uwrC Gene Function in Excision Repair in Toluene-Treated Escherichia coli

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We have examined the role of the uwrC gene in UV excision repair by studying incision, excision, repair synthesis, and DNA strand reformation in Escherichia coli mutants made permeable to nucleoside triphosphates by toluene treatment. After irradiation, incisions occur normally in uwrC cells in the presence of nicotinamide mononucleotide (NMN), a ligase-blocking agent, but cannot be detected otherwise. We conclude that repair incisions are followed by a ligation event in uwrC mutants, masking incision. However, a uwrC polA12 mutant accumulates incisions only slightly less efficiently than a polA12 strain without NMN. Excision of pyrimidine dimers is defective in uwrC mutants (polA+ or polA12) irrespective of the presence or absence of NMN. DNA polymerase I-dependent, NMN-stimulated repair synthesis, which is demonstrable in wild-type cells, is absent in uwrC polA+ cells, but the uwrC polA12 mutant exhibits a UV-specific, ATP-dependent repair synthesis like parental polA12 strains. A DNA polymerase I-mediated reformation of high-molecular-weight DNA takes place efficiently in uwrC polA+ mutants after incision accumulation, and the uwrC polA12 mutant shows more reformation than the polA12 strain after incision. These results indicate that normal incision occurs in uwrC mutants, but there appears to be a defect in the excision of pyrimidine dimers, allowing rescaling via ligation at the site of the incision. The lack of NMN-stimulated repair synthesis in uwrC polA+ cells indicates that incision is not the only requirement for repair synthesis.

The repair mechanisms that are independent of visible radiation are excision repair and post-replication repair. The excision pathway is a major source of low-error repair of UV-induced damage, defined by the Escherichia coli uwr mutants. (12, 28, 42). This repair pathway appears to involve sequential enzymatic events of scission of the DNA chain close to UV-induced pyrimidine dimers, excision of the dimers, reinsertion of nucleotides, and finally sealing of the nicks (12). Although the uwr genes represent separate loci on the E. coli genetic map, the enzymatic events in excision repair seem to be performed in a coordinated process.

Among these loci at least three, uwrA, uwrB, and uwrC, are required for the excision process (3, 12, 36). The uwrA and uwrB mutants have been identified as defective in incision (36). The reported absence of UV-specific endonuclease in uwrA and uwrB mutants supports the involvement of these gene functions in the incision step (4; A. Braun and L. Grossman, Fed. Proc. 33: 1599, 1974). However, no role has been determined for the uwrC gene function in excision repair. The excision of pyrimidine dimers has been found defective in uwrC mutants in vivo, as in uwrA and uwrB mutants, although the excision data do not distinguish between the functional characteristics of these mutants (3, 12, 36). Studies with host cell reactivation show that uwrC mutants, like uwrA and uwrB mutants, do not catalyze the formation of single-strand breaks in transfecting foreign (phage) DNA, suggesting that uwrC mutants are defective in incision (40). Other studies on the formation of breaks in covalently closed λ DNA molecules in lysogenic repair-proficient and -deficient host cells after UV irradiation also demonstrate the absence of incision in uwrC mutants (28). In contrast, other in vivo studies show the presence of single-strand breaks in DNA of uwrC mutants after treatment with UV (13) or mitomycin C (29). In vivo results with uwrC lig7(Ts) mutants suggest that the uwrC gene product prevents sealing of the incised DNA by polynucleotide ligase (34). The same work detected no incisions in uwrC polA12 cells after irradiation. The uwrC gene function has also been seen to be responsible for the modification of the termini of incised DNA in UV-irradiated cells (13). Studies in a sucrose-plasmolyzed cell system demonstrated the absence of ATP-dependent incisions in the DNA of UV-irradiated uwrC mutants (35), although uwrC mutants have been shown to possess nor-
unal levels of uvrA,B endonuclease (4). Studies using cell extract complementation assays have indicated that the uvrA, uvrB, and uvrC gene products are required to catalyze repair incisions (32, 33). In uvrC toluene-treated cells a lack of UV-specific DNA repair synthesis has been interpreted as being secondary to a failure of incision in such mutants (1, 39). Thus, no conclusive role of uvrC gene function in excision repair has been determined, and the data from various in vivo and in vitro studies do not agree.

To gain a clear understanding of the defect in uvrC mutants, it is essential to study individual events, which is difficult in vivo because the repair process is performed rapidly. Toluene-treated cells are permeable to low-molecular-weight compounds and allow manipulation of DNA repair reactions (26). Previous work from this laboratory and others has shown that the toluene-treated cell system is useful in demonstrating the involvement of various DNA polymerases in repair (2, 6, 7, 20, 25, 37), the requirement for ATP for incision (6, 41; J. W. Dorson and R. E. Moses, Fed. Proc. 33:1599, 1974), excision (5), and synthesis (2, 20), and the requirement for the uvrA,B gene products in UV-specific incision (21, 25). In this report, we studied the functional characteristics of uvrC mutants in the toluene-treated cell system. Since DNA polymerase I influences excision and reformation (6), we constructed isogenic uvrC polA12 mutants for evaluation. Our observations demonstrate the occurrence of incision and failure of excision of pyrimidine dimers in vitro in uvrC mutants after irradiation. These observations suggest that the uvrC gene function may be necessary for the performance or regulation of excision reactions.

MATERIALS AND METHODS

Chemicals and reagents. Nicotinamide mononucleotide (NMN), ATP, and nonradioactive deoxyribo- nucleoside 5'-triphosphates (dNTP's) were purchased from P-L Biochemicals. [methyl-3H]thymidine (specific activity, 47 Ci/mmol) was from Schwarz/Mann. a-[32P]dATP was purchased from ICN. Novobiocin was a gift from The Upjohn Co. Bacterial culture media, lysozyme, Sarkosyl, and N-ethylmaleimide were purchased from Difco, Worthington, Sigma, and Eastman Kodak, respectively. Cellulose sheets for pyrimidine dimer analysis were from Brinkman Instruments Inc. Pronase was obtained from Calbiochem.

Bacterial strains. The sources and characteristics of the strains used in this work are detailed in Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td>W3623</td>
<td>gal trp Str' pro+ (wild type)</td>
<td>J. Tomizawa (3)</td>
</tr>
<tr>
<td>N17-7</td>
<td>uvrC56 (W3623 derivative)</td>
<td>J. Tomizawa (3)</td>
</tr>
<tr>
<td>MM383</td>
<td>polA12 thy Str' [pol(T)]</td>
<td>M. Monk (25)</td>
</tr>
<tr>
<td>RM 76</td>
<td>uvrC polA12</td>
<td>This paper</td>
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MetE), donated by M. Murgola (M. D. Anderson Hospital and Tumor Institute, Houston, Texas). Transductants to Val' and Met' phenotype were selected by growth on selective media, including correction of Met' phenotype by B12. P1 was grown on MM383 (polA12 thy Str') twice, and the stock was titrated on the same strain using soft (0.7%) L-agar overlay. This stock was then used to transduce Met' to the N17-7 recipient at a multiplicity of 0.02. After 20 min the culture was made 0.1 M in citrate, and the cells were collected by centrifugation and suspended in minimal medium for plating on medium containing proline and tryptophan but no methionine. Individual colonies were picked and checked for other markers including UV'. Individual colonies were grown and assayed as described (27) for DNA polymerase activity. From 17 individual isolates, 2 were identified as polA' in extracts. Both double mutants grow at 42°C. MM383 was used to avoid possible lethality in the double mutants.

Growth of cells and labeling of DNA. Cells were routinely grown to a concentration of 5 x 10^9 to 1 x 10^10 cells per ml in L-broth containing, per liter of water: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 10 mg of thymine. [methyl-3H]thymidine (5 µCi/ml) was added to label DNA during exponential growth of the cells. For thymine dimer analysis, cells were grown in L-broth containing 2 µg of thymine per ml and M9 (22) medium (50:50%). M9 medium contained, per liter of water: Na_2HPO_4, 7H_2O, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; MgSO_4, 1 mmol; CaCl_2, 0.1 mmol; and glucose, 4 g; supplemented with 2 µg of vitamin-free Casamino Acids per ml. For thymine dimer analysis, DNA was labeled using 10 µCi of [methyl-3H]thymidine per ml for several generations.

Toluene treatment and UV irradiation. Exponentially growing cells were harvested and suspended in 50 mM KPO_4 (pH 7.4) and treated at 10^10 cells per ml with 1% toluene as described (26). Cells were washed and resuspended in 50 mM KPO_4 (pH 7.4) at the same concentration. Cell suspensions in petri dishes (5-cm diameter) were placed on a platform shaker and irradiated at 254 nm from an unfiltered GE G8T5 lamp at a distance of 50 cm. A dose rate of 0.8 J/m² per s at the surface was measured by a UV intensity meter (Ultraviolet Products, Inc., San Gabriel, Calif.). Subsequent manipulation of the cells was performed in subdued light.

Conditions for in vitro repair synthesis. Toluene-treated and irradiated cells were diluted to 3 x 10^9 cells per ml in reaction mixtures (0.3-ml) containing 66 mM KPO_4, buffer (pH 7.4), 13 mM MgCl_2, 1.3 mM ATP, and 33 µM each of dNTP's. One of the dNTP's was radioactive, as required, at 90 cpn of a-[32P]dATP or 40 cpn of [3H]dTTP per pmol. NMN and nicotinamide adenine dinucleotide (NAD) were added to final concentrations of 5 mM and 0.5 mM, respectively,
when required. DNA synthesis was monitored by measuring radioactivity in trichloroacetic-acid-insoluble radioactive material determined by liquid scintillating counting (26). Strain MM383 (polA12) manifests polA repair synthesis levels in vitro at 37 °C (25).

**Two-dimensional thin-layer chromatography.** The analysis of excision of photo-products was performed by two-dimensional thin-layer chromatography on precoated cellulose plastic sheets as described earlier (5). In brief, after trichloroacetic-acid precipitation, the insoluble material was hydrolyzed with 6N formic acid. The hydrolysates were cooled and evaporated to dryness in a vacuum desiccator. The samples were dissolved in a minimal volume of water (10 μl). The chromatography was performed using a solvent mixture of butanol–water (86:14) for the first dimension and a saturated solution of ammonium sulfate–1 M sodium acetate–isopropanol (80:18:2) for the second dimension. Dimers were produced at a 40-J/m² UV dose.

**Alkaline sucrose gradient sedimentation.** Alkaline sucrose gradient analysis was performed as described (6, 17). The reactions were stopped by chilling and adding 0.2 M tris(hydroxymethyl)amino methane (free base) and 0.04 M ethylenediaminetetraacetic acid. Samples of 5 × 10⁷ cells were layered onto 5 to 20% alkaline sucrose gradients, already overlaid with 0.2 ml of lysing solution containing 0.5 M NaOH, 0.5 N NaCl, 1% Sarkosyl, and 4 mM ethylenediaminetetraacetic acid. After 30 min at room temperature, the gradients were centrifuged at 25,000 rpm in an SW50.1 rotor at 20 °C for 2 h, collected on paper strips, precipitated with cold 10% trichloroacetic acid–0.1 M NaPP₆, and then washed with 5% trichloroacetic acid and 0.01 M HCl for 10 min each. The gradients contained 2 × 10⁶ to 5 × 10⁶ cpm and are plotted as percent recovered counts per fraction. The weight average molecular weight was calculated with reference to co sedimenting λX-174RF DNA according to Rupp and Howard-Flanders (30) using the equation

\[ M_w = \frac{M_s \times (C_s - C_f)}{C_f - C_s} \]

where \( M_w \) is the molecular weight of the DNA in the corresponding fraction and \( C_s \) represents the amount of the radioactivity in that fraction. The number average molecular weight was calculated by assuming \( M_s = M_w/2 \) if the breaks are randomly distributed in the DNA (35).

**Reformation of high-molecular-weight DNA.** Reformation of high-molecular-weight DNA was analyzed on alkaline sucrose gradients. Single-strand breaks were accumulated by incubating tolune-treated and UV-irradiated cells with ³H-labeled DNA in a reaction mixture containing ATP and NMN (5 mM) but lacking dNTP's for 15 min. The NMN and ATP were removed by centrifugation for 45 s in a Beckman microfuge. Cells were reincubated for 2 min in a complete reaction mixture containing 1.3 mM ATP, 33 μM dNTP's, and 0.5 mM NAD. The termination of reactions and alkaline sucrose gradient sedimentation were performed as described above.

**Isopionic centrifugation.** The DNA of exponentially growing cells was labeled with 0.01 μCi of [methyl-³H]thymidine per ml, harvested by centrifugation, and toluene treated. Unirradiated and UV-irradiated cells were incubated in standard reaction mixtures (0.6 ml) containing bromodeoxyuridine triphosphate in place of dTPP and α-[³²P]dATP at 200 cpm/pmol. After 30 min of incubation at 37 °C, the cells were lysed in Sarkosyl–Pronase, and the synthesized DNA was analyzed by buoyant density centrifugation as described (8).

**RESULTS**

**Incisions in uvrC mutants.** A widely accepted model for excision repair proposes incision by an endonucleolytic activity that recognizes the dimers and makes a single-strand cut on the 5' side of the dimer (10). As noted above, there is conflicting evidence on whether *uvrC* mutants catalyze incisions. To investigate this, we have taken advantage of the fact that incisions can be readily accumulated in toluene-treated cells by omitting dNTP's or through the addition of NMN (6, 41; Dorson and Moses, Fed. Proc. 33:1599, 1974). Cells with ³H-labeled DNA were toluene treated, exposed to UV at 30 J/m², and incubated in reaction mixtures lacking dNTP's, and the DNA was analyzed on alkaline sucrose gradients as described in Materials and Methods. Figure 1 shows the comparative results for formation of UV-specific, ATP-dependent, single-strand breaks in wild-type and *uvrC* cells. Under standard incision conditions (no dNTP's), both polA⁺ (Fig. 1A) and polA12 (Fig. 1B) cells executed incisions resulting in the reduction in the size of the DNA from irradiated cells. In these studies we found no indication of nonspecific DNA cleavage (6). To ensure that no ligation of single-strand breaks occurred, studies on incision were also done using NMN, an inhibitor of ligase activity (16, 24). The addition of NMN to reaction mixtures did not cause appreciable reduction in the size of DNA in irradiated *polA*⁺ or *polA12* cells, indicating little religation of the incisions even in the absence of NMN. These results confirm our previous findings in toluene-treated wild-type cells (6). In contrast, we failed to detect single-strand breaks in the DNA of irradiated *uvrC polA⁺* cells (Fig. 1C) under the normal conditions for ATP-dependent incision. However, when NMN was added to reaction mixtures, the *uvrC polA⁺* cells exhibited incisions almost to the same extent as wild-type cells. The *uvrC polA12* cells catalyzed incisions only slightly less efficiently than the parental *polA12* strain under normal conditions, and in the presence of NMN virtually the same reduction in the size of DNA was detected (Fig. 1B and D). This result is in contrast to observations in other reports based on *uvrC lig-7(Ts)* cells in vivo (34) and plasmolyzed cells (35). In the absence of ATP, the addition of NMN did not induce appreciable formation of single-strand breaks (see Fig. 2) in either wild-type or *uvrC* cells, indicating that the incision
was part of a specific repair response. These studies were repeated using another uvrC polA+ strain, AB1884 (uvrC94, from the E. coli Genetic Stock Center, New Haven, Conn.), again demonstrating NMN-dependent incisions.

The above results show that incisions do occur in uvrC mutants, and we have evaluated whether they occur at the same rate in wild-type and uvrC cells in vitro. Toluene-treated cells were irradiated at 30 J/m², incubated in standard reaction mixtures (no dNTP's) containing or lacking NMN for various time periods, and analyzed on alkaline sucrose gradients (Fig. 2). In both polA+ and polA12 cells, incisions accumulated in the presence or absence of NMN (Fig. 2A and B). The uvrC polA+ cells did not show any reduction in the size of DNA during a 15-min incubation in the presence of ATP alone. However, in the presence of NMN, uvrC polA+ cells followed the same kinetics of incision (Fig. 2C) as observed in polA+ cells (Fig. 2A). The uvrC polA12 cells exhibited intermediate levels of incision during incubation in the absence of NMN. These cells showed the same kinetics of incision (Fig. 2D) as polA12 cells in the presence of NMN (Fig. 2B). Thus uvrC (polA+ or polA12) cells exhibited UV-dependent breaks in their DNA in the presence of NMN at the same rate as wild-type cells, suggesting a ligation of the breaks in uvrC mutants as the cause of failure to detect incisions.

**Repair synthesis in uvrC cells.** The results...
presented thus far led us to expect that once incisions occurred in uvrC mutants, they might be followed by subsequent events of excision repair, unless the uvrC gene function was modulating these events. As a result of incision, there should be repair synthesis in the DNA of irradiated uvrC cells. We have shown that, after irradiation, toluene-treated cells deficient in the polymerizing activity of DNA polymerase I exhibit a level of repair synthesis approximately 30% of replication. We have concluded that this synthesis is dependent on DNA polymerase III (2, 25). In contrast, toluene-treated polA+ cells demonstrate very low levels of repair incorporation. The low repair incorporation in polA+ cells has been related to the efficient interaction of polymerase I and ligase activities and efficient excision of dimers in these cells, resulting in short patch repair synthesis (6, 8).

Figure 3 shows the comparative results of repair synthesis in toluene-treated cells of strains MM383 (polA12) and RM 76 (uvrC polA12). It is important to note that uvrC polA12 cells exhibited normal levels of UV-induced, ATP-dependent repair synthesis (Fig. 3B) compared with polA12 cells (Fig. 3A), supporting the previous data that uvrC mutants execute incisions. These results further indicate that the defect in uvrC polA12 cells does not inhibit repair synthesis. Novobiocin inhibits DNA replication by blocking DNA gyrase activity (9), but does not inhibit UV repair synthesis in toluene-treated cells (31). DNA synthesis observed in uvrC polA12 cells persisted in the presence of novobiocin (Fig. 3B) as it did in polA12 cells (Fig. 3A), again indicating the occurrence of incisions in uvrC mutants, and showing that the repair synthesis is independent from replication.

NMN causes a stimulation of repair incorporation in polA+ toluene-treated cells, presumably by preventing effective sealing of breaks in the DNA of irradiated polA+ cells during repair (8). The stimulation of repair synthesis is ATP dependent and comparable with the repair synthesis observed in polA cells. Figure 4 shows typical results for repair synthesis in toluene-treated W3623 (polA+) and N17-7 (uvrC polA+) cells. The results presented in Fig. 4A agree with the previous findings in toluene-treated polA+ cells. UV irradiation of the cells resulted in inhibition of replicative DNA synthesis. Stimulation of repair incorporation was observed only in the presence of NMN. Surprisingly, the NMN-induced stimulation of repair synthesis was negligible in uvrC polA+ cells (Fig. 4B),
FIG. 3. DNA synthesis in toluene-treated (A) MM383 (polA12) and (B) RM 76 (uvrC polA12). Toluene-treated cells were exposed to UV, and DNA synthesis was measured as described in the text. Novobiocin was present at 15 μg/ml when used. One hundred percent represents 86 pmol of DNA synthesis in MM383 and 57 pmol in RM 76. α-[32P]dATP was the labeled substrate. Symbols: (●) +ATP; (▲) +ATP, +novobiocin; (△) -ATP.

FIG. 4. Comparison of NMN stimulation of repair synthesis in wild-type (W3623) and uvrC polA+ (N17-7) cells. Toluene-treated cells were exposed to UV, and DNA synthesis was determined as described in the text. NMN and novobiocin were present at 5 mM and 15 μg/ml, respectively. One hundred percent represents 78 pmol of DNA synthesis in W3623 (polA+) and 63 pmol in N17-7 (uvrC polA+). Symbols: (●) +ATP; (▲) +ATP, +NMN; (▲) +ATP, +NMN, +novobiocin; (△) -ATP, +NMN, +novobiocin; (○) -ATP.

compared with that in wild-type cells (Fig. 4A). Incorporation in the absence of ATP was low in both the strains in unirradiated or irradiated cells whether or not NMN was present. These experiments were also done in the presence of novobiocin. The addition of novobiocin did not perturb stimulation of repair incorporation in polA+ cells (Fig. 4A), indicating the independence of repair incorporation from replicative DNA synthesis. It is worth noting that in the presence of NMN there was a significant level of novobiocin-resistant synthesis even with no irradiation. This indicates that NMN induces some repair synthesis and inhibits replication somewhat, in agreement with data presented below in Fig. 5 and 6. In uvrC polA+ mutants novobiocin-resistant synthesis did not rise above this level, in contrast to wild-type cells, indicating very low UV-stimulated repair synthesis in such mutants. The lack of stimulation of repair synthesis in uvrC polA+ cells in the presence of NMN suggests a block of repair synthesis in the absence of uvrC gene function. However, our studies do not rule out incorporation of a small number of nucleotides at the site of incision.

To clarify these observations, we took another experimental approach, that of following repair synthesis in the presence of a suitable density-
FIG. 5. Isopycnic analysis of DNA synthesis in vitro in W3623 (polA+). DNA synthesis was analyzed as described in the text. (A) No UV; (B) no UV, +NMN; (C) UV (90 J/m²); (D) UV, + NMN. NMN was present at 5 mM. Symbols: (●) 32P-labeled newly synthesized DNA; (□) 3H-prelabeled DNA. Arrows indicate the density of the corresponding fraction.

labeled nucleotide precursor to monitor the mode of DNA synthesis. Figures 5 and 6 present the results of isopycnic analysis of repair synthesis in wild-type (polA+) and uvrC polA+ cells. Cells with 3H-labeled DNA were toluene treated, irradiated at 90 J/m², and incubated with bromodeoxyuridine triphosphate in place of TTP under standard conditions for DNA synthesis. Figure 5A represents semiconservatively synthesized DNA appearing at hybrid density. Addition of NMN in the case of unirradiated cells allowed the continuation of replicative DNA synthesis, although NMN sometimes brought down the levels of DNA synthesis (Fig. 5B). UV irradiation caused inhibition of replication, but repair synthesis was not prominent because of efficient repair (Fig. 5C). Results shown in Fig. 5D confirmed our previous findings that NMN stimulates the repair synthesis in irradiated polA+ cells.

In contrast, irradiated uvrC polA+ cells did not exhibit UV stimulation of synthesis in the presence of NMN (Fig. 6D). These mutants exhibited normal replicative DNA synthesis when unirradiated or in the presence of NMN (Fig. 6A and B). The lack of NMN-stimulated repair incorporation in uvrC polA+ toluene-treated cells (cf. Fig. 5D) leads to the conclusion that in polA+ cells incision is not the only prerequisite for efficient repair synthesis, and that a subsequent step is defective in uvrC mutants.

Defective excision in uvrC mutants. Toluene treatment does not interfere with the production and recovery of dimers, but the extent of excision is less than in vivo. The excision reaction in vitro requires ATP and the uvrA,B gene products (5). We studied the rate and extent of excision of dimers in toluene-treated uvrC cells. Cells were highly labeled with [3H]thymidine during growth, harvested, toluene treated, and irradiated at 40 J/m² as described in Materials and Methods. The excision reactions were
FIG. 6. Isopycnic analysis of DNA synthesis in vitro in N17-7 (uvrC polA+). Experimental conditions were the same as described in the legend to Fig. 5. (A) No UV; (B) no UV, +NMN; (C) UV (80 J/m²); (D) UV, + NMN. Symbols: (●) 32P-labeled newly synthesized DNA; (○) 3H-prelabeled DNA.

done in the presence of dNTP's so that simultaneously occurring repair synthesis could enhance the excision of dimers (6). To maximize the sites of incision and repair synthesis, we studied the excision of pyrimidine dimers in the presence or absence of NMN (5 mM). Figure 7 shows typical results for excision of pyrimidine dimers in wild-type cells and uvrC mutants. Under the conditions employed a 30% removal of dimers was observed. The removal of dimers in W3623 (polA+) was rapid and occurred within 5 min (Fig. 7A). No excision occurred in the absence of ATP (data not shown). NMN did not appreciably affect either the rate or extent of the removal of thymine dimers in polA+ cells (Fig. 7A). Strain MM383 (polA12) showed a slower excision of dimers during 5 min, although to the same extent as observed in polA+ cells (Fig. 7B). This is in agreement with past observations (25). No excision was detected in uvrC polA+ (Fig. 7A) or uvrC polA12 (Fig. 7B) cells whether NMN was present or not. Nonspecific degradation of the DNA of irradiated cells was also followed for each reaction mixture (by acid precipitation), and our results showed no appreciable degradation in any cells (data not shown). These results demonstrate that excision in vitro requires the uvrC gene function. The failure of excision in the presence of NMN indicates that a lack of incisions in uvrC mutants is not the cause for this defect, since incisions accumulate in NMN.

Reformation of high-molecular-weight DNA in uvrC mutants. Our observation that uvrC mutants are defective in excision of pyrimidine dimers raised the question of whether they might provide a different structural environment for the restoration of high-molecular-weight DNA from wild-type cells. To investigate whether the incisions accumulated in uvrC mutants (in the presence of ATP and NMN but in the absence of dNTP's) can be rejoined or not, the cells with incised DNA were incubated in a second reaction in the presence of NAD and dNTP's. Figure 8 shows the reformation of high-molecular-weight DNA in toluene-treated uvrC
cells and wild-type cells. Irradiated, toluene-treated cells with \(^3\)H-labeled DNA were incubated in a reaction mixture (no dNTP’s) for 15 min to accumulate incisions. NMN was removed, and the cells were reincubated in complete reaction mixtures containing dNTP’s and NAD. It is evident from the data presented in Fig. 8A that, after incision accumulation, incubation of toluene-treated poLA\(^+\) cells in the presence of NAD and dNTP’s resulted in the rapid restoration of high-molecular-weight DNA. In contrast, a very poor reformation was observed in poLA12 cells after incision accumulation (Fig. 8B), agreeing with previous reports (6, 19). The \(wurC\) poLA\(^+\) cells showed efficient restoration of high molecular weight after incision accumulation (Fig. 8C), and the \(wurC\) poLA12 cells showed better reformation of high-molecular-weight DNA (Fig. 8D) than poLA12 cells (Fig. 8B). These results indicate that proper terminus configuration for ligation persists in the DNA of irradiated \(wurC\) mutants after 15 min of incision accumulation.

**DISCUSSION**

The results presented in this paper show that (i) the incision process is intact in vitro in \(wurC\) mutants; (ii) the \(wurC\) mutants are defective in the excision of pyrimidine dimers in vitro; and (iii) we cannot detect any repair synthesis in \(wurC\) poLA\(^+\) cells. The conclusion that the incision process is intact in \(wurC\) mutants is based on the findings that ATP-dependent incisions occur normally in \(wurC\) poLA\(^+\) cells in the presence of NMN and in \(wurC\) poLA12 cells with or without NMN. Our results agree with previous in vivo findings of incisions in DNA of irradiated \(wurC\) cells (13). In contrast, results obtained by other investigators using the sucrose-plasmolyzed in vitro cell system indicated a lack of incisions in \(wurC\) mutants (35). The requirement for gene functions \(urrA\), \(urrB\), and \(wurC\) in the form of a multienzyme complex to catalyze incision has been suggested using a cell extract complementation assay (32, 33). However, our results demonstrate the occurrence of incisions in toluene-treated \(wurC\) mutants, indicating the presence of an active incision process. It is interesting that, in the absence of NMN, no incisions could be detected in \(wurC\) poLA\(^+\) cells, in contrast to wild-type cells. This failure to detect incisions could be due to resealing of the breaks. The possibility of premature ligation of incisions by polynucleotide ligase in \(wurC\) mutants has been investigated using \(wurC\) lig-7(Ts) double mutants in vivo (34). These studies led to the conclusion that the \(wurC\) gene controls or interferes with ligase activity, although no incisions were seen in \(wurC\) poLA12 cells. Our studies on reformation of high molecular weight in \(wurC\) mutants (Fig. 8) showed that termini are still available for ligation even at a late time after incision. We did not find any evidence for super-ligation. On the contrary, the appearance of incisions in \(wurC\) poLA12 cells in the absence of NMN would seem to argue against rapid ligation.

Our results of defective excision of pyrimidine dimers in \(wurC\) mutants agree with the in vivo findings of other investigators (3, 12, 13, 36). We conclude that the failure of excision in vitro is not secondary to a failure of incision, in contrast to the conclusions of some. For the experiments
reported here we added NMN, which would maximize the accumulation of incisions and thereby repair synthesis, favoring excision (6). However, we failed to detect excision of pyrimidine dimers in uvrC mutants. Where dimers are not excised, ligase would have opportunity to seal the incision, masking the occurrence of incisions. The UV endonuclease of E. coli leaves termini susceptible to ligase action (Braun and Grossman, Fed. Proc. 33:1599, 1974). We propose that the event of resealing at the site of incision occurs secondarily to the failure of excision of pyrimidine dimers in uvrC mutants.

Results presented in Fig. 4 and 6 suggest that the lack of NMN-stimulated repair synthesis in uvrC polA+ cells could be accounted for by a defect in excision of pyrimidine dimers. The accumulation of incisions in the presence of NMN with no repair synthesis in uvrC polA+ cells argues that repair synthesis in polA+ cells requires not only incisions but other events. One proposition is that the failure in the excision of
dimers in uvrC polA* cells may block the nick translation process, resulting in a lack of repair synthesis (8, 14). Another possibility is that the defect in uvrC mutants may affect the strand displacement or local denaturation of the nucleotides containing pyrimidine dimers, restricting repair incorporation, although DNA polymerase I has been shown to perform strand displacement by itself (11, 18). Another possibility suggested by Kato (13) is that the uvrC gene function may be required in terminus modification to give a suitable configuration (3'-hydroxyl) for DNA polymerase. However, the known UV-specific endonucleases have been shown to induce breaks with 3'- hydroxyl termini (10). Recently, it has been reported that DNA polymerase I-dependent, NMN-stimulated repair synthesis is blocked in tolune-treated uvrC mutants (1, 39). This result was explained as being secondary to a lack of incision in uvrC polA* mutants. On the other hand, we observed the accumulation of incisions in uvrC polA* mutants in the presence of NMN. Thus the absence of NMN-stimulated repair synthesis in uvrC polA* cells could be due to a failure in nick translation or strand displacement resulting from the defect in excision of dimers.

The observation that uvrC polA12 mutants exhibited the same levels of repair synthesis as observed in polA12 cells is in contrast with the results of repair synthesis in uvrC polA* cells. One possibility allowing such synthesis could be degradation in the 3'-5' direction from the incision. This would be compatible with the ease of detection of incisions in uvrC polA12. Presumably the lack of DNA polymerase I for efficient gap closure would antagonize ligation. The defect in incision of dimers would be a limiting factor for the degradation in the 5'-3' direction; such a situation might favor religation under the conditions of repair synthesis and gap closure. This is in agreement with our observation of somewhat better reformation of high-molecular-weight DNA in uvrC polA12 cells as compared with that in parental polA12 cells. Furthermore, the uvrC polA12 cells also exhibit a defect in excision of pyrimidine dimers even in the presence of greatly increased repair synthesis, suggesting that in polA1 cells repair synthesis and excision of pyrimidine dimers may not be performed in a coupled manner.

The evidence reported in this paper suggests that uvrC gene function may not control the incision process or modulate ligase activity. The failure of excision of pyrimidine dimers in uvrC mutants, however, suggests that the uvrC gene product may be a pre-excision function, such as an unwinding protein, or an excision nuclease, or regulate the activities of the excision nucleases.

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