Excision of Pyrimidine Dimers in Toluene-Treated
Escherichia coli

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Toluene-treated cells were used for examining excision of pyrimidine dimers in Escherichia coli strains W3110, DM845 (uvrA−), P3478 (polA−), and KS5064 (polAexi). Excision occurring in toluene-treated cells is rapid, adenosine 5′-triphosphate dependent, and requires the uvrA gene function. In strains lacking either the polymerizing or 5′→3′ exonucleolytic activity of deoxyribonucleic acid polymerase I, excision does occur. However, both in vivo and in vitro, the excision in such strains is initially slower than wild type.

The excision of pyrimidine dimers, produced by exposure to ultraviolet (UV) irradiation, requires an orchestrated response of several enzymes. Current information supports a model which involves a UV-specific endonucleolytic incision made on the 5′ side of the pyrimidine dimer. This is followed by the excision of an oligonucleotide containing the dimer. Correct nucleotides are inserted in the repair region by a polymerase, using the opposite intact strand as a template. Polynucleotide ligase then seals the remaining nick.

Mutants substantially more sensitive than wild type to UV have been shown to be frequently associated with a defect in one of the obligatory steps of excision repair. The Escherichia coli uvrA and uvrB mutants do not incise their deoxyribonucleic acid (DNA) after irradiation (11), and uvrA has been shown to be defective in an endonucleolytic activity (4). In contrast, the uvrC mutant does carry out incision, at a very slow rate, but excision of dimers is not observed (12). UV-sensitive mutants of Micrococcus luteus (15) and mutant T4v of bacteriophage T4 (21) have been shown to lack endonucleases specific for UV-irradiated DNA. Mutants lacking DNA polymerase I are also UV sensitive and have a lowered rate of dimer excision (2, 7, 18). In addition, from studies using purified enzyme, DNA polymerase I has been implicated in the excision and resynthesis steps (13).

The discrete processes of excision repair have remained obscure, partly due to the inability to monitor the substrates and separate the individual steps of incision, excision, repair synthesis, and ligation. Various in vitro systems have been developed to overcome the above obstacles. One such system, toluene treatment, has provided a permeable system for investigations on replicative and repair synthesis (1, 17, 19). Using toluene-treated cells, it has been shown that the incision step is rapid and is adenosine 5′-triphosphate (ATP) dependent (J. W. Dorson and R. E. Moses, Fed. Proc., p. 1599, 1974; 20).

In this paper we report that excision of pyrimidine dimers takes place in toluene-treated cells, and this excision is in response to levels of pyrimidine dimers compatible with data observed in other systems. Excision is rapid, requires ATP, and is absent in the UV-sensitive uvrA mutant. The results with mutants deficient in polymerizing or 5′→3′ exonuclease activity of DNA polymerase I were also investigated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. E. coli K-12 strains W3110 and P3478 (polAI), a DNA polymerase I-deficient mutant (5), were used. Strain KS5062 (polAexi), a mutant temperature sensitive for the 5′→3′ exonuclease function of DNA polymerase I, was obtained from B. Konrad (14). DM845 (uvrA−), a K-12 strain containing the uvrA6 mutation (10), was obtained from D. Mount (10).

Bacteria were routinely diluted from overnight cultures and grown at 37°C with shaking (32°C for polAexi strain) in M9 medium containing, per liter of water: Na2HPO4·7H2O, 6.0 g; KH2PO4, 3.0 g; NaCl, 0.5 g; NH4Cl, 1.0 g; MgSO4·7H2O, 1 mmol; CaCl2, 0.1 mmol; glucose, 4 g supplemented with 2.0 μg of vitamin-free Casamino Acids per ml, 100 μg of deoxyadenosine per ml, and 1.0 μg of thymine per ml. Cells were labeled for three generations with [3H]thymidine (Schwarz/Mann, Div. of Becton, Dickison & Co., Orangeburg, N.Y.). The label utilized in all cases had been refined by one-dimensional, thin-layer chromatography in butanol-water (86:14).

Toluene treatment. Exponentially growing cells
were harvested at concentrations of $5 \times 10^8$/ml of culture, toluene treated as previously described (19), washed free of toluene, and resuspended in 50 mM KPO$_4$, pH 7.4, buffer.

**UV irradiation.** For in vivo studies, cells were resuspended in M9 salts, without glucose, at a concentration of $10^{10}$ cells/ml. For in vitro studies, cells were resuspended at the above concentration in 50 mM KPO$_4$, buffer, pH 7.4. Cell suspensions (2.5 ml by volume in a 10-cm diameter glass petri dish at 4°C) were then placed on a platform shaker rotating at 120 rpm and exposed to radiation largely at 254 nm from an unfiltered GE G8T5 lamp at a distance of 100 cm. A dose rate of 1 erg/mm$^2$ per s at the surface was measured by a UV intensity meter (Ultra-Violet Products, Inc., San Gabriel, Calif.). All subsequent steps were performed in subdued light or in the dark.

**Incubations for in vivo repair.** Irradiated cells were added to prewarmed M9 medium (containing glucose and 100 μg of deoxyadenosine and 1 μg of thymine per ml) at a concentration of $10^8$ cells/ml. At all times during incubation the cell suspension was shielded from the light.

**Incubations for in vitro repair.** Incubation mixtures (0.3 ml) contained 70 mM KPO$_4$, pH 7.4; 13 mM MgCl$_2$; 1.3 mM ATP; 33 μM deoxythymidine 5’-triphosphate, deoxyguanosine 5’-triphosphate, deoxyadenosine 5’-triphosphate, and deoxycytidine 5’-triphosphate; and $10^8$ toluene-treated, irradiated cells. Reactions were initiated by addition of cells and terminated by addition of 1 ml of cold 10% trichloroacetic acid-0.1 M pyrophosphate.

**Thymine dimer analysis.** After trichloroacetic acid precipitation, the acid-insoluble fraction of irradiated and nonirradiated toluene-treated cells were hydrolyzed with 98% formic acid and subjected to two-dimensional chromatography, as described by Goldmann and Friedberg (8). The photoproducts analyzed were presumed to constitute dimers of thymine-thymine and thymine-cytosine. For reference, the dimers produced at 300 ergs/mm$^2$ yielded from 110 to 150 counts/min above the counts per minute obtained from a nonirradiated control (from 20 to 40 counts/min for each region of the second dimension analyzed). Values for counts per minute in thymine were maintained at 150,000 to 200,000.

**RESULTS**

**Production of pyrimidine dimers in toluene-treated cells.** To demonstrate that toluene treatment does not interfere with the production and recovery of dimers, wild-type strain W3110 was toluene treated and exposed to varying doses of UV. The production of pyrimidine dimers is linear up to doses of 1,000 ergs/mm$^2$ (Fig. 1). The percentages of pyrimidine dimers in relation to UV dose corresponds to 6 dimers formed per erg for the genome of W3110. Expected and observed values for production per erg in _E. coli_ have been reported between 5 and 6 (3, 10).

**Excision of pyrimidine dimers in vitro.**

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**Fig. 1.** The production of pyrimidine dimers at different doses of UV. Strain W3110 was toluene treated and exposed to UV at the indicated doses. Values reported are calculated as a ratio of counts per minute in dimers to total counts per minute in thymine. The counts per minute for total thymine at each dose reported was from 150,000 to 200,000 counts/min. A nonirradiated control was routinely run and subtracted from values obtained for dimers in irradiated samples.

Dimer content was determined after cells were toluene treated, exposed to UV, and incubated in the dark for various periods of time. Incubation mixtures contained deoxynucleoside triphosphates so that repair synthesis could occur. In vitro excision of pyrimidine dimers occurs only when incubation mixtures contain ATP (Fig. 2). Repair synthesis is known to be ATP dependent in toluene-treated cells (1, 17).

The in vitro removal of dimers in W3110 (Fig. 2 and 3) is rapid, with the most pronounced effect in the first 5 min. Thereafter, only a slight change in dimer content is observed. The data represent an initial 1,800 dimers produced by exposure to UV, with approximately 1,200 dimers remaining after 30 min of dark repair or, as seen in Fig. 3, 30% removal of dimers from the acid-insoluble fraction.

Seventy percent of the dimers consistently remain in the acid-insoluble fraction in wild-type cells, even with conditions of higher ATP concentration and UV doses up to 2,000 ergs. Thus, under our experimental conditions, W3110 appears to have a limited extent of
Figure 2. ATP dependence of in vitro dimer excision. Toluene-treated W3110 were irradiated (300 ergs/mm²) and incubated at 37°C in the presence (1.3 mM) or absence of ATP.

Figure 3. In vitro dimer excision for strains W3110 (O), DM845 (●), P3478 (Δ), and KS5064 (▲). The individual strains were toluene treated, irradiated at 300 ergs/mm², and incubated in complete reaction mixtures at 37°C (42°C in the case of KS5064).

repair in vitro, in which 30% of the dimers are removed rapidly. A residual dimer content is also found when W3110 is incubated in vivo (Fig. 4), although the extent of removal is greater.

Lack of excision of pyrimidine dimers in a uvrA⁻ strain. uvrA mutants are defective in incision and, since the excision pathway is sequential, excision of dimers should not occur. Figure 3 shows that in toluene-treated and irradiated DM845 (uvrA⁻) excision was not detected. This is consistent with in vivo observations (Fig. 4). These results indicate that the excision of dimers is repair pathway dependent. Moreover, the removal of dimers by nonspecific functions is undetectable. The in vitro results agree with the in vivo results in this respect.

Participation of DNA polymerase I in excision. It is possible to examine the role of DNA polymerase I in excision repair by use of the polA1 mutant, which displays intermediate UV sensitivity. This property of the mutant, as well as the demonstrated ability of the enzyme to remove dimers, suggests that DNA polymerase I may play a role in excision repair. The polA1 mutant (P3478) has lost the polymerization function but retains the 5' → 3' exonucleolytic activity of DNA polymerase I (16). Another mutant has been identified which has polymerizing activity but a thermolabile 5' → 3' exonucleolytic function (14). Using these mutants, it is possible to examine the effect on excision of each activity of DNA polymerase I.

Strain P3478 (polA⁻) in the first 5 min exhibits a slower in vitro rate of excision than does wild type (Fig. 3). For strain KS5064 (polAex1) there is no detectable excision in the first 5 min of incubation at the restrictive temperature. Both mutants exhibit comparable levels of excision by 30 min. uvrA⁻ cells exhibit...
no observable excision at the restrictive temperature employed for KS5064.

The results in vivo again demonstrate a slower rate of dimer removal in the mutants defective in either of the DNA polymerase I functions (Fig. 4). It appears in vivo and in vitro that the polymerization, as well as the 5' → 3' exonucleolytic activity, must be present for a normal rate of dimer removal.

**DISCUSSION**

We have shown that excision of pyrimidine dimers occurs in toluene-treated cells and that it requires both ATP and the uvrA gene product. This is consistent with previous results in which it was shown that the incision step is dependent upon the presence of ATP and the uvrA gene product (J. W. Dorson, W. A. Deutsch, and R. E. Moses, Fed. Proc., p. 516, 1975; J. W. Dorson and R. E. Moses, Fed. Proc., p. 1599, 1974; 20). The absence of a functional uvrA gene product blocks the sequential multienzyme pattern of excision repair at incision, resulting in no excision. These results also indicate that toluene treatment does not introduce excision that is not repair pathway related.

A criterion for interpreting the in vitro removal of dimers is comparison with in vivo analyses. Work presented here and elsewhere shows that, for wild-type strains, the initial rate of dimer excision is rapid, followed by a slower or negligible rate of removal (2). In the case of a polA strain, the initial in vivo rate of dimer excision is slower than that of wild type, but proceeds for a longer period of time. A mutant lacking of 5' → 3' exonuclease function excises dimers at a slower rate than a polA strain. Both mutants ultimately approximate the excision seen in wild type. The conditional lethality of polA+ makes it difficult to assess UV sensitivity at the restrictive temperature in vivo, but dimer removal can be investigated. Quantitation of the dimer content in this strain is difficult at longer times due to apparent DNA degradation at the restrictive temperature.

Mutant defective in functions of DNA polymerase I can excise dimers in toluene-treated cells, much in the same manner as is seen in vivo. The decreased initial rate of excision is compatible with the decreased survival following UV irradiation. Because both mutants demonstrate a slower rate of dimer excision initially, both DNA polymerase I functions may be required in concert to achieve the initial rate of excision seen in wild type.

The pattern of in vitro excision for wild type is similar to the pattern of in vivo excision; a short period of excision is followed by no further excision. Efforts to increase the extent of dimer excision in vitro were unsuccessful. Seventy percent of the dimers remain unexcised even at UV doses up to 2,000 ergs/mm², although the absolute number of dimers removed is eight times greater than that removed at 300 ergs. Increasing the ATP concentration also does not increase the final extent of dimer removal. Data from other sources indicate that the in vitro removal of dimers is only partial, with the majority of dimers remaining in the acid-insoluble fraction. In the case of freeze-treated *Bacillus subtilis* (9), the preponderance of dimer removal occurs in the initial 15 min (31% removal at 45 min). In another case, Ganesan (6) found that incision only occurred at 33% of the expected dimer sites. She suggested several explanations for this observation, such as dimer clustering and sequestering of dimers by cellular components. Alternatively, it is possible that only 30% of the dimer sites are accessible to repair enzymes in an in vitro system.

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