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Dose-dependent Differences in the Profile of Mutations Induced by (+)-7*R*,8*S*-Dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo-(*a*)pyrene in the Coding Region of the Hypoxanthine (Guanine) Phosphoribosyltransferase Gene in Chinese Hamster V-79 Cells[1]

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ABSTRACT

Chinese hamster V-79 cells were exposed to a high dose (0.30–0.48 μM; 32% cell survival), an intermediate dose (0.04–0.10 μM; 100% cell survival) or a low dose (0.01–0.02 μM; 97% cell survival) of (+)-7*R*,8*S*-dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene [(+)-BPDE] which is the ultimate carcinogenic metabolite of benzo(*a*)pyrene. The mutation frequency for cells treated with dimethyl sulfoxide vehicle or with low, intermediate or high doses of (+)-BPDE were 1, 10, 52 or 514 8-azaguanine-resistant colonies/10^5 survivors, respectively. Independent 8-azaguanine-resistant clones were isolated, and complementary DNAs were prepared by reverse transcription. The coding region of the hypoxanthine (guanine) phosphoribosyltransferase (*HPRT*) gene was amplified by the polymerase chain reaction and sequenced. Altogether, 368 (+)-BPDE-induced mutant clones were examined. At all doses, base substitutions were the most prevalent mutations observed (about 72% of the mutant clones), followed by exon deletions (about 26% of the mutant clones) and frame-shift mutations (about 6% of the mutant clones). At the high cytotoxic dose, 7 of 120 base substitutions occurred at AT base pairs (6%) and 113 at GC base pairs (94%). At the intermediate noncytotoxic dose, 20 of 82 base substitutions occurred at AT base pairs (24%) and 62 at GC base pairs (76%). At the low noncytotoxic dose, 27 of 76 base substitutions were at AT base pairs (36%) and 49 were at GC base pairs (64%). The results indicated that decreasing the dose of (+)-BPDE decreased the proportion of mutations at GC base pairs and increased the proportion of mutations at AT base pairs. As the dose of (+)-BPDE was decreased, there was a dose-dependent decrease in the proportion of GC→TA transversions (from 69% to 42% of the base substitutions) and a dose-dependent increase in the proportion of AT→CG transversions (from 1% to 25% of the base substitutions). The data also indicated dose-dependent differences in (+)-BPDE-induced exon deletions and hot spots for base substitutions at GC and AT base pairs.

Although more than 99% of the (+)-BPDE-induced mutations at guanine occurred on the nontranscribed strand of DNA, (+)-BPDE-induced mutations at adenine occurred on both the transcribed and nontranscribed strands. The ratio of mutations at adenine on the transcribed strand to mutations at adenine on the nontranscribed strand was 35:19 in (+)-BPDE-treated V-79 cells. These observations suggest different mechanisms of mutation induction at GC and AT base pairs and/or differences in repair mechanisms for premutagenic lesions at GC and AT base pairs. Several nucleotide sequences with frequent (+)-BPDE-induced mutations targeted at guanines in the coding region of the *HPRT* gene were identified. These included AGGGGGGC, TGGA, AGGA, TGGT, AGGC, TGGGA, AGGGA, and TGGGGA. Seventy % of all base substitution mutations targeted at guanine were on these sequences. (+)-BPDE-induced mutations in *ras* motifs (corresponding to *ras* codons 12, 13, 61) in the coding region of the *HPRT* gene were more commonly observed than by chance.

INTRODUCTION

Since mutations play an important role in carcinogenesis (1–4), studies on factors that influence the formation of mutations in endogenous genes should enhance our understanding of the etiology of cancer. Earlier studies from our laboratory demonstrated that (+)-BPDE[2] is the major ultimate carcinogenic metabolite of benzo(*a*)pyrene, and this optically active diol-epoxide was also shown to be the most potent mutagenic metabolite of benzo(*a*)pyrene at the *HPRT* locus in Chinese hamster V-79 cells (5–7). Studies with the 4 metabolically possible optically active bay-region 7,8-diol-9,10-epoxides of benzo(*a*)pyrene demonstrated that only (+)-BPDE was both a potent mutagen in V-79 cells and a potent carcinogen in mice (5–7).

We recently reported that 8-azaguanine-resistant mutant clones obtained from treating Chinese hamster V-79 cells with a low noncytotoxic dose (0.04 μM) of (+)-BPDE had a higher proportion of base substitution mutations at AT base pairs in the coding region of the *HPRT* gene than did mutant clones from cells treated with a high cytotoxic dose (0.48 μM) of (+)-BPDE (8). In this earlier study, we examined the types of mutations in ninety-two 8-azaguanine-resistant clones from V-79 cells treated with two doses of (+)-BPDE. In the present study, we have greatly extended our preliminary observations by analyzing the kinds of mutations in the coding region of the *HPRT* gene in three hundred sixty-eight 8-azaguanine resistant mutant clones obtained by treating V-79 cells with a high cytotoxic dose, an intermediate noncytotoxic dose, and a low noncytotoxic dose of (+)-BPDE. The results demonstrated dose-dependent differences in the profile of mutations induced by (+)-BPDE. The proportion of GC→TA transversions decreased and the proportion of AT→CG transversions increased when the dose of (+)-BPDE was decreased. Dose-dependent differences in hot spots for base substitutions and for exon deletions were also observed.

MATERIALS AND METHODS

Materials. All reagents, polymerase chain reaction primers, sequencing primers and enzymes were obtained and used as described previously (8). (+)-BPDE was synthesized as described previously (9) and handled under subdued light.

Exposure of V-79 Cells to (+)-BPDE and Isolation of 8-Azaguanine-resistant Mutant Clones. Chinese hamster V-79-4 cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured and exposed to (+)-BPDE for 1 h as described earlier (8, 10). Resistance to the lethal effects of the purine analogue 8-azaguanine was used as the mutagenic marker. The details for assessing cytotoxicity and isolating independent 8-azaguanine-resistant mutant clones after (+)-BPDE treatment have been described (8).

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[2] The abbreviations used are: (+)-BPDE, (+)-7*R*,8*S*-dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo(*a*)pyrene (benzylic hydroxyl group *trans* to the epoxide oxygen); HPRT, hypoxanthine (guanine) phosphoribosyltransferase; cDNA, complementary DNA; DMSO, dimethyl sulfoxide.

Table 1 *Mutagenicity and cytotoxicity of (+)-BPDE in Chinese hamster V-79 cells*

Chinese hamster V-79 cells were treated with a high (0.30–0.48 μM), intermediate (0.04–0.10 μM) or low (0.01–0.02 μM) dose of (+)-BPDE or with DMSO vehicle alone, and mutation frequency and cytotoxicity were determined as described in the text. Each number represents the mean ± SE from 8–16 experiments.

Parameter measured	High dose	Intermediate dose	Low dose	DMSO control
Mutations/10^5 surviving cells	514 ± 112	52 ± 13	10 ± 1	1.0 ± 0.2
% of cell survival	32 ± 3	100 ± 3	97 ± 1	100

Table 2 *Kinds of mutations in the coding region of the HPRT gene in (+)-BPDE-induced 8-azaguanine-resistant mutant clones*

Chinese hamster V-79 cells were treated with a high (0.30–0.48 μM), intermediate (0.04–0.10 μM) or low (0.01–0.02 μM) dose of (+)-BPDE or with DMSO vehicle alone. Independent 8-azaguanine-resistant clones were isolated, and mutations in the coding region of the *HPRT* cDNA were characterized by polymerase chain reaction amplification and direct DNA sequencing. Some mutant clones had more than one type of mutation.

Mutant clones	High dose	Intermediate dose	Low dose	DMSO control
No. analyzed	157	104	107	25
% with base substitutions	75	73	69	56
% with exon deletions	23	25	29	20
% with ±1 frame shifts	6	5	7	24

Preparation, Amplification, and Sequencing of *HPRT* cDNA. cDNA from each independent mutant clone was synthesized and amplified as described (8, 11). First-strand cDNA was prepared from mRNA in the crude cell lysate of 100–1000 cells by reverse transcription. Double-stranded cDNA was synthesized and amplified from the first-strand cDNA by two rounds of polymerase chain reaction with nested primer sets as described previously (8, 11). Amplified cDNA was sequenced directly by the dideoxy termination reaction (12) with Sequenase and three sequencing primers as described previously (8).

RESULTS

Dose-dependent Cytotoxic and Mutagenic Effects of (+)-BPDE. Treatment of Chinese hamster V-79 cells with a low dose (0.01–0.02 μM), an intermediate dose (0.04–0.10 μM), or a high dose (0.30–0.48 μM) of (+)-BPDE for 1 h resulted in 97, 100, and 32% cell survival, respectively, and the frequency of mutations (8-azaguanine resistant colonies/10^5 surviving cells) was increased 9-, 51-, and 513-fold, respectively, over the mutation frequency for control cells treated with DMSO (Table 1). The amount of DNA binding immediately after treatment of V-79 cells with 0.48, 0.04, or 0.01 μM [^{3}H]-(+)-BPDE for 1 h was 344, 35, or 10 adducts/10^7 nucleotides, respectively.[3]

Kinds of Mutations in the Coding Region of the *HPRT* Gene in (+)-BPDE-induced 8-Azaguanine-resistant Clones. Independent 8-azaguanine-resistant colonies (mutant clones) were isolated from (+)-BPDE-treated V-79 cells, cDNAs were prepared, and the coding region of the *HPRT* cDNA was sequenced. The kinds of mutations were determined for 157 mutant clones from the high dose group, 104 mutant clones from the intermediate dose group, and 107 mutant clones from the low dose group. Base substitution mutations in the coding region of the *HPRT* gene were the most common mutation observed and were found in 69–75% of the mutant clones analyzed (Table 2). Exon deletions (aberrant splicing mutations) were observed in 23–29% of the mutant clones analyzed and ±1 frame-shift mutations were observed in 5–7% of the mutant clones analyzed (Table 2). Varying the dose of (+)-BPDE did not influence the percentage of mutant clones with base substitutions, exon deletions, or ±1 frame-shift mutations (Table 2).

Dose-dependent Differences in (+)-BPDE-induced Base Substitutions in the Coding Region of the *HPRT* Gene. Decreasing the dose of (+)-BPDE decreased the proportion of base substitution mutations at GC base pairs and increased the proportion of base substitutions at AT base pairs (Table 3). At the high dose, 7 of 120 base substitutions occurred at AT base pairs (6%) and 113 at GC base pairs (94%). At the intermediate dose, 20 of 82 base substitutions occurred at AT base pairs (24%) and 62 at GC base pairs (76%). At the low dose, 27 of 76 base substitutions were at AT base pairs (36%) and 49 were at GC base pairs (64%). The results indicated that as the dose of (+)-BPDE was decreased, the proportion of mutations at AT base pairs was increased. The dose-dependent differences in mutations at GC and AT base pairs are statistically significant ($P < 0.0001$; χ^2 test). As the dose of (+)-BPDE was decreased, there was a dose-dependent decrease in the proportion of GC→TA transversions (from 69% to 42% of the total base substitutions) and a dose-dependent increase in the proportion of AT→CG transversions (from 1% to 25% of the base substitutions). The dose-dependent effects of (+)-BPDE on GC→TA and AT→CG transversions are statistically significant ($P < 0.0001$; χ^2 test). A smaller increase in the proportion of AT→GC transitions was also observed as the dose of (+)-BPDE was decreased. When additional base substitution data from aberrant splicing mutants were added to our results on base substitutions in the coding region of the *HPRT* gene, the percentage of total base substitutions as GC→TA transversions decreased from 71% for the high dose group to 43% for the low dose group, the percentage of total base substitutions as AT→CG transversions increased from 0.7% for the high dose group to 20% for the low dose group, and the percentage of total base substitutions as AT→GC transitions increased from 3.5% for the high dose group to 15% for the low dose group.[3]

Dose-dependent Differences in Hot Spots for (+)-BPDE-induced Mutations. An evaluation of the frequency of mutations at specific bases in the coding region of the *HPRT* gene indicated differences in the profile of hot spots induced by the different doses of (+)-BPDE (Fig. 1; Table 4). Hot spots were defined as target bases that had at least 3.3% of the total base substitutions at the dose level studied (significantly different from random mutations; $P < 0.01$). Eleven hot spots were observed for the high dose group, 7 hot spots were observed for the intermediate dose group, and 6 hot spots were observed for the low dose group. These results are consistent with more extensive damage of the *HPRT* gene with high doses of (+)-BPDE than with intermediate or low doses. Hot spots found in the high dose group that were not seen in the low dose group occurred at G-47, G-130, G-135, G-199, G-209, G-355, G-358, G-418, and G-569. Hot spots that were found in the low dose group that were not seen in the high dose group occurred at G-152, G-229, G-539, and T-578. A hot spot at G-634 was observed in all three dose groups.

Table 3 *Kinds of base substitutions in the coding region of the HPRT gene in (+)-BPDE-induced-8-azaguanine-resistant mutant clones*

Chinese hamster V-79 cells were treated with a high (0.30–0.48 μM), intermediate (0.04–0.10 μM) or low (0.01–0.02 μM) dose of (+)-BPDE or with DMSO vehicle alone. Independent 8-azaguanine-resistant clones described in Table 2 were examined for base substitution mutations. Numbers in parentheses represent the percentage of total base substitutions.

	No. of mutations observed							
Mutations	High dose		Intermediate dose		Low dose		DMSO control	
At G·C base pairs								
G·C → T·A	83	(69)	43	(52)	32	(42)	2	(14)
G·C → C·G	23	(19)	12	(15)	11	(14)	0	(0)
G·C → A·T	7	(6)	7	(9)	6	(8)	1	(7)
Total	113	(94)	62	(76)	49	(64)	3	(21)
At A·T base pairs								
A·T → G·C	4	(3)	8	(10)	5	(7)	10	(71)
A·T → T·A	2	(2)	6	(7)	3	(4)	0	(0)
A·T → C·G	1	(1)	6	(7)	19	(25)	1	(7)
Total	7	(6)	20	(24)	27	(36)	11	(79)

[3] Unpublished observations.

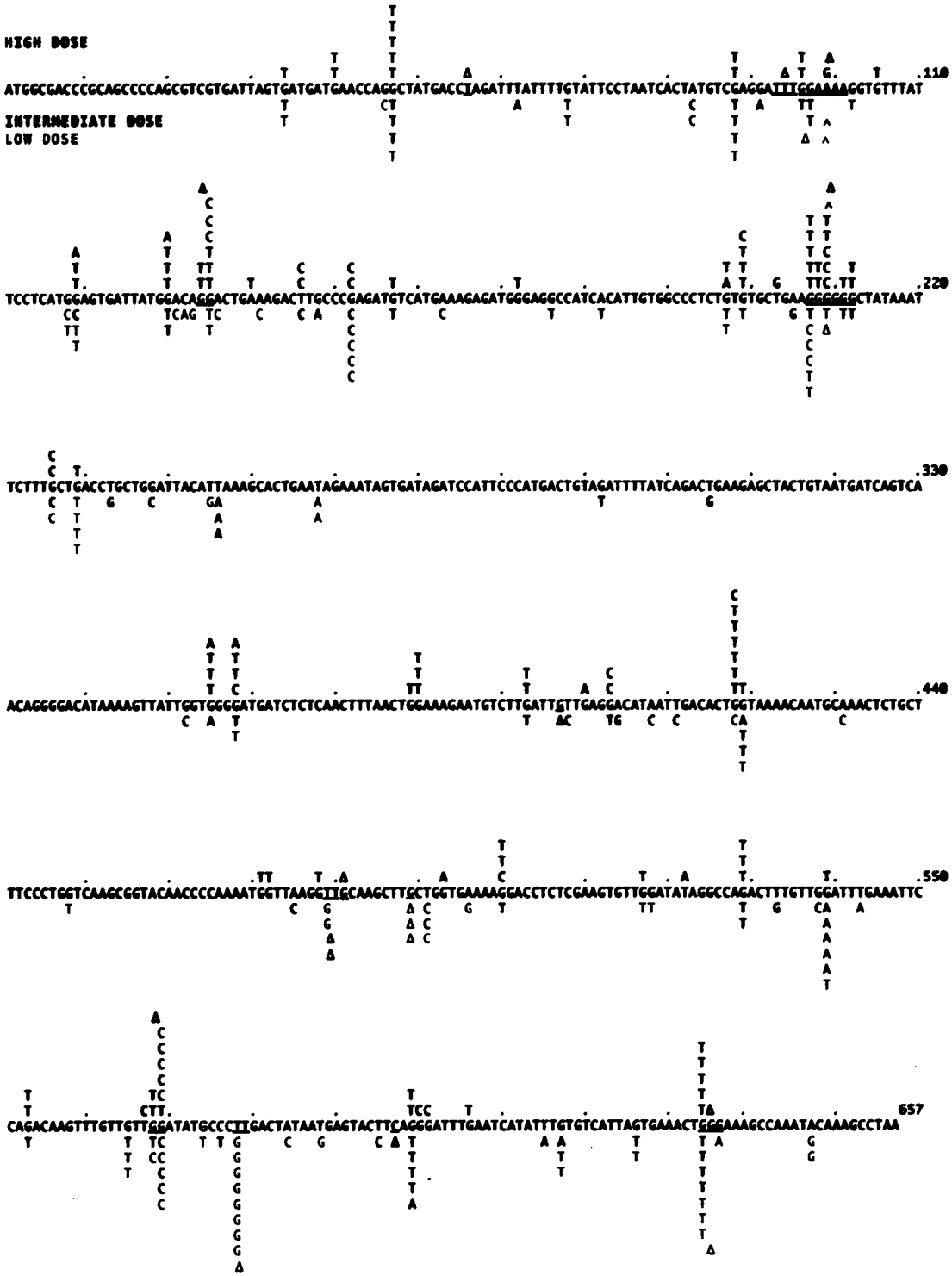

Fig. 1. (+)-BPDE-induced mutations in the coding region of the *HPRT* gene in V-79 cells. Mutations obtained from the high dose of (+)-BPDE (0.30–0.48 μM) are shown in *green* above the wild-type sequence, mutations from the intermediate dose of (+)-BPDE (0.04–0.10 μM) are shown in *reddish brown* below the wild-type sequence, and mutations from the low dose of (+)-BPDE (0.01–0.04 μM) are shown in *blue* below the wild-type sequence. The numbers indicate the nucleotide positions relative to the first base of the start codon. The letters used for the base substitutions indicate the new bases found in the *HPRT* mutant clones. Caret (^) indicates a single base addition; indicates a single base deletion in the region *underlined*.

It was of interest that more hot spots were observed for the high dose group than for the intermediate or low dose groups and that hot spots observed in the high dose group were more likely to have multiple base substitutions than hot spots in the intermediate or low dose groups (Table 4; Fig. 1). Eight of the 11 hot spots (73%) from the high dose (+)-BPDE group had multiple base substitutions whereas only 4 of 13 (31%) hot spots from the combined intermediate and low dose groups had multiple base substitutions (Table 4).

Strand Distribution for Base Substitutions in the Coding Region of the *HPRT* Gene of V-79 Cells Treated with (+)-BPDE. Examination of the strand distribution for (+)-BPDE-induced base substitutions revealed that 222 of 224 mutations at guanine (99%) were on the nontranscribed strand (Table 5). In marked contrast to these observations, 35 of 54 (+)-BPDE-induced mutations at adenine (65%) occurred on the transcribed strand (Table 5). Although the ratio of adenine to guanine mutations increased with decreasing dose, the

Table 4 *Hot spots for (+)-BPDE-induced mutations in the HPRT gene*

Dose	No. of base substitutions analyzed	Hot spot[a]	% of total base substitutions	Type of base substitution	Target sequence in nontranscribed strand[b] 5'→3'	Purine-containing putative target strand	Codon no. of *ras* sequence with homology to the HPRT hot spot sequence
High (0.30–0.48 μM)	120	G-47 (5×)	4.2	G→T (5×)	CC**AGGC**TAT	NT	H-13 (mouse)
		G-130 (4×)	3.3	G→T (3×); G→A (1×)	TA**TGGA**CAG	NT	H-12 (mouse)
		G-135 (6×)	5.0	G→T (3×); G→C (3×)	AC**AGG**ACTG	NT	H-12 and H-13 (mouse)
		G-199 (4×)	3.3	G→T (3×); G→C (1×)	CTG**TGT**GCT	NT	N-61 (human and mouse)[c]
		G-207 (5×)	4.2	G→T (5×)	TGA**AGGGGG**	NT	H-13 (mouse); N-12 (human and mouse)
		G-209 (5×)	4.2	G→C (3×); G→T (2×)	AAGGGGGGC	NT	None
		G-355 (4×)	3.3	G→T (3×); G→A (1×)	**TGGTGG**GGA	NT	K-13 (mouse)
		G-358 (4×)	3.3	G→T (2×); G→A (1×); G→C (1×)	TGG**GGA**TGA	NT	H-12 (mouse)
		G-418 (7×)	5.8	G→T (6×); G→C (1×)	CA**CTGGT**AA	NT	K-12 (mouse)
		G-569 (6×)	5.0	G→C (5×); G→T (1×)	GT**TGGA**TAT	NT	H-12 (mouse)
		G-634 (5×)	4.2	G→T (5×)	AA**CTGGG**AA	NT	H-12 (mouse); K-12 (human and mouse)
Intermediate (0.04–0.10 μM)	82	G-47 (3×)	3.7	G→T (3×)	CC**AGGC**TAT	NT	H-13 (mouse)
		G-88 (3×)	3.7	G→T (3×)	TGTC**GAGG**A	NT	H-12 (mouse)
		T-246 (3×)	3.7	T→A (3×)	ACATTAAAG	T	None
		G-419 (3×)	3.7	G→T (2×); G→A (1×)	A**CTGGT**AAA	NT	K-12 (human and mouse)
		G-569 (4×)	4.9	G→C (4×)	GT**TGGA**TAT	NT	H-12 (mouse)
		G-599 (5×)	6.1	G→T (4×); G→A (1×)	TT**CAGG**GAT	NT	N-12 (human and mouse); H-61 (human)
		G-634 (4×)	4.9	G→T (4×)	AA**CTGG**GAA	NT	H-12 (mouse); K-12 (human and mouse)
Low (0.01–0.02 μM)	76	G-152 (3×)	3.9	G→C (3×)	GCCC**GAG**AT	NT	H-12 (mouse)
		G-207 (5×)	6.6	G→C (3×); G→T (2×)	TGA**AGGGGG**	NT	H-13 (mouse); N-12 (human and mouse)
		G-229 (4×)	5.3	G→T (4×)	TGC**TGACCT**	NT	K-61 (human and mouse)[c]; H-61 (mouse)[c]
		G-539 (6×)	7.9	G→A (5×); G→T (1×)	GT**TGGA**TTT	NT	H-12 (mouse)
		T-578 (8×)	10.5	T→G (8×)	GCC**CTTGAC**	T	K-61 (human and mouse)[c]; H-61 (mouse)[c]
		G-634 (3×)	3.9	G→T (3×)	AA**CTGG**GAA	NT	H-12 (mouse); K-12 (human and mouse)

[a] A hot spot is defined as a target base with at least 3.3% of the total base substitutions at the dose studied (significantly different from random mutations; $P<0.01$). Numbers in parentheses, number of occurrences of mutations. NT and T, nontranscribed and transcribed strands, respectively.

[b] Nucleotides that were mutated are shown in **bold type** and sequence homologies to *ras* hot spot sequences are underlined.

[c] The homologous sequence is on the transcribed strand.

Table 5 *Strand distribution for base substitutions in the coding region of the HPRT gene in (+)-BPDE-induced 8-azaguanine-resistant mutant clones*

Chinese hamster V-79 cells were treated with a high (0.30–0.48 μM), intermediate (0.04–0.10 μM) or low (0.01–0.02 μM) dose of (+)-BPDE or with DMSO vehicle alone. Independent 8-azaguanine-resistant clones described in Table 2 were examined for base substitutions at guanine and adenine in the transcribed and nontranscribed strands.

Putative target strand for mutations	No. of base substitutions observed			
	High dose	Intermediate dose	Low dose	DMSO control
At guanine				
Transcribed strand	0	1	1	0
Nontranscribed strand	113	61	48	3
Total	113	62	49	3
At adenine				
Transcribed strand	5	14	16	0
Nontranscribed strand	2	6	11	11
Total	7	20	27	11

ratio of adenine mutations on the transcribed/nontranscribed strand was independent of dose, and essentially all of the guanine mutations were found on the nontranscribed strand at all doses. The strand distribution for base substitutions at adenine in the DMSO-treated control group was very different from that in the (+)-BPDE-treated groups. All 11 base substitutions at adenine in the DMSO-treated control group occurred on the nontranscribed strand (Table 5). These results with DMSO-treated V-79 cells differ from earlier studies which indicate that spontaneous mutations at adenine were equally divided between the transcribed and nontranscribed strands (13).

Dose-dependent Differences in Exon Deletions in the Coding Region of the *HPRT* Gene. Thirty-six exon deletion mutations were observed in the high dose (+)-BPDE group, 26 were observed in the intermediate dose group, 31 were observed in the low dose group, and 11 were observed in the DMSO control group (Table 6). Comparison of exon deletions in V-79 cells treated with a high or low dose of (+)-BPDE suggested a smaller proportion of exon 2 and 5 deletions in the low dose group and an increased proportion of exon 4 and 7 deletions in the low dose group when compared with the high dose group. The differences in the distribution of exon deletions at exon 2, 4, 5, 7, and 9 among the three dose groups are statistically significant ($P = 0.02$, Fisher exact test).

Preferential (+)-BPDE-induced Base Substitution Mutations at *ras* Hot Spot Sequences in the Coding Region of the *HPRT* Gene. It is of interest that all but 2 of the hot spots for (+)-BPDE-induced base substitutions described in Table 4 are located in nucleotide sequences associated with codons 12, 13, or 61 of human or mouse H-, K-, or N-*ras* protooncogenes (Table 4). Ninety-three % of the base substitution mutations (101 of 109) described in Table 4 occurred in motifs (≥3 nucleotides) associated with a *ras* hot spot. The results of additional analysis of base substitutions in the coding region of the *HPRT* gene for all doses of (+)-BPDE combined indicated that 88% of all (+)-BPDE-induced base substitution mutations (245 of 278) occurred in a motif (≥3 nucleotides) with homology to a *ras* hot spot and adjacent bases. Only 61% of the mutable bases (345 of 567) in the coding region of the *HPRT* gene are in a motif (≥3 nucleotides) with homology to a *ras* hot spot and adjacent bases. The data indicate that (+)-BPDE has a preference for causing mutations in motifs in the

Table 6 *(+)-BPDE-induced exon deletions in the coding region of the HPRT cDNA in Chinese hamster V-79 cells*

Chinese hamster V-79 cells were treated with a high (0.30–0.48 μM), intermediate (0.04–0.10 μM) or low (0.01–0.02 μM) dose of (+)-BPDE or with DMSO vehicle alone. Independent 8-azaguanine-resistant clones described in Table 2 were examined for exon (E) deletions in the coding region of the *HPRT* cDNA. Numbers in parentheses, percentage of exon deletions.

Exon deletions	Base no.	No. of exon deletions observed							
		High dose		Intermediate dose		Low dose		DMSO control	
E1	1–27	1	(3)	0	(0)	0	(0)	0	(0)
E2	28–134	8	(22)	7	(27)	4	(13)	3	(27)
E3	135–318	2	(6)	0	(0)	1	(3)	3	(27)
E4	319–384	4	(11)	3	(12)	12	(39)	3	(27)
E5	385–402	10	(28)	2	(8)	4	(13)	1	(9)
E6	403–485	1	(3)	2	(8)	0	(0)	0	(0)
E7	486–532	4	(11)	2	(8)	7	(23)	1	(9)
E8	533–609	2	(6)	5	(19)	0	(0)	0	(0)
Part of E9	610–626	4	(11)	5	(19)	3	(10)	0	(0)
Total		36	(100)	26	(100)	31	(100)	11	(100)

coding region of the *HPRT* gene that have sequence homology to codons 12, 13, and 61 of H-, K-, and N-*ras* protooncogenes ($P < 0.0001$).

Nucleotide Sequences with Frequent (+)-BPDE-induced Mutations in the Coding Region of the *HPRT* Gene. Examination of all 278 (+)-BPDE-induced base substitutions (for all doses combined) in the coding region of the *HPRT* gene revealed several sequences that are frequent targets for mutagenesis (Fig. 1). A particularly strong hot spot region for (+)-BPDE-induced mutations at guanine is AGGGGGGC (G-207 to G-212). Twenty-four base substitutions, as well as two single base deletions and one single base addition, were observed in this string of guanines. Other nucleotide sequences that have many (+)-BPDE-induced base substitution mutations targeted at guanines include TGGA, AGGA, TGGT, AGGC, TGGGA, AGGGA, and TGGGGA (Fig. 1; Table 7). Mutations at guanine in the above 8 sequences accounted for 56% of the total base substitutions and 70% of the substitutions at guanine. Additional information on frequently mutated sequences is given in Table 8. The data in Table 8 indicate specific hot spots for (+)-BPDE-induced base substitution mutations in nucleotide tetramers that appear in the coding region of the *HPRT* gene. GGTA, CTGG, CGAG, GGCT, AGGG, and TGGG (mutated base is *underlined*) were particularly good G hot spots and CTTG was an excellent A hot spot. The putative target sequence for the A hot spot is CAAG.

DISCUSSION

Although experimental carcinogenesis and mutagenesis studies are often done with high doses of chemicals, humans and other organisms are usually exposed to only small amounts of carcinogens in their natural environment. The present study was done to evaluate the mutagenic profile of low, intermediate, and high doses of (+)-BPDE at the *HPRT* locus of Chinese hamster V-79 cells. In this study, we treated V-79 cells with a high cytotoxic dose (0.30–0.48 μM; 32% cell survival), an intermediate noncytotoxic dose (0.04–0.10 μM; 100% cell survival), or a low noncytotoxic dose (0.01–0.02 μM; 97% cell survival) of (+)-BPDE. When independent 8-azaguanine-resistant clones were isolated for molecular analysis of the mutation profile, we observed dose-dependent differences in the spectrum of mutations induced by (+)-BPDE. At the high cytotoxic dose of (+)-BPDE, most base substitutions were at GC base pairs (predominantly GC→TA transversions) with only an occasional mutation observed at an AT base pair (Table 3). As the dose of (+)-BPDE was decreased, however, there was a dose-dependent increase in the proportion of base substitutions observed at AT base pairs and a corresponding decrease in the proportion of base substitutions at GC base pairs (Table 3). The percentage of base substitutions observed at AT base pairs was 6% for the high dose group, 24% for the intermediate dose group, and 36% for the low dose group (Table 3). As the dose of (+)-BPDE was decreased, there was a dose-dependent decrease in the proportion of GC→TA transversions and a dose-dependent increase in the proportion of AT→CG transversions. Additional observations indicated the presence of dose-dependent differences in exon deletions (Table 6) and dose-dependent differences in hot spots for base substitutions (Fig. 1; Table 4). Examination of the mutational profile for the DMSO control group indicated that it was different from that observed for the (+)-BPDE groups (Tables 3 and 6). The results of the present study, which analyzes 368 independent mutants from (+)-BPDE-treated V-79 cells, confirm and greatly extend the results of our earlier preliminary study that indicated dose-dependent differences in the profile of (+)-BPDE-induced mutations in V-79 cells (8). A recent report on the mutational profile of cytotoxic doses of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in the coding region of the *HPRT* gene of human fibroblasts also suggests dose-dependent effects (14). Treatment of the cells with a very high cytotoxic dose of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10–12 μM; 8–15% cell survival) resulted in base substitution mutations predominantly at GC base pairs whereas cells treated with a lower cytotoxic dose (6–8 μM; 26–40% cell survival) had an increased proportion of mutations at AT base pairs.

Previous studies on the mutational specificity of racemic BPDE in mammalian cells were done with a shuttle vector in COS (a SV40-transformed monkey kidney cell line) (15) and human (16) cells, with the endogenous adenine phosphoribosyltransferase (17) and dihydrofolate reductase (18) genes in Chinese hamster ovary cells, and with the endogenous *HPRT* gene in human fibroblasts (19–21). The studies with endogenous mammalian genes (17–21) were done utilizing high cytotoxic doses of racemic BPDE, and mutations observed in these studies were predominantly at GC base pairs (87–100% of the base substitution mutations). The major mutations observed were GC→TA transversions, which are similar to the results of our present study with (+)-BPDE (Table 3). Similar results were obtained in a recent study on the effect of a high cytotoxic dose of (+)-BPDE on the *HPRT* gene in human T-lymphocytes (22).

An important question is the nature of the premutagenic lesions that occur after treating cells with (+)-BPDE. Major adducts of (+)-

Table 7 *Nucleotide sequences with frequent (+)-BPDE-induced base substitution mutations in the coding region of the HPRT gene*

Two hundred seventy-eight (+)-BPDE-induced base substitutions from all doses were evaluated. Mutations at Gs in the indicated sequence were counted.

Target sequence	No. of sites available	No. of sites with mutations	No. of mutations observed	% of total base substitutions
AGGA	4	4	20	7.2
AGGC	3	2	11	4.0
TGGA	8	8	52	18.7
TGGT	5	5	20	7.2
AGGGA	1	1	9	3.2
TGGGA	2	2	14	5.0
TGGGGA	1	1	11	4.0
AGGGGGGC	1	1	24	8.6

Table 8 *Hot spots for (+)-BPDE-induced base substitution mutations in nucleotide tetramers in the coding region of the HPRT gene*

Two hundred seventy-eight (+)-BPDE-induced base substitutions from all doses were evaluated.

Target sequence[a]	Positions of putative target base[b]	No. of sites available	No. of mutations observed	No. of mutations observed/site
GGTA	418	1	8	8.0
CTGG	379, 418, 634	3	21	7.0
CGAG	88, 152	2	13	6.5
GGCT	47, 212	2	12	6.0
AGGG	207, 334, 599	3	18	6.0
TGGG	171, 355, 634	3	17	5.7
GGTA	419	1	5	5.0
GCTG	229	1	5	5.0
GGTG	355	1	5	5.0
GTGG	355	1	5	5.0
GTGC	199	1	5	5.0
GGGG	207, 208, 209, 334, 355	5	24	4.8
TGGA	97, 119, 130, 238, 380, 518, 539, 569	8	37	4.6
CAGG	47, 135, 335, 600	4	17	4.3
TTGG	97, 353, 518, 539, 569	5	21	4.2
GGAT	91, 238, 358, 518, 539, 569, 601	7	28	4.0
GGGG	209, 211	2	8	4.0
GATA	268, 518, 569	3	12	4.0
AAGG	102, 207, 477, 500	4	16	4.0
GGGA	171, 335, 356, 599, 634	5	19	3.8
ACTG	139, 289, 317, 379, 418, 634	6	21	3.5
CCCG	11, 152	2	7	3.5
AGGG	209, 601	2	7	3.5
GGAC	130, 135, 337, 403, 501	5	17	3.4
AGGC	47, 176, 525	3	10	3.3
TGGT	352, 418, 471	3	10	3.3
GGGC	212	1	3	3.0
GGAG	119, 173	2	6	3.0
AGGA	91, 135, 403, 501	4	12	3.0
TGTG	187,197, 199, 617	4	12	3.0
CTTG	146, 391, 487, 578	4	11	2.8

[a] Frequently mutated bases in the nontranscribed strand are underlined.

[b] Positions of the putative target bases are as shown in Fig. 1. Mutated bases are underlined.

BPDE with the exocyclic amino group of guanine and to a considerably lesser extent with the exocyclic amino group of adenine in DNA are known to occur (Refs. 23 and 24 and references therein). In addition, other minor adducts from the benzo(*a*)pyrene diol-epoxides that have been proposed include those at N^7 of guanine (24, 25), phosphotriester adducts (26), and cytosine adducts (24, 27). It is not known which, if any, of the above adducts are responsible for the transformation of cells. Recent studies in our laboratory and by others have shown that addition of (+)-BPDE to Chinese hamster V-79 cells or to calf thymus DNA resulted in high selectivity for covalent binding to guanine (24, 28).[3] About 98% of the DNA adducts from (+)-BPDE were with guanine (at the exocyclic amino group) and 2–3% of the adducts were with adenine (at the exocyclic amino group). The effect of dose of (+)-BPDE on the distribution of these adducts is currently under investigation.

There are several possible reasons for differences in the spectrum of mutations that occur after treating V-79 cells with different doses of (+)-BPDE. One possible explanation would be dose-dependent differences in the covalent binding of (+)-BPDE to DNA bases [Is the ratio of adenine adducts to guanine adducts higher in cells treated with a low dose of (+)-BPDE than in cells treated with a high dose?]. Another possible explanation would be dose-dependent differences in repair activities for the removal of guanine and adenine adducts from DNA [Does a high dose of (+)-BPDE selectively induce the removal of adenine adducts to a greater extent than guanine adducts? Does a high dose of (+)-BPDE inhibit cell division and DNA replication, thereby allowing more time for selective repair of adenine adducts? Is repair activity for guanine adducts saturated or inactivated at the high dose of (+)-BPDE, thereby increasing the proportion of stable guanine adducts at high dose levels?]. A third possible explanation would be dose-dependent differences in the death of cells with adenine adducts [Does the high dose of (+)-BPDE cause preferential death of cells with adenine adducts?]. An additional explanation would be dose-dependent differences in the kinds of cellular lesions induced by (+)-BPDE [perhaps (+)-BPDE-induced DNA adducts are important premutagenic lesions at high dose levels and (+)-BPDE-induced damage of proteins that participate in faithful replication and/or repair may become increasingly more important for generating mutations at low doses (*i.e.*, modulation of DNA repair enzymes or fidelity of DNA polymerases)]. More studies are needed to determine whether these or other mechanisms are responsible for the dose-dependent differences in mutational spectra that were observed. It is of interest to note the results of an earlier study by Pegg and Hui that indicated dose-dependent differences in the O^6-methylguanine/N^7-methylguanine ratio in liver DNA at 4–24 h after treating rats i.p. with different doses of nitrosodimethylamine (29). The O^6-methylguanine/N^7-methylguanine ratio was about 10-fold higher with a high dose (10 mg/kg i.p.) than with a low dose (<0.5 mg/kg i.p.) of nitrosodimethylamine. These results were attributed to more extensive repair of O^6-methylguanine adducts at the low dose than at the high dose.

It is of considerable interest that virtually all (>99%) of the (+)-BPDE-induced mutations at guanine occurred on the nontranscribed strand (Table 5) which suggests efficient preferential repair of the transcribed strand. Marked strand bias for mutations at guanine was observed for all doses of (+)-BPDE. Strand specificity for diol-epoxide-induced mutations at guanine in the coding region of endogenous genes in mammalian cells has been reported earlier for racemic BPDE (17–19, 21) and more recently for (+)-BPDE (22). Preferential repair of racemic BPDE adducts in the transcribed strand of the *HPRT* gene of human fibroblasts has also been reported (30), and strand bias for mutations at guanine was markedly decreased in excision repair-

deficient cells (20). The results of our studies and those by others (cited above) strongly suggest that the observed strand specificity for (+)-BPDE-induced mutagenesis results from preferential excision repair of (+)-BPDE-guanine adducts on the transcribed strand. The results of our studies indicating that (+)-BPDE-induced mutations at adenine occurred on both the transcribed and nontranscribed strand were unexpected and novel. Indeed, the ratio of (+)-BPDE-induced mutations at adenine on the transcribed strand to that on the nontranscribed strand was almost 2/1 (Table 5). Assuming that (+)-BPDE-induced premutagenic lesions are on purines, our results suggest that repair mechanisms for (+)-BPDE-adenine adducts in DNA are different from those for (+)-BPDE-guanine adducts and that (+)-BPDE-adenine adducts are less efficiently removed from the transcribed strand than (+)-BPDE-guanine adducts. A possible alternative explanation for differences in strand specificity for mutations at guanine and adenine is that mutagenesis at GC base pairs is caused predominantly by (+)-BPDE-guanine adducts whereas mutations at AT base pairs may result from other kinds of (+)-BPDE-induced cellular changes that are independent of covalent binding to DNA (altered DNA polymerase fidelity, changes in DNA repair activity etc.).

Examination of 278 (+)-BPDE-induced base substitutions in V-79 cells revealed hot spot regions with very high (+)-BPDE-induced mutagenic selectivity. We found that guanines in TGGA, TGGT, AGGA, AGGC, AGGGA, TGGGA, TGGGGA, and AGGGGGGC were particularly excellent targets for (+)-BPDE-induced mutations (Table 7; Fig. 1). Mutations at guanines in the above 8 sequences accounted for 56% of all base substitutions and 70% of the substitutions at guanine. It is of considerable interest that TGGA is a particularly good target for (+)-BPDE-induced mutations (18.7% of total base substitutions; Table 7) and that this sequence corresponds to that of codon 12 and the last base of codon 11 in the mouse H-*ras* protooncogene. The high mutagenic selectivity for the above eight sequences could be the result of preferential covalent binding of (+)-BPDE, since polycyclic aromatic hydrocarbon diol-epoxides are known to target purine-rich sequences in DNA (31). Additional studies indicated that a guanine residue with an adjacent guanine or cytosine was a preferential target for (+)-BPDE binding in 8 and 10 base oligonucleotides (32). The prevalence of mutations at runs of guanine is in agreement with other studies on the effect of racemic BPDE on endogenous mammalian genes (17, 18, 21). In these studies, hot spot regions were reported to be $AG(G)_nGA$ in the adenine phosphoribosyltransferase gene (17) and AGG or GGA in the dihydrofolate reductase gene of Chinese hamster ovary cells (18) and a run of six Gs in the *HPRT* gene of human fibroblasts (21). In a recent study with (+)-BPDE, an AGG sequence was identified as a hot spot region in the *HPRT* gene of human T-lymphocytes (22). Although the above studies identified hot spot regions for racemic BPDE or (+)-BPDE-induced mutations in the adenine phosphoribosyltransferase, dihydrofolate reductase, and *HPRT* genes in cells treated with high cytotoxic doses of the diol-epoxide, no specific bases were identified as hot spots.

The results of our study identified several bases in the coding region of the *HPRT* gene that are hot spots for (+)-BPDE-induced base substitution mutations (Table 4). It was of interest that more hot spots were observed for the high dose group than for the intermediate and low dose groups, and the location of hot spots appeared to depend on the dose. For some hot spots there was only one kind of base substitution whereas for other hot spots there was more than one kind of base substitutions (Fig. 1; Table 4). For example, only G→T transversions were observed at G-47, while both G→C and G→T transversions were detected at G-135. Different types of substitutions induced by (+)-BPDE at the same target base could result from (*a*) different premutagenic adducts on the same base, (*b*) a single premutagenic adduct that results in multiple base pairing due to several different conformations that are possible for the modified base, (*c*) the same or different premutagenic adducts on the base adjacent to the mutated base, and (*d*) the modulation of the fidelity of DNA polymerase or DNA repair enzymes. Examples of a single adduct that can induce several different base substitutions have been reported. An O^6-benzylguanine at the second base of codon 12 of H-*ras* in a plasmid induced G→C, G→T and G→A mutations at the same position when the plasmid was transfected into Rat 4 cells (33); 8-hydroxyguanine at the second position of codon 12 in a synthetic c-H-*ras* gene induced G→T, G→C and G→A mutations at the modified site as well as G→T and G→A mutations at the first base of codon 12 when the plasmid was transfected into NIH3T3 cells (34). In addition, the adduct formed on *trans*-opening of (+)-BPDE at C-10 by the exocyclic N^2-amino group of deoxyguanosine at G-437 in plasmid pUC 19 induced both G→T and G→C transversions at the modified site when the plasmid was replicated in *Escherichia coli* (35). Our findings that some mutagenic hot spots had a single kind of substitution and some had multiple kinds of substitutions suggest the presence of several mutagenic mechanisms.

Analysis of our data indicated that (+)-BPDE-induced mutations in the coding region of the *HPRT* gene had a marked preference for motifs with sequence homology to codons 12, 13, and 61 of H-, K-, and N-*ras* (Table 4). The results of *in vivo* studies indicate the presence of activated *ras* oncogenes in tumors from mice treated with benzo(*a*)pyrene. Although the number of tumors examined in these studies was quite small, G→T transversions were the most prominent mutation, and G→A and A→T mutations were also observed (36–38). There is a need for additional studies on the profile of mutations in tumors obtained from animals treated with benzo(*a*)pyrene and (+)-BPDE. Although the results of our studies indicate dose-dependent differences in the mutagenic profile of a carcinogen in cultured cells, it is not known whether there are dose-dependent differences in the mutational spectra of carcinogens *in vivo* in animals. The finding that there are dose dependent differences in the O^6-methylguanine/N^7-methylguanine ratio in liver DNA from rats treated with nitrosodimethylamine (29) suggests that dose-dependent differences in the mutational profile of chemical carcinogens may exist *in vivo*. It would be of interest to determine whether there are dose-dependent differences in the profile of mutations in oncogenes and tumor suppressor genes in tumors obtained from animals and to determine whether or not the profile of mutations described in the present report will be similar to that in animals or humans exposed to benzo(*a*)pyrene.

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