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NOTES

Kinds of Mutations Formed when a Shuttle Vector Containing Adducts of Benzo[a]pyrene-7,8-Diol-9,10-Epoxide Replicates in COS7 Cells

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We have investigated the kinds of mutations induced when a shuttle vector containing covalently bound residues of the (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) replicates in the monkey kidney cell line COS7. The target for detecting mutations was the 200-base pair gene for a tyrosine suppressor tRNA (*supF*), inserted at the *Eco*RI site in shuttle vector p3AC (Sarkar et al., Mol. Cell. Biol. 4:2227–2230, 1984). When introduced by transformation, a functioning *supF* gene in progeny plasmid recovered from COS7 cells allows suppression of a *lacZ* amber mutation in the indicator *Escherichia coli* host. Treatment of p3AC with BPDE caused a linear increase in the number of BPDE residues bound per plasmid. Untreated plasmids and plasmids containing 6.6 BPDE residues were transfected into COS7 cells, and the progeny were assayed for mutations in the *supF* gene. The frequency of mutants generated during replication of the BPDE-treated plasmids was not higher than that from untreated plasmids, but the two populations differed markedly in the kinds of mutations they contained. Gel electrophoresis analysis of the size alterations of 77 mutant plasmids obtained with untreated DNA and 45 obtained with BPDE-treated DNA showed that the majority of the mutant progeny of untreated plasmids exhibited gross alterations, principally large deletions. In contrast, the majority of the mutants generated during replication of the BPDE-treated plasmids contained only minor alterations, principally point mutations. Sequence analysis of progeny of untreated plasmids containing putative point mutations showed insertions and deletions of bases and a broad spectrum of base substitutions; in those from BPDE-treated plasmids, all base substitutions involved guanosine · cystosine pairs.

As part of a study of the mechanisms of carcinogenesis, we are investigating at the sequence level the specific kinds of mutations induced in mammalian cells by carcinogens, including (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE), a major reactive metabolite of the widely distributed environmental carcinogen benzo[a]pyrene (26). Several investigators have examined the kinds of mutations induced by BPDE in bacteria, but not in mammalian cells. For example, Eisenstadt et al. (8) treated nucleotide excision repair-deficient *Escherichia coli* with BPDE and genetically analyzed a large number of nonsense mutations in the *lacI* gene for the kinds of base substitutions induced. The majority proved to be G · C → T · A. Chakrabarti et al. (6) treated plasmid with BPDE, isolated a specific fragment in the gene for tetracycline resistance, ligated it back into the complementary part of unmodified plasmids, and then transformed *E. coli* with the chimeric plasmid containing the localized patch of BPDE adducts to determine their effect on plasmid survival and mutagenesis. They found that the majority of the mutations did not involve major alterations in the target gene. Sequence analysis of five mutants showed that three had G · C pair deletions, one had a G · C → T · A transversion, and one had a A · T → G · C transition. Mizusawa et al. (18) conducted a similar type of study, treating plasmids with BPDE and transfecting them into various *E. coli* strains with different repair capacities to study the effects of BPDE on plasmid survival in the various recipients and on the frequency of mutants (*galK*$^+$ → *galK*). Sequence analysis was not carried out because of the large size of the gene, but gel electrophoresis analysis of the mutant plasmids taken from the bacteria showed that the majority (15 of 16) did not involve detectable alterations in the size of the DNA fragment of interest. In a related study involving a *galK* → *galK*$^+$ selection system and a 230-base pair (bp) transcription termination sequence as the target for mutations, sequence analysis of the DNA from 15 *galK*$^+$ colonies revealed three with mutations in the region of interest (19). Two of these showed insertion of T · A into a cluster of T · A base pairs; the third showed a deletion of G · C from a cluster of G · C base pairs.

Indirect evidence suggests that the mechanisms of mutagenesis by certain carcinogens in mammalian cells can differ from that in bacteria (2). Therefore, we treated a shuttle vector with tritium-labeled BPDE and transfected it into the monkey kidney cell line COS7 to allow replication to occur. Mutant progeny plasmids were identified by transforming an *E. coli* indicator host and analyzed for mutations in the target gene, the 200-bp tyrosine suppressor tRNA (*supF*). We found that the frequency of gross alterations formed in the progeny of BPDE-treated plasmids was significantly lower than with untreated plasmid, and DNA sequence analysis showed that, unlike the case for the untreated plasmid, the majority of the point mutations obtained with BPDE-treated

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TABLE 1. Frequency of *supF* mutants obtained with BPDE-treated or untreated p3AC that had replicated in COS7 cells

Treatment	No. of transformants	No. of mutants	Mutant frequency
None	21,300	77	36×10^{-4}
BPDE	13,500	45	33×10^{-4}

plasmid were base substitutions, and all of these involved a GC base pair transition or transversion.

The 6.6-kbp shuttle vector p3AC, constructed by Sarkar et al. (23) and provided by W. C. Summers, contains parts of a pBR322 plasmid including the origin of replication and the gene for ampicillin resistance (*amp*), the *Bam*HI and *Hpa*II fragments of the early region of simian virus 40 DNA, and the 200-bp *supF* gene, which serves as the target gene for mutagenesis and subsequent sequencing. The ampicillin-sensitive indicator bacterial host, *E. coli* SY204, carries an amber mutation in the β-galactasidase gene (23). The eucaryotic host of the shuttle vector, COS7 simian cells (10), were grown in Eagle minimal essential medium supplemented with 0.2 mM L-serine, 0.2 mM L-aspartic acid, 1 mM sodium pyruvate, and 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.).

Preparation of plasmid DNA-containing BPDE adducts. Plasmid was prepared by an alkaline lysis procedure (16) and purified by ethidium bromide-CsCl density centrifugation. A small volume (1 to 16 μl) of generally tritiated BPDE (692 mCi/mmol; 0.3 mg/ml in tetrahydrofuran; 96% pure [Midwest Research Institute, Kansas City, Mo.]), was diluted in anhydrous acetone immediately before use and added to 200 μl of a 0.5-mg/ml solution of DNA in 10 mM Tris hydrochloride–1 mM EDTA buffer, pH 8.0. The mixture, protected from light, was incubated at room temperature for 2 h. Unbound BPDE was removed by three successive ethanol precipitations, and the moles of BPDE residues bound per mole of p3AC was calculated from the A_{260} profile of the DNA and the specific activity. The number of BPDE adducts per molecule of plasmid was proportional to the concentration of BPDE, with 5 μM giving 6.6 BPDE residues per plasmid.

Transfection of COS7 cells and assay of progeny plasmid for *supF* mutations. COS7 cells in suspension were transfected with untreated plasmid or plasmid containing 6.6 BPDE, using the DNA-calcium phosphate coprecipitation method of Chu and Sharp (7). After 48 h, plasmid DNA was extracted by the procedure of Hirt (13), purified with phenol, treated with RNase A (50 μg/ml) at 37°C for 1 h, followed by proteinase K (100 μg/ml) at 50°C for 2 h, and then extracted with phenol-chloroform, precipitated with ethanol, and further purified by drop dialysis (25). The purified plasmid was treated with *Dpn*I to digest any input plasmid and then was used to transform SY204 bacterial cells to ampicillin resistance, using the method of Hanahan (11). Transformants were selected on Luria-Bertani (LB) broth plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (40 mg/liter), an inducer, isopropyl-β-D-thiogalactoside (20 mg/liter), and ampicillin (50 mg/liter). Cells containing plasmids with a functioning *supF* gene which suppresses the amber mutation can form blue colonies on X-Gal plates, whereas cells containing plasmids with a mutated, inactive *supF* gene form white colonies. The frequency of mutants was determined by dividing the number of white colonies by the total number of colonies. The results are shown in Table 1.

TABLE 2. Characterization of the kinds of mutations generated in p3AC during replication in COS7 cells

Characterization of mutant p3AC vectors	No. (%) of mutants	
	No treatment (controls)	BPDE treated (6.6 adducts/p3AC)
Minor alteration		
Putative point mutations	12 (15.6)	15 (33.3)
Small deletions or insertions (delete or insert <30 bp)	5 (6.5)	8 (17.8)
Gross alteration		
Deletions (delete ~200 bp)	25 (32.5)	12 (26.7)
Large deletions (delete >1 kb[a])	24 (31.1)	4 (8.9)
Large insertions (insert >1 kb)	11 (14.3)	6 (13.3)

[a] kb, Kilobase.

Characterization of mutant plasmids by gel electrophoresis and sequencing. Bacterial cells from white colonies were restreaked on fresh X-Gal plates containing isopropyl-β-D-thiogalactoside and ampicillin to confirm their phenotype, and their DNA was extracted, purified, and analyzed by electrophoresis on 0.8% agarose gels for altered DNA mobility (gross alterations). Plasmids with normal agarose gel patterns were digested with *Eco*RI and analyzed by electrophoresis on 6% polyacrylamide gels for changes in the size of the *supF* gene. The data for 77 mutants from untreated p3AC and 45 mutants obtained from the progeny of BPDE-treated plasmid are summarized in Table 2. The majority of the mutants obtained after transfection of COS7 cells with untreated p3AC exhibited gross rearrangements, predominantly deletions but also large insertions. In contrast, the majority of the mutant plasmids obtained with BPDE-treated DNA exhibited only minor alterations, and most of these were putative point mutations since they did not show any visible alteration in size on agarose or polyacrylamide gels.

Plasmids with a normal polyacrylamide gel pattern were considered to contain putative point mutations in the *supF* gene. Seven unambiguously independent mutants of this type derived from untreated plasmids and nine derived from BPDE-treated plasmids were sequenced, using a modification of the dideoxyribonucleotide method of Sanger et al. (22). DNA was prepared and purified through CsCl gradients as described above and denatured by alkali to generate single-stranded DNA template as described previously (28). Polymerization from a pBR322 *Eco*RI site primer was carried out with the Klenow fragment of DNA polymerase I. [^{35}S]dATP (034S; New England Nuclear, Boston, Mass.) and buffer gradient-denatured polyacrylamide gels were used for greater resolution (3). The results are shown in

TABLE 3. Analysis of the kinds of base changes found in the *supF* gene of the mutant p3ACs analyzed[a]

Type of change	Untreated p3AC		BPDE-treated p3AC	
	Times occurring	% of total	Times occurring	% of total
GC → TA	2	14	6	46
GC → AT	5	36	3	23
CG → GC	1	7	2	15
AT → GC	1	7	0	0
AT → CG	2	14	0	0
Insert an A	2	14	1	8
Insert a T	0	0	1	8
Delete a C	1	7	0	0

[a] Only those p3ACs containing putative point mutations were analyzed at the sequence level.

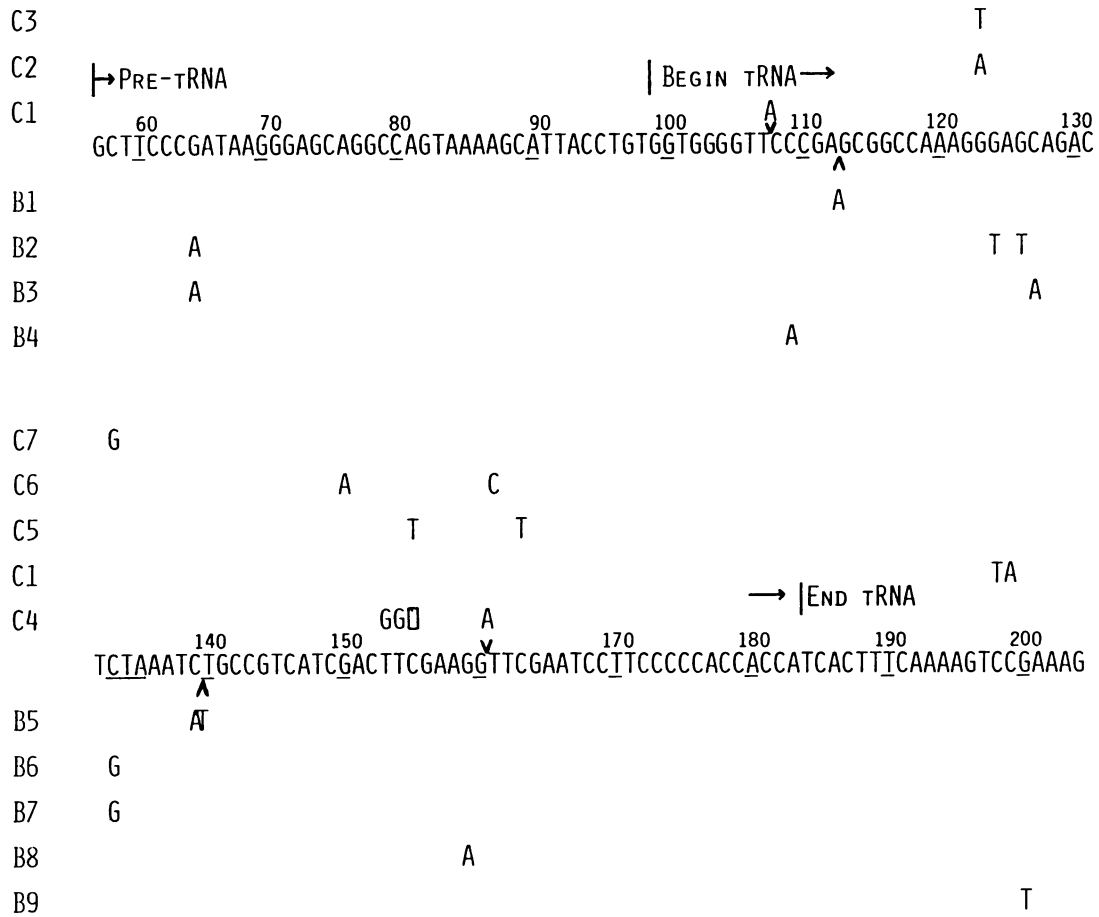

FIG. 1. Distribution of mutants in the *supF* gene of p3AC. The DNA strand shown is the strand synthesized during the DNA sequencing reaction, using the *Eco*RI rightward-sequencing primer. It corresponds to the tRNA sequence. C1 to C7 refers to mutants obtained with untreated plasmids; B1 to B9 refers to those from treated plasmids.

Table 3 and Fig. 1. Among the untreated controls, three of the seven mutants showed a single base substitution G → A, G → T, and C → T; two had two base substitutions; and two had several changes, including single base insertions and deletions. In contrast, five of the nine mutants from BPDE-treated plasmids showed a single base substitution; three of nine had two base substitutions; and one had three base substitutions. All of these substitutions involved G · C base pairs. One mutant had a base substitution, but also the insertion of a nucleotide; one had just the insertion of a nucleotide. The location of each change in the gene can be seen in Fig. 1.

For comparative purposes, it is useful to calculate the mutant analysis data of Tables 1 and 2 on the basis of the frequency of each type of mutation. This calculation shows that the frequency of mutant progeny from untreated plasmids carrying minor alterations was 8×10^{-4}, but for the BPDE-treated plasmids it was 17×10^{-4}. This indicates that BPDE induces point mutations, which supports the findings of King and Brookes (14), who studied the kinds of mutations induced in V79 Chinese hamster cells as detected by DNA hybridization, and those of Aust et al. (2), who showed in human diploid fibroblasts that BPDE does not cause the kinds of mutations (deletions, rearrangements) expected to completely inactivate the gene coding for elongation factor 2, which is involved in diphtheria toxin resistance. That all base substitutions observed with progeny of BPDE-treated plasmid involved G · C pairs is in keeping with the hypothesis that BPDE adducts were involved since this carcinogen predominantly binds to guanine (26, 27).

A similar analysis of the data in Tables 1 and 2 for the frequency of mutants carrying gross alterations shows that for the progeny of untreated plasmid this value was 28×10^{-4}, with 23×10^{-4} being deletions and 5×10^{-4} being insertions. This result is not surprising. Evidence from several groups of investigators indicates that, when simian virus 40-based plasmids are introduced into mammalian cells, they are subjected to strand breaking, deletions, duplications, recombination, and more complex rearrangements, including the insertion of sequences from the mammalian cell genome (1, 4, 5, 15, 17, 20, 21). However, with the progeny of BPDE-treated plasmids, the frequency of mutants carrying such gross alterations was significantly lower than background, only 16×10^{-4}, with 12×10^{-4} being deletions, compared to 23×10^{-4} in the control. (The frequency of insertions was about the same in both populations.) The failure to recover the background level of plasmids carrying deletions suggests that the presence of BPDE adducts interferes in some way with the process that produces these gross rearrangements. Another explanation is that the BPDE adducts increase the size of the deletions so that they extend into the *amp* gene. Such plasmids would not be recovered in our assay.

Gross rearrangements were rarely seen when we trans-

formed *E. coli* SY204 directly with BPDE-treated or untreated p3AC without first transfecting COS7 cells (data not shown). This result agrees with the result of Chakrabarti et al. (6), who did not observe rearrangements following transformation of *E. coli* with plasmid. Neither did Glazer et al. (9) find rearrangements in the *supF* gene when it was integrated into a mammalian cell chromosome and the host cell was exposed to mutagens.

One important advantage of using the *supF* gene as the target for mutation studies at the sequence level is its small size. Another is that the tRNA gene is responsive to base substitutions in many different positions (9, 12, 23). Recently, Seidman et al. (24) reported construction of a shuttle vector, pZ189, in which the *supF* gene is strategically located between the origin of replication of the plasmid in *E. coli* and the gene for ampicillin resistance so that the possibility of recovering mutants containing these spontaneous gross rearrangements is greatly decreased. This plasmid will make it easier to study the types of minor alterations induced by various mutagens (12) and we are currently using it to obtain such data for BPDE. Nevertheless, use of p3AC in the present study made it possible for us to detect the effect of BPDE on the process responsible for generating the spontaneous gross alterations.

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