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Genetics

Mutagenic specificities of four stereoisomeric benzo[*c*]phenanthrene dihydrodiol epoxides

(shuttle vector/polycyclic aromatic hydrocarbon/mutagenic specificity/enantiomers)

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ABSTRACT **The pS189 shuttle vector carrying a *supF* target gene was used to compare the mutagenic specificities of the four configurational isomers of benzo[*c*]phenanthrene 3,4-dihydrodiol 1,2-epoxide. One of these isomers is the most tumorigenic dihydrodiol epoxide tested to date and another is essentially inactive as a tumorigen. Overall mutagenicities were not correlated with tumorigenicities, but each configurational isomer induced a unique spectrum of mutational hot spots in the *supF* target gene, which monitors primarily point mutations. It is suggested that the demonstrated isomer-specific selectivity for mutation targets within the *supF* gene may be indicative of a similar selectivity for one gene versus another and that such selectivity may be one determinant of relative tumorigenicity.**

Polycyclic aromatic hydrocarbon carcinogens are prevalent in the environment and require metabolic activation (1) to mutagenic bay region dihydrodiol epoxides (2–5) in order to initiate carcinogenesis. That a mutagenic event is required for the initiation of carcinogenesis by hydrocarbons is also suggested by a correlation between tumorigenic potency and levels of binding to DNA (6), the finding that the specific metabolites responsible for DNA binding are tumorigenic (2, 5), and the observation that a point mutation is responsible for activation of *ras* protooncogenes in hydrocarbon-induced tumors (7). In recent years, new approaches, including the use of shuttle vectors (8–10), have begun to allow detailed questions about mutagenesis mechanisms for hydrocarbon metabolites to be addressed.

The bay region dihydrodiol epoxide metabolites of hydrocarbon carcinogens can exist as any one of four optically active stereoisomers, as illustrated for benzo[*c*]phenanthrene (BcPhDE) in Fig. 1. These isomers comprise two pairs of enantiomers wherein the benzylic hydroxyl group and the epoxide oxygen are cis (*syn*) in one pair (designated BcPhDE-1) and trans (*anti*) in the other pair (designated BcPhDE-2). Cellular systems examined so far preferentially generated one enantiomer of each pair [i.e., with the stereochemistry of (+)-BcPhDE-1 and (−)-BcPhDE-2] and more of the trans than of the cis diastereomer (11). When all four optically active isomers were prepared by synthesis and tested for biological activity, the dihydrodiol epoxide isomer preferentially formed by metabolism was found to be the most tumorigenic (4, 11).

It is not immediately obvious why the tumorigenic activity of the isomeric dihydrodiol epoxides varies so widely. For example, (−)-BcPhDE-1 is essentially devoid of tumorigenic activity, whereas (−)-BcPhDE-2 is the most active hydrocarbon dihydrodiol epoxide tested to date, and the other stereoisomers exhibit intermediate activities (12). To investigate the basis for these tumorigenic activities, relative mutagenicities in bacterial and mammalian cells have been reported (13), as have detailed studies of the chemistry of their interactions with DNA (14, 15). These studies have shown that the exocyclic amino groups of deoxyadenosine and deoxyguanosine are the principal sites of reaction and that opening of the dihydrodiol epoxide occurs both cis and trans at the benzylic position. We now extend our previous report on the mutational specificity of one of the configurational isomers of BcPhDE in a simian virus 40-based shuttle vector (16) to include all four configurational isomers in order to provide further information with which their relative biological activities might be clarified.

MATERIALS AND METHODS

Plasmid pS189 (17), human embryonic adenovirus-transformed kidney cells (Ad293) (18), and *Escherichia coli* strain MBM7070, which carries a *lacZ* amber mutation, were gifts from Michael M. Seidman (Otsuka Pharmaceutical, Rockville, MD). The four configurational isomers of BcPhDE were synthesized as described (19).

pS189 DNA (10 μg) in Tris·HCl buffer was treated with various amounts of each BcPhDE isomer. Based on calibration curves from work at higher levels of reaction, a solution of BcPhDE was prepared and diluted to yield solutions expected to modify 1 nucleotide in either 200, 300, 400, 500, or 1000 base pairs (bp) in the vector DNA. This DNA was then transfected into Ad293 cells, which were incubated for 48 h to allow replication of the vector. Vector DNA was isolated, digested with *Dpn* I to remove unreplicated DNA, and used to transform *E. coli* MBM7070 grown on ampicillin, 5-bromo-4-chloro-3-indolyl β-D-galactoside, and isopropyl β-D-thiogalactopyranoside to distinguish bacteria-containing vector with an intact *supF* gene (blue colonies) from those containing vector with a mutated *supF* gene (white and pale blue colonies). Bacteria from colonies with mutant genes were purified by restreaking and vector DNA was isolated and sequenced to define specific mutations. All of the procedures used were as described earlier (16), except that in some experiments vector DNA was isolated from human cells by a rapid alkaline extraction procedure (20). A hot spot was defined as a site at which the number of mutations found exceeded those expected from a Poisson distribution by a factor of 5 or more. The χ^2 goodness-of-fit test was applied to determine that the distribution of mutations over the 85-bp sequence for the mature tRNA was nonrandom.

RESULTS

A summary of the mutant classes obtained after treatment of the pS189 plasmid with each BcPhDE configurational isomer,

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Abbreviation: BcPhDE, benzo[*c*]phenanthrene dihydrodiol epoxide.

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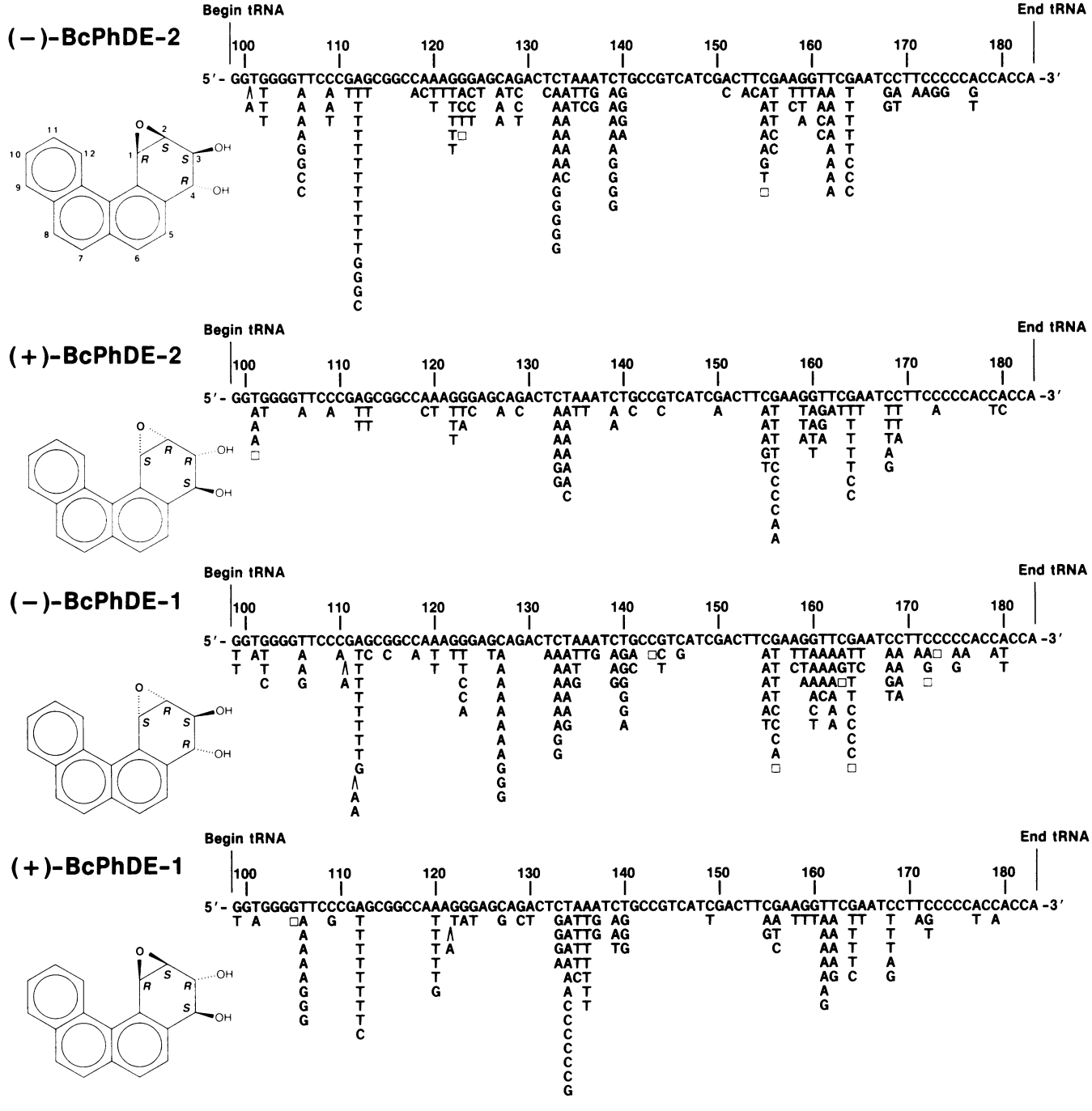

FIG. 1. Distribution of BcPhDE-induced point mutations in the *supF* DNA sequence. BcPhDE-induced mutations are shown below the sequence. ∧, Insertion; □, deletion. Eight of 10 spontaneous mutants contained single mutations as follows (number represents site): 123 G → C; 129 G → C,C,C; 133 C → G; 136 A → T; 156 G → T; 159 G → T. The pairs of mutations found in the remaining two mutants were as follows: 124 □ + 164 G → C; 167 T → A + 168 C → A. One hundred and twenty-three of 137, 70 of 80, 114 of 132, and 96 of 104 mutants induced by (−)-BcPhDE-2, (+)-BcPhDE-2, (−)-BcPhDE-1, and (+)-BcPhDE-1, respectively, contained a single mutation. The 50 remaining mutants, of which 48 contained two mutations, 1 contained three mutations, and 1 contained four mutations, are as follows: 1, (−)-BcPhDE-2-induced, 95 C → T + 112 A → T, 100–101 ∧A + 102 G → T, 102 G → T + 111 G → T, 102 G → T + 112 A → T, 109 C → A + 139 C → A, 109 C → T + 155 C → T, 118 C → A + 120 A → T, 121 A → T + 128 A → T, 124 G → T + 125 A → T, 127 C → A + 129 G → T, 151 A → C + 164 G → T, 153 T → A + 155 □, 154 T → C + 155 C → G, 174 C → G + 177 A → T; 2, (+)-BcPhDE-2-induced, 21 A → G + 164 G → C, 65 G → A + 123 G → T, 101 T → A + 134 T → A, 101 T → A + 134 T → A, 101 T → A + 134 T → A, 102 G → T + 179 C → T, 123 G → A + 150 G → A, 155 G → T + 163 C → T + 191 C → T, 165 A → T + 180 A → C; 3, (−)-BcPhDE-1-induced, 69 A → T + 165 A → T, 99 G → T + 102 G → C, 99 G → T + 164 G → T, 101 T → A + 134 T → A, 102 G → T + 126 G → T, 102 G → T + 180 A → T, 106 T → G + 140 T → A, 110 C → A + 112 A → T, 115 G → C + 180 A → T, 132 T → A + 162 T → A, 143 □ + 137 A → G, 146 C → G + 163 C → A, 155 C → T + 156 G → C, 162 T → A + 163 C → G, 169 C → A + 171 T → A, 172–176 □C + □C, 175 C → G + 110–111 ∧A, 175 C → A + 176 C → A; 4, (+)-BcPhDE-1-induced, 99 G → T + 164 G → C, 101 T → A + 134 T → A, 122 G → T + 121–122 ∧A, 124 G → T + 130 A → T, 136 A → T + 186 A → T, 139 C → T + 149 C → T + 155 C → G + 191 C → T, 161 T → A + 197 T → A, 179 C → A + 176–177 ∧AA. Additional point mutations occurring outside mature tRNA sequences and not listed above under multiple mutations are as follows: 1, (−)-BcPhDE-2-induced, 49 A → T; 2, (+)-BcPhDE-1-induced, 45 T → A, 50 T → A.

at doses estimated to yield approximately 5, 11, 13, 18, and 27 adducts per plasmid in each case, is presented in Table 1. (When the same mutation arose more than once from the same transfection it was counted as one independent mutation.) The first entry shows that the background mutations, resulting from examination of 465,872 revertants, were roughly equally distributed between point mutations (base substitutions or the insertion or deletion of 1 base pair) and mutations involving larger deletions, additions, or rearrangements. In contrast, the mutants that arose from treatment with the dihydrodiol epoxides were predominantly in the point mutation category and, at all doses, mutant frequency was significantly increased over background frequency ($P < 0.0001$). The frequency for point mutations (but not for other mutations) increased fairly convincingly with increasing adducts per plasmid. Thus, the values of the slopes of the regression lines, representing the point mutation frequency for one adduct per plasmid, and the corresponding correlation coefficients for the (−)-1, (+)-1, (−)-2, and (+)-2 isomers, respectively, were 1×10^{-4} ($r = 0.94$), 0.7×10^{-4} ($r = 0.93$), 0.4×10^{-4} ($r = 0.98$), and 0.3×10^{-4} ($r = 0.79$).

Although a few point mutations were deletions or insertions, 99%, 98%, 97%, and 94% for the (+)-2, (−)-2, (+)-1, and (−)-1 isomers, respectively, arose from the base substitutions shown in Table 2. The distribution of mutations within the six different base substitution categories did not vary significantly (by linear regression analysis) with dose for any of the four isomers. For example, slopes and correlation coefficients derived from plots of the sum of all G·C base substitution mutations as a percentage of total mutations versus adducts per plasmid are given in Table 3 (results for A·T pairs are the same except of opposite sign). In contrast, the slopes of regression lines for mutation frequency at G·C pairs and at A·T pairs versus adducts per plasmid (also in Table 3), like those for the overall mutation frequencies, indicated that these frequencies were mostly significantly positively correlated with dose—i.e., $r > 0.88$. The poor dose–response for mutation frequency at G·C pairs (and overall) for the (+)-2 isomer is largely attributable to the data obtained at the 100 ng/ml dose (Table 1), where mutation frequency at G·C pairs was ≈3 times that expected based on the other groups.

The differences in the distributions of mutations for each isomer (shown in Table 2) were mostly statistically significant. Thus, mutations at G·C pairs as a percentage of total base change mutations gave the following mean ± SD over the five doses used in each case: (+)-2, 77% ± 8%; (−)-1, 62% ± 10%; (−)-2, 53% ± 4%; (+)-1, 32% ± 13%. Comparison of these means by analysis of variance and Duncan's multiple range test indicated that, except for the comparison of the (−)-1 and (−)-2 isomers, all others were significantly different from one another ($P < 0.05$). The preference of most of the isomers for mutation at G·C pairs, but of the (+)-1 isomer for mutation at A·T pairs, was also apparent from the relative slopes in the frequency analyses in Table 3.

Differences in selectivities for mutation at G·C versus A·T pairs exhibited by the different BcPhDE enantiomers correlated reasonably well with the chemical selectivities measured earlier for reaction with guanine versus adenine residues in DNA (4) (Table 4). Although the correlation between preference for reaction at guanine versus adenine residues in DNA and preference for mutation at each purine was not perfect, it certainly suggested that most mutations were targeted to the adducts formed. For example, (+)-BcPhDE-1 is quite remarkable in that it reacts principally with adenine residues in DNA (14). As shown in Table 2, 68% of the mutations it induced were correspondingly at A·T pairs.

The stereochemical differences among the four configurational isomers not only determined different distributions of mutations among the 6 base substitution possibilities, as

Table 1. Analysis of spontaneous and BcPhDE-induced *supF* mutants

Inducing compound	BcPhDE, ng/ml	Mutant class		Point mutations,[a] frequency × 10^4
		Point	Other	
Solvent only	0	10[b]	13	0.2
(−)-BcPhDE-2	40	16[c]	2	1.6
	80	19[c]	0	4.1
	100	21	2	3.2
	135	23[d]	1	6.1
	200	58[e]	4	9.6
(+)-BcPhDE-2	40	10[f]	1	2.9
	80	12[c]	7	2.4
	100	7	3	8.6
	135	15[b]	2	4.4
	200	36[g]	4	8.1
(−)-BcPhDE-1	60	10[c]	0	3.3
	120	24[h]	0	5.2
	150	32[h]	3	7.5
	200	32[d]	1	11.2
	300	34[i]	0	28.0
(+)-BcPhDE-1	65	22[j]	6	3.4
	125	26[c]	1	7.8
	160	17	4	5.3
	210	23[b]	3	8.6
	315	16[b]	1	21.5

BcPhDE concentrations were estimated to substitute 1 in 1000, 500, 400, 300, and 200 bp in the 5337-bp plasmid—i.e., adducts per plasmid should be ≈5, ≈11, ≈13, ≈18, and ≈27, respectively. Point mutations are defined as base substitutions or insertions and deletions of 1 bp. Numbers of transformants analyzed averaged 40,235 at any dose of any isomer. Numbers from any particular group can be derived from the number of mutants and the frequencies given. Other mutations, defined as deletions, insertions >1 bp, and gross rearrangements, are listed below with numbers representing sites in the *supF* sequence. Deletions or insertions ≥120 bp were detected by agarose gel electrophoresis and were not sequenced. Spontaneous other mutations were as follows: *i*, deletion of bp 162 and 163 (□162–163); *ii*, □159–162; *iii*, □102–106; *iv*, □153–167; *v*, □148–167; *vi*, □84–107; *vii*, □123–147; *viii*, □78–129; *ix*, □43–108; *x*, insertion (∧) of 3 bp between positions 112 and 113; *xi*, ∧ of 40 bp between positions 118 and 119; *xii*, rearrangement (R) 3′ to position 106; *xiii*, R 3′ to position 121. BcPhDE-induced other mutations are listed for each compound in the order presented in the table—i.e., from lowest to highest dose. The (−)-BcPhDE-2-induced other mutations were as follows: *i*, □≥120 bp; *ii*, ∧≥120 bp; *iii*, □≥120 bp; *iv*, R 3′ to position 162; *v*, □144–152; *vi*, □73–160; *vii*, ∧ of 2 bp between positions 130 and 131; *viii*, □≥120 bp; *ix*, R 3′ to position 32. The (+)-BcPhDE-2-induced other mutations were as follows: *i*, ∧ of 21 bp between positions 69 and 70; *ii*, □126–132; *iii*, □50–100; *iv*, □43–137; *v*, □31–191; *vi*, □155–318; *vii*, ∧ of 75 bp between positions 161 and 162; *viii*, R 3′ to position 66; *ix*, □105–204; *x*, □167–313; *xi*, R 3′ to position 56; *xii*, □ of 3 bp between positions 142 and 147; *xiii*, □48–269; *xiv*, □159–161; *xv*, □ of 7 bp between positions 154 and 164; *xvi*, □33–227; *xvii*, □88–147. The (−)-BcPhDE-1-induced other mutations were as follows: *i*, □≥120 bp; *ii*, □≥120 bp; *iii*, R 3′ to position 31; *iv*, ∧ of 4 bp between positions 137 and 138. The (+)-BcPhDE-1-induced other mutations were as follows: *i*, R 3′ to position 98; *ii*, □117–121; *iii*, □159–165; *iv*, □145–153; *v*, □79–101; *vi*, □37–101; *vii*, □89–196; *viii*, □159–164; *ix*, □61–146; *x*, R 3′ to position 89; *xi*, R 3′ to position 38; *xii*, □135–136; *xiii*, □88–109; *xiv*, □77–128; *xv*, □142–147.

[a]Determined by dividing the number of mutants containing point mutations by the total number of transformants.
[b]Two mutants contained two point mutations each.
[c]One mutant contained two point mutations.
[d]Four mutants contained two point mutations each.
[e]Eight mutants contained two point mutations each.
[f]One mutant contained three point mutations.
[g]Six mutants contained two point mutations each.
[h]Three mutants contained two point mutations each.
[i]Seven mutants contained two point mutations each.
[j]Two mutants contained two point mutations each and one mutant contained four point mutations.

Table 2. Base-substitution mutations in the *supF* gene generated by replication of BcPhDE-treated and untreated pS189 vector in mammalian cells

Base change	(−)-BcPhDE-2	(+)-BcPhDE-2	(−)-BcPhDE-1	(+)-BcPhDE-1	Untreated
Transversions					
G·C → T·A	49 (33%)	35 (39%)	54 (38%)	16 (14%)	3 (27%)
G·C → C·G	24 (16%)	14 (16%)	26 (18%)	11 (10%)	6 (55%)
A·T → T·A	46 (31%)	18 (20%)	38 (27%)	55 (50%)	2 (18%)
A·T → C·G	10 (7%)	4 (4%)	9 (6%)	11 (10%)	0
Transitions					
G·C → A·T	6 (4%)	17 (19%)	10 (7%)	9 (8%)	0
A·T → G·C	13 (9%)	2 (2%)	4 (3%)	9 (8%)	0
Total	148	90	141	111	11

listed in Table 2, but also determined a different distribution of mutations through the target gene sequence. With the exception of 12 point mutations described in the legend to Fig. 1, the rest of the 506 mutations induced by the four BcPhDE isomers (these were identified by sequencing all 453 mutants, some of which had more than 1 mutation, in Table 1) were located within the 85-bp *supF* sequence coding for the mature tRNA as shown in Fig. 1. All the mutations from treatments at different doses were pooled for this analysis, assuming that distribution through the gene (like distribution through different base substitution types) was independent of dose.

The distributions of mutations over the target gene (Fig. 1) were nonrandom in each case and the sites and numbers of hot spots of mutation (sites with ≥5 times more mutations than expected from a Poisson distribution) varied with each dihydrodiol epoxide isomer. Hot spots were identified as follows: for (−)-BcPhDE-2, sites 106, 112, 133, 134, 139, 155, 162, and 164, each with seven or more mutations; for (+)-BcPhDE-2, sites 133, 134, 155, 156, 164, and 168, each with five or more mutations; for (−)-BcPhDE-1, sites 112, 127, 133, 134, 140, 155, 156, 160, and 164, each with six or more mutations; for (+)-BcPhDE-1, 106, 112, 120, 134, 135, 161, 162, 164, and 168, each with five or more mutations. Two sites—134 and 164—were hot spots for all isomers and three sites—112, 133, and 155—were hot spots for three of the four isomers. However, six sites—120, 127, 139, 140, 160, and 161—were hot spots for only one of the four isomers, so that overall the mutation spectra for each isomer were unique. It can also be shown from Fig. 1 that the trinucleotide sequence in which the central nucleotide is most frequently mutated is TAG for the (+)-1 and (+)-2 isomers, AGA for the (−)-2 isomer, and TGC for the (−)-1 isomer.

Table 3. Independence of mutation distribution and dependence of mutation frequency on dose level of dihydrodiol epoxide

BcPhDE isomer	(Change in % mutation at G·C)[a]/(adducts per plasmid)[c]	(Frequency of mutation × 10^4)[b]/(adducts per plasmid)[d]	
		G·C	A·T
(−)-2	0.3 (0.63)	0.22 (0.97)	0.16 (0.96)
(+)-2	−0.8 (−0.77)	0.13 (0.45)	0.10 (0.96)
(−)-1	−0.1 (−0.08)	0.64 (0.98)	0.54 (0.91)
(+)-1	0.1 (0.07)	0.29 (0.91)	0.52 (0.88)

[a]Slope of linear regression plot of percentage of total base change mutations at G·C pairs versus estimated number of adducts per plasmid. Correlation coefficients are given in parentheses.
[b]Slope of linear regression plot of frequency of mutation at G·C or A·T pairs versus estimated adducts per plasmid. Correlation coefficients are given in parentheses.
[c]Correlation coefficients indicate slopes are not significantly different from zero.
[d]Correlation coefficients indicate a significant positive correlation except for the (+)-2 isomer at G·C pairs.

DISCUSSION

The majority of the mutations found in this study were base substitutions even though the system used does detect insertions and small deletions (21). Particular advantages of the pS189 system are that there are few sites within the coding sequence for the tRNA where base substitutions are not expressed as mutations (21) and that the forward mutation system allows a more extensive mutation target to be explored than a reversion assay. In addition, since the lowest mutation frequency recorded in this work was 8 times background, the induced mutants analyzed should include very few spontaneous mutants.

The rank order of mutagenicity found [(−)-1 > (+)-1 > (−)-2 > (+)-2] was different from that reported in *Salmonella typhimurium* where (+)-BcPhDE-2 was the most active, and was also different from that found in a mammalian cell assay (13) or in tumorigenesis assays in mouse skin (12), where (−)-BcPhDE-2 was the most active. Since the shuttle vector comparison is made per adduct, the relative mutagenicities indicate that a dihydrodiol epoxide 1 adduct is 2–3 times more likely to yield a mutation than a dihydrodiol epoxide 2 adduct. The most likely reasons would be either that the dihydrodiol epoxides 1 react at more mutation-prone sites or that the conformation of the corresponding adducts more readily leads to base substitution, because of either less facile repair or more facile replication errors.

In the present studies and in our earlier chemical investigations (14, 15) where, in both cases, reaction between dihydrodiol epoxide and DNA occurred under analogous aqueous conditions, the findings parallel one another—i.e., the chemical preference for reaction at adenine or guanine residues is reflected in preferential mutation at either A·T or G·C pairs (Table 4). The extensive mutagenesis at A·T pairs, noted earlier for the (−)-2 isomer (16) and for racemic BcPhDE-2 (22), has not been seen with other hydrocarbons but it is consistent with reports of *ras* gene activation by a hydrocarbon at adenine residues in codon 61 (23). The (+)-1

Table 4. Mutagenic and chemical selectivity for adenine and guanine residues in DNA

BcPhDE	Mutagenic selectivity[a]		Chemical selectivity[b]	
	Adenine	Guanine	Adenine	Guanine
(−)-2	1.1	0.9	1.2	0.8
(+)-2	0.7	1.2	0.9	1.1
(−)-1	0.8	1.1	1.0	0.9
(+)-1	1.6	0.6	1.6	0.2

[a]The percentage of total mutations at A·T or G·C pairs was divided by the percentage of A·T or G·C pairs in the 85-bp sequence coding for the mature tRNA (41% A·T, 59% G·C) to yield mutagenic selectivities.
[b]Chemical selectivity was determined from previous data (4) by dividing the percentage reaction with A·T or G·C pairs in calf thymus DNA by the percentage occurrence of those pairs in the DNA (56% A·T, 44% G·C).

isomer is particularly effective at generating mutations at A·T pairs and it exhibits some similarities with melphalan in this regard (24).

For all isomers, irrespective of whether the target for mutation was a G·C or an A·T pair, the product of mutation was most frequently a T·A pair. This would arise as a consequence of inserting an adenine residue opposite either a hydrocarbon-guanine or a hydrocarbon-adenine adduct, in concert with the adenine insertion rule (25, 26). Some evidence for such a mechanism has been obtained by using model hydrocarbon adducts with DNA polymerase *in vitro* (27).

From the mutation spectra obtained by combining the data obtained at different doses, it is clear that each spectrum is unique, but that some similar features are present. Position 134 corresponding to the central base in the anticodon in the tRNA was a hot spot for all four isomers. With hydrocarbon derivatives that react preferentially with G·C pairs, the neighboring position 133 has been found to be a common hot spot (28). Position 164 in the pseudouridine loop also is a hot spot for most of the hydrocarbons studied so far (this work and ref. 28). While these findings suggest that these two locations are highly mutable, the importance of the specificity of the chemical mutagen is apparent from the presence of hot spots at position 133 or position 134 or both, depending on the mutagen used.

The most notable overall conclusion is that, as previously found for the chemistry of reactions of the configurational isomers of BcPhDE with DNA (4, 14, 15), the mutagenic specificity of each individual isomer is unique. Although we have not yet measured the sequence selectivity of the reaction of each isomer with the *supF* gene, it is likely that the latter together with the subtly different propensities of each isomer to form adducts by cis or trans opening of the epoxide upon reaction with a given nucleotide ultimately determine the differences in mutagenic specificities for the different isomers. Given that here we clearly demonstrate differences in mutagenic selectivity within a given gene, the *supF* gene, for different isomers, it should follow that these isomers may also be able to selectively mutate one gene versus another. Given the lack of correlation between overall mutagenicity and tumorigenicity for these isomers, it seems likely that differences in selectivity for mutation in key genes associated with initiation of tumorigenesis may be the major factor determining the relative tumorigenicities of these four isomers.

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