Deoxyribonucleic Acid Repair Replication after Ultraviolet Light or X-Ray Exposure of Bacteria

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A comparison of repair synthesis after ultraviolet light (UV) or X-ray exposure was made in Escherichia coli strains 15T\(^-\) (555-7) and B/r by use of a D,\(^{14}\)N, \(^{13}\)C density labeling system. During the initial 15 min of incubation after UV irradiation, both a "repair" synthesis and a reduced semiconservative deoxyribonucleic acid (DNA) synthesis occurred. In the so-called "physiological" dose range used, the latter was greater than the former. X-irradiation of cells, at doses producing similar levels of cell death as in the UV-exposed cultures, did not lead to a similar repair replication process. However, a density heterogeneity of the DNA synthesized in the initial 10 min after exposure was observed. This is interpreted in terms of X-ray-induced DNA degradation. Normal cells showed only a semiconservative type of replication and, therefore, within the limits of resolution of the system used (the incorporation of 1,000 to 5,000 nucleotides per replicating chromosome could be measured), DNA in normal cells did not appear to undergo a repair synthesis involving thymine exchange. These results indicate that not all repair mechanisms mimic that found after UV exposure.

"Dark repair" of damaged deoxyribonucleic acid (DNA) is currently conceived as a two-step process. The defective region must be first sensed and removed, and then the excised nucleotide sequence replaced by bases complementary to the remaining strand. The excision of pyrimidine dimers induced by ultraviolet (UV) exposure by those bacteria genetically competent to do so has been firmly established (4, 16, 18). Pettijohn and Hanawalt (13) reported evidence of the resynthesis of the excised region, using the thymine analogue 5-bromouracil (BU) as a density marker. Similar repair synthesis phenomena in bacteria have been claimed for other types of challenge, including pretreatment with the alkylating agents, nitrogen mustard (7) or methyl methane-sulfonate (14), and prior thymine starvation (12). Although it has long been known that X-ray damage to bacteria can be reversed (17), evidence for an excision and repair synthesis of DNA, similar to that following UV exposure, has not been forthcoming. Recent evidence based on molecular weight measurements suggests that a "repair" of DNA breaks as measured by molecular size does occur after X-irradiation (10, 11).

We wished to determine whether "repair synthesis" (defined as a limited incorporation of nucleotides into pre-existing DNA strands) could be demonstrated in Escherichia coli after X-ray exposure. We believed that this could best be accomplished by a simultaneous study of UV-induced repair synthesis, preferably at equal levels of cell survival, by using procedures in which the incorporation of BU into DNA was avoided because of the well-established toxic (19) and synergistic (3) effects attributable to BU.

In this communication, we report experiments in which we used D\(^2\)O-\(^{14}\)N-\(^{13}\)C as a density label to examine "repair" synthesis following both UV and X-irradiation. The data show that, in two radiation-resistant strains of E. coli, strain 15T\(^-\) (555-7) and strain B/r, the repair synthesis exhibited after UV exposure was not observed after X-ray exposure.

MATERIALS AND METHODS

Growth of bacteria and isotope labeling. The details of the methods used have been previously described (3) and only the additional pertinent details will be included here.

A description of the origin and growth requirements of E. coli strains 15T\(^-\) (555-7) and B/r has been published (8). All media were supplemented with the appropriate amino acids and thymine in the following concentrations. thymine, 2 \(\mu\)g/ml; methionine, 30 \(\mu\)g/ml; arginine, 38 \(\mu\)g/ml; and tryptophan, 14 \(\mu\)g/ml.
Density labeling was achieved by growing the cells at 37°C in a modified medium C (15) in which D₂O (99 moles %) was substituted for H₂O, 0.1% ¹⁵N HCl, and 0.1% ¹³C-glucone (60 atom % ¹³C, random label) was used as the carbon source. As a convenience for discussion, this medium will be subsequently referred to as "heavy Med C," "light Med C" will refer to the modified Med C consisting of H, ¹⁴N, ¹²C components.

Uniform radioisotopic labeling of the DNA was accomplished by growing the cells for three or more generations in heavy Med C containing 0.05 or 0.10 μL of thymine-¹⁴C per ml and a final concentration of 2.0 μG of thymine per ml. The generation time during this period was approximately 130 min. When the cell concentration reached 10⁸ cells per ml, the organisms were harvested by filtration, washed two or three times with prewarmed, light Med C salts, and resuspended at 37°C in light Med C for 30 min. At this point the cells were harvested and washed with cold, light Med C salts. They were then resuspended in one-fourth to one-half the original volume of cold, light Med C salts and irradiated. After irradiation, the cells were incubated at 37°C in the original volume of light Med C, which contained 20 μG of thymine-normal-H₃ per ml (specific activity, 11 c/mmole). At specified times, 5- or 10-ml samples were removed and added to saline to stop growth.

Lysate preparation, density gradient equilibrium sedimentation, and DNA denaturation. Cell lysates were obtained by the lysozyme-pronase procedure already described (3). Density gradient equilibrium sedimentation analysis of isotope distribution in CsCl was reported earlier (3). At least two 5-min counts were made for each gradient fraction. Denatured DNA was prepared as follows. DNA fractions from whole-cell lysates that had undergone CsCl gradient separation were pooled according to density position. They were then dialyzed overnight against standard saline-citrate buffer. The DNA was then heated in a boiling-water bath for 15 min and rapidly cooled.

Irradiation of bacteria. X-ray exposure of E. coli B/r was accomplished by subjecting 5 or 10 ml of a cell suspension, in equilibrium with air, to the irradiation delivered by a General Electric Maxitron, X-ray Therapy Machine (230 kv, 20 ma, 0.5-mm Cu filter, 800 to 1,000 R/min). E. coli 15T (555-7) was irradiated in a Westinghouse Quadrocondrex X-ray machine (250 kv, 15 ma, 1-mm Al plus 0.5-mm Cu filtration) at a dose rate of approximately 600 R/min.

UV light exposure was carried out with two General Electric 15-w germicidal lamps. The cell suspension, containing 10⁹ to 2 × 10⁹ cells per ml, was positioned 70 cm from the UV source and agitated by a magnetic stirrer. A dose of 10 ergs per mm² per sec was measured at this position by a Jagger-type (9) ultraviolet dose-rate meter. Viability was determined on properly supplemented minimal salts-medium agar plates (2). Colonies were counted after 24 hr of incubation at 37°C.

RESULTS

Repair-synthesis following irradiation of E. coli 15T (555-7). To avoid the use of BU as a density label while obtaining sufficient buoyant density resolution of bacterial DNA fragments, deuterium (D), ¹⁴N, and ¹³C isotopes were employed to density-label E. coli DNA. The organisms were first trained, and then grown on heavy Med C in the presence of ¹³C-thymine. In the DNA obtained from such cells, both strands were "heavy" and ¹³C-labeled (DD-DNA).

To deplete pools of "heavy" and radioactive precursors and to move the chromosomal growing point away from the DD-DNA, a 30-min chase in light Med C preceded irradiation of the cells. The effect of such a shift on generation time during the transition is not known. CsCl gradient analysis of lysates from these cells (Fig. 1) showed that about 25% of the parental ¹³C-DNA had been replicated during the "chase." Thus, prior to irradiation, a minor portion of the DNA was of hybrid nature (DL-DNA, buoyant density of about 1.733 g/cc) in that one strand was "heavy" and ¹³C-labeled (D), the other was "light" and not radioactive (L), and the major portion of the DNA was in the DD-DNA form (buoyant density about 1.755 g/cc).

The cells were then divided into three portions: control, UV-exposed, and X-irradiated. After exposure, these cells were incubated in "light" Med C containing ¹⁴H-thymine of high specific activity. The control cells were incubated for 5 min; the UV-exposed cells for 15 min; and the X-irradiated cells, 10 min. The predicted consequences of either a semiconservative or repair-type synthesis subsequent to irradiation on the distribution of ¹⁴H-labeled DNA is as follows. If ¹⁴H-thymine is incorporated by the repair synthesis pathway, then ¹⁴H label should be found in both the DD- and DL-DNA bands. However, if only the normal semiconservative mode of synthesis occurs, then ¹⁴H should be found solely in the DL-DNA band.

It was observed that the only lysate showing repair synthesis was that from the UV-exposed cells (Fig. 2). This was indicated by the appearance of ¹⁴H in the DD-DNA as well as in the DL-DNA band. In contrast, the ¹⁴H in the control and X-irradiated cells appeared primarily in the DL-DNA.

Since resolution of the DNA fragments was incomplete in the cell lysates, and in order to quantitate the extent of repair synthesis, the banded DD- and DL-DNA fractions were separately pooled and reband for further purification. The resulting "purified" DD- and DL-DNA were dialyzed against normal saline-citrate buffer to remove CsCl, and the DNA was then denatured by immersion in a boiling-water bath for 15 min followed by rapid cooling. The results of rebanding the denatured DNA are shown in Fig. 3. In the
Fig. 1. Density distribution of DNA fragments isolated from Escherichia coli 555-7 after a “light” chase. The cells were first grown in a D, $^{14}$N, $^{13}$C medium containing $^{14}$C-thymine. They were then shifted to an H, $^{14}$N, $^{12}$C medium for 30 min. The cell lysate was then subjected to CsCl gradient analysis. Approximately 25% of the $^{14}$C counts were in the newly synthesized DNA (buoyant density of 1.730 g/cc). Two drops collected per fraction.

Control, the denatured single-stranded D-DNA, obtained from the native DD-DNA, banded at the expected density of 1.770 g/cc. The small quantity (note scale) of single-stranded L-DNA carrying $^3$H label and banding at 1.725 g/cc represents contamination by native DL-DNA found in the “purified” DD-DNA. This was shown by rebanding a portion of the “purified” native fraction. The denatured DL-DNA preparation gave both the expected D and L single-stranded DNA.

With the DNA from the UV-exposed cells, the parental D-DNA strands contained $^3$H label, whether obtained from the native DD or DL-DNA (Fig. 3), as would be expected from the “excision-repair” synthesis model.

During the 15 min of incubation following a UV dose of 159 ergs/mm², approximately 7% of the thymine incorporated into the chromosome was via a repair synthesis and about 93% by semiconservative replication (Table 1). However, 20% or less (depending on pool clearance rates) of the DNA present at the time of irradiation was the L-DNA synthesized during the 30-min “light” chase. After UV exposure, repair synthesis as measured by $^3$H-thymine uptake in this L-DNA could not be differentiated from the $^3$H-thymine uptake into L-DNA due to semiconservative replication. Therefore, the 7% of $^3$H label observed in the D-DNA and counted as repair synthesis is an estimated minimum, which could be as high as 8.4% if the L-DNA present at the time...
of irradiation participates equally in repair synthesis.

The DNA synthesized by the X-irradiated cells exhibited a more complex banding pattern (Fig. 3). Denaturation of the "purified" DD-DNA resulted in the expected banding of the $^{14}$C-labeled D-DNA strands, but there was also a small quantity of $^{3}$H-labeled DNA which banded...
TABLE 1. Characterization of DNA synthesis following irradiation of E. coli 15T− (555-7)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Survival</th>
<th>Incubation time with 3H-thymine (min)</th>
<th>Normalized 3H counts per min</th>
<th>DNA synthesized (%)</th>
<th>Semiconservative</th>
<th>Repair</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>5</td>
<td>62,300</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>159 ergs of UV/mm²</td>
<td>35</td>
<td>15</td>
<td>34,700</td>
<td>93</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>5,000 R of X rays</td>
<td>50</td>
<td>10</td>
<td>72,000</td>
<td>95</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>

* The total 3H counts in the DNA, analyzed by gradient banding, were corrected for sampling error by multiplying the number of 3H counts in a given sample (from Fig. 3) by the fraction obtained by dividing the 14C counts in the control by the 14C counts in the irradiated DNA.

† That portion of the 3H label banding with the light denatured DNA strand.

‡ That portion of the 3H label banding with the heavy denatured DNA strand. These results are not corrected for repair synthesis in the light, unlabeled DNA (approximately 20% of the total DNA) which had been synthesized during the 30-min "light" chase and which appears with the L-DNA band.

§ The density distribution was intermediate between the heavy (parental) strand and the light (newly synthesized) strand, unlike either UV or control.

intermediate between that expected for D-DNA and L-DNA. This heterogeneous DNA constituted less than 5% of the DNA synthesized during the 10-min postirradiation incubation (Fig. 3, Table 1). The banding profile from the denatured DL-DNA exhibited the D-DNA, as well as the bulk of the newly synthesized 3H-labeled L-DNA expected from a semiconservative synthesis.

Of the DNA replicated in 10 min after exposure to 5,000 R of X rays (N/N₀ = 0.50), approximately 95% was by a semiconservative mechanism and the remaining 5% showed the density heterogeneity mentioned earlier.

Repair-synthesis after irradiation of E. coli B/r.

A similar study was carried out with E. coli B/r, since it is apparently free of detectable prophage by tests which proved their presence in E. coli 15T− strains (Hewitt et al., Radiation Res., in press), and as an extension of repair-synthesis studies to other strains of E. coli. The experimental procedure was identical to that described for E. coli 15T− (555-7). During the 30-min light chase, approximately 30% of the DD-DNA was replicated. The CsCl banding profiles of "purified" denatured DD and LD-DNA of the controls are given in Fig. 4 and 5, respectively.

In E. coli B/r, exposed to 473 ergs of UV per cm² (approximately three times that dose used in the E. coli 15T− experiment), approximately 44% of the thymine incorporated was by a semiconservative synthesis, and 56% by repair synthesis (Fig. 4 and 5, and Table 2).

In bacteria exposed to 11,300 R of X rays, all of the DNA synthesized after irradiation (3H-DNA) showed a broad band with a mean buoyant density close to that expected for single-strand fragments made up of "light" precursors (Fig. 4 and 5). However, a decided skew to a higher buoyant density was noted. The mean density displacement of the 3H-containing DNA strand is between 20 and 40% to the heavy side of that expected for the density position of light single-stranded DNA. Thus, approximately 20 to 40% of the DNA in the newly synthesized strand consists of heavy bases.

In the denatured DNA obtained from the DD-DNA fraction of the lysate (Fig. 4), a portion of the 14C was heterogeneously distributed to the

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**FIG. 4.** CsCl gradient profiles of "purified" and then denatured heavy DD-DNA from Escherichia coli B/r. Procedure for preparation was as given in Fig. 3, except that only one purification rebanding was carried out before denaturation of the DNA. Stippled band represents the position of a 14C-labeled T2 marker DNA (ρ = 1.700 g/cc). Dose of irradiation and survival are given in Table 2. Five drops collected per fraction.
and light denatured strands. The distribution in CsCl of the denatured DNA is shown in Fig. 6. Again, a UV-like repair synthesis was not observed. At 40,000 R the mean density of the radiolabeled strand indicated an average 60 to 70% content of heavy bases. In this experiment the T2 reference DNA was melted along with the E. coli DNA to insure that melting had occurred. The T2 DNA banded at 1.715 g/cc as expected for denatured T2 DNA (Fig. 6).

If the peculiar density banding pattern of the DNA synthesized after irradiation was due to a repair synthesis of a magnitude sufficient to account for the intermediate density of the single-strand DNA fragments, then sonic treatment should result in a shifting of the radiolabel to the L-DNA position in CsCl. Accordingly, a lysate of cells exposed to 40,000 R was subjected to sonic oscillation, heat-denaturation, and then analyzed in a CsCl gradient. The nonsonic-treated lysate (Fig. 7A) showed a 14C and 3H distribution similar to that seen in Fig. 6. The sonic-treated lysate (Fig. 7B) exhibited only the broadening of the DNA bands expected from the fragment size reduction due to shearing. Therefore, reducing the DNA strand size does not result in any apparent resolution of "light" DNA from the DNA of intermediate buoyant density.

**DISCUSSION**

**UV repair synthesis.** These data show that, when radioresistant strains of *E. coli* in exponential growth are exposed to UV light, resulting in 50 to 90% cell death, repair as well as semiconservative DNA synthesis occurs in the initial 15 min of growth after irradiation. At a UV dose below 200 ergs/mm², most of the thymine uptake exhibited by cells in the initial 15 min of post irradiation incubation results from semiconservative DNA replication.
FIG. 6. Effect of 40,000 R of X rays on the density distribution of DNA strands synthesized in Escherichia coli after irradiation. In this experiment, like all others, the cell lysate was subjected to CsCl gradient centrifugation as an initial purification step. However, because of the heterogeneous nature of the 3H-label distribution, all the DNA fractions were pooled as one sample; the DNA was then denatured and then reband. The stippled band represents denatured T2 DNA marker. Five drops collected per fraction.

Since a D, 14N, 13C density system was employed in these studies, repair synthesis is not an artifact caused by the added insult of BU substitution for thymine during or after irradiation. A similar type of synthesis was not observed in the unirradiated cells.

In addition, repair synthesis is herein shown to occur in E. coli B/r which, by the tests applied, does not contain a detectable prophage as found in the 15T- derivatives (Hewitt et al., Radiation Res., in press).

Nature of DNA synthesis after X-irradiation. E. coli 15T- exposed to 5,000 R or E. coli B/r exposed to 11,300 or 40,000 R of X rays did not show a repair synthesis of the type seen in UV-exposed cells. Since repair of DNA breaks appears to occur (10, 11) after X-irradiation, the following possible explanations are apparent: (i) repair involves a simple rejoining of a break in the DNA backbone which may or may not involve phosphate exchange; (ii) repair synthesis is less extensive than that following UV exposure; or (iii) relatively long fragments of DNA are excised as compared to UV.

The first possibility is a reasonable one in that it allows either spontaneous rejoining of broken ends or enzymatically controlled rejoining. There is little evidence for or against spontaneous rejoining of phosphate ends to the sugar carbon. Hurwitz, Gefter, and Becker (J. Cellular Comp. Physiol., in press) reported the presence of an enzyme in E. coli B/r capable of converting the circular form of lambda DNA to a covalent form by the insertion of phosphate. This “sealing” enzyme would thus accomplish the rejoining of “ends” without the incorporation of nucleotides.

Limited base excision and synthesis could also occur and escape detection. From Friefelder's data obtained with phage (5), the maximal number of single-strand breaks in the replicating
chromosome will be 1.0 per chromosome per R if the data obtained with "free DNA" are used, and one-hundredth as much or 0.01 break per chromosome if the "DNA in phage" data are used. At 40,000 R the maximal number of breaks would be approximately 40,000 and the minimal number 400 breaks per bacterial chromosome. If a UV-type excision and repair synthesis sequence occurred, it would have been observed in our system even at the minimal number of breaks estimated.

However, our data eliminate only the possibility that a repair synthesis quantitatively similar to that observed for UV repair occurs after ionizing radiation.

Finally, the possibility of extensive excision and repair must be considered. Although this explanation cannot be unequivocally discarded, two facts make it an unlikely candidate to explain our data. The fact that sonic treatment of the isolated strands does not result in shifting of the 14C (heavy) and 3H (light) label of the intermediate-density strand formed after 40,000 R eliminates the possibility of an extensive "repair" synthesis of sufficient proportions to enable a shift in the density of the parental, heavy strands. An equally strong argument against this possibility is the observation that, with increasing dose, the mean buoyant density in CsCl of the DNA synthesized after irradiation (3H label) increases. If repair synthesis were occurring, the opposite result would be expected, since increasing quantities of light precursors should be incorporated into the heavy strand as the DNA damage increases. Pool mixing by precursors derived from DNA degradation products is a more likely explanation for the density behavior of the DNA newly synthesized after X-irradiation.

These results suggest that the chemistry of the repair mechanism may differ depending on the nature of the damage to DNA and that not all repair processes are necessarily like that following UV exposure.

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