REPAIR OF ULTRAVIOLET-INDUCED DAMAGE IN TRANSFORMING DNA OF *BACILLUS SUBTILIS*

NOBUO MUNAKATA

Institute of Applied Microbiology, University of Tokyo, Tokyo*

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After a series of investigations, Setlow and Setlow (1962, 1963) concluded that the formation of pyrimidine dimers by ultraviolet (UV) irradiation may be the major cause of UV inactivation of transforming DNA in *Haemophilus influenzae*. Studies on the UV sensitivity of *Escherichia coli* revealed the importance of the repair of DNA damage, especially the excision-resynthesis repair of pyrimidine dimers (Setlow and Carrier 1964; Boyce and Howard-Flanders 1964; Howard-Flanders, Boyce, and Theriot 1966). The isolation of mutants of *Bacillus subtilis* Marburg sensitive to UV light was reported previously by Okubo and Roming (1965) and Reiter and Strauss (1965), and the latter authors suggested that repair reactions may also occur in *B. subtilis*, since extractable transforming activity increased when UV-resistant cells were incubated after UV irradiation under appropriate conditions.

We have previously reported several UV-sensitive mutants of *B. subtilis* Marburg (Munakata and Ikeda 1969). The mutants were classified as *uvs* (Hcr⁻), *uvs* (Hcr⁺), and *uvss* (Hcr⁻) by their UV sensitivity and host-cell reactivation ability. UV-irradiated transforming DNA gave smaller numbers of transformants when assayed with the Hcr⁻ strains than when assayed with the Hcr⁺ strains.

This work was undertaken to study the relationship between the UV sensitivity of a strain and its ability of restore the activity of UV-inactivated transforming DNA *in vivo*. Restoration of the transforming activity, measured by *his*₃₁⁺ marker activity, was apparent in UV-resistant (Hcr⁺) (*uvr*) strain, but not in *uvs* (Hcr⁻) strains. In a *uvs* (Hcr⁺) strain, post-irradiation inactivation of transforming activity was observed.

MATERIALS AND METHODS

Strain: *Bacillus subtilis* Marburg *uvr thy ind*₁₆₈ was the parental UV-resistant strain. Several UV-sensitive (*uvs*) and supersensitive (*uvss*) mutants were derived from this strain as reported previously (Munakata and Ikeda 1969). They were designated as *uvs-1*, *uvs-19*, etc. These *uvs* and *uvss* strains, except *uvs-80*, are host-cell reactivation negative (Hcr⁻); they do not reactivate UV-inactivated phage M2. From these strains, *uvs thy his*₃₁ strains were produced by the transformation technique, where a *his*₃₁ strain was used as the donor and *uvs thy ind*₁₆₈ strains were used as the recipients. Since the *ind*₁₆₈ locus and the *his*₃₁ locus are closely linked on the chromo-

*present address; Department of Microbiology,
The University of Chicago, Chicago, U.S.A.

some, *uvr thy his₃₁* transformants are easily selected. Three auxotrophic mutants requiring two nutrients for growth, *try₁₆₀ met₁₄*, *try₁₆₀ arg₁₅*, and *try₁₆₀ asp₆*, were also used (Munakata, Saito, and Ikeda 1966).

Media: TY broth contained in 1,000 ml; 10 g Bacto-tryptone (Difco), 2.5 g yeast extract (Difco), and 2.5 g NaCl (pH 7.2).

Dilution buffer contained in 1,000 ml; 10 ml of trishydroxymethylaminomethane (Tris) buffer (1M, pH 7.2), 10 ml of MgCl₂(1M), and 5 g NaCl.

Labeling of Cells: An overnight culture was diluted one to fifty with fresh TY broth containing 2 μ Ci/ml of H³-thymidine (specific activity 5 μ Ci/ μ mole, from Radiochemical Center) and incubated at 37° for 8 hours by which time the culture was in the stationary phase. The labeled cells were collected by centrifugation, washed five times with dilution buffer, and then suspended in dilution buffer to be exposed to UV light.

Irradiation: Ten ml of dilution buffer containing ca. 5×10^9 cells per ml was exposed to UV light from 15 W germicidal lamp. The dose rate was 39 erg/mm²/sec at a distance of 19 cm. During the irradiation, the fluid in a Petri dish (diameter 9 cm) was agitated vigorously.

Post-irradiation treatment: After irradiation, the cells were diluted to the original volume with TY broth containing 10 μ g/ml of unlabeled thymidine. The inoculated broth was incubated at 37° with shaking. Samples were withdrawn at intervals and served for assay of radioactivity as well as for the preparation of transforming DNA.

Acid-precipitation: One ml samples were withdrawn at intervals from the post-irradiation culture and separated into cells and medium by centrifugation. Cells were assayed by suspension in cold 5 % trichloroacetic acid (TCA) solution. The medium was assayed, after the addition of non-labeled carrier cells (ca. 5×10^8) to ensure precipitation, by mixing with 0.1 ml of 50 % TCA solution. After holding one hour at ice temperature, the mixtures (final concentration, 5 % TCA) were centrifuged at 4,000 rpm for 15 min. The supernatants were extracted five times with an equal volume of ether. The pellets were suspended in 5 % ammonia solution. Counting of radioactivity in each fraction was carried out with a liquid scintillation counter (Packard Tri-Carb Spectrometer).

Paperchromatography: Determination of photoproducts was carried out by slight modification of the methods of Wacker (1963) and Smith (1964). Tritium-labeled cells collected by centrifugation were suspended in 1 ml TCA solution (5 %) and kept at ice temperature for one hour. Precipitates were collected by centrifugation and washed with cold 95 % ethylalcohol, ethylalcohol-ether, and ether in sequence. Dried samples were hydrolysed with 70% perchloric acid at 100° for 45 min and portions of the hydrolysates were spotted on Whatman filter (3 MM). The solvent used was a mixture of *n*-butanol, acetic acid, and water (80 : 12 : 30 by volume). Positions of the radioactive materials on the paperchromatogram were determined by cutting the chromatogram into strips and determining the radioactivity of water extracts of each strip. The followings were used as standard markers; thymine dimer, thymine-uracil dimer, and uracil dimer. These marker materials were produced from a frozen mixture of C¹⁴-thymine (0.7 μ Ci/ μ mole) and C¹⁴-uracil (0.7 μ Ci/ μ mole), 1 mg/ml each, by UV irradiation for one hour. Radioactivity in spots of R_f 0.41, 0.31, and 0.19 was considered to be in thymine dimer, thymine-uracil dimer, and uracil dimer, respectively.

Preparation of transforming DNA from UV-irradiated cell: Cells (ca. 5×10^9) harvested at intervals from a post-irradiation culture were treated at 37° for 15 min with $100 \mu\text{g/ml}$ of lysozyme in 0.5 ml of saline-EDTA solution (0.15 M NaCl plus 0.1 M ethylenediaminetetraacetate, pH 8) and then frozen in dry-ice acetone. After several cycles of freezing and thawing, an equal volume of Tris-SLS solution (1M Tris buffer, pH 9.0, 2% sodiumlaurylsulfate, and 0.1 M NaCl) was added. The lysate was digested at 37° for 15 min with 0.05 ml of pronase (1 mg/ml) and then added with 1 ml of phenol that had been saturated with Tris-SLS. The mixture was rotated for 30 min at room temperature and the upper layer obtained by centrifugation was dialysed overnight at room temperature against 0.15 M NaCl supplemented with 0.015 M trisodium citrate (pH 7.2). Recovery of the radioactivity was 60-70%. The concentration of the DNA used for the transformation experiments, which were performed as described by Saito and Miura (1963), was $0.2 \mu\text{g/ml}$.

RESULTS

Degradation of DNA during post-irradiation incubation

UV-induced degradation of DNA was studied as described in Materials and Methods. Figure 1 shows the relative amounts of acid-soluble radioactivity released from the DNA in the UV-irradiated cells during the incubation. The acid-soluble radioactivity indicated was present in the medium. As shown by Shuster (1967), almost all UV-induced degradation products from *B. subtilis* DNA appear in the medium in contrast

Fig. 1. Degradation of DNA in UV-treated *thy ind₁₆₈* strains during post-irradiation incubation.

uvr (○), *Uvs-1* (△), *Uvs-19* (+), *uvss-19-8* (×), *Uvs-80* (□), *Uvs-105* (▼).

to the case of *E. coli*. The acid-soluble radioactivity in the cells was negligibly small throughout the whole course of post-irradiation incubation.

As shown in the figure, the UV-induced degradation of DNA is large in *uvr*, *uvr-80*, and *uvr-105*, intermediate in *uvr-19*, and small in *uvr-1* and *uvr-19-8*. These results may be understood if we assume that the release of acid-soluble material from DNA can only occur in strains which have excision system. The mutants *uvr-1* and *uvr-19-8* may have defects in early steps of excision-repair reaction, while the mutants *uvr-80* and *uvr-105* may have defects in some later steps.

Fate of thymine dimers in uvr-80

Shuster (1967) reported that the thymine dimers in DNA of a UV^r (Hcr⁺) strain of *B. subtilis* were excised in the dark but those in DNA of a UV^s (Hcr⁻) mutant were not. A similar experiment was performed with strain *uvr-80* which inspite of its UV-sensitive character released as large an amount of acid-soluble material as did the *uvr*. Figure 2 shows chromatographic profiles of the photoproducts of DNA in *uvr-80* irradiated with UV and the incubated for 120 min. The profiles show that after 5 min UV irradiation the amount of thymine dimers in the acid-insoluble fraction is approximately 3.8 % of the total thymine residues but the amount of thymine dimers is reduced

Fig. 2. Thymine-containing photoproducts in strain *uvr-80 thy ind₁₆₈*. DNA extracted from irradiated cells was subjected to chromatography as described in the text. The radioactivity on each strip cut from the paperchromatogram is given as per cent of the total radioactivity.

□ unirradiated, □ irradiated for 5 min, ▨ irradiated for 5 min and then incubated for 120 min.

The arrows indicate the positions of the bases and authentic photoproducts, thymine, uracil, thymine dimer, thymine-uracil dimer, and uracil dimer.

to approximately 1.8 % after 120 min incubation. This suggests that more than half of the thymine dimers produced by UV in the irradiated cells disappeared from the acid-insoluble fraction during post-irradiation incubation. The profiles were indistinguishable from those of a *uvr* strain and it was presumed that strain *uvs-80* retained almost normal excision ability.

Restoration of transforming activity during post-irradiation incubation

Restoration of transforming activity during post-irradiation incubation was compared among the three strains, *uvr thy ind₁₆₈*, *uvs-19 thy ind₁₆₈*, and *uvs-1 thy ind₁₆₈*; these strains released, respectively, a large, intermediate, and small amount of acid-soluble material from DNA after UV irradiation. As recipient of transforming DNA prepared from these strains, *uvr thy his₃₁*, *uvs-19 thy his₃₁*, or *uvs-1 thy his₃₁* was used. The activity of the DNA prepared from *uvr* cells immediately after 5 min irradiation was 10 % of that from unirradiated cells when assayed with *uvr* and 1-2 % when assayed with *uvs-19* or *uvs-1*. As shown in the previous paper (Munakata and Ikeda 1969), this difference in the activity of DNA when assayed in a *uvr* or a *uvs* recipient is due to the repair mechanism existing only in the *uvr* recipient. The activity of UV-irradiated DNA, extracted from a *uvr* donor and assayed with a *uvs* recipient, increases when cultures are incubated after UV irradiation and prior to DNA extraction until it reaches the values obtained with the *uvr* recipient (Fig. 3). Such post-irradiation re-

Fig. 3. Restoration of transforming activity during the course of post-irradiation incubation. UV-irradiated cells of *uvr thy ind₁₆₈* (a), *uvs-1 thy ind₁₆₈* (b), and *uvs-19 thy ind₁₆₈* (c) were incubated for the indicated time. The DNA was then extracted and used to transform *his₃₁⁺* in *uvr thy his₃₁* (○), *uvs-1 thy his₃₁* (△), and *uvs-19 thy his₃₁* (+).

covery did not occur in *uvr* donors (Fig. 3 (b) and (c)). Furthermore, the figures suggest that no post-irradiation inactivation or replication of the marker region has taken place during the incubation, and that UV damage occurring in the DNA of *uvr-1* or *uvr-19* cells remains unrepaired during the post-irradiation period.

Attempt to classify the processes of post-irradiation repair by complementation experiments

If the processes of post-irradiation repair consist of sequential reactions, the genetic defect in an early step mutant should be rescued by the action of a late step mutant. Figure 3 (b) and (c) show that such DNA-mediated complementation may not take place at least between *uvr-1* and *uvr-19*. To test the possibility further, various *uvr thy his₃₁* cells were exposed to DNA prepared from a post-irradiation culture of *uvr-19 thy ind₁₆₈* or *uvr-105 thy ind₁₆₈*. The results in Fig. 4 suggest that UV damage in irradiated donor DNA is not repaired in the recipient *uvr* cells. The failure of these complementation experiments will be discussed later.

*Decline of transforming activity in strain *uvr-80* during post-irradiation incubation*

The UV-induced damage in the *his₃₁⁺* marker region of *uvr-1*, *uvr-19*, and *uvr-105* strains was repaired in the recipient *uvr* cells to a level of about 10 % surviving activity

Fig. 4. Results of complementation experiments performed between two *uvr* strains. DNA extracted from UV-irradiated cells of *uvr-19 thy ind₁₆₈* (a), *uvr-105 thy ind₁₆₈* (b) was used to transform *his₃₁⁺* marker into *uvr thy his₃₁*. Recipient strains; *uvr* (○), *uvr-1* (△), *uvr-19* (+), *uvr-19-8* (×), *uvr-42* (▽), *uvr-46* (■), *uvr-105* (▼), and *uvr-114* (▲).

for the marker and this value remained constant during post-irradiation incubation of donor cells (Fig. 3, 4). However, this was not the case with the DNA of strain *uvr-80*. The transforming activities of various tested markers in *uvr-80* assayed with *uvr* recipients decreased with post-irradiation incubation (Fig. 5). The phenomenon will be called post-irradiation inactivation. Post-irradiation inactivation was not so evident when assayed with a *uvr* recipient.

Fig. 5. Inactivation of transforming activity in strain *uvr-80* during post-irradiation incubation. DNA extracted from an irradiated culture of *uvr-80 thy ind*₁₆₈, incubated for the indicated time, was used to transform markers in *uvr* strains, *met*₁₄⁺ (□), *arg*₁₅⁺ (○), *his*₃₁⁺ (■), *try*₁₆₀⁺ (●), and *asp*₆⁺ (▼), and *his*₃₁⁺ marker in *uvr-1* (×).

DISCUSSION

Demonstration of the post-irradiation recovery of transforming activity in *B. subtilis* illustrates the fact that cellular sensitivities are primarily determined by the repair reactions acting upon DNA damage. All the tested *uvr* (*Hcr*⁻) strains are characterized by their inability to restore UV-inactivated transforming DNA during the course of post-irradiation incubation. This incapability of post-irradiation recovery can presumably be attributed to a defect in the *Hcr* mechanism. The processes of post-irradiation repair may consist of several biochemical steps. The existence of *uvr* strains which release

different amounts of acid-soluble material from DNA during post-irradiation incubation might be taken to support the view. However, an attempt to sort out the different steps by complementation experiments ended in failure. These complementation experiments suggest that the repair processes may not be so simple as to permit the detection of particular intermediates by the DNA-mediated complementation method used. In *E. coli* at least three distinct classes of UV-sensitive (Hcr^-) mutants are known and an attempt to sort out these classes by a DNA-mediated complementation method has failed (Howard-Flanders, Boyce, and Theriot 1966).

Strain *uvs-80* (Hcr^+) is sensitive to UV light presumably because it has defect in a mechanism different from the *Hcr* mechanism. This strain releases a large amount of acid-soluble material from DNA after UV irradiation, the excision of thymine dimers occurs as in *uvr* strain, and, moreover, the extractable transforming activity declines remarkably during the post-irradiation period. A simple explanation is that DNA degradation proceeds in this mutant without being accompanied by recovery reactions. In other *uvs* (Hcr^-) strains, the excision reaction may not take place or may proceed to a limited extent and the DNA may be protected against further degradation.

SUMMARY

UV damage induced in the DNA of *uvr* or *uvs* strains of *Bacillus subtilis* was repaired in *uvr* recipient cells, but no such repair occurred in *uvs* cells. Post-irradiation repair of UV damage induced in the DNA of the *uvr* strain was inferred from the increase in extractable transforming activity observed during post-irradiation incubation carried out before assaying with *uvs* recipient strains. No post-irradiation restoration of UV-irradiated transforming DNA was observed in *uvs* cells. Strain *uvs-80* was able to excise thymine dimers from the DNA as did the *uvr* strain but lost extractable transforming activity rapidly during post-irradiation incubation.

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