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# Splicing mutations in the CHO *DHFR* gene preferentially induced by (±)-3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene

(polycyclic aromatic hydrocarbon/mutagenesis/mRNA splicing/DNA sequencing)

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ABSTRACT **Cultured Chinese hamster ovary (CHO) cells were treated with the polycyclic aromatic hydrocarbon racemic 3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[*c*]-phenanthrene. Mutants deficient in dihydrofolate reductase activity were isolated. A carcinogen treatment at 0.1 μM yielded a 46% survival of the treated population and an induced frequency of mutation of $1.7 \times 10^{-4}$, $10^3$-fold greater than the spontaneous rate. By polymerase chain reaction amplification and direct DNA sequencing, we determined the base changes in 38 mutants. Base substitutions accounted for 78% (30/38) of the mutations. We obtained, in addition, four frameshift and four complex mutations. The preferred type of mutation was transversion (A·T → T·A and G·C → T·A) occurring in 69% of the analyzed mutants. A purine was on the 3′ side of the putative adduct site in every mutant. Mutations were favored at sequences AGG, CAG, and AAG (the underlined base is the target). Surprisingly, 42% of the mutations created mRNA splicing defects (16/38), especially at splice acceptor sites for each of the five introns. Thus, this chemical carcinogen may recognize some aspect of DNA structure in regions corresponding to pre-mRNA splice sites.**

Polycyclic aromatic hydrocarbons are a widespread class of environmental pollutants that are metabolized by microsomal enzymes to chemically reactive and ultimately carcinogenic products (1). Benzo[*c*]phenanthrene (B[*c*]PH), a weakly tumorigenic polycyclic aromatic hydrocarbon, is metabolized to diastereomeric pairs of bay-region epoxides. Whereas the diol epoxides represent only minor metabolites of B[*c*]PH, they are exceptionally tumorigenic (2) and mutagenic (3); most potent in this regard are diastereomers with trans stereochemistry. DNA adducts derived from each of the configurational B[*c*]PH diol epoxide isomers have been characterized and occur at exocyclic amino groups of guanine (N-2) and adenine (N-6) (4). The optically active (−)-*anti*-dihydroxy 2-epoxide of 3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrahydrobenzo[*c*]phenanthrene (B[*c*]PHDE) reacts more extensively with adenine (5). The mutagenic specificity of this isomer was investigated using the *supF* shuttle vector system (6). Results from that study showed that the (−)-*anti*-dihydroxy 2-epoxide of B[*c*]PHDE primarily induced G·C → T·A and A·T → T·A transversions.

We have used the dihydrofolate reductase (*DHFR*) locus in cultured Chinese hamster ovary (CHO) cells as a target to study spontaneous (7) and carcinogen-induced mutations at the DNA level (8–10). The gene is 25 kilobase pairs in size and contains six exons (11). Recessive $DHFR^-$ mutants can be readily selected from a hemizygous cell line (UA21) that carries a single *DHFR* gene (12). In the present study, we have used racemic B[*c*]PHDE for mutagenesis; the structure of this compound is shown in Fig. 1. We determined the induced sequence changes in 38 $DHFR^-$ mutants. A remarkably large proportion (16/38) of the induced mutations occurred at pre-mRNA consensus splice sites, resulting in splicing defects.

## MATERIALS AND METHODS

**Materials.** Reagents, media, and materials used in this work were the same as indicated (8). B[*c*]PHDE was synthesized as described (13).

**Mutagenesis and Selection.** Cultures of UA21 cells (12), which are hemizygous at the *DHFR* locus were initiated from an inoculum of 100–200 cells to eliminate possible preexisting spontaneous mutants from the population. The cells were treated with 0.1 μM B[*c*]PHDE (stock dissolved in dimethyl sulfoxide) for 90 min; they were then treated with trypsin, counted, and plated on 15 150-mm dishes. Selection for $DHFR^-$ clones (14) followed a 6-day expression period. Individual colonies were isolated and tested for glycine, thymidine, and hypoxanthine auxotrophy. Subclones of auxotrophs were expanded and assayed for ability to bind [$^3$H]methotrexate (15). Three colonies (designated A, B, and C) per dish were analyzed. Mutants arising on the same dish were deemed independent if DNA sequencing showed different base changes for each mutant. Presumed sisters were discarded (six altogether).

**DNA Sequence Analysis.** *DHFR* fragments from genomic DNA or *DHFR* cDNA from each mutant were amplified by the polymerase chain reaction (PCR) (16) as described (9, 17). Direct DNA sequencing reactions of PCR-amplified templates used *Thermus aquaticus* polymerase and [$\gamma^{32}$-P]ATP-labeled primers (17).

**Analysis of mRNA from Splicing Mutants.** Splicing mutant mRNA was analyzed by RNase protection as described (8). The splicing patterns of particular mutants deduced from the RNase protection experiments were confirmed by electrophoresis and/or sequencing of PCR-amplified cDNA. Sequencing through exon joints provided an exact description of the aberrant splice. In cases where the mutation caused complex mixtures of spliced products, dominant species were isolated by preparative NuSieve GTG agarose gel electrophoresis (FMC) and reamplified prior to sequencing.

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Abbreviations: B[*c*]PHDE, 3α,4β-dihydroxy-1α,2α-epoxy-1,2,3,4-tetrabenzo[*c*]phenanthrene; PCR, polymerase chain reaction; B[*c*]PH, benzo[*c*]phenanthrene; B[*a*]PDE, benzo[*a*]pyrene diol epoxide.

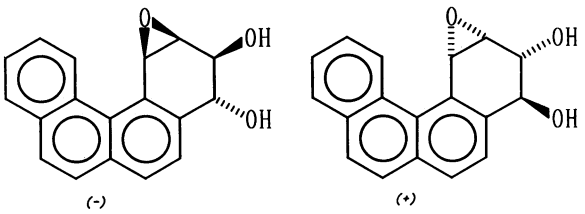


FIG. 1. Structures of (−)- and (+)-B[*c*]PHDE.

## RESULTS

Treatment of CHO UA21 cells (12) with 0.1 μM B[*c*]PHDE killed about half of the population and yielded DHFR$^-$ mutants at an induced frequency of $1.7 \times 10^{-4}$, an induction of 100-fold over the spontaneous frequency and 1000-fold over the spontaneous mutation rate (7). Thirty-eight mutants were analyzed for DNA sequence changes in the protein coding portion of the gene. In most cases, each of the six exons plus flanking intronic regions was amplified from genomic DNA (9) and directly sequenced (17). Alternatively, transcripts were analyzed by reverse transcribing the DHFR mRNA, amplifying the cDNA by the PCR (16), and directly sequencing the PCR-amplified product (17). All 38 mutations occurred within the protein coding region or at splice site consensus sequences (Table 1). Most of the mutations were single-base substitutions (30/38) with a few single-base insertions (4/38) and tandem 2- or 3-base substitutions (4/38). Deletions or gene disruptions were not found in this collection of mutants. Of the 38 mutants, 18 (47%) contained nonsense codons that cause premature translation termination, 14 by direct base substitution and 4 indirectly as a result of single-base insertions. Four mutants (11%) carried missense mutations, one of which destroyed the translational

Table 1. Characteristics of B[*c*]PHDE-induced DHFR$^-$ mutants

| Designation | Exon | Target sequence | Change | Position(s) | Phenotype |
|---|---|---|---|---|---|
| | | Single-base change | | | |
| DP3B | 1 | GTC **G** AAC* | G → A | 7 | Nonsense |
| DP15B | 1 | AAC **G** GAG | G → T | 61 | Nonsense |
| DP12C | 2 | TTC **A** GGA | A → T | −2 | Splice acceptor defect |
| DP7B | 2 | TC**A** **G** GAA | G → C | −1 | Splice acceptor defect |
| DP9B | 2 | GTG **G** AAG | G → T | 133 | Nonsense |
| DP15C | 2 | GTG **G** AAG | G → T | 133 | Nonsense |
| DP10C | 2 | **GGT** **A** ATT | A → T | +3 | Splice donor defect |
| DP11B | 3 | TGT **A** GGT | A → T | −2 | Splice acceptor defect |
| DP1C | 3 | TGT **A** GGT | A → T | −2 | Splice acceptor defect |
| DP13C | 3 | GGT **A** AAC | A → T | 139 | Nonsense |
| DP14A | 3 | CAG **A** ACC | A → T | 145 | Missense Asn-48 → Tyr |
| DP9A | 3 | TTA **A** AGG | A → T | 205 | Nonsense |
| DP5C | 3 | AGT **A** GAG | A → T | 232 | Nonsense |
| DP13A | 3 | **GTA** **A** GTG | A → T | +4 | Splice donor defect |
| DP8C | 4 | TTC **A** GGG | A → T | −2 | Splice acceptor defect |
| DP4A | 4 | TC**A** **G** GGA | G → A | −1 | Splice acceptor defect |
| DP1B | 4 | CCA **G** AGT | G → T | 313 | Nonsense |
| DP10B | 4 | GGA **G** GCA | G → T | 352 | Missense Gly-117 → Cys |
| DP12B | 5 | TAA **A** GGA | A → T | −2 | Splice acceptor defect |
| DP7C | 5 | AA**A** **G** GAA | G → T | −1 | Splice acceptor defect |
| DP8A | 5 | CTC **A** GAC | A → T | 397 | Nonsense |
| DP5B | 5 | GAG **A** AAT | A → T | 466 | Nonsense |
| DP11A | 5 | TAT **A** AAC | A → T | 472 | Nonsense |
| DP2A | 5 | TAT **A** AAC | A → T | 472 | Nonsense |
| DP6A | 5 | **AGT** **A** AGT | A → C | +3 | Splice donor defect |
| DP5A | 6 | TCC **A** GGT | A → T | −2 | Splice acceptor defect |
| DP14C | 6 | TCC **A** GGT | A → G | −2 | Splice acceptor defect |
| DP15A | 6 | CC**A** **G** GTA | G → A | −1 | Splice acceptor defect |
| DP3A | 6 | CAG **G** AGG | G → T | 514 | Nonsense |
| DP6C | 6 | CAG **G** AGG | G → T | 514 | Nonsense |
| | | Single-base frameshift | | | |
| DP7A | 4 | ACC **A**↓GAG | +T | 312 | Nonsense at position 312 |
| DP1A | 6 | AAA **A**↓GGC | +T | 522 | Nonsense at position 534 |
| DP12A | 6 | AAA **A**↓GGC | +T | 522 | Nonsense at position 534 |
| DP2C | 6 | AAA **A**↓GGC | +T | 522 | Nonsense at position 534 |
| | | Complex mutation | | | |
| DP10A | 1 | CCA **TG** ACA* | TG → AT | −1, 1 | Start codon destroyed |
| DP6B | 3 | GT**A** **G** GTA | G → TAT | −1 | Splice acceptor defect |
| DP9C | 3 | CAG **A** ACC | AA → TT | 145, 146 | Missense Asn-48 → Phe |
| DP2B | 4 | CCC **TG** AAA* | TG → AT | −2, −3 | Splice acceptor defect |

Unless otherwise indicated, the target sequence represented is the nontranscribed (coding) strand in a 5′ to 3′ orientation. Mutated bases are underlined. The GT and AG dinucleotides at splice sites are shown in bold lettering. Position numbers refer to the protein coding region of the cDNA where the first base is the adenine of the ATG translation initiation codon. For mutations that occurred in introns, the position relative to the nearest exon is given with + indicating downstream from the exon and − meaning upstream from the exon. An exception is DP10A; −1 refers to the nucleotide preceding the first coding base. Arrow indicates the position of the +1 frameshift insertion.

*Sequence shown is the transcribed (template) strand assuming adduct formation occurred at a purine.

start codon. The remaining 16 mutants (42%) carried base changes near exon–intron joints that reduced homology with splice consensus sequences. These splicing mutations were distributed throughout all five introns in the gene.

B[*c*]PHDE yields adducts to both adenine and guanine in DNA (4). Consistent with this specificity, we found that base changes arose at both A·T (61%) and G·C (39%) base pairs. The putative modified purine was on the nontranscribed (coding) strand in 92% of the mutants. Repeated occurrence of the same mutation was found at five sites in the gene. The same transversions occurred twice at positions 133, 472, and 514 and the −2 splice acceptor site of exon 3. A single-base insertion occurred three times at a purine-rich sequence containing four tandem adenines at position 522. Moreover, the same base was substituted at the −2 splice acceptor site of exon 6 but in two ways and a single and tandem double substitution occurred at position 145. Thus, the 38 mutants represent attack at only 30 sites. Among the single-base substitutions, the majority (25/30) were transversion of either A·T or G·C to T·A. A summary of the types of mutations induced by B[*c*]PHDE is presented in Table 2.

Examination of the sequences surrounding the presumed target purine at the 30 mutation sites revealed some sequence specificity for B[*c*]PHDE mutagenesis. At all 30 of these sites, a purine occupied the position to the 3′ site of the target base. Table 3 shows that in 79% of the mutations, the next base 3′ is also a purine. No apparent pattern is present upstream or farther downstream of the target base. Hence, the consensus B[*c*]PHDE mutagenic target is 5′-R<u>R</u>R-3′ (where R is a purine and the mutated base is underlined).

The DHFR pre-mRNA splicing patterns of all mutants carrying base changes at splice sites were determined by RNase protection analysis, PCR amplification and sequencing of cDNA, or both. The results of this detailed analysis will be presented elsewhere. The 15 mutations all resulted in aberrant splicing. Exon skipping was the predominant phenotype for sites bounding internal exons, whereas splicing to cryptic sites occurred when sequences at the first or last exon were mutated. Little or no correctly spliced mRNA was produced by the three mutants (DP10C, DP13A, and DP6A, Table 1) that carried base changes falling outside the nearly invariant GT and AG dinucleotides.

Table 2. Summary of types of B[*c*]PHDE-induced mutations in the *DHFR* gene

| Mutation | Number |
|---|---|
| DNA base change | |
| Transversion (68.5%) | |
| G·C → T·A | 9 |
| A·T → T·A | 16 |
| G·C → C·G | 1 |
| A·T → C·G | 1 |
| Transition (10.5%) | |
| G·C → A·T | 3 |
| A·T → G·C | 1 |
| Single-base frameshift (10.5%) | |
| (+1 insertion of thymine) | 4 |
| Complex mutation (10.5%) | |
| Double transversion | 3 |
| Substitution + insertion (G → TAT) | 1 |
| Total (100%) | 39 |
| Phenotypic consequence | |
| Nonsense mutation (47%) | |
| Base substitution | 14 |
| Amber | 4 |
| Ochre | 7 |
| Opal | 3 |
| Frameshift | 4 |
| Missense mutation (11%) | |
| Amino acid substitutions | 3 |
| Start codon destroyed | 1 |
| Mutation at splice site (42%) | |
| Donor (5′ end of intron) | 3 |
| Acceptor (3′ end of intron) | 13 |
| Total (100%) | 52 |

Table 3. B[*c*]PHDE consensus target sequence

| | % sequence | | | | | |
|---|---|---|---|---|---|---|
| Base | −2 | −1 | 0 | +1 | +2 | +3 |
| A | 14 | 13 | 23 | 17 | 14 | 10 |
| G | 7 | 7 | 15 | 21 | 16 | 10 |
| C | 8 | 8 | 0 | 0 | 4 | 10 |
| T | 9 | 10 | 0 | 0 | 4 | 8 |
| %R | 55 | 53 | 100 | 100 | 79 | 53 |

Position 0 is the site of the mutated purine.

## DISCUSSION

**The Mutagenic Action of B[*c*]PHDE.** We have shown here that B[*c*]PHDE is a powerful direct mutagen in cultured mammalian cells, in agreement with its potent action as a carcinogen (2). Indeed, B[*c*]PHDE is the most effective of the seven mutagens tested at the *DHFR* locus in CHO cells, being somewhat more active than ethyl methanesulfonate (15) and UV irradiation (18). The potency of B[*c*]PHDE as a mutagen may be attributed to its ability to efficiently form adducts to both adenine and guanine in DNA (4). The spectrum of mutations induced in the *DHFR* gene is consistent with this mode of action in chromatin. Furthermore, B[*c*]PHDE-induced transversion of either purine to thymine is the preferred mechanism of mutation (Table 2) accounting for 69% of the mutants. A similar proportion (64%) was found in the *supF* shuttle vector system where naked DNA was the target (6).

Despite the versatility of B[*c*]PHDE in forming adducts, the interaction of this carcinogen with cellular DNA is apparently constrained by a limited sequence specificity. In all 38 mutants, another purine occupied the position to the 3′ side of the mutated base, and nearly 80% of the mutations occurred at the triple purine sequence 5′-R<u>R</u>R-3′ (the underlined nucleotide being the target). A dipurine (but not tripurine) preference can also be seen in the plasmid mutagenesis data of Bigger *et al.* (6), where 83% of the B[*c*]PHDE-induced mutations in the *supF* gene followed this pattern. These authors also reported hotspots for mutation at the trinucleotides A<u>G</u>A, A<u>A</u>C, and G<u>A</u>G (the mutated base is underlined). However, only one (DP1B, Table 1) of 38 mutations in *DHFR* occurred at any of these targets and none was a hotspot. These distinctions may reflect differences in DNA accessibility or conformation in chromatin as opposed to supercoiled plasmid DNA or differences in repair processes between chromosomal and episomal DNA (19). It is unlikely that these differences are entirely due to the fact that the pure enantiomer was used in the shuttle vector study.

Maintenance of the B[*c*]PHDE adduct in genomic DNA may be promoted by binding at runs of purines. The adduct possibly is stabilized by hydrophobic interactions with the aromatic rings of 3′ flanking purines. Alternatively, the misinsertion of dATP by DNA polymerase may depend on stacking interactions created by the 3′ base(s) neighboring the target. Singer *et al.* (20) have shown such an effect *in vitro* using a template that contained an alkylated guanine. However, we did not see the requirement for a 3′ purine among mutants induced by the racemic polycyclic aromatic hydrocarbon benzo[*a*]pyrene diol epoxide (B[*a*]PDE, ref. 10), possibly due to the greater hydrophobicity of this compound

relative to B[*c*]PHDE. B[*a*]PDE and B[*c*]PHDE have similar relative sterochemistry; however, B[*a*]PDE preferentially binds to and induces mutations at guanine residues. It is interesting that the two compounds yielded different sets of mutated sites. Among the 13 sites of B[*c*]PHDE-induced guanine mutations and 14 B[*a*]PDE-induced mutations at guanine residues, only one site was in common [DB21 (10) and DP10B, Table 1].

All but 3 of the 38 mutations induced by B[*c*]PHDE fell on the nontranscribed DNA strand of the *DHFR* gene. This type of asymmetry has been seen (8, 21), prompting the suggestion of a linkage between transcription and repair (19). Alternatively, the strand preference may merely reflect a selection bias, with one strand being far richer than the other in the target base that can give rise to a deleterious amino acid substitution. This idea is difficult to test since one cannot reliably predict which amino acid changes destroy enzyme activity. However, for *DHFR* the most common coding change found in negative mutants has been the generation of a translational termination codon. Presumably, the selection for a negative phenotype is quite stringent here, and thus splicing mutations, frameshifts, and nonsense mutations predominate. Nonsense mutations eliminate DHFR activity even when near the carboxyl terminus (9). Therefore, one can ask whether the strand bias reflects a selection bias for this class of mutations. As in the complete set, analysis of the coding sequence shows that 90% of potential nonsense codons that arise by a purine-to-thymine transversion, in fact, falls on the nontranscribed strand (43 to 47). Thus, the strand bias here can be ascribed to selection bias, and it is not necessary to postulate a mutagenic mechanism to account for it.

**Preferential Mutation at Splice Acceptor Sites.** A surprising aspect of the sequence or site specificity of B[*c*]PHDE mutagenesis was its remarkable predilection for splice sites, especially splice acceptors sites. A predominance of splice site mutations was not found with any of the other mutagens studied at the *DHFR* locus: $\gamma$-rays (12), *N*-acetoxy-2-acetylaminofluorene (8), UV (18), or B[*a*]PDE (9). The propensity of B[*c*]PHDE to mutate double-purine tracts *per se* cannot explain the preference for splice sites; considering both strands, there are 59 double purines in the 561-base-pair *DHFR* protein-coding sequence that can produce, upon single-base substitution, a nonsense codon. In comparison, the five AG dinucleotides at *DHFR* splice acceptors form 10 sites. In this combined set, the splice acceptors represent 14% of the sites yet account for 44% of the mutants (11/25). By $\chi^2$ analysis, the probability that these nonsense and splicing targets are equally mutable is <0.001. A similar statistic results if one considers only the predominant mutational change, a purine-to-thymine transversion or only triple purine targets. Thus there must be a characteristic of the splice acceptor sequence that predisposes the AG double purine to mutation by B[*c*]PHDE.

A characteristic common to all splice acceptor sites is the polypyrimidine tract that lies upstream of the AG (22). As can be seen in Table 4, the polypurine tracts on the opposite strand at *DHFR* splice acceptor regions contain poly(A) runs with an average length of 4 bases (8). A possible explanation for B[*c*]PHDE-induced splicing mutations may relate to the DNA conformation at these regions. If there is bending of the helix in these locations, the tilt at the two ends of the adenine tract compresses the minor groove; this effect, in turn, is ameliorated by a roll at the 5′ end opening the minor groove there (23, 24). The 5′ end of the adenine tract abuts the splice acceptor sites and is adjacent to the sites of mutation. If B[*c*]PHDE, like B[*a*]PDE (25), is located in the minor groove, then the DNA conformation at splice acceptor sites may afford a favorable environment for binding. For this idea to hold, the conformational change at the 5′ end of the adenine tract must extend up to 4 bases upstream of the bend–B-DNA interface. Interestingly, the discrimination of precisely these structural features has been shown for the intercalation of ethidium bromide, which also interacts at the minor groove (26).

Table 4. Oligo(A) tracts at DNA regions corresponding to DHFR pre-mRNA splice sites

| Exon | Mutation | B[*c*]PHDE-induced splice acceptor mutants, no. |
|---|---|---|
| 2 | 5′-CTTT C**A**G/GAA-3′<br>3′-GAAA GTC CTT-5′ | 2 |
| 3 | 5′-ATTTT GT**A**G/GTA-3′<br>3′-TAAAA CATC CAT-5′ | 3 |
| 4 | 5′-CTTTTTT C**A**G/GGA-3′<br>3′-GAAAAAA GTC CCT-5′ | 3 |
| 5 | 5′-ATTTT A**A**AG/GAA-3′<br>3′-TAAAA TTTC CTT-5′ | 2 |
| 6 | 5′-CTTTT CC**A**G/GTA-3′<br>3′-GAAAA GGTC CAT-5′ | 3 |

Boldface letters indicate bases altered by mutation. The space distinguishes the interface at the 5′ end of the oligo(A) tract. The slash indicates the location of pre-mRNA splice site.

Inactivation of genes plays an important role in cancer progression. Allele loss can arise by various means (point mutation, deletion, translocation, inversion, etc.). However, chemical carcinogens primarily induce point mutations. In this study, we have shown that metabolites of an environmental carcinogen B[*c*]PH mainly induced point mutations (30/38) in an endogenous mammalian gene. The *anti*-dihydroxy epoxides of B[*c*]PH are the most potent tumorigenic diol epoxides ever tested in both mouse skin and newborn mice assays (27). B[*c*]PHDE in the *DHFR* gene preferentially mutated splice sites. In general, splicing mutations generate deletions in the specified protein and thus are likely to cause a null phenotype. Perhaps there is a correlation between the tumorigenicity of a compound and its ability to yield a null as opposed to a partial phenotype. Similar mutations have been described in the human retinoblastoma susceptibility gene (28) in which 9 of 10 mutations were splicing (3/10) or nonsense (6/10) mutations, presumably leading to a null phenotype. For tumor suppressors, the production of a null phenotype may be necessary for loss of growth control. The propensity of a mutagen to attack splice sites may thus magnify its potency as a carcinogen.

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