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Genetics

Carcinogenic epoxides of benzo[*a*]pyrene and cyclopenta[*cd*]pyrene induce base substitutions via specific transversions

(mutagenesis/polycyclic aromatic hydrocarbons/*lac* operon)

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ABSTRACT **We have determined the spectrum of base-pair substitution mutations induced in the *lacI* gene of a *uvrB*⁻ strain of *Escherichia coli* by two polycyclic aromatic hydrocarbons—(±)7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10 tetrahydrobenzo-[*a*]pyrene (BPDE), and 3,4-epoxycyclopenta[*cd*]pyrene (CPPE). Approximately 10% of all *lacI* mutations induced by either BPDE or CPPE are nonsense mutations, suggesting that base-pair substitutions are a large fraction of the mutational events induced by these agents in the *uvrB*⁻ bacteria. Both carcinogens specifically induced the G·C → T·A and, to a lesser extent, the A·T → T·A transversions. One possible mechanism for transversion induction at G·C sites by BPDE might involve carcinogen binding to the exocyclic amino group of guanine in the template strand followed by a rotation of the modified base around its glycosylic bond from the *anti* to the *syn* conformation. This could allow specific pairing of modified bases with an imino tautomer of adenine.**

Although the mutagenicity of most carcinogens is well documented (1), little is known about the spectrum of mutagenic events that carcinogens are capable of inducing. The most widely utilized mutagenicity test, the Ames test (1), detects the ability of chemicals to revert any one of three different point mutations (two frameshift mutations and a base-substitution mutation) but does not yet provide information about the specific changes in DNA sequence that are induced at these loci. Because there is a wide range of possible base sequence alterations it seems of fundamental importance to document the spectrum of mutations induced *in vivo* by carcinogens. Miller and his colleagues have developed a genetic system in *Escherichia coli* which is useful for rapidly and rigorously identifying base-pair substitutions that generate nonsense mutations at 64 sites within the *lacI* gene (2). This system detects all possible base-pair substitution events with the exception of the A·T → G·C transition. We have begun to use the *lacI* system to explore the spectrum of base-pair substitutions induced *in vivo* by carcinogens.

In the study reported here we focused on two polycyclic aromatic hydrocarbons which are environmental carcinogens: benzo[*a*]pyrene (BP) and cyclopenta[*cd*]pyrene (CPP) (3–5). Many carcinogens are metabolized to highly reactive species which bind covalently to DNA (6) and induce mutations in bacteria or in mammalian cells (7, 8). The reactive metabolite of BP that accounts for most of the binding to DNA is a *trans* diol epoxide, (±)7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (BPDE) (9). Fig. 1 depicts the structure of BPDE. The reactive metabolite of CPP is 3,4-epoxycyclopenta[*cd*]pyrene (CPPE). The structure of CPPE is also depicted in Fig. 1. The reactive carbonium ions derived from the opening of BPDE and CPPE are identical (5).

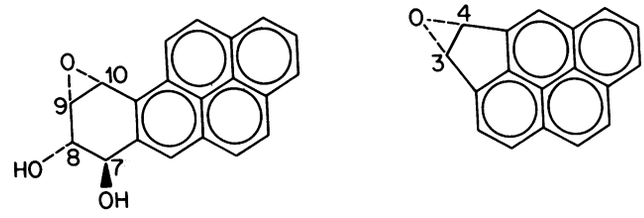

FIG. 1. Structure of BPDE (*Left*) and CPPE (*Right*).

Several BP adducts to DNA have been identified *in vivo* and *in vitro*. The principal adduct to DNA is to the exocyclic N2 amino group of guanine (10). Adducts of the *trans* diol epoxide with the exocyclic N6 amino group of adenine (11) and with cytosine (12) have also been reported. There is also indirect evidence that an adduct may be formed between the *trans* diol epoxide and the N7 position of guanine (13). CPP adducts to DNA have not been identified.

Which of the BPDE–DNA adducts represent biologically significant premutational lesions? The mutational spectrum induced by BPDE should indicate which premutational lesions are biologically relevant. We have determined the spectrum of base substitutions induced in the *lacI* gene of *Escherichia coli* by BPDE and CPPE and have found that both agents induce specific transversions, leading us to consider a mechanism of action dependent on adducts to the exocyclic amino group of guanine (N2).

MATERIALS AND METHODS

Bacterial Strains. The set of *E. coli* strains for identifying *lacI* nonsense mutants has been described in detail (14). The *lacI* gene was carried on an F′ episome. Strains EE122 and EE124 are episome-bearing derivatives of strain P90C [Δ(*gpt-proA-lac*)*ara thi*] which carry two additional mutations rendering them defective in excision-repair (by introducing a deletion through the *uvrB* locus) and more permeable to high molecular weight carcinogens (by introducing an uncharacterized mutation which made them sensitive to the dye crystal violet). The *uvrB* deletion was introduced by selecting for chlorate-resistant derivates of P90C and then screening the survivors for their sensitivity to UV light. The $1/e$ dose for the mutant we isolated is approximately 2 J/m^2. The permeability defect was intro-

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Abbreviations: BP, benzo[*a*]pyrene; BPDE, (±)7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; CPP, cyclopenta[*cd*]pyrene; CPPE, 3,4-epoxycyclopenta[*cd*]pyrene.

Table 1. Incidence of Lac constitutive mutants induced by BPDE or CPPE

Bacterial strain (relevant genotype)	Treatment	Frequency of Lac constitutives	% nonsense
EE122 (*uvrB*$^-$)	None	5×10^{-6}	2
	CPPE	6×10^{-5}	7
	BPDE	8×10^{-5}	11
EE124 (*uvrB*$^-$ + pKM101)	None	5×10^{-6}	2
	CPPE	5×10^{-4}	13

duced by selecting for phage-resistant bacteria and screening survivors for their sensitivity to crystal violet and deoxycholate.

Media. The preparation of selective and indicator plates has been described by Coulondre and Miller (14). The minimal medium used to culture bacteria for the mutagenicity experiments contained E salts (15), 0.2% glucose, and 5 μg of thiamin and 1 μg of biotin per ml. Bacteria grew in this medium with a generation time of 1 hr at 37°C.

Chemicals. BPDE was obtained from the National Cancer Institute's chemical carcinogen repository; we used lot NC120-6-1. The UV spectrum of BPDE was confirmed. BPDE concentrations were calculated by using molar extinction coefficients of 43,652 at 344 nm and 28,184 at 327 nm. CPPE was synthesized as described by Gold *et al.* (16).

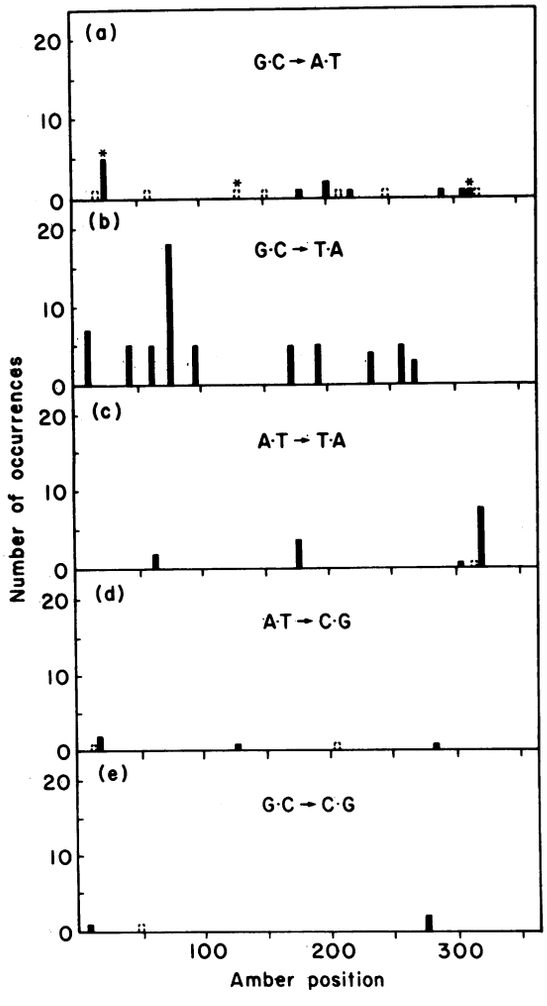

FIG. 2. The amber sites in the *lacI* gene induced by BPDE. Solid bars, individual sites at which we detected mutations; open bars, sites at which we did not detect mutations; asterisks (in *a*), sites at which the target codon contains 5-methylcytosine.

Mutagenesis. Bacteria were grown in minimal medium to a cell density of 3.5×10^8/ml and then washed and concentrated by centrifugation to a cell density of 2×10^9/ml in E salts. Bacteria were exposed to epoxide (10 μg/ml) for 20 min at room temperature and then washed once by centrifugation. Washed cells were diluted 1:50 into culture tubes containing 5 ml of minimal medium. Seventy cultures were generated from CPPE-treated cells and 139 cultures were generated from BPDE-treated cells. Cultures were incubated on a roller drum at 37°C until stationary phase had been reached. Aliquots from each culture were spread on selective plates containing phenyl β-D-galactoside as the sole carbon source. Twenty-five I$^-$ colonies from each culture were picked and gridded onto fresh

Table 2. Distribution of *lacI* amber mutations induced by CPPE or BPDE

Base Substitutions	Site	Independent occurrences at each site, no. CPPE −pKM101	CPPE +pKM101	BPDE −pKM101
G·C → A·T	*A5*	0	0	0
	*A6**	1	0	5
	A9	1	0	0
	*A15**	0	0	0
	A16	0	0	0
	A19	1	0	1
	A21	1	0	2
	A23	0	0	0
	A24	0	0	1
	A26	0	0	0
	A31	0	0	1
	A33	0	0	1
	*A34**	2	3	1
	A35	0	0	0
G·C → T·A	*A2*	8	1	7
	A7	0	5	5
	A10	1	3	5
	A12	5	4	18
	A13	3	2	5
	A17	10	4	5
	A20	5	9	5
	A25	0	0	4
	A27	0	1	5
	A28	3	5	3
A·T → T·A	*A11*	1	0	2
	A18	2	1	4
	A32	0	3	1
	A36	0	0	8
A·T → C·G	*A3*	0	0	0
	A4	2	0	2
	A14	0	0	1
	A22	0	1	0
	A30	1	0	1
G·C → C·G	*A1*	1	0	1
	A8	0	0	0
	A29	0	0	2
Total	36 sites	48	42	96

* A6, A15, and A34 all arise from codons containing 5-methylcytosine (CAG → UAG). These are hotspots for spontaneous and 2-aminopurine-induced transitions.

selective plates which then served as master grids for the transfer into a set of suppressor strains of episomes bearing *lacI*$^-$ alleles. We used suppressors Su2, Su3, SuB, and SuC (14) for screening BPDE- and CPPE-induced Lac constitutives. One amber and one ochre mutant from each culture was ultimately chosen and identified by mapping the mutations and determining their suppression pattern (2, 14).

RESULTS

Table 1 summarizes the yield of Lac constitutive mutants and *lacI* nonsense mutants after treatment with BPDE and CPPE. The detailed analysis of the *lacI* nonsense mutants generated by each of these agents is presented below.

BP. To characterize the mutational spectrum induced by BP, we used BPDE, the BP metabolite that reacts with and covalently binds to DNA. The use of BPDE obviated the need for a metabolic activation system and simplified our task of interpreting the mutational specificity of a single chemical species whose binding to DNA *in vivo* and *in vitro* has been well characterized. *lacI* nonsense mutants induced by treating cells with BPDE were collected from 138 independent cultures from which 3450 colonies were screened for suppressible mutations. Out of this collection, 381 (11%) were found to carry mutations suppressible by either amber or ochre suppressors. The first amber and ochre mutants detected from a culture were collected and further analyzed, leading eventually to the identification of 96 amber and 89 ochre mutants. Because the BPDE-treatment induced a greater than 80-fold increase in the incidence of suppressible mutations over the spontaneous background, we expect few spontaneous amber or ochre mutants in this collection.

Table 3. Distribution of *lacI* ochre mutations induced by CPPE or BPDE

Base substitutions	Site	Independent occurrences at each site, no. CPPE −pKM101	CPPE +pKM101	BPDE −pKM101
G·C → A·T	*O10*	0	0	0
	O9	0	0	0
	O11	0	0	0
	O13	1	4	0
	O17	0	1	0
	O21	0	0	3
	O24	0	0	3
	O27			2
	O28	1	1	1
	O29	0	0	1
	O34	1	1	0
	O35	0	1	0
G·C → T·A	*O3*	8	15	12
	O8	1	5	4
	O14	0	0	2
	O15	8	7	7
	O19	8	1	4
	O20	4	3	12
	O25	0	1	0
	O26	4	5	4
	O30	0	0	1
	O31	3	1	2
	O32	0	0	1
	O36	1	6	12
A·T → T·A	*O1*	0	0	2
	O2	0	0	5
	O4	0	0	0
	O5	0	0	1
	O12	0	0	0
	O16	0	0	6
	O18	0	0	0
	O22	1	0	4
	O23	0	1	0
	O33	0	0	0
Total	36 sites	44*	54†	89

* Includes three occurrences of a transversion that is either G·C → T·A or A·T → T·A.

† Includes one occurrence of a transversion that is either G·C → T·A or A·T → T·A.

Fig. 2 and Table 2 summarize the distribution of BPDE-induced amber mutations. Transversions account for 88% of these mutations. The G·C → T·A substitution was the most frequent transversion found. There are 10 sites within the *lacI* gene at which this substitution can generate amber mutations detectable in this system. Amber mutations at all 10 sites were found, with multiple independent occurrences at all sites ranging from 3 to 18. The average number of transversions per site was 6.2. Excluding one site with 18 occurrences, the average number of transversions per site was 4.9. There are 14 sites at which the G·C → A·T substitution can be detected. This transition was detected at only seven of these sites, and only one of these sites was represented by multiple occurrences. [This last site, *A6*, is known to be a hotspot for the spontaneously occurring G·C → A·T transition (17).] The average number of transitions per site was 0.9. Excluding the spontaneous hotspot *A6* (with five occurrences), the average number of transitions per site was 0.6. Thus, hotspots aside, the G·C → T·A transversion appeared more than 8 times as frequently as the G·C → A·T transition in the BPDE-induced amber collection.

The distribution of BPDE-induced ochre mutants (Table 3) reveals a similar dominance by mutations arising via transversions. Mutants arising via the G·C → T·A substitution account for 69% of BPDE-induced ochres. G·C → T·A transversions occurred 6 times as frequently as G·C → A·T transitions.

The only other base substitution frequently induced by BPDE is the A·T → T·A transversion (Fig. 2; Tables 2, 3, and 4). The A·T → C·G and the G·C → C·G transversions were not significantly induced. An interesting comparison involves the three base pairs in the *lacI* gene at which either the G·C → T·A (UAC to UAA, ochre) or G·C → C·G (UAC to UAG, amber) substitution can be detected. BPDE induced 17 ochre mutations at these three sites compared to only 3 ambers (Table 5), although the 17 ochre mutations at these sites were distributed unevenly. The 12:1 preference for G·C → T·A transversions over G·C → C·G transversions at the same G·C base pair in the codon for tyrosine-7 argues against the possibility that occur-

Table 4. Mutations induced by BPDE

Substitution	Amber: Available sites	Amber: Sites found	Amber: Total occurrences	Ochre: Available sites	Ochre: Sites found	Ochre: Total occurrences
G·C → A·T	14	7	12	12	5	10
G·C → T·A	10	10	62	12	11	61
A·T → T·A	4	4	15	14	5	18
A·T → C·G	5	3	4	—	—	—
G·C → C·G	3	2	3	—	—	—

The 96 amber and 89 ochre mutations induced by BPDE in strain EE122 are divided into different categories based on the base substitution required to generate each mutation.

Table 5. BPDE-induced mutations at three coding positions

Site	Coding position	Independent occurrences, no. UAC → UAA (G·C → T·A)	UAC → UAG (G·C → C·G)
A1, O3	Tyr-7	12	1
A8, O8	Tyr-47	4	0
A29, O30	Tyr-273	1	2
Total		17	3

The UAC codon for Tyr at amino acid residues 7, 47, and 273 in the *lac* repressor can mutate to either an amber codon by the G·C → C·G transversion or an ochre codon by the G·C → T·A transversion.

rences of G·C → C·G transversions are underestimated because sites at which this event can be scored are not targets for BPDE.

CPPE. CPPE is an arene oxide metabolite of CPP which shares chemical features with BPDE (5, 16). Although CPPE–DNA adducts have not yet been identified, CPPE is expected to react with DNA as does BPDE. We have determined the spectrum of nonsense mutations induced in the *lacI* gene by CPPE treatment of the same bacterial strain used to isolate BPDE-induced mutants and in an isogeneic strain carrying the mutation-enhancing plasmid pKM101 (18). The results of our analysis of the CPPE-induced nonsense mutants are presented in Tables 2, 3, and 6.

Although the total number of mutants analyzed from each genetic background is half the number analyzed for BPDE, the results are clear. CPPE and BPDE have nearly identical mutational specificities. This implies that they induce the same premutational lesion. The presence of pKM101 had no effect on the spectrum of mutational events, even though it resulted in greater than 8-fold enhancement of the yield of CPPE-induced Lac constitutives and greater than 15-fold enhancement of CPPE-induced *lacI* nonsense mutants (Table 1).

DISCUSSION

BPDE and CPPE induced close to 10% nonsense mutants among the total I^- mutants generated in the Uvr^- bacteria. Because the nonsense codons represent less than one-fifth of the *lacI* codons and because only certain base-pair substitutions in these codons generate nonsense mutations, base-pair substitutions must account for a major fraction of the induced mutations.

Knowledge of the mutational specificity of a chemical, when taken together with specific information concerning its reactions with DNA, may permit identification of the mutagenically relevant lesions. For mutagens such as ultraviolet light, which require the functions controlled by the *recA* and *lexA* gene products—the SOS response (19)—there is the added complication of determining whether and to what extent mutations occur at sites that are not the site of a lesion, because one of the phenotypes associated with this response is an increase in mutation rate in the absence of any exposure to chemicals or radiation. Although this increase is small (19), there is still the formal possibility that untargeted errors during replication may increase substantially when part of the genome sustains damage. However, if untargeted mutagenesis were a major contributor to mutations induced by chemical or radiation treatment of cells, one might expect the mutational specificity of SOS-dependent mutagens to be similar.

Table 6. Mutations induced by CPPE

Substitutions	Amber: Available sites	Amber: Sites found	Amber: Total occurrences	Ochre*: Available sites	Ochre*: Sites found	Ochre*: Total occurrences
G·C → A·T	14	5	9	12	5	11
G·C → T·A	10	9	69	12	9	81
A·T → T·A	4	3	7	11	2	2
A·T → C·G	5	3	4	—	—	—
G·C → C·G	3	1	1	—	—	—

The 90 amber and 94 ochre mutations induced by CPPE in strains EE122 and EE124 have been pooled and divided into different categories based on the base substitution required to generate each mutation.

* Four occurrences of an ochre mutation that may be either G·C → T·A or A·T → T·A are not included in the summary.

Two SOS-dependent mutagens—ultraviolet radiation and 4-nitroquinoline-1-oxide—have been examined in the *lacI* system (in a Uvr^+ background) and they were shown to induce different mutational spectra (17). BPDE probably is an SOS-dependent mutagen because its ability to induce reversion of a *trp* ochre mutation in *E. coli* was shown to require the $recA^+$ $lexA^+$ genotype (20). Although the BPDE-induced base-pair substitutions in *lacI* are $recA^+$ $lexA^+$ dependent, the high frequency of induced mutations and unique spectrum of base substitutions generated by BPDE argues that the transversions are targeted mutations determined by some feature of the BPDE-induced premutational DNA adduct.

Can we account for the unique transversion specificity of both BPDE and CPPE? We have considered the possibility that apurinic sites generated by BPDE or CPPE subsequent to formation of adducts at the N7 position of guanine or adenine might specifically induce transversions if purines (in particular, adenine) are preferentially selected by the replication machinery when apurinic sites are encountered. Although apurinic sites might be the premutational lesions (21) for some of the BPDE-induced nonsense mutations, we are unaware of evidence that such sites can direct specific mutational events.

Topal and Fresco (22) have presented hypothetical purine·purine pairing schemes to explain how specific transversions occur. In these schemes (Fig. 3), purine residues in the *syn* configuration (instead of the normal *anti*) pair specifically with tautomeric forms of adenine or guanine. Thus, pairing between A_{imino} and G_{syn} yields the G·C → T·A transversion. Similarly, pairing between A_{imino} and A_{syn} yields the A·T → T·A transversion, pairing between $G_{enol,imino}$ and G_{syn} yields the G·C

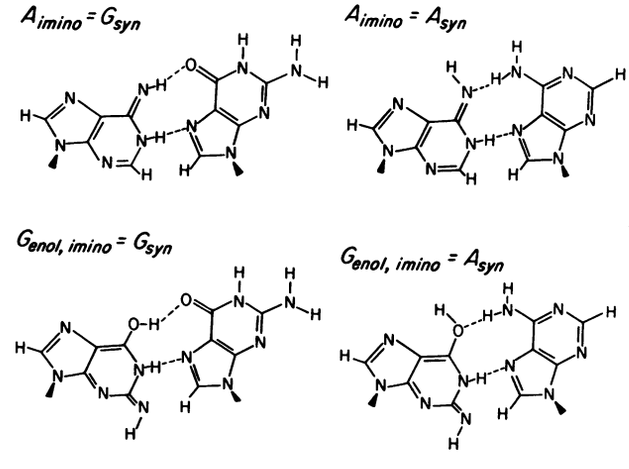

FIG. 3. The complementary DNA base pairs that can be formed between tautomeric and isomeric forms of purines [after Topal and Fresco (22)]. The *syn* conformer of guanine might be generated in the template strand subsequent to adduct formation at the N2 position.

→ C·G transversion, and pairing between $G_{enol,imino}$ and A_{syn} yields the A·T → C·G transversion. This theory has received qualitative support from recent *in vitro* studies on the accuracy of ϕX174 replication mediated either by DNA polymerase III (23) or by proteins encoded by T4 replication genes (24).

The major BPDE–DNA adduct is formed by *trans* addition of the N2 exocyclic amino group of guanine to C10 of BPDE. This adduct occupies the minor groove of the DNA double helix where it might interfere specifically with the normal hydrogen bonding between the N2 hydrogen of guanine and the oxygen at the C2 position of cytosine. Rotation of BPDE-modified guanine around the glycosylic bond from the normal *anti* to the *syn* conformation would place the adduct in the major groove where it would not interfere with specific pairing with tautomers of adenine or guanine. Thus, if the N2 BPDE adduct of guanine is in fact responsible for the observed mutations at G·C base pairs in the *lacI* gene, these could be accounted for by isomerization of modified guanine to the *syn* conformer on the template strand and its subsequent pairing with an imino tautomer of adenine. This scheme accounts for the rarity of the G·C → C·G transversion because it requires formation of the rare *enol*-imino tautomer of guanine. Kadlubar (25) has argued from both model building and physicochemical data that the N2 BPDE–adduct could promote the conversion of purines in the template strand from the *anti* to the *syn* conformation. We considered the possibility that BPDE modification of guanine in the template strand induces rare guanine tautomers that mispair with precursor purine triphosphates in the *syn* conformation. We think that this can be ruled out because the explanation predicts that G·C → C·G transversion would occur as frequently as G·C → T·A and is clearly not borne out by our data.

Although this model could account both for the specific G·C → T·A transversion and the rarity of G·C → C·G transversion, it is not directly applicable to the A·T → T·A transversion. BPDE forms an adduct to adenine at the N6 amino group of adenine (11). Adducts at this position occupy the major groove and could interfere with hydrogen bonding between adenine and thymine. Rotation of adenine from the *anti* to the *syn* conformation leaves the exocyclic amino group with its adduct in the major groove and in a position where it could still interfere with base pairing. Perhaps BPDE-induced DNA lesions such as apurinic sites (13, 21) or gaps (26) account for the occurrences of mutations other than G·C → T·A transversions.

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