We have developed a method to quantify (6-4) photoproducts in genes and other specific sequences within the genome. This approach utilizes the following two enzymes from Escherichia coli: ABC excinuclease, a versatile DNA repair enzyme which recognizes many types of lesions in DNA, and DNA photolyase, which reverts pyrimidine dimers. DNA is isolated from UV irradiated Chinese hamster ovary cells and digested with a restriction enzyme. Pyrimidine dimers, the major photoproduct produced at biological UV fluences, are then completely repaired by treatment with DNA photolyase. The photoreactivated DNA is treated with ABC excinuclease, electrophoresed in an alkaline agarose gel, transferred to a support membrane and probed for specific genomic sequences. Net incisions produced by ABC excinuclease following photoactivation are largely due to the presence of (6-4) photoproducts. These adducts are quantitated by measuring the reduction of intensity of the full length fragments on the autoradiogram. Using this approach we have shown that (6-4) photoproducts are produced at equal frequency in the dihydrofolate reductase coding sequence and in its 3' flanking, noncoding sequences and that the formation of (6-4) photoproducts is linear in both sequences up to a UV dose of 60 J/m2. The repair of (6-4) photoproducts in these DNA sequences was measured after a dose of 40 J/m2 over 4-, 8-, and 24-h time periods. The (6-4) photoproducts are repaired more efficiently than pyrimidine dimers in both sequences and there is preferential repair of (6-4) photoproducts in the dihydrofolate reductase gene compared with the downstream, noncoding sequences.

It is well established that ultraviolet (UV) light induces specific DNA lesions in bacteria and higher organisms and that this damage is correlated with mutagenesis, carcinogenesis, and cell killing. From work done with Escherichia coli, it has been estimated that 85-90% of the lesions after a physiological UV dose are cyclobutane dimers (= pyrimidine dimers) and 10-15% are (6-4) photoproducts (6-4 PP) (1). While the involvement of pyrimidine dimers in the biological effects of UV is well established (2), the role of 6-4 PP in mutagenesis and lethality has been of much current interest and controversy (3). Whereas much is known about the formation and repair of pyrimidine dimers in mammalian cells, little is known about the fate of 6-4 PP. There are several well-established techniques to study pyrimidine dimers, but studies on the formation and repair of 6-4 PP in mammalian cells have been impeded by the lack of readily available techniques. There are no known frequencies of irradiation or sources of light known exclusively or predominantly to produce 6-4 PP, and there are to date no known enzymes which specifically recognize 6-4 PP. Although 6-4 PP clearly are important both as mutagenic and lethal lesions, there has been no method to measure the formation and repair of these lesions at subgenomic levels in vivo in mammalian cells and only one assay for these measurements in vivo in the overall genome.

The original values for frequencies of 6-4 PP are now being challenged. Mitchell et al. (4) developed a radioimmunoassay to detect 6-4 PP and have recently found that the frequency of this adduct is about 1/5 of that of pyrimidine dimers for the overall genome at doses less than 2 kJ/m2 (3). Using the radioimmunoassay it was found that 6-4 PP were removed from the genome of normal human and rodent cells but not from UV sensitive mutants of hamster or human cells (3).

Bohr et al. (5) developed an assay for measuring pyrimidine dimers formation and repair in specific genomic sequences. Using this approach it was found that pyrimidine dimers are removed preferentially from actively transcribing sequences in rodent and human cells (6), and it was suggested that the repair of pyrimidine dimers in active genes was responsible for the relatively high survival of rodent cells when exposed to UV irradiation (9). A considerable body of work has been done using this approach, but the repair measurements in specific genes after UV damage have been based exclusively on measurements of formation and removal of pyrimidine dimers (6-8). Although 6-4 PP are important lesions and appear to be repaired faster in rodent cells than pyrimidine dimers, the fate of these photoproducts in specific sequences such as active or inactive genes has not been investigated. Thomas et al. (9) extended the range of the assay for intragenomic repair by using E. coli ABC excinuclease to probe DNA damage. This enzyme recognizes most DNA adducts that create significant helical distortions (10). The enzyme incises DNA at 6-4 PP in vitro (11) and is required for removal of these adducts from bacterial chromosomes in vitro (12). In this report we have used ABC excinuclease in conjunction

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with another DNA repair enzyme from E. coli, DNA photolyase, to measure 6–4 PP in the dihydrofolate reductase (DHFR) gene of CHO cells. Photolyase plus light repairs pyrimidine dimers but not 6–4 PP (13, 14). The remaining photoproducts, which almost exclusively are 6–4 PP, are then probed with ABC excinuclease. We find that the DHFR gene is preferentially repaired compared with downstream, noncoding sequences and that the efficiency of repair of 6–4 PP is consistently higher than pyrimidine dimer repair in the sequences studied.

**EXPERIMENTAL PROCEDURES**

**Materials**—The CHO cell line CHO-B11, which contains an amplified DHFR gene (E), was maintained in Dulbecco's minimum essential medium supplemented with 10% dialyzed fetal calf serum and methotrexate. The cells were subcultured 1 day before irradiation to ensure exponential growth at the time of irradiation. ABC excinuclease, T4 endonuclease V (T4 endo) and DNA photolyase were purified as described (16–18). The DNA probes were as previously described (19). The pMB5 probe was used to detect the CHO DHFR 14-kb KpnI fragment and subclone of the Cs-14 probe was used to detect the downstream, noncoding 14-kb KpnI fragment of the DHFR gene (Fig. 1). This subclone was generated by selecting fragments which contained a minimum of repetitive sequences.

**Methods**—The initial steps of the assay were as described by Bohr and Okumoto (20). The CHO cells, grown in monolayer, were irradiated with UV doses (254 nm) ranging from 20 to 60 J/m². Cells harvested to 0 h repair were lysed immediately, whereas those for repair time points were incubated in bromodeoxyuridine (BrdUrd) and fluorodeoxyuridine to density label the DNA replicated after damage. High molecular weight DNA was isolated and digested to completion with KpnI restriction endonuclease using 5 units/μg DNA, and centrifuged in a CsCl equilibrium gradient to fractionate parental and replicated DNA. The DNA sequences were analyzed for the presence of pyrimidine dimers and 6–4 PP by exposure to photoreactivating light with or without DNA photolase, followed by treatments with T4 endo or ABC excinuclease. Samples treated with photolyase were preincubated for 5 min at room temperature in the dark in a buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.25 μg of photolyase/μg of DNA. The sample was then placed in a small reaction tube and irradiated in a monochrometer at 405 nm for 30 min at room temperature. The DNA was extracted with phenol followed by ether, precipitated with ethanol, and resuspended in TE to a concentration of approximately 0.2 μg/μl. The treatment of the DNA with T4 endo and ABC excinuclease and the preparation of the samples for alkaline gel electrophoresis, Southern transfer, and hybridization have been described in detail (9, 20). Sometimes, samples were recut with ABC excinuclease. For CHO DNA the samples were deproteinized and dialyzed as described (9, 20) before recutting with the enzyme. When labeled pBR 322 was used, the DNA (100 ng) was extracted twice in phenol and twice with ether after the first round of cutting, and then recut with the enzyme. The samples were run on a 1% agarose gel and the bands corresponding to Form I and II DNA were excised, solubilized, and quantitated by scintillation counting.

**RESULTS**

**Method for Measuring 6–4 PP in Specific Genomic Sequences**—We have previously described a general method to quantitate base adducts (damage) in specific genomic sequences (9). Utilizing ABC excinuclease, damage induced by a wide variety of DNA damaging agents, both chemical and physical, can be probed. UV light produces two major photoproducts, the pyrimidine dimers and the 6–4 PP. A direct method was previously developed for pyrimidine dimer quan-

**THE QUANTITATION OF 6-4 PHOTOPRODUCTS IN GENES**

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**Fig. 1. Flow diagram of the assay.**

*PD*, pyrimidine dimers.
Preferential DNA Repair of (6-4) Photoproducts

We have now extended this assay to measure nondimer photoproducts by the use of DNA photolyase to repair all pyrimidine dimers before treatment of the DNA sample with ABC excinuclease. The remaining, nondimer photoproducts are almost exclusively 6-4 PP (14).

Fig. 1 shows a flow diagram of the procedures. Cells are irradiated when they are in logarithmic growth phase to standardize conditions and to ensure that the active genes are actively transcribed. After UV irradiation of the cells they are incubated for repair in the presence of bromodeoxyuridine in order to later separate the semiconservatively replicated DNA from the parental. The DNA is isolated, samples are treated with a restriction endonuclease, and the parental DNA is separated on CsCl gradients (20). The DNA in each sample is divided into two portions. The first is treated with T4 endo to measure pyrimidine dimers as described (20). The other portion is treated with DNA photolyase, which specifically and quantitatively reverts pyrimidine dimers (10). The photoreactivated DNA is further divided into three aliquots. One aliquot is treated with T4 endo to demonstrate that virtually all of the pyrimidine dimers were repaired by the photoreactivation treatment. Another is treated with ABC excinuclease to excise the DNA at the remaining adducts. A third is used as a control. The three samples are run in parallel on an alkaline agarose gel, transferred to a support membrane, and probed for a specific DNA sequence. The relative intensities of parallel bands representing full length fragments are compared to determine adduct frequency (20). We have measured the repair of 6-4 PP in two KpnI restriction fragments, both with a length of 14 kb. One fragment constitutes the 5′-coding portion of the DHFR gene, and another the 3′-flanking non-coding region (Fig. 2).

Formation of Pyrimidine Dimers and 6-4 PP in the DHFR Gene and Downstream Sequences—CHO cells were irradiated with increasing doses of UV light, the DNA extracted and treated as described above, and divided into aliquots for treatment either with T4 endo or DNA photolyase. The samples treated with T4 endo were run on an alkaline gel, transferred to a support membrane, and probed first for the DHFR gene (Fig. 3A), and after deprobing, for a downstream flanking sequence (Fig. 3C). This represents a measure of pyrimidine dimer formation at 20, 40, and 60 J/m². Based on densitometry of autoradiograms, the pyrimidine dimer frequency of both sequences were determined and found to increase in a linear fashion with dose, and to be similar in both sequences at all doses, Table I. The samples treated with DNA photolyase were digested with either T4 endo or ABC excinuclease, run on an alkaline gel, transferred, and probed for the DHFR coding sequences (Fig. 3B). After exposure, the probe was removed, and the membranes were probed for the downstream sequences (Fig. 3D). Densitometric scans of these autoradiograms indicate that fewer than 0.08 pyrimidine dimers remain in either KpnI fragment (compare T4 endo-treated lanes with the untreated samples at each dose in Fig. 3, B and D). As a control, a sample irradiated with 60 J/m² that was not photoreactivated was digested with T4 endo which resulted in 3.03 pyrimidine dimers/DHFR fragment.

![Fig. 2. Map of the DHFR gene of Chinese hamster ovary cells showing the regions analyzed.](image)

![Fig. 3. Formation of pyrimidine dimers and (6-4) photoproducts in transcribed and nontranscribed sequences.](image)
and 3.24 pyrimidine dimers/noncoding fragment (this is less than expected, but the measurement is difficult due to the small zero class represented by fragments containing many pyrimidine dimers). At 60 J/m², photoreactivated DNA treated with T4 endo yielded only 0.08 remaining pyrimidine dimers/DHFR fragment and 0.06 pyrimidine dimers/noncoding fragment. At 20 J/m², fewer than 0.01 pyrimidine dimers were detected in either sequence after photoreactivation. This demonstrates that the photoreactivation is more than 98% complete and establishes a base line from which to observe and quantify 6-4 PP in these fragments. As can be seen in the lanes representing ABC excinuclease treatment after photoreactivation in Figs. 3, B and D, the full length DHFR or noncoding fragment is increasingly sensitive to ABC scission in a UV dose-dependent manner. Densitometric scans of autoradiograms were used to calculate incisions by the enzyme and these are listed in Table I and plotted in Fig. 4 at different UV doses. It is seen that 6-4 PP are formed in a linear fashion and to a similar extent in both sequences, a parallel finding with pyrimidine dimers in coding and noncoding sequences.

Table I
Formation of pyrimidine dimers and (6-4) photoproducts in DHFR and downstream sequences of CHO cells

<table>
<thead>
<tr>
<th>Fluence, J/m²</th>
<th>Dimers (incisions)</th>
<th>(6-4) photoproducts (incisions)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gene</td>
<td>Downstream</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>1.67</td>
<td>1.69</td>
</tr>
<tr>
<td>40</td>
<td>2.55</td>
<td>2.48</td>
</tr>
<tr>
<td>60</td>
<td>3.15</td>
<td>3.00</td>
</tr>
</tbody>
</table>

The exact number of 6-4 PP cannot be derived directly from the figure, since ABC excinuclease does not cleave 100% of the adducts under in vitro conditions (9). From previous work (see “Discussion”), we estimate that ABC excinuclease cleaves approximately 50% of the adducts. This implies that approximately twice as many 6-4 PP as indicated are present at each dose level in Fig. 4. Based on this estimate, 6-4 PP are formed in these fragments at about ½ of the level of pyrimidine dimers or at a level of about 40% of total photoproducts.

To further support this argument, we did some experiments on recutting with the ABC enzyme. If the fraction of lesions cleaved with ABC excinuclease is not qualitatively different from the fraction that is not, it should be possible to deproteinize the DNA after one round of cutting, and obtain further cleavage by the enzyme of the remaining adducts. This experiment was performed using both CHO DNA and ³H-labeled plasmid pBR322 and the results are presented in Table II. The CHO DNA was irradiated and photoreactivated as described while the plasmid was irradiated at 125 J/m² and then photoreactivated. After one round of cutting with ABC excinuclease followed by deproteinization, further incision is seen by recutting with the enzyme. If the incisions made during
After UV, it can be regarded as a measure of 6-4 PP since it has been shown that 6-4 PP are by far the major non-pyrimidine dimers photoproduct (1, 14). From the above experiments we conclude that 6-4 PP are preferentially and efficiently repaired from the DHFR gene in CHO cells. This is similar to the situation for pyrimidine dimers which we have previously reported (5, 19), although the extent of repair of 6-4 PP is slower than that reported for the repair of 6-4 PP from the overall genome in wild-type CHO cells by the use of the radioimmunoassay (3). However, this is probably due to the different doses used. Mitchell's experiment was done at a UV dose of 10 J/m², and our experiment is at 40 J/m². The relatively high dose may also be the reason for our result that only about 35% of the 6-4 PP are removed from the downstream sequences. Although it would be desirable to use lower UV doses in these experiments, our procedure requires relatively high UV doses since the ABC excinuclease does not cleave 100% of the lesions. Myles et al. (14) compared the intensities of bands generated by alkali cleavage to those generated by ABC excinuclease cleavage of terminally labeled DNA that was photoreactivated before exposure to chemical or enzymatic (ABC excinuclease) cleavage. They found that ABC excinuclease incises at about 50% of the adducts in a manner that is essentially sequence independent.

It is important to emphasize that we are convinced that the ABC excinuclease is not detecting a special subset or modification of the adducts. This is supported by the previous studies (5, 14) which have reported rates for the repair of 6-4 PP (like pyrimidine dimers) that are random in coding and non-coding sequences. In order to determine the fate of 6-4 PP in these two sequences, DNA was isolated from cells irradiated at a dose of 40 J/m² and allowed to repair for up to 24 h. The DNA samples were processed as above to estimate 6-4 PP in the DHFR and noncoding sequences. The autoradiograms of such an experiment is presented in Fig. 5. As in the dose experiments above, the photoreactivation is very efficient, as very few (<0.04) pyrimidine dimers are detected by T4 endo in either fragment. Photoreactivated samples treated with ABC excinuclease were highly susceptible to cleavage (0.96 adducts/fragment) at 0 h, but became increasingly more resistant to cleavage after 4-, 8-, and 24-h post-irradiation (Table III). In the DHFR gene, 46% repair is seen after 4 h, increasing to 66% after 24 h (Fig. 6). In contrast, in the non-coding downstream sequence, the repair at 4 h is only 21%, and it only reaches about 35%. This preferential repair of the coding sequences resembles that seen for pyrimidine dimers in these sequences when measured for a 20 J/m² dose (19). The extent of repair of 6-4 PP is not quite as high as that for pyrimidine dimers at 20 J/m². It is, however, higher than the extent of pyrimidine dimers repair at the same dose of 40 J/m²: 24 h after a dose of 40 J/m² we find 66% repair of 6-4 PP versus 40% repair of pyrimidine dimers in the gene (Fig. 3).

**DISCUSSION**

We here describe the first method to measure the formation and removal of 6-4 PP in specific mammalian sequences such as defined genes. In conjunction with this assay we have also measured the formation and repair of pyrimidine dimers at the same dose by use of T4 endo. Although our assay for 6-4 PP detects total photoproducts other than pyrimidine dimers


We have previously proposed that the repair of pyrimidine dimers is preferentially repaired. We still do not know the mechanism that governs the preferential repair of active genes. The recognition by certain DNA repair enzymes may be dependent on the degree of distortion of the adduct introduced. The repair of various adducts in the transcribed and non-transcribed strand of an active gene, as has been demonstrated for pyrimidine dimers in the CHO DHFR gene, is non-transcribing, but it may not be the case for the repair of 6-4 PP. This fragment might be unusual and not reflective of the overall genome as regards 6-4 PP repair.

Studies on preferential repair have for a while been limited to measurements of pyrimidine dimer removal after UV damage. However, by use of related approaches, it has been possible to examine the repair of other adducts including methylated purines (21) and N-acetoxyaminofluorene (22) from the CHO DHFR gene. For both of these damaging agents, there appears to be no preferential DNA repair in the gene compared with the non-transcribed, downstream fragment. It is therefore of particular importance that we can now document the preferential DNA repair in this biological system of a lesion other than pyrimidine dimers. And it is of obvious importance to further investigate whether other adducts are preferentially repaired. We still do not know the mechanism that governs the preferential repair of active genomic regions. One interesting approach, for example, is to examine whether the preferential repair is related to the size of the adduct introduced. The recognition by certain DNA repair enzymes may be dependent on the degree of distortion introduced into the double helix by the damage. It is also of importance to determine whether a difference exists in the formation and repair of various adducts in the transcribed versus non-transcribed strand of an active gene, as has been demonstrated for pyrimidine dimers in the CHO DHFR gene (23).

Little is known about the repair mechanism for 6-4 PP. In human cells there appears to be a better correlation between repair of 6-4 PP and of pyrimidine dimers than is the case in rodent cells. In order to further elucidate the repair mechanism of 6-4 PP, we need to examine the repair in specific genes in various human and CHO-hypersensitive mutants and transfectants. This may also help understand the role of 6-4 PP repair in specific genes in relation to cellular survival. We have previously proposed that the repair of pyrimidine dimers in active genes may play an important role in cellular survival and in some cases correlate better with UV resistance than the overall genome, average repair (17). We now need to investigate the role of 6-4 PP repair in active genes in relation to cell survival.

REFERENCES