Role of DNA Polymerases in Excision Repair in *Escherichia coli*§

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The excision repair process following ultraviolet irradiation has been fractionated into its individual steps in toluene-treated cells. Incision can be examined in *vitro* independently of other reactions by omission of the deoxyribonucleoside triphosphates which prohibits repair synthesis and causes incisions to accumulate. Incision requires ATP, continues from 10 to 15 min at 37°C, and is specific for the excision repair pathway in toluene-treated cells. The excision of pyrimidine dimers in strains containing DNA polymerase I is rapid when all components are present and results in 30 to 40% excision in the first 5 min. When the deoxyribonucleoside triphosphates are omitted, the excision rate, but not extent, is much reduced. This pattern of excision is comparable to that observed in intact cells deficient in DNA polymerase I. Neither DNA polymerase II nor III appears to influence the rate of dimer removal. The requirement for repair synthesis in excision repair has been evaluated by the addition of the deoxyribonucleoside triphosphates subsequent to incision accumulation, thus allowing repair synthesis and ligation to return the DNA to its original size. The reformation of the DNA to high molecular weight is rapid and nearly complete by 2 min in cells containing DNA polymerase I. The reformation is slower and less complete in the absence of DNA polymerase I. This slower reformation is apparently catalyzed by DNA polymerase III as synthesis is observed in a mutant lacking both DNA polymerase I and II.

Ultraviolet (UV) radiation causes the formation of dimers between adjacent pyrimidines in DNA (1). Excision repair is one mechanism whereby this damage is removed and corrected in the DNA (2, 3). This repair pathway is characterized genetically in *Escherichia coli* by the *uvr* mutants (4, 5). At the minimum, the pathway must have individual steps of incision near the site of the UV damage, excision of the damage, insertion of correct nucleotides by repair replication, and ligation of these new nucleotides to the old strand. We have attempted to manipulate this process in order to gain a clearer understanding of these individual steps. We have utilized toluene-treated cells for this study (6-10). This system has been useful in demonstrating the involvement of various DNA polymerases in repair (6, 10, 11), the requirement for ATP during repair synthesis (6, 11-13), and the requirement for ATP in UV-specific incision (7, 14).

Incision is difficult to study in live cells as an independent event because the nicks are rapidly repaired. However, incision can be followed independently of subsequent repair events *in vitro* by omitting the deoxyribonucleoside triphosphates (dNTPs) to prohibit repair replication. Since repair replication is blocked, the incisions can not be repaired and thus accumulate during the postirradiation incubation. Incision can be studied in toluene-treated cells by following changes in the size of the DNA on alkaline sucrose gradients. Methods that block ligase such as NMN addition (15) or the use of a ligase mutant (16) also allow incision study (17, 18). The advantage of allowing incision accumulation by dNTP starvation rather than the introduction of inhibitors is that it is not necessary to remove the inhibitor for the study of events subsequent to incision.

We have examined the influence of repair synthesis on the excision and reformation of high molecular weight DNA following incision accumulation. This was done by utilizing cells deficient in the different polymerases and by manipulation of the dNTPs. Dimer excision requires ATP (9) and proceeds in the absence of repair synthesis (dNTPs), but at a rate simulating polA excision (9). The reformation experiments indicate that the repair synthesis mediated by DNA polymerase I leads to efficient ligation and a rapid reformation of intact DNA strands. Reformation in the absence of DNA polymerase I is slow. In strains lacking both DNA polymerase I and II, reformation is observed as a result of repair synthesis by DNA polymerase III. These data suggest that there are two alternatives for repair synthesis and reformation following incision.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth** - The following strains of *Escherichia coli* have been used:

* E. coli K12 (wild type)
* E. coli B (polA)
* E. coli B (polA-)
* E. coli B (polA, polB)

These strains were obtained from the American Type Culture Collection (Rockville, Md.).

* DNA polymerase I ( terminal activity from calf thymus)*
* DNA polymerase II ( terminal activity from calf thymus)*
* DNA polymerase III ( terminal activity from calf thymus)*

These enzymes were obtained from Boehringer-Mannheim (Mannheim, Germany).

* alkaline sucrose gradient centrifugation*

This technique was used to determine the size of the DNA. The DNA was isolated from the cells and run on alkaline sucrose gradients.

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* The abbreviation used is: dNTPs, a mixture of the four deoxyribonucleoside 5'-triphosphates.

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In Vitro DNA Repair

richia coli K-12 were used: W3110 (polA-) (19), P3478 (polA+) (20), HMS88 (polA-, polB-) (21), HMS85 (polA+, polB-) (21), HMS434 (polA-, polB+, polC+) (12).

Cells were grown in L-broth (10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and 1 liter of water) supplemented with 20 ml of thymine and the DNA was labeled with tritiated thymidine (2 μCi/ml, 6 Ci/mmol). Cells which were to be used for dimer analysis received a second supplement of tritiated thymidine after 30 min of growth.

Toluene Treatment—Cells were toluene treated as previously described (22). Fifty-milliliter cultures were harvested at a cell density of 0.5 to 1 x 10^9 cells/ml and resuspended in 0.05 M potassium phosphate buffer (pH 7.4). The cells were exposed to 1% toluene at room temperature for 5 to 10 min with slow stirring. After centrifugation the cells were resuspended at a concentration of 5 x 10^9 to 10^10 cells/ml in potassium phosphate buffer.

Irradiation and Assay for Repair Synthesis—The tolune-treated cells were exposed to radiation emitted largely at 254 nm from an unfiltered GE G8T5 lamp at a distance of 100 cm. Radiant energy was 6 J/m^2/min as measured by an ultraviolet intensity meter (Ultraviolet Products, Inc.) and resulted in the expected pyrimidine dimer production (9). A thin layer of cells was gently agitated on a rotating platform shaker at 4° during irradiation.

Following irradiation, cells were incubated in 0.15-ml reaction mixtures containing 66 mM potassium phosphate buffer (pH 7.4), 13 mM MgCl2, 1.3 mM ATP, and 33 μM each of the four deoxyribonucleoside triphosphates when added.

Thymine Dimer Analysis—After trichloroacetic acid precipitation, the acid-insoluble fraction of irradiated and unirradiated tolune-treated cells was hydrolyzed with 99% formic acid and subjected to two-dimensional chromatography as described earlier (9). The photoproducts analyzed were presumed to be dimers of thymine-thymine and thymine-cytosine.

Alkaline Sucrose Gradient Sedimentation—The reactions were terminated by placing on ice and the addition of 0.2 M Tris (free base) and 0.04 M EDTA. Fifty microliters of the reaction mixture was loaded onto an alkaline sucrose gradient, essentially as described by McGrath and Williams (23). The gradients (4.8 ml) were 5 to 20% (w/v) sucrose in 0.3 M NaOH, 0.7 M NaCl, 4 mM EDTA, and overlayed with a lysing solution (0.2 ml) containing 0.5 M NaOH, 0.5 M NaCl, 1% Sarkosyl, and 4 mM EDTA. After 20 min at room temperature, the gradients were centrifuged for 2 h at 25,000 rpm in an SW 50.1 rotor at 20°. The tubes were punctured in the bottom and 7 drop fractions collected on a continuous paper strip (24). The paper strips were washed in 10% trichloroacetic acid, 0.1 M NaOH, and overlayed with a lysing solution (0.2 ml) containing 0.5 M NaOH, 0.5 M NaCl, 1% Sarkosyl, and 4 mM EDTA. After drying the strips were cut, placed into scintillation vials, and counted in a liquid scintillation counter. The results are expressed as percentage of recovered counts per fraction. The gradients usually contained 10 to 50% total input and recovery averaged 95%. The number-average molecular weight calculations were performed according to Rupp and Howard-Flanders (25) using 4X-174 RFI DNA as reference.

RESULTS

Incision—The first step in excision repair appears to be the introduction of a break in the phosphodiester backbone of the DNA near the pyrimidine dimer. In polA- cells, it has been shown that there is a higher persistent level of breaks in the DNA during repair than in polA+ cells (26-28). In tolune-treated cells, it is possible to block repair synthesis by omitting dNTPs from the reaction mixture. When tolune-treated cells are irradiated and incubated in reaction mixtures lacking dNTPs and then centrifuged on alkaline sucrose gradients, the breaks in the DNA accumulate with time (Fig. 1). DNA from unirradiated cells is unaffected by these conditions. DNA incision accumulate rapidly for up to 15 min. Comparison of strains which are isogenic except for the polA locus suggests an apparently faster rate and greater extent of incision in the polA- strains (Fig. 1). The ATP dependence of incision is demonstrated for both strains but the DNA of the irradiated polA- cells undergoes a low level of ATP-independent breakage. This breakage is not the result of UV-specific incisions as unirradiated polA- controls also show a slow ATP-independent decrease in the size of the DNA during comparable incubations (data not shown).

The number-average molecular weight of the irradiated sample incubated with ATP indicates approximately a 100-fold change from intact strands. From the dose given of 30 J/m^2, and the estimate of 50 to 60 dimers produced/genome/J/m^2 (25), there should be approximately 600 to 900 dimers/ DNA strand. Thus, the molecular weight change suggests that incision is taking place at approximately 10 to 15% of the sites in the DNA. Tolune-treated uvrA- or uvrB- cells exhibit a lack of incision when incubated under the strict conditions employed above (7, 14), demonstrating that the ATP-dependent incision observed in tolune-treated cells is dependent upon the excision repair pathway.

Excision—We have shown that the removal of dimers from irradiated DNA requires ATP in tolune-treated cells (9). Excision does not require repair synthesis but in polA+ cells there is a marked effect on the rate of excision when dNTPs are absent. This reduced rate is similar to that seen in polA- cells (Fig. 2). This suggests that in tolune-treated cells, repair synthesis may drive dimer excision and that synthesis and excision are coupled in polA+ cells. The dNTP effect on the rate of excision is consistent with the stimulation of the 5’
3’ exonuclease activity by dNTPs or synthesis for purified DNA polymerase I (29, 30).

Influence of DNA Polymerases on Excision of Dimers — We have measured the influence of the DNA polymerases on excision in vivo. Our results indicate two distinguishable rates of excision (Fig. 3). The faster rate requires synthesis by DNA polymerase I (Refs. 9 and 31 and Fig. 2). In polB- strains deficient in DNA polymerase II, the patterns of excision (polA+ or polA-) are identical with those in polB+ strains. These data indicate that DNA polymerase II is not critical for either fast or slow dimer excision. To examine the influence of DNA polymerase III, it was necessary to use conditional mutants as the polC gene function is required for survival. Under the nonpermissive condition, the polC defect does not alter the pattern of excision seen in the polA strain. The loss of DNA polymerase II function again does not affect the rate. These data suggest that DNA polymerase I alone can interact in the excision of dimers.

Influence of DNA Polymerases on Reformation — Following incision accumulation subsequent addition of dNTPs allows repair synthesis to proceed and if the appropriate configuration exists in the repaired DNA, then ligation will result in the reformation of high molecular weight DNA. The influence of DNA polymerase I on repair synthesis is examined in Fig. 4. The data in the left panel for the polA+ strain demonstrate that after the incision period, the addition of dNTPs results in rapid reformation. Longer incubation results in a profile which is close to that obtained from unirradiated cells carried through the same protocol. In the polA- cells, there is a much slower and less complete reformation with only a small proportion of the DNA achieving a molecular weight similar to the unirradiated control. It is important to keep in mind that it is the parental DNA which is followed in this experiment and not newly incorporated repair synthesis. The amount of synthesis during the postirradiation incubations is insufficient to account for the production of high molecular weight material de novo; rather, the labeled parental strands must be joined as a result of the repair synthesis and ligation.

The participation of DNA polymerase II in excision repair was examined in polB- cells (Fig. 5). In a polB- strain with an active DNA polymerase I, the reformation is fast and

![Fig. 2. Influence of deoxynucleosides on dimer excision rate.](image)

PRELABELED TOLENE-TREATED CELLS WERE EXPOSED TO 42 J/m² UV dose and incubated in reaction mixtures containing ATP and 33 μM each of the dNTPs when present. The reactions were stopped at various times by the addition of trichloroacetic acid and the amount of dimer content determined by thin layer chromatography as described under "Materials and Methods." ○, W3110, wild type strain with dNTPs; △, W3110 without dNTPs; □, P3478, deficient in DNA polymerase I, plus or minus dNTPs.

![Fig. 3. Influence of DNA polymerase on dimer excision in vivo.](image)

EXPERIMENT AS IN FIG. 2, EXCEPT CELLS WERE NOT TOLENE TREATED; FOLLOWING UV EXPOSURE THE CELLS WERE PLACED IN MEDIA AND INCUBATED AT 37° C EXCEPT FOR THE POLC STRAINS WHICH WERE INCUBATED AT 42° C. ○, W3110; △, P3478; ▲, HMS83; △, HMS85; ■, HS434; □, HS432.
FIG. 5. Influence of DNA polymerase II on reformation of high molecular weight DNA after incision accumulation. The results with HMS85, deficient in DNA polymerase II but containing DNA polymerase I, are to the left and HMS83, deficient in both DNA polymerase I and II, to the right. Experiment was as in Fig. 4. O, 10 min without dNTPs; •, dNTPs added for 2 min; △, dNTPs added for 20 min; Δ, unirradiated cells 10 min without dNTPs followed by 20 min with dNTPs.

complete as in other polA+ strains (left panel). This indicates that DNA polymerase II is not required for fast reformation. This is consistent with the lack of UV sensitivity observed for this strain (21). In a strain deficient in both DNA polymerases I and II, a slow and less complete reformation is observed, as in Fig. 4. These data suggest that DNA polymerase III may be responsible for the low level of reformation that is seen in the absence of DNA polymerase I.

DISCUSSION

We have fractionated the multistep process of excision repair into individual reactions (Fig. 6). The incision reaction is a consequence of the UV irradiation and requires ATP but neither dNTPs nor repair synthesis. Incisions accumulate at 37° for approximately 15 min in vitro. This time period is the same as that seen for excision of pyrimidine dimers (9) and incorporation during repair synthesis (6). In irradiated polA- cells, there is a slow rate of strand breakage in the absence of ATP (Fig. 1) which is also present in unirradiated control cells. The lower stability of the DNA in polA- cells may account for an indication that incision was ATP-independent since DNA polymerase I-deficient cells were used in an earlier study (17). We have demonstrated that the low level of repair synthesis seen in the absence of ATP does not vary appreciably with UV dose (32).

The number of breaks introduced into the DNA is less than the number of pyrimidine dimers produced by the given doses. We observe incisions at 10 to 15% of the dimer sites. This value is less than the 30% observed by Ganesan (33) for in vitro incision with T4 UV endonuclease in Brij-treated cells but about the same as values observed by Waldstein et al. (14). We observe approximately 30% excision in vivo under the same conditions.

The excision of pyrimidine dimers from irradiated DNA does not require either dNTPs or repair synthesis. However, the excision rate in cells that contain DNA polymerase I is stimulated by simultaneous repair synthesis. The 5' → 3' exonuclease activity of DNA polymerase I is also stimulated by simultaneous polymerization (29, 30). We have also shown a requirement for the 5' → 3' exonuclease function of DNA polymerase I for fast excision in the presence of dNTPs (9). These data taken together suggest the fast excision in the presence of simultaneous repair synthesis is catalyzed by DNA polymerase I, probably by a nick translation mechanism. DNA polymerase I is suited to participate in an efficient
repair as it has been shown in vitro to be capable of dimer excision (29, 34), and it can carry out nick translation synthesis in which both the polymerizing and 5'→3' exonucleolytic functions are simultaneously active (35, 36). The absence of excision stimulation by dNTPs in poLA strains might indicate that removal of dimers and polymerization are uncoupled, since these cells contain nearly normal levels of the 5'→3' exonuclease activity of DNA polymerase I.

We reported earlier that toluene-treated wild type cells maintain their DNA as a high molecular weight form following irradiation when incubated in a complete reaction mixture (37). For this to occur following incision, the steps of excision and repair synthesis that require a nick in the DNA must be completed rapidly so that ligation will limit the number of breaks present in the DNA at any one time. That is, the incisions are repaired and resealed before they can accumulate. DNA polymerase I-mediated repair, resulting in breaks remaining in the DNA for the shortest time, is consistent with the observation that a small number of nucleotides are inserted into the damage site in polA+ cells (38) and with low levels of synthesis in vitro (32). The rapid reformation and small patch size in polA+ cells indicate an effective interaction with the observation that a small number of nucleotides are repair incorporation (6) which is dependent on the has also been shown to be responsible for postirradiation breaks present in the DNA at any one time. That is, the breaks present in the DNA at any one time. That is, the nuclease is responsible for dimer excision in DNA polymerase I. Since the repair of these lesions is independent of the presence of DNA polymerase III, it is concluded that excision by DNA polymerase I is not rate-limiting for the complete process (8). This is consistent with the lack of stimulation by dNTPs in poLA+ cells. At this point, it is not evident what nuclease is responsible for dimer excision in poLA strains.

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