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Kinetics of Pyrimidine(6-4)Pyrimidone Photoproduct Repair in *Escherichia coli*

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We compared the removal of pyrimidine(6-4)pyrimidone photoproducts [(6-4) photoproducts] and cyclobutane pyrimidine dimers (CPDs) from the genome of repair-proficient *Escherichia coli*, using monoclonal antibodies specific for each type of lesion. We found that (6-4) photoproducts were removed at a higher rate than CPDs in the first 30 min following a moderate UV dose (40 J/m^2). The difference in rates was less than that typically reported for cultured mammalian cells, in which the removal of (6-4) photoproducts is far more rapid than that of CPDs.

Irradiation of organisms with UV produces lesions in DNA which are genotoxic and mutagenic. UV is also a risk factor in the etiology of human skin cancers (reviewed in reference 17). The two primary lesions produced by UV are the *cis*,*syn*-cyclobutane pyrimidine dimer (CPD) and the pyrimidine(6-4)pyrimidone photoproduct, often simply called (6-4) photoproduct. Less frequent than CPDs, (6-4) photoproducts account for about one-quarter of the lesions produced in DNA by moderate doses of UV [for a review of the (6-4) photoproduct, see reference 23].

There is a large body of research concerning the induction, repair, and mutagenicity of CPDs in microorganisms and mammalian cells, while less is known about the (6-4) photoproduct because of greater technical difficulties in studying this lesion. Nevertheless, there is considerable evidence that the (6-4) photoproduct is a biologically relevant lesion, involved in both the toxic and the mutagenic effects of UV (23). Reported evidence that the (6-4) photoproduct may be one of the UV-induced lesions responsible for mutations in the human p53 gene leading to nonmelanoma skin cancer (2, 8, 38) has renewed interest in this photoproduct.

It has been known for a number of years that (6-4) photoproducts are removed from the total mammalian genome at a much higher rate than are CPDs (e.g., references 22 and 27). The induction of (6-4) photoproducts in mammalian chromatin is in fact nonrandom; lesions are formed in nucleosome linker DNA about six times more frequently than in nucleosome core DNA (24). Induction of CPDs, though modulated by DNA associations with core histone proteins, is much more uniform (10, 11). This has led to speculation that the faster repair of (6-4) photoproducts may be due in part to greater accessibility of nucleosome linker DNA to DNA repair enzymes. Additionally, in human cell extracts a (6-4) photoproduct stimulates repair synthesis in a defined substrate more readily than a CPD, which may indicate a greater affinity of the excision repair apparatus for (6-4) photoproducts (33).

We have examined the kinetics of removal of both (6-4) photoproducts (TC and TT) and CPDs (TT and CT) from the *Escherichia coli* genome, using monoclonal antibodies (30, 32). After a UV dose of 40 J/m^2, the repair rate of (6-4) photoproducts exceeded that of CPDs in the first 30 min following irradiation. This may be a result of a greater affinity of the Uvr(A)BC nucleotide excision repair complex for (6-4) photoproducts than for CPDs, which has been shown in vitro (31). After a dose of 200 J/m^2, the repair of (6-4) photoproducts was similar to that after 40 J/m^2 but the rate of removal of the more numerous CPDs was reduced. We consider the significance of these findings and discuss the differences and similarities between bacterial and mammalian photoproduct repair.

MATERIALS AND METHODS

E. coli K-12 strain SR108 (F^- λ^s *thyA deo*) was grown at 37°C in Davis minimal medium with 0.4% glucose, 0.05% casein hydrosylate, and 2 μg of thymine per ml (19, 29), irradiated with UV (primarily 254 nm, from a General Electric G15T8 germicidal bulb) in the medium at room temperature, and then incubated at 37°C in the same medium. Samples were removed for lysis (29) at various times, and DNA was purified by phenol-chloroform extraction and ethanol precipitation. No attempt was made to remove replicated DNA, since no significant replication occurs within 30 min after a UV dose of 40 J/m^2 (6).

In order to reduce its viscosity, purified *E. coli* DNA in 1 ml of Tris-EDTA buffer was lightly sonicated (30 s at power setting 2) with a Branson Sonifier 200 equipped with a microtip. DNA was then quantified by fluorometry with Hoechst 33258 (4), and 0.75 μg of DNA from each time point was loaded, in triplicate, onto a Hybond N^+ membrane (Amersham) by using a slot blot apparatus.

The membrane was probed with mouse monoclonal antibodies against TT and CT CPDs (TDM-2 [25]) or TC and TT (6-4) photoproducts (64M-2 [25]) as described elsewhere (30, 32). Primary-antibody binding was detected by using a secondary ^{35}S-labeled sheep anti-mouse immunoglobulin G antibody (Amersham). The radioactivity bound to the various DNA samples on the membrane was measured with a Bio-Rad model GS-363 phosphorimager and analyzed with the associated Molecular Analyst software.

Four complete, independent experiments were performed, three at a dose of 40 J/m^2 and one at a dose of 200 J/m^2. Data for triplicate DNA samples within each experiment were averaged, and the mean and standard error were calculated for the three experiments done at 40 J/m^2 [standard error = (standard deviation) $\cdot (\sqrt{n})^{-1}$].

RESULTS

Repair-proficient *E. coli* (K-12 strain SR108) cells were irradiated with UV and then incubated in the dark. Aliquots were taken at various times following irradiation (see Materials and Methods), and equal amounts of purified DNA from each time point were bound to a nylon membrane and probed for the presence of photoadducts (30, 32) with either of two monoclonal antibodies, one specific for TC and TT (6-4) photoproducts (64M-2 [25]) and the other specific for TT and CT CPDs (TDM-2 [25]). These primary antibodies were then detected with a ^{35}S-labeled secondary antibody.

Images of the radioactivity from two rows on one membrane are shown in Fig. 1. DNA repair is revealed by a reduction in the radioactive signal with time. Each experiment had three

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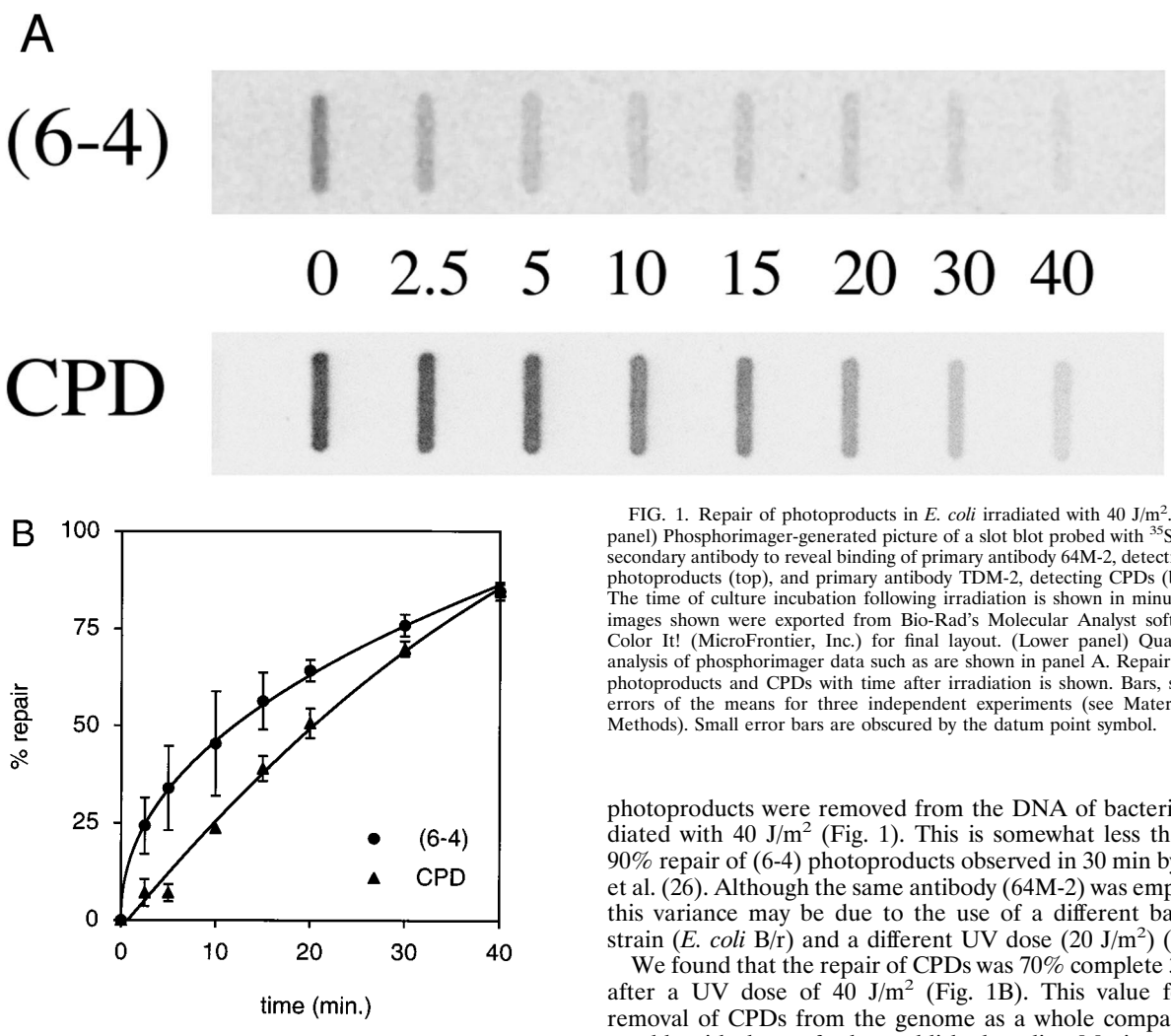

FIG. 1. Repair of photoproducts in *E. coli* irradiated with 40 J/m^2. (Upper panel) Phosphorimager-generated picture of a slot blot probed with ^{35}S-labeled secondary antibody to reveal binding of primary antibody 64M-2, detecting (6-4) photoproducts (top), and primary antibody TDM-2, detecting CPDs (bottom). The time of culture incubation following irradiation is shown in minutes. The images shown were exported from Bio-Rad's Molecular Analyst software to Color It! (MicroFrontier, Inc.) for final layout. (Lower panel) Quantitative analysis of phosphorimager data such as are shown in panel A. Repair of (6-4) photoproducts and CPDs with time after irradiation is shown. Bars, standard errors of the means for three independent experiments (see Materials and Methods). Small error bars are obscured by the datum point symbol.

such rows for each of the two primary-antibody probes (DNA was loaded in triplicate), whose signals were averaged in the quantitative analysis. DNA repair was calculated as the percentage of radioactive secondary-antibody signal lost relative to the signal at time zero. Unirradiated DNA loaded as a control for nonspecific antibody binding produced no signal significantly above background (not shown).

Three experiments starting with separate cultures were performed to examine the repair of (6-4) photoproducts and CPDs after a UV dose of 40 J/m^2 (Fig. 1B). The fraction of (6-4) photoproducts removed from the genome exceeded that of the CPDs at each time point up to 30 min, but by 40 min 85% of both lesions were repaired.

We also performed one experiment at a UV dose of 200 J/m^2 (Fig. 2). Increasing the UV dose fivefold had little effect on the repair of (6-4) photoproducts, perhaps reducing the rate slightly at times less than 10 min. However, the rate of repair of CPDs, as a percentage of starting lesions, was reduced at the higher dose (compare Fig. 2 with Fig. 1B).

DISCUSSION

We have used a monoclonal antibody-based method (25, 30, 32) to examine the kinetics of repair of (6-4) photoproducts and CPDs in the *E. coli* K-12 genome. In 30 min, 76% of (6-4) photoproducts were removed from the DNA of bacteria irradiated with 40 J/m^2 (Fig. 1). This is somewhat less than the 90% repair of (6-4) photoproducts observed in 30 min by Mori et al. (26). Although the same antibody (64M-2) was employed, this variance may be due to the use of a different bacterial strain (*E. coli* B/r) and a different UV dose (20 J/m^2) (26).

We found that the repair of CPDs was 70% complete 30 min after a UV dose of 40 J/m^2 (Fig. 1B). This value for the removal of CPDs from the genome as a whole compares favorably with those of other published studies. Mori et al. (26) found that 60% of CPDs were removed from a B/r strain 30 min after a UV dose of 20 J/m^2, as detected with the same antibody (TDM-2). With a dose of 20 J/m^2, Setlow and Carrier (28) found that *E. coli* B/r and *E. coli* B removed 65 and 80% of thymine-containing CPDs, respectively, in 30 min. For an *E. coli* K-12 strain irradiated with 40 J/m^2, Cooper (5) found about 80% repair of thymine-containing CPDs in 30 min. Although the dose was the same as that used in our study, the cells in Cooper's experiments were incubated at 42°C (instead of 37°C), which may account for the slightly higher repair rate. Even more rapid repair of CPDs has been observed at low UV doses. Ganesan and Hanawalt (12) used the enzyme T4 endonuclease V to incise bacterial DNA specifically at the sites of CPDs and found about 80% repair in 20 min in an *E. coli* K-12 strain irradiated with a dose of 2 J/m^2. Similarly, Green et al. (14) used *Micrococcus luteus* UV endonuclease to cleave DNA specifically at CPDs and found about 90% repair in 20 min in *E. coli* B/r irradiated with 3 J/m^2.

The closest comparison of our results can be made with the data reported by Mellon and Hanawalt (19), whose protocol for the growth, irradiation, and harvesting of bacteria was followed in our experiments. Indeed, the same bacterial strain (SR108), the same UV dose, and the same UV source were used. Mellon and Hanawalt employed T4 endonuclease V to measure the repair of CPDs in the transcribed and nontranscribed strands of the *lac* operon. Under conditions in which

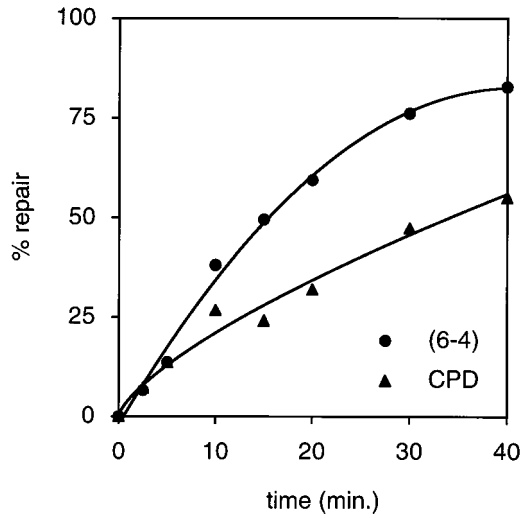

FIG. 2. Repair of (6-4) photoproducts and CPDs in *E. coli* with time after irradiation with 200 J/m^2.

expression of the operon was repressed, the repair of CPDs in the transcribed and nontranscribed strands of the operon was essentially identical to our results shown in Fig. 1, with about 8% repair attained in 5 min, 22% repair attained in 10 min, and 50% repair attained in 20 min (19). When the *lac* operon was derepressed with isopropyl-β-D-thiogalactoside (IPTG), repair of the transcribed strand increased dramatically, demonstrating the phenomenon known as transcription-coupled repair (15, 20), while repair in the nontranscribed strand remained similar to that in the repressed state. We conclude that the repair of CPDs in silent genes, or the nontranscribed strand of active genes, closely resembles the repair in the genome overall, mirroring the results seen for mammalian cells (for examples, see references 1, 18, and 20).

We performed one experiment at a UV dose of 200 J/m^2 (Fig. 2) in order to compare our data directly with the high-performance liquid chromatography studies of Franklin and Haseltine (9). While the kinetics for repair of (6-4) photoproducts was not significantly changed compared with that at the 40-J/m^2 dose, the removal of CPDs, as a percentage of starting lesions, was reduced. This may reflect a limiting of cellular resources needed to remove the more numerous CPD lesions from DNA at higher doses. Our measurement of 47% repair of CPDs at 30 min is somewhat lower than the 57% repair of thymine-containing CPDs observed by Franklin and Haseltine (9). Our measurements for (6-4) photoproduct repair at 200 J/m^2, however, differ quite significantly from those of Franklin and Haseltine (9). They isolated TC (6-4) photoproducts which, in addition to the minor TT (6-4) photoproduct, are detected by the 64M-2 antibody used in our experiments (25). They observed just 21% repair at 30 min and 25% repair at 60 min. With both 40 and 200 J/m^2, we observed very rapid repair of (6-4) photoproducts, exceeding 75% loss of starting lesions in 30 min (Fig. 1 and 2).

We consider our (6-4) photoproduct repair data at the higher UV dose to be more consistent with the known biology of *E. coli* than those of Franklin and Haseltine (9). In liquid holding experiments, in which plating efficiency is enhanced by holding the bacteria in minimal media after irradiation, Doudney and Haas (7) found that following a UV dose of 241 J/m^2, *E. coli* B began recovering in 30 min and that survival had increased 10-fold by 60 min. It was later discovered that liquid holding recovery (in the dark) is due to excision repair of UV-induced lesions (13). Since both (6-4) photoproducts and CPDs are toxic (23), we believe these liquid holding recovery data are more consistent with our observed rapid repair at 200 J/m^2 (Fig. 2).

Repair of (6-4) photoproducts in mammalian cells has been the subject of much investigation (for examples, see references 21, 22, 27, 34, and 36). These photoproducts are removed from the total mammalian genome at a vastly greater rate than are CPDs. Repair of (6-4) photoproducts in human cells reaches completion in 6 h (22), while repair of CPDs is 30 to 50% complete in 6 h (35) and 70 to 90% complete in 24 h (18, 35). Repair of (6-4) photoproducts in hamster cells reaches completion in 3 h (16, 27), but overall repair of CPDs is severely deficient (1, 35, 37).

One reason for this difference in human cells could be the affinity of the excision repair complex for different types of lesions. Szymkowski et al. (33) examined the repair replication which occurred when plasmids containing defined lesions were treated with a human cell extract. They found that repair synthesis was 10-fold greater in a plasmid containing a single TT (6-4) photoproduct than for one containing a TT *cis*,*syn*-CPD. Another factor involved in the rate difference could be in the pattern of induction of photoproducts in chromatin. CPDs are induced in nucleosome core and linker DNAs at roughly the same frequencies (24), although induction in the core region is affected by DNA-protein interactions with histones (10). (6-4) photoproducts, however, are reportedly induced sixfold more frequently in linker DNA than in core DNA (24). If linker DNA is more accessible to repair enzymes, this could result in faster recognition and repair of photodimers in linker DNA. Thus, (6-4) photoproducts, being mainly in the linkers, would be repaired faster overall than the more evenly distributed CPDs (11).

E. coli does not contain clearly defined nucleosomes of the kind seen in mammalian cells (3), although its DNA is found to be associated with histone-like proteins. It is not known how these proteins affect DNA damage induction or repair. We observed that CPDs are repaired at a lower rate than (6-4) photoproducts in the first 30 min following irradiation, but the difference is small in *E. coli* compared with that in human cells. A possible reason for this difference may be the affinity of the Uvr(A)BC excision repair complex for different types of photodimers. With purified UvrA, UvrB, and UvrC proteins, Svoboda et al. (31) measured the cleavage of DNA substrates each containing one of several types of photoadducts, including a TT *cis*,*syn*-CPD and a TT (6-4) photoproduct. They found that the rate of excision of the (6-4) photoproduct was about nine times greater than that of excision of the *cis*,*syn*-CPD. This could account for the rate difference we see in vivo if the rate of excision is tempered by the actions of other proteins in the cell or if the difference is less pronounced for the more frequent TC and CC (6-4) photoproducts.

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