNA. In the case of both 5 and 10 lethal hits, the UV-irradiated heavy DNA cosedimented with the reference DNA (Fig. 2A). Thus, in this dose range, UV irradiation of heavy DNA does not cause double-stranded breaks.

On the other hand, alkaline sucrose gradient analysis revealed dose-dependent single-stranded breakage of UV-irradiated heavy DNA (Fig. 2B). For DNA receiving 5 lethal hits, the D_2/D_1 ratio equaled 0.56, corresponding to seven randomly distributed single-stranded breaks. For DNA receiving 10 lethal hits, D_2/D_1 equaled 0.48, indicating an average of 13 single-stranded breaks. Therefore, UV irradiation of heavy T4 DNA causes 1.3 to 1.5 single-stranded breaks per lethal hit, regardless of whether the radiation is delivered to the phage or to the extracted DNA.

Additional breakdown of UV-irradiated BUdR-substituted DNA after infection. The integrity of the intracellular parental DNA of UV-irradiated heavy phage 5 min after infection was examined by alkaline sucrose gradient analysis. The re-

FIG. 1. Alkaline sucrose gradient analysis of ^{32}P -labeled DNA extracted from heavy T4 phage which had received 0 (A), 5 (B), or 10 (C) hits of UV

sults documented here show that UV-irradiated heavy DNA acquires additional single-stranded breaks after injection into the host cell.

As shown above (Fig. 1C), the DNA of heavy phage irradiated with 10 lethal hits had approximately 15 single-stranded breaks before infection, resulting in an average size of approximately one-eighth that of unit-length T4 DNA. However, alkaline sucrose gradient analysis of the intracellular phage DNA extracted 5 min after infection (multiplicity of infection [MOI] = 6) revealed a bimodal distribution of the parental label (Fig. 3A). The faster sedimenting portion (which consisted of 25% of the recovered parental label) corresponded to a size approximately one-eighth that of unit-length heavy T4 DNA. In other words, it appeared unchanged in size from that of the UV-irradiated parental DNA before infection. The remaining 75% of the parental DNA was very small and located near the top of the gradient, where resolution was too poor to allow a precise size determination. It is possible to state, however, that the strands were less than 1/100 the size of unit-length T4 DNA.

Clearly, this bimodal size distribution could not be due to a generalized degradation of the total parental DNA. Rather, the data suggest that, although most DNA of highly irradiated heavy phage is additionally and extensively broken after injection into the host cell, a small portion is somehow protected from this process. Indeed, experiments described below implicate the DNA-protein replicative complex in the mechanism of this protection.

To determine whether host- or phage-coded enzymes mediate the observed intracellular breakdown, the protein synthesis inhibitor chloramphenicol was added to a portion of the bacterial suspension at the time of infection to prevent the production of phage-coded proteins. Figure 3B shows the alkaline sucrose gradient sedimentation pattern of DNA extracted 5 min after infection with UV-irradiated (10 hits) heavy phage. The pattern was very similar to

irradiation. ^3H -labeled unit-size heavy T4 DNA was added as reference. Note the distance sedimented through the gradient by the UV-irradiated DNA compared to that by the reference DNA. The D_2/D_1 ratio has been shown by computer simulation and in vitro experiments to be a useful index for approximating the average number of random breaks inflicted on an experimental population of DNA molecules (16). This figure demonstrates a linear, dose-dependent relationship between the number of lethal hits of UV irradiation and the resulting number of single-stranded breaks in the DNA of the irradiated phage. Symbols: ○, ^3H ; ●, ^{32}P .

FIG. 2. Sucrose gradient analyses of ^{32}P -labeled heavy T4 DNA extracted from phage and then exposed to 0, 5, or 10 hits of UV irradiation. ^3H -labeled unit-size heavy T4 DNA was added as reference. Upon analysis in neutral sucrose gradients (NSG) (A), the irradiated heavy phage DNA bands coincidentally with genomic length reference DNA. Thus, UV irradiation in this dose range does not cause any double-stranded breaks in heavy T4 DNA. Note that the sedimentation patterns in alkaline sucrose gradients (ASG) (B) look very similar to those shown in Fig. 1. Again, 1.3 to 1.4 single-stranded breaks per lethal hit are introduced by UV irradiation of genomic length heavy T4 DNA. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

that seen without chloramphenicol, with 73% of the parental label sedimenting as very small fragments and 27% as larger (one-eighth of T4 unit length) pieces. This strongly suggests that the breakdown of UV-irradiated phage DNA after infection is performed by host-coded enzymes.

Extent and nature of DNA replication of UV-irradiated heavy phage as examined by CsCl density gradient analysis. The experiments described above demonstrate (i) the immediate effect of UV irradiation on the structural integrity of BUdR-substituted phage DNA and (ii) the

further breakdown of the irradiated DNA subsequent to infection. To determine the replicative competency of the damaged heavy phage DNA, we examined its ability to undergo replication (as evidenced by a change in density) under a number of infection conditions in light bacteria: low and high multiplicity and in the presence of unirradiated, rescuing phage.

First, we observed phage DNA replication in *E. coli* infected at a low multiplicity with UV-irradiated heavy phage. Separate portions of bacteria were infected at an MOI of 0.2 with ^{32}P -labeled heavy phage irradiated with 0, 5, or 10

FIG. 3. Alkaline sucrose gradient analysis of intracellular ^{32}P -labeled heavy parental DNA isolated 5 minutes after infection with phage irradiated with 10 lethal hits (A). (B) The same experiment but in the presence of chloramphenicol (CM) added at the time of infection. ^3H -labeled unit-length heavy T7 DNA is the reference. The two panels show similar size distributions of parental DNA: in both cases the majority (75%) is very small, about 1/100 the size of unit-length T4 DNA, and the minority (25%) bands at a location corresponding to one-half T7 unit-length or approximately one-eighth unit-length T4 DNA. The similarity of patterns indicates the intracellular breakdown of UV-irradiated T4 DNA is not dependent on phage proteins and suggests it is host mediated. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

lethal hits. Samples were taken at 20 min after infection, at which time, even at low MOI, the DNA synthesis of unirradiated phage was well underway. The DNA was extracted, supplemented with ^3H -labeled heavy and light T4 DNA as density references, and subjected to CsCl

FIG. 4. CsCl gradient analysis of ^{32}P -labeled heavy parental DNA 20 min after low-multiplicity ($\text{MOI} = 0.2$) infections with phage that had received 5 (A) or 10 (B) hits of UV irradiation. ^3H -labeled heavy and light T4 DNA were added as references. Note the shift in density of the parental label away from the heavy reference location to an almost-light location. The E value is considerable and significantly higher for phage exposed to higher doses of irradiation. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

gradient analysis. In addition, portions of the extracted DNA were precipitated with trichloroacetic acid. Despite the significant damage done to heavy DNA exposed to high doses of UV irradiation, the parental label was never more than 20% decomposed to trichloroacetic acid-soluble fragments (data not shown). Treatment of separate portions of extracted DNA with nuclease S1 (data not shown) revealed no detectable digestion by this single strand-specific endonuclease. Thus, there was no large proportion of parental DNA in single-stranded form.

Twenty minutes after infection with unirradiated heavy phage at an MOI of 0.2, 73% of the parental label moved from the heavy toward the light location (data not shown). This density shift was accepted as a normal (or 100%) efficiency of DNA replication (21).

Figure 4A documents CsCl analysis of the DNA extracted 20 min after the low-multiplicity infection with heavy phage which had received five hits of UV irradiation. Forty-three percent of the ^{32}P label moved from the heavy location toward the light reference location, indicating an efficiency of replication of 58%. In the case of the low-multiplicity infection with heavy phage irradiated with 10 hits, 58% of the parental label shifted toward the light location, reflecting an efficiency of replication of 70% (Fig. 4B).

Note that the efficiency of replication was greater for the phage receiving the higher dose of UV irradiation. This is in seeming conflict with the fact that approximately 30% of T4 genes are involved in DNA replication (24), and, therefore, 10 hits delivered to a phage will result in damage, on average, to three of these genes. Although not all these genes are absolutely required, one should expect that, without the full complement of genes necessary for replica-

FIG. 5. CsCl gradient analysis of ^{32}P -labeled heavy parental DNA 20 min after a high-multiplicity (MOI = 5) infection with phage that had received 5 hits of UV irradiation. ^3H -labeled heavy and light T4 DNA were added as references. Panel (A) shows a significant density shift of parental label, representing an E value of 70%. Panel (B) shows the CsCl gradient distribution of the same DNA after sonication. About half of the almost-light parental label moved to the hybrid location. This represents relatively long stretches of parental sequences, whereas the parental label remaining almost-light after sonication must be disposed as very short stretches among much longer light ones. Panel (C) shows the extracted phage DNA after alkali denaturation. This treatment fails to shift the replicated parental label back toward the heavy location, indicating that these parental regions are covalently joined to newly synthesized progeny DNA. Symbols: ○, ^3H ; ●, ^{32}P .

tion, one would observe a lower efficiency of DNA replication in an infection with heavy phage irradiated with higher doses. However, these data are consistent with host-mediated repair replication of the UV-induced damages, and a body of evidence in support of this hypothesis will be presented in later sections.

In a separate experiment, *E. coli* were infected at high multiplicity (MOI = 5) with heavy phage which had received 0 or 5 hits of UV irradiation. As shown in Fig. 5A, DNA replication of the irradiated phage DNA was demonstrated by the shift of the heavy parental label toward the light location. Accepting as 100% efficiency the extent of density shift undergone by unirradiated heavy parental label, this corresponds to an efficiency of replication of 70%.

In a separate branch of the experiment, we coinfect with cold (non-radiolabeled), unirradiated rescuing phage to ensure that the infective cycle in each bacterium would be completed. *E. coli* were coinfect with UV-irradiated ³²P-labeled heavy phage (MOI = 0.2) and unirradiated cold heavy phage (MOI = 5). Figures 6A and 7A document the CsCl gradient analysis of the DNA extracted 20 min after coinfection. In the rescue experiment with phage irradiated with 5 lethal hits, 64% of the ³²P label moved from the heavy toward the light reference location, reflecting an efficiency of replication of 75%. Note that this efficiency of replication is not significantly different from that seen in the high-MOI experiment with heavy phage irradiated with 5 lethal hits. In the case of DNA extracted after coinfection with heavy phage irradiated with 10 lethal hits and the rescuing phage, 60% of the parental label underwent a shift toward the light location, reflecting an efficiency of replication of 70%.

We have demonstrated that UV-irradiated heavy phage undergo a considerable amount of DNA replication under the conditions of low

FIG. 6. CsCl gradient analysis of ³²P-labeled parental DNA 20 min after coinfection with heavy phage which had received 5 hits of UV irradiation (MOI = 0.2) and unirradiated cold heavy phage (MOI = 5). ³H-labeled heavy and light T4 DNA were added as references. (A) The efficiency of replication is slightly higher than in cells infected without rescuing phage. The replicated parental now bands very close to the light reference, indicating it has acquired a larger progeny contribution. (B) Sonication reveals (similar to Fig. 5) that half of the parental contribution in the replicated parental exists as short stretches and half exists as long stretches, relative to surrounding light progeny sequences. (C) Denaturation demonstrates covalent attachment of replicated heavy parental and light progeny DNA. Symbols: ○, ³H; ●, ³²P.

MOI, high MOI, and in the presence of unirradiated rescuing phage. These data are in contrast with previous experiments with irradiated light phage, which showed that during low MOI infections no detectable DNA replication (assayed by density shift of parental label) occurred for phage which had received 10 lethal hits (21). The high efficiency of replication documented above, as well as the increased efficiency seen in infections with heavy phage exposed to higher doses, make it highly unlikely that incomplete injection of phage DNA is a secondary consequence of UV irradiation, since, if this were the case, efficiency of replication would decrease with increasing doses of UV.

Close scrutiny of the CsCl gradient data reveals a notable difference in the density of the replicated moieties when one compares the low- and high-MOI experiments with the rescue experiments. In the rescue experiments, a portion of the parental label shifted to a location almost identical to the light reference DNA (Fig. 6A and 7A). In contrast, in the experiments without rescuing phage, a portion of the parental label banded at a discernibly heavier location than the light reference DNA. This indicates that in the low- and high-MOI experiments, the proportional contribution of ^{32}P -labeled heavy DNA to the progeny DNA was greater than that seen in the presence of rescuing phage. In the high-MOI experiment, the proportional contribution of parental to progeny DNA was calculated by a method previously published (8) and found to be approximately 15% of the mass of the replicated segment. In the two rescue experiments, the close overlap of the light reference DNA and the parental label precluded a precise calculation of the small fractional amount of the parental contribution to the replicated segments.

To further analyze the form of the parental contribution to the replicated molecule, samples

FIG. 7. CsCl gradient analysis of DNA extracted after coinfection with heavy phage which had received 10 hits of UV irradiation ($\text{MOI} = 0.2$) and unirradiated cold heavy phage ($\text{MOI} = 5$). ^3H -labeled heavy and light T4 DNA were added as references. (A) The magnitude and position of the density shift of parental label show that (i) much of the irradiated heavy phage DNA has replicated ($E = 70\%$) and (ii) that the replicated parental DNA is virtually indistinguishable from light density; therefore, light progeny sequences constitute the vast majority of the replicated parental. (B) Sonication does not change the banding pattern, therefore virtually all parental sequences in the replicated DNA exist as very short segments dispersed among light progeny. (C) Heavy parental and light progeny sequences are covalently joined. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

of extracted DNA from the above experiments were sonicated to a size range of 5×10^5 to 1×10^6 daltons and banded in CsCl equilibrium gradients. The degree of density shift of the parental label after sonication, relative to the position of the unsheared DNA in the original CsCl gradient, is related to the length of the parental segment in the replicated moiety. If the lengths of the parental segments were larger than those of the fragments resulting from sonication, the label would band at the hybrid location. On the other hand, very little shift of any kind would indicate dispersion of the parental label as very small subunits, significantly shorter than the fragments generated by sonication. (For mathematical reasoning and equations, see the appendix in reference 5.)

Figures 5B, 6B, and 7B show the results of CsCl gradient analysis of the sonicated DNA. In the case of moderately irradiated heavy phage (5 hits), DNA was isolated after the high-MOI experiment (Fig. 5) and the rescue experiment (Fig. 6). In each case, approximately half of the replicated parental DNA underwent a shift to the hybrid location after sonication. This suggests there is a bimodal distribution of lengths of heavy parental DNA within the replicated fragments. It is possible that relatively long stretches of DNA in the moderately UV-irradiated heavy genome remain intact after infection and replicate semiconservatively. These stretches of parental DNA were still small in proportion to the amount of light density DNA which was synthesized; thus, the replicated molecules were of almost-light density. Upon sonication, however, these stretches of replicated parental DNA shifted to the hybrid location. The remainder of the parental label, which after sonication still banded at the light or almost-light location, represented heavy parental DNA which was dispersed in very small pieces within the replicated molecule.

The DNA extracted from cells infected with heavy phage irradiated with 10 lethal hits banded at the same position in the CsCl gradient both before and after sonication (Fig. 7). In this case, the parental DNA is present within the replicated moiety in minute stretches which are much shorter than the lengths of fragments produced by sonication.

Further analysis was performed to test whether the heavy parental DNA and the light progeny DNA were covalently linked or merely joined via hydrogen bonding between complementary areas. The extracted DNA from the high-MOI and rescue experiment was denatured, neutralized, and analyzed in CsCl gradients. A shift of the parental label back to the heavy location in the gradient would reflect a lack of covalent joining of progeny to parental DNA. Figures 5C,

6C, and 7C represent CsCl gradient analysis of the denatured DNA. In all cases, the parental label remained at the light or slightly heavier than light location. Therefore, we conclude there is covalent attachment between the parental and newly synthesized progeny DNA.

Effect of chloramphenicol on the replication of UV-irradiated heavy DNA. Upon a low-multiplicity infection with light phage exposed to 10 hits of UV irradiation, small gaps are repaired by host polymerase (2), but no phage DNA replication sufficient to produce a density shift of parental label in a CsCl gradient is observed (21). However, we showed above (Fig. 4B) a significant amount of DNA replication ($E = 70\%$) upon a low-multiplicity infection with heavy phage irradiated with 10 lethal hits.

When unirradiated heavy or light phage is used to infect bacteria in the presence of chloramphenicol, there is neither phage DNA synthesis nor replication of parental label as measured by a density shift (9, 10). Since 10 lethal hits are likely to render three phage genes for DNA replication nonfunctional, we considered the possibility that the extensive phage DNA replication observed upon infection with heavy phage is performed by host enzymes. To test this hypothesis, we measured DNA replication when chloramphenicol was added at the time of infection (MOI = 8) with ^{32}P -labeled heavy phage irradiated with 3, 5, or 10 lethal hits. If host enzymes are responsible for the DNA replication observed after infection of UV-irradiated heavy phage, then chloramphenicol should not prevent the replication. Moreover, if radiation-induced damage is indeed repaired by host enzymes, then in the presence of chloramphenicol one would expect more DNA replication for phage receiving higher doses of UV irradiation.

Figure 8 depicts the CsCl gradient analysis of the DNA extracted 45 min after infection. Panel A shows that 15% of the heavy parental DNA exposed to 3 lethal hits shifted toward the light location, banding just to the heavy side of the light reference DNA. This indicates that 15% of the parental label has become incorporated into fragments composed of mostly progeny DNA. After infection with heavy phage exposed to 5 or 10 lethal hits, 24 or 32%, respectively, of the DNA shifted to a location slightly heavier than the light reference.

In summary, UV-irradiated heavy phage undergo a significant amount of DNA replication despite the presence of a protein synthesis inhibitor. In agreement with our prediction, the magnitude of this replication increases with the dose of irradiation to which the phage were exposed. This strongly suggests host enzymes are responsible for the observed DNA replication and that the replication is in response to UV damage.

Formation of parental DNA-protein complexes after infection with UV-irradiated heavy phage. Soon after infection, light replicative T4 parental DNA can be found in a DNA-protein complex which sediments rapidly to the bottom of a neutral sucrose gradient (11, 18, 19). To examine the effect of UV irradiation of heavy phage on the formation of the fast-sedimenting replicative complex, *E. coli* were infected (MOI = 6) with heavy phage exposed to 0 or 10 lethal hits of UV irradiation. Five minutes after infection, the cells were gently lysed by the Triton-lysozyme method, and the lysates were analyzed in neutral sucrose gradients underlaid with a pad of saturated sucrose solution. (Part of the lysate was supplemented with ^3H DNA [as a reference] and Mg and digested with pancreatic DNase, at 10 $\mu\text{g}/\text{ml}$. The extent of solubilization [95%] was identical for both isotopes. Thus, parental DNA of UV-irradiated phages was injected completely.)

After infection with unirradiated heavy phage (Fig. 9A), approximately 90% of the parental DNA sedimented to the bottom of the gradient, resting on the pad. In contrast, examination of the lysates from cells infected with heavy phage which received 10 lethal hits revealed that only 26% of the parental DNA reached the pad (Fig. 9B). The remainder of the DNA banded near the top of the gradient. Thus, at 5 min after infection the percentage of irradiated heavy parental DNA associated with the fast-sedimenting complex is significantly reduced when compared with that of unirradiated DNA.

The sedimentation pattern in a neutral sucrose gradient is dependent upon the three-dimensional structure of the DNA-protein complexes. Hence, the relative position of the moieties in the gradient does not yield information concerning the size of the DNA. To determine the sizes of the DNA containing a parental contribution in the two moieties of the neutral sucrose gradient, the appropriate fractions were dialyzed against 1 mM EDTA, denatured with alkali, and then sedimented through alkaline sucrose gradients. Note the striking difference between the size

FIG. 8. CsCl gradient analyses of ^{32}P -labeled parental DNA isolated 45 min after infection, in the presence of chloramphenicol (CM; added at the time of infection), with heavy phage (MOI = 8) which had received 3 (A), 5 (B), or 10 (C) hits of UV irradiation. ^3H -labeled heavy and light T4 DNA were added as references. In all cases there was a density shift of some of the parental label to an almost-light location, despite the presence of a protein synthesis inhibitor during the infection. The extent of density shift, which reflects repair replication, increases with increasing dose of UV irradiation. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

FIG. 9. Padded neutral sucrose gradient (NSG) analysis of lysates from cells 5 min after infection with heavy phage which received 0 (A) or 10 (B) hits of UV irradiation. The gradients were underlaid with a pad of saturated sucrose, and ^3H -labeled unit-size heavy T4 DNA was added as reference. Ninety percent of unirradiated heavy parental sediments to the pad, indicating its association with the DNA-protein replicative complex. Only 26% of the irradiated heavy parental label becomes associated with the fast-sedimenting complex. The rest sediments very slightly, remaining near the top of the gradient. Symbols: ○, ^3H ; ●, ^{32}P .

profiles of the parental DNA associated with the fast-sedimenting complex (Fig. 10A) and the parental DNA isolated from the top of the neutral sucrose gradient (Fig. 10B). The majority of the DNA isolated from the pad was of considerable size, approximately one-eighth the size of unit-length T4 DNA, whereas that from the top of the gradient was very small, less than 1/100 the size of unit-length T4 DNA.

We also wished to measure the extent of DNA replication undergone by the parental DNA residing in these two moieties. Heavy phage which had received 15 lethal hits were used to infect *E. coli* (MOI = 8). At 20 min after infection, the bacteria were gently lysed, and the sample was sedimented through a neutral sucrose gradient underlaid with a pad of saturated sucrose solution. The overall sedimentation pattern (data now shown) of the parental label in the neutral sucrose gradient was similar to that seen at 20 min after infection with heavy phage which received 10 lethal hits. Fractions from the top of the gradient and from the pad were recovered and dialyzed, and the DNA was extracted with phenol and analyzed in CsCl density gradients.

As seen in Fig. 11, the DNA from the fast-sedimenting complex replicated to a greater extent than did the DNA from the top of the neutral sucrose gradient. The majority (64%) of the heavy parental label isolated from the pad underwent a shift toward the light location, whereas only 28% of the DNA from the top underwent such a shift. In summary, after infection with UV-irradiated heavy phage, the parental DNA exists in two classes. One resides in the fast-sedimenting DNA-protein complex, is of considerable size, is apparently protected against host-mediated degradative processes, and has a high percentage of replicative DNA. The other class is not associated with the fast-sedimenting complex and consists of very small fragments with a much smaller proportion of replicative DNA.

Association of progeny DNA with the fast-sedimenting complex. In an earlier paper, we documented the sizes of the progeny DNA synthesized at various times after infection with UV-irradiated heavy phage (15). The size of progeny DNA early after infection, approximately one-eighth of unit-length T4 DNA, is similar to that of the UV-irradiated parental DNA residing in the fast-sedimenting complex. We therefore proposed that progeny DNA is generated from those parental DNA fragments in the complex which contain origins of DNA replication. In this section, we will show that the majority of newly synthesized progeny is indeed associated with the fast-sedimenting complex.

E. coli were infected at an MOI of 8 with heavy phage which had received 15 hits of UV

FIG. 10. Alkaline sucrose gradient (ASG) analysis of DNA isolated from the pad and top of a preparative padded neutral sucrose gradient (see Fig. 9). ³²P-labeled parental DNA was extracted 5 min after infection with heavy phage which had received 10 hits of UV irradiation. ³H-labeled unit-size heavy T7 DNA was added as a reference. The DNA from the pad (A) is of variable size, but the predominant peak corresponds to one-half T7 unit-length or approximately one-eighth T4 unit-length. The DNA from the top of the neutral sucrose gradient (B) is very small, less than 1/100 unit-size T4 DNA. Symbols: ○, ³H; ●, ³²P.

FIG. 11. CsCl gradient analysis of ³²P-labeled parental DNA isolated from the top and pad of a preparative padded neutral sucrose gradient. DNA was extracted 20 min after infection with heavy phage which had received 15 hits of UV irradiation. ³H-labeled unit-size heavy T7 DNA was added as a reference. The DNA from the pad (A) is more extensively replicated than is DNA from the top (B) of the neutral sucrose gradient as indicated by the proportion of parental label undergoing density shift. Symbols: ○, ³H; ●, ³²P.

FIG. 12. Padded neutral sucrose gradient (NSG) analysis of progeny (^3H -labeled) DNA from the lysate of cells infected with heavy T4 phage which had received 15 hits of UV irradiation. A [^3H]thymidine-containing package was added at 3.5 min after infection and the sample was taken at 20 min after infection. Note that 60% of the incorporated progeny label, in contrast to only 26% of the parental label (see Fig. 9), is associated with the fast-sedimenting complex at the pad.

irradiation. A [^3H]thymidine-containing package (specific activity, 1 mCi/mg) was added at 3.5 min after infection, and samples were analyzed at 20 and 40 min after infection. The infected cells were gently lysed and the lysates sedimented through padded neutral sucrose gradients. At 20 min after infection, the majority (60%) of the [^3H]thymidine-labeled progeny DNA was associated with the fast-sedimenting complex (Fig. 12), whereas, as documented in the previous section (Fig. 10B), only 26% of the parental DNA resided there. Importantly, even 40 min after infection (data not shown), approximately 50% of the progeny DNA still resided in the fast-

sedimenting complex, possibly replicating autonomously.

Comparison of multiplicity reactivation of light and heavy T4 bacteriophage. To compare the extent of multiplicity reactivation of heavy and light bacteriophage, suspensions of each were exposed to one of a range (0 to 27 lethal hits) of doses of UV irradiation. The phage were used to infect separate portions of *E. coli* at an MOI of 6. The level of multiplicity reactivation was calculated by dividing the number of infective centers produced by the UV-irradiated phage by the number of infective centers produced by unirradiated (0 hits) control phage.

Fig. 13 shows that, in the range of low doses (2 to 5 lethal hits), multiplicity reactivation was nearly identical in light and heavy phage. However, heavy phage irradiated with more than 5 lethal hits clearly showed a marked deficiency in multiplicity reactivation. With increasing doses of UV irradiation, there was a progressive divergence between the two lines. The relationship of this deficiency in multiplicity reactivation to the intracellular fate of the UV-irradiated heavy DNA will be discussed below.

DISCUSSION

In this report, we have attempted to characterize the molecular events after the damage of BUdR-labeled T4 phage by UV irradiation. The

FIG. 13. Multiplicity reactivation in *E. coli* infected (MOI = 6) with heavy or light T4 phage which had received one of a range (0 to 27 hits) of doses of UV irradiation. At doses of 5 hits or lower, the levels of multiplicity reactivation are very similar in heavy and light phage. At higher doses, however, heavy phage undergo progressively less multiplicity reactivation than do their light counterparts. Symbols: ○, light phage; ●, heavy phage.

direct effect of irradiating heavy phage can be distinguished from those further insults which ensue after infection, resulting from the interaction of the damaged phage DNA with the host cell. In concert, these sequelae of irradiation not only determine the degree of phage lethality but also bias the genetic representation among newly synthesized progeny phage DNA in favor of areas containing origins of replication (15).

UV irradiation of heavy phage or of heavy phage DNA results in the introduction of single-stranded breaks in the phage DNA. As we have shown here by analyzing the sedimentation profile of the phage DNA in alkaline sucrose gradients, the number of single-stranded breaks increases linearly with the number of lethal hits. Specifically, over the dose range tested, 1.3 to 1.5 single-stranded breaks per genomic strand result from each lethal hit. When analyzed in neutral sucrose gradients, UV-irradiated heavy phage DNA shows no evidence, for doses up to 10 lethal hits, of double-stranded breaks.

Exposure of heavy DNA to UV irradiation causes debromination of the BUdR, resulting in uracil (23; H. H. Vogelbacker and S.-K. Ling, Fed. Proc. 40:1764, 1981). The relationship between the debromination and the induction of single-stranded breaks has not yet been clarified (22; H. H. Vogelbacker, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1982).

After infection of *E. coli* with UV-damaged heavy phage, the DNA is subjected to further breakdown by host-mediated processes. At high doses of irradiation (10 lethal hits), the intracellular parental phage DNA clearly exists as two size classes, one apparently unchanged from its preinjection size (representing 25% of the parental label) and the other class (75%) smaller by more than 10-fold. (Note, however, that the breakdown is not accompanied by a substantial amount of solubilization to trichloroacetic acid-nonprecipitable material.) Since a similar amount and pattern of breakdown occur in the presence of a protein synthesis inhibitor added at the time of infection, we believe the post-infection breakdown is host mediated. (Of course, we cannot rule out the possibility that, in the presence of chloramphenicol, some phage-coded proteins are synthesized in such small quantities that they cannot be detected by pulse-labeling with radioactive amino acids.)

The bimodality of the distribution of intracellular heavy parental label in alkaline sucrose gradients is clearly not consistent with the operation of a single random intracellular cutting event applied equally to all phage DNA. We interpret these two distinct size classes of intracellular phage DNA as representing the products of two competing processes within the host cell. On one hand there exists a process which de-

grades UV-damaged DNA to an extent directly related to the amount of damage. A series of experiments recently undertaken in this laboratory have specifically implicated the removal of uracil by the host-coded enzyme uracil glycosylase as instrumental in this degradative process (H. H. Vogelbacker, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1982). Events after uracil removal, e.g., counternicking, may produce double-stranded cuts which subsequently interfere with replication by preventing elongation from the 3' end of the fragment. On the other hand, other simultaneous events protect some of the damaged DNA from further breakdown or, alternatively, ensure prompt and efficient repair. A more trivial interpretation would suggest that the degradative process is saturated when 75% of the UV-damaged DNA is broken down. However, the data presented above suggest the existence of an active protective process.

First, the percentage of heavy irradiated parental DNA not subjected to the intracellular breakdown is the same as that percentage found associated with a fast-sedimenting DNA-protein complex. Second, the size of this minority class parental DNA is the same as that found residing in the complex. Third, the parental label residing in larger fragments associated with the complex undergoes more extensive replication than does the parental label in the very small parental pieces not associated with the complex. (Of course, phage which did not inject, if sedimented to the pad, should contain nonreplicated DNA.)

The protection afforded damaged parental DNA associated with the replicative complex probably does not entail the prevention of all corrective excisions in that DNA. If those excisions, by uracil glycosylase, for instance, were not introduced to DNA in the complex, then upon sonication of the replicative DNA, one should be able to resolve the parental contribution in the form of a hybrid moiety. However, for high doses of irradiation, when a majority of parental DNA in the complex is replicated, sonication does not result in the shift of the parental label to hybrid density. Hence, "protection" refers to the providing of a molecular milieu in which large stretches of DNA can be maintained despite the infliction of transient breaks. This could be accomplished by holding damaged DNA strands in close linear proximity, or by facilitating recombination between fragments, thus resulting in relatively long subunits. Previous work from this laboratory showed that even double-stranded cuts introduced by ^{32}P decay in vitro do not dissociate DNA from the replicative complex (19). Therefore, it was postulated that the protein components of the com-

plex are firmly associated with the DNA at multiple sites. This is consistent with the protective role we believe the complex plays.

To explain our data, we propose that after infection with UV-irradiated heavy phage, some areas become preferentially associated with the replicative complex and are able to serve as templates for DNA synthesis (in a manner to be explained below). On the other hand, those areas not associated with the complex are more likely to be exposed to host degradative processes and are further extensively broken. This proposition is consistent with our earlier hybridization data, which demonstrated gene amplification in the progeny DNA synthesized after infection with UV-irradiated heavy phage (15). The biased representation of areas containing origins among the progeny sequences suggests that origins are also over-represented in the parental DNA associated with the complex. Not surprisingly, we showed in this report a large proportion (60%) of progeny DNA associated with the complex, in contrast with the association of only 26% of the irradiated heavy parental DNA with the complex. This report also presents evidence that UV irradiation greatly decreases the association of heavy parental DNA with the replicative complex, which is in keeping with the concept of competition between the degradative process directed at radiation-damaged DNA and the protective process related to the DNA-protein replicative complex.

In our view, the most interesting implications of our data concern the nature of DNA replication after infections with irradiated heavy T4 phage. In an earlier report, we presented experiments in which we labeled the newly synthesized progeny DNA. A considerable amount of light progeny DNA (36 phage equivalent units per infective center) was synthesized after infection with heavy phage irradiated with 10 lethal hits. The progeny label was not covalently attached to the parental molecules, and its representation of the T4 genome was strongly biased in favor of areas containing known origins (15). In the present series of experiments, we followed the progress of radiolabeled heavy parental DNA through the course of an infection. Despite moderate or high doses of UV irradiation, the heavy parental DNA underwent extensive replication, as evidenced by the magnitude of density shifts in CsCl equilibrium gradients. The efficiency of replication in low MOI, high MOI, and rescue experiments was between 58 and 75% of that after infection with unirradiated heavy phage. In contrast, irradiated light phage does not show any DNA synthesis detectable by density shift analysis. It does, however, undergo repair synthesis to fill in the very short gaps generated by excision of damaged areas (21; E.

Shahn, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1965).

Our results illustrate four features of the phage DNA replication during infections with irradiated heavy phage. First, we observed a higher efficiency of replication in infections with phage receiving more irradiation. Second, the replication took place (and with comparable efficiency) in the presence of chloramphenicol. Together, these data indicate that the replication is in response to UV damage, i.e., is a form of repair replication, and that this repair replication is host mediated.

The third notable feature concerns the amount of distribution of the heavy parental contribution in repair-replicated molecules. Conventional repair consists of damage recognition and excision followed by gap recognition, filling in, and ligation. In this mode of repair, molecular integrity is restored by the addition of a few nucleotides and therefore without any appreciable change in density of the parental subunit. However, in the case of irradiated heavy phage, the parental label undergoes a dramatic density shift, moving to a location very close to the light reference DNA (Fig. 4 through 7). This indicates that the parental contribution constitutes a very small percentage of the mass of the replicated subunit, with the remainder contributed by newly synthesized light DNA. In high-multiplicity infections, the estimated proportional contribution of parental label to the repaired molecule was only 15%. An even smaller parental contribution was seen when bacteria were coinfecting with cold unirradiated and radiolabeled UV-irradiated phage. (The almost coincidental banding of the parental label with the light reference may reflect recombination between UV-damaged heavy DNA and the undamaged progeny DNA of the rescuing phage.)

By conventional repair replication, the minimal parental contribution to the repaired molecule is 50%, i.e., hybrid, unless there is homologous recombination between the repaired parental and whole progeny. However, since progeny synthesis in infections with irradiated heavy phage is site-specific (15), many areas of the parental genome would be unable to recombine in this way. The exceptionally small parental contribution suggests that the repair replication seen after infection with UV-irradiated heavy phage is qualitatively very different from conventional gap-filling repair synthesis.

The parental contribution in the repaired template is not only small, but also widely dispersed among stretches of light parental sequences as revealed by the sonication data. At moderate doses (5 lethal hits) the parental nucleotides exist partly as relatively long (significantly longer than 10^6 daltons) stretches and partly as very

small (significantly shorter than 5×10^5 daltons), highly dispersed units. At higher doses, the parental contribution in the almost-light repaired molecule is virtually entirely disposed as very small segments scattered among long stretches of light sequences.

These heavy parental segments are covalently linked to newly synthesized light progeny DNA as evidenced by the failure of denaturation to change the CsCl banding pattern of the parental label (Fig. 5 through 7). This covalent attachment between parental and progeny sequences clearly distinguishes this type of replication from that detected when one follows the progeny label. The majority of newly synthesized ^3H -labeled light progeny DNA is not covalently attached to the parental molecule (15).

Hence, the phage DNA synthesis occurring upon infection with UV-irradiated heavy phage consists of two types of replication which take place simultaneously. The first type is the usual replication aimed at generating numerous progeny phage segments with which to complete the infective cycle. Since initiation of DNA synthesis occurs preferentially at origins of replication, and since the parental DNA is fragmented, elongation of the progeny strands is limited and gene amplification results (15). This amplified, biased progeny DNA is not covalently linked to the parental template. The second type is repair replication of UV-induced damage to the parental molecule. We believe this occurs preferentially in areas protected from host-mediated disintegration by virtue of their association with a replicative complex. The products of this replication are short, heavy parental fragments scattered among long stretches of newly synthesized light progeny to which they are covalently attached. Note that this repair replication contributes a small fraction to net DNA synthesis and can only be observed by following the parental label.

Before considering two models which might explain this unusual repair replication, a fourth important aspect must be emphasized. As previously mentioned, the production of mostly-light repaired molecules does not involve a considerable loss of heavy parental nucleotides, as evidenced by lack of extensive solubilization of the parental label throughout the course of an infection. Therefore, we conclude there is no considerable exonuclease digestion extending from areas of UV damage or from gaps produced by the excision of uracil (H. H. Vogelbacker, Ph.D. thesis, University of Pennsylvania, Philadelphia, 1982).

In attempting to explain the features of repair replication of UV-damaged heavy templates mentioned above, we considered two models (Fig. 14). In both models, areas of UV-induced

damage are excised from the heavy DNA molecule. According to model 1 (Fig. 14, left panel), the gaps created by the excision are simply filled in with newly synthesized light progeny DNA. Since simultaneous excision of all damages would result in the segment's disintegrating into small fragments, the excisions would have to occur in a partly step-wise fashion. To be consistent with the CsCl data showing replicated molecules of almost-light density, model 1 has as a requirement the excision of very long stretches of heavy parental DNA (surrounding or starting from each area of damage), which would result in a high degree of solubilization of the parental label. Since we observed relatively little solubilization, model 1 is not a tenable interpretation of our data.

To explain the repair of UV-irradiated heavy parental DNA such that the resulting subunits are of almost-light density, yet without an appreciable amount of solubilization of the parental DNA, we postulate model 2 (Fig. 14, right panel). In this model, excision also proceeds in a sequential manner but the area of parental nucleotides removed at each damage is very small. This would result in relatively little solubilization of the parental label and is therefore more consistent with our findings. The model further proposes that, as the progeny DNA elongates and the gap is essentially filled in, the leading progeny strand displaces, instead of ligating to, the 5' end of the parental strand and continues elongating. The displaced strand could itself then act as a template for progeny synthesis. The synthesis on this lagging strand is presumably discontinuous. UV damages (i.e., uracil) still remaining in either parental strand will not impede their use as templates; the uracil can be removed in a later cycle of the process. As the schematic diagram indicates, the gradual removal of UV damages followed by repair synthesis ultimately results in a large proportion of subunits in which the parental contribution is very small and flanked by much longer stretches of covalently attached progeny DNA. For any given fragment of damaged parental DNA, the fractional length of stretches of parental label found in repair-replicated fragments will depend on the number and distribution of uracil excisions. At high doses, a larger proportion of repaired fragments will have shorter parental contributions, as is demonstrated by our sonication data.

The proposed scheme of strand displacement during repair synthesis of UV-irradiated heavy phage is reminiscent of DNA replication at late times after infection with unirradiated phage, when reinitiation occurs at random locations along the parental molecule (4). This late reinitiation contrasts with that occurring early after

FIG. 14. Two proposed models for repair replication after infection with UV-irradiated heavy DNA. According to model 1, the removal of uracil is accompanied by excision of large stretches of neighboring parental nucleotides, resulting in a high level of solubilization of parental label (1-A). The gap is subsequently filled in with newly synthesized progeny DNA which ligates to the encountered 5' parental end (1-B). According to model 2, a much smaller gap is introduced after the excision of uracil (2-A). As the 3' end of the elongating progeny strand approaches the end of the gap, the encountered 5' end is displaced and replication proceeds to the end of the original parental template strand. In addition, the displaced strand is also replicated, possibly after a lag and presumably in a discontinuous manner (2-B). In both models, additional rounds of excision and repair generate repair-replicated fragments containing (i) a majority of progeny sequences and (ii) parental label widely dispersed through most of the fragments as relatively short stretches. Since model 2 postulates relatively little solubilization of parental label, it is more in keeping with our data.

infection, which has been shown to initiate at specific locations on the T4 genome (3) and which results in amplification and accumulation of these areas (12). To equalize the genetic representation of the progeny DNA, which must occur before viable phage are produced, it was proposed that recombinational intersections act as random initiation sites of DNA replication (4, 6). We believe the recombinational intersections of unirradiated DNA and the small gaps created by the excision of uracil in UV-damaged heavy DNA may be very similar with respect to initiation of DNA synthesis.

We wish to emphasize the novelty of the proposed model of repair replication. To the best of our knowledge, there is no precedent for repair replication which leads to segments in which the repaired areas constitute the majority of the length of the repaired DNA segments. Nor do we know of any other example of strand displacement occurring as a secondary consequence of irradiation of DNA. Despite this lack

of precedent, the model gains some credibility from in vitro observations of strand displacement synthesis beginning at a single-stranded nick in duplex T4 DNA (20). One should also recall that in the past, strand displacement was also observed for *E. coli* polymerase I (17) and T7 polymerase (7), though in neither case was the displaced strand replicated to an appreciable extent.

The outcome of competition between destructive and protective processes is likely to influence whether an infection with irradiated heavy phage is productive. Infections with irradiated heavy phage exhibit a marked deficiency of multiplicity reactivation at doses greater than 5 lethal hits (Fig. 13). Our data suggest two explanations for this phenomenon which are not mutually exclusive. First, there may be a limit to the number of damaged areas which can be protected. Perhaps this limit is exceeded at higher doses of irradiation, resulting in an ever-increasing number of areas which undergo fur-

ther breakdown, become detached from any segments containing origins, and therefore never replicate. A second explanation concerns the possibility of a time lag before the displaced strand is replicated. At high doses, when damages are close together and steps in the repair synthesis pathway are occurring simultaneously, a displaced strand may fall off (e.g., when the leading strand encounters another gap) and therefore not ever be used as a template. This second explanation seems less likely, since the leading strand would have already made a repaired copy of the displaced strand. The balance between destruction and protection is extremely precarious, possibly because both processes are related to uracil glycosylase. Experiments showing less repair replication of UV-irradiated heavy phage in uracil excision-defective hosts indicate uracil excision is necessary both for susceptibility to extensive breakdown and for extensive repair replication (Vogelbacker and Ling, *Fed. Proc.* **40**:1764, 1981).

In summary, we have demonstrated a host-mediated repair synthesis in cells infected with UV-damaged heavy T4 phage which differs drastically from that observed with irradiated light phage. Repair begins with the excision of debrominated BUdR (i.e., uracil) and is followed by an extensive repair replication of the parental DNA carried out by host enzymes. The resulting repaired fragments consist of a majority of newly synthesized progeny sequences covalently linked to a minority of widely dispersed parental sequences. To account for this phenomenon, we have postulated a new model for radiation damage-induced synthesis. According to this model, replication initiates at the 3' end of a gap, and the elongating, leading progeny strand displaces, rather than ligates to, the 5' end of the parental DNA at the other side of the gap.

ACKNOWLEDGMENTS

This research was supported by the National Science Foundation grant PCM 78-19971 (awarded to A.W.K.). H.H.V. was supported by training grant 2T32GM 07229-06. L.L.R. was supported by National Institutes of Health Medical Scientist training grant GM 07170.

This research resulted partially from the efforts of the students participating in the course (551) given by the Graduate Group in Genetics.

L.L.R. is a member of the Graduate Group of Genetics, and H.H.V. and T.M. are members of the Graduate Group of Microbiology.

LITERATURE CITED

1. Carlson, K., and A. W. Kozinski. 1974. Nonreplicated DNA and DNA fragments in T4 r^- bacteriophage particles: phenotypic mixing of a phage protein. *J. Virol.* **13**:1274-1290.
2. Carlson, K., Z. K. Lorkiewicz, and A. W. Kozinski. 1973. Host-mediated repair of discontinuities in DNA from T4 bacteriophage. *J. Virol.* **12**:310-319.
3. Halpern, M. E., T. Mattson, and A. W. Kozinski. 1979. Origins of T4 DNA replication as revealed by hybridization to cloned genes. *Proc. Natl. Acad. Sci. U.S.A.* **76**:6137-6141.
4. Halpern, M., T. Mattson, and A. W. Kozinski. 1982. Late events in T4 bacteriophage DNA replication. III. Specificity of DNA reinitiation as revealed by hybridization to cloned genetic fragments. *J. Virol.* **42**:422-431.
5. Howe, C. C., P. J. Buckley, K. M. Carlson, and A. W. Kozinski. 1973. Multiple and specific initiation of T4 DNA replication. *J. Virol.* **12**:130-148.
6. Hutchinson, N., T. Kazic, S. J. Lee, C. Rayssiguier, B. S. Emanuel, and A. W. Kozinski. 1979. Late replication and recombination in the vegetative pool of T4. *Cold Spring Harbor Symp. Quant. Biol.* **43**:517-523.
7. Kolodner, R., and C. C. Richardson. 1977. Replication of duplex DNA by bacteriophage T7 DNA polymerase and gene 4 protein is accompanied by hydrolysis of nucleoside 5'-triphosphates. *Proc. Natl. Acad. Sci. U.S.A.* **74**:1525-1529.
8. Kozinski, A. W., and P. B. Kozinski. 1963. Fragmentary transfer of 32 P-labeled parental DNA to progeny phage. II. The average size of the transferred parental fragment. Two-cycle transfer. Repair of the polynucleotide chain after fragmentation. *Virology* **20**:213-229.
9. 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.
10. Kozinski, A. W., P. B. Kozinski, and P. Shannon. 1963. Replicative fragmentation in T4 phage: inhibition by chloramphenicol. *Proc. Natl. Acad. Sci. U.S.A.* **50**:746-753.
11. Kozinski, A. W., and T. H. Lin. 1965. Early intracellular events in the replication of T4 phage DNA. I. Complex formation of replicative DNA. *Proc. Natl. Acad. Sci. U.S.A.* **54**:273-278.
12. Kozinski, A. W., S. K. Ling, N. Hutchinson, M. E. Halpern, and T. Mattson. 1980. Differential amplification of specific areas of phage T4 genome as revealed by hybridization to cloned genetic segments. *Proc. Natl. Acad. Sci. U.S.A.* **77**:5064-5068.
13. Kozinski, A. W., and Z. K. Lorkiewicz. 1967. Early intracellular events in the replication of T4 phage DNA. IV. Host-mediated single-stranded breaks and repair in ultraviolet-damaged DNA. *Proc. Natl. Acad. Sci. U.S.A.* **58**:2109-2116.
14. Kozinski, A. W., and W. Szybalski. 1959. Dispersive transfer of the parental DNA molecule in the progeny of phage ϕ X174. *Virology* **9**:260-274.
15. Ling, S.-K., H. H. Vogelbacker, L. L. Restifo, T. Mattson, and A. W. Kozinski. 1981. Partial replication of UV-irradiated T4 bacteriophage DNA results in amplification of specific genetic areas. *J. Virol.* **40**:403-410.
16. Litwin, S., E. Shahn, and A. W. Kozinski. 1969. Interpretation of sucrose gradient sedimentation pattern of deoxyribonucleic acid fragments resulting from random breaks. *J. Virol.* **4**:24-30.
17. Masamune, Y., and C. C. Richardson. 1971. Strand displacement during deoxyribonucleic acid synthesis at single strand breaks. *J. Biol. Chem.* **246**:2692-2701.
18. Miller, R. C., Jr., and P. Buckley. 1970. Early intracellular events in the replication of bacteriophage T4 deoxyribonucleic acid. VI. Newly synthesized proteins in the T4 protein-deoxyribonucleic acid complex. *J. Virol.* **5**:502-506.
19. Miller, R. C., Jr., and A. W. Kozinski. 1970. Early intracellular events in the replication of bacteriophage T4 deoxyribonucleic acid. V. Further studies on the T4 protein-deoxyribonucleic acid complex. *J. Virol.* **5**:490-501.
20. Nossal, N. G., and B. M. Peterlin. 1979. DNA replication by bacteriophage T4 proteins. The T4 43, 32, 44-62 and 45 proteins are required for strand displacement synthesis at nicks in duplex DNA. *J. Biol. Chem.* **254**:6032-6037.
21. Rayssiguier, C., A. W. Kozinski, and A. H. Doermann.

1980. Partial replicas of UV-irradiated bacteriophage T4 genomes and their role in multiplicity reactivation. *J. Virol.* **35**:451-465.
22. **Rupp, W. D., and W. H. Husoff.** 1965. Photochemistry of iodouracil. II. Effects of sulfur compounds, ethanol and oxygen. *Biochem. Biophys. Res. Commun.* **18**:53-64.
23. **Smelts, L. A., and J. J. Cornelis.** 1971. Repairable and irreparable damage in 5-bromouracil-substituted DNA exposed to ultra-violet radiation. *Int. J. Radiation Biol.* **19**:445-457.
24. **Wood, W. B., and H. R. Revel.** 1976. The genome of bacteriophage T4. *Bacteriol. Rev.* **40**:847-868.