Preferential repair and strand-specific repair of benzo[a]pyrene diol epoxide adducts in the HPRT gene of diploid human fibroblasts

(nucleotide excision repair/human cells/UvrABC excinuclease)

Ruey-Hwa Chen*, Veronica M. Maher*, Jaap Brouwer†, Pieter van de Putte‡, and J. Justin McCormick*

*Carcinogenesis Laboratory, Fee Hall, Departments of Microbiology and Biochemistry, Michigan State University, East Lansing, MI 48824-1316; and
‡Laboratory of Molecular Genetics, Department of Biochemistry, Gorlaeus Laboratories, Leiden, University, RA Leiden, The Netherlands

Communicated by Philip C. Hanawalt, March 2, 1992 (received for review November 25, 1991)

ABSTRACT If excision repair-proficient human cells are allowed time for repair before onset of S phase, the premutagenic lesions formed by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (benzo[a]pyrene diol epoxide, BPDE) are lost from the transcribed strand of the hypoxanthine (guanine) phosphoribosyltransferase (HPRT) gene faster than from the nontranscribed strand. No change in strand distribution is seen with repair-deficient cells. These results suggest strand-specific repair of BPDE-induced DNA damage in human cells. To test this, we measured the initial number of BPDE adducts formed in each strand of the actively transcribed HPRT gene and the rate of repair, using UvrABC excinuclease in conjunction with Southern hybridization and strand-specific probes. We also measured the rate of loss of BPDE adducts from the inactive 754 locus. The frequencies of adducts formed by exposure to BPDE (1.0 or 1.2 μM) in each strand of a 20-kilobase fragment that lies entirely within the transcription unit of the HPRT gene were similar; the frequency in the 14-kilobase 754 fragment was ≈20% lower. The rates of repair in the two strands of the HPRT fragment differed significantly. Within 7 hr after treatment with 1.2 μM BPDE, 53% of the adducts had been removed from the transcribed strand, but only 26% from the nontranscribed strand; after 20 hr, these values were 87% and 58%, respectively. In contrast, only 14% of the BPDE adducts were lost from the 754 locus in 20 hr, a value even lower than the rate of loss from the overall genome (i.e., 38%). These results demonstrate strand-specific and preferential repair of BPDE adducts in human cells. They suggest that the heterogeneous repair of BPDE adducts in the human genome cannot be accounted for merely by the greatly increased rate of the repair specific to the transcribed strand of the active genes, and they point to a role for the chromatin structure.

UV-induced cyclobutane dimers are excised more rapidly—i.e., preferentially—in the actively transcribed genes of rodent and human cell lines than in inactive genes or in nontranscribed segments of DNA or in the overall genome of the cells (1–3). Furthermore, in these mammalian cells as well as in Escherichia coli, such lesions are excised faster in the transcribed strand of actively transcribed genes than in the nontranscribed strand of these genes (4–6). These results led the investigators to hypothesize that nucleotide excision repair is coupled to gene transcription. Indeed, a factor responsible for such coupling has recently been partially purified from E. coli cell extracts (7).

Although the existence of such heterogeneous repair of cyclobutane dimers from the DNA of mammalian cells is well documented, the applicability of this model to bulky chemical residues covalently bound to DNA (adducts) is unclear and controversial. The results of our studies of the effect of excision repair in diploid human cells on the strand distribution of the principal premutagenic lesions induced by (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (benzo[a]pyrene diol epoxide, BPDE) showed that in the absence of repair, such lesions are located in both strands of the coding region of the hypoxanthine (guanine) phosphoribosyltransferase (HPRT) gene, but after 12 hr of repair, no premutagenic lesions remain in the transcribed strand (8, 9). These results provide biological evidence of strand-specific repair of BPDE adducts in the human HPRT gene.

Strand-specific repair could also account for the strand bias for BPDE-induced premutagenic lesions observed in the adenosine phosphoribosyltransferase gene (10) and the dihydropolate reductase (dhfr) gene of repair-proficient Chinese hamster ovary (CHO) cells (11). However, Tang et al. (12), using the technique developed by Bohr et al. (1) to detect lesions present in specific genes, but using E. coli UvrABC excinuclease to recognize and incise bulky chemical adducts, found no difference in the rate of repair of DNA adducts formed by a structurally related compound, N-acetoxyacetylaminofluorene, in the active and nonactive (nontranscribed) regions of the dhfr gene of CHO cells. More recently, Tang and Zhang (13) reported that BPDE-induced DNA adducts also are not preferentially removed from the active region of the dhfr gene of CHO cells.

The present study was designed to measure the ability of human cells to remove BPDE adducts from either strand of the HPRT gene to see if strand-specific repair occurs. We also tested for preferential repair of BPDE adducts by comparing the rate of their removal from an active gene, HPRT, and an inactive locus (14) on the same chromosome, the 754 locus. To investigate these questions, we synchronized repair-proficient diploid human fibroblasts and treated them with BPDE in early G phase so there would be a long period for excision repair before semiconservative DNA replication. The cells were harvested immediately or after various times, and the DNA was extracted and assayed for lesions in the HPRT gene. Using UvrABC excinuclease, which specifically and quantitatively incises at least 80% of BPDE-DNA adducts in human genomic DNA (15), and using Southern blotting and hybridization with probes specific for the individual strands of the HPRT gene, we quantified the initial numbers of BPDE adducts formed in the two strands and the numbers remaining at various times after treatment.

We found no significant difference in the initial frequency of BPDE adducts formed in the two strands of the HPRT fragment, but the rates of removal of adducts from the two

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: BPDE, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (benzo[a]pyrene diol epoxide).

†To whom reprint requests should be addressed.
strands differed markedly. The transcribed strand was repaired significantly faster than the complementary strand; furthermore, the rate of excision repair of BPDE adducts in the 754 locus was slower still, even slower than the rate of loss of radiolabeled BPDE adducts from the genome overall.

MATERIALS AND METHODS

Cell Culture and Synchronization. Diploid human male fibroblasts from foreskin of a neonate (16) were cultured in Eagle’s medium containing 10% supplemented bovine calf serum (HyClone). Cells were driven into the Go state by density inhibition and nutrient depletion as described (17). To stimulate the cells to reenter the cell cycle, they were released from confluence and plated in culture medium at a density of 106 cells per cm2. The time of onset of S phase after release from Go was determined by the incorporation of [3H]thymidine into acid-insoluble material as described (17).

BPDE Treatment and Post-Treatment Incubation. Five hours after release from Go (in early G1), the cells were rinsed with phosphate-buffered saline (PBS) and treated with 1.0 or 1.2 μM BPDE in 0.6% albumin, denatured salmon sperm DNA (50 μg/ml). E. coli RNA (100 μg/ml), and 2–3 × 106 cpm of 32P-labeled probe at 42°C for 20–24 hr. The riboprobes used for strand-specific hybridizations to the BamHI fragment covering the 5’ half of the HPRT genomic DNA were generated by using the method described by Melton et al. (20), with the modifications suggested by Boehringer Mannheim. DNA probes for the 754 locus were labeled with [32P]dNTPs by random primer extension (21). After hybridization, the membranes were washed at 60°C with a final wash step at 0.1× SSPE/0.1% SDS and exposed to Kodak XAR-5 x-ray films with intensifying screens.

Hybridization Probes. Plasmid pG2Pa (22), constructed by subcloning a 1.4-kilobase (kb) EcoRI-Xho I fragment containing sequences from the first intron of the human HPRT gene into the vector pGEM2, was provided by A. C. Chinnault (Baylor College, Houston). It contains the SP6 and T7 promoters. RNA transcripts synthesized by the SP6 and T7 polynucleases hybridize with the transcribed and nontranscribed strands of HPRT genomic fragment, respectively. The 2.0-kb HindIII fragment of plasmid pG2Pa was used by L. H. F. Mullenders (Leiden University, The Netherlands).

Quantitation. The intensities of bands were quantified by using the Bio-Image Visage 110 system (Millipore). The intensity of the full-length fragment band was normalized with the internal standard band referred to above. The average number of UvrABC-sensitive sites per fragment was calculated by the Poisson distribution equation as described by Bohr and Okumoto (24). These calculations took into consideration the nonspecific incisions produced by the UvrABC excinuclease, which ranged from 0.05 to 0.15 incisions per 10 kb in the present experiments.

Determination of the Number of BPDE Adducts Bound to Cellular DNA. Generally tritiated BPDE (specific activity, 464.7 mCi/mmol; 1 Ci = 37 GBq) was obtained from Chem sync Science Laboratories, Lenexa, KS, as a tetrahydrofuran solution. The methods used to determine the number of adducts remaining in the genome have been described (25).

RESULTS

Determination Delay in Onset of DNA Replication Induced by BPDE. In our study of the effect of excision repair on the strand distribution of premutagenic BPDE adducts in the human HPRT gene (8, 9) we synchronized the cells by releasing them from the Go state and plating them at 106 cells per cm2. Using incorporation of tritiated thymidine, we showed previously (17) that under these conditions, untreated cells begin DNA replication ∼16 hr after release from confluence, but exposure to BPDE should delay the onset of S phase. Before analyzing the rate of removal of BPDE adducts from the DNA of treated cells, we synchronized the cells, plated them into a series of dishes at 106 cells per cm2, and after 5 hr, exposed them to 1.0 or 1.2 μM BPDE and measured incorporation of tritiated thymidine at various times after treatment for up to 24 hr. In the untreated population, DNA replication began 11 hr later—i.e., ∼16 hr after release from Go; in the treated population there was no incorporation of thymidine whatsoever at any time during the next 24 hr—i.e., during the 29 hr after release from Go (data not shown). The total absence of semiconservative DNA replication after exposure of the synchronized cells to 1.0–1.2 μM BPDE simplified the assay of repair because there was no dilution of parental DNA with daughter DNA in the BPDE-treated populations during the period of interest—i.e., for at least 24 hr after treatment.

Determination of the Rate of Excision Repair of BPDE Adducts. Synchronized cells were plated at 106 cells per cm2 into a series of 150-mm-diameter dishes, using 10–15 dishes per
repair time period to be assayed. After 5 hr, the cells were treated with BPDE and populations were harvested immediately or after the designated times. For each time point, DNA was extracted from treated and control populations, digested with the appropriate restriction enzyme, and treated with UvrABC excinuclease or incubation buffer. The samples were denatured under neutral conditions as described (12) to avoid the possibility of DNA strand breaks formed nonenzymatically at the sites of any alkali-labile N7-guanine adducts (26). The samples were then separated on agarose gels and analyzed by Southern hybridization using gene- or strand-specific probes.

Evidence of Strand-Specific Repair of BPDE Adducts in Human Cells. Using this method, we measured the initial number of BPDE-induced lesions formed in the individual strands of the 20-kb BamHI fragment of the human HPRT gene, as well as their rate of disappearance. This fragment is located in the 5' half of the gene and is entirely within the transcription unit (Fig. 1). To avoid detection of homologous pseudogenes containing HPRT exon sequences (27), we used riboprobes complementary to intron sequences of the gene. Representative autoradiograms of such repair studies are shown in Fig. 2 A and B. The intensities of the full-length fragments were determined by densitometric scanning, and the number of incisions and the percentage of adducts removed were calculated as described. The data from two separate experiments are given in Table 1 and plotted in Fig. 3. The level of adduct formation by BPDE increased with dose and was similar in each strand of the HPRT fragment. However, the rates of adduct removal from the transcribed and nontranscribed strand differed significantly. Within 7 hr, 53% of the adducts had been removed from the transcribed strand; only 26% had been removed from the nontranscribed strand. By 20 hr, 87% of the adducts had been removed from the transcribed strand, whereas 58% still remained in the nontranscribed strand of the HPRT gene.

Evidence of Preferential Repair of BPDE Adducts in Human Cells. To see if there was a difference in the rate of repair of BPDE adducts from a nontranscribed (inactive) genomic sequence compared with that from the actively transcribed HPRT gene, we measured the formation of such adducts and their rate of repair in a 14-kb EcoRI fragment (Fig. 1) of the transcriptionally inactive 754 locus, using a 2.0-kb probe labeled by the random priming method. The autoradiogram of that study is shown in Fig. 2C. The data are given in Table 2 and plotted in Fig. 3. The initial frequency of adducts formed by 1.2 μM BPDE was 0.92 per 14-kb fragment or 1.31 per 20 kb, which is ~20% less than that observed in either strand of the HPRT fragment. However, the efficiency of repair in the 754 locus was markedly reduced. During the 20-hr period after

![Table 1. Formation of BPDE adducts in the individual strands of the 20-kb BamHI fragment and their rates of removal](https://example.com/table1)

<table>
<thead>
<tr>
<th>BPDE dose, μM</th>
<th>Repair time, hr</th>
<th>Transcribed strand</th>
<th>Nontranscribed strand</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incisions/fragment*</td>
<td>% repaired</td>
<td>Incisions/fragment*</td>
</tr>
<tr>
<td>1.2</td>
<td>0</td>
<td>1.73 ± 0.08</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.81 ± 0.08</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.25 ± 0.04</td>
<td>87</td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>1.30 ± 0.02</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.58 ± 0.03</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*Calculated from the densitometric scanning as described. The nonspecific incisions, ranging from 0.09 to 0.3, have been subtracted. The errors represent SEM derived from two separate experiments at each dose.

---

![Fig. 1. Molecular organization of the HPRT gene and the 754 locus. The BamHI and EcoRI restriction fragments analyzed for repair are indicated. Vertical bars in the HPRT and 754 maps indicate exons and restriction sites, respectively. The locations of the probes are shown as rectangles. H, HindIII; R, EcoRI.](https://example.com/f1)

![Fig. 2. Autoradiograms illustrating extent of repair in the transcribed (A) and nontranscribed (B) strand of the HPRT gene and in both strands of the 754 locus (C). DNA was isolated from untreated human cells (first two lanes of each panel) or from cells incubated for the indicated repair period (hr) after treatment with 1.2 μM BPDE. DNA samples were digested with the appropriate restriction enzyme. DNA fragments containing the sequences to be probed were included in each DNA sample as internal markers. The samples were then treated (+) or not treated (−) with UvrABC and subjected to electrophoresis and Southern hybridization with 32P-labeled probes. (Upper) Bands corresponding to the 20-kb fragment of the HPRT gene or the 14-kb fragment of the 754 locus. (Lower) Bands corresponding to the DNA fragments serving as internal markers.](https://example.com/f2)
found to be significantly faster than that in the nontranscribed strand (28). Strand-specific repair of DNA damage induced by cisplatin and psoralen plus near UV has been demonstrated in an E. coli cell-free extract (7). Such repair has been suggested to explain the fact that essentially all of the premutagenic lesions induced by (±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene in the dhfr gene of CHO cells were located in the nontranscribed strand (29). Such strand-specific repair suggested a specific coupling between transcription and repair (4, 5). Recently, an assay for such repair by cell-free extracts of E. coli was developed, and a candidate "transcription-repair coupling factor" was partially purified from the extracts (7). This coupling factor could lead to faster repair in the transcribed strand than in the opposite strand by associating with component(s) of the transcriptional complex via protein–protein interactions and facilitating the assembly of the repair complex in the vicinity of the transcriptional complex, so that repair enzymes can scan the strand that is being transcribed. Another possibility is that this factor directly or indirectly recognizes a unique DNA structure or other signal(s) generated by the stalled transcriptional complex at the site of DNA damage and targets the repair enzymes to the template strand.

Our finding that the removal of BPDE adducts from the transcriptionally inactive 754 locus was much less efficient than that from either strand of the HPRT gene demonstrates that there are at least three different rates of repair of BPDE adducts from the genome of human cells. The slow repair we observed in the 754 locus agrees with the hypothesis that DNA damage located in condensed, inactive, chromatin is less accessible to repair enzymes than that in active chromatin (1). Our finding that repair of BPDE adducts in both strands of the inactive 754 locus is even slower than that observed in the nontranscribed strand of the active HPRT gene is consistent with what was recently found for the repair of aflatoxin B$_1$ adducts in the active and inactive human metallothionein genes (28). However, the rate of removal of pyrimidine dimers from the nontranscribed strand of active genes was similar to that from both strands of the 754 locus (23, 30). Little is known about the mechanism(s) of repair of damage in the compact chromatin where the inactive genes are located. However, the lack of repair of dimers in the inactive genes of group C xeroderma pigmentosum cells (23, 31), which repair active genes at a normal rate, is consistent with a requirement for an additional factor to enable the repair enzymes to reach DNA damage in such chromatin. If so, our results suggest that this factor functions less efficiently for processing some bulky adducts, such as the BPDE adducts, than for dimers. But we cannot rule out the possibility that the observed difference in the rates of repair of BPDE adducts in the nontranscribed strand of HPRT and in the 754 locus resulted from preferential removal of BPDE adducts from the nontranscribed strand of the active gene compared with the removal from the genome overall.

Since the majority of the genome contains nontranscribed sequences, the average rate of repair of BPDE adducts in the overall genome should be similar to that in the inactive 754 locus if the rate of repair of such adducts is homogeneous in all of the inactive regions. However, we found the removal of adducts from the 754 locus to be even slower than that from the genome overall, suggesting that the efficiency of repair varies among the inactive regions. Heterogeneous rates of repair among various inactive regions have also been reported with aflatoxin B$_1$ (28) and methylglyoxal (32), suggesting that the rate of repair of certain adducts in the inactive regions is influenced by the structure of the DNA and/or the compactness of the chromatin where the sequence is located.

In contrast to our results, Tang and Zhang (13) reported no difference in rate of removal of BPDE adducts from the
transcriptionally active and the 3' nonactive regions of the dhfr gene of CHO cells. The reason for the discrepancy between these two studies is not known, but the cell types used and the genes examined differ, and Tang and Zhang used a higher concentration of BPDE. Furthermore, we compared the rate of repair of damage in an active gene to that in an inactive locus, while they assayed the actively transcribed segment of the dhfr gene and its 3' nontranscribed region. In certain genes the region in which the preferential repair occurs is larger than the entire transcription unit (33). Therefore, it may be that, unlike repair of cyclobutane dimers (34), repair of BPDE adducts extends beyond the transcribed section of the dhfr gene into its 3' nontranscribed flanking regions. Another possible reason for the discrepancy is that Tang and Zhang used probes from double-stranded DNA, whereas we used single-stranded riboprobes. Use of the former gives information on the average rate of repair in the two DNA strands, which would lessen the difference detected.

To use Southern blotting for studies of repair rates, one needs to introduce an average of one UvrABC efficiently recognizable lesion per fragment analyzed, but not more than two. Under our conditions, 1.0 μM BPDE yielded a mean of 1.4 UvrABC sites per 20-kb fragment. This level of damage reduced the colony-forming ability of the cells to <0.01% of the control, but during the 20 or 24 hr after BPDE treatment, ≈90% of the cells remained attached to the dishes. Note that such cells were able to remove almost all the UvrABC sites from the HPRT gene within the 24 hr after BPDE treatment, even though the majority of them would eventually die.

The levels of BPDE adduct formation in each strand of the HPRT gene were very similar, indicating no strand-specific modification. However, the frequency in the 754 locus was only 80% of that in the HPRT gene. Certain chemical carcinogens, including BPDE, react preferentially with regions of DNA that are sensitive to DNase I (35, 36), suggesting a binding preference for actively transcribed genes. But it has also been reported that reactive metabolites of benzo[a]pyrene bind preferentially to DNase I-resistant regions of chromatin (37). Still others report that a transcriptionally active gene and an inactive gene are similarly modified by a series of polycyclic aromatic compounds (38). In any event, chromatin accessibility need not be the only explanation for the slightly lower binding frequency we observed in the 754 locus. The GC content of a fragment can also affect the binding, since BPDE binds almost exclusively to guanine (39). Information on the GC content of the 754 locus is not yet available.

In summary, our data show that human fibroblasts carry out preferential and strand-specific repair of BPDE adducts in the HPRT gene. Our finding that adduct removal from the inactive 754 locus is slower than from the genome overall indicates that the efficiency of repair in the various inactive regions of the genome is also heterogeneous.

We thank Drs. A. C. Chinnault and L. H. F. Mullenders for the probes and Dr. J. Venema for advice on Southern blotting. The expert technical assistance of M. de Ruijter and G. Moolenaar in preparing the UvrABC enzyme subunits is greatly appreciated. The research was supported in part by Grant CA21253 from the National Cancer Institute.