MOLECULAR AND CELLULAR BIOLOGY, Aug. 1988, p. 3364–3372
0270-7306/88/083364-09$02.00/0

Vol. 8, No. 8

Kinds and Spectrum of Mutations Induced by 1-Nitrosopyrene Adducts during Plasmid Replication in Human Cells

JIA-LING YANG,* VERONICA M. MAHER, AND J. JUSTIN McCORMICK

Carcinogenesis Laboratory, Departments of Microbiology and Biochemistry, Michigan State University, East Lansing, Michigan 48824-1316

Received 19 February 1988/Accepted 9 May 1988

1-Nitropyrene has been shown in bacterial assays to be the principal mutagenic agent in diesel emission particulates. It has also been shown to be mutagenic in human fibroblasts and carcinogenic in animals. To investigate the kinds of mutations induced by this carcinogen and compare them with those induced by a structurally related carcinogen, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetra-hydrobenzo[a]pyrene (BPDE) (J.-L. Yang, V. M. Maher, and J. J. McCormick, Proc. Natl. Acad. Sci. USA 84:3787–3791, 1987), we treated a shuttle vector with tritiated 1-nitrosopyrene (1-NOP), a carcinogenic mutagenic intermediate metabolite of 1-nitropyrene which forms the same DNA adduct as the parent compound, and introduced the plasmids into a human embryonic kidney cell line, 293, for DNA replication to take place. The treated plasmid, pZ189, carrying a bacterial suppressor tRNA target gene, *supF*, was allowed 48 h to replicate in the human cells. Progeny plasmids were then rescued, purified, and introduced into bacteria carrying an amber mutation in the β-galactosidase gene in order to detect those carrying mutations in the *supF* gene. The frequency of mutants increased in direct proportion to the number of DNA–1-NOP adducts formed per plasmid. At the highest level of adduct formation tested, the frequency of *supF* mutants was 26 times higher than the background frequency of 1.4×10^{-4}. DNA sequencing of 60 unequivocally independent mutants derived from 1-NOP-treated plasmids indicated that 80% contained a single base substitution, 5% had two base substitutions, 4% had small insertions or deletions (1 or 2 base pairs), and 11% showed a deletion or insertion of 4 or more base pairs. Sequence data from 25 *supF* mutants derived from untreated plasmids showed that 64% contained deletions of 4 or more base pairs. The majority (83%) of the base substitutions in mutants from 1-NOP-treated plasmids were transversions, with 73% of these being G · C → T · A. This is very similar to what we found previously in this system, using BPDE, but each carcinogen produced its own spectrum of mutations. Of the five hot spots for base substitution mutations produced in the *supF* gene with 1-NOP, two were the same as seen with BPDE-treated plasmids. However, the three other hot spots were cold spots for BPDE-treated plasmids. Conversely, four of the other five hot spots seen with BPDE-treated plasmids were cold spots for 1-NOP-treated plasmids. Comparison of the two carcinogens for the frequency of *supF* mutants induced per DNA adduct showed that 1-NOP adducts were 3.8 times less effective than BPDE adducts. However, the 293 cells excised 1-NOP-induced adducts faster than BPDE-induced adducts.

Many carcinogens have been found to induce tumors only at specific sites in the body of the treated animal. For example, when injected intraperitoneally, benzo[a]pyrene, a widely distributed polycyclic aromatic hydrocarbon formed by incomplete combustion, forms tumors predominantly in the lung (24, 39), whereas the structurally related nitro aromatic compound 1-nitropyrene primarily forms liver (39) or mammary tumors (17). One explanation for such specificity is that the carcinogen is metabolized into a reactive form only in specific target organs. Another is that the carcinogen is capable of inducing only certain kinds of mutations and that a specific change in DNA is required in a particular organ to bring about the necessary alteration leading ultimately to tumorigenesis. The latter hypothesis is supported by recent studies on the specific mutational changes needed to activate specific oncogenes (7, 37, 44). Therefore, we and others (3, 4, 8, 9, 11, 12, 14, 21, 22, 30, 32, 40, 41) have begun to study the specific kinds of mutations induced by carcinogens in mammalian cells.

Attempts to deduce the nature of the molecular mechanisms by which carcinogens induce mutations in mammalian cells have previously been hampered by the inability to isolate and analyze newly mutated genes at the sequence level. But the development of shuttle vectors, i.e., plasmids carrying a defined target gene and capable of replicating in mammalian cells and also in bacteria, provides a solution to this problem (8, 9, 21, 32, 33). We are using the shuttle vector pZ189, containing the *supF* gene which codes for a tyrosine suppressor tRNA, to investigate at the DNA sequence level the kinds of mutations induced when DNA containing covalently bound carcinogen residues replicates in human cells. The advantage of the *supF* gene as the target for mutation studies at the sequence level is its small size and the fact that it is highly responsive to base changes. Examination of data from a number of studies, including those in references 3, 4, 6, 12, 14, 30, 40, and 41, indicates that a change in any one of at least 63 of the 85 bases which make up the tRNA structure will result in a mutant phenotype. Use of the human cell line 293 as the eucaryotic host for replicating the plasmid offers the advantage of a background mutant frequency of 1.4×10^{-4} (41), which is low enough to allow one to observe an increase in mutant frequency induced by carcinogen treatment of the plasmids.

Using this system, we recently determined the specific kinds of mutations induced by the major reactive metabolite of benzo[a]pyrene, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (41), which has been shown to form lung tumors when injected intraperi-

* Corresponding author.

toneally into newborn mice (24). In the present study we investigated the mutagenic specificity of 1-nitrosopyrene (1-NOP), a partially reduced metabolite of 1-nitropyrene, which forms the same DNA adducts as the parent compound (1, 29), induces mutations in bacteria (16) and mammalian cells (15, 28, 29), and forms liver tumors in animals (39). Both 1-NOP and BPDE form covalently bound adducts in DNA principally with guanine (16, 19, 26, 38). 1-NOP binds only at the C-8 position (1, 15, 16, 29), whereas BPDE forms its principal guanine adduct at the N^2 position (38). Since these guanine adducts are located in very different positions in the DNA helix and only one, the N^2 position, is involved in the base-pairing part of the molecule, we were particularly interested in comparing the specific kinds of mutations induced by these two agents and their location in the target gene, as well as their biologic effectiveness, i.e., their ability both to induce mutations when the treated plasmids replicate in human cells and to interfere with bacterial transformation. The results indicated that the adducts formed by the two carcinogens are equivalent in decreasing bacterial transformation and the kinds of mutations induced by 1-NOP adducts are similar to those induced by BPDE. However, the frequency of mutants induced per DNA adduct was 3.8 times lower for 1-NOP than for BPDE, and each compound exhibited a specific spectrum of base changes in the target gene.

MATERIALS AND METHODS

Cells and plasmid. The human embryonic kidney cell line 293 was grown in modified Eagle minimal essential medium containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) (culture medium) as described previously (41). The ampicillin-sensitive indicator bacterial host was *Escherichia coli* SY204, which carries an amber mutation in the β-galactosidase gene (32). The 5.5-kilobase-pair shuttle vector, pZ189, contains the *supF* tyrosine suppressor tRNA gene flanked by two genes essential for recovery in *E. coli*, i.e., the ampicillin gene and the bacterial origin of replication (33). It also carries the origin of replication and large-T antigen gene from simian virus 40.

Formation of 1-NOP adducts on the plasmid. Plasmid DNA, prepared by using an alkaline lysis procedure (25) and purified by ethidium bromide-CsCl density centrifugation, was suspended in 190 μl of helium-purged sodium citrate (10 mM) buffer, pH 5.0, at a concentration of 300 μg/ml. A 5-μl aliquot of a freshly prepared solution of ascorbic acid (20 μM in H_2O) was added to provide needed reduction of the 1-NOP (16), followed by 5 μl of a stock solution of tritiated 1-NOP (specific activity, 217 mCi/mmol; purity, 99%) dissolved in dimethyl sulfoxide at a concentration of 0.05 to 0.08 μM. The radiolabeled 1-NOP was supplied by F. A. Beland of the National Center for Toxicological Research, Jefferson, Ark. The samples were immediately mixed and incubated at 37°C for 2 h. Unbound 1-NOP was removed by phenol-chloroform extraction and three successive ethanol precipitations. The moles of 1-NOP residues bound per mole of plasmid was calculated from the A_{260} profile of the DNA and the specific activity.

Transfection and rescue of replicated plasmid. The 293 cells in culture medium were plated into a series of 150-mm-diameter dishes at 10^6 cells per dish. After 24 h, the cells were transfected with 6 μg of plasmid, using a modification of the $CaPO_4$ coprecipitation technique described before (41). After 48 h, the cells from each dish were harvested separately and progeny plasmids were extracted and separated from cellular DNA as described previously (18, 41). The DNA was purified as described before (41), including drop dialysis (34). To distinguish between independent mutants with identical mutations and putative siblings derived from a single event, progeny plasmids obtained from each dish of cells were maintained and assayed separately. Before being used to transform indicator bacteria, they were treated with 20 U of *Dpn*I, a restriction enzyme which digests any input DNA that still has the bacterial methylation pattern generated when the plasmids were first prepared in bacteria. As a control, an aliquot was also treated with *Mbo*I, which digests plasmids generated during replication in human cells, and tested for residual transforming ability. The results showed that >99.9% of the purified DNA was derived from plasmids that had replicated in the human cells.

Bacterial transformation and mutant identification. Progeny plasmids were assayed for mutant *supF* genes by transforming *E. coli* SY204 to ampicillin resistance and selecting the transformants on plates containing ampicillin (50 mg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and an inducer of the β-galactosidase gene, as described previously (41). Transformants containing plasmids lacking a functioning *supF* gene, which is needed to suppress the amber mutation in the β-galactosidase gene of the bacteria, could be identified because they form white or light-blue colonies rather than dark-blue colonies on X-Gal plates. Each white or light-blue putative mutant colony was restreaked on fresh plates to confirm the phenotype.

Characterization of mutants. Plasmid DNA from bacteria in these white or light-blue colonies was amplified, extracted, dissolved in buffer, and analyzed by electrophoresis on 0.8% agarose gels for altered DNA mobility (gross alteration). Plasmids without evidence of gross alterations were amplified further, purified by CsCl centrifugation, and analyzed by a secondary bacterial transformation to ensure that the observed inability of the bacteria to utilize X-Gal was the result of inactivation of the *supF* gene rather than a mutation in the β-galactosidase gene of *E. coli* (41). Putative *supF* mutants were sequenced by the dideoxyribonucleotide method (31) modified as follows. Plasmids were denatured with alkali to generate single-stranded templates (43), and polymerization from a pBR322 *Eco*RI site primer was carried out with the Klenow fragment of DNA polymerase I. The primer and polymerase were purchased from New England BioLabs, Beverly, Mass. [α-^{35}S]dATP (NEN-034S; New England Nuclear Corp., Boston, Mass.) and buffer gradient denatured polyacrylamide gels (2) were used for greater resolution of the sequencing gel.

Determination of sites of carcinogen-induced adducts. The positions of 1-NOP or BPDE adducts in the *supF* gene of carcinogen-treated plasmids were determined by the in vitro DNA polymerase-stop assay of Moore and Strauss (27). Briefly, double-stranded plasmid containing 1-NOP or BPDE adducts (10 to 70 adducts per molecule of plasmid) was denatured and annealed with the pBR322 *Eco*RI site primer. The length of the DNA from the primer site to the end of the *supF* gene is ~230 nucleotides, so the average number of adducts per strand of *supF* gene was 0.2 to 1.5. The polymerization reaction was then carried out as described for the sequencing reaction, except that the dideoxynucleotides were omitted. DNA from the four dideoxy sequencing reactions, carried out on an untreated template, was electrophoresed on the same gel to serve as DNA size markers. The relative intensities of the bands on the autoradiograph of the gel were determined by a laser densitometer (LKB 2222-010, Ultrascan XL; Pharmacia LKB Biotechnol-

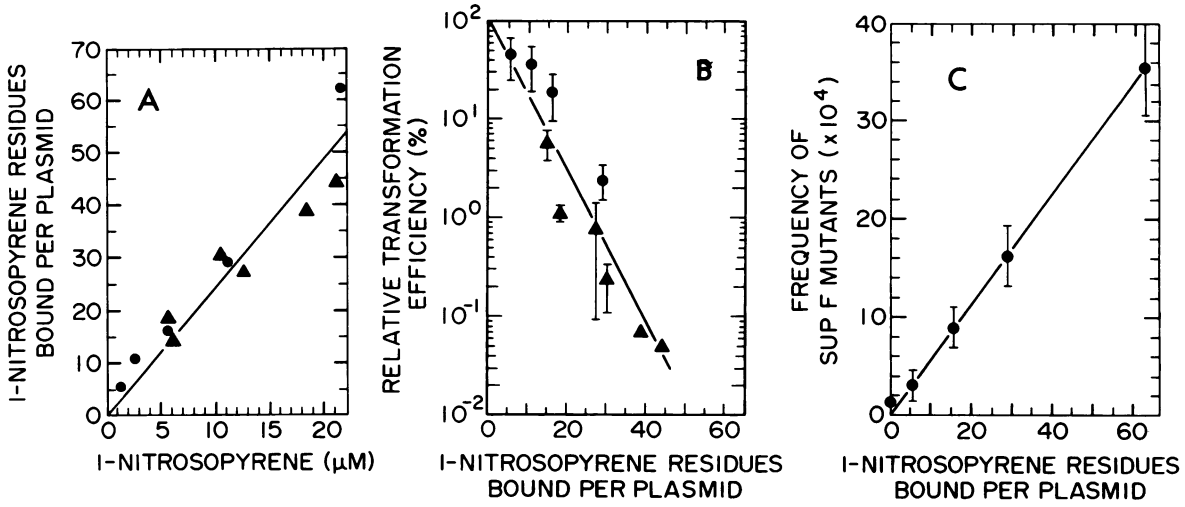

FIG. 1. Number of 1-NOP adducts per plasmid as a function of concentration of 1-NOP in the presence of ascorbic acid (A) and relative frequency of transformation of bacteria to ampicillin resistance as a function of the number of 1-NOP adducts per plasmid (B). (Error bars indicate the standard errors of four determinations.) Also shown is the frequency of *supF* mutants as a function of the number of 1-NOP adducts per plasmid (C). The error bars refer to the standard errors of the *supF* mutant frequencies obtained from a series of individual human cell transfection experiments made with each set of treated plasmids. Symbols (● and ▲) indicate data from plasmids treated with 1-NOP in two separate experiments.

ogy, Inc., Piscataway, N.J.) and were corrected for position in the gene by taking into account the number of ^{35}S-labeled adenine bases that would be present in each length of newly synthesized DNA.

Determination of number of carcinogen residues bound to DNA of human 293 cells. Cells were plated at a density 25% that of confluence in culture medium containing ^{14}C-labeled thymidine (50 μCi/ml, 51.3 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) at 5×10^{-3} μCi/ml and incubated for 48 h to prelabel the DNA. They were then pooled and plated at 2×10^4 to 4×10^4 cells per cm^2; after attachment, they were treated for 1 h with tritium-labeled 1-NOP or BPDE. The BPDE (specific activity, 692 mCi/mmol) was obtained from Midwest Research Institute, Kansas City, Mo. as a tetrahydrofuran solution. Just prior to use, an aliquot was dried under nitrogen gas and redissolved in anhydrous acetone. At the end of the 1-h treatment, the medium containing carcinogen was removed and the cells were rinsed twice with culture medium. One set of cells (6×10^6 to 10×10^6 per dose) was assayed immediately for the number of carcinogen residues bound to DNA. The rest were incubated at 37°C with fresh culture medium for the designated period before being similarly analyzed as follows. The cells were washed, lysed (in their original dishes) with a 0.5% solution of sodium lauryl sarkosine, transferred into tubes, and incubated with protease K (100 μg/ml) at 50°C for 12 h. The DNA was extracted three times with phenol-chloroform and precipitated with ethanol. The DNA was dissolved in buffer, treated with RNase A (100 μg/ml) and RNase T_1 (50 U/ml) at 37°C for 2 to 3 h, extracted several times with phenol-chloroform until no interface material could be seen, and then precipitated with ethanol. The initial number of covalently bound residues per 10^6 DNA nucleotides was determined from the UV absorption profile of the DNA solution at 260 nm and the specific radioactivity. The percentage of adducts remaining on the parental DNA after 18 or 30 h was calculated from the $^3H/^{14}C$ ratio at the time of interest, divided by the ratio at time zero.

RESULTS

Characterization of 1-NOP-treated plasmid. Vector pZ189 was treated with various concentrations of tritium-labeled 1-NOP in the presence of ascorbic acid as the reducing agent. The number of residues bound per plasmid was determined, and their ability to interfere with transformation of bacterial cells to ampicillin resistance was determined by the transformation method of Hanahan (13). The number of 1-NOP-induced adducts per molecule of plasmid increased linearly with the concentration of 1-NOP used (Fig. 1A). The transforming activity of the modified plasmid decreased in direct proportion to the number of 1-NOP residues bound (Fig. 1B). Approximately seven 1-NOP residues bound were required to lower the transforming activity of the treated plasmid to 37% of the untreated control plasmid.

Frequency of mutants induced by 1-NOP adducts. Plasmids containing various levels of 1-NOP adducts and untreated plasmids were introduced into human cells by transfection and allowed to replicate for 48 h. The progeny plasmids were harvested and assayed for mutations in the *supF* gene by introducing them into *E. coli* SY204 indicator bacteria. There was a linear increase in the frequency of *supF* mutants as a function of the number of 1-NOP-induced adducts per plasmid (Fig. 1C). At the highest level of 1-NOP adduct formation tested, i.e., 63 adducts per pZ189, the frequency of *supF* mutants was 35.8×10^{-4}, which is 26 times higher than the background frequency of 1.4×10^{-4}.

A total of 30 mutant plasmids derived from untreated plasmids and 88 from 1-NOP-treated plasmids were analyzed for altered gel mobility to see if they contained gross alterations, i.e., deletions or insertions of >150 base pairs (bp). The majority of those that did not were further analyzed by DNA sequencing for the small deletions or insertions and for point mutations, i.e. base substitutions or deletions or insertions of 1 or 2 bp. The results are summarized in Table 1, which includes data from 14 experiments in which human cells were transfected with untreated plasmids

TABLE 1. Analysis of mutants obtained by transformation of *E. coli* with progeny of 1-NOP-treated pZ189 generated during replication in 293 cells

Adducts per plasmid	No. of human cell transfection expts	*supF* mutants[a]/ transformants	Frequency of *supF* mutants (10^4)	Plasmids with altered gel mobility[b]/no. examined	Total plasmid *supF* genes sequenced	Characterization of sequenced mutants			Frequency of mutants with point mutations (10^4)[f]
						No. with deletions[c]	No. with insertions[d]	No. with point mutations[e]	
0	14	31/218,750	1.4	5/30	25	13	3	9	0.4
5.7	2	4/12,985	3.1	0/4	4	1	0	3	2.3
16.2	4	18/20,200	8.9	3/17	10	1	0	9	6.2
29.1	4	27/16,705	16.2	1/26	20	2	0	18	13.9
62.5	4	42/11,720	35.8	2/41	30	1	2	27	30.2

[a] Plasmid from each mutant was assayed by a secondary transformation to ensure that the inability of the cell to metabolize X-Gal resulted from inactivation of the *supF* gene (see text).
[b] Alteration visible on agarose gel (>150 bp).
[c] Deletion of 9 to 150 bp.
[d] Insertion of 10 to 20 bp.
[e] Substitution, deletion, or insertion of 1 or 2 bp.
[f] Calculated from fraction of mutants with point mutations times the observed frequency (column 4). The fraction of mutants with point mutations is the number in column 9 divided by that in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

to determine the background frequency of *supF* mutants and to generate control mutants for analysis: 8 of these accompanied studies with BPDE-treated plasmids; 6 accompanied studies with 1-NOP-treated plasmids. The analysis of these control mutants was reported earlier (41) and is included in Table 1 for comparative purposes.

The majority of the mutants obtained with 1-NOP-treated plasmids contained point mutations. Of 67 analyzed mutants derived from plasmids carrying 29.1 or 62.5 adducts per plasmid, only 3 (4.5%) exhibited altered gel mobility, and only 5 (10%) of the sequenced mutants from that group proved to contain deletions or insertions of 4 bp or more. In contrast, 70% of the control mutants (21 of 30) contained such mutations. Calculation of the fraction of mutants containing point mutations (Table 1, last column) showed that the frequencies derived from plasmids carrying the two highest levels of 1-NOP adducts were, respectively, 35 and 75 times higher than the control (0.4×10^{-4}).

Nature and location of specific mutations induced by 1-NOP-treated plasmids in 293 cells. Sequence analysis of 60 unequivocally independent mutants derived from 1-NOP-treated plasmids indicated that 88.3% contained point mutations (Table 2); i.e., 80% (48 of 60) contained a single base substitution, 5% (3 of 60) contained two base substitutions, and 3.3% (2 of 60) had deletion or insertion of 1 bp. These data contrast with the control mutants in which only 38% showed such point mutations. Table 3 shows the specific kinds of base substitutions induced in the *supF* gene by replication of 1-NOP-treated plasmids in 293 cells. The majority (45 of 54) of the changes were transversions, with 33 of 45 being G · C → T · A. The majority (87%) of base changes (47 of 54) involved G · C pairs.

The specific locations of 1-NOP-induced point mutations (spectrum) are shown in Fig. 2. Three strong hot spots were found at positions 109, 123, and 127. All of the base substitutions at these three hot spots were G · C pair transversions (16 G · C → T · A; 4 G · C → C · G). Two less prominent hot spots were seen at positions 156 and 159, with transitions occurring twice as frequently as transversions (6 G · C → A · T; 3 G · C → T · A). All five hot spots were located at nucleotides corresponding to the stem of the cloverleaf structure of the *supF* tRNA (Fig. 3). All seven of the A · T pair base substitutions were located at positions in the gene specifying the loops of the tRNA cloverlead structure.

Mutagenic effectiveness of 1-NOP compared with BPDE. Because 1-NOP binds almost exclusively to guanine to form adducts at the C-8 position, whereas the structurally related carcinogen BPDE binds predominantly to guanine at the N^2 position, we compared the two carcinogens for the fre-

TABLE 2. Analysis of sequence alterations generated in the *supF* gene by replication of 1-NOP-treated or untreated pZ189 in 293 cells

Sequence alterations	No. of times occurring	
	Untreated	1-NOP treated
Single base substitution	3	48
Two base substitutions		
Tandem	0	2
≤20 bases apart	2	0
>20 bases apart	0	1
Deletions		
Single G · C pair	2	1
Single A · T pair	1	0
Tandem base pairs	0	0
4–20 bp	4	3
>20 bp	7	2
Insertions		
Single A · T pair	0	1
4–20 bp	2	2

TABLE 3. Kinds of base substitutions generated by replication of 1-NOP-treated or untreated pZ189 in 293 cells

Base change	No. of mutations observed	
	Untreated	1-NOP treated
Transversions		
G · C → T · A	6	33
G · C → C · G	1	8
A · T → T · A	0	3
A · T → C · G	0	1
Transitions		
G · C → A · T	0	6
A · T → G · C	0	3

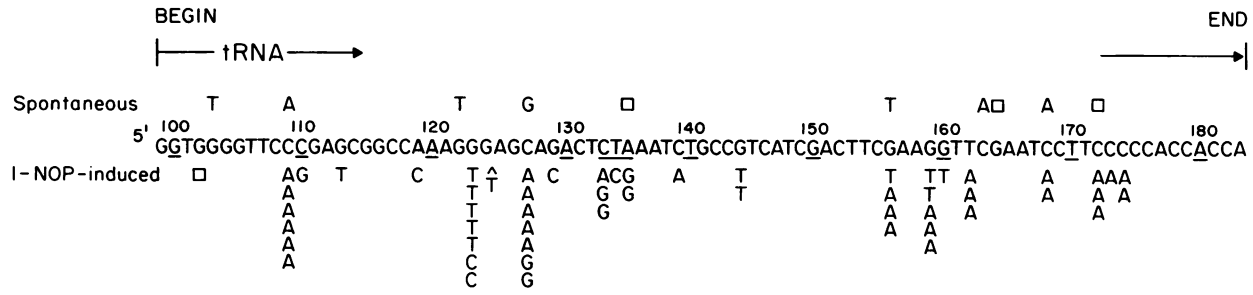

FIG. 2. Location of independent point mutations in the coding region of the *supF* tRNA gene. The DNA strand shown is the 5'-to-3' strand synthesized from the *Eco*RI rightward primer. The point mutations observed in the progeny of 1-NOP-treated plasmids are placed below the sequence. The rectangle represents a deleted guanine; the caret shows the location of an inserted thymidine. Every 10th residue and the anticodon triplet are underlined.

quency and kinds of mutations induced on the basis of equal numbers of adducts per plasmid. Interpolation of the data in Fig. 1C indicates that 20 1-NOP adducts per plasmid increased the *supF* mutant frequency from 1.4×10^{-4} to 11.6×10^{-4}. A similar interpolation of our published data for BPDE shows that 20 adducts per plasmid increased the frequency to 40×10^{-4} (41). These data indicate that BPDE adducts are 3.8-fold more effective than 1-NOP adducts in causing mutations, even though both carcinogens predominantly induce point mutations, with the majority being G · C → T · A transversions. This difference may reflect the intrinsic difference in the nature of the adducts formed. However, it could reflect a difference in the rate of excision of 1-NOP and BPDE adducts from the plasmid by the host 293 cells. Therefore, we examined the ability of 293 cells to remove these adducts from their DNA. The DNA of the human cells was prelabeled with [^{14}C]thymidine, and the cells were exposed to doses of tritiated 1-NOP or BPDE known to give cell survival levels between 30 and 60% that of an untreated population. The number of carcinogen residues covalently bound to DNA immediately after the 1-h treatment was determined, as well as the number remaining after 18 or 30 h. BPDE initially formed 9.5 and 10.7 adducts per 10^6 nucleotides and 1-NOP formed 48 and 61 adducts per 10^6 nucleotides, values in good agreement with published results with normal diploid human fibroblasts (28, 42). The rate of removal of 1-NOP adducts was significantly faster than that of BPDE adducts (Fig. 4).

Sites of 1-NOP or BPDE adducts. To determine whether there was a correlation between the positions and frequencies of the carcinogen-induced mutations and the sites and frequencies of carcinogen-DNA adducts in the *supF* gene, we carried out the DNA synthesis-stop assay of Moore and Strauss (27), in which bulky adducts interfere with DNA replication. The template DNA used in the assay contained 10 to 70 1-NOP or BPDE adducts per 5,500-bp plasmid, which represents 0.2 to 1.5 adducts per strand of the *supF* gene, if binding to guanine is essentially random. This protocol for estimating the percentage of carcinogen adducts formed at particular sites on the gene assumes, first, that the density of the bands in a sequencing gel, after adjustment for the extent of [α-^{35}S]dATP incorporated, is proportional to the number of DNA molecules of a particular length and, second, that the length of the fragments reflects the chance of adduct-induced premature termination of the polymerization.

The gel pattern of the DNA bands obtained in this assay for DNA containing 1-NOP or BPDE residues corresponded to positions 1 nucleotide prior to virtually every cytosine in

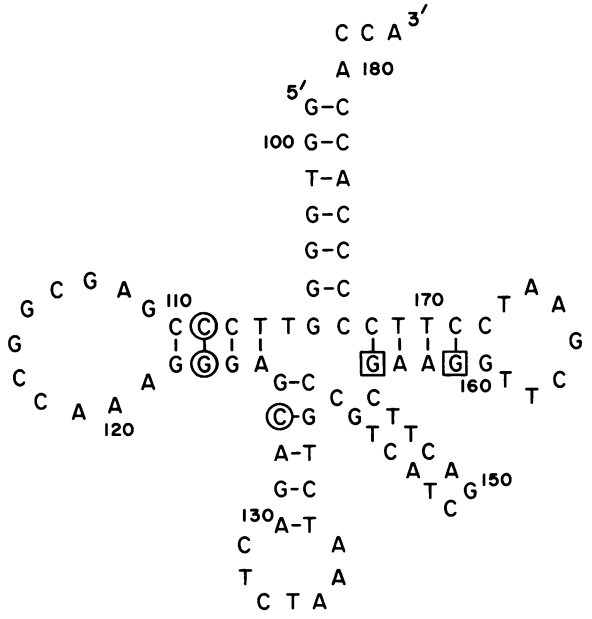

FIG. 3. Location of 1-NOP-induced hot spots on the cloverleaf structure of the *supF* tRNA. The nucleotide T shown in the tRNA represents uridine for the purpose of simplification. Circles indicate the three strong hot spots. Squares indicate the two less prominent hot spots.

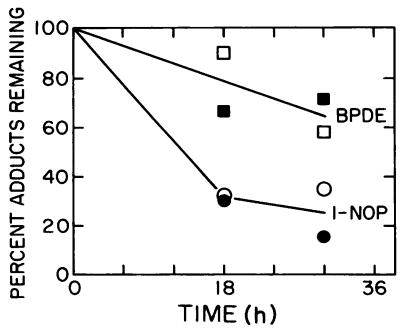

FIG. 4. Rate of removal of 1-NOP or BPDE adducts by 293 cells. The cells were labeled with [^{14}C]thymidine for 2 days before 1-h treatment with tritiated carcinogens. The percentage of adducts remaining on the DNA was calculated by dividing the ^{3}H/^{14}C ratio at 18 or 30 h by that at time zero. Open and closed symbols represent data taken from two separate experiments.

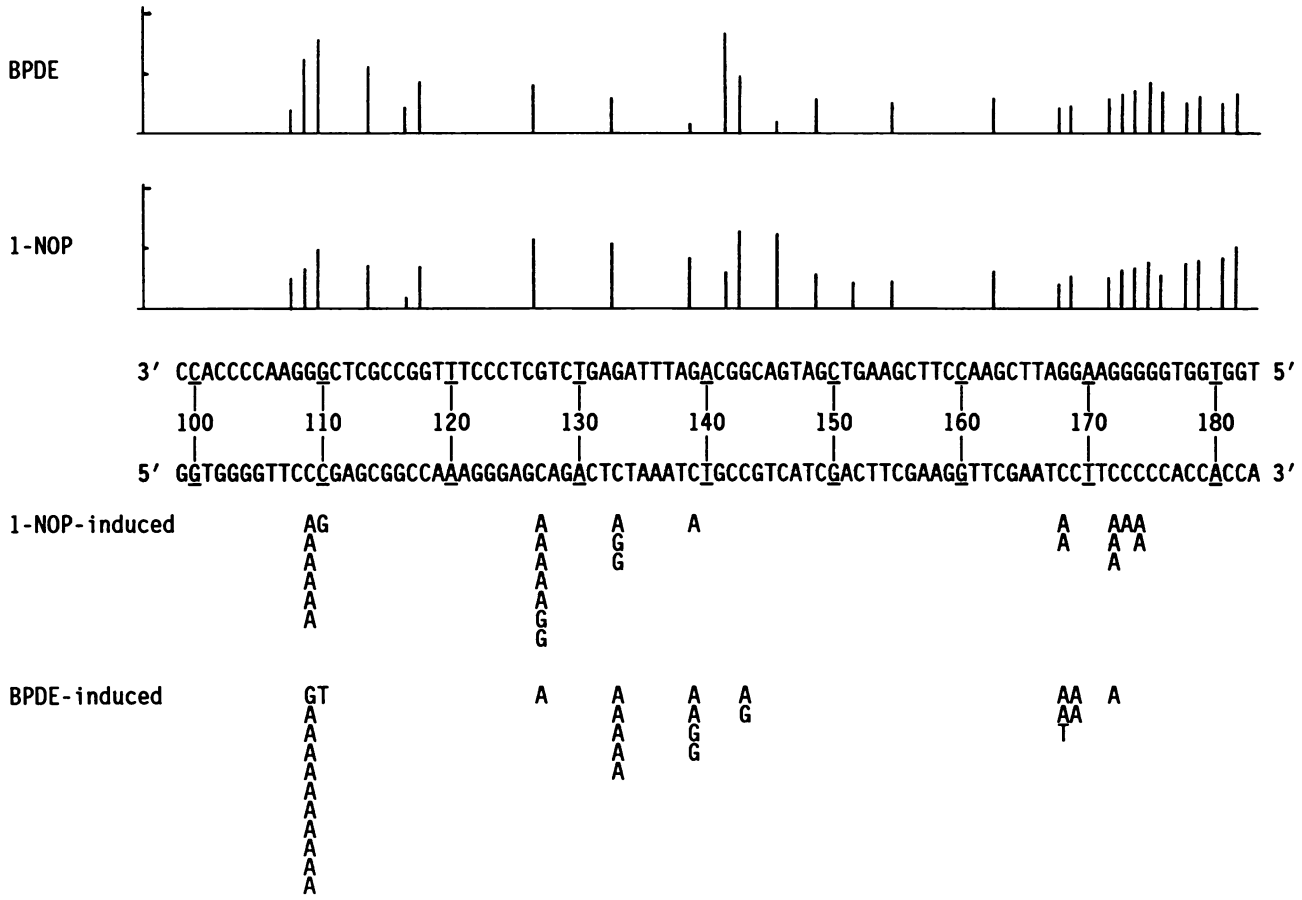

FIG. 5. Relative frequency of 1-NOP or BPDE adducts in the 3'-to-5' strand of the coding region of the *supF* gene, as judged by the polymerase-stop assay, and location of 1-NOP- or BPDE-induced base substitutions in the corresponding 5'-to-3' strand. The Klenow fragment of DNA polymerase I was used with the *Eco*RI rightward sequencing primer to determine polymerase-stop sites (panel shown above the coding sequence of the *supF* gene). The relative intensities of the bands on the autoradiograph were determined by densitometer and were corrected for position in the gene by taking into account the number of ^{35}S-labeled adenine bases that would be present in each length of newly synthesized DNA. The base substitutions shown below the *supF* tRNA gene are the cytosine base changes found in the corresponding 5'-to-3' strand (cf. Fig. 2 and 3 of reference 41).

the DNA-sequencing standard lane, indicating that DNA synthesis was terminated 1 base prior to each guanine in the template. No bands corresponding to positions 1 nucleotide away from any base other than guanine were seen, and there was no evidence of any interference with polymerization when untreated template was used. The pattern of bands did not vary significantly with the number of 1-NOP or BPDE adducts per molecule of plasmid. The relative intensities of the bands in the 85 nucleotides making up the tRNA of the 3'-to-5' template, using the pBR322 *Eco*RI site primer, are shown in Fig. 5. The frequency of DNA adducts, as estimated with this assay, ranged from 0% (position 131) to 6.6% (position 143) for 1-NOP adducts and from 0% (positions 131 and 152) to 12.9% (position 110) for BPDE adducts. The 1-NOP–guanine adducts were most frequently located at position 143 (Fig. 5), but the data in Fig. 2 show that no mutations were observed at this position. The frequency of 1-NOP–guanine adducts located at positions 109, 114, 118, 142, 149, 163, and 169 was approximately the same (~3%), but only at position 109 did mutation occur, and, in fact, position 109 represents a very strong mutational hot spot for 1-NOP. This apparent lack of correlation between the hot spots for mutation induction and hot spots for adduct formation, as judged from the intensity of the bands in the stop assay gel, was also observed for BPDE. The mutation data for BPDE in Fig. 5 are taken from reference 41.

DISCUSSION

The data in Fig. 1A indicate that the number of 1-NOP adducts per plasmid increased linearly with the concentration of 1-NOP used in the presence of ascorbic acid. The amount bound per applied concentration of carcinogen was three times higher than what we found previously with BPDE (41), but unlike the direct-acting BPDE, 1-NOP requires reducing agents such as ascorbic acid for activation (16). Once the 1-NOP adducts had formed, they were very similar to BPDE adducts in their ability to interfere with the processes involved in bacterial transformation; i.e., approximately seven 1-NOP adducts per plasmid (Fig. 1B) and nine BPDE adducts per plasmid (41) were sufficient to lower the transforming activity of the treated plasmid to 37% of that of untreated plasmid.

The data in Table 3, indicating that 1-NOP adducts caused base substitutions mainly at G · C base pairs (87%) just like BPDE does (41), strongly suggest that the mutagenesis was targeted to sites where adducts occur. Targeted mutagenesis is also indicated for 1-NOP-induced base substitutions in-

volving A · T base pairs. Our results (Table 3) showed that 13% of the base changes involved A · T base pairs, and Kinouchi and Ohnishi (19) recently demonstrated with bacterial nitroreductases that a minor adduct formed by 1-NP involved deoxyadenosine. Therefore, we consider the base substitutions induced by these two carcinogens to be targeted to adducts rather than being the result of apurinic sites (24). Additional data in support of this conclusion for BPDE were given in our earlier paper (41).

The majority of the 1-NOP- or BPDE-induced base changes we observed were G · C → T · A transversions. It may be that, as suggested by Strauss et al. (35), the DNA polymerase in the human 293 cell line preferentially inserts an adenine nucleotide opposite a noninstructional base containing a bulky adduct ("A rule"). However, G · C → C · G transversions were also found (15% of the base substitutions). Another explanation for the predominance of G · C → T · A transversions, and the one which we favor, is that with some frequency stable purine · purine base pairing occurs when the guanine carries a 1-NOP residue (or a BPDE residue) and that this mispairing goes undetected. Eisenstadt et al. (10) invoked the model of Topal and Fresco (36), in which such mispairing occurs by having the guanine carrying a BPDE residue at the N^2 position be oriented in the *syn* position and pair with an incoming adenine in the rare tautomeric imino form. However, Kennard and her colleagues (5) recently reported that stable $G_{anti} \cdot A_{syn}$ base pairs are formed in a synthetic deoxydodecamer and are accommodated in the DNA double helix with little or no disruption of the local or global conformation. Their model does not require the formation of the very rare enol or imino tautomeric forms. 1-NOP binds predominantly to the C-8 position of guanine, which is not involved in base pairing. The presence of this bulky residue may direct guanine in the normal *anti* configuration to pair with an incoming adenine nucleotide triphosphate, with adenine adopting the *syn* configuration. This could result in a stable base pair which would represent a mutation.

Using the polymerase-stop assay (Fig. 5), we did not find a high correlation between the frequency of 1-NOP or BPDE adduct sites and the frequency of base substitution mutations at these sites. Although the A rule as well as the purine · purine mispairing mechanism may explain G · C → T · A transversions, they cannot easily explain the location of the mutational hot spots we observed with either carcinogen. This lack of correlation is not caused by the inability of changes at these sites to cause detectable phenotypic changes in the tRNA gene, since base substitutions at all but six of these positions have been shown previously by a number of workers to cause such a phenotype. One possible explanation is that excision repair by the host cells plays a role and preferentially removes adducts from some sites in the plasmid faster than from other sites.

Fuchs and his co-workers (20) proposed the "mutation-prone sequences" model to explain the lack of correlation they observed between the sites of acetylaminofluorine-induced frameshift mutations in a bacterial plasmid and the sites at which T4 DNA polymerase exonuclease III activity was blocked by acetylaminofluorine residues. Similarly, Brash et al. (3) proposed the "pass/fail" model to explain the lack of correlation of mutation frequency with UV-induced photoproduct frequency at different sites in the *supF* gene. Such mutation-prone sequence- or "pass site"-determined mutational hot spots, rather than mere DNA lesion-determined hot spots, also fit our findings, but do not explain why some sites are very hot for mutation occurrence.

Although both 1-NOP and BPDE induced point mutations, with single base substitutions predominating and with G · C → T · A transversions being the most common mutation, the spectrum of mutations in the *supF* tRNA gene induced by 1-NOP differed from that induced by BPDE. The spectrum of the two carcinogens (cf. Fig. 2 and 3 of reference 41) exhibited two hot spots in common (positions 109 and 123). However, three 1-NOP-induced hot spots (positions 127, 156, and 159) were cold spots for BPDE, and four BPDE-induced hot spots were cold spots for 1-NOP (positions 112, 139, 160, and 164). These differences are not likely to be the result of differential binding of the two carcinogens at those positions, at least not as determined by the DNA polymerase-stop assay. For example, adducts were formed at position 139 more frequently by 1-NOP than by BPDE, but this site was a mutational hot spot for BPDE, not for 1-NOP. These differences may, as suggested by Brash et al. (3), reflect the fact that structural features differ between hot spots and non-hot spots in DNA sequences and that the ability of carcinogen-modified nucleotides to alter DNA structure at specific sites differs for 1-NOP and BPDE. They may also reflect differential repair.

When the mutagenicity of these two structurally related carcinogens was compared on the basis of equal numbers of adducts per plasmid, 1-NOP was 3.8 times less effective than BPDE in inducing mutations during the replication of the modified plasmid in the human host cells (Fig. 1C, compared with Fig. 2 of reference 41). However, the results in Fig. 4 indicate that 293 is a repair-proficient cell line and excises these two carcinogen-induced adducts at different rates. That 1-NOP adducts were lost from genomic DNA at a rate three times faster than BPDE adducts suggests that 1-NOP adducts may also be removed from plasmid DNA faster than BPDE adducts and disappear before replication of the plasmid has occurred. If this were the case, 1-NOP adducts may actually be equal to BPDE adducts in their effectiveness in inducing *supF* mutants during replication in human cells.

It may be important to point out that, unlike Seidman, Kraemer, and their collaborators, who studied UV-induced *supF* mutations in pZ189 replicating in simian virus 40-transformed human cells (3, 4), or Hauser et al., who studied this in a monkey cell line (14), we observed very few multiple mutations occurring in a single mutant plasmid, i.e., 1 of 60 (Table 2). The reason for this significant difference in results between UV-induced lesions and adducts formed by bulky carcinogens is currently under investigation.

ACKNOWLEDGMENTS

We thank Frederick A. Beland of the National Center for Toxicological Research for providing us with radiolabeled 1-NOP and for his helpful advice with the ascorbic acid reaction procedures. The excellent technical assistance of Bernard Schroeter is gratefully acknowledged.

This research was supported in part by Public Health Service grant CA21253 from the National Cancer Institute and by contract 87-2 from the Health Effect Institute, an organization jointly funded by the U.S. Environmental Protection Agency (assistance agreement x812059) and automotive manufacturers.

LITERATURE CITED

1. **Beland, F. A., M. Ribovich, P. C. Howard, R. H. Heflich, P. Kurian, and G. E. Milo.** 1986. Cytotoxicity, cellular transformation and DNA adducts in normal human diploid fibroblasts exposed to 1-nitrosopyrene, a reduced derivative of the environmental contaminant, 1-nitropyrene. Carcinogenesis 7:1279–1283.

2. **Biggen, M. D., T. L. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
3. **Brash, D. E., S. Seetharam, K. H. Kraemer, M. M. Seidman, and A. Bredberg.** 1987. Photoproduct frequency is not the major determinant of UV base substitution hot spots or cold spots in human cells. Proc. Natl. Acad. Sci. USA **84**:3782–3786.
4. **Bredberg, A., K. H. Kraemer, and M. M. Seidman.** 1986. Restricted ultraviolet mutational spectrum in a shuttle vector propagated in xeroderma pigmentosum cells. Proc. Natl. Acad. Sci. USA **83**:8273–8277.
5. **Brown, T., W. N. Hunter, G. Kneale, and O. Kennard.** 1986. Molecular structure of the G · A base pair in DNA and its implications for the mechanism of transversion mutations. Proc. Natl. Acad. Sci. USA **83**:2402–2406.
6. **Celis, J. E., and P. W. Piper.** 1982. Compilation of mutant suppressor tRNA sequences. Nucleic Acids Res. **10**:R83–R91.
7. **Dandekar, S., S. Sukumar, H. Zarbl, L. J. T. Young, and R. D. Cardiff.** 1986. Specific activation of the cellular Harvey-*ras* oncogene in dimethylbenzanthracene-induced mouse mammary tumors. Mol. Cell. Biol. **6**:4104–4108.
8. **Drinkwater, N. R., and D. K. Klinedinst.** 1986. Chemically induced mutagenesis in a shuttle vector with a low-background mutant frequency. Proc. Natl. Acad. Sci. USA **83**:3402–3406.
9. **DuBridge, R. B., P. Tang, H. C. Hsia, P.-M. Leong, J. H. Miller, and M. P. Calos.** 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. **7**: 379–387.
10. **Eisenstadt, E., A. J. Warren, J. Porter, D. Atkins, and J. H. Miller.** 1982. Carcinogenic epoxides of benzo[a]pyrene and cyclopenta[cd]pyrene induce base substitutions via specific transversions. Proc. Natl. Acad. Sci. USA **79**:1945–1949.
11. **Gentil, A., A. Margot, and A. Sarasin.** 1986. 2-(*N*-acetoxy-*N*-acetylamino)fluorene mutagenesis in mammalian cells: sequence-specific hot spot. Proc. Natl. Acad. Sci. USA **83**:9556–9560.
12. **Glazer, P. M., S. N. Sarkar, and W. C. Summers.** 1986. Detection and analysis of UV-induced mutations in mammalian cell DNA using a lambda phage shuttle vector. Proc. Natl. Acad. Sci. USA **83**:1041–1044.
13. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
14. **Hauser, J., M. M. Seidman, K. Sidur, and K. Dixon.** 1986. Sequence specificity of point mutations induced during passage of a UV-irradiated shuttle vector plasmid in monkey cells. Mol. Cell. Biol. **6**:277–285.
15. **Heflich, R. H., N. F. Fullerton, and F. A. Beland.** 1986. An examination of the weak mutagenic response of 1-nitropyrene in Chinese hamster ovary cells. Mutat. Res. **161**:99–108.
16. **Heflich, R. H., P. C. Howard, and F. A. Beland.** 1985. 1-Nitrosopyrene: an intermediate in the metabolic activation of 1-nitropyrene to a mutagen in *Salmonella typhimurium* TA1538. Mutat. Res. **149**:25–32.
17. **Hirose, M., M.-S. Lee, C. Y. Wang, and C. M. King.** 1984. Induction of rat mammary gland tumors by 1-nitropyrene, a recently recognized environmental mutagen. Cancer Res. **44**: 1158–1162.
18. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
19. **Kinouchi, T., and Y. Ohnishi.** 1986. Metabolic activation of 1-nitropyrene and 1,6-dinitropyrene by nitroreductases from *Bacteroides fragilis* and distribution of nitroreductase activity in rats. Microbiol. Immunol. **30**:979–992.
20. **Koffel-Schwartz, N., J. M. Verdier, M. Bichara, A. M. Freund, M. P. Daune, and R. P. P. Fuchs.** 1984. Carcinogen-induced mutation spectrum in wild-type, *uvr*A, and *umu*C strain of *Escherichia coli*. J. Mol. Biol. **177**:33–51.
21. **Lebkowski, J. S., S. Clancy, J. H. Miller, and M. P. Calos.** 1985. The *lacI* shuttle: rapid analysis of the mutagenic specificity of ultraviolet light in human cells. Proc. Natl. Acad. Sci. USA **82**: 8606–8610.
22. **Lebkowski, J. S., J. H. Miller, and M. P. Calos.** 1986. Determination of DNA sequence changes induced by ethyl methanesulfonate in human cells, using a shuttle vector system. Mol. Cell. Biol. **6**:1838–1842.
23. **Levin, W., A. W. Wood, P. G. Wislocki, R. L. Chang, J. Kapitulnik, H. D. Mah, H. Yagi, D. M. Jerina, and A. H. Conney.** 1978. Mutagenicity and carcinogenicity of benzo[a]pyrene and benzo[a]pyrene derivatives, p. 189–202. *In* H. V. Gelboin and P. O. Ts'O (ed.), Polycyclic hydrocarbons and cancer, vol. 1. Academic Press, Inc., New York.
24. **Loeb, L. A., and B. D. Preston.** 1986. Mutagenesis by apurinic/apyrimidinic sites. Annu. Rev. Genet. **20**:201–230.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 86–94. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Meehan, T., K. Straub, and M. Calvin.** 1977. Benzo[a]pyrene diol epoxide covalently binds to deoxyguanosine and deoxyadenosine in DNA. Nature (London) **269**:725–727.
27. **Moore, P., and B. S. Strauss.** 1979. Sites of inhibition of *in vitro* DNA synthesis in carcinogen- and UV-treated ϕX174 DNA. Nature (London) **278**:664–666.
28. **Patton, J. D., V. M. Maher, and J. J. McCormick.** 1986. Cytotoxic and mutagenic effects of 1-nitropyrene and 1-nitrosopyrene in diploid human fibroblasts. Carcinogenesis **7**:89–93.
29. **Patton, J. D., V. M. Maher, and J. J. McCormick.** 1987. Cytotoxicity, mutagenicity, and transformation of diploid human fibroblasts by 1-nitropyrene and 1-nitrosopyrene, p. 678–698. *In* M. Cooke and A. J. Dennis (ed.), Polynuclear aromatic hydrocarbons: a decade of progress. Battelle Press, Columbus, Ga.
30. **Protić-Sabljić, M., N. Tuteja, P. J. Munson, J. Hauser, K. H. Kraemer, and K. Dixon.** 1986. UV light-induced cyclobutane pyrimidine dimers are mutagenic in mammalian cells. Mol. Cell. Biol. **6**:3349–3356.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
32. **Sarkar, S., U. B. Dasgupta, and W. C. Summers.** 1984. Error-prone mutagenesis detected in mammalian cells by a shuttle vector containing the *supF* gene of *Escherichia coli*. Mol. Cell. Biol. **4**:2227–2230.
33. **Seidman, M. M., K. Dixon, A. Razzaque, R. J. Zagursky, and M. L. Berman.** 1985. A shuttle vector plasmid for studying carcinogen-induced point mutations in mammalian cells. Gene **38**:233–237.
34. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments in gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Strauss, B., S. Rabkin, D. Sagher, and P. Moore.** 1982. The role of DNA polymerase in base substitution mutagenesis on noninstructional templates. Biochimie **64**:829–838.
36. **Topal, M. D., and J. R. Fresco.** 1976. Complementary base pairing and the origin of substitution mutations. Nature (London) **263**:285–289.
37. **Vousden, K. H., J. L. Bos, C. J. Marshall, and D. H. Phillips.** 1986. Mutations activating human c-Ha-*ras1* protooncogene (*HRAS1*) induced by chemical carcinogens and depurination. Proc. Natl. Acad. Sci. USA **83**:1222–1226.
38. **Weinstein, I. B., A. M. Jeffrey, K. W. Jennette, S. H. Blobstein, R. G. Harvey, C. Harris, H. Autrup, H. Kasai, and K. Nakanishi.** 1976. Benzo[a]pyrene diol epoxides as intermediates in nucleic acid binding *in vitro* and *in vivo*. Science **193**:592–595.
39. **Wislocki, P. G., E. S. Bagan, A. Y. H. Lu, K. L. Dooley, P. P. Fu, H. Han-Hsu, F. A. Beland, and F. F. Kadlubar.** 1986. Tumorigenicity of nitrated derivatives of pyrene, benzo[a]anthracene, chrysene and benzo[a]pyrene in newborn mouse assay. Carcinogenesis **7**:1317–1322.
40. **Yang, J.-L., V. M. Maher, and J. J. McCormick.** 1987. Kinds of mutations formed when a shuttle vector containing adducts of benzo[a]pyrene-7,8-diol-9,10-epoxide replicates in COS7 cells. Mol. Cell. Biol. **7**:1267–1270.
41. **Yang, J.-L., V. M. Maher, and J. J. McCormick.** 1987. Kinds of mutations formed when a shuttle vector containing adducts of (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydro-

benzo[a]pyrene replicates in human cells. Proc. Natl. Acad. Sci. USA **84**:3787–3791.

42. **Yang, L. L., V. M. Maher, and J. J. McCormick.** 1980. Error-free excision of the cytotoxic, mutagenic N^2-deoxyguanosine DNA adduct formed in human fibroblasts by (±)-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene. Proc. Natl. Acad. Sci. USA **77**:5933–5937.
43. **Zagursky, R. J., K. Baumeister, N. Lomax, and M. L. Berman.** 1985. Rapid and easy sequencing of large linear double-stranded DNA and supercoiled plasmid DNA. Gene Anal. Tech. **2**:89–94.
44. **Zarbl, H., S. Sukumar, A. V. Arthur, D. Martin-Zanca, and M. Barbacid.** 1985. Direct mutagenesis of Ha-*ras*-1 oncogenes by *N*-nitroso-*N*-methylurea during initiation of mammary carcinogenesis in rats. Nature (London) **315**:382–385.